Dissertation

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

Florian Kohlhepp

Diploma-Biologist
born in Lohr am Main
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Oral examination: .............................................
EHD proteins in Apicomplexan Parasites

Referees:  Prof. Dr. Michael Lanzer
          Dr. Ann-Kristin Müller

.............................................    ................................................... ..........
Datum       Florian Kohlhepp
Contents

Danksagung

Summary

Zusammenfassung

Abbreviations

1 Introduction

1.1 Apicomplexa

1.1.1 General

1.1.2 Specific subcellular structures of apicomplexa

1.1.3 Replication of the apicomplexan pathogens

1.1.4 Epidemiology and pathogenicity

1.2 Eps15-homology domain proteins

1.2.1 Protein architecture and function

1.2.2 Function

1.3 Aim of this study

2 Materials and Methods

2.1 Laboratory equipment

2.2 Consumables

2.3 Strains

2.3.1 Bacteria strains

2.3.2 Cell lines

2.3.3 Parasite strains

2.3.4 Mosquito strains

2.3.5 Mouse strains

2.4 Chemicals and reagents

2.5 Oligonucleotides

2.6 Antibodies

2.6.1 Primary antibodies

2.6.2 Secondary antibodies

2.7 Media, buffers and solutions

2.7.1 Media and buffers for molecular biological methods

2.7.2 Media and buffers for cell culture

2.7.3 Media, buffers and solutions for parasitological methods

2.7.4 Buffers and solutions for biochemical methods

2.7.5 Antibiotics

2.8 Molecular biological methods

2.8.1 Cloning of the targeting constructs for parasite transfection

2.8.2 Stage-specific RNA isolation and cDNA synthesis for RT PCR
2.9 Cell culture ................................................................. 49
  2.9.1 *In vitro* liver-stage development of *P. berghei* in human hepatoma cells Huh7 ...................................................... 49
  2.9.2 *P. falciparum* culture - asexual parasites .................. 49
  2.9.3 *P. falciparum* culture - gametocytes ....................... 50
  2.9.4 Cultivation of *Toxoplasma gondii* in human foreskin fibroblasts (HFFs) ......................................................... 50

2.10 Parasitological methods ............................................. 51
  2.10.1 *Plasmodium* methods ........................................... 51
  2.10.2 *Anopheles* mosquito methods ............................... 56
  2.10.3 *Toxoplasma* methods ......................................... 57

2.11 Animal experimental methods ...................................... 60
  2.11.1 Administration of anaesthesia ............................... 60
  2.11.2 Infection of rodents with *Plasmodium* parasites ......... 60
  2.11.3 Blood withdrawal by heart puncture ....................... 60

2.12 Biochemical methods .................................................. 61
  2.12.1 Preparation of parasite lysate for SDS-PAGE .............. 61
  2.12.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE) .... 61
  2.12.3 Western blot analysis ......................................... 62

2.13 *In silico* analysis of apicomplexan EHD-proteins ........... 62

3 Results ........................................................................... 63
  3.1 Establishment of a combined *in vitro/in vivo* *P. falciparum* life-cycle 63
    3.1.1 *Plasmodium falciparum* asexual and sexual bloodstage *in vitro*
        culture ................................................................. 63
    3.1.2 Membrane feeding of mature gametocytes to *Anopheles stephensi*
        mosquitoes .......................................................... 65
    3.1.3 Liver-stage development of *Plasmodium falciparum* *in vitro* . 66
  3.2 Characterization of EHD-proteins in apicomplexan parasites .... 67
    3.2.1 RME-1 encodes for a conserved EHD-family member in apicomplexan parasites ..................................................... 67
    3.2.2 *Tg*RME-1 localizes to not yet known subcellular structures
        within *Toxoplasma gondii* ....................................... 67
    3.2.3 Functional analysis of RME-1 in *Toxoplasma gondii* ...... 71
    3.2.4 Localization of *Plasmodium falciparum* EHD-protein *Pf*EHD 76
    3.2.5 Protein localization of *Tg*RME-1 and *Pf*EHD is interchange-
        able between apicomplexans, but differs according to the intrinsic protein architecture .............................................. 78
    3.2.6 Expression and localization of the EHD-protein in *P. berghei* 81
    3.2.7 EHD gene deletion in murine *P. berghei* is not essential for
        blood-stage development ........................................... 81
    3.2.8 EHD mutant *P. berghei* parasites develop indistinguishable
        from wildtype parasites during the intra-mosquito life-cycle . 88
3.2.9 Depletion of EHD results in developmental slow-down during late liver-stage development in *P. berghei* and protection of severe pathology in C57BL/6 mice ................. 92

4 Discussion 95
   4.1 *Plasmodium falciparum* transmission ................. 95
   4.2 Characterization of EHD-proteins in apicomplexa .......... 97
      4.2.1 The apicomplexan EHD-protein family ............... 98
      4.2.2 Structure of the apicomplexan EHD-proteins .......... 99
      4.2.3 Localization and function of EHD-proteins in apicomplexa . 102
      4.2.4 Virulence of the *P. berghei* ANKA strain depleted of *PbEHD* 112

5 Supplementary data 117

6 References 118
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Summary

A bottle neck in malaria research is the investigation of *Plasmodium falciparum* liver stage parasites because of technical issues in the infection of *Anopheles* mosquitoes with these parasites and subsequent generation of infectious sporozoites. Therefore I, in close collaboration with a colleague, established a combined *in vitro/in vivo* *P. falciparum* life-cycle in our lab. For that a protocol was established that included the generation of sexual *P. falciparum* stages in cell culture that were subsequently transmitted to *Anopheles* mosquitoes utilizing a special membrane feeding system. Later in the life-cycle sporozoites were extracted from the mosquito salivary glands to finally infect liver cells for further studies. We were able to establish a constant mosquito-infection rate for several months to perform experiments on *P. falciparum* sporozoites and exo-erythrocytic forms.

To help decipher the in apicomplexans so far mostly uncharacterized cellular process endocytosis I investigated the function and localization of an EH-domain containing dynamin-like protein in *Toxoplasma* and *Plasmodium*. It belongs to a family of eukaryotic Eps15-homology domain containing proteins (EHDs) that have been characterized in higher eukaryotes and especially vertebrates to be part of endocytic events such as vesicular trafficking and endocytic recycling. I was able to show by an *in silico* analysis that in contrast to vertebrates (four different EHDs) there is only one protein member of this family existing in each apicomplexan. Nevertheless, the apicomplexan EHD-protein has similar to all other EHD-proteins a predicted characteristic ATPase-domain (dynamin-like G-domain) and the Eps15-homology domain (EH). Through a fluorescent tagging approach I was able to show a dynamic localization of the *Toxoplasma* EHD-protein member *TgRME-1* (named after its ortholog in *C. elegans receptor-mediated endocytosis protein 1*) within the parasites. It localized to a vesicular compartment within the parasites that did not colocalize with known organelles so far. The compartment fragmented upon cellular division and is most likely involved in vesicular trafficking of supply vesicles that transport lipids or other nutrients to the newly forming daughter-cells. From the data obtained in this thesis it can be hypothesized that the TgRME-1 labelled compartment represents a storage compartment that is filled up during the non-replicative phase and during endodyogeny helps to form daughter-cells. Structural analysis of the protein by deletion of either the G-domain or the EH-domain revealed a similar architecture of the protein compared to published data on mammalian EHDs. Investigation of the *Plasmodium berghei* EHD (*PbEHD*) with an antibody generated against the protein revealed a different localization in different parasite stages. Whereas the protein localized to several vesicular compartments in the sporozoite stage it concentrated to a single organelle-like compartment in liver-stages 24 hours after invasion. This compartment later (48 hours after invasion) also fragmented and was distributed to the newly forming merozoites during schizogony, similar to TgRME-1. This subcellular localization indicated that both proteins might share a similar function in tachyzoites of *Toxoplasma* and *Plasmodium* liver stage parasites. A phenotypical
analysis of *PbEHD* via generation of a *pbeh* (-) parasite revealed a putative function for the protein during intrahepatic development. The *pbeh* (-) liver stage parasite showed a reduced growth rate *in vivo* and *in vitro* but was still able to complete the life-cycle. *In vivo*, C57BL/6 mice infected with *pbeh* (-) parasites showed a prolonged prepatency period and did not develop experimental cerebral malaria in contrast to wildtype-infected mice. I was able to narrow down this protective effect solely to both the prolonged liver-stage phase and the involvement of the immune-modulator cytokine IL-10.

Even though a defined role for the EHD-protein in the apicomplexan parasites could not be determined in this thesis I was able to characterize its architecture and localization in *Toxoplasma gondii* and *Plasmodium berghei*. I was able to identify a so far uncharacterized compartment in these parasites that is most likely involved in endocytic-recycling and storage of nutrients such as lipids for the parasites. In addition, my studies showed that the apicomplexan EHD-protein is involved in processes of the cellular division. A better understanding of these and other mechanisms of endocytosis will lead to anti-parasitic strategies that may reduce the burden caused by apicomplexan parasites.
Zusammenfassung


## Abbreviations

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<th>Description</th>
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<td>aa</td>
<td>amino acids</td>
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<tr>
<td>ACP</td>
<td>acyl-carrier protein</td>
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<td>ampicillin</td>
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<td>Adenosin monophosphate</td>
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<td>Cytoplasm membrane/Cerebral malaria</td>
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<td>carboxy-terminus</td>
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<td>double distilled water (Millipore)</td>
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<td>diethylpyrocarbonate</td>
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<td>dihydrofolate reductase</td>
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<td>dihydrofolate reductase-thymidylate synthase</td>
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<td>DIC</td>
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<td>Dulbecco’s Modified Eagle Medium</td>
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<td>E. coli</td>
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<td>Dynamin-related protein B</td>
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<td>experimental cerebral malaria</td>
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<td>EDTA</td>
<td>ethylenediaminetetra acetic acid</td>
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<td>EE</td>
<td>early endosome</td>
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<td>extra-erythrocytic form</td>
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<td>EH</td>
<td>Eps15-homology domain</td>
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<td>Eps15-homology domain containing protein</td>
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<td>ER</td>
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<td>FCS</td>
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</tr>
<tr>
<td>for</td>
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</tr>
<tr>
<td>g</td>
<td>gram or gravitational acceleration</td>
</tr>
<tr>
<td>G</td>
<td>gauge</td>
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<tr>
<td>GFP</td>
<td>green-fluorescent protein</td>
</tr>
<tr>
<td>GOI</td>
<td>gene of interest</td>
</tr>
<tr>
<td>GPI</td>
<td>glycophosphatidylinositol</td>
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<td>GRA</td>
<td>dense granule protein</td>
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<td>GSH</td>
<td>Glutathion</td>
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<td>GTP</td>
<td>Guanosin triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin fibroblast</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>iEM</td>
<td>immuno-electron microscopy</td>
</tr>
<tr>
<td>IFA</td>
<td>immuno-fluorescence analysis</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin 10</td>
</tr>
<tr>
<td>IMC</td>
<td>inner membrane complex</td>
</tr>
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<td>Ig</td>
<td>immuno-globuline</td>
</tr>
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<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
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<td>i.v.</td>
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<td>iRBC</td>
<td>infected red blood cell</td>
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<td>liter</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LE</td>
<td>late endosome</td>
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<td>L-FABP</td>
<td>liver fatty acid-binding protein</td>
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<td>LS</td>
<td>liver stage</td>
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<td>m</td>
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<td>molar</td>
</tr>
<tr>
<td>mA</td>
<td>milli Ampere</td>
</tr>
<tr>
<td>MHC</td>
<td>major histo-compatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>micronemal protein</td>
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<td>min</td>
<td>minute</td>
</tr>
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<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<td>MPA</td>
<td>Mycophenolic acid</td>
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<td>MTOC</td>
<td>microtubule-organizing center</td>
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<td>n</td>
<td>nano</td>
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<tr>
<td>N-terminus</td>
<td>amino-terminus</td>
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<td>OD</td>
<td>optical density</td>
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<td>ODS</td>
<td>oocyst-derived sporozoites</td>
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<td>ORF</td>
<td>open-reading frame</td>
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<td>PbA</td>
<td><em>Plasmodium berghei</em> strain ANKA</td>
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<tr>
<td>PABA</td>
<td>para-Aminobenzoic acid</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
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<td><em>Plasmodium falciparum</em></td>
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<td>PFA</td>
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<tr>
<td>PM</td>
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<td>P/S</td>
<td>penicillin and streptomycin</td>
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<td>PV</td>
<td>parasitophorous vacuole</td>
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<td>PVM</td>
<td>parasitophorous vacuolar membrane</td>
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<td>RBC</td>
<td>red blood cell</td>
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<td>Rhooptry protein</td>
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<td>Rhooptry neck protein</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>tris-buffered saline/tween</td>
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<td>TEMED</td>
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<td><em>Toxoplasma gondii</em></td>
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<td>TM</td>
<td>transmembrane domain</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>TRIS</td>
<td>tris (hydroxymethyl)-aminomethane</td>
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<td>tubovesicular membranous network</td>
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<td>U</td>
<td>units</td>
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<td>UIS</td>
<td>upregulated in infective sporozoites</td>
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<td>untranslated region</td>
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<td>WT micro</td>
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<td>Xanthin</td>
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<td>YFP</td>
<td>yellow-fluorescent protein</td>
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Chapter 1

Introduction

1.1 Apicomplexa

1.1.1 General

Apicomplexans are unicellular eukaryotic organisms (protists) that belong, together with their two well-studied sister-lineages dinoflagellates and ciliates, to the higher order of the assemblage of the alveolates. Alveolates are characterized by a common feature, the so-called cortical alveoli, membrane-bound vesicles that can be found in close association with the plasma membrane of the cell. Apicomplexans and dinoflagellates probably evolved from a group of predatory flagellates, the colpodellids, that were shown to represent a sister group of the apicomplexans. Both, apicomplexans and flagellates, share a common mechanism of attaching to the host cell (apicomplexans) or the prey (colpodellids), respectively, that is mediated by similar organelles. Whereas both sister groups, the apicomplexans and the colpodellids, kept these organelles within the course of evolution, dinoflagellates lost them. But all three clades still share an endosymbiontically taken up red alga, the apicoplast, a remnant chloroplast, that in some alveolates still is photosynthetically active. Apicomplexans belong to a monophyletic group that is almost exclusively obligate intracellular parasitic and affect human life in terms of veterinary medicine and agriculture (Babesia, Theileria and Eimeria) and in terms of human health (e.g. Toxoplasma, Plasmodium) on a daily basis. For an overview of pathogenic apicomplexa infecting humans see Tab. 1.1.

1.1.2 Specific subcellular structures of apicomplexa

Apical secretory organelles

Unique to the invasive stages of apicomplexan protozoa are three types of electron-dense secretory organelles, namely rhoptries, micronemes and dense granules (Fig. 1.1). These organelles are carrying characteristic secreted proteins that are

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involved in adhesion (AMA-1, MICs) and both the formation of the moving junction and the PV/PVM (RONs, ROPs, GRAs)\(^7\), respectively. A number of key parasite ligands that are located and released from these secretory organelles mediate the interaction of the parasite with the host cell to facilitate and trigger invasion. Studies on *Plasmodium falciparum*, e.g., have shown that the invasion process requires several sequential steps like attachment to the erythrocyte surface, apical re-orientation and release of proteins of the apical organelles, with micronemes being secreted first\(^8\)\(^9\). **Micronemes** are the smallest in size among the apicomplexan apical secretory organelles and their number per cell varies highly between different genera, species and developmental stages of apicomplexan parasites\(^10\). In general, parasite stages that are able to glide and to invade cells have more micronemes in comparison to non-invading cells who contain few if not none. The majority of microneme proteins (MICs) that are secreted upon host-cell contact have multiple adhesion-domain types that lead to interactions between the parasite and the host-cell. For this reason micronemes are secreting before all other secretory organelles since they provide the initial contact through secreting them onto the parasite surface. Not much is known about the factors triggering secretion but it has been shown that micronemal protein secretion is rapidly upregulated upon contact with the host cell\(^8\)\(^4\). In case of *P. falciparum* external low potassium ion concentrations both internal elevated cyclic AMP (cAMP) and Ca\(^{2+}\) concentrations are involved in triggering this process\(^20\). **Rhoptries** are club shaped organelles that exist in the number of 8-12 per parasite cell and are located at the apical end of the parasite. The extended apical end of these organelles, the so-called neck, is connected to the apical pole of the parasite where rhoptry proteins can be released from the cell. Rhoptries are acidified organelles, with a pH of about 3.5-7 (depending on the maturity of the organelle)\(^11\) and occupy about 10-30% of the total cell volume\(^7\). Rhoptries harbour about 30 different proteins, most of them located either within the bulbous part (rhoptry proteins, ROPs) or at the neck of the organelle (rhoptry neck proteins, RONs)\(^12\). Some of these proteins are important kinases that are essential virulence factors and are secreted upon invasion of the parasites\(^13\)\(^14\)\(^15\)\(^16\)(Fig. 1.2). Rhoptry proteins are produced as pro-peptides at the ER and then processed, packaged and sorted at the Golgi-apparatus into vesicular immature rhoptries. Some proteins are also delivered to the rhoptries via the endosomal pathway in multivesicular bodies and might therefore represent analogous organelles of secretory lysosomal granules of mammalian cells\(^17\)\(^18\)\(^19\). The external signals and signaling mechanisms responsible for secretion of rhoptry proteins are so far unknown in apicomplexan parasites\(^20\). **Dense granular** proteins are released during and after invasion of the parasites into the host cell and they remain either soluble within the PV or are integrated into the PVM or the TVM, a tubovesicular membranous network within the PV\(^21\). Dense granule proteins are thought to modify the environment within the PV, are release through an "open IMC-window"\(^22\) (Fig. 1.2) and have been identified as excretory/secretory antigens in *Toxoplasma*\(^23\).
CHAPTER 1. INTRODUCTION

1.1 Figure 1.1: The morphology of apicomplexan parasites. Apicomplexa invasive stages possess several clade-specific cellular organelles in addition to the eukaryotic standard repertoire Golgi-apparatus, mitochondria (not shown in this figure), endoplasmic reticulum and the nucleus. Secretory organelles such as rhoptries, micronemes and dense granules contain secretable proteins that are required for motility, invasion, formation of the PVM and establishment of the PV milieu. An additional organelle is the apicoplast, a secondary endosymbiotic red alga, that supplies the parasite with fatty acids. The conoid is a spiral structure of undefined material and facilitates invasion into the host cell. It can protrude from or retract into the apical polar ring, a MTOC for the subpellicular microtubules that help to keep the parasite's elongated shape. The parasites are bounded by the pellicle, which consists of the IMC and the parasite membrane. PVM: parasitophorous vacoule membrane; PV: parasitophorous vacuole; MTOC: microtubule-organizing center; IMC: inner membrane complex; Adapted from Morrissette and Sibley, 2002.

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CHAPTER 1. INTRODUCTION

Figure 1.2: 3-D reconstruction of the secretory apical organelles during initiation of invasion. Rhoptries (green) are secreted one at a time through the conoid ring at the apical tip. Micronemes (red) dock to the posterior polar conoid ring at the tip. Dense granules (dark blue) are secreting through “open IMC-windows” at the side of the apical tip. The polarity and shape of the cells is maintained by subpellicular microtubules (grey net of bundles). (Adapted from Paredes-Santos et al., 2012)

Apicoplast

An apicomplexa-specific organelle first identified in 1975 harbors a circular extrachromosomal genome of prokaryotic origin, quite similar to that of plastids of plants and algae. In contrast to the confirmed plant- or algae-like origin it has been shown that this plastid-like organelle lacks any genes involved in photosynthesis. Comparisons of the apicoplast with photosynthetic plastids of the ancestral apicomplexan parasite, Chromera velia, show that these plastids all share the same ancestry and undoubtedly are of red algae origin. This alga was taken up into the parasites by endosymbiosis. Dinoflagellates also possess this plastid and therefore the endosymbiosis of the red alga took place before dinoflagellates and apicomplexa separated (see 1.1). Another indicator that clearly supports the endosymbiosis theory is the fact that the apicoplast possesses 3-4 membranes as observed in electron-microscopy studies. The apicoplast occurs in all members of the phylum apicomplexa with exception of Cryptosporidium spp. and possibly gregarines, as well. For a long time no function of the apicoplast other than keeping itself alive has been suggested, in addition to the observation of the obvious loss of the photosynthetic activity. On the other hand disrupting the integrity of the apicoplast by chemical or genetic intervention leading to delayed death showed that the plastid is essential for the parasite. Already in 1998 parasite genome projects made it possible to identify apicoplast-specific genes involved in parasite metabolism. Waller...
et al. identified genes for an apicoplast fatty acid biosynthesis system in *Toxoplasma* and *Plasmodium*, the type II fatty-acid synthesis (FASII) pathway. Previously it had been believed that apicomplexans were not able to synthesize their own fatty acids *de novo* and these metabolites had to be scavenged from the host cell *de novo*. One of the key proteins involved in the FASII pathway in apicoplasta is the acyl-carrier protein ACP. Conditional mutagenesis of this protein in *Toxoplasma* effects apicoplast biogenesis and results in death of the parasite. But on the other hand this pathway was not essential for blood- and mosquito-stages, but essential for liver-stages, of *Plasmodium*, indicating for different needs of fatty-acids in these cells or other mechanisms to *de novo* synthesize or salvage these metabolites from host cells in different stages. Some other *de novo* mechanisms like a FASI-pathway (most apicomplexans except *Theileria* and *Babesia*) and a fatty acid elongation pathway (*Toxoplasma*) have been identified, recently. In addition to that other apicomplexan parasites harbouring an apicoplast (*Babesia, Theileria*) are completely lacking the FASII synthesis machinery suggesting that they are dependent on fatty acid salvage from the host. This leads to the question what is the potential role of the apicoplast in these organisms, something that still remains to be experimentally addressed. Since the human FASI pathway components differ from the apicomplexan FASII, this makes it, at least for the stages depending on this pathway, an interesting target for apicomplexan parasite prevention strategies. For an overview of the processes involved in the fatty acid metabolism in *Plasmodium* liver stage parasites see Fig. 1.3.

**Cytoskeleton**

The cytoskeleton of apicomplexa consists of three different types of structural elements: **Microtubules** (and associated proteins), the **subpellicular network** (including the inner membrane complex IMC) and the combination of **actin/myosin**. The haploid stages of apicomplexa have two different forms of **microtubules**: Spindle microtubules and subpellicular microtubules (Fig. 1.4A). The **subpellicular microtubules** are spirally arranged and radiate from the apical polar ring down to the region of the nucleus (approximately 2/3 of the length of the parasite), where they end. Underlying the inner membrane complex of the parasite the subpellicular microtubules confer both polar orientation and elongated shape of the parasite (compare Fig. 1.1, Fig. 1.2 and Fig. 1.4). Therefore replicative parasite stages lacking the subpellicular microtubules are non-motile, non-polar and non-invasive. The apical polar ring (APR) (Fig. 1.1) acts as a microtubule organizing center (MTOC) and laterally associates with the minus end of the subpellicular microtubules. **Spindle microtubules** are employed by replicative stages during mitosis. Since nuclear division of the parasites occurs without nuclear breakdown spindle microtubules associate with a spindle organizing structure located within nuclear envelope invaginations during this process, the so-called centrocone. Close to the centrocone located is the centriol (except...
CHAPTER 1. INTRODUCTION

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Figure 1.3: **Schematic overview of the fatty acid metabolism pathways in Plasmodium liver-stages.** Fatty acids in the liver-stages can be obtained in 4 different ways: Through 1) uptake from the host cell, 2) *de novo* synthesis via the FASII-pathway taking place in the apicoplast, 3) recycling of membrane lipids or 4) modification and integration into membranes. 1) The parasite-specific PVM resident protein UIS3 is interacting with the liver host cell specific lipid carrier liver fatty acid binding protein (L-FABP) and uptake is proposedly mediated by UIS3. 2) For the *de novo* synthesis of fatty acids in the apicoplast the main precursor is acetyl-CoA, which can be either synthesized from pyruvate by the pyruvate-dehydrogenase complex (PDH) or from acetate via the acetyl-CoA synthetase (ACS). Before going into the FASII elongation cycle, where the fatty acid chain is elongated by two carbons a cycle, acetyl-CoA is condensed with the acyl-carrier protein (ACP). Several enzymes are involved in the several steps before either octanoyl-ACP (important co-factor of the PDH) or C_{10} to C_{14} fatty acid chains are produced. 3) Free fatty acids can be released from membrane turnover events. 4) The activation of fatty acids to Acyl-CoA thioesters is needed for most cellular processes fatty acids are involved in. The process is mediated by the enzymes ACL (acyl-CoA synthase) and ABP (acyl-CoA binding protein). The fatty acids can then be modified by other enzymes in the endoplasmic reticulum (ER). (Adapted from Tarun et al., 2009.)
in *Plasmodium* merozoites and *Theileria* sporozoites), a specialized MTOC, that does not seem to be necessary for nuclear division but might be necessary for apicoplast division or serve as a nucleation site for the basal body of the flagellum of microgametes \(^{59}\). The **subpellicular network** was first identified in *Toxoplasma gondii* (Fig. 1.4B). It was shown that a network of filaments of 8-10 nanometers in diameter is underlying the plasma membrane (PM) of the parasites creating a three-layered pellicle (PM, 2x IMC)\(^{60}\). Two of the main network proteins are members of the inner membrane complex (IMC) in *Toxoplasma*, *TgIMC-1* and *TgIMC-2*. These proteins are predicted to from coiled-coils similar to the ones formed by myosin in order to stabilize the network\(^{47}\). The IMC is directly underlying the parasite’s plasma membrane and is made of large flattened vesicles in *Plasmodium* sporozoites. In other apicomplexans it is made of many cortical bound large vesicles, the alveoli (Alveolata-specific), aligned in longitudinal rows\(^{61}\) \(^{62}\). The membranes of the IMC are spiked with intramembranous particles (IMPs) that are also organized in rows and might represent transmembrane domains of proteins attached to intermediate filaments, giving the whole cytoskeleton more stability (Fig. 1.4B)\(^{47}\).

**Actin** is expressed by two different genes in *Plasmodium*\(^{63}\), whereas Toxoplasma actin is only expressed by one\(^{64}\). Most of the actin molecules in apicomplexa seem to be in a monomeric state rather than existing as a polymerized form. *Toxoplasma* tachyzoites for example only have about 2% of their actin in assembled filaments\(^{64}\) and only just a few years ago actin microfilaments could be observed in electron microscopy studies for the first time\(^{95}\). However, artificially stabilizing actin in *Toxoplasma* with the drug jasplakinolide showed that indeed actin monomers can form filaments in these cells and this most notably at the apical end of tachyzoites\(^{65}\) \(^{66}\) (Fig. 1.4C). In addition to that, more recent studies have shown that actin filaments in *Toxoplasma* and *Plasmodium* are rather short, between 50 and 150 nm in length\(^{67}\) and undergo rapid structural reorganization and turnover\(^{68}\), mediated by polymerizing and depolymerizing factors such as ADF, ARP2/ARP3 etc.\(^{69}\) \(^{70}\). In artificially hypothonically swelled *Toxoplasma* parasites \(\alpha\)-actin antibodies labeled the region between the plasma membrane and the IMC. Actin filaments have been shown to be linked to the substrate extracellular parasites are binding to by transmembrane adhesins that are reaching from the outside of the cell through the plasma membrane to the actin filaments\(^{71}\). One of the major roles of actin filaments in extracellular apicomplexan stages is the interaction with the substrate and formation of the motor complex together with **myosin** and other accessory proteins (Fig. 1.5) (Fig. 1.5). This actin-myosin based motor complex enables the parasites to glide on substrates (gliding motility) and to penetrate and invade cells. Apicomplexans harbor only a limited and rather uncommon repertoire of myosins including myosins of the unusual class XIV, which is restricted to this phylum\(^{72}\). One of the most important roles for apicomplexan myosins is exhibited by myosin A (MyoA). MyoA is anchored within the IMC by accessory proteins\(^{73}\) \(^{74}\) and forms the motor protein complex for gliding motility that directly interacts with the actin-filaments (Fig. 1.5). It has long
been thought that MyoA is absolutely essential for gliding motility and invasion, since tetracyclin-inducible conditional knock-out studies showed complete blockage of invasion, before recently new knock-out studies made it possible to completely knock-out MyoA while invasion to a certain degree is still possible (Nicole Andenmatten and Markus Meissner, unpublished observations).

Figure 1.4: Components of the apicomplexa cytoskeleton. A) Microtubules. Subpellicular microtubules radiate from the apical polar ring (black ring at the upper end of the parasite) in close association with the cytosolic face of the IMC towards the basal end of the parasite. Spindle microtubules nucleate at the centrocones (invaginations of the nuclear membrane) during mitosis and are associated with centrioles (dotted circular structure) that might be needed for apicoplast division or serve as nucleation site for the basal body of the flagellum of sexual forms. B) The subpellicular network is an association of intermediate filaments that underlie the inner membrane complex (IMC) and is connected with this by transmembrane domains of membrane receptors to give stability and rigidity to the parasite. C) Actin and myosin are located between the IMC and the plasma membrane, actin associated with the IMC, myosin anchored within the plasma membrane, and form, together with other associated proteins, the motor machinery of the parasites. (Adapted from Morrissette and Sibley, 2002.)

1.1.3 Replication of the apicomplexan pathogens

Life-cycle

Apicomplexa grow and replicate within parasitophorous vacuoles in host cells and do not undergo extracellular cell division. This means apicomplexa have to rapidly invade host cells, followed by parasite replication including cellular division, host cell
Figure 1.5: **The apicomplexan gliding machinery.** This caption shows a model for the interaction of the MyoA motor complex with the micronemal protein-host receptor complex. The MyoA motor complex is anchored within the membrane of the IMC and can bind to and move along the actin microfilaments. The actin-filaments themselves are connected with microneme-secreted transmembrane proteins located in the parasite membrane via an aldolase protein. The transmembrane proteins on the outside of the parasite can bind to host receptors thereby promoting parasite-host cell interaction and gliding of the parasite since the driving force of the MyoA-complex can be transmitted to the substrate. Black arrows indicate the movement of the complexes. Open arrows show proteolitic cleavage sites. MyoA: Myosin A; IMC: Inner membrane complex; (Adapted from Soldati and Meissner, 2004)
lysis and reinvasion, in order to multiply and survive. Apicomplexa have developed complex life-cycles in order to colonize their hosts and to be transmitted to a new host (Fig. 1.6). Whereas *Toxoplasma* can be transmitted directly from vertebrate to vertebrate via sporozoites stages within oocysts shed within the feces from cats or via ingestion of tissue cysts by canine animals (Fig. 1.8) *Plasmodium* needs an invertebrate vector to be transmitted from one host to the other. In order to produce replicative stages, that multiply within the host cells, apicomplexa produce extracellular invasive stages (*Toxoplasma*: Tachyzoites, Bradyzoites, Sporozoites; *Plasmodium*: Merozoites, Ookinete, Sporozoites) that possess specific organelles required for invasion. They are motile to reach and invade new host cells (Fig. 1.6). Whereas *Toxoplasma* can invade any nucleated cell of warm-blooded vertebrates, *Plasmodium* is restricted to specific cell types such as hepatocytes and red blood-cells to productively replicate within.

**Parasitophorous vacuole (PV)**

After invasion of the apicomplexans into their host cell these parasites reside within a parasitophorous vacuole surrounded by a membrane, the parasitophorous vacuole membrane (PVM)\(^78\). The PVM forms a physical barrier between the parasites and the host cell cytosol protecting the parasite from host defense mechanisms. But since the parasites are surrounded by this, and in addition to that, at least one other membrane (host cell cytoplasmic membrane) and therefore can not directly take up nutritional factors from the surrounding medium the PVM plays also a specific role in parasite-host-specific exchange. The entry of apicomplexan parasites is an active process driven by the parasites' ability to glide and to form a moving junction at the connection point between the parasite and the host cell. The invasion is therefore termed "active" instead of "induced" (bacteria) since the host cell does not take part in this process\(^79\) \(^80\) \(^81\). Therefore the parasite first attaches to the host cell and subsequently penetrates into a vacuolar invagination of the host cell membrane by forming a moving junction between the parasite and the host cell surface\(^82\). It has long been thought that the driving force of the invasion of apicomplexan parasites is the motor complex, the so-called glideosome, an acto-myosin motor located between the parasite plasma membrane and the inner membrane complex\(^83\) (Fig. 1.5). But this biological dogma recently seems to have been proven wrong: Knock-out studies of protein-members of the motor-complex of *Toxoplasma gondii*, previously said to be essential for parasite invasion, did not completely abolish invasion of the parasites (Nicole Andenmatten and Markus Meissner, WTCMP Glasgow; unpublished observations). The attachment of the parasites to the host cell membrane and the invasion into it requires sequential secretion of specific proteins from apical parasite organelles (1.1.2 Apical secretory organelles), termed micronemes, dense granules and rhoptries\(^84\). These proteins and lipids, together with proteins and lipids provided by the host cell, are incorporated into the newly forming PVM\(^81\) \(^85\) \(^86\) \(^87\) \(^88\) \(^89\). The mature PVs in *Toxoplasma* and *Plasmodium* differ to some extend.
CHAPTER 1. INTRODUCTION

1.1 Figure 1.6: Comparison of the life-cycles of Toxoplasma and Plasmodium.

The inner and the outer white circles represent the generic apicomplexan and the Plasmodium life-cycle, respectively. The inner grey-shaded circle represents the Toxoplasma life-cycle. Rapidly proliferating haploid asexual stages of apicomplexans are able to produce sexual stages (gametocytes). Gametocytes transform into gametes of both, male and female gender, that fuse to form a diploid zygote. Via meiosis the zygotes are transformed into haploid sporozoites that are able to initiate infection in a new host individual. The sexual reproduction of Toxoplasma via sexual stages can take place in the intestinal epithelium of cats only, their definite host. Asexual reproduction, in contrast, can take place in many other warm-blooded intermediate hosts (vertebrates) and the replication of tachyzoites happens in many different cell-types and organs. Tachyzoites can differentiate into slowly replicating bradyzoites that form dormant cyst stages in muscle and brain tissues. These tissue cysts are then infectious to carnivorous hosts again by bypassing the sexual phase and reactivating tachyzoite production from the cysts. In contrast to Toxoplasma, Plasmodium species are restricted to a specific host and a mosquito vector that is transmitting the sporozoites to a new host individuum. Whereas the Toxoplasma zygotes directly transform into sporozoites within the intestinal epithelium of cats Plasmodium zygotes transform into motile ookinetes first, that cross the midgut epithelium of the mosquito. Afterwards they transform into resident oocysts, where meiosis is going on and sporozoites are produced, that travel to the salivary glands of the vector. After maturing there, the parasites are injected upon blood-meal of the mosquito into the skin and bloodstream of a new host, to finally establish infection within liver-cells. Whereas the asexual Toxoplasma tachyzoites are able to infect any nucleated cell of their vertebrate host, Plasmodium merozoites, released by the liver cell, can only infect non-nucleated red blood-cells, where they undergo several rounds of asexual replication before they produce sexual stages again. (The figure was adapted from Morrissetti and Sibley, 2002\textsuperscript{47}.)
PV in *Toxoplasma* is a wide compartment with the parasites being connected at the center and space remaining at the periphery. The *Toxoplasma* PVM is tightly associated with host cell mitochondria and endoplasmic reticulum (ER) and can be prolonged by long extensions reaching into the host cell cytoplasm. In *Plasmodium*, in contrast, the parasite membrane and the PVM are always tightly associated and therefore difficult to distinguish from each other in fluorescence microscopy analyses. *Plasmodium* parasites differ in terms of the composition of their PVM according to which host-cell type they are residing in. Liver-stage parasites have, in comparison to blood-stages, different proteins residing in the PVM to achieve protein export into the PVM, the host-cell cytoplasm or beyond. In addition to that liver-stage parasites are closely associated with the host-cell ER via the PVM to scavenge host-derived lipids, something that is not possible in intraerythroctic.

**Replication**

The replication of apicomplexans in general to create two daughter-cells or multiple progenie can occur through two different mechanisms: Whereas *Plasmodium*, *Eimeria*, *Babesia* and *Theileria* replicate through a mechanism called schizogony creating up to 64 daughter parasites, *Neospora* and *Toxoplasma* replication occurs by endodyogeny. In both mechanisms the nuclear membrane remains intact throughout the whole nuclear division (cryptomitosis) and the caryokinesis occurs without chromatin condensation. Both processes, endodyogeny and schizogony, are very similar and are differing mainly in the preservation of the mother cell specialization (Fig. 1.7).

**Endodyogeny** During endodogeny two daughter-cells are formed within the mother cell. The mother cell remains polarized during the whole replication cycle and keeps the integrity of the inner membrane complex (IMC) and the subpellicular membranes. Therefore the mother cell preserves the ability to glide and to invade new host cells throughout the complete life-cycle even if the host cell lyses before the generation of the daughter-cells is complete. Each daughter-cell is enclosed by its own IMC and associated subpellicular microtubules and filled with the apical organelles in addition to a nucleus, mitochondrion, Golgi-apparatus and the plastid.

**Schizogony** In parasites undergoing schizogony subpellicular membranes and the IMC are disassembled after invasion of the parasites into host cells and several rounds of nuclear divisions take place. At the end of each cycle up to 64 (in blood stages) or up to 30,000 (in liver stages) daughter nuclei have been formed and move to the periphery of the cell where they assemble together with new sets of the organelles, are incorporated each with their own newly formed IMC, and finally bud off from the mother cell as merozoites. During the process of schizogony, in contrast to parasites undergoing endodyogeny, parasites are not invasive any more because
Figure 1.7: **Schematic comparison of apicomplexan endodyogeny and schizogony.**

**A)** Endodyogeny is producing two daughter-cells inside the mother cell without losing the shape and polarity of the mother cell. The inner membrane complex (IMC, thick black line) and subpellicular microtubules of the mother cell remain intact throughout the whole process and the daughter-cells are forming surrounded by their own IMC. When the daughter-cell formation is complete the newly formed cells bud off from the remnants of the mother cell.

**B)** During schizogony the integrity and polarity of the mother cell is lost, the parasite rounds up and subpellicular microtubules and the IMC are lost. Extensive cell growth and nuclear division takes place before single nuclei align with single sets of apical organelles, the IMC and subpellicular microtubules at the periphery of the replicating cell. (represented as white circles) (Figure adapted from Morrissetti and Sibley, 2002.)
they lack cell polarity and the integrity of the organization of apical organelles.

1.1.4 Epidemiology and pathogenicity

Toxoplasmosis and Malaria

Toxoplasmosis  
Toxoplasma replication and transmission follows a predator-prey system that alternates between definite (sexual reproduction) and intermediate (asexual reproduction) hosts (Fig. 1.6). It is very unique for Toxoplasma in its coccidian group that transmission of the parasites can occur not only between definite and intermediate host (sexual cycle), but also between two intermediate hosts by carnivorism (asexual cycle) and even between definite hosts by oocyst-contaminated feces (Fig. 1.8). But the proportion of asexual and sexual cycles taking part in transmission vary between given environments according to the structures of the definite and intermediate host populations. In general, Toxoplasma infection has been described for more than 350 host species, mostly mammals and birds, and the majority of these animals are living in a wild environment. The contamination of this environment is linked by the stray, domestic or wild feline definite hosts shedding oocysts that are than taken up by intermediate hosts (Fig. 1.8), and therefore the seroprevalence of Toxoplasma in the intermediate hosts mostly depends on the felids in their environment. Most definite hosts have a seroprevalance of close to 100%, but this depends on many different environmental factors such as climatic conditions, susceptibility of the host, lifespan and feeding behaviour. Seroprevalence in humans also varies quite dramatically: In general, it is assumed that about 25-30% of the world’s human population is infected with Toxoplasma parasites. Whereas low seroprevalences (10-30%) have been found in North America, South East Asia, Northern Europe and Sahelian countries of Africa, moderate prevalences (30-50%) have been found in Central and Southern Europe and high prevalences in Latin America and in tropical African countries. Important factors for the seroprevalence of human populations are climatic conditions that affect the survival of oocysts in the environment, infection rates in meat producing animals, dietary habits (cooking of meat, handwashing, fertilization and collection of vegetables, kinds of meat and vegetables consumed etc.) and the domestication of cats. In addition to that, water contaminated with feces also plays a very important role in transmission of Toxoplasma to humans. In general, most human infections are acquired through horizontal transmission via ingestion of tissue cysts in infected meat (30-63% of all risks in Europe) or by ingestion of feline feces-contaminated soil (6-17% of all risks in Europe), water, or food with sporulated oocysts. The fact that most cases of new Toxoplasma infections in Europe are caused by contaminated food is especially surprising considering the fact that tissue cysts are usually killed in deeply frozen food (below −12°C) after 3 days or immediately by heating them up to 67°C. Two rather rare sources for an infection are the horizontal transmission of tachyzoites through the plazenta from the mother to the
unborn child (congenital), happening only if the mother acquired a primary acute infection during the pregnancy, and infections related to organ transmission. For a summary of all infection risks see Fig. 1.8. **Pathogenesis during the course of the infection in humans:** After ingestion of cysts or oocysts into the human body the respective forms, bradyzoites or sporozoites, invade the small intestinal epithelium where they convert into the rapidly growing tachyzoites. The acute early steps of the infection of this tissue finally lead to a transmigration of the parasites through the epithelium to the basolateral side, where the parasite invades monocytes. These are the key cells for disseminating the parasites through the blood flow to all organs, using them as shuttle service to cross biological barriers almost "unseen" by the immune system, being able to infect almost any nucleated cell. Nevertheless, shortly after ingestion of the parasite into the human digestive tract there is a local release of chemokines by the infected cells which then leads to attraction of cells of the innate immune system. Phagocytotic immune-cells are recruited and a Th1-based immune-reaction is generated, which, if not tightly regulated by IL-10 and TGF-β, can result in severe tissue damage in non-healthy patients. An overshooting Th1-immune-response might also be responsible for the fatality of acute *Toxoplasma* infection during pregnancy. Normally a Th2-based immune-response leads to maternal-fetal tolerance but is destroyed by IFN-γ secretion during a *Toxoplasma* infection which can lead to serious damage if not abortion of the unborn child. On the other hand, in contrast to generating an overwhelming immune-response during acute infection, *Toxoplasma* has evolved many different techniques to avoid the host cell immune system and thereby establishing a lifelong persistent infection in its host. Some of the parasite strains for example are able to get some of the secreted proteins transported into the host cell nucleus, where they interfere with pathways of the host immune system. In addition to that it has also been demonstrated that the parasites are able to inhibit apoptotic mechanisms of the cells they are residing in, thereby ensuring protection from rapid clearance of intracellular tachyzoites from macrophages and oocysts from tissues and prevent alerting of the immune system. In 80% of all cases in immunocompetent individuals an acquired infection is asymptomatic. In all other cases in this group patients may experience fever, a swelling of cervical lymphnodes or other non-clinical symptoms. Yet, the severity of the disease outcome might be dependend on the parasite strain since recent observations showed that countries that have more virulent strains predominating generally have higher incidence rates of severe disease. This severity is usually expressed by the existence of high rates of corioretinitis, an inflammation of the patient’s eye. But these strains can also be responsible for the generation of lethal infections in immunocompetent patients. In contrast to immunocompetent individuals, Toxoplasmosis is always life-threatening in immunocompromised patients. Among these patients people with an HIV-infection or under immunosuppressive therapies are considered at the highest risk. Especially people receiving an organ via transplantation are at a
very high risk since they can either develop Toxoplasmosis through reactivation of cysts during their immuno-suppressive therapy or via the transplanted organ, that may carry tissue-cysts itself\textsuperscript{123, 134, 135}. In HIV patients the incidence of Toxoplasmosis is closely related to the number of CD4\textsuperscript{+} T-cells, the risk increasing with decreasing cell numbers. The most affected organ in these patients is the brain, where toxoplasmic encephalitis (TE) can occur. This inflammation of the brain can lead to symptoms ranging from headache, lethargy and incoordination to loss of memory, major motor seizures and finally death\textsuperscript{136}. But also other organs can be involved such as the lung, the eyes and the heart resulting in inflammation of the tissue in these organs\textsuperscript{137, 138}. As described above one fatal outcome of the primary infection in immunocompetent individuals can be the \textit{congenital Toxoplasmosis} in pregnant women. Here, tachyzoites that are circulating in the mother during a recently acquired infection are transmitted to the child via vertical transmission (Fig. 1.8) through the placenta, a target tissue for parasite replication\textsuperscript{139}. The severity of the disease thereby is highly dependent on the timepoint of the infection of the mother during her pregnancy. During the first trimester the placental barrier is highly efficient and only in 10\% of the cases parasites pass through. This changes with the 2nd and 3rd trimester when in 30\% and 60-70\% of the cases, respectively, parasites can pass the barrier\textsuperscript{140}. Nevertheless parasite replication at all times can lead to inflammation of the placenta and in the fetus’ eye and brain tissue causing severe damage to the child and finally may lead to abortion of the child.

\textbf{Malaria} To date malaria is still one of the most dangerous and deadly neglected infectious diseases in the world. Even though the World Health Organization (WHO) reported a 50\% reduction of reported malaria cases in 43 of 99 countries with ongoing transmission (Fig. 1.9) between 2000 and 2010 in their World Malaria report 2011, there are still more than 200 million malaria cases estimated each year\textsuperscript{141}. Of these, more than 80\% are reported in subsaharan Africa resulting in 600.000 deaths per year on this continent, mostly amongst children under the age of 5 years\textsuperscript{141}. Malaria is caused by at least five different species of the apicomplexan parasite \textit{Plasmodium} (see 1.1): \textit{P. vivax}, \textit{P. malariae}, \textit{P. ovale}, \textit{P. knowlesi} and \textit{P. falciparum}, the latter of which causes Malaria tropica, the most severe form of all malaria diseases. Today, more than 100 different \textit{Plasmodium} species are known, infecting a broad variety of vertebrates including mammals like humans and rodents, but also birds and reptiles. The transmission of the \textit{Plasmodium} parasites from host to host occurs through an infectious blood meal of mosquitoes (see 1.1.3) of the genera \textit{Culex}, \textit{Anopheles}, \textit{Culiceta}, \textit{Mansonia} and \textit{Aedes}. After transmission of the parasites in form of sporozoites by a mosquito they reach the liver (see 1.1) where they replicate and remain clinically silent. In \textit{P. malariae}, \textit{P. ovale} and \textit{P. vivax} liver-stage parasites can form persistent stages, so-called hypnozoites, that can lead to disease relapses years after the initial infection. After replication within the liver-cells merozoites buds-off and detach from the surrounding tissue\textsuperscript{142}. These merozoite filles vesicles are though to be derived from the host liver cell and its membranes\textsuperscript{143}. 

16
Figure 1.8: Sources of *Toxoplasma gondii* infection for humans. (Adapted from Robert-Gangneux and Darde, 2012.)
They release merozoites into the bloodstream of the host, where they undergo asexual replication (see 1.1). It is during this phase of the parasite’s life-cycle that the human host is suffering from clinical symptoms. The classical clinical symptoms of malaria are intense chills, fever and sweating. The chills will often be accompanied by headaches, nausea and fatigue. One of the most characteristic symptoms of a malaria infection in humans is the re-occurrence of fever attacks. The frequency of these fever attacks is dependent on the speed of the asexual replication and the timing of the erythrocyte bursting. This bursting leads to a release of merozoites as well as free antigens alerting the immune system and leading to an inflammatory reaction resulting in fever. All Plasmodium species infecting humans except *P. falciparum* can only invade erythrocytes of a certain age. Therefore their replication cycles and as a result the burst of the mature infected red blood cells (iRBCs) are synchronized leading to a defined pattern of fever attacks. In a *P. vivax* and a *P. ovale* infection the fever attacks are re-occurring every 48 hours (Malaria tertiana) whereas in *P. malariae* they do every 72 hours (Malaria quartana). *P. falciparum*, in contrast, can infect RBCs of any age and therefore the replication and burst of iRBCs is not synchronized, the fever-attacks are irregular. Since replication of the *Plasmodium* parasites leads to the burst of erythrocytes one of the complications of malaria is anemia. *P. falciparum*, in contrast to the other parasite strains, can also cause additional complications of the infection called severe malaria. Severe malaria is characterized by acute renal failure, lung edema, severe anemia and acidosis. One reason for the higher virulence of *P. falciparum* is its ability to express variant surface antigens like PfEMP-1 on the iRBC surface that subvert immunity and mediate sequestration from blood circulation to avoid spleen-dependent killing mechanisms by sticking to endothelial cells. This ability to sequester is one of the most important features involved in both cerebral malaria (described below) and pregnancy-associated malaria (PAM), both complications of severe malaria. *PfEMP-1* protein variants are expressed by members of the var gene family that have different exons lying in subtelomeric regions of the chromosomes. The var genes undergo genetic recombinations of different subregions that can more or less randomly be recombined through antigenic switching to express different protein variants that are selected throughout the course of the infection. Some of the variants lead to adhesion in different tissues like the placental condroitin-sulfate A (CSA) or the brain. The different adhesion properties are thought to be mainly determined by specific *PfEMP*s that can bind to ICAM-1, CD36, CSA or other endothelial receptors and lead to agglutination and rosetting and other interactions in the tissue capillaries. Var genes can be separated into different groups according to the genome architecture and genes within these groups are more likely to recombine with each other than with others. Variants expressed by different groups seem to have different influence on the clinical outcome of the disease. Whereas some of the var gene groups seem to be more associated with mild malaria others are with severe malaria. For example variants that do not bind to
CD36, a tissue marker that is only expressed in low numbers in the brain, are associated with severe complications such as cerebral malaria\textsuperscript{156} \textsuperscript{157}. In addition, most of these variants also do support iRBC rosetting, which is consistently associated with severe malarial infections in children and partly with the development of cerebral malaria\textsuperscript{158} \textsuperscript{159} \textsuperscript{153}. This led to the conclusion that at least partially specific surface proteins expressed by variant parasite genes (var) on iRBCs involved in binding to specific tissues like the brain (ICAM-1) or the placenta (CSA) are responsible for the clinical outcome of malaria infections\textsuperscript{152}.

Figure 1.9: Countries and territories affected by Malaria in 2010. This map was adapted from the WHO Malaria report 2010.

Cerebral malaria and experimental cerebral malaria

Cerebral malaria (CM) is a life-threatening complication of severe malaria infections (see 1.1.4) in humans. The mechanisms are not fully understood in detail, yet. One of the reasons for this is the difficult to study the parasitological and immunological events within the affected organ, the brain, ante-mortem\textsuperscript{160}. Therefore a mouse model system has been found that is supposed to mimic cerebral malaria events and was called experimental cerebral malaria (ECM). It is currently highly under debate if this ECM model system is of added value to the scientific CM community and if its study is at all relevant to the humane CM disease\textsuperscript{160}. Nevertheless, it is the only system generating a similar \textit{Plasmodium}-caused severe disease phenotype in a model organism closely related to humans. Therefore, even if not directly comparable to human CM, can give clues to what mechanisms may be underlying the generation of the disease\textsuperscript{161}.

Human cerebral malaria The WHO defines CM as an unrousable coma that is not attributable to other causes than a severe malaria (see 1.1.4) infection\textsuperscript{162}. Whereas
adults generally are resistant to developing severe malaria, very young children are at a high risk of developing severe malarial anemia. In contrast to that, older children, who have had at least one previous malaria infection already, seem to be at increasing risk of developing cerebral malaria. Therefore the epidemiology of severe malaria leads to the assumption of a major role of the immune system in both initiation of (in children) and protection from (adults) cerebral malaria. Fields studies have shown that about 1% of all diagnosed *P. falciparum* infections progress into CM. In about 10-20% of these CM patients the outcome is fatal leading to 300,000-500,000 deaths per year. In the last few years it has been shown that the generation of CM is most likely caused by a combination of a host-immune response to the parasite and the parasites’ ability to sequester in the brain of its host. Cerebral malaria can rapidly develop in infected patients after 2-3 days of fever with coma being the standard definition of CM. Early clinical symptoms like headache, fits, vomiting and diarrhea can not be distinguished from symptoms generated by meningitis, encephalitis and febrile convulsions. But these can rapidly progress into more severe symptoms and finally coma if immediate medical treatment is not provided. Two of the main pathological characteristics of CM have been seen in post-mortem analyses of brain sections of patients having died of CM. In these sections haemorrhaging into the white matter of the brain tissue, indicating a leakage of the blood-brain barrier (BBB) and sequestration of iRBCs within cerebral capillaries could be observed. In addition to that, accumulated and adherent leukocytes like monocytes and macrophages could be found sequestered together with the infected cells and infiltrated into the brain tissue. It is known, that endothelial receptors in the brain play a very important role for sequestration of malaria parasites during CM within the brain. One of the best studied receptors involved in iRBC sequestration is ICAM-1. It has been shown that ICAM-1 is upregulated on cerebral vasculature endothelium during malaria infections and that iRBC are binding to ICAM-1 molecules *in vitro*. In addition to that, there is evidence that the binding of iRBCs to ICAM-1 *in vivo* is associated with the risk of developing CM, even though there is conflicting data on that matter. Therefore other receptors like VCAM-1, E-Selectin and ELAM-1 might be involved since they have been shown to be upregulated in CM as well. Rather than just single receptor molecules being involved most likely *P. falciparum*-cytoadherence is a multi-step process in which many different receptors are involved initiating first contact, mediating rolling of the parasites and finally firm adhesion. A widely accepted model assumed for a long time that symptoms of CM were solely due to blockage of the blood flow in brain microvessels by sequestered iRBCs. In this model parasites adhere to brain endothelial cells via surface proteins that interact with endothelial receptors, combined with rosetting of infected and non-infected red blood cells, thereby impairing blood flow leading to hypoxia, hypoglycemia and built up of toxic waste products. This rapidly would lead to irreversible tissue damage. This being the only cause for CM symptoms is currently under debate.
cause, even though parasite sequestration is usually seen in CM brains, there is also deaths attributable to CM according to WHO guidelines that do not show sequestration\textsuperscript{179 180 164}. In addition, parasite sequestration has also been observed in patients that did not develop CM\textsuperscript{172 181}. One of the other factors that might contribute to the development of CM during severe malarial infections is the host immune system. Highly characteristic cytokine profiles associated with acute severe malaria prove evidence for the involvement of the immune response in the generation of CM. Elevated levels of pro-inflammatory cytokines in the patients plasma such as TNF\textalpha, IFN\gamma and increased production of anti-inflammatory cytokines such as IL-10 have been observed consistently during the course of cerebral malaria\textsuperscript{182 183}. Also high concentrations of inflammatory cytokines in the cerebrospinal fluid could be associated with the severity of the disease\textsuperscript{184}. Nevertheless, most of the data acquired on immune-cells and factors involved in the development of human CM were gained from peripheral fluids. This is especially crucial when it comes to the analysis of the involvement of T-lymphocyte subsets in the development of CM. So far only data comparing peripheral blood T-cell populations in CM and non-CM cases could be analyzed due to the inability to collect cells from crucial tissues at key time-points\textsuperscript{185}. Since it is hypothesized that there has to be a major migration of T-cell subsets to the brain tissue in order to play a prominent role there, this explains the lack of knowledge still present how exactly the immune system is involved in the development of CM\textsuperscript{160}. Therefore an experimental system is still needed to understand the mechanisms behind the disease.

**Experimental cerebral malaria** The best accepted experimental model of CM is the *Plasmodium berghei* ANKA (PbA) model\textsuperscript{160}. Infection of susceptible inbred mice, including the strains C57BL/6 and CBA, with this rodent malaria strain leads to severe cerebral pathological symptoms such as ataxia, fitting, respiratory distress and ultimately coma\textsuperscript{186}. Depending on the genetic background of the host and on the specific parasite clone the onset of clinical signs after infection is typically between 5-10 days post-infection\textsuperscript{186}. As seen in humans upon the first occurence of clinical signs of the infection usually there is a rapid decline of the condition of the animals with death occuring within 4 or 5 hours after the onset of neurological signs\textsuperscript{160}. It has been observed in mice showing signs of ECM that there is disruption of the blood-brain barrier with blood-leakage into the cortex and other regions of the brain\textsuperscript{177 187}, accumulation of iRBCs in blood vessels\textsuperscript{188 189} and signs of perivascular inflammation in these regions\textsuperscript{190}. Other factors, like cognitive dysfunction and impaired visual memory\textsuperscript{191} during the course of a PbA infection lead to a progressing decline of the general animal condition that has recently been made evaluable by the definition of a "coma and behaviour scale" documenting the progress for the assessment of ECM in a murine model\textsuperscript{192}. As shown for human CM, also the susceptibility of mice to ECM is depending on genetic and environmental factors, such as the genetic background (BALB/C vs. C57BL/6) and the age . It has been shown, for example, that differences in the expression profile of genes in the brain of
susceptible mice, compared with resistant mice, involve metabolic energy pathways, immune activation, apoptosis and neuroprotection/toxicity. But most of the characteristics involved are yet to be determined. Since parasite sequestration in the brain is assumed to be one of the main factors involved in generation of CM in humans the obvious question arises: Do PbA parasites also sequester in the brain of mice showing signs of ECM and if yes, which parasite and host receptors are involved in this? Indeed, PbA infected RBCs have been shown to be accumulated in brain capillaries of mice showing signs of ECM on light and electron microscopy level. But it is not yet understood if the sequestration of the parasites indeed is mediated by strong adherence, comparable to human CM, or rather tight junctions or even only weak interactions. In terms of receptors possibly involved in PbA sequestration it has recently been shown that CD36-mediated sequestration is involved in blocking of blood vessels in lung and adipose tissue but not in the brain. This has been interpreted as evidence for PbA-sequestration not being important for the development of ECM in the PbA model. Nevertheless, even in human CM the role of CD36 for the sequestration in the brain is under debate and the possibility of other endothelial receptors like ICAM-1 being involved is very high (as discussed above). Additionally, it has been shown that at least the number of parasites residing in the brain, shown by the parasite biomass present, is directly correlated with the risk of ECM. In line with the observation that CD36-mediated sequestration does not play a major role in the generation of ECM it has been shown that other endothelial receptors like ICAM-1, VCAM-1 and P-selectin are upregulated on brain endothelial cells in ECM-susceptible mice during a PbA infection. These results are in good agreement with the observations in human brains affected by CM. In addition, ICAM-1 or P-selectin deficient mice did not develop ECM, while leukocyte attachment in these mice in the brain was not impaired, suggesting a major role of both ICAM-1 and P-selectin for iRBC sequestration in these mice. As described above, parasite proteins involved in sequestration of the human malaria parasite P. falciparum include variant antigenic proteins expressed by var genes. There is no known homolog of var genes in other malaria species but a protein family exists, the Plasmodium interspersed repeat (pir) family, that is believed to be involved in antigenic variation and has been identified in P. vivax a decade ago. Since then members of the pir family have been investigated also in rodent malaria parasites like the cirs (P. chabaudi), the birs (P. berghei) and the yirs (P. yoelii). But a connection to P. berghei-induced ECM could so far not be made. The lack of information about receptors involved in the generation of the cerebral symptoms makes a detailed comparison of CM and ECM quite difficult, but the majority of immunological features of human CM are recapitulated during a PbA infection in C57BL/6 mice and can be compared. Like in human CM, the susceptibility of mouse strains to ECM has been directly correlated with the strength of a pro-inflammatory immune environment in response to the parasite. A great variety of experiments in the field of ECM immunology
has led to the conclusion, that a balance between Th-1 to regulatory T-cell responses is determining the outcome of a PbA infection\textsuperscript{212 200 213}, whereas manipulation of the Th2-response (for example through ablation of the IL-4R) does not have a major influence\textsuperscript{214}. As already observed in human CM, also in ECM circulating cytokines lead to an upregulation of the expression of endothelial receptors as well as increased expression of chemokines in leukocytes. Thereby, one of the most important family of leukocytes involved in the development of ECM seems to be CD8\textsuperscript{+}-T cells. It has been shown that CD8\textsuperscript{+}-T-cells accumulate in the brain of susceptible but not resistant mice immediately before the onset of neurological signs and it is believed that these cells can directly cause the disruption of the blood brain barrier via secretion of perforin, a pore-forming protein\textsuperscript{215}. In addition to that depletion of CD8\textsuperscript{+}-T cells either early (from start of the infection) or late (between day 4 and 5 post-infection) completely inhibits the development of ECM in these mice\textsuperscript{216 217 218}. The interplay between the innate and the adaptive immune response also seems to play a major role since it has recently been shown that NK cell-derived IFN\textsuperscript{γ} is required for the upregulation of co-stimulatory receptors on CD8\textsuperscript{+} T cells and for their subsequent migration to and sequestration in the brain of susceptible mice\textsuperscript{219 220}. Even though CD4\textsuperscript{+}-T cell depletion during early stages protects from development of ECM, their role during coma stage of the infection is somehow contradictory. Whereas they do not seem to play a role in some studies\textsuperscript{217}, in others their depletion also at later timepoints of an PbA infection prevents from ECM and an adoptive transfer of PbA-specific CD4\textsuperscript{+}-T cells has been shown to reduce parasite burden and prevent ECM in susceptible mice\textsuperscript{220 221}. Therefore a role of CD4\textsuperscript{+}-T cells during the generation ECM can not be ruled out and this topic needs some further investigation.

### 1.2 Eps15-homology domain proteins

For the uptake of nutrients and signalling molecules membrane-bound receptors on mammalian cells and other organisms need to be internalized and processed. In addition to that, the expression of adhesion molecules on the surface, ion channels and the retrieval of synaptic vesicles in neurons need to be fine regulated\textsuperscript{222}. But not all of the receptors and membrane-bound proteins that are internalized are directed to protein degradation. Some of the receptors need to be recycled back to the surface of the cell by a process called endocytic recycling. Here, first receptors are collected into the early endosome (EE) and then transported back to the plasma membrane. This can happen either directly (fast recycling) or through an organelle first (slow recycling), that is called endocytic recycling compartment (ERC)\textsuperscript{223 224} (Fig. 1.10). One of the key groups of proteins involved in endocytic recycling is the family of the Rab-GTPases (Rabs)\textsuperscript{225 226}. Rabs are cycling between an inactive (GDP-bound) and an active state (GTP-bound), which has a high affinity for effector proteins, such as SNARE proteins e.g. These proteins mediate fusion between vesicles and target organelles and they themselves promote vesicular transport, fission of and fusion with membranes\textsuperscript{227}. Recently, another protein family has been identified that is
involved in the regulation of endocytic recycling events, the Eps15-homology domain (EHD) protein family. EHD-proteins have been identified in many different eukaryotic organisms but are best studied in mammalian cells and in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*. EHD-proteins have been linked to Rab-proteins in a few studies not because of direct interaction but through mutual interaction partners\(^\text{228} 229 230\). Whereas there are four different members of EHD-proteins in mammals, EHD1 to EHD4, there is only one ortholog in both, *Drosophila* (Past-1) and *C. elegans* (Receptor-mediated endocytosis protein 1, Rme-1). In the following, I will mainly focus on the mammalian EHD-protein family members since most functional and structural data have been obtained for this family.

### 1.2.1 Protein architecture and function

EHD-proteins have a molecular mass of about 60 kDa and contain several distinct architectural features: They harbor a **G-domain** at the N-terminal part of the protein, followed by a **helical middle domain** and an **EH-domain** at the C-terminus (N-terminus in plant orthologs)\(^\text{282}\) (Fig. 3.5). The proteins are able to bind and hydrolyze ATP, oligomerize around membrane tubules and use the energy created by ATP-hydrolysis to pinch off vesicles from membranes.

#### G-domain

The G-domain harbours the nucleotide binding site of EHD-proteins and is predicted to be similar to the nucleotide binding site-domain found in Ras and dynamin family GTPases in the first place\(^\text{231}\), which is the reason why the domain has been named G-domain. Later, it could be shown that EHD2 indeed is a P-loop-containing NTPase (Guanosin-recognition motive: NKxD; P-loop motive: GQYSTGKT), binding and hydrolyzing nucleoside-triphosphates, but instead of binding to GTP alone, it has a way higher affinity for ATP\(^\text{283} 282\). Upon nucleotide binding EHD-proteins are able to dimerize (Fig. 1.11 A) which then makes them able to bind to membranes. It has been shown that nucleotide-binding of the G-domain is a prerequisite for the protein to dimerize and to oligomerize in vivo since nucleotide-free mutants of EHD2 could not bind to lipids anymore and the protein remained cytosolic\(^\text{282}\).

A recently developed model\(^\text{232}\) (Fig. 1.11 B) proposes a mechanism in which the binding of ATP to the G-domain of an EHD-protein leads to an initial dimerization step(Fig. 1.11 A), upon which the dimer undergoes conformational change and becomes able to bind to lipid bilayers. The dimerization is mediated by a highly conserved hydrophobic interphase within the G-domain (Fig. 1.11 A). After the binding the dimer is able to oligomerize with other dimers to form a ring around membrane tubules and is finally able to pinch off vesicles upon ATP-hydrolysis (Fig. 1.11 B). This function as so-called "membrane bender" has been characterized for EHD2 in vitro, because it was shown that this protein tubulates liposomes, forms ring-like oligomers around the tubules and induces the ATP-hydrolysis upon
Figure 1.10: Regulation of endocytic transport and proteins involved. Internalized receptors are transported to the sorting/early endosome (EE) where their future fate will be determined. They can either be directly recycled back to the surface via fast recycling or slowly, going into the perinuclear endocytic recycling compartment (ERC) first. Proteins destined for degradation can either be transported into the late endosome, from where they are moving on into lysosomes, or they are undergoing retrograde transport to the Golgi-apparatus. In all of these processes vesicles need to be labeled and transported to their final destination, which is mediated by trafficking proteins such as EHD-proteins, Rab-GTPases and Rab-like proteins. The figure was adapted from Naslavsky and Kaplan, 2011.
CHAPTER 1. INTRODUCTION

1.2

the binding of the membrane\textsuperscript{282} \textsuperscript{233}. Nevertheless it is not sure, yet, if the tubulation event also happens under endogenous gene expression (not overexpressed) \textit{in vivo}. EHD1 and EHD4 are the only EHD-proteins that have been found on endogenous tubules in mammalian cells so far. EHD1 mutants that lacked the ability to bind to tubules created an impaired ability in these cells to recycle receptors indicating for a receptor-recycling function of these tubules\textsuperscript{234}. But there is a question that remains to be answered: Do these EHD-proteins tubulate/stabilate the liposomes within the cells or do they only bind to the tubules after they have already formed\textsuperscript{232}?

**Helical middle domain**

The helical domain is located downstream of the G-domain and can bind to lipids with a polybasic stretch close to its N-terminal tip. Upon dimerization of the protein via the G-domain the lipid-binding site is exposed and forms an interaction site for lipids together with the second protein within the dimer (Fig. 1.11 A).

**EH-domain**

The C-terminal Eps15-homology domain was named after three homologous domains at the N-terminus of the epidermal growth factor receptor tyrosine kinase substrate Eps15\textsuperscript{235} \textsuperscript{236}. EH-domains contain two calcium-binding helix-loop-helix motifs (EF-hands) that give them a stable secondary structure. It has been shown that EH-domains are protein-protein-interaction domains that bind specifically to the tripeptide Asparagine-Proline-Phenylalanine (NPF)\textsuperscript{237} \textsuperscript{238}. In addition to that, it has been shown that EH-domains can also interact with phosphorylated phosphoinositides\textsuperscript{239}. Even though EH-domains of EHD-proteins and other proteins containing EH-domains are quite similar and all can possibly bind to NPF-motifs, there is a clear need for selectivity of the domains in these organisms. There need to be other factors involved in selecting to bind a specific protein via their EH-domain other than just the NPF-motif. There is a clear difference between EH-domains in EHD-proteins and in other proteins, respectively. In proteins where the EH-domain is located N-terminally, such as in Eps15, the surface of the domain is generally negatively charged, whereas it is positively charged in EHD-proteins\textsuperscript{240}. Therefore both proteins can only bind to NPF-motifs that are surrounded by amino acids of the opposite charge\textsuperscript{241}. It has been shown in addition, that EH-domains of EHD-proteins can not only bind to NPF- but also to GPF-motifs, even though with a much lower affinity\textsuperscript{242}. During formation of homo-dimers of EHD2 the EH-domain of one monomer binds to a GPF motif in the linker region between the EH-domain and the helical domain of the other monomer\textsuperscript{282}. In addition to the GPF motif in the linker of EHD2 there is also a KPFxxxNPF-motif within the G-domain that might be the connection point between the EH-domain of one dimer with the G-domain of another dimer during oligomerization\textsuperscript{282}.
1.2.2 Function

The understanding of the function of EHD-proteins in mammalian cells have mainly derived from point mutations observed in the protein and changes in the expression level that lead to diseases in humans. It has been shown for example that in oral squamous cell carcinoma cells there is a more than 10-fold increase of the expression of EHD3 transcripts\(^{243}\). But EHD-proteins also seem to be involved during infections of cells with pathogens since for example human brain endothelial cells binding to \(P. falciparum\) infected red blood cells exhibit an almost 5-fold increase of EHD1 transcripts than compared to controls\(^{244}\). In general, the knock-out of EHD1 and EHD4 leads to only minor phenotypes in mouse models \textit{in vivo}, leading to the conclusion that other EHD-proteins can take over the function and are at least partially redundant\(^{245} 246 247\).

\textbf{EHD1}

EHD1 presents the highest homology to the orthologs in invertebrate organisms and is the best characterized member of the four mammalian EHDs. It was shown to be involved in receptor-recycling processes regulating the recycling of transferrin receptors (Tfr) through a clathrin-dependent process\(^{248}\) and other receptors such as major histocompatibility complex (MHC) class I and class II, the insulin-regulated GLUT4 glucose transporter, potassium-channels, \(\beta\) integrins and other receptors through a clathrin-independent process\(^{231} 249 250 251 252\). EHD1 can interact with Rab11-FIP2 and localizes to peripheral endosomes\(^{229}\). Together with the fact that it is also linked to dynein motors that drive early endosome (EE) to ERC transport this suggests that EHD1 might be involved in the receptor-recycling transports from EE to ERC (Fig. 1.10)\(^{253} 254 255\). But EHD1 has also been shown to be involved in the retrograde transport of protein complexes from endosomes to the Golgi and endocytic trafficking events in neuronal cells in addition to its involvement in the regulation of cholesterol homeostasis and lipid droplet storage\(^{256}\). Together with EHD2, EHD1 has also recently been shown to interact with FER1L5, a ferlin-like protein, that mediates myoblast fusion for the generation and repair of muscle cells in mammalians\(^{257}\).

\textbf{EHD2}

EHD2 appears to be unique among the mammalian EHD-proteins and shares only about 70\% sequence identity with EHD1. EHD2 so far has only been found to form homo-oligomers\(^{282}\), whereas all other EHDs have been shown to form hetero-oligomers\(^{258} 259 283\), as well. Even though EHD2’s function is not quite clear yet, it has been shown to be involved in a few different pathways of endocytic trafficking, most of them starting from the plasma membrane (Fig. 1.10). It was found to be associated with the cell’s plasma membrane mainly, a localization crucially depending on ATP-binding of the G-domain\(^{260}\), and to be a binding partner of EHBP1,
a protein harbouring five different NPF-motifs, that is involved in internalization events of transferrin receptors and GLUT4\textsuperscript{261}. Additionally, depleting EHD2 from mammalian cells leads to delayed recycling of transferrin receptors from the ERC in these cells\textsuperscript{262}. It is likely, that in some tissues EHD2 shares a functional redundancy with EHD1 since also EHD2 has been shown to be involved in myoblast fusion and membrane resealing events by interacting with Fer1L5 and also Myoferlin\textsuperscript{263}. More recently, it has been shown that in addition to trafficking events EHD2 oligomers can also act as scaffolding proteins stabilizing caveolae, special membrane invaginations and part of lipid rafts, at the plasma membrane via interaction with actin filaments and regulating the dynamics of the formation of these membrane invaginations\textsuperscript{264, 265}. Many endocytic proteins have been shown to regulate gene expression by undergoing nucleoplasmic shuttling into the nucleus. Whereas EHD1 and EHD3 did not show such a behaviour, EHD2 indeed possesses a nuclear localization signal (NLS), enters the nucleus and represses transcription\textsuperscript{266}.

**EHD3**

EHD3 is quite similar to EHD1 according to their sequence identities (86%), but nevertheless it seems to be more variably expressed in mammalian tissues, to a rather weak degree in most tissues. It is strongly expressed in the brain, the liver, the kidney and also myocytes of mice, where it seems to be important for the expression and function of the sodium/calcium exchangers on the heart muscle cells\textsuperscript{267}. EHD3 has been shown to form hetero-oligomers with EHD1 and seems to cooperate with this protein and Rab8a to form a complex with Myosin Vb motors\textsuperscript{268}. Like EHD1, EHD3 was found to be involved in retrograd transport from the EE to the Golgi (Fig. 1.10). This might represent a redundancy of function of both proteins and explain, why EHD1 knock-out mice do only show a modest phenotype. Therefore a generation of EHD1/EHD3 double-knock out mice would be interesting to study\textsuperscript{245, 262}.

**EHD4**

Like EHD3, also EHD4 can hetero-oligomerize with EHD1\textsuperscript{259, 246}. Both proteins cooperate in the control of receptor-recycling events\textsuperscript{269} in the nervous system and EHD4 has been assigned a tissue-specific role in the brain, even though no severe neurological symptoms have been seen in ehd4 knockout mice. EHD4 depletion leads to enlarged EEs and has been shown to be involved in the regulation of receptor transport from EE to the ERC and from the ERC to the lysosomal pathway (Fig. 1.10) and it has been suggested that EHD4 functions at the EE, upstream of EHD4 \textsuperscript{232}.
CHAPTER 1. INTRODUCTION

1.3 Aim of this study

Apicomplexan protists are among the most important human and domestic animal parasites influencing our lives on an almost daily basis. Two of these organisms, *Toxoplasma* and *Plasmodium*, can cause severe diseases in humans but a promising vaccine against these parasites is still not available. Both parasites have a complex life-cycle involving both, asexual and sexual reproduction, that comes along with a change in hosts. The different environments in these hosts led to the evolutionary development of different parasite stages that are able to adapt to these different environmental conditions. The investigation of these stages will hopefully lead to the development of different anti-parasitic strategies to eradicate these diseases.

The investigation of *Plasmodium falciparum* exo-erythrocytic stages is a bottle neck in malaria research because of the rare availability of these stages under laboratory conditions. Only a few laboratories in the world have established a combined *in vivo*/*in vitro* *P. falciparum* life-cycle in their facilities to both generate and allow for the accessibility of the full array of *P. falciparum* stages. This is mainly because of technical issues. Therefore one aim during my thesis was to establish a constant combined *P. falciparum in vitro/in vivo* life-cycle that will enable researchers in the lab to work on different stages of *P. falciparum*, especially the elusive liver-stages.

One of the major obstacles for the apicomplexa parasites is the fact that they are replicating intracellularly. Even though this protects them from eradication by factors of the immune system of their hosts it prevents them from free access to sur-
rounding nutrients via several membranous barriers. Therefore the parasites have evolved specialized organelles and cellular mechanisms that eventually supply them with nutrients from the host cell to ensure their survival. One of the mechanisms by which nutrients can cross the parasite membrane is endocytosis. This process is not very well understood in apicomplexans so far. Therefore the aim of the second project of my thesis was to characterize an Eps15-homology-domain containing protein (EHD-protein) in both *Toxoplasma* and *Plasmodium*. Proteins of the EHD-protein family have been shown in other eukaryotic cells already to take part in endocytic trafficking and receptor-recycling. Therefore I hypothesized that the protein may be involved in endocytic events in *Plasmodium* and *Toxoplasma* parasites, respectively. One of my goals was to characterize the localization of the protein in different stages of *Toxoplasma gondii* and *Plasmodium berghei* by fluorescent tagging and immuno-fluorescence assays. By colocalization studies with proteins of subcellular localization, i.e. with organelles of the endocytic system of the parasites, will assist in confirming a putative role for Apicomplexan EHD in endocytic trafficking. Furthermore, endogenous depletion of the gene in both parasites will facilitate the phenotypical characterization and hence function for the parasite life cycle. A detailed characterization of this protein family in apicomplexan parasites will help to further understand the process of how these organisms can survive intracellularly and will ultimately pave the way to develop novel anti-parasitic strategies.
Chapter 2

Materials and Methods

2.1 Laboratory equipment

AMAXA Nucleofector II electroporation machine
Analytical scales BL510
Autoclave
Binocular Niko SMZ 1500
Camera, DC 120 Zoom digital
Citation manager Endnote X
Electrophoresis System Horizon 11.14
Electrophoresis Power Supply EPS 301
Electroporation cuvettes plus
Film developer Hyperprocessor
Film developing cassettes
Freezer $-80^\circ$C
Freezers $-20^\circ$C
Fridges
Heat block thermomixer comfort
Haemocytometer (Neubauer)
Ice machine AF 30
Imaging software Image J 1.45s
Hera Cell Incubator
Shaking Incubator Innova 4000/4300
Multi-gas incubator ($O_2$, $CO_2$)
Liquid nitrogen tank
Magnetic stirrer, Heidolph MR3001
Megafuge 1.0R
Microcentrifuges 5415 R, 5415 D
Light optical microscope, Axiostar plus
Light optical microscope, Axioskop
Light optical microscope, Axiocvert 25
DeltaVision Epifluorescence microscope
Microwave oven
Mosquito cages
Nikon TE200 Inverted Microscope
GeneAmp PCR system 9700
Mastercycler Gradient
pH-meter

Lonza, Koeln
Sartorius GmbH, Goettingen
Systec GmbH, Wettenberg
Nikon, Tokyo, Japan
Kodak, New York, USA
Thomso Scientific, USA
Whatman Inc., USA
Amersham Pharmacia Biotech, Freiburg
BTX, San Diego, USA
Amersham Pharmacia Biotech, Freiburg
Dr Goos suprema GmbH, Heidelberg
Sanyo
Liebherr, Biberach
Liebherr, Biberach
Eppendorf, Hamburg
Labotec, Labor-Technik, Goettingen
Scotsman, Milano, Italy
National Institutes of Health, USA
Heraeus Instruments, Hanau
New Brunswick Scientific Co. Inc.
Mytron, Heiligenstadt
CBS, USA
NeoLab, Heidelberg
Heraeus Instruments, Hanau
Eppendorf, Hamburg
Zeiss, Jena
Zeiss, Jena
Zeiss, Jena
Applied Precision; Washington, USA
MDA
BioQuip Products Inc, USA
Nikon, Tokyo, Japan
Applied Biosystems, CA USA
Eppendorf, Hamburg
Inolab, Heidelberg
CHAPTER 2. MATERIALS AND METHODS

2.2 Consumables

- Photometer
- Single channel pipettes
- 12-channel pipette 200 µl
- Pipetting aid pipetus
- Precision balance
- SDS-PAGE system Heidelberg
- SDS-PAGE system small Glasgow
- SDS-PAGE system big Glasgow
- Semi-dry blot apparatus
- Sterile work bench Gelaire X
- Transmission electron microscope, 900
- Ultra cryo-ultramicrotome
- Vortex Genie 2
- Water bath Julabo U3
- Wet blot system
- Zeiss Axiovert 200M microscope

- Eppendorf, Hamburg
- Abimed, Langenfeld
- Abimed, Langenfeld
- Hirschmann Laborgeraete, Eberstadt
- Mettler Toledo, Switzerland
- Amersham Pharmacia Biotech, Freiburg
- CTI GmbH, Idstein
- Biorad, Munich
- CTI GmbH, Idstein
- Flow Laboratories, Meckenheim
- Zeiss, Jena
- Leica
- Scientific Industries Roth, Karlsruhe
- Julabo, Seelbach
- Amersham Pharmacia Biotech, Freiburg
- Carl Zeiss, Obernkirchen

14 ml polystyrene round bottom tubes with lid
15 ml polypropylene tubes with lid
50 ml polypropylene tubes with lid
6-well cell culture plates, Cellstar
8-well chamber slides
96-well round bottom plates
Cell culture flasks:
- Cellstar (Filter Cap, 75 cm²)
- Nunc Flasks Nuclon (Filter Cap, 25 cm²)

- Greiner Bio-one, Frickenhausen
- Sarstedt, Nuembrecht
- Sarstedt, Nuembrecht
- Greiner Bio-one, Frickenhausen
- Nunc, Langenselbold
- Greiner Bio-one, Frickenhausen
- Nunc, Langenselbold

- Cell strainer (70 µm)
- Cuvettes
- Cryovials
- Dialysis tube membrane Nadir
- Filter paper Whatman TM 3MM
- Gloves, Peha-soft satin
- Immersion oil
- Microscope cover slips
- Needles, BD Microlance
- Nitrocellulose membrane, Hybond ECL
- Glass slides
- Parafilm
- Pasteur capillary pipettes

- BD Biosciences, Heidelberg
- Sarstedt, Nuembrecht
- Greiner Bio-one, Frickenhausen
- Carl Roth GmbH, Karlsruhe
- Whatman, GE Healthcare, Dassel
- Hartmann, Heidenheim
- Zeiss, Jena
- Marienfeld, Lauda-Koenigshofen
- Becton Dickinson; Heidelberg
- Amersham, GE Healthcare, Freiburg
- Marienfeld; Lauda-Koenigshofen
- Pechiney Plastic Packaging; USA
- Wu; Mainz
CHAPTER 2. MATERIALS AND METHODS

2.4

Petri dishes (94/16 mm) | Greiner Bio-one, Frickenhausen
Pipette filter tips, Biosphere | Sarstedt, Nuembrecht
Pipette tips | Sarstedt, Nuembrecht
Reaction tubes (0.5 ml, 1.5 ml, 2.0 ml) | Sarstedt, Nuembrecht
Sterile filtration devices (500 ml) | Nalagene, Wiesbaden
Sterile pipettes (1 ml-25 ml) Cellstar | Greiner Bio-one, Frickenhausen
Sterile syringe filter, Filtropur (0.22 µm pore size) | Sarstedt, Nuembrecht
Syringe, BD Microlance | Becton Dickinson, Heidelberg
Thermo well PCR tubes (0.2 ml) | Sarstedt, Nuembrecht

2.3 Strains

2.3.1 Bacteria strains

*Escherichia coli* XL1 blue | Stratagene; Agilent Technologies Sales & Services GmbH & Co. KG

*Escherichia coli* XL10 Gold | Stratagene; Agilent Technologies Sales & Services GmbH & Co. KG

2.3.2 Cell lines

Huh7 | human hepatoma cell line
HFF | human foreskin fibroblasts

2.3.3 Parasite strains

*Plasmodium falciparum* NF54 | (Ponndurai et al. 1981)\(^{270}\)

*Plasmodium berghei* ANKA GFPcon | (Franke-Fayard et al. 2004)\(^{271}\)

*Plasmodium berghei* ANKA cl15cy1 | (Hall et al. 2005)

*Plasmodium berghei* NK65 | (Yoeli and Most 1965)\(^{272}\)

*Toxoplasma gondii* RHxgprt(-) | (Donald et al. 1996)\(^{273}\)

2.3.4 Mosquito strains

*Anopheles stephensi* NIJ | Nijmegen, Niederlände

2.3.5 Mouse strains

Naval Medical Research Institute (NMRI), outbred | Charles River Laboratory, Sulzfeld, Germany

C57BL/6, inbred mice | Charles River Laboratory, Sulzfeld, Germany


CHAPTER 2. MATERIALS AND METHODS

2.4 Chemicals and reagents

Chemicals were typically purchased in p.a. quality from the companies Roth, Merck, Sigma, Serva and AppliChem. Chemicals and reagents from other companies are listed below.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>AP Conjugate Substrate Kit</td>
<td>Biorad Laboratories, Muenchen</td>
</tr>
<tr>
<td>Bacto™-Agar</td>
<td>Difco Laboratories, Augsburg</td>
</tr>
<tr>
<td>Bacto™-Trypton</td>
<td>Difco Laboratories, Augsburg</td>
</tr>
<tr>
<td>Bacto™-Pepton</td>
<td>Difco Laboratories, Augsburg</td>
</tr>
<tr>
<td>Cellulose powder CF11 (fibrous)</td>
<td>Whatman, GE Healthcare, Dassel</td>
</tr>
<tr>
<td>CFSE</td>
<td>Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Heparin</td>
<td>Braun, Melsungen</td>
</tr>
<tr>
<td>Nycodenz powder</td>
<td>Axis-Shield PoC, Oslo</td>
</tr>
<tr>
<td>PBS-pellets</td>
<td>Gibco Invitrogen, Karlsruhe</td>
</tr>
<tr>
<td>Sea salt</td>
<td>Alnatura</td>
</tr>
<tr>
<td>Streptavidin-ALP</td>
<td>Mabtech, Sweden</td>
</tr>
</tbody>
</table>

2.5 Oligonucleotides

Oligonucleotides were ordered as custom DNA oligonucleotides in a desalted purity from Invitrogen, Karlsruhe. Lyophilised oligonucleotides were dissolved in ddH$_2$O in a concentration of 100 µM and stored at $-20^\circ$C.
### 2.6 Antibodies

#### 2.6.1 Primary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Dilution</th>
<th>IFA</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-pro <em>Tg</em> M2AP-AK (Harper et al. 2006)</td>
<td>R</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Tg</em> Catalase (Ding et al. 2000)</td>
<td>R</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Tg</em> DrpB-AK (Breinich et al. 2009)</td>
<td>M</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Tg</em> IMC-AK (Mann and Beckers 2001)</td>
<td>R</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Tg</em> Mic8EGF-AK (Meissner et al. 2002)</td>
<td>R</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Tg</em> NtRhop5-AK (T53E2; El Hajj et al. 2007)</td>
<td>M</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ddFKBP12-AK (ABR, Rockford, USA)</td>
<td>R</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Pf</em> EHD (T. Spielmann, BNI, Hamburg)</td>
<td>M</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-<em>Pb</em> HSP70 (Hybridoma, supernatant)</td>
<td>M</td>
<td>undiluted</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6.2 Secondary antibodies

ALEXA Fluor antibodies were ordered from Invitrogen (Frankfurt). The gold labeled antibody for immuno-EM was ordered from BBInternational.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Dilution</th>
<th>IFA/iEM</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALEXA Fluor 488 α-mouse</td>
<td>goat</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALEXA Fluor 594 α-mouse</td>
<td>goat</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALEXA Fluor 488 α-rabbit</td>
<td>goat</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALEXA Fluor 594 α-rabbit</td>
<td>goat</td>
<td>1:1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-rabbit IgG, HRP</td>
<td>goat</td>
<td></td>
<td>1:5000</td>
<td></td>
</tr>
<tr>
<td>gold (15nm) α-mouse IgG</td>
<td>goat</td>
<td></td>
<td>1:500</td>
<td></td>
</tr>
</tbody>
</table>

2.7 Media, buffers and solutions

2.7.1 Media and buffers for molecular biological methods

**LB (Luria Broth) Medium:**

- 10 % trypton
- 5 % yeast extract
- 10 % NaCl
- pH 7.5; autoklave

**LB Agar:** LB-medium; 15 g/l agar

**TAE (50 x):**

- 2 M TRIS
- 1M acetic acid
- 50mM EDTA; pH 8.0

**PBS (10 x):**

- 0.01 M KH2PO4
- 1.37 M NaCl
- 0.027 M KCl
- pH 7.2; autoklave

or dissolve 20 PBS tablets (Gibco) in 1 l ddH2O; autoklave

**PBS (1 x):**

- 100 ml 10 x PBS, filled up with ddH2O to 1 l
- or purchased from Invitrogen

**TfB I:**

- 30 mM potassium acetate
- 50 mM MnCl2
2.7

100 mM KCl
10 mM CaCl2
15 % glycerine

The pH was adjusted to 5.8 with acetic acid. The buffer was sterile filtered and stored at 4°C.

**TfB II**: 10 mM MOPS
75 mM CaCl2
10 mM KCl
15 % glycerine

The pH was adjusted to 7.0 with NaOH. The buffer was sterile filtered and stored at 4°C.

**3 M sodium acetate (NaAc)-solution**: 24.06 g NaAc

add 100 ml H2O

pH 5.2

**IFA-solutions**

**PFA-fixation solution**: 4% PFA (w/v) in PBS

**Permeabilisation solution**: 0.2% Triton X-100 in PBS

**Blocking solution**: 2% BSA in permeabilization solution

or 10% FCS in PBS

**Washing solution**: 1% FCS or BSA in PBS

2.7.2 Media and solutions for cell culture

Cell culture media and supplements were purchased from Invitrogen or Gibco, Karlsruhe.
CHAPTER 2. MATERIALS AND METHODS

2.7

Hepatocyte culture medium: 1 x Dulbecco’s Modified Eagle Medium (DMEM complete)
- 10 % FCS
- 1 % penicillin/streptomycin;
- sterile, stored at 4°C

DMEM HFF culture: 500 ml DMEM
- 10% FCS (v/v)
- 1% Glutamine (v/v)
- 1x Gentamycin (20 μg/ml)

Cell freezing solution: 80 % FCS
- 20 % DMSO

or

- 90 % FCS
- 10 % DMSO
mixed 1 : 1 with complete culture medium

P. falciparum culture medium: 500 ml 1 x RPMI 1640
- 50 ml human serum A+
- 550 μl 1000 x Hypoxanthine
- 550 μl 1000 x Gentamycin (opt.)
- sterile filtered

P. falciparum thawing solutions: Sterile 0.2% dextrose/0.9% NaCl
- Sterile 1.6% NaCl
- Sterile 12% NaCl

2.7.3 Media, buffers and solutions for parasitological methods

P. berghei transfection medium: 160 ml RPMI 1640 medium (T-medium)
- 40 ml FCS (US certified)
- heat inactivated for 30 minutes 56°C
- 60 μl Gentamycin;
- sterile filtered

P. berghei freezing solution: 10 % glycerine in Alsever’s solution (Sigma)
Nycodenz stock solution: 110.4 g Nycodenz powder
5 mM TRIS/HCl; pH 7.5
3 mM KCl
0.3 mM EDTA; pH 8.0
fill up to 400 ml with ddH_2O; autoclave; store at 4°C

Pyrimethamine stock solution: 7 mg/ml Pyrimethamine in DMSO
store at 4°C

Electroporation buffer/Cytomix: 10 mM K2HPO4/KH2PO4
25 mM HEPES
2 mM EGTA pH 7.6
120 mM KCl
0.15 mM CaCl2
5 mM MgCl2
together with
5 mM KOH to pH 7.6
2 mM ATP
3 mM GSH

ATP (30 µl/ml) 100 mM in water

GSH (30 µl/ml) 100 mM in water

2.7.4 Buffers and solutions for biochemical methods

Saponin buffer: 1x PBS, 2% BSA, 0.5% Saponin; prepare fresh

PAA-Stock solution: 30% PAA
0.8% Bis-AA

Stacking gel buffer (SDS, 4x): 0.5 M Tris/HCl pH 6.8
0.4% SDS (w/v)
sterile filter

Stacking gel: 4% PAA (v/v)
25% 4x stacking gel buffer (v/v)
0.1 % APS 10%(v/v)
0.2% TEMED (v/v)
Separating gel buffer (SDS, 4x): 1.5 M Tris/ HCl auf pH 8.8
0.4% SDS (w/v)
sterile filter

Separating gel: 8-15% PAA (v/v)
25% 4x separating gel buffer
0.1% APS 10% (v/v)
0.2% TEMED (v/v)

5x SDS-PAGE running buffer: 33 mM Tris/HCl pH 6.8
190 mM Glycin
0.1% SDS

4x SDS-PAGE sample buffer: 50% 4x Sammelgelpuffer (v/v)
40% Glycerol (v/v)
8% SDS (w/v)
0.2% Bromphenolblau (w/v)
400 mM DTT (w/v)

RIPA lysis buffer: 50 mM Tris-HCl, pH 7.5
150 mM sodium chloride (NaCl)
5 mM EDTA
50 mM sodium fluoride (NaF)
0.5% sodium deoxycholate (NaDOC)
0.1% SDS
1% Triton X-100
freshly added 1 mM DTT
protease inhibitor

Semidry-Blot-Transfer buffer: 48 mM Tris
39 mM Glycin
20% Methanol

10x TBS: 20 ml 1M Tris, pH 7.6
80 g sodium chloride (NaCl)
add 1 l H₂O
CHAPTER 2. MATERIALS AND METHODS

2.8

1x TBST: 100 ml 10x TBS
900 ml ddH₂O
1 ml Tween 20

2.7.5 Antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin 1000x</td>
<td>100 mg/ml in ddH₂O</td>
</tr>
<tr>
<td>Tetracyclin 1000x</td>
<td>5 mg/ml in 70% Ethanol</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1000x 10 mg/ml in Ethanol</td>
</tr>
<tr>
<td>MPA 500x</td>
<td>12.5 mg/ml Methanol</td>
</tr>
<tr>
<td>Xanthin 500x</td>
<td>20 mg/ml 1 M KOH</td>
</tr>
<tr>
<td>Pyrimethamin 1000x</td>
<td>1 mM in Ethanol</td>
</tr>
<tr>
<td>Gentamycin 500x</td>
<td>10 mg/ml in H₂O</td>
</tr>
</tbody>
</table>

2.8 Molecular biological methods

2.8.1 Cloning of the targeting constructs for parasite transfection

For the *Toxoplasma* RME-1 expression construct pTub8DDmCherry*Tg*RME-1FL (Fig. 2.1) the full length coding sequence of the gene TGME49_031210 was amplified from *Toxoplasma gondii* RHΔHX cDNA using the oligo set pDLPfullsenseAvrII and pDLPfullantisensePacI. The PCR-fragment was introduced into the Tub8DDmCherry-HXGPRT plasmid via the restriction sites AvrII and PacI.

For the *Toxoplasma* RME-1 over-expression ATPase-domain deletion construct pTub8DDmCherry*Tg*RME-1∆ATPase (Fig. 2.2) the coding sequence of the gene was amplified downstream of the sequence coding for the predicted ATPase domain using the oligo set pDLP∆ATPasesenseAvrII and the same reverse primer as mentioned above for the full length construct. The PCR-product was subsequently introduced into the Tub8DDmCherry-HXGPRT plasmid via the restriction sites AvrII and PacI.

For generation of the *Tg*RME-1 EH-domain over-expression deletion mutant construct pTub8DDmCherry*Tg*RME-1∆EH (Fig. 2.3) the coding sequence of the gene was amplified upstream of the sequence coding for the predicted EH-domain with a stop-codon added to the reverse Primer. Primers pDLP∆EHashsensePacI and the full-length forward primer mentioned above were used to amplify the fragment which was introduced into the plasmid pTub8DDmCherry-HXGPRT via the restriction sites AvrII and PacI.

For generation of the construct pTub8DDmCherryPfc0190c (*Pf*EHD) (Fig. 2.4) the full-length ORF of the gene PFC0190c/ PF3D7_0304200 (*Pf*EHD) was amplified from *Plasmodium falciparum* cDNA using the oligo set pPFC0190cAvrIIfor and pPFC0190cPacIrev. The PCR-product was subsequently introduced into the pTub8DDmCherry-HXGPRT plasmid via the restriction sites AvrII and PacI.
transfection into *Toxoplasma gondii* parasites all Tub8DDmCherry-constructs were linearized by *Kp*NI restriction enzyme.

For generation of the single-crossover *pbehd (-)* targeting construct pFK01 (Fig. 2.5) a 1kb fragment of the middle part of the ORF of PbANKA040280 was amplified from genomic DNA using the oligo set PbEHDkointegSacIIfor and PbEHDkointegSpeIrev. The PCR-product was subsequently introduced into *P. berghei* transfection vector b3D.DT/H./D274 via the restriction sites *Sac*II and *Spe*I. For transfection the plasmid was linearized by *Hpa*I restriction.

All vector maps were created using the *Serial Cloner Tool* (Version 2.5; Franck Perez, SerialBasics). Other used vectors in this thesis:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tub8ddmycGFP-TgDrpB</td>
<td>Breinich et al., 2009</td>
</tr>
<tr>
<td>Tub8ddmyc-Rab5a</td>
<td>AG Meissner</td>
</tr>
<tr>
<td>Tub8ddmyc-Rab7</td>
<td>AG Meissner</td>
</tr>
<tr>
<td>Tub8IMC-YFP</td>
<td>AG Meissner</td>
</tr>
</tbody>
</table>

**Amplification of the specific DNA fragments by Polymerase chain reaction (PCR)**

Specific DNA fragments were amplified by Polymerase chain reaction (PCR) (Saiki et al. 1985) using specific oligonucleotides. All used oligonucleotides and their sequences are noted in section 2.5. As templates served *P. falciparum, P. berghei* or *T. gongii* gDNA or cDNA. For a standard PCR reaction 0.5 - 2 µl parasite
CHAPTER 2. MATERIALS AND METHODS

2.8

Figure 2.2: Vector map **Tub8DDmCherryTgRME-1ΔATPase**.

Figure 2.3: Vector map **Tub8DDmCherryTgRME-1ΔEH**.
Figure 2.4: Vector map Tub8DDmCherryPfc0190c.

Figure 2.5: Vector map pFK01.
gDNA/cDNA was used in a reaction volume of 10-50 µl. Furthermore the reaction contained 50 pmol of the specific oligonucleotides (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl2, 1 x Taq reaction buffer (+ KCl) and 2.5 U Taq polymerase (all Fermentas) filled up to 10-50 l with ddH2O. Reaction tubes were placed into a thermal cycler running the program with an initial denaturation of the dsDNA for 5 minutes at 95°C, followed by 30 cycles with a denaturation at 94°C for 30 seconds, annealing of the specific oligonucleotides at 45-60 C for 30 seconds and extension of the newly synthesised DNA strand 60°C (72°C in case of P. falciparum and T. gondii) for 1 minute per amplified kilobase. A final extension for 10 minutes at 72°C was carried out and amplified DNA was stored at 4°C for short-term and at −20°C for long-term storage.

Analysis of DNA fragments by agarose gel electrophoresis

Amplified DNA fragments were separated by size by agarose gel electrophoresis. For this 1% agarose was dissolved in 1 x TAE buffer by heating in the microwave. Ethidium bromide (Sigma, Taukirchen) was added to a final concentration of 50 ng/ml. Samples were mixed with 1/5 volume of 6 x Orange Loading dye (Fermentas) and loaded along with 1 µg GeneRuler 1 kb DNA Ladder (Fermentas) into the wells. The gel was run for 1 hour at 100-120 V in 1 x TAE buffer in a Whatman Horizon 11.14 electrophoresis chamber. Negatively charged DNA thereby migrates in direction of the anode. Separated DNA fragments were visualized by exposure of the DNA-intercalating ethidium bromide to UV-light and fluorescence was recorded with the Electrophoresis Documentation and Analysis System 120 (Kodak).

Purification of DNA with Qiaquick PCR purification Kit

Amplified DNA fragments were purified with Qiaquick PCR purification Kit (Qiagen, Hilden) prior to digestion with restriction endonucleases and digested DNA fragments as well as plasmid DNA also prior to ligation according to the manufacture’s manual.

Digestion of double-stranded DNA

Purified DNA fragments or plasmid DNA were digested with specific restriction endonucleases (New England Biolabs NEB, Frankfurt) prior to ligation (preparative digest) or after plasmid isolation (control digest). For the preparative digest the amplified and purified DNA fragments and the respective plasmid DNA were cut with the same restriction endonucleases. The complete purified DNA fragment or 1 - 3 µg plasmid DNA were mixed with 10 - 20 Units of the respective restriction enzymes, 5 µl of the matching buffer (10x) and 5 µl of BSA (10x), if necessary, and filled up to 50 µl with ddH2O. The digest was typically incubated over night at 37 °C (if not otherwise suggested by NEB). For the control digest of isolated plasmids the approach was typically scaled down to 20 l reaction volume, using only 0.5 - 1 µg
DNA. The reaction was then incubated for 2 hours at 37°C. All digested DNA was analysed by agarose gel electrophoresis as described in section 2.8.1 and preparative digested DNA was purified prior to ligation as described in section 2.8.1. Prior to parasite transfections 10 µg of the generated plasmids were digested completely with up to 40 units of the respective enzymes in a volume of 100 µl over night at 37°C. Subsequently digested plasmid DNA was ethanol precipitated as described below.

Ligation of DNA

Digested and purified DNA fragments were ligated with equally restricted plasmids in a ratio of 7:1 or 6:2 in a total reaction volume of 10 µl. 5 Units of the T4 ligase were used to catalyse the reaction in 1 x of the provided T4 ligase reaction buffer (10 x, Fermentas). The ligation reaction was incubated for 2 hours at room temperature or at 16°C over night. Subsequently competent *E. coli* XL1 blue were directly transformed with the ligated DNA constructs.

Preparation of transformation competent *E. coli* XL1 blue

*E.coli* XL1 blue were cultured over night in LB medium with tetracycline (5 µg/ml). The over night culture was diluted 1 : 100 in LB medium with tetracycline (5 µg/ml) and cultivated shaking at 37°C until it reached an OD600 of 0.5. The culture was cooled down on ice and spun for 5 minutes at 1500 x g at 4°C. The cell pellet of 100 ml culture was resuspended in 30 ml cold TBI, incubated for 15 minutes on ice and spun again for 5 minutes at 1500 x g. The pellet was resuspended in 8 ml TIBII and incubated another 15 minutes on ice. 200 µl aliquots of the suspension were frozen at −80°C.

Transformation of competent *E. coli* XL1 blue

Transformation competent *E. coli* XL1 blue, either purchased from Stratagene or prepared as described above, were thaw on ice. 35 hboxµl of the purchased cells or 200 µl of the self-made competent cells were used for one transformation. Competent cells were either treated for 10 minutes on ice with 0.68 hboxµl -mercaptoethanol (1.42 M; Stratagene) and subsequently mixed with 2 µl of the ligation (Stratagene competent cells) or directly mixed with 10 µl ligation (self-made competent cells). The cells were incubated for 30 minutes on ice, followed by a heat-shock for 45 seconds at 42°C in the water bath. Cells were incubated subsequently another 2 minutes on ice, then 1 ml pre-warmed LB medium was added and cells were incubated, shaking at 37°C for 1 hour. The cells were spread on LB agar plates with ampicillin (100 µg/ml) and incubated over night at 37°C.

Plasmid-isolation with Quiaprep Spin Miniprep Kit

After transformation single colonies were used to inoculate 3 ml LB medium with ampicillin (100 µg/ml) each. These cultures were incubated shaking at 37 C over
night. The over night cultures were spun for 2 minutes at 16,000 x g in a table-top microcentrifuge. The plasmid isolation from the cell pellet was performed as described in the manufacturer’s manual. Isolated plasmids were control digested as described under 4 and resulting DNA fragments were analysed by agarose gel electrophoresis (described above) to confirm successful integration of the DNA fragments.

**Preparation of bacteria glycerol-stocks**

For conservation of transformed E. coli containing the generated plasmids over night cultures were prepared as described under 8. 850 µl of the over night culture were mixed with 150 µl glycerine (99.5 %, Roth) in a cryovial and stored at −80°C.

**Determination of DNA concentration by photometric measurement**

In order to determine the DNA concentration of a plasmid preparation the DNA was typically diluted 1 : 100 in ddH₂O. The absorption of the DNA at 260 nm was measured in a photometer (Eppendorf) and DNA concentration was calculated according the following relation:

\[
\text{OD}_{260} \times 50 \times \text{dilution factor} = \text{concentration dsDNA [g/ml]}
\]

The purity of the DNA preparation was shown by the ratio of OD260/OD280 and typically ranged from 1.8 to 2.0.

**Ethanol precipitation of DNA**

Digested DNA was ethanol precipitated prior to parasite transfection. For this purpose 2.5 volumes 100 % ethanol and 1/10 volume 3 M sodium acetate, pH 5.2, were added to the DNA solution and incubated at least 30 minutes at −80°C. After spinning for 15 minutes at 16,000 x g the DNA pellet was washed once with ice cold 70 % ethanol and again spun for 5 minutes at 16,000 x g. The ethanol was carefully removed and the DNA pellet was air dried. Finally the DNA was resuspended in 1 x PBS to a final DNA concentration of 1 µg/µl. Digested and precipitated DNA was once more analysed by agarose gel electrophoresis and stored at −20°C until parasite transfection.

**2.8.2 Stage-specific RNA isolation and cDNA synthesis for RT PCR**

Different parasite stages were isolated from infected mosquitos (2.10.2), in-vitro hepatocyte cultures (2.9) or infected blood (2.10.1).

**Isolation of total RNA with RNeasy Mini Kit (Quiagen)**

Parasite cells were disrupted by addition of in 350 µl RLT buffer (supplied, Qiagen) supplemented with 1 % - mercaptoetanol (14.3 M) and homogenised by vortexing for 1 minute. Total RNA was isolated from the cell lysate according to the ”Animal Cell Spin” protocol supplied in the manufacturer’s manual. Isolated RNA was stored at −80°C.
**Trizol extraction of total RNA**

Parasites were resuspended in 1 ml Trizol Reagent (Invitrogen), 200 µl chloroform was added and carefully mixed by inverting. After 3 minutes incubation at room temperature it was mixed again and subsequently spun for 15 minutes at 12,000 x g at 4°C. The top aqueous layer, containing the RNA, was carefully transferred to a fresh RNase-free tube (Eppendorf). After addition of 500 µl isopropanol (2-propanol) the solution was incubated for 10 minutes at room temperature and spun for 10 minutes at 12,000 x g at 4°C. The supernatant was discarded and the RNA pellet was washed with 75 % ethanol and thoroughly mixed by vortexing. After a final centrifugation for 5 minutes at 7,500 x g at 4°C the RNA pellet was air dried, resuspended in 10 µl DEPC-treated H2O and incubated for 10 minutes at 60°C. Isolated RNA was stored at −80°C.

**DNase treatment of total RNA**

After RNA isolation remaining DNA contaminations were removed by digestion with DNase. The RNA was mixed with 1/10 volume of 10 x TURBO DNase buffer (Ambion) and 1 µl TURBO DNase (Ambion) and incubated for 30 - 45 minutes at 30°C. 1/10 volume of the resuspended DNase Inactivation Reagent (Ambion) was added, mixed thoroughly and incubated for 2 minutes at room temperature. The reaction was centrifuged for 1 minute at 10,000 x g and the supernatant containing the RNA was transferred to a fresh RNase-free tube (Eppendorf). DNase treated RNA was stored at −80°C.

**First strand cDNA synthesis**

RNA was transcripted into complementary DNA (cDNA) with the Fermentas "First Strand cDNA Synthesis Kit" according to the manufacturer’s manual using random hexamer oligonucleotides. The initial optional denaturation step of the total RNA for 5 minutes at 65°C was performed. For each transcripted RNA one approach without reverse transcriptase (-RT) was run to exclude DNA contaminations. The transcripted product was directly used for reverse transcriptase PCR (below) or stored at −20°C.

**Reverse transcriptase PCR (RT PCR)**

A standard PCR approach was prepared as described above. In a reaction volume of 20 µl 0.5 - 2 µl first strand cDNA of the different parasite stages were used as template together with *P. berghei* or *T. gondii* gene specific oligonucleotides. For each sample one tube with cDNA synthesised with reverse transcriptase (+ RT) and one - RT control tube were prepared. Oligonucleotides specific for the *P. berghei* aldolase, expressed constantly throughout the life cycle, were used as control. The amplification was performed in a thermal cycler using the standard PCR program as described above.
2.9 Cell culture

2.9.1 In vitro liver-stage development of P. berghei in human hepatoma cells Huh7

Frozen cell stocks of the human hepatoma cell line Huh7 were kept in 80% FCS, 20% DMSO in liquid nitrogen. Thawed cells were immediately transferred into pre-warmed DMEM culture medium containing 10% FCS and 1% Penicillin/Streptomycin (DMEM complete) and centrifuged for 5 minutes at 200 x g. The cell pellet was resuspended in fresh DMEM complete and transferred into a cell culture flask (25 cm$^2$ or 75 cm$^2$). Cells were incubated at 37°C, 5% CO$_2$ until a confluent monolayer was reached. For further cultivation medium was removed, attached cells were washed once with HBSS and detached with 0.25% Trypsin/EDTA (Gibco) for 3 - 5 minutes at 37°C. After addition of 10 ml DMEM complete cells were transferred to a 15 ml tube and centrifuged for 5 minutes at 200 x g. The cell pellet was washed once with HBSS and finally resuspended in 10 ml DMEM complete. Depending on the approach an aliquot of 200 µl up to 2 ml of the cell suspension were transferred to a new culture flask and filled up to 15 ml with DMEM complete.

For the Plasmodium berghei in vitro liver cell development assay Huh7 cells cultivated to a confluent monolayer were detached with 0.25 % Trypsin/EDTA and washed as described. The cell pellet was resuspended in DMEM complete and counted diluted in Trypan blue (0.4 %) using a haemocytometer. Either 25,000 cells per well on a 8 well chamber slide (nunc) or 200,000 cells per well on a 6 well plate (greiner) were seeded and cultivated over night. Mature salivary gland sporozoites were purified (described in section 2.10.1) and 25,000 - 35,000 sporozoites or 100,000 - 200,000 sporozoites were added on the cultivated cells on the 8 well chamber slide or 6 well plate, respectively. Sporozoites were allowed to invade for at least 90 minutes, afterwards the sporozoite suspension was removed and cells were incubated in DMEM complete plus anti-contamination cocktail. Liver-stage development was stopped after different time points by either fixing them for 10 minutes with 4% PFA or Methanol (icecold) for IFA-studies or harvesting the cells with 0.25 % Trypsin/EDTA as described before. The harvested cells were then transferred into a 1.5 ml tube and centrifuged for 2 minutes at 16,000 x g and depending on the application resuspended in RLT buffer, Trizol or PBS.

2.9.2 P. falciparum culture - asexual parasites

A small culture flask (25 cm$^2$) with 5 ml complete Medium (10% human heat inactivated A+ Serum, Gentamycin 50 µl/ml, Hypoxanthin; filtered through a 0,22 µm filter) and a final haematocrit of 5% (1:10 dilution out of stock: 50% weekly freshly purified erythrocytes in complete medium) was inoculated with a freshly thawed P. falciparum stock solution. Parasites were grown for several days while culture was checked every second day for its parasitemia. If erythrocytes reach a parasitemia between 5-10% the culture was split down to a new small culture flask.
to a concentration of 1% parasitized erythrocytes per flask. Therefore the volume of the old culture that had to be transferred to a new culture flask was calculated as follows: \( y = 11.1 \times X \times 500 \mu l \) (\( X \) is the needed dilution to obtain 1% parasitaemia; for example 1:5 if the parasitaemia of the old culture is 5%). \( y \) was diluted in 5 - \( y \) ml complete medium and 500 \( \mu l \) - \( y/11.1 \) fresh erythrocyte solution (see above) was added. After at least 10 subculturing rounds the asexual culture was used to set up a gametocyte culture. The parasite culture was maintained at 37°C and 5% \( O_2/5% CO_2/90% N_2 \).

2.9.3 P. falciparum culture - gametocytes

To set up a gametocyte culture an asexual \( P. falciparum \) culture was grown to a parasitaemia of 6-10%. This culture was then transferred into a big culture flask (75 cm\(^2\)) with the volume calculated as follows: \( y = 11.1 \times X \times 2000 l \) (\( X \) is the needed dilution to obtain 1% parasitaemia; for example 1:8 if the parasitaemia of the asexual culture was 8%). \( y \) was diluted in 20 ml - (\( y+blood \)) complete medium (10% human heat inactivated A+ Serum, without Gentamycin, Hypoxanthin; filtered through a 0.22 m filter) and 2000 \( \mu l \) - \( y/11.1 \) blood was added. The culture medium was changed every day, first week after setting up 15 ml, later 25 ml. Occasional blood smears of the cultures were done from day 7 on to check for gametocyte production.

2.9.4 Cultivation of Toxoplasma gondii in human foreskin fibroblasts (HFFs)

The virulent \( Toxoplasma gondii \) strain RH\( \Delta HX \) was cultivated in vitro in human foreskin fibroblast (HFF) cells. HFF cells are primary culture cells that can not be maintained for longer than 30 passages in culture. They form a single confluent layer in cell culture flasks. The cells were kept in DMEM complete medium and were splitted into new flasks every 3-4 days in a ratio of 1:3 or seeded into 6-cm dishes in a ratio of 1:5 for Toxoplasma cultures. For parasites cultures a confluent HFF dish was infected with extracellular parasites and kept until complete lysis of the host cells under the conditions described above. After the lysis parasites keep viable for another 12-24 hours before they die if they can not invade new cells. If intracellular parasites are needed to be extracted from host cells for experiments the infected cells can be scratched off from the dish bottom. Afterwards they can be pressed through a 26G needle to release the parasites from the host cells. A washing step of about 5-30 min centrifugation at 1000 x g and RT can purify the parasites from surrounding medium for further experiments.
2.10 Parasitological methods

2.10.1 Plasmodium methods

Determination of parasitemia in giemsa stained blood smears

A small drop of blood was obtained from the tail of the infected mouse or the *P. falciparum* cell culture (spun down at 7000 rpm for 1 min) and a thin blood film was prepared on a glass slide. The blood smear was air dried and fixed for 10 seconds with methanol. The fixed blood smear was stained with giemsa, diluted 1:10 in deionised water, for 15 minutes. The stained blood smear was washed with water and parasites were examined under the light microscope using the 100 x objective with oil immersion. In order to determine the parasitemia the number of all erythrocytes and the infected erythrocytes were counted in 25 fields. Subsequently the parasitemia was calculated using the following formula:

\[
\text{Number of infected erythrocytes/ total number of erythrocytes} \times 100 = \text{parasitemia (\%)}
\]

Examination of exflagellating gametocytes

The hatching of male gametes from erythrocytes is called exflagellation, a process that can be observed under the light microscope. A high number of exflagellating parasites is important for a successful transmission to the mosquito and was hence examined routinely before parasite transmission. For this a drop of tail blood or a small volume (about 200 µl) of the *P. falciparum* cell culture was placed on a glass slide and carefully covered with a cover slip. The slide was incubated for 10 minutes at room temperature. Exflagellation was examined under the light microscope using the 40 x objective lenses with phase contrast (Ph2). For a successful transmission at least 3 to 5 exflagellation centres should be observed per field (P. berghei) or 1 exflagellation centre per field in the case of *P. falciparum*.

Cryopreservation of Plasmodium parasites

Plasmodium parasites were conserved during blood stage development, because infected blood can be stored for long periods in liquid nitrogen. For this purpose 100 µl freshly withdrawn blood was mixed with 200 µl parasite freezing solution (10% glycerine in Alsever’s solution) in a cryo vial and immediately frozen in liquid nitrogen. Thawed parasite stocks can be reinjected into mice to continue the parasite growth.

Thawing of Plasmodium falciparum

Parasites vial (frozen culture) were taken from cold storage and thawed at 37°C for 1-2 min. Culture was transferred to a 50ml centrifuge tubes with a sterile serological pipette. Blood volume = V was measured and 0.1x V of 12% NaCl slowly added,
drop wise while shaking the tube gently. Subsequently the tube rested 5 min before 10x V of 1.6% NaCl was added slowly, drop wise swirling the tube. Afterwards the tube was centrifuged at 1,500 rpm at room temperature for 5 min. To remove the supernatant a Pasteur pipette and the vacuum pump were used, carefully not to remove any red blood cells. 10X V of 0.2% dextrose/0.9% NaCl in a 10ml syringe was measured and added to the parasites slowly, drop wise mixing gently. The tube was again centrifuged at 1,500 rpm at room temperature for 5 min. To remove the supernatant a Pasteur pipette and the vacuum pump was used again, being careful not to remove any red blood cells. Finally the pellet was re-suspended with 3mL of complete RPMI medium with gentamycin, transferred to a 25cm² tissue culture flask and supplemented with two drops of 50% hematocrit washed blood. Incubation followed at 37°C.

**Plasmodium falciparum membrane feeding**

At day 16 after setting up a gametocyte culture, two cultures (16 and 14 days old) were mixed and centrifuged in a 50 ml falcon tube at 2000 rpm/ 37°C. After that the resulting supernatant was removed and the pellet diluted 1:1 with freshly purified erythrocyte concentrate (O+, 37°C). Finally this mixture of erythrocytes was diluted to a final haematocrit of 60% with pre-warmed (37°C) heat inactivated human serum (O+), mixed by pipetting up and down and filled into a membrane glass feeder system by using a 1000-µl-ppendorf pipette (prewarmed tip). *A. stephensi* mosquitoes were then allowed to feed through a parafilm membrane from the bottom of the feeder for 20 min. After the bloodmeal the mosquitoes were maintained in a humidity and temperature controlled incubator (80% humidity, 37°C) and checked for oocysts at day 7. At day 8 after infection a second bloodmeal with freshly purified human uninfected blood (see asexual culture above) was performed to supply the mosquitoes with nutrients. An additional nutrient supply was provided every day through applied cotton pads that were soaked in glucose/PABA solution (10% glucose, 0.05% Para-aminobenzoic acid). At day 17 SGs were dissected from the infected mosquitoes and SG sporozoites were extracted.

**Plasmodium berghei transfection**

The rodent *Plasmodium* parasite *P. berghei* was transfected with linearised DNA using the AMAXA transfection system (Lonza). By a crossing-over event between homolog regions the targeting constructs inserted into the targeted genomic loci.

**Overnight culture and merozoite purification** For one transfection typically 2 - 3 NMRI mice with a high level parasitemia (3 - 5% for *P. berghei* ANKA) were used. Mice were sacrificed and blood was collected by heart puncture (see below). The blood were combined in a 50 ml tube with 10 ml T-medium containing 250 μl Heparin (200 p.i. in PBS) and centrifuged for 8 minutes at 400 x g without brake. The medium was removed and the blood pellet was resuspended in 20 ml fresh T-
The blood suspension was carefully dropped by gravity into a conical flask containing already 100 ml pre-warmed T-medium. The 50 ml tube was washed with 30 ml fresh T-medium and this was also dropped carefully into the flask without swirling the blood. The parasites were cultivated at 37°C, 5% O₂, 5% CO₂ and 90% N₂, shaking at 70 rpm. To enrich schizonts in the blood culture the incubation took 16 to 18 hours for *P. berghei*. Mature schizonts were purified by a Nycodenz density gradient centrifugation. The Nycodenz stock solution was diluted to 55% or 60% working solutions in PBS for *P. berghei* purification. The over night parasite culture was transferred into four 50 ml tubes (approximately 35 ml per tube) and each tube was under-laid with 10 ml Nycodenz solution pre-warmed to room temperature. Tubes were exactly balanced and centrifuged for 25 minutes at 200 x g at room temperature without brake. The mature schizonts appeared as a brown ring at the interface and were carefully collected to two new 50 ml tubes. Tubes were filled up to approximately 40 ml with T-medium (from top of the gradient) and centrifuged for 8 minutes at 400 x g. The schizont pellet was resuspended in fresh T-medium, the volume depended on the size of the pellet and the number of constructs the parasites should be transfected with. For one transfection 1 ml resuspended schizonts were transferred in a 1.5 ml microcentrifuge tube and spun for 15 seconds.

**AMAXA transfection and selection for transformants** For each transfection, 100 µL of AMAXA human T cell nucleofector solution (Lonza) was added to 5 - 10 µg of digested and precipitated plasmid DNA. The DNA solution was then added to the schizont pellet, mixed well, transferred to the AMAXA cuvette and pulsed once using the U-033 pre-programmed setting on the AMAXA machine. After pulsing 50 µl fresh T-medium was directly added into the cuvette and transfected parasites were immediately injected i.v. in nave NMRI mice. Typically 2 mice per construct were used. 24 hours post-infection a giemsa stained blood smear was prepared to record the starting parasitemia and pyrimethamine treatment of the mice was started. For this the pyrimethamine stock solution was diluted 1 : 100 in tap water (final concentration 70 µg/ml) and provided as drinking water. Parasitemia typically decreased to undetectable levels (blood smear) on day 2 post infection and first resistant parasites appeared in giemsa stained blood smears from day 7. When parasitemia reached at least 0.5 % mice were sacrificed and blood was withdrawn by heart puncture. Blood was transferred to new mice, cryopreserved and parasite gDNA was isolated (parental population). The transfer animals were further treated with pyrimethamine and mice were sacrificed as soon as parasitemia reached sufficient levels. The infected blood was again cryopreserved and purified for parasite DNA isolation (transfer population).

**Isolation of blood stage parasites for genomic DNA purification** To isolate parasites from infected blood a column was made using a 5 ml syringe. The syringe was closed with cotton wool at the bottom. Thereon a 2 - 3 cm thick layer of cellulose powder CF11 (Whatman) was put and the column was completed with around 1
cm glass beads (diameter 212 - 300 µm, unwashed; Sigma). The column was equilibrated with 2 column volumes 1 x PBS and subsequently the infected blood was transferred on the column. The erythrocytes were washed off the column with 1 x PBS. Starting from the first red drop 15 ml erythrocyte suspension were collected in a respective tube. The suspension was centrifuged for 8 minutes at 400 x g, without brake, and the supernatant was carefully removed. The erythrocyte pellet was resuspended in 10 - 15 ml 0.2% saponin in PBS to lyse the red blood cells. The suspension was again centrifuged for 8 minutes at 1500 x g and the supernatant was discarded. The parasite pellet was resuspended in 1 ml PBS and transferred to a 1.5 ml microcentrifuge tube. The isolated parasites were once more centrifuged for 2 minutes at 4500 x g and finally resuspended in 200 µl PBS. The parasite genomic DNA (gDNA) was subsequently isolated using the QIAamp Blood Mini Kit with the "Blood or Body Fluid Spin Protocol". For this 20 µl Qiagen protease and 200 µl buffer AL were added to 200 µl parasites in PBS and thoroughly mixed by vortexing. All following steps were performed according to the manufacturer’s instructions. The parasite gDNA was finally eluted in 100 - 150 µl elution buffer AE (10 mM TRIS/HCl; 0.5 mM EDTA; pH 9.0) and stored at −20°C.

**Genotyping PCR of transfected parasites**  The isolated gDNA of parental and transfer transfectants was tested for integration of the targeting constructs by PCR. Templates for this genotyping PCR were typically 1 - 2 µl parasite gDNA of the transfectants or wildtype (WT) parasites as control. As additional control the targeting constructs were diluted 1 : 100 in ddH₂O and also used as template for the PCR. Usually three different oligonucleotide pairs were used for genotyping PCR. The integration test oligonucleotides (test) typically bind inside the selectable marker inserted and the parasite’s genome. Resulting fragments therefore verify successful integration of the targeting construct inside the targeted genomic locus. Control oligonucleotide pairs are usually WT or open-reading frame (ORF) specific oligonucleotides and vector specific oligonucleotides (episomal). The PCR approach was prepared as described under above using the standard PCR program with an annealing temperature of usually 54°C. Resulting DNA fragments were analysed by agarose gel electrophoresis.

Genotyping of the obtained parasite populations for the *pbehd (-)* parasite was performed by specific PCRs using the following primer combinations:

- **PbANKA_040280_KO_5UTR_for_KpnI-HF/** PbANKA_040280_KO_3UTR_rev_XbaI for the Wildtype population
- **TgDHFRTS_for** PbANKA_040280_KO_5UTR_for_KpnI for the Integration population
CHAPTER 2. MATERIALS AND METHODS

Parasite cloning

Successfully transfected parasite populations were selected for clonal parasites by limited dilution. For this a frozen blood stock of the parental or transfer population was injected i.p. into a naive NMRI mouse. Once the parasitemia ideally reached 0.3 - 0.5% the mouse was sacrificed and blood was withdrawn by heart puncture. Parasitemia was determined very exactly by counting at least 80 fields. Assuming on average $7 \times 10^6$ erythrocytes per µl mouse blood the number of parasites was determined using the following formula:

$$7 \times 10^6 \times \text{parasitemia} \times 10^{-2} = \text{parasites/µl}$$

A dilution series was prepared in RPMI medium to inject theoretically one parasite per mouse. For this 100 µl blood was diluted 1 : 10 in RPMI, this dilution was again diluted 1 : 10 in RPMI and so on. Typically the 1 : $10^6$ dilution contained less then one parasite per µl. The calculated amount of this dilution that theoretically held one or in some cases three parasites was mixed with fresh RPMI medium and 10 naive NMRI mice were injected i.v. each with 100 l medium containing one parasite. Mice usually became blood stage positive from day 7 post infection on and as soon as sufficient levels of blood stage parasites were reached mice were sacrificed and blood was collected. Not all injected mice developed a blood stage parasitemia, typically mice were declared negative if there were no parasites visible in a giemsa-stained blood smear up to 21 days post infection. Clonal parasites were isolated from infected blood as described (above) and gDNA was tested for integration of the respective targeting construct by PCR (see above).

Imuno-fluorescence analysis (IFA)

For the analysis of the expression and localization of the EHD-protein in Plasmodium berghei blood stages, sporozoites and liver stages the parasites were fixed and incubated with a primary α-PfEHD antibody. For the investigation of the P. berghei liver-stage development a primary α-PbHSP70 antibody was used, similar to the P. falciparum liver-stage development assay where a α-PbHSP70 antibody was used. These primary antibodies were then detected by a secondary fluorescently labeled antibody. All antibodies used are listed in section 2.6.1 and 2.6.2.

In general, the parasites were fixed with 4% paraformaldehyde (PFA) at RT for 10-20 minutes and subsequently permeabilized with permeabilization solution for 20 minutes. Afterwards unspecific binding of the primary antibody was prevented by blocking unspecific binding sites via incubating the parasites with blocking solution for 20-60 minutes. Afterwards the primary antibody was incubated on the cells diluted in blocking solution and subsequently removed by washing the cells with washing solution 3 times for 5 minutes. Detection of the primary antibody via a fluorescent secondary antibody (diluted in blocking solution) followed for 30-60 minutes. Afterwards unbound secondary antibody was washed away by incubating the samples in PBS for 3 times for 5 minutes before the samples were mounted with a 50% glycerol/PBS solution and covered with a coverslip. If a nuclear staining was
desired a Hoechst-staining step incubating the cells with Hoechst diluted 1:1000 for one minute was added before the second last washing step of the protocol. The analysis of the fixed fluorescent samples was performed using the microscopes listed in section 2.1.

**Immuno-electron microscopy (iEM)**

For cryo-immunolabelling, overnight *P. berghei* schizonts were fixed in 0.1 M sodium cacodylate buffer, pH 7.2, containing 4% freshly prepared formaldehyde and embedded in gelatin. The samples were infiltrated overnight in 2.1 M sucrose and rapidly frozen by immersion in liquid nitrogen. Cryosections were obtained at $-90^\circ$C using an Ultracut cryo-ultramicrotome (Reichert). Cryosections were thawed in methylcellulose, blocked in PBS-3% bovine serum albumin and then incubated in the presence of the antibody $\alpha$-PfEHD. The cryosections were then incubated with 15 nm, gold-labeled $\alpha$-mouse IgG (BBInternational), and observed in a Zeiss 900 transmission electron microscope. For a detailed protocol see Lemgruber et al., 2011.

### 2.10.2 Anopheles mosquito methods

**Mosquito breeding**

*Anopheles stephensi* mosquitoes were bred at 28°C, 75% humidity under a 12-h light/12-h dark cycle. Larvae were raised in 1% sea salt ddH$_2$O, pupae were collected and allowed to hatch in mosquito cages (BioQuip Products Inc; USA). Adult mosquitoes were fed on a 10% sucrose/PABA solution provided on cotton wool pads. In order to maintain the mosquito life cycle, female *Anopheles* mosquitoes were blood fed on nave anaesthetized NMRI mice. Four days after the blood meal dishes with 1% sea salt ddH$_2$O soaked filter paper were put into the cages and female mosquitoes laid their eggs. Eggs were washed with 70% ethanol and twice with 1% sea salt ddH$_2$O and again put in trays filled with 1% sea salt ddH$_2$O. Hatched larvae were fed on cat food (Brekkies) and split depending on the density.

**Parasite transmission**

For transmission of *P. berghei* and *P. falciparum* parasites 4-7 day old female mosquitoes were blood fed on anaesthetized NMRI mice that had been infected i.p. with parasite blood stocks. Mice were assayed for high levels of parasitemia and the percentage of exflagellating male gametocytes was observed under the microscope. After the infective blood meal, mosquitoes were maintained at 21°C, 80% humidity. On day 10 post feeding, mosquitoes were dissected in RPMI 1640 medium/3% BSA, and isolated midguts were examined for the infection rate. From day 17 post infection mature sporozoites could be isolated from the salivary glands (below). In order to maintain a continuous *Plasmodium* cycle nave rodents could be exposed to bites of infected mosquitoes from day 17 post feeding, respectively.
Mosquito dissection

Midguts of infected mosquitoes were dissected 10 days after the blood meal in order to observe oocyst formation, 12 - 14 days post feeding midgut sporozoites were isolated from midgut oocysts. And 17 - 21 days post feeding salivary glands were isolated and infectious sporozoites were extracted (below). For all dissections infected mosquitoes were anaesthetised on ice. The dissection was performed in RPMI 1640 medium with 3% BSA under a stereo microscope in the insectary at 15°C using two needles (27 G and 23 G). Midguts and salivary glands were kept in RPMI 1640 medium with 3% BSA on ice until sporozoite extraction.

Sporozoite extraction from salivary glands

Mosquito midguts or salivary glands were disrupted mechanically in RPMI 1640 medium with or without 3% BSA using a pestle and spun for 3 minutes at 90 x g, 4°C. The supernatant containing the sporozoites was transferred to a fresh tube and the pellet was again squeezed with a pestle in fresh RPMI. A second centrifugation was performed for 3 minutes at 100 x g, 4°C and the supernatant was combined with the first collected. Extracted sporozoites were subsequently count under the microscope in a haemocytometer using the 40 x objective lenses with phase contrast (Ph2). For counting the sporozoite solution was typically diluted 1/10 in RPMI. The number of sporozoites was calculated with the following formula:

\[
\text{Number of sporozoites in 4 large squares} / 4 \times \text{dilution factor} \times 10^4
\]

The quantity of a mosquito infection was typically expressed as number of sporozoites per mosquito.

2.10.3 Toxoplasma methods

Transient transfection of T. gondii

For a transient transfection of a plasmid into Toxoplasma parasites the plasmid was not linearized to allow the parasites to maintain it extra-chromosomally. Therefore, after some rounds of replication, the parasites lose the plasmid again since it lacked an origin of replication (parasite-specific) that would have allowed autonomous replication of the plasmid. Since the transient transfection does not require a selection procedure (described for the stable transfection below) it is a quicker procedure as the stable transfection and produces faster results. But in contrast to a stable transfection the transient one produces a very heterogenous transient population of parasites that can not be cloned out.

The DNA was transfected into the parasites via electroporation. Therefore freshly lysed tachyzoites were washed once with Cytomix and subsequently resuspended in 850 µl Cytomix. About 1x10^7 parasites were used for one single transfection with about 60 µg of DNA that was ethanol precipitated, resuspended in 50 µl Cytomix and supplemented with 25 µl ATP (100 mM) and 25 µl GSH (100 mM). This mix was then added to 700 µl of parasite suspension and the whole volume (800 µl) was
then transferred into electroporation cuvettes. The electroporation was subsequently
done in an Electro Square Por 830 machine (BTX) with two pulses at 1,7 kV for
176 µs. With the transfected parasites finally confluent HFF cells were inoculated.

Stable transfection of T. gondii

For a stable transfection of a second copy of an endogenous gene into Toxoplasma
parasites a linear plasmid (linearized in the backbone of the plasmid) was transfected
and randomly integrated into the genome. To select parasites that successfully inte-
grated the plasmid a selection marker (dhfrts or hxgprt) was cloned into the plasmid
that carried the gene of interest. Via this selection marker the addition of selective
drugs (pyrimethamine or mycophenolic acid, respectively) only leads to the death
of parasites that do not possess the selection marker. In case of the mycophenolic
acid (MPA) selection only the parasites carrying the HXGPRT-gene are able to use
Xanthin (additionally added to the medium) instead of Hypoxanthin, that is blocked
by MPA. The transfection procedure in general was carried out as described above
for the transient transfection. But in contrast to this procedure for the stable trans-
fection only 10 - 30 µg of DNA was transfected that was also mixed with 10 U of the
linearization enzyme previously to the transfection, a technique named REMI (Re-
striction Enzyme Mediated Insertion). After the transfection the parasites were
inoculated on HFF cells and maintained under drug pressure starting 12-24 hours
after inoculation. The selective drugs were used in the following concentrations:

Mycophenolic acid: 25 µg/ml
Pyrimethamine: 1 µM
Xanthin: 40 µg/ml

After about 1 week, depending on the type of selective drug, all parasites not carrying
the plasmid died and left a pool of transgenic parasites.

Isolation of stable clones via limiting dilution

To isolate parasite clones from the stable transfections mentioned above a limiting
dilution was performed in 96-well cell culture mikrotiter plates. After 5-7 dayes
plaques of lysed cells are visible in the wells that represent a parasite clone, each.
Only the wells that contained one plaque only from the beginning of the plaque
formation are wells that containe only one clone. These clones were then isolated
and expanded to 24-well cell culture plates and further characterized.

Induction of expression of DD-tagged proteins

To induce overexpression of DDmCherry TyRME-1 protein variants in Toxoplasma
gondii a destabilization domain (ddFKBP) was cloned in front of the mCherry-tag
under a strong promotor. In the absence of the ligand Shield-1 (Shld-1) the fusion
protein is not stable within the parasites and is degraded\textsuperscript{288}. Thereby parasites carrying the modified or overexpressed protein of interested that might be toxic to the parasites can be maintained in culture. When Shld-1 is added to the culture medium it binds to the fusion protein and stabilizes the protein expression. Thereby protein localization and influence of the overexpression of the respective protein can be studied. Shld-1 was used in a concentration of 1 µM to overexpress the TgRME-1 fusion proteins in this thesis.

**Plaque-assay**

The intracellular replication of \textit{T. gondii} leads to a lysis of the infected cell. The emerged parasites then infect surrounding cells and the ongoing replication finally leads to the generation of cell-free areas within a confluent cell layer, so-called \textit{Plaques}. In a Plaque-assay\textsuperscript{277} the ability of different parasite lines to grow, replicate and finally lyse the host cells can be investigated. By comparing the numbers and sizes of Plaques of WT parasites and parasites expressing the (modified) gene of interest an influence of the protein of interest on the replication of the parasites can be visualized. In this thesis 6-well titer plates with a confluent HFF cell layer were inoculated with 50 cells per well. The parasites were maintained under Shld-1 protein expression or without (control) to investigate the influence of the overexpression of DDmCherry \textit{Tg}RME-1 and the deletion mutants of the protein on parasite replication. After 6-8 days the cell layers were fixed with Methanol for 10 minutes and subsequently stained with Giemsa-solution (see section 2.10.1).

**Fluorescence-analysis of intracellular parasites**

For the investigation of the fluorescent proteins investigated in this thesis in \textit{Toxoplasma} intracellular parasites were induced with Shld-1 for 4-8 hours to express the DDmCherry-fusion proteins. If no co-labelling with an antibody-staining was performed the parasites were immediately fixed with 4% PFA for 10 minutes, shortly washed afterwards and finally mounted as described in section 2.10.1 for the \textit{Plasmodium} IFAs. If an immuno-labelling was performed in addition to the expression of a fluorescent protein an IFA was done as described in section 2.10.1 for \textit{Plasmodium} IFAs.

**Live-imaging**

For live-imaging of \textit{Toxoplasma} parasites HFF cells were grown in µ-Dish\textsuperscript{35mm,high} ESS coated live-cell dishes (ibidi, Martinsried). These dishes had a really thin bottom allowing an inverse objective of a fluorescent microscope to image through the bottom. The HFF coated dishes were one day prior to imaging infected with the DDmCherry \textit{Tg}RME-1 expressing parasites. About 4 hours before the live-imaging protein expression was induced via addition of Shld-1 to the medium. To image processes the mCherry-tagged \textit{Tg}RME-1 is involved in the dish carrying the infected
cells was transferred into a live-cell chamber at a DeltaVision RT epifluorescence microscope imaging system (Applied Precision; Washington, USA) at the imaging facility of the Institute of Infection, Immunity and Inflammation of the University of Glasgow (Glasgow, UK). This live-cell chamber was heated to 37°C and in addition connected to a CO2 supply. In a long-term imaging experiment pictures of the YFP-signal (IMC-YFP construct) and the mCherry-signal (DDmCherryTgRME-1) of a parasite cell of interest were taken every 10 or every 30 minutes for several hours or over night. An autofocus mode provided by the microscope software (SoftWx) was used to keep the moving cells in focus over time. An autotracking mode was switched off since it did not prove to be useful. Images were taking with a CoolSnap HQ camera and later put together to a movie with ImageJ. The same procedure was done for a short-term imaging study taking pictures of the DDmCherryTgRME-1 expressing parasites every 3-5 seconds to visualize rapid vesicular movement within the parasites.

### 2.11 Animal experimental methods

Mice were purchased from Charles River Laboratories, Germany or Janvier, France with an age of 18 - 20 days and animal care was done in a central facility of the University of Heidelberg (Interfakultaere Biomedizinische Forschungseinrichtung; IBF). All animal experiments were conducted according to the European regulations and approved by the state authorities (Regierungspraesidium Karlsruhe).

#### 2.11.1 Administration of anaesthesia

Mice were anaesthetised with Ketamine (100 mg/ml)/Xylazin (3 mg/ml) (K/X) administered into the abdominal cavity (intraperitoneal; i.p.).

#### 2.11.2 Infection of rodents with Plasmodium parasites

In order to transmit *Plasmodium* parasites to the rodent host the mice were either anaesthetized and exposed to bites of infected mosquitoes or isolated salivary gland sporozoites were injected in various numbers (1,000 - 10,000 spz) into the tail veins (i.v.). Furthermore blood stages injected either i.v. or i.p. established a *Plasmodium* infection, isolated schizonts, freshly isolated infected blood or frozen blood stocks were used.

#### 2.11.3 Blood withdrawal by heart puncture

Mice were terminally anaesthetized with diethyl ether and the heart was uncovered preferably fast. Blood was withdrawn using a heparinized needle (23 G) and a 2 ml or 5 ml syringe from the right ventricle. Infected blood was immediately put on ice and processed as fast as possible. Typically 0.7 - 1.5 ml blood could be obtained from one mouse.
2.12 Biochemical methods

2.12.1 Preparation of parasite lysate for SDS-PAGE

For the preparation of parasite material for the detection of the DDmCherry TgRME-1 protein on a western blot (time-dependent expression in response to Shield1) a confluent layer of HFF cells in a number of 6-cm cell culture dishes was inoculated with the recombinant parasites. These parasite were allowed to replicate until a great number of parasite vacuoles within the cell layer was visible but without allowing the generation of plaques in this layer. When a great number of intracellular parasites had formed Shield1 in a concentration of 1 M was added to the cell culture medium in the dishes. Thereby the expression of DDmCherry TgRME-1 was induced in these parasites and the parasites could be harvested after the desired time of induction.

The HFF cells including the intracellular parasites of each dish were then scratched of from the bottom of the dish and pressed through a syring supplied with a 26G needle (BRAUN). Thereby the parasites were released from the host cell, could be washed with icecold PBS and then taken up in 1 ml PBS in an 1,5-ml-Eppendorf tube. Washed parasites were then counted in a Haemocytometer and subsequently pelleted in a centrifuge at 1000x g for 10 minutes at 4°C. Afterwards the pellet was either frozen down at −80°C or resuspended in RIPA buffer and incubated on ice for 5 minutes. The volume of the RIPA buffer was variable and adjusted to reach a final concentration of 1,25 x 10^5 parasites per µl. After the incubation on ice the lysate was pelleted at full speed for 60 minutes at 4°C and the supernatant was transferred to a new reaction tube and frozen down or directly mixed with SDS-PAGE loading buffer and loaded onto a protein gel (see below). Per lane 6 x 10^6 of the DDmCherry TgRME-1 parasites were loaded onto the gel.

2.12.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their size by SDS polyacrylamide gel electrophoresis. Components of the gels are described in section 2.7.4 and were mixed in the indicated order there. The addition of ammonium persulphate (APS) and TEMED starts the polymerisation. The anionic detergent SDS denatures the protein and applies a negative charge therefore by SDS-PAGE proteins run towards the anode. First the separating gel was poured between the two glass plates fixed in the gel caster (Amersham). The gel was overlaid with a thin layer of isopropanol and allowed to polymerise. The isopropanol was removed, the loading gel was poured on top of the separating gel and a comp was placed in order to create the wells. As soon as the loading gel is polymerized as well the comb can be removed and the gel is ready for electrophoresis. Protein samples were mixed 1 : 4 with 4 x SDS loading buffer and heated to 95°C for 5 minutes prior to loading on the gel. Alongside 15 - 30 µl of the samples 10 µl PageRuler Prestained Protein Ladder (Fermentas) was loaded on the gel. The gel was fixed in the electrophoresis chamber (Amersham) and surrounded with running
buffer. Electrophoresis was performed for 1 hour at 80 V, 25 mA and subsequently for further 1 - 2 hours at 100 V. The proteins were then transferred to a nitrocellulose membrane via western blot and detected with specific antibodies.

2.12.3 Western blot analysis

For specific detection of PbEHD and DDmCherry TgRME-1 with specific antibodies the separated proteins were transferred from the SDS gel to a nitrocellulose membrane. For this 4 x Whatman paper and 1 x nitrocellulose membrane were cut in gel size and wet together with 4 blotting sponges in 1 x transfer buffer. The Western blot sandwich was built starting with 2 blotting sponges on the cathode side of the blotting cassette (Amersham), followed by 2 x Whatman paper, the SDS gel, the nitrocellulose membrane, 2 x Whatman paper and finally 2 more blotting sponges. The cassette was closed with the anode plate and protein transfer occurred for 1 - 1.5 hours at 125 mA, approximately 20 V in 1 x transfer buffer. The membrane was subsequently blocked over night at 4°C with 5% milk in TBST and washed three times for 10 minutes with 1 x TBST at room temperature. The incubation with the primary antibodies was carried out for 1 hour at room temperature or again over night at 4°C under continuous shaking. The membrane was again washed three times for 10 minutes with TBST and the secondary HRP conjugated goat antibody (1 : 10,000 in TBST; Sigma) was added for 1 hour at room temperature, shaking. Washing was repeated as before. The chemiluminescent detection of the labeled protein was performed with the "ECL Western Blotting Detection Reagents" (GE Healthcare). For this the substrate solutions 1 and 2 were mixed 1 : 1 and incubated on the membrane for 1 minute. The membrane was subsequently transferred in a film cassette, a film (Kodak) was incubated in the dark for 5 seconds up to 25 minutes on the membrane and the light signal on the film was developed (Hyperprocessor Amersham Pharmacia Biotech, Freiburg).

2.13 In silico analysis of apicomplexan EHD-proteins

The in silico analysis of apicomplexan EHD-proteins was based on the published nucleotide and protein sequences of Toxoplasma, Plasmodium and vertebrate EHD-proteins using the online databases Genebank, Uniprot, ToxoDB and PlasmoDB and the prediction algorithms used in these databases. For alignments and further analysis of the sequences the program CLC genomics workbench (CLC bio EMEA, Denmark) was used.
Chapter 3

Results

3.1 Establishment of a combined in vitro/in vivo P. falciparum life-cycle

The investigation of *Plasmodium falciparum* exo-erythrocytic stages is a bottle neck in malaria research. To obtain these stages infectious sporozoites first have to develop in *Anopheles* mosquitoes that previously have been infected with sexual stages of the parasite. This includes the generation of a high number of *P. falciparum* gametocytes in an *in vitro* culture system and subsequently transferring these into the *in vivo* situation in *Anopheles* mosquitoes. Even though there are standard protocols\(^{278}\) existing for that process only a few labs in the world are able to perform it and to successfully establish this process in our lab several factors had to be taken into account: 1) We had to generate a constantly high gametocytemia in the sexual *P. falciparum* cultures by optimizing the culture conditions to generate high infections of the mosquitoes. 2) A perfect timing of the gametocyte cultures and the mosquito breeding had to be established to match mature gametocyte cultures with the right age of the *Anopheles* mosquitoes. 3) The best breeding conditions and age for the susceptibility of the *Anopheles* mosquitoes for a *P. falciparum* infection had to be evaluated. 4) A membrane feeding protocol had to be established that is allowing the transfer of viable mature gametocytes from the cell culture into the midgut of the mosquitoes.

The aim of the whole project was to generate a constant combined *P. falciparum* *in vitro/in vivo* life-cycle (Fig. 3.1) that enables researchers in the lab to work on different stages of *P. falciparum*, especially liver-stages. In one of the projects the sporozoites obtained from the infected mosquitoes were administered to primary human hepatocytes to identify and characterize liver stage antigens of *P. falciparum*, a project I was not involved in (R. Frank. et al.)

3.1.1 *Plasmodium falciparum* asexual and sexual bloodstage *in vitro* culture

To establish an *in vitro* *P. falciparum* life-cycle in our lab parasites of the NF54-strain were cultivated *in vitro* under gametocyte-inducing conditions such as high parasitemia to induce gametocytogenesis and a protocol was established to enrich mature gametocytes within the *in vitro* culture (see section 2.10.1). The highest gametocyte yields could be obtained when gametocyte-cultures were inoculated from an asexual culture of parasitemias between 4-5% (small culture flask, 25 cm\(^2\)) and split down to a starting parasitemia of 1% in big culture flasks (75 cm). Mature gametocytes were checked for maturity at day 15-17 via exflagellation assay and
CHAPTER 3. RESULTS

3.1

Figure 3.1: Combined in vitro/in vivo *Plasmodium falciparum* life-cycle. To investigate *Plasmodium falciparum* liver-stage antigens a combined *in vitro/*in *vivo* lifecycle was established. **Inner circle:** *In vivo* life-cycle. **Outer circle:** Combined life-cycle established in the lab. The generation of liver-stages started with cultivation of gametocytes in *in vitro* blood cell cultures followed by membrane feeding of *Anopheles stephensi* mosquitoes. *P. falciparum* sporozoites were later extracted from *A. stephensi* salivary glands and added to cultivated primary human hepatocytes, extracted beforehand from liver resections. Liver-stage development was finally confirmed by immuno-fluorescence analysis (IFA) detecting liver-stage specific antigens.
CHAPERT 3. RESULTS

3.1 subsequent transferred to *A. stephensi* mosquitoes via membrane-feed.

3.1.2 Membrane feeding of mature gametocytes to Anopheles stephensi mosquitoes

To transfer mature gametocytes from the *in vitro* cell culture to *A. stephensi* mosquitoes a standard membrane feeding protocol was optimized for our lab conditions (see section ). Throughout the course of the experiments described in Frank, R. et al. (*in preparation*) a stable transmission rate could be maintained that ceased again afterwards for reasons discussed in chapter 5. To evaluate the success of the *P. falciparum*-transmissions midguts and later salivary glands of a proportion of the infected mosquitoes were analyzed for the presence of oocysts and salivary gland sporozoites, respectively (Fig. 3.2). A high prevalence of infected midguts with a high number of oocyst per midgut could be achieved that led to a sufficient number of salivary glands sporozoites for subsequent experiments (Tab. 3.1).

![Image](image_url)

Figure 3.2: Membrane transfer of *Plasmodium falciparum* *in vitro* cultures to *A. stephensi* mosquitoes and analysis of parasite development within the anophelene host. A) Membrane-feeding. Mature gametocytes were supplemented with fresh human blood and injected into a glass-feeder sealed by a parafilm membrane on the bottom. Mosquitoes were kept in paperboard cups and allowed to feed on the blood distributed over the membrane for up to 20 minutes. B) Evaluation of the infection of mosquito midguts. *P. falciparum* infected *A. stephensi* midguts were extracted from the mosquito carcass and stained with mercurochrome red. Black arrows show some of the parasite oocysts present in the midgut. C) *P. falciparum* salivary gland sporozoites. Salivary gland sporozoites were extracted from the mosquitoes and stained in an immuno-fluorescence assay (IFA) with rabbit α-*PfCSP*/alexa594 α-rabbit antibodies. (Bar: 5 µm)

<table>
<thead>
<tr>
<th>Total amount</th>
<th>Oocysts</th>
<th>Sporozoites (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>78.5% prevalence</td>
<td>2,700.00</td>
<td></td>
</tr>
<tr>
<td>Average number/individual</td>
<td>27</td>
<td>28,000</td>
</tr>
<tr>
<td>Total no. of individuals</td>
<td>14</td>
<td>93</td>
</tr>
</tbody>
</table>
CHAPTER 3. RESULTS

3.2

Figure 3.3: Liver-stage development of *P. falciparum* parasites in vitro in hepatoma cells. *P. falciparum* sporozoites were allowed to invade Huh7 hepatoma cells for 30-120 minutes and to develop for 24h (upper panel) or 48 h (lower panel), respectively. After liver-stage development parasites were fixed with Methanol and subsequently stained with mouse α-\(Pf\)HSP70/alexa488 α-mouse antibodies. As shown in both panels parasites were able to generate exo-erythrocytic forms in human hepatoma cells and have started to undergo schizogony as shown by nuclear staining (Hoechst) in the lower panel. (Bar: 8 µm)

3.1.3 Liver-stage development of *Plasmodium falciparum* in vitro

To obtain *P. falciparum* liver-stage parasites for further experiments described elsewhere (Roland Frank, PhD thesis; Frank, R. et al. in preparation) sporozoites were extracted from *A. stephensi* salivary glands and added to cultivated primary human hepatocytes (Frank, et al., in preparation). An isolation and cultivation of primary human hepatocytes was necessary since infection of immortalized liver cells such as HuH7 cells did not work effectively. After invasion of hepatocytes for 30-120 minutes for evaluation of liver-stage development liver-stage parasites were allowed to grow for 24-48 hours in primary cell culture and hepatoma cell lines (Huh7 cells) and were subsequently fixed and stained for immuno-fluorescence analysis (IFA; Fig. 3.3). The evaluation of the IFAs showed that *P. falciparum* sporozoites were able to invade both primary human hepatocytes and hepatoma cell lines and to develop into liver-stage trophozoites (Fig. 3.3, upper panel) and early schizonts (Fig. 3.3, lower panel).
3.2 Characterization of EHD-proteins in apicomplexan parasites

3.2.1 RME-1 encodes for a conserved EHD-family member in apicomplexan parasites

In an attempt to identify new Dynamin-related proteins in apicomplexan parasites we performed an *in silico* analysis of the *Toxoplasma gondii* genome searching for genes harboring a predicted G-domain-coding sequence in apicomplexan parasites. We identified a so far unknown member of the Eps15 homology (EH) domain-containing protein family in these organisms. Whereas mammalian cells and other higher eukaryotes possess several different EHD-proteins, *C. elegans* possesses only one member, i.e. *Ce*RME-1 (Receptor-mediated endocytosis protein 1)\(^{280}\). A phylogenetic analysis (kindly provided by Dr. Markus Meissner, Glasgow, UK) shown in Fig. 3.4 demonstrates that also apicomplexan parasites possess only one EHD-protein member, clustering tightly within this clade, that shows the characteristic primary structure of EHD-proteins (Fig. 3.5).

The apicomplexa protein is harboring a predicted p-loop containing nucleoside triphosphate hydrolase motif\(^{281}\), known to bind and hydrolase ATP in EHD-proteins instead of GTP in other G-domain proteins\(^{282}\)\(^{283}\), and a predicted EH-domain. It was named in *Toxoplasma* and *Plasmodium Tg*RME-1 and *Px*EHD, respectively.

3.2.2 TgRME-1 localizes to not yet known subcellular structures within *Toxoplasma gondii*

According to the ToxoDB database, *Tg*RME1 transcripts as well as protein peptides are readily detected in tachyzoites and oocysts of the *Toxoplasma gondii* RH stain \(^{284}\)\(^{285}\). Additionally, transcriptomic analysis of the *Toxoplasma* tachyzoite intracellular replication cycle shows that the relative RNA-expression of the gene peaks with the mitosis phase of the parasite and decreases again upon G-phase entry\(^{286}\). In order to analyze the subcellular localization of the RME-1 protein, I generated a parasite line that expressed *Tg*RME-1 that was N-terminally fused to a fluorescent mCherry-tag. Therefore the full-length coding sequence of the gene was cloned downstream of the sequence of a destabilization-domain (ddFKBP) and a mCherry-tag (Fig. 3.6), controlled by a strong promotor (p5RT70), and the vector was subsequently transfected and randomly integrated into the genome of *Toxoplasma gondii* RHΔHX parasites as a second copy of the gene. A selection marker within the plasmid carrying the just described genetic sequences allowed to select for parasites that successfully took up the linearized plasmid (restriction digest in the backbone) after transfection of the parasites. To remove any non-integrated episomal plasmids the selective drug was removed after resistant parasites were growing in a normal replication rate in cell culture. Since expression levels of genes in the integrated plasmids can vary depending on their genomic environment because of
CHAPTER 3. RESULTS

3.2

Figure 3.4: Phylogenetic tree of EHD-proteins. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.). Both, ATPase- and EH-domain, were included into the analysis. The bootstrap consensus tree inferred from 500 replicates (Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.) is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.). The evolutionary distances were computed using the JTT matrix-based method (Jones D.T., Taylor W.R., and Thornton J.M. (1992). The rapid generation of mutation data matrices from protein sequences. Computer Applications in the Biosciences 8: 275-282.) and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter 0.7693). The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 242 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura K., Dudley J., Nei M., and Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.).
Figure 3.5: **General EHD-protein domain structure in apicomplexans.** The single predicted domains are represented by colored boxes. The aminoacid motifs of the G-domain that represent the P-loop nucleotide binding site (G...T) and the KPF, NKAD and PF protein-protein-interaction motifs that are all conserved between the apicomplexan EHDs and human EHD2 are shown in letters below the boxes (figure adapted from Daumke et al., 2007 [1]). aa: Amino-acids

Figure 3.6: **Tagging approach for subcellular localization analysis.** *Tg*RME-1 and *Pf*EHD were N-terminally tagged with a mCherry-tag and a destabilization-domain (DD) and expression of the gene was driven by a strong promotor (p5RT70). After addition of the Shield-1 ligand to the culture medium expression was induced via stabilization of the protein. To create dominant-negative effects two deletion mutants lacking either the ATPase-domain (∆ATPase) or the EH-domain (∆EH) were cloned and expressed within the parasites. FL, full length; aa, amino-acids
the random integration single parasite clones were selected for further analysis by limiting dilution. By adding the synthetic ligand Shield-1 to the cell culture medium a fluorescence could be observed in the parasite. The protein expression was analyzed by both Western blot and fluorescence assay. Through binding of the soluble ligand Shield-1 to the ddFKBP-domain the protein was stabilized within the parasite and could be detected as early as 2h hours after induction by Western blot (Fig. 3.7). In a time-course fluorescence assay parasites where fixed with 4% paraformaldehyde (PFA) and the mCherry signal was fluorescence-microscopically analyzed subsequently in response to the duration of Shield-1 exposure. Only a very weak background cytosolic mCherry signal could be seen for the first two hours after induction of protein expression. When the amount of protein increased over time a distinct spot labeled by the mCherry signal within the parasite became apparent (Fig. 3.7). In indirect immuno-fluorescence assay (IFA) colocalization studies, where the parasite’s inner membrane complex (IMC) was labeled with an α-IMC antibody detected by a green-fluorescent secondary antibody, we could show that the tagged TgRME-1 localizes to an organelle-like compartment. This compartment within the parasite does not always correspond to the same coordinates within the cell (Fig. 3.8). The protein can be found in a single compartment at the apical end (arrow heads) as well as in a single compartment at the basal end of the parasite (arrows). In other occasions it localizes to the newly forming daughter-cells or can also be found in several smaller vesicle-like structures distributed over the whole parasite (Fig. 3.8). The localization of the over-expressed TgRME-1 does also not seem to be synchronized between the single cells within the same vacuole. These
observations already led to the hypothesis that \( Tg \)RME-1 localization within the parasite may be dynamic. To investigate this, we applied two different live-imaging approaches, long- and short-term, to study localization of the tagged \( Tg \)RME-1 protein during parasite replication within the host cell. Via short-term imaging, taking a picture of the parasites every 4 seconds, we observed small mCherry-labeled vesicular structures within the parasites that are rapidly moving in a multi-directional manner, mainly circulating between the two poles of the parasite (suppl. video 2). These vesicles had not been observed previously in fixed samples. This movement of the \( Tg \)RME-1 protein-labeled vesicles dramatically increases upon formation of the daughter-cells within the mother cell. During long-term imaging studies, by taking a picture of the parasites every 10 minutes, mainly in parasites undergoing cellular division we observed dramatic structural re-organization of the \( Tg \)RME-1-labeled compartment (Fig. 3.9). During an early time point of the formation of daughter-cells the \( Tg \)RME-1 compartment fragments and smaller vesicles are transported to the apical tip of the newly forming IMC (arrow heads, Fig. 3.9). At a late stage of the endodyogeny the main \( Tg \)RME-1 compartment within 30-40 minutes moves from the apical to the basal pole of the parasite, upon a so far unknown trigger (arrows, Fig. 3.9). When cytokinesis is completed \( Tg \)RME-1-labeled compartments can be found again at the apical pole of the two newly formed daughter-cells as well as at their connection point at the residual body.

In order to confirm correlation of the long-term movement of the \( Tg \)RME-1 compartment with the formation of daughter-cells in live-imaging studies we co-transfected the DDmCherry \( Tg \)RME-1-expressing parasites with a construct that leads to the expression of an IMC-YFP fusion protein. Through that we were able to image the formation of the IMC in daughter-cells and the movement of the \( Tg \)RME-1 compartment at the same time in living cells. In this approach again we were able to show a reorganization of the \( Tg \)RME-1 compartment upon formation of the IMC of the daughter-cells. A smaller mCherry-labeled structure is embodied by the IMC of the daughter-cells and the bigger compartment of the mother cell squeezes alongside the daughter IMC to the basal pole of the mother cell (Fig. 3.10). My attempts to colocalize the \( Tg \)RME-1 protein with any organelle of the endosomal system, the secretory system or any other organelle known in Toxoplasma failed so far (exemplary organelles shown in Fig. 3.11). Together my findings propose a localization of \( Tg \)RME-1 to a so far unknown organelle-like structure in \textit{Toxoplasma gondii} that is undergoing dramatic reconstruction during formation of daughter-cells.

### 3.2.3 Functional analysis of RME-1 in \textit{Toxoplasma gondii}

To study the biological function of \( Tg \)RME-1 in \textit{Toxoplasma} parasites I created two different over-expression constructs where either the sequence coding for the predicted ATPase-domain or the predicted EH-domain was deleted. The truncated protein was fused to a N-terminal DD/mCherry-tag in order to create an inducible dominant-negative effect (Fig. 3.6). After transfection, random integration into
Figure 3.8: Localization of DDmCherry TgRME-1 in *Toxoplasma*. mCherry-tagged TgRME-1 expression was induced via addition of Shield-1 for 4 hours. After fixation of the cells an indirect immuno-fluorescence assay was performed labeling the inner membrane complex (IMC) of the parasites with an α-IMC antibody. Localization of the tagged TgRME-1 was directly imaged via the mCherry signal. Localization of the mCherry-signal appeared in a dynamic vesicular labeling of the apical part of the parasites (arrow heads) as well as the basal part (arrows). (Bar: 4 µm.)
Figure 3.9: **Time-lapse analysis of DDmCherry TgRME-1.** 4h before imaging DDmCherry TgRME-1 expression was induced via addition of Shield-1 to the culture. Intracellular parasites were then imaged in a live-cell chamber taking a picture of the mCherry-signal every 10 min starting at timepoint 0 (4h after induction of expression). Upon formation of daughter-cells (0+90min) the mCherry-labeled apical compartment fragments and smaller vesicles move to the apical end of the daughter-cells (arrow heads). About 40 min after initiation of daughter-cell formation (0+120min) the main apical TgRME-1 compartment starts to move to the basal end of the parasite (arrows). (Bar: 4 μm.)
Figure 3.10: **Time-lapse analysis to investigate** $T_g$RME-1-IMC colocalization. DDmCherry $T_g$RME-1 parasites were transiently co-transfected with a plasmid that allows constitutive expression of an IMC-YFP fusion protein within the parasites. This expression was used to visualize the formation of daughter-cells by live-cell imaging via visibility of the IMC (Yellow-fluorescent protein; YFP). Upon initiation of formation of the daughter-cells ($T=0$ min) the $T_g$RME-1 compartment (mCherry panel) fragments and smaller vesicular structures are incorporated by the IMC of the forming daughter-cells ($T=30$ min). About 60 min after initiation of daughter-cell formation the apical $T_g$RME-1 compartment starts to squeeze along the daughter-cell-IMC to move to the basal end of the mother cell. (Bar: 4 µm.)
CHAPTER 3. RESULTS

3.2

Figure 3.11: Exemplified images of the colocalization of TgRME-1 with organelles of the secretory and endosomal system, respectively. Pictures show a selection of colocalization studies of TgRME-1 with the secretory and the endosomal system. Co-localization was either analyzed via indirect immuno-fluorescence assay (IFA) of the organelar markers or via co-expression (DD-tag) of tagged proteins (Rhop5: Rhoptries; Mic8: Microneme subset; ProM2AP: Early endosome; Rab5A: Endosomal-like compartment; Rab7: late-endosomal like-compartment). No colocalization could be observed for any of the tested organelles. (Bar: 4 µm.)
Figure 3.12: Expression of the \textit{TgRME-1∆EH} deletion mutant. \textit{TgRME-1} lacking the Eps15-homology domain (EH) was N-terminally tagged with a destabilization domain (DD) and a mCherry-tag. Over-expression of the deletion mutant was induced via addition of 1 µM Shield-1 for 6 h and the parasites were subsequently fixed with 4% PFA. The inner membrane complex (IMC) was detected via an indirect immunofluorescence assay (IFA) using an α-IMC antibody. The localization of \textit{TgRME-1∆EH} was comparable to the full-length protein. (Bar: 4 µm)

the genome and successful cloning of single parasite lines I initially examined the localization of the protein in the deletion mutants generated. Interestingly, whereas localization of the \textit{TgRME-1∆EH} protein was comparable to the wild-type localization (Fig. 3.12), the subcellular localization of the \textit{TgRME-1∆ATPase} protein differed dramatically from the wild-type, in a sense that it appeared mainly cytosolic (Fig. 3.13). When the parasites were subsequently analyzed for their ability to grow while expressing the deletion mutant no growth defect was observed in standard plaque-assays (Fig. 3.14). All attempts to generate a potent knock-out of the \textit{TgRME-1} gene in \textit{Toxoplasma} parasites failed due to the inability to amplify the 3'UTR of the gene from genomic DNA for cloning.

3.2.4 Localization of \textit{Plasmodium falciparum} EHD-protein \textit{PfEHD}

To analyze the localization of the \textit{Plasmodium falciparum} ortholog of \textit{TgRME-1}, \textit{PfEHD}, in collaboration with Florian Kruse (PhD-student, AG Spielmann, BNI-Hamburg), the \textit{pfehd} open-reading frame was fused C-terminally to a sequence coding for a GFP-tag and stably expressed in \textit{P. falciparum} blood-stage parasites. Analysis of the fixed transgene parasites under the fluorescence microscope revealed a localization of the GFP-fusion protein to the periphery of the parasites, in close association of the parasite plasma membrane (Fig. 3.15). A faint staining of the cytoplasm as well as a strong focal staining of some areas of the plasma membrane could be observed also.
Figure 3.13: **Expression of the $Tg$RME-1ΔATPase deletion mutant.** $Tg$RME-1 lacking the ATPase-domain was N-terminally tagged with a destabilization domain (DD) and with a mCherry-tag, respectively. Expression of the deletion version was induced via addition of 1 µM Shield-1 for 6 h and parasites were subsequently fixed with 4% PFA. The inner membrane complex (IMC) was detected via an indirect immunofluorescence assay (IFA) using an α-IMC antibody. Localization of $Tg$RME-1ΔATPase was mainly cytosolic. (Bar: 4 µm)

Figure 3.14: **Plaque-Assay of DDmCherry $Tg$RME-1-expressing *Toxoplasma* parasites.** Human foreskin fibroblast monolayers were infected with *Toxoplasma* parasites expressing DDmCherry $Tg$RME-1 in presence or absence of Shield-1 for 7 days, fixed with Methanol and subsequently stained with Giemsa-stain solution. No difference in parasite growth could be observed between parasites that are expressing the fusion protein and that are not expressing it, respectively. (Bar: 1 mm)
CHAPTER 3. RESULTS

3.2

Figure 3.15: Localization of PfEHD-GFP in Plasmodium falciparum trophozoites. PfEHD was C-terminally tagged with a GFP-tag and expressed as additional copy to the endogenous protein in P. falciparum. Stably expressing parasites were fixed with 4% PFA and the GFP-signal was analyzed subsequently under a fluorescent microscope. The GFP-signal could be observed at the periphery of P. falciparum trophozoites closely and homogenously associated with the plasma membrane of the parasite, as well as at distinct focal spots. (Bar: 2 μm)

3.2.5 Protein localization of TgRME-1 and PfEHD is interchangeable between apicomplexans, but differs according to the intrinsic protein architecture

To analyze the overall conservation of the single EHD-protein for apicomplexan parasites regarding both its subcellular localization and hence functionality we carried out complementation studies by integrating the TgRME-1 gene into Plasmodium falciparum and vice versa. Interestingly, when we transiently expressed PfEHD in Toxoplasma applying the exact same genetic strategy as for TgRME-1 (DDmCherry-Tag, N-terminally, described in section 3.2.1), we observed a localization of PfEHD mainly concentrated to the parasite’s plasma membrane (Fig. 3.16). This is consistent with the localization of PfEHD in P. falciparum as shown in Fig. 3.15 and described in section 3.2.4. When we stably expressed TgRME-1 in P. falciparum fused to a C-terminal GFP-tag the protein localized to a distinct spot within the Plasmodium parasite, similar to its defined localization observed in Toxoplasma parasites (Fig. 3.17; in collaboration with Florian Kruse, AG Spielmann, BNI-Hamburg). Both experiments suggest a localization of each protein specific for a distinct structure in apicomplexan parasites, that is interchangeable between both parasites, meaning the cellular localization for PfEHD and TgRME-1, respectively, is the same in both organisms. Each apicomplexan EHD-protein localizes to the same structure within both parasites, but this localization differs from the other apicomplexan EHD-protein member, respectively. Therefore the localization of the protein seems to be defined by its intrinsic architecture rather than its parasite-specific interaction partners. This is consistent with the above mentioned finding that deletion of the EH-domain of TgRME-1, generally known to be an important site for protein-protein-interactions in other EHD-proteins289, does not lead to a
Figure 3.16: Localization DDmCherry\textit{P}f\textit{EHD} in \textit{Toxoplasma}. \textit{Plasmodium falciparum} EHD was tagged N-terminally with a destabilization domain (DD) and an mCherry-tag and transiently transfected into \textit{Toxoplasma gondii} RHΔHX GFPcon (conditional cytosolic GFP-expressing) parasites. \textit{Pf}EHD-protein expression was induced with Shield-1 for 6 h and parasites were fixed subsequently. The mCherry-tagged protein localized to the cytoplasm membrane of the \textit{Toxoplasma} parasites. (Bar: 4 \textmu m)

different localization of the protein. Furthermore, an alignment of the three apicomplexan EHD-protein family members of the human malaria pathogen \textit{P. falciparum}, the murine malarial parasite \textit{P. berghei} and human Toxoplasmosis causing agent \textit{T. gondii} shows that, even though all three proteins are very similar regarding their primary protein architecture, solely \textit{Tg}RME-1 harbors a long C-terminal stretch downstream of the EH-domain (Fig. 3.18). This stretch seems to be highly phosphorylated as described recently by Treek et al.\textsuperscript{290}.
Figure 3.17: Comparison of the expression of GFP-tagged $T_g$RME-1 ($T_g$EHD) and $P_f$EHD in $Plasmodium falciparum$ 3D7 parasites. $T_g$RME-1 and $P_f$EHD were both C-terminally GFP-tagged and this version of the protein was stably expressed in addition to the endogenous protein in $Plasmodium falciparum$. A) Expression of $T_g$RME-1/$T_g$EHD-GFP in $P. falciparum$. $T_g$EHD-GFP is expressed in distinct organelle-like compartments within the parasite as shown by fluorescence microscopy (lower panel) and confocal 3D-reconstruction (upper panel) in blood-stage trophozoites. Some of the labeled spots seem to be associated with the nucleus of the parasite. The parasite cytoplasm was co-stained with bodipy-stain in addition to DAPI-staining of the nucleus (Bar: 2 µm). B) Expression of $P_f$EHD-GFP in $Plasmodium falciparum$. $P_f$EHD-GFP localizes to the periphery, likely the plasma membrane, of the parasite and concentrates at few distinct spots at this membrane. In addition to that some of the protein can also be found in the parasite cytoplasm (Bar: 2 µm). C) Western blot detecting the fusion proteins $T_g$EHD-GFP and $P_f$EHD-GFP with an α-GFP antibody. The marker on the lefthand side indicates protein sizes in kDa.
3.2.6 Expression and localization of the EHD-protein in P. berghei

In order to analyse expression and localization of the EHD-protein in the rodent malaria parasite *Plasmodium berghei* an antibody against the amino-acids 339-533 (compare Fig. 3.18) of the *Pf*EHD-protein was generated (kindly provided by Dr. Tobias Spielmann, BNI Hamburg) and tested against the *P. berghei* protein in IFA studies. In summary it could be shown that *PbEHD* is expressed on protein level in bloodstages (BS), salivary gland sporozoites (SGS) and liver-stages (LS). When BS were analysed on immuno-EM level a gold labeling of the parasite’s plasmamembrane (PM) as well as structures within the parasite could be observed (Fig. 3.19). When a number of EM-sections (\(N \approx 100\)) were analyzed for the number of events in which certain organelles were labeled by gold particles combined with \(\alpha\)-*PfEHD* antibodies in a semi-quantitative analysis (Fig. 3.20) a preferred localization of *PbEHD* to the parasite cytoplasm, vesicles, the endoplasmic reticulum (ER) and the parasite nucleus could be shown (Fig. 3.21). In less frequent occasions also a labeling of the parasite’s mitochondria, the cytostome, the apicoplast and the surrounding membranes (PM/PVM) and even a labeling of the red blood-cell lumen and membrane could be seen (Fig. 3.21 and Fig. 3.20). When blood-stage parasites (schizont overnight culture) were analyzed in an IFA only a very weak expression of the protein could be observed (Fig. 3.22). When SGS were labeled with the antibody a vesicular staining could be observed that is homogenously distributed over the whole cytoplasm of the parasite (Fig. 3.23). This staining changes after the parasites invade HuH7 hepatoma cells *in vitro* and develops into early extra-erythrocytic forms (EEFs). After development of EEF trophozoites for 24h the protein can be found in a single compartment of the parasite (Fig. 3.24). This compartment is aligning with the plasma membrane (PM) of the parasite and appears to be dynamic since it is forming extensions into the cytoplasm of the parasite. In most of the images observed the localization of this compartment is polarized within the parasite towards the host cell nucleus, were the labeling of the compartment by \(\alpha\)-*PfEHD* antibodies appears to be stronger. After 48 h of EEF development, when schizogony has taken place and first-generation merozoites begin to form, the EHD-labeled compartment splits up into vesicular structures that are distributed non-homogenously over both the whole parasite and the PM (Fig. 3.25). Similar to what was observed for *TgRME-1* the localization of *PbEHD* seems to be highly dynamic, as well, since tubular structures could also be seen in *P. berghei* (Fig. 3.25, bottom panel).

3.2.7 EHD gene deletion in murine *P. berghei* is not essential for blood-stage development

Since functional studies in *Toxoplasma* failed (described above) and in order to study the importance of EHD during the malarial life-cycle, I generated loss-of-function mutants in the rodent *Plasmodium* parasite, *P. berghei*. I therefore applied an integration strategy in order to disrupt the endogenous EHD gene locus by a single cross-over homologous recombination\(^\text{291}\). For targeted gene-disruption a plas-
CHAPTER 3. RESULTS

3.2

Figure 3.18: **Alignment of selected apicomplexan EHD-proteins.** **Pf**EHD, **Pb**EHD and **Tg**RME-1 were aligned according to their primary structure. Main sequence motifs coding for known functional domains are shown in colored bars above the sequences (predicted p-loop containing nucleoside triphosphate hydrolase motif, green; KPF-binding motif; short yellow bar; EH-domain, long yellow bar; phosphorylation sites, red bars).

Figure 3.19: **Immuno-EM of** **P. berghei** bloodstage parasites labeling **Pb**EHD. **P. berghei**-infected red blood cells (iRBC) were fixed and embedded using the Tokuyasu-method, cryo-sectioned (100nm) and labeled using an anti-**Pf**EHD primary antibody detected by 10nm gold-bead conjugated secondary antibodies. Gold-beads can be found at the parasite’s plasma membrane as well as in the cytoplasm and at vesicles.
Figure 3.20: **Semiquantitative analysis of PbEHD localization in *P. berghei* blood-stages in electron microscopy studies.** The number of events of labeled organellar structures on the EM sections were counted and plotted as percentage of the total number of events ($N \bar{1}00$). Labeling of one organellar structure by several gold particles within the same section was counted as only one event. ER: Endoplasmic reticulum; RBCM: Red blood cell membrane; RBCL: Red blood cell lumen; PM: Parasite membrane; PVM: Parasitophorous vacuole membrane;

![Figure 3.20: Semiquantitative analysis of PbEHD localization in *P. berghei* blood-stages in electron microscopy studies.](image)

Figure 3.21: **Overview of the labeling of different organelles by α-PbEHD antibodies in immuno-EM studies of blood-stages.** Arrows point to the gold-beads attached to antibodies labeling PbEHD. Pictures A-F show examples of the organelles labeled by the antibodies. A: Mitochondrion; B: ER (endoplasmic reticulum) profiles; C: Cytostome; D: Apicoplast; E: PM/PVM (parasite membrane and parasitophorous vacuole membrane) and nucleus - arrowhead points to nuclear pore; F: Vesicle.
Figure 3.22: **Localisation of PbEHD in blood-stage parasites.** Blood-stage parasites enriched during a 15h over-night culture were fixed with 4% PFA and subsequently stained with mouse α-PbEHD/α-mouse Alexa488 antibodies. Only a weak staining of the parasites could be observed in ring-stage trophozoites (upper and middle panel) and blood-stage schizonts (arrow, lower panel). (Bar: 4 µm)
Figure 3.23: **Localisation of PbEHD in salivary gland sporozoites.** PbANKA sporozoites were extracted from salivary glands of *A. stephensi* mosquitoes and allowed to glide on BSA-coated slides for 30 min. After fixation in PFA an IFA was performed detecting PbEHD with the mouse α-PfEHD/α-mouse Alexa488 antibody. A vesicular staining covering the entire cytoplasm of the parasites was observed. (Bar: 4 µm)
Figure 3.24: **Localization of *Pb*EHD EEFs after development of 24h *in vitro*. *P. berghei* parasites were allowed to develop within Huh7 hepatoma cells *in vitro* for 24h post invasion. In an IFA α-*Pf*EHD antibodies were applied after PFA-fixation to visualize localization of the protein within the parasites. A strong labeling of one compartment within the parasites could be observed that has a polar orientation directed to the host cell nucleus. Extensions of the compartment into the parasite cytoplasm can be detected. (Bar: 8 µm)
Figure 3.25: **Localization of PbEHD EEFs after development of 48h in vitro.** *P. berghei* parasites were allowed to develop for 24h post invasion and an IFA was performed as described in Fig. 3.24. A labeling of vesicular structures within the parasites can be seen as merozoites start to form. The structure is dynamic (vesicular and tubular; bottom panel) and non-homogenously distributed over the whole parasite. (Bar: 8 µm)
mid carrying a fragment of the pbehd open-reading frame (ORF) and a selectable marker (tdhfr/ts) was linearized within the homologous region and transfected into parasites of the P. berghei ANKA (PbA) reference line (Fig. 3.26A). By successful integration of the full-length plasmid into the pbehd locus via homologous recombination the gene was truncated making it unlikely for the full-length mRNA of the gene of interest (GOI) to be expressed. Applying a double cross-over strategy to replace the endogenous locus of the GOI was however not possible, since the 3’ untranslated region of the gene could not be amplified by PCR-reaction. This is consistent with recent data from Pfander et al. who were also not able to include the 3’ part of the pbehd ORF into their plasmoGEM database recombination vectors\textsuperscript{292}. Transfected parasites were selected with the antifolate Pyrimethamine and the parental blood-stage population from a successful transfection, verified by genotyping-PCR, was subsequently used to isolate four independent knock-out clonal lines, two of which were used for phenotypical analyses (pbehd(-)#4/1 and pbehd(-)#4/2; Fig. 3.26B). Since stable clones of the pbehd (-) strain could be obtained after transfection the protein does not seem to be essential for the blood-stage phase of the parasite.

### 3.2.8 EHD mutant P. berghei parasites develop indistinguishable from wildtype parasites during the intra-mosquito life-cycle

Blood-stage positive pbehd (-) and WT infected mice were analyzed for the existence of mature gametocytes via exflagellation assay. When at least 2-5 exflagellation centers per field could be observed at 400x magnification 5-7 days old Anopheles stephensi mosquitoes were allowed to feed for 15 min on the anesthetized infected mice. After 12 days 10 mosquitoes per group were killed and midguts were analyzed for the numbers of oocysts present. The same midguts were afterwards combined and mashed within an eppendorf reaction tube. Through that oocyst derived sporozoites (ODS) were extracted and could be counted afterwards. At day 19 after the blood meal 15-20 mosquitoes were killed, salivary glands combined and mashed in an eppendorf reaction tube. After that salivary gland sporozoites (SGS) were counted at 400x magnification.

In summary we observed no significant difference between wildtype parasites and the pbehd (-) strain during development of the parasites within A. stephensi mosquitoes as shown by similar numbers of oocysts, ODS and SGS (Fig. 3.27).
Figure 3.26: **A) Endogenous gene depletion resulted in *pbehd* (-) parasites.** *pbehd* was deleted via single cross-over integration strategy. For transfection pFK01 was linearized with *Bst*BI and the homologues sequence of the *pbehd* ORF cloned into pFK01 (*Sac*II/*Spe*I) was allowed to recombine with the endogenous gene locus. Thereby the *pbehd* 5' and 3' fragments were separated and a resistance gene (*Tg*dhfr/ts) was integrated. Parasites stably carrying the integrated plasmid were selected with Pyrimethamine and successful integration was confirmed via PCR (WT fragment 2.2 kb, Integration fragment 2.0 kb). **B) Confirmation of the integration of pFK01 into the *pbehd* locus.** Clonal parasite lines from two independent transfections and limited dilutions were tested for the existence of the wildtype or the *pbehd* (-) locus (integration) via PCR. Depicted below the lanes are the respective templates chosen for the PCR. Numbers 4/1 and 4/2 represent clones from the 1st transfection; Numbers 1/1 and 5/3 represent clones from the 2nd transfection. Left: Primer combination to detect the WT locus chosen. Right: Primer combination to detect the recombined locus chosen. WT: wildtype; V: vector.
Figure 3.27: Analysis of pbehd (-) parasite development in the mosquito vector. A) Analysis of the oocyst formation in A. stephensi midguts. B) Number of oocyst derived sporozoites per mosquitoes (numbers x 1000). C) Number of mature sporozoites per A. stephensi salivary gland at day 19 after blood meal.
### Figure 3.28: Analysis of pbehd (-) parasite development throughout the lifecycle (significant value differences shown in bold)

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Oocyst, no. d12 mean (range)</th>
<th>Sporozoite no. per oocyst d12 (mean)</th>
<th>Sporozoite injection (10,000); no. of infected mice</th>
<th>Sporozoite injection (1,000); no. of infected mice</th>
<th>Liver trophozoites at 48 h in vitro, size Area (Pixel², mean)</th>
<th>Prepatency after Sporozoite challenge (days, mean)</th>
<th>No. of infected mice developing ECM, spz challenge</th>
<th>No. of infected mice developing ECM, BS challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT PbANKA</strong></td>
<td>40.0 (0-120)</td>
<td>24750</td>
<td>14300</td>
<td>9/9</td>
<td>3/3</td>
<td>145±12</td>
<td>0.35±0.013</td>
<td>3.5±0.5</td>
</tr>
<tr>
<td><strong>pbehd (-)</strong></td>
<td>37.78 (0-110)</td>
<td>16750</td>
<td>14450</td>
<td>9/9</td>
<td>2/3</td>
<td>127±15</td>
<td>0.28±0.013</td>
<td>4.5±0.5</td>
</tr>
</tbody>
</table>

Figure 3.28: Analysis of *pbehd* (-) parasite development throughout the lifecycle (significant value differences shown in bold)
3.2.9 Depletion of EHD results in developmental slow-down during late liver-stage development in P. berghei and protection of severe pathology in C57BL/6 mice

When C57BL/6 mice were infected with salivary gland sporozoites (SGS) intravenously a significant delay in prepatency of approximately 1 day in mice infected with pbehd (-) parasites in comparison to the WT could be shown by examination of blood smears. An in vitro liver-stage development assay showed a slowed down development of pbehd (-) parasites during the liver phase since the knock-out parasites were significantly smaller than the wildtype at 48h after invasion (Fig. 3.29). When C57BL/6 mice infected with sporozoites became blood-stage patent interestingly, in addition to the delay in prepatency, also a striking difference in disease outcome was observed. Infection of C57BL/6 mice with *Plasmodium berghei* ANKA parasites represents a well-accepted murine model of experimental cerebral malaria (ECM), which reflects symptoms observed in patients suffering from human cerebral malaria (HCM)\(^\text{293}\). Mice in this model usually suffer from a neurological syndrome characterized by paralysis, deviation of the head, ataxia, convulsions and coma between six and 10 days after inoculation with parasitized red blood cells. Infection leads to death in 60-100% of mice despite relatively low parasitemia\(^\text{294}\). In our approach, whereas all PbANKA (PbA) wildtype-infected mice developed ECM around day 8 post infection at parasitemias not higher than 5%, none of the pbehd (-) infected mice did show any signs of ECM rather going into hyper-parasitemia instead and dying much later in result of anaemia (Fig. 3.30A and Fig. 3.30B). The protection from ECM in pbehd (-) infected mice could be shown to be solely dependent on the liver phase of the parasite since animals challenged with 1x10\(^6\) iRBC of pbehd(-) (blood-stage knock-out) parasites were not protected from developing ECM similar to those infected with PbA iRBC (blood-stages) (Fig. 3.28). When brains of infected mice were analyzed via Evan’s blue staining at the coma stage (in the case of a PbA wildtype infection) or after the mice were sacrificed (pbehd(-) infection), a leakage of the blood brain barrier was observed for the PbA-infected mice as had been shown previously\(^\text{295}\), whereas no leakage could be observed for the pbehd (-) infected mice (Fig. 3.30D). All of these results were confirmed for a second clone and are summarized in Fig. 3.28. Since observations by Lewis et al. (unpublished observations) indicated that IL-10 induction in the host might play a role in the protection from ECM during the liver stage we depleted IL-10 via an IL-10 depletion antibody in 3 mice during the liverstage phase of pbehd (-) infected mice. Interestingly, 2 out of 3 pbehd (-) infected mice developed ECM when depleted from IL-10 confirming a protective role for IL-10 in this model.
CHAPTER 3. RESULTS

3.2

Figure 3.29: Liverstage development assay comparing pbehd (-) and WT parasites. Parasites were treated as described in Fig.3.30 C and sizes of the EEFs were analyzed at 24h and 48h post invasion.
Figure 3.30: **A) Parasitemia curve after challenge of C57BL/6 mice with 10,000 sporozoites of WT and pbehd (-)**. 10,000 sporozoites were injected intravenously into C57BL/6 mice (N=6). WT parasites died at low parasitemia around day 7 whereas pbehd (-) parasites developed slower, were delayed in reaching the blood-stage phase, finally developed hyperparasitemia and died later of anemia symptoms.

**B) Survival rate of mice after sporozoite inoculation.** 1,000 and 10,000 sporozoites of wt and pbehd (-), respectively, were intravenously injected into 3 C57BL/6 mice each. Whereas WT mice died around d7-8 post infection, showing strong symptoms of experimental cerebral malaria (ECM), pbehd (-)-infected mice died of hyper-parasitemia around day 17, at no point during infection showing symptoms of ECM. Spz: Sporozoites.

**C) In vitro liver-stage development.** 10,000 sporozoites of wt and pbehd (-) parasites where inoculated on Huh7 cells and developed for 24 h and 48 h. Parasites where stained with α-HSP70 antibodies and analyzed by indirect immuno-fluorescence assay (IFA). A highly significant difference (P < 0.001) was detectable between liver-stage sizes of WT parasites and pbehd (-) at 48 h post invasion.

**D) Evans Blue staining of brains of infected mice.** Evan’s Blue Dye (EBD) was injected into mice when mice where showing ECM symptoms in the wt group and 2 hours later brains where taken out and pictures shot. Leakage of EBD into the brains of wt infected C57BL/6 mice indicates damage of the blood brain barrier (BBB) in these mice, whereas the BBB in pbehd (-) infected mice seems to be intact. Statistical analysis and plotting of the results was performed with GraphPad Prism V.6.00.
Chapter 4

Discussion

The aim of the work presented in this thesis was a twofold approach: 1) The establishment of a *P. falciparum* in vitro life-cycle in the host department and 2) the characterization of a newly identified protein in apicomplexans of the EHD-protein family in the two parasites *Toxoplasma* and *Plasmodium*.

4.1 Plasmodium falciparum transmission

The protocol established in this thesis led to *P. falciparum* infections of *A. stephensi* mosquitoes that were sufficient (sporozoite numbers and prevalence) to infect primary human hepatocytes as well as immortalized hepatoma cell lines and to isolate *Plasmodium* liver-stage RNA from these to investigate gene expression. We were able to keep the mosquito infections high enough during the course of the experiments but could not maintain a constant infection rate for more than just a few months. Since the feeding procedure, the membrane transfer of mature gametocyte cultures onto *A. stephensi* mosquitoes, was standardized only the change in the quality of the *Plasmodium* gametocyte cell culture or the mosquitoes may have led to the decline in the infection rate. Since it has been observed before that the age of the mosquitoes at the feeding day is quite crucial (Van de Vegte-Bolmer, M. and van Gemert, G.J., Radboud University Nijmegen Medical Center, personal communication) we tried to standardize the feeding procedure in regard to that. Unfortunately, the periodic mosquito breeding routine and the fluctuating availability of mosquitoes in the lab did not always allow to create the perfect timing of the generation of a mature *P. falciparum* gametocyte culture (characterized by a certain number of exflagellating gametes) with the correct mosquito age. Therefore the susceptibility of the mosquitoes to a *P. falciparum* infection for some feeding procedures might not have been high enough, even though it was always made sure that the mosquitoes are feeding enough blood. The susceptibility of mosquitoes is not only influenced by their pure age alone, but also by other factors. One of it being the innate-immune system of the *Anopheles* mosquito, for example, that has been shown to get alerted by *Plasmodium* ookinetes when these try to traverse the midgut epithelium. As a result many of the ookinetes are killed by complement-like mosquito-proteins or reactive oxygen species. The immune system is determined by genetic factors of the mosquito strains and might be influenced by long rounds of inbreeding under laboratory conditions. In addition to that, a recently published study showed that the gut microbiota of *Anopheles gambiae* has a strong impact on the susceptibility of the mosquitoes to *P. falciparum* infections. In this study the authors could show that the gut microbiota in different adult wild-bred mosquito populations differed widely due to bacterial contamination of their differ-
ent larval habitats. The difference in the gut microbiota also correlated with the ability of these mosquitoes to transmit *Plasmodium* parasites. Indeed, some of the bacteria were actually needed by the parasites for a successful transmission, whereas others correlated with non-susceptibility. The authors also compared insectary-bred mosquitoes to field-collected ones and found great difference in the bacteria strains mainly prominent in these mosquitoes. The authors speculate that the reason for that might be the diverse mosquito-diet under both conditions is favouring different bacteria species, something that has been shown to influence the gut microbiota before already\textsuperscript{301 302 303}. Quite recently the inhibitory effect on *Plasmodium* parasite transmission through destroying commensal gut microbes or alerting the mosquito immune system by introducing unusual alpha-proteobacteria (Wolbachia) in *Anopheles* has also been described as a transmission blocking tool\textsuperscript{304 305}. In summary, it is not surprising that our insectary-bred mosquitoes are influenced by environmental factors such as their diet, the larvae breeding water etc., that vary over time and thereby lead to a change in the mosquito immune system and their gut microbiota. This might have influenced the susceptibility of the mosquitoes to a *Plasmodium* infection and thereby might explain the drop in the infection rate in our experiments. This leads to the conclusion that for constantly high *Plasmodium falciparum* transmission rates the mosquito-breeding needs to be standardized and factors such as the pH of the breeding water, contamination of the larvae trays with bacteria and sources of other bacterial contaminations that might influence the growth of a gut ”*Plasmodium*-unfavorable” microbiota need to be controlled and maintained at a constant level. Another factor that might have influenced the *P. falciparum* infection rate in our experimental setup is the quality of the gametocyte culture produced \textit{in vitro} for the feeding of the mosquitoes. The aim for a sexual stage *P. falciparum* gametocyte culture is to create a large percentage of infected erythrocytes that form gametocytes within the culture. Therefore an asexual culture flask that is usually kept in a small volume and by replacement of iRBCs with fresh RBCs is maintained at a parasitemia below 5 %, is expanded into a larger volume and no fresh RBCs are added anymore. Thereby the parasitemia increases dramatically and gametocytes are formed as a result of density stress\textsuperscript{306}. The maturity of a gametocyte culture is represented by a high number of stage V gametocytes\textsuperscript{307} that are able to form gametes when activated by external triggers such as pH-change of the surrounding medium, temperature drop and a mosquito molecule, the xanthurenic acid\textsuperscript{308 309 310 311 312}. During this process that can be visualized under a light microscope the male gametocytes form eight exflagellated gametes (exflagellation center) that possess highly motile flagella\textsuperscript{309}. We used this process to evaluate the maturity of our gametocyte culture by examining the number of exflagellation centers per volume induced by temperature drop (cell culture to lab room temperature) under a light microscope. Even though we kept this number at a constant range for the feeding over time and therefore the quality of the culture should have been constant, this procedure might have been misleading. The exflagellation rate is only provid-
ing information about the maturity of male gametocytes, not female. As has been shown, though, a ratio of at least 50% of female gametocytes is needed to confer high infection rates\textsuperscript{313}. It has been reported, for example, that sex ratios in \textit{Plasmodium} sexual parasites vary in response to environmental factors such as host hormones etc. and that this influences the transmission success\textsuperscript{314}. Important to note hereby is, that the human serum taken for the parasite \textit{in vitro} culture in our setup was ordered from the Heidelberg University Hospital Blood Bank. This serum is expired donated material from donors of various genetic and health background and therefore the content is not standardized. Hence, the different batches of serum used for the parasite cultures might have had an influence on the gametocyte production. Therefore our evaluation of the maturity of our cultures by checking exflagellation may have been wrong since the sex ratio of the gametocytes may have been wrong. This may also have been influenced by the fact that the culture maintenance (exchange of the medium, inoculation of the cultures) was performed manually and not standardized. Other laboratories that have established a constantly running \textit{P. falciparum}-transmission system therefore installed an automated cell-culture system that allows them to rely on perfectly mature cultures at certain timepoints and to time their maturity with the maturity of the \textit{Anopheles} mosquitoes. In this setup they can also control the number of passages that have been made for maintaining the asexual cultures and check, if the rate of the gametocytogenesis, the formation of gametocytes, has dropped. This can happen if a parasite population has been passaged for many times in asexual cultures without having had to go through the mosquito cycle from time to time\textsuperscript{315} (Marga van de Vegte-Bolmer, Radboud University Nijmegen Medical Center, personal communication).

### 4.2 Characterization of EHD-proteins in apicomplexa

In this thesis I was able for the first time to identify a member of the EHD-protein family in apicomplexan parasites by \textit{in silico} analysis. Subsequently, I was able to characterize the protein in regard to its localization and function in the parasites \textit{Toxoplasma gondii} and \textit{Plasmodium berghei/Plasmodium falciparum}. The results of my thesis have contributed to a better understanding of EHD-proteins in apicomplexan parasites. It is not quite clear yet, if the results obtained for the single investigated organisms can be combined to one full picture or rather need to be viewed seperately. Especially, if it comes to the comparison of EHD-proteins in \textit{Toxoplasma} and \textit{Plasmodium} it might not be possible to compare localization and function of EHD-proteins in both organisms, because of the different lifestyle of both. Therefore most of the interesting results will be discussed individually in the following.
4.2.1 The apicomplexan EHD-protein family

The in silico analysis in this thesis has revealed that the apicomplexan parasites Toxoplasma and Plasmodium each possess a single ortholog of proteins of the EHD-protein family (Fig. 3.4). The protein clusters tightly within the apicomplexan clade and is closely related to their orthologs in C. elegans and in vertebrates (Fig. 3.4). While C. elegans and apicomplexa only seem to have one predicted member of the EHD-protein family each, vertebrates have four paralogs. One of the reasons for the number of different paralogous proteins in vertebrates could be their higher organisation into different tissues. It has been shown for mammalian EHD-proteins that some have different functions in different tissues (see 1.2.2). EHD3 and EHD4 for example are specifically expressed in the glomerular endothelium of kidney cells whereas the other two family members are not\textsuperscript{316}. In other mammalian cells at least some of the EHD-protein functions are redundant and depletion of just one member does not lead to major defects in these cells (see 1.2.2). The fact that also other protozoan single cellular organisms like Entamoeba are also predicted to have more than just one EHD-protein member (Fig. 3.4) seems to argue against this ”complexity-theory”. But so far only in silico data are existing for Entamoeba EHD-proteins making it difficult to interpret their existence. One could argue further against it, that also the multicellular worm C. elegans possesses only one EHD-protein, Rme-1, a protein shown to be involved in receptor-recycling events and also other cellular processes\textsuperscript{280}. But it has been shown that C. elegans can produce several different isoforms of Rme-1 by alternative splicing that can likely exert different functions\textsuperscript{280}. In addition to that, also the Drosophila ortholog Past-1 has shown to be differentially expressed in different transcripts\textsuperscript{317}. Most of the studies on mammalian EHD-proteins have lead to a clear picture of the EHD-protein function during one specific process, the endocytic recycling in cells. Here, different EHD-proteins take over a function in different endocytic trafficking events such as trafficking from the plasma membrane to the early endosome (EE), from EE to the endocytic-recycling compartment (ERC) or from the ERC back to the membrane or into protein degradation (see Fig. 1.10). Human EHD-1 exhibits the highest level of sequence homology to the single EHD ortholog expressed in invertebrate organisms and the sequence similarity of these orthologs is higher than between the EHD-paralogs in humans themselves. This leads to the conclusion that an ancestral cell had only one protein, quite similar to EHD-1, that later became multiplied in higher organisms to fulfill different roles in the increasingly complex organisms. It seems to be very unlikely that a complex process like endocytic recycling could be driven by just one EHD-protein member in apicomplexans alone, since protein and receptor-recycling needs to be tightly regulated. And it is not known wether isoforms of the EHD-protein in apicomplexa may exist, something that will have to be investigated in the future. But if there is only one EHD-protein version in these single-celled protozoans this is leading me to the following conclusion: Either the EHD-protein has only one specialized function in these organisms in a process that is not as complex as the
endocytic-recycling of higher eukaryotes, or different proteins than EHDs are taking part in this complex process.

4.2.2 Structure of the apicomplexan EHD-proteins

The in silico analysis performed in this thesis predicted that all characteristic domains of EHD-proteins are also present in the apicomplexa member. The analysis of the predicted amino-acid sequence showed a characteristic P-loop containing nucleotide binding site in the G-domain that possibly binds and hydrolyses ATP, even though this was not investigated in the present study. It has been shown for human EHD2 that the binding of ATP by the G-domain is required for the dimerization of two monomers (see chapter 1.2.1 and Fig. 1.11). Only upon dimerization the EHD2 homo-dimer is then able to bind to membranes of target organelles or structures via the helical middle domain of the protein. The ATP-binding and dimerization/oligomerization of the apicomplexan EHD-protein itself has not been investigated in this thesis but there are other results that lead to the assumption that also the apicomplexan EHD-protein might be able to dimerize: I was able to show that the deletion of the G-domain of TgRME-1 leads to a cytosolic localization of the fluorescently tagged protein in Toxoplasma gondii (see chapter 3.2.3 and Fig. 3.13)). This stood in contrast to the localization of the full-length protein tagged by the same strategy which localized to distinct punctated and tubular structures within the parasite (discussed in 4.2.3). A similar phenotype has been shown for the non-ATP-binding mutant T72A of human EHD2. In this study the mutation of Threonin to Alanin in the nucleotide-binding site prevented ATP-binding of the protein and thereby inhibited the dimerization of the protein in vitro. The overexpression of a tagged version of the same mutant in vivo led to a cytoplasmic localization of the protein in HeLa cells instead of a localization at tubules and punctated structures as seen for the full-length protein. The authors assumed that the lacking ability of the mutated protein to bind ATP and to oligomerize prevented it from binding to membranes. Therefore the protein did not show a localization to membranous structures anymore. The ability of membrane binding can also be assumed for the apicomplexan EHD-protein from its primary structure and homology to the human EHD-proteins and also from the localization studies shown in section 3.2. Certainly the depletion results in this thesis show that the G-domain of TgRME-1 is essential for its localization. But in addition, similar to human EHD2, TgRME-1 depleted from its G-domain and thereby from its ATP-binding site is loosing its ability to bind to membranes and remains cytosolic, indicating also for a need of the protein to dimerize via the G-domain to bind to membranes. One might argue that deleting the whole G-domain of TgRME-1 might destroy a lot more interactions of the protein by removing parts of its secondary structure than just preventing the ATP-binding of the protein. This is certainly the case and in future studies a mutation of just the ATP-binding site in this protein seems to be a more elegant method to narrow down the domain-function on its ability to bind ATP. Nevertheless, the depletion of the
G-domain in this thesis gives a first hint on the function of the G-domain in the *Toxoplasma Tg*RME-1 and probably the orthologous EHDs in apicomplexa in general, as well. Another important feature of the G-domain is the side-to-side KPFxxxNPF amino acid motif. This motif is important in the mammalian EHD2 protein for the oligomerization of the homodimer with other EHD2 proteins\(^{282}\). In this oligomer the EHD-domain of one dimer is interacting with the NPF motif of another to form the oligomer. Upon oligomerization the ATPase-activity is stimulated by the interaction. Interestingly, the apicomplexan EHD-protein ortholog does possess only the KPF motif, not the NPF (see chapter 1.2.1 and Fig. 3.18). Even more surprising is the fact that the second amino acid in the NPF tripeptide in both organisms, *Toxoplasma* and *Plasmodium*, is mutated from the hydrophobic amino acid Proline to a hydrophilic amino acid. In *Toxoplasma* Proline is mutated into Asparagine and in *Plasmodium* into Serine. This is surprising since EH-domains have been found to form a hydrophobic pocket that binds the hydrophobic residues of the xPF motif. This leads to the hypothesis that oligomerization does not play a major role in the working mechanism of the apicomplexan EHD or rather a weak interaction of the oligomers is present since only one of the side-to-side xPF motifs is conserved in this protein. An additional hypothesis is presented by the fact that, whereas the first of the two side-to-side xPF motifs is also present in EHD1, EHD3 and EHD4, the NPF motif is, like in apicomplexa, also not found in these paralogs of EHD2 even though they are thought to form oligomers\(^{318}\). Therefore it has been hypothesized that in these oligomers maybe other proteins that possess NPF motifs and interact with the EH-domains of the dimers provide a scaffold for oligomer-formation, something that still has to be experimentally confirmed. The G-domain of EHD-proteins is followed downstream of the domain by a helical middle domain and further downstream by an **EH-domain** (see chapter 1.2.1). The helical middle domain, that is known to bind to lipid membranes, and the EH-domain are connected by a linker sequence. This sequence comprises another xPF motif in all mammalian EHDs (GPF) that was shown to be an interaction site for both EH-domains in the dimer with the opposite monomer (see chapter 1.2.1). Interestingly, this xPF motif within the linker is also present in the apicomplexan EHD-protein which could be another indicator for the apicomplexan EHD-protein also being able to dimerize (Fig. 1.11). Nevertheless, in both, *Toxoplasma* and *Plasmodium*, respectively, the first amino acid of the xPF motif has mutated from a hydrophobic amino acid in mammalian EHDs (Glycin) to a hydrophilic amino acid (Serine or Threonine, respectively). It is not quite sure, yet, which impact this could have for the binding properties since most of the interaction is mediated by the Proline and the phenylalanine\(^{289}\). To investigate the function of the EH-domain of *Tg*RME-1 in this thesis I generated an overexpression-mutant of the protein tagged with a N-terminal fluorescent tag that lacked the EH-domain (see chapter 3.2.3). The experiments showed, that deletion of the EH-domain in this protein did not change its localization to a punctate structure within the parasite, similar to the full-length protein (localization discussed in chapter 4.2.3). From the
literature about human and other EHD-proteins it is known that the deletion of the EH-domain could have several impacts on the protein: 1) The EH-domain is known to be the interaction domain of an EHD-protein through which it binds to other proteins that harbour NPF motives. A deletion of the EHD-domain therefore would stop the proteins’ ability to interact with possible interaction partners. So far no data are present suggesting a specific interaction partner of the apicomplexan EHD-protein. Nevertheless, since the localization to the specific punctum structure of the parasite is not affected in the EH-domain deletion mutant this localization does not seem to be dependent on interaction partners mediated by an EH-domain-NPF motif-interaction. 2) The EH-domain has been shown to be important for oligomerization of human EHD-proteins as discussed above. Additionally, without certain amino acid motives present in the apicomplexan EHD-protein it is not quite clear, to which extend oligomerization is happening for these proteins at all. Nevertheless, if at all happening in the full-length protein, deletion of the EH-protein should inhibit oligomerization of the apicomplexa protein. This means, that also oligomerization is not necessary for the localization of the apicomplexan EHD-protein to the punctum structure within the parasite. This is in agreement with a third consideration: 3) EH-domains, in addition to binding to NPF motives in proteins, have also been shown to be interacting with lipid-molecules such as phosphoinositides. The binding of these lipids leads to a localization of EHD1 to long tubular endosomal membranes. I was able to show a localization of $T_g$RME-1 in tachyzoites and $P_b$EHD in liver-stage parasites on tubular structures, as well (discussed in chapter 4.2.3). Both, the mutation of the lipid-binding site in the EH-domain and also the truncation of the whole domain of mammalian EHD1 led to a change in the cellular localization of the protein from a punctum-tubular intermediar phenotype to a solely punctated phenotype\textsuperscript{239}. This indicates, that the EH-domain, via the binding to phosphoinositides, is mediating the binding of EHD-proteins to tubular structures in the cell, whereas the localization to punctate endosomes is not affected by this\textsuperscript{239}. The same seems to be true for the *Toxoplasma* $T_g$RME-1 that also still showed localization to the punctum when the EH-domain was deleted (Fig. 3.12). Since localization of the $T_g$RME-1\text{Δ}EH deletion mutant to tubules was not further investigated in this thesis a future investigation will be needed to confirm the assumptions made above (Fig. 3.12). Most data gained in this thesis about the structural features of the apicomplexan EHD-protein were obtained in *Toxoplasma*. Can they be transferred to Plasmodium as well? Is there a difference in both proteins? Even though both proteins share a high sequence similarity of almost 60% and their similarity of the G- and EH-domains is even higher there is one striking difference about both proteins and even all other EHD-proteins (Fig. 3.18). *Toxoplasma* RME-1, in contrast to all other EHD-proteins investigated so far, has a long C-terminal stretch downstream of its EH-domain (Fig. 3.18). This stretch is more than 60 amino acids long and possesses 6 experimentally confirmed phosphorylation sites\textsuperscript{290}. Since all other EHD-proteins do not have this stretch it seems unlikely that *Toxoplasma* kept it if
it does not fulfill a defined purpose. The strong phosphorylation and its expression profile (upregulation during cytokinesis) could indicate for a function in signal transduction, cell-cycle related activation or an interaction of this C-terminal tail with other proteins via the phosphorylated sites. But an in silico analysis of this C-terminal stretch did not show significant homology with special protein domains known or any other protein sequence in other organisms. Therefore it will need further analysis to narrow down a specific function on this protein sequence.

4.2.3 Localization and function of EHD-proteins in apicomplexa

The localization of the apicomplexan EHD-protein was investigated in this thesis in the three different organisms Toxoplasma gondii, Plasmodium berghei and Plasmodium falciparum. The studies were performed applying different techniques in different organisms (T. gondii and P. falciparum: Fluorescently tagged EHD, live-imaging and fixed parasites; P. berghei: EHD antibody detection, fixed parasites only) and in different parasite stages. Therefore the results will be discussed separately for each organism in the following and in the end compared to each other, as far as possible. To localize the Toxoplasma TgRME-1 within intracellular tachyzoite cell cultures a second copy of the endogenous rme-1 gene was integrated randomly into parasite’s genome. Upstream of the open-reading frame of the gene a sequence encoding a destabilization domain (DD) followed by a sequence encoding a mCherry fluorescent tag were cloned (compare Fig. 3.6). A time-course assay of the DDmCherry TgRME-1-expression after Shield-1-induction (1µM) showed a time dependent expression of the protein by western blot in relation to the amount of time spent after addition of Shield-1 (Fig. 3.7). The fusion protein blotted on a western blot under reducing conditions revealed a molecular weight of slightly higher than 100 kDa which is in the range of the calculated weight (TGME49.031210 66.7 kDa; mCherry-tag 28.8 kDa; DD-tag 12 kDa). After two hours of protein expression only a weak cytosolic mCherry signal in IFA-studies could be observed within the parasite. This changed after 4 hours of expression when few fluorescently labeled puncta occurred within the parasites (Fig. 3.7). Over time these puncta became more apparent but even though there was still an increasing amount of protein produced between 8 and 24 hours (ON) after start of the induction as seen on the western blot, there was no change in the localization of the fluorescent signal of the parasites after 8 hours. This indicates for a saturated location of the protein on the cellular level and that further overexpression of the protein does not change the phenotype anymore. This was supported by the fact that also the increase of the Shield1-concentration did not change the localization of the protein in the cell. Most of the times in fixed parasites the mCherry signal could be found on only few puncta within the parasites, one bigger punctum (a few hundred nanometer in diameter) and some smaller ones. These structures could either be located at the apical end of the parasites or at the basal end. To determine the origin of the labeled puncta a colocalization study was performed co-labeling known parasite organelles of the
secretory and endosomal system in addition to the fluorescently tagged \( TgRME-1 \) (Fig. 3.11). The endosomal system was chosen since EHD-proteins in mammalian cells have been shown to localize to this network. No colocalization of the mCherry-labeled organelle could be found for parasite secretory organelles such as Rhoptries, Micronemes and compartments of the endosomal system such as the early endosome, endosomal-like compartment and the late endosomal-like compartment. Nevertheless, the resolution of the images might have been too low to show partial colocalization of the smaller vesicular mCherry-labeled puncta with some of these organelles. But the big punctum labeled by the mCherry-tagged protein could not been seen colocalizing with one of the mentioned organelles in any parasite. There is no hint so far which compartment within the parasite \( TgRME-1 \) could localize to since also another round shaped compartment, the apicoplast, of about the same size of the big RME-1-punctum could not be colocalized. Another similar structure has been seen before for the EHD-protein related protein dynamin-related protein B (DrpB)\(^{319}\) (both share a dynamin-like G-domain). A tagged overexpressed version of this protein in \( Toxoplasma \) tachyzoites also showed an accumulation of the DrpB close to but distinct from the Golgi-apparatus. The protein, in addition, has been proven to be important for the biogenesis of secretory organelles in this study. During replication of the parasites the DrpB accumulation breaks up and reassembles again within the newly formed daughter-cells. The same dynamics were shown for the DDmCherry \( TgRME-1 \) labeled compartment in this thesis when living parasites were imaged live under a fluorescent microscope in a live-cell chamber. Most of the time of the replication cycle the big punctum remained at a distinct focus at the apical part of the parasite. Via short-term live-imaging I was able to show that during this phase small mCherry-labeled vesicles are rapidly shuttling between the big punctum and the back end of the parasite (supplementary movie 2). When the parasites start to form daughter-cells, analogous to DrpB, the big punctum fragments and smaller fragments localize to the apical tips of the daughter-cells, as shown by long term imaging (Fig. 3.9 and Fig. 3.10; supplementary movie 1). At a later timepoint during the replication phase the apical mCherry-labeled compartment of the mother cell moved to the back end of the cell, squeezing alongside the IMC of the newly forming daughter parasites. After cytokinesis the \( TgRME-1 \) compartment has reemerged at the apical tip of the parasite but also remained at the basal part of the vacuole of the two newly formed parasites. Since dynamics and localization of DrpB and \( TgRME-1 \) appeared quite similar a colocalization of both proteins was investigated in this thesis (supplementary data; Fig. 5.1). Unfortunately, no colocalization could be observed for both protein compartments. This leads to the conclusion that both proteins probably localize to different cytosolic pool-like structures but nevertheless underly similar dynamics and might exhibit comparable functions during endodyogeny of the parasites. Another recently identified new compartment in \( Toxoplasma gondii \) (VAC) was shown to harbour a cathepsin-like protease, \( TgCPL \)\(^{320}\). The authors showed a function for the protease in the proteolytic processing of prepeptides.
destined to the secretory organelles of the parasite. They speculated about the
punctate structure it localizes to it might be a lysosome or a lytic vacuole. Whereas
the VAC did not colocalize with the DrpB-accumulation within the parasite either,
it showed at least partial colocalization with endocytic organelles. Interestingly, also
this VAC-compartment was shown to be a dynamic organelle that undergoes struc-
tural reorganisation during the cell cycle in their study. Comparable to \( TgRME-1 \) in
this thesis, also small \( TgCPL \) positive structures in the study of Parussini et al.\(^{320} \)
are forming during fragmentation of the VAC upon daughter-cell formation and can
soon be found in the apical part of the daughter-cells. The authors could not distin-
guish in their study, if these structures move to the apical end of the daughter-cells
or rather to the posterior end of the mother cell where they might take part in the
proteolytical processing of proteins in the basal body. In the latter case the VAC
compartment in the daughter-cells would be formed and loaded with \( TgCPL \) \textit{de novo}
or filled up with material from the compartment at the basal end. The same
question could also not be answered for the \( TgRME-1 \) labeled compartment in this
thesis where at least a movement of the compartment to the basal end of the para-
site could be shown. But here also smaller compartments appear at the apical pole
of the daughter-cells before the movement of the big compartment to the posterior
pole has been initiated. It will be interesting to study in the future, if the \( TgRME-1 \)
labeled compartment might be the VAC identified in the study mentioned above.
But the comparison of the localization and colocalization in both studies, the one
from Parussini et al. and results of my thesis, lead to the hypothesis that both
might rather be two different vacuolar structures within the \textit{Toxoplasma} parasite
and both possibly members of the endosomal system. The similar dynamics during
the replicative phase of the parasite leads to the hypothesis that there might be a
general inheritance mechanism used in \textit{Toxoplasma} to distribute organelle members
of the endosomal system into the daughter-cells or a common specialized function
for the proteins inhabited by these organelles during replication. If VAC and the
\( TgRME-1 \) compartment are not the same structure then what the latter consists of
could not be defined in this thesis but will be interesting to study in the future.

One possibility for the functionality of the compartment identified in this thesis
would be that of an \textbf{endosomal recycling compartment (ERC)}. Since verte-
brate EHD-proteins have been found to localize to the ERC (see chapter 1.2.2) a
colocalization with this organelle might be possible. Nevertheless, an ERC has never
been identified in \textit{Toxoplasma} so far and its existence would raise the question: What
would be recycled or transported to the ERC? The intracellular forms of apicom-
plexan parasites reside within a parasitophorous vacuole in mammalian cells. This
means their internal organelles are surrounded by three membranes that block them
from nutrients in the surrounding medium: The plasmamembrane of the host cell
(HPM), the parasitophorous vacuole membrane (PVM) and the parasite membrane
(PM) (see chapter 1.1.2). To acquire nutrients they can not synthesize themselves
\textit{de novo}. Therefore the parasites need to take up nutrients from their host cell cross-
ing the lipid bilayers of the PVM and the PM. Whereas the PVM contains pores facilitating passive diffusion of low molecular weight molecules into the PV the PM is not permeable and needs active processes to be crossed\textsuperscript{321}. One of these active processes is endocytosis, still a mystery in apicomplexa today and hence not fully understood. Nevertheless, first evidence has been found almost two decades ago already that endocytosis is in fact occurring at a special site in the anterior half of the parasite at the parasite membrane, the micropore\textsuperscript{322}. This invagination of the plasma membrane penetrates the parasite cytoskeleton, the IMC, to allow vesicular trafficking into the parasite cytosol. In eukaryotic cells the endocytosis of nutrients from the outside of the cells can be mediated either via fluid-phase endocytosis or receptor-mediated endocytosis. During receptor-mediated endocytosis a receptor on the surface of the cell is internalized via endocytosis while having bound a ligand. This allows a specific uptake of molecules that are existing in a low concentration at a high rate from outside the cell. The vesicles containing the receptors having bound (or not having bound) their ligand are then transported to the early endosome (EE) from where their content will be further transported to the final destination (see Fig. 1.10). The receptors internalized together with their ligands will be recycled back to the surface for several additional rounds of receptor-mediated endocytosis via the fast route directly back from the early endosome, or through a slower process that is organized at the endosomal recycling compartment (ERC)\textsuperscript{223}. In these receptor-mediated endocytosis and receptor-recycling processes many proteins of the EHD-family are involved (see chapter 1.2.2). It is not clear because of the lack of knowledge about endocytosis in apicomplexa, though, if receptor-mediated endocytosis also occurs in these organisms and which receptors would be recycled. But a conserved sorting motif in apicomplexa for intracellular trafficking known from mammalian cells indeed indicates this process might also be conserved in apicomplexans\textsuperscript{325}. Neither is it known, if an ERC exists in these parasites in addition to early and late endosomes. But in addition to recycling receptors back to the cell surface the ERC and recycling endosomes have been shown to play an important role in cytokinesis, as well\textsuperscript{324, 325}. By trafficking of recycling endosomes and Golgi-derived vesicles the cells contribute membrane to the cleavage furrow. In addition, they have been shown to facilitate abscission during the last phase of the cytokinases, an ability that is also known for EHD-proteins. Interestingly, the live-imaging studies of the \textit{TgRME-1} in Toxoplasma have shown a localization of the protein to the very end of the parasites after the cellular division has occured. Here the protein localized to the connection point of the two daughter-cells and the residual body where abscission might be facilitated by the \textit{TgRME-1} protein.

Another nutrient uptake different to receptor-mediated endocytosis the apicomplexan EHD-protein might be involved in is the scavenging and uptake of lipids from host cells. It has been shown for \textit{Toxoplasma gondii} for example that the parasite can mobilize lipid resources from the host cell for its intracellular growth. The PVM of the parasite after formation inside the host cell becomes rapidly associated to sites
of the host cell lipid biosynthesis such as the ER and mitochondrial membranes. In addition, it has been shown that *Toxoplasma gondii* scavenges lipid resources such as low-density lipoprotein (LDL) and cholesterol from the host cell to use them as a lipid source for the mass production of membranes. Therefore the parasite stores the uptaken lipids in so-called lipid bodies and endomembranes and thereby maintains its lipid homeostasis. During replication neutral lipids are transported from the lipid body to the ER and the Golgi to incorporate them into the lipid metabolism. But the exact composition of lipid bodies is not yet known.

Even though the parasites are able to produce fatty acids, precursors of all kinds of lipids, through a special FASII-pathway, some parasite stages and some parasite strains do need take up host-cell derived lipids to ensure optimal growth (see chapter 1.1.2). EHD-proteins in apicomplexans might be mediators of the uptake, storage and/or trafficking of these lipids before and especially during cellular division. At this timepoint in the lifecycle there is a very high demand of lipids for the production of membranous structures in daughter-cells. Because of their lipid-binding ability and their ability to pinch off vesicles from membranes apicomplexan EHD-proteins might be a very good motor to help sort and distribute lipid resources during replication of the parasite. EHD1 for example has already been shown to be involved in the regulation of cholesterol homeostasis and lipid droplet storage in mammalian cells. The *TgRME-1* labeled compartment together with the structures seen for *Plasmodium* liver stages discussed below might represent lipid storage compartments of these parasites that are reorganized and trafficked during replication to the newly forming membranous structures. To investigate localization of the EHD-protein in *Plasmodium berghei* an antibody, generated against the amino-acids 339-533 of the *Plasmodium falciparum* EHD-protein (kindly provided by Dr. Tobias Spielmann, BNI Hamburg), was used in IFA studies on sporozoites, liver-stages and blood-stages. Since the respective amino acid sequences of both orthologous proteins, PbEHD and PfEHD, respectively, are highly similar the PfEHD-specific antibody promised to be detecting the protein in *P. berghei*, as well. Indeed, using the antibody on PFA-fixed *P. berghei* sporozoites led to a specific signal of PbEHD in a vesicular pattern within the sporozoites (see Fig. 3.23). This staining looked very similar in all salivary gland sporozoites observed, indicating for rather static dynamics, and the vesicles seemed to be distributed more or less homogenously over the whole body of the parasite. Most of the vesicles located at the periphery of the cytosol of the parasite in close association with the plasma membrane, even though a lack of antibodies generated in a background other than mouse did not allow a co-staining with this membrane and other cellular markers. Depleting parasites from PbEHD (discussed in detail further below) did not lead to a impaired development of *P. berghei* salivary gland sporozoites which leads to the conclusion that the protein is not needed during this phase of the parasites’ lifecycle. Therefore the abundance of the PbEHD-labeled vesicles suggests them being a storage compartment harboring proteins or nutrients such as lipids etc. that are needed in later stages. It has
been shown before that parasite stages already prepare themselves molecularly before they are transmitted to a new host that has different environmental conditions. There they establish as different stages that require a rapid molecular and cellular re-programming of the parasite. For example gametocytes have been shown to store translationally repressed mRNAs and proteins that are required much later during formation of ookinet in the mosquito. In addition, protein storage has been shown in Plasmodium sporozoites, as well. Most of these stored proteins known so far are required for gliding motility, invasion of liver cells and formation of the parasitophorous vacuole of the parasites and are released only by specific triggers in the mammalian host. For example TRAP, a protein essential for the sporozoite motility and cell invasion is stored in secretory organelles, the micronemes, and released from the parasite upon a calcium trigger. Most of the known proteins stored in sporozoites are secretory proteins released from secretory apical organelles, since they are needed immediately upon contact with host cells. The fact that the PbEHD pattern in sporozoites suggests no localization of the protein to apical organelles leads, together with the lack of any indication that it might be secreted at all, to the assumption, that the protein is not secreted and needed upon host cell contact.

Nevertheless, the presence and abundance of the protein in IFA studies in this thesis during the sporozoite stage argues for a function of the protein shortly after the entry into the host. This is in concordance with the results obtained during liver-stage development for a pbeh (-) parasite created in this thesis. In these parasites the pbeh open reading frame (ORF) was disrupted by integrating a plasmid carrying a resistance marker via single-crossover into it. Thereby, even though transcription might still occur, translation of the protein is most unlikely. A double crossover replacement strategy was also followed up to completely remove the open-reading frame from the parasite but was not successful because of an inability to amplify the 3'UTR from the ORF of the gene. This goes in line with the results of Pfander et al. who were also not able to include the 3'-part of the gene into their recombination vectors. A reason for that might be a tight regulation of the gene expression of this gene and the resulting tight packaging of the gene by histones and other DNA-binding proteins on genomic level. Thereby the secondary structure of the locus at the 3' part of the gene might not be accessible for amplification and cloning strategies. In this thesis, by integration of the whole plasmid into the pbeh ORF the sequence was disrupted. A single-crossover integration can be reversible and removed by the parasite from its genome. Therefore the parasites where checked again for the existence of a wildtype population after having completed a lifecycle.

No reversion to the wildtype could be observed in these cases. The recombinant parasites did not show any growth defect during in vivo blood-stage and mosquito-stage development and during in vitro liver-stage development 24 hours after invasion of hepatocytes. But they were significantly smaller after 48 hours growth in vitro in comparison to wildtype parasites. The invasion of sporozoites into hepatoma cells
CHAPTER 4. DISCUSSION

4.2 did not seem to be affected since the numbers of liver-stages in this setup did not differ from the ones obtained for wildtype parasites. This also indicates that PbEHD has a function for Plasmodium parasites during the liver phase. In addition, the delay in liver stage growth was also confirmed by in vivo data showing a significant delay of prepatency of mice infected with the pbehd (-) sporozoites in comparison to wildtype-infected mice.

When the localization of PbEHD was investigated in wildtype liverstages in vitro using the antibody described above an interesting pattern could be observed (see Fig. 3.24 and Fig. 3.25). The dynamics of this pattern seemed to be similar to the ones observed for the TgRME-1 compartment in Toxoplasma gondii discussed above. In liver stage trophozoites 24 hours after invasion the protein localized to a single compartment within the parasite. This compartment had a strongly antibody-labeled core, indicating for a great amount of protein, and tubulated structures that emerged from the core into the cytosol of the parasite. Interestingly, in most of the parasites the core located to the periphery of the parasites in close association with the host cell nucleus. In later stages 48 hours after invasion, when schizogony is taking place, the PbEHD compartment fragmented and split up into vesicular structures and tubules that seemed to localize around the nuclei of the newly forming daughter merozoites. Together with the fact, that in pbehd (-) parasites the growth of the liver parasites between 24 and 48 hours is slowed down I would hypothesize a function of PbEHD in liverstages for the supply of the parasites with nutrients. Scavenging of nutrients from the host cell has already been shown for P. berghei liver stages. Quite recently, it has been shown that an essential cofactor for enzymes, lipoic acid, is most likely taken up via the close association of the parasite and the host cell endoplasmic reticulum, though the exact mechanism is not known, yet. In this study the blockage of the lipoic acid uptake via an lipoic acid analogue did have a great impact on the liver stage parasites during later stages of development, when schizogony was occurring, and led to an impaired but not complete blockage of full liver stage development. The fact that these data are quite similar to the ones observed in this thesis for PbEHD does not necessarily mean that PbEHD is involved in lipoic acid uptake. The localizations of lipoic acid and PbEHD in liver stages differ to some extend, anyways. But it underlines again how important nutrient uptake for schizogony and maturation of liver stages is and that depletion of some nutrients can lead to a phenotype observed for pbehd (-) parasites.

Another process that eukaryotic cells are using for nutrient supply is autophagy. Even though the process is one of three different cell-death mechanisms it can also be used to degrade the cell’s own constituents upon nutrients starvation or changing surrounding conditions. During macroautophagy cytoplasmic components or organelles are engulfed by lysosomal membranes for degradation. Autophagosome formation is a process that is mediated by autophagy-related proteins (Atg) that have interestingly also been identified in apicomplexan parasites. It has been shown for Toxoplasma
for example that depletion of TgAtg3 causes growth inhibition of the parasites\textsuperscript{343}. Also, the mitochondria in these parasites show anomalies indicating for a defect in the autophagy of these organelles\textsuperscript{343}. Furthermore, \textit{Plasmodium berghei} liver-stage parasites have been shown to harbour autophagosome-like double-membrane structures that might be involved in degradation of of micronemes\textsuperscript{344 345}. In addition to that, Atg8 localizes to abundant vesicles that are organized in a reticular network in \textit{P. berghei}\textsuperscript{345}. During the erythrocytic stage, in contrast, Atg8 was found to localize to the apicoplast\textsuperscript{340}. Autophagy in exo-erythrocytic stages, even though not well studied yet, might represent an important step of the transition of Sporozoites into liver-stages and from these into merozoites. During both transformation steps organelles and structures such as the secretory organelles, the IMC, the nucleus and mitochondria need to be degraded or built up and segregated. In \textit{Plasmodium berghei} liver-stage parasites it has been shown for example that during a so-called cytomere-stage the parasite apicoplast begins to reorganize and to be segregated to the newly forming merozoites\textsuperscript{346}. The segregation pattern of the apicoplast shown in the study of Stanway et al.\textsuperscript{346} looks very similar to that of \textit{PbEHD} in liver-stages. In addition to that, also in \textit{Toxoplasma} parasites autophagic vesicles have been shown to exist especially in intracellular dividing parasites\textsuperscript{343}, similar to TgRME-1. Therefore the TgRME-1-labelled compartment in \textit{Toxoplasma} and the PbEHD-1 compartment in \textit{Plasmodium berghei} liver-stage trophozoites might represent either an autophagosomal-like compartment or might at least take part in the degradation and segregation of organelles during the replication of the parasites. The PbEHD-protein’s catalytic abilities might help to structure the complex membrane dynamics of these processes.

The ability of EHD-proteins to bind and bend membranes and to transport lipids as discussed above for TgRME-1 would make it likely to assume a lipid supply function for PbEHD. \textit{Plasmodium} parasites, like \textit{Toxoplasma} as well, are in great need of lipids throughout their lifecycle. During schizogony in the liver one mothercell can produce thousands of merozoites that all are individual cells requiring membranes to surround them. This phase represents one of the fastest growth rates among eukaryotic cells known so far\textsuperscript{93}. To provide itself with enough lipids (and precursors such as fatty acids) to built membranes the parasite has established two different methods, a \textit{de novo} synthesis and in addition a mechanism to scavenge lipid resources like fatty acids from the host cell (see chapter 1.1.2 and Fig. 1.3). The scavenging of lipid resources does likely involve host cell specific proteins that interact with parasite-specific proteins to recruit lipids to the parasite. It has been shown for example that UIS3, a parasite-specific protein upregulated in invasive sporozoites and known to be essential for the liver stage development\textsuperscript{347 348}, is located within the PVM\textsuperscript{349} with its C-terminal tail reaching into the cytoplasm of the liver cell. Thereby it can interact indirectly with fatty acids via direct interaction with liver fatty acid binding protein (L-FABP)\textsuperscript{349 350}. Deletion of UIS3 in the parasites also renders the parasites unable to develop into fully mature liver shizonts. Furthermore, downregulation of
L-FABP in liver cells by RNAi greatly inhibits proliferation of *Plasmodium* parasites whereas overexpression promotes growth\(^{449}\). By binding to L-FABP UIS3 may help to recruit fatty acids to the PVM and into the PV, but how these are reaching the cytosol of the parasite is not known. The further investigation of *Pb*EHD may help to solve this mystery. Besides, other proteins like EXP-1 (Nyboer et al., unpublished observations) and *Pf*E1590w\(^{351}\) have been shown to interact with apolipoproteins in liver cells. Both parasite proteins are located within the PVM and even though apolipoproteins have rather shown to be involved in parasite invasion into host cells it cannot be ruled out that they are also serving as a lipid source for liver stage parasites. The data acquired in this thesis together with the recent literature suggest that *Pb*EHD might be involved in storage and trafficking of lipid storage compartments/vesicles or liposomes within the liver stage parasite and in later liver stages to help tubulate and form membranes around the merozoites building up within the mother cell.

Localization of the EHD-protein in *Plasmodium* blood stages was carried out in two different ways: *Plasmodium falciparum* EHD was N-terminally tagged with a GFP-tag and its localization was analyzed in fixed parasites (see chapter 3.2.5). This strategy revealed a localization mainly to the plasma membrane of the parasites and more prominent to a distinct spot close to it. In contrast to that, using an antibody against the protein in *Plasmodium berghei* in IFA and immuno-EM (iEM) studies showed a localization of the protein distributed over the whole parasite, but mainly associated with membranous structures (compare Fig. 3.19). The signal obtained from both was rather low, though, indicating for a low abundance of the protein in blood stages. A semi-quantitative analysis of the structures labeled by anti-EHD antibodies in the iEM revealed that most of the protein seems to be locating to structures in the cytoplasm followed by vesicular structures/endoplasmic reticulum and the nucleus. Interestingly, also mammalian EHD2 has recently shown to be travelling to the nucleus where it represses transcription\(^{266}\). Most interestingly, the iEM analysis also showed a localization of *Pb*EHD to the red blood cell membrane and the red blood cell lumen, leading to the assumption that this protein might also be exported in blood stages. Even though both techniques to identify the localization of the EHD-protein in both, *P. berghei* and *P. falciparum*, differed, a similar localization to membranes could be observed. Whereas in *P. falciparum* the tagging revealed a prominent localization of the protein to the parasite membrane and likely the cytostome (Dr. Tobias Spielmann, BNI Hamburg, *personal communication*), an assumed hotspot for endocytosis in blood stages, the data obtained with antibody labeling from *Pb*EHD indicated the protein might be located at different intra- and extracellular organelles, probably shuttling between them. For the comparison of both, *Pf*EHD and *Pb*EHD in blood stages, it has to be kept in mind that the tagging of the *Pf*EHD (data provided by Dr. Tobias Spielmann, BNI Hamburg) was carried out C-terminally. This strategy might interfere with the interaction of the protein with interaction partners and therefore may lead to a mislocalization of the protein.
Therefore future studies in *P. falciparum* should also include antibody labeling of the PfEHD in blood stages. The only low amount of data obtained for blood stages in this study does not explain the protein’s function in these stages. But the normal viability of the pbehd (-) blood stages leads to the conclusion that the protein is not essential for these stages. Nevertheless, the affects might be more subtle and not visible in a parasite growth curve.

In a complementation experiment I tried to investigate if both proteins, the *Toxoplasma Tg*RME-1 and the *Plasmodium falciparum* PfEHD can be transferred into the other respective organism. If so the localization of both was to be compared. This localization then should have led to a conclusion about the comparability of the function and similarity of the protein structure of both orthologs. Meaning, that if the orthologus protein localizes in a same pattern than the endogenous protein both probably have the same architecture, the same interaction partners and the same structures they can localize to and thereby they would be functional in the respective other apicomplexan parasite, as well. This complementation project was performed together with Florian Kruse (BNI, Hamburg) who conducted the experiment in *P. falciparum*. To allow the comparison between the orthologs, both proteins, the TgRME-1 and the PfEHD, were expressed with the exact same strategy within the respective organism (DDmCherry N-terminal tag in *T. gondii* tachyzoites; GFP-tag C-terminal in *P. falciparum* blood-stages). Interestingly, the orthologous proteins did not localize to the same compartment within the same organism but each protein localized to the same parasite structure regardless of the parasite. This means, first of all, that both proteins are determined to localize to distinct subcellular structure by their intrinsic protein architecture. Alternatively it could mean, that, if interaction proteins are also needed to direct the proteins to distinct structures, these interaction partners also seem to be present in the sister apicomplexan parasite. Nevertheless, I was never able to stably express the tagged version of PfEHD in *Toxoplasma gondii* and the parasites did not seem to grow very well. This could indicate that, even though PfEHD does not localize to the same spot within *Toxoplasma* as the endogenous protein, it still might be inhibiting processes within the parasite or block interaction partners away from the endogenous protein and therefore is toxic to the parasite. One of the problems of complementing both, TgRME-1 and PfEHD, might have been the selection of the wrong stage for the comparison. Both parasites, *Toxoplasma* and *Plasmodium*, have different life-cycles with different developmental stages and host-cells they are residing in (chapter 1.1.3). *Toxoplasma gondii* tachyzoites and *Plasmodium falciparum* asexual blood-stages where choosen for complementation and localization studies because of technical issues. But even though they are both asexual intracellular stages of their life-cycle tachyzoites are residing in nucleated humane foreskin fibroblasts (in cell culture) whereas *P. falciparum* asexual stages are growing within non-nucleated human red blood cells. Both cells represent totally different environments for the parasites and under such different conditions the need and function of EHD-proteins might be different. From the
localization studies of PbEHD in *Plasmodium berghei* liver-stages and from the more similar cellular environment of liver-stage parasites and tachyzoites it seems likely that they might share a more common EHD-protein profile than the one observed for tachyzoites and blood-stages. Therefore a complementation study of *Tg*RME-1 in *Plasmodium* liver-stages in future might lead to a more secure hypothesis about the comparability of function and localization of both orthologs in apicomplexa. **In summary** it can be concluded from this thesis that EHD-proteins in apicomplexan parasites most likely take part in the nutrient supply of the intracellular parasites and/or in autophagocytotic-like events to promote organelle-segregation. During the growth phase of the parasite the protein localizes to a single compartment within the parasite that may represent a storage compartment such as a lipid droplet or an ERC-like compartment for lipids, recycling endosomes or other nutrients. During this phase the EHD-protein takes part in vesicular trafficking most probably from hotspots of endocytosis to the storage compartment and may also be involved in the uptake of lipids from the host cell. Upon cellular division of the parasites, during endodyogeny and schizogeny, the storage or autophagosome-like compartment fragments and is distributed to the forming daughter-cells and locates to tubules as well as vesicular structures. During this phase the protein is most-likely assisting to distribute nutrients via recycling endosomes to the forming cells or building up new membranes and segregating organelles. In addition, it is creating a new pool of nutrients within the daughter-cell. During the late stage of the cytokinesis the apicomplexan EHD-protein might also assist with the abscession of the parasites from each other.

**4.2.4 Virulence of the *P. berghei* ANKA strain depleted of PbEHD**

When *pbehd (-)* sporozoites (discussed in chapter 4.2.3) were injected into C57BL/6 naive mice a delay in prepatency in these mice of about one day in comparison to wildtype PbA infected mice was observed. This is in agreement with the observations made for the *pbehd (-)* liver stage development observed in *in vitro* development assays where I could show that the deletion of PbEHD leads to a slowed-down liver-stage development of the parasites. The fact that the parasites reach the blood of the mice *in vivo* at all, only later than the wildtype, shows that they are in general able to fully mature in the liver cell to produce merozoites and to infect RBCs. The *pbehd (-)* parasites were generated in the *Pb*ANKA (PbA) background. The wildtype PbA parasites normally are able to induce experimental cerebral malaria (ECM) in C57BL/6 and CBA mice (ECM model explained in chapter 1.1.4). Very surprisingly, when C57BL/6 mice were infected with *pbehd (-)* sporozoites they were protected from developing ECM in 100% of the cases, went into hyperparasitaemia and died several days later than expected. In contrast all of the mice inoculated with PbA wildtype parasites developed ECM and died around day 7-8. But what is the connection between the development of ECM and *PbEHD*? From what is known about the generation of CM in the mouse model (chapter 1.1.4) two scenarios can
be envisaged: Either \textit{pbeh}d (-) parasites are different to the wildtype in their ability 1) to \textit{sequester} as bloodstages or 2) to alert the \textbf{immune system} of their host.

1) Which role could \textit{P}b\textit{EHD} play in the sequestration of bloodstages? Even though in this thesis a role for \textit{P}b\textit{EHD} in receptor-recycling was not investigated, there is still the possibility that the protein is involved in this process, at least in blood-stages. As discussed in chapter 1.1.4 a role for parasite-specific receptors on the surface of infected red blood cells (iRBCs) has been shown in the generation of human cerebral malaria. By binding to endothelial receptors via parasite-specific surface proteins the iRBCs sequester and block capillaries in the brain that block blood the flow and lead to the recruitment of immune cells. Even though the role of sequestration for the generation of ECM is not as clear as in human CM a few observed factors have led to the conclusion that parasite-specific receptors might be involved in this model as well (compare chapter 1.1.4). Therefore the possibility exists that \textit{P}b\textit{EHD} is involved in the receptor-recycling of adhesion molecules on the surface of RBCs. The depleted expression of the protein in \textit{pbeh}d (-) parasites would then lead to a reduced expression of surface markers of the iRBC because the recycling and trafficking back to the surface is not possible anymore. It has been shown for example, that siRNA knock-down experiments of human EHD-proteins resulted in a blockage of the transferrin receptor-recycling of HeLa cells that remained within the perinuclear region of the cell instead of trafficking to the cytoplasm membrane as shown for EHD-expressing wildtype cells\textsuperscript{262}. Nevertheless, since no receptor has been identified in \textit{P. berghei} so far that is mediating iRBC-endothelium contact this receptor-recycling hypothesis could not be tested in this thesis. But to test if solely the bloodstage phase is responsible for the protection from ECM symptoms in \textit{pbeh}d (-) infected mice I circumvented the liver phase by directly transferring blood containing 1x10\textsuperscript{6} iRBCs to naive C57BL/6 animals. But surprisingly 2 out of 3 mice in this setup were not protected from severe symptoms and died from ECM. This means that even though the bloodstage phase and receptor-recycling therein might play a partial role in the protection from ECM in the \textit{pbeh}d (-) C57BL/6 model the main protection is mediated by the liverstage phase. During the liverstage the second factor that is involved in the generation of human CM and ECM might play a role, the immune system.

2) The immune system has been shown to play an important role in the generation of human CM and ECM (chapter 1.1.4). But it is mostly unknown so far which role the immune-reaction generated by the liverstage parasites is playing in the generation of CM. Nevertheless, the results obtained from the \textit{pbeh}d (-) bloodstage transfer discussed above indicate for a strong correlation between the liverstage and protection from ECM. But what is the difference between the liverstages of \textit{pbeh}d (-) and wildtype liverstage parasites and how could this difference influence the outcome of ECM? The only difference between both parasite strains determined
in this thesis was the growth rate during their liverstage development and as a result the time they spent within the liver cell. In contrast to the wildtype PbA parasites \textit{pbehd (-)} parasites spent about 24 hours longer within these cells in average. Could that lead to a difference in the generation of an immune reaction against the parasites? Yes it could. Comparing the ECM-generating strain PbA to the non-ECM strain NK65 shows, that also NK65 develops slower within the liver. Comparing the immunological profile of both parasites showed that NK65 is inducing a higher level of the anti-inflammatory cytokine IL-10 in infected mice than PbA and that could then lead to a prevention of the generation of ECM symptoms (Lewis and Joschko, \textit{unpublished observations}). In fact, when IL-10 knockout mice were infected with NK65 parasites ECM symptoms were induced (Lewis and Joschko, \textit{unpublished observations}). To test, if IL-10 also plays a role for the protection of mice infected with \textit{pbehd (-)} parasites from ECM, mice were depleted from IL-10 via an anti-IL10 depletion antibody during the liverstage phase. Indeed, 3 out of 3 mice infected with \textit{pbehd (-)} parasites and depleted from IL-10 did develop ECM whereas control mice not depleted did not show ECM symptoms. These results indicate that the slow down of the liverstage development of \textit{pbehd (-)} parasites does lead to a prevention of ECM in infected C57BL/6 mice. This prevention is mediated by the anti-inflammatory cytokine IL-10. It is not clear, if the prolonged development of the parasites in the liver generates a higher level of IL-10 during this phase or if the later appearance of the parasites in the blood gives the immune system more time to act against an overshooting immune-response resulting in a cytokine storm. Higher mouse numbers for the statistical analysis and an evaluation of the immunological profile of the mice will lead to a better understanding of this phenomenon in the future.
Figure 4.1: **Summary of the working hypothesis for apicomplexan EHD-proteins in parasite stages residing in nucleated cells - *Toxoplasma*.** I) After invasion of the host cell a *Toxoplasma* tachyzoite is scavanging lipids and other nutritional factors from the host cell that cannot be synthesized *de novo* by the parasite itself. These factors are passively diffusing into the PV by pores inside the PVM or actively transported by transmembrane transporters. From the PV the factors are then endocytosed by the parasite into the parasite cytosol, most likely at the micropore of the parasite. The endocytosed or autophagic vesicles are trafficked to the endosomal system via EHD-proteins (red small circles) and other proteins and some will end up in the storage compartment labeled by the *Tg*RME-1-protein (red oval-shaped structure) or will be distributed by *Tg*RME-1-vesicles to other organelles. II) During cytokineses the storage compartment fragments and *Tg*RME-1 distributes nutrients (possibly lipids etc.) to the cleavage furrow. In addition, a part of the storage compartment migrates to the back end of the parasite and also appears in the apical part of the newly formed daughter-cells (shown by the green IMC). It is not clear if this compartment in the daughter-cells is synthesized *de novo* (possibly as an autophagosome-like structure) or transported into the cell in step III. III) After cellular division the daughter-cells are still connected via a residual body at the basal part and the *Tg*RME-1 protein localizes to the locus where abscission takes place. Through oligomerization *Tg*RME-1 might assist during abscission of the membranes. In addition, a compartment in the back end *Tg*RME-1 localizes to might be used to fill up a new storage compartment at the apical end of the daughter cells with nutrients. (dark blue lines: membranes; red: *Tg*RME-1; green: IMC; dark blue circle: nucleus; light blue space: PV)
4.2 Figure 4.2: Summary of the working hypothesis for apicomplexan EHD-proteins in parasite stages residing in nucleated cells - *Plasmodium* liver stages. **I** After invasion *Plasmodium* liver stages scavenge lipids and other nutrients from the host cell. Uptake of lipids into the PV might occur through the interaction of PVM resident proteins (EXP-1, UIS3) with L-FABP or apolipoproteins. From the PV the taken up factors are then most likely endocytosed and stored inside the parasite, a process likely mediated by the apicomplexan EHD-protein. The storage compartment (possibly an autophagosome-like structure) is directed towards the host cell nucleus and possibly closely associated with the host cell ER. It is not known, yet, if there is a mechanism for direct trafficking between the host cell ER and the EHD-storage compartment. **II** During schizogony the EHD-labeled compartment is fragmenting and trafficking to the newly forming daughter merozoites and either assisting with organelle segregation (ER, apicoplast) or transporting lipids to form the new merozoite membranes. The protein can also be found on tubules that are not identified yet, but might represent membrane accumulations or the mother cell ER that are/is segregated/autophagocytosed and separated by the EHD-protein. (dark blue lines: membranes; red: *P*xEHD; dark blue circle: nucleus; light blue space: PV)
Chapter 5

Supplementary data

Figure 5.1: Colocalization study of TgRME-1 with DrpB. Toxoplasma gondii Dynamin-related protein B tagged with a DDmycGFP-tag was episomally expressed in stably DDmCherry TgRME-1 expressing parasites. Protein expression was induced with 1 µM Shield-1 in intracellular parasites 6 hours before the parasites were fixed with 4% PFA and imaged under a epifluorescence microscope. (Bar: 4 µm)

Supplementary movie 1: Long-term live-imaging of DDmCherry TgRME-1 expressing parasites. Parasites were imaged as described in section 2.10.3. A picture was taken every 10 minutes and subsequently put together as a video sequence in ImageJ.

Supplementary movie 2: Short-term live-imaging of DDmCherry TgRME-1 expressing parasites. Parasites were imaged as described in section 2.10.3. A picture was taken every 3 seconds and subsequently put together as a video sequence in ImageJ.

The supplementary movies can be found on the attached DVD.
Chapter 6

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