Molecular interactions of the malaria parasite *Plasmodium falciparum* during the sexual reproduction in the mosquito midgut

Molekulare Wechselwirkungen des Malariaparasiten *Plasmodium falciparum* während der sexuellen Fortpflanzung im Mitteldarm der Mücke

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submitted by

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1 Introduction

1.1 Malaria

Malaria is a vector-borne disease which is caused by the protozoan parasite *Plasmodium* and is transmitted from human to human by the bite of an infected female *Anopheles* mosquito. Five human pathogenic species of *Plasmodium* have so far been described: *P. falciparum, P. vivax, P. ovale, P. malariae* and more recently *P. knowlesi* (Cox-Singh *et al.*, 2008). Of these *P. falciparum* is responsible for 98 % of all malaria cases in the African region (WHO, 2011) and causes the severe form Malaria tropica. *P. falciparum* is a unicellular eukaryote which belongs to the Apicomplexa phylum (Fig. 1.1).

Domain: Eukaryota
Kingdom: Chromalveolata
Superphylum: Alveolata
Phylum: Apicomplexa
Class: Aconoidasida
Order: Haemosporida
Family: Plasmodiidae
Genus: *Plasmodium*

Figure 1.1: **Classification of *Plasmodium*** (from http://en.wikipedia.org/).

According to the World Malaria Report 2011 released by the WHO, there were 216 million cases of malaria worldwide and an estimated 655 000 deaths in 2010. Children under the age of 5 years affect for 91 % of all malaria deaths in Africa (WHO, 2011; Fig. 1.2).

Figure 1.2: **Malaria distribution.** Areas at risk of transmission 2010 (from http://www.malaria.com).

Reductions in malaria burden have been observed in all regions observed by the WHO, with the largest proportional decreases noted in the European Region, followed by the American region. The largest absolute decreases in deaths were observed in Africa (Fig. 1.2; WHO, 2010). While progress in reducing the malaria burden has been remarkable, there has been evidence of an increase in malaria cases in three African countries in 2009 (Rwanda, Sao Tome and Principe, and Zambia). The reasons for the resurgences are uncertain and regional changes in climate are overly simplistic (Hay *et al.*, 2002). Increases in malaria cases highlight the fragility of malaria control and the need to maintain control programs, even if numbers of cases have been reduced substantially (WHO, 2010). Therefore, the work on the development of economical and effective drugs and vaccines is still an important objective of scientific research. Mounting evidence has revealed pathological interactions between HIV and malaria in dually infected patients, but the public health implications of the interplay have remained unclear (Abu-Raddad *et al.*, 2006). Malaria has adverse impacts on regions affected through many avenues. Detrimental effects include decreased fertility, population growth, saving and investment, worker productivity, absenteeism, increased premature mortality and medical costs (reviewed in Sachs and Malaney, 2002).

Malaria is transmitted by the female *Anopheles* mosquito, one of the most accomplished vectors of human diseases. Various species have been found as vectors for *Plasmodium* in different parts of the world. *A. gambiae* is the major vector in Africa. In addition, species like *A. stephensi* (Fig. 1.3) are highly adaptable and are found to be very potent vectors of human malaria in Asia. Currently *A. gambiae* is responsible for approximately 80 % of the global malaria morbidity and mortality that occurs in sub-saharan Africa (Kessler and Guerin, 2008).

Figure 1.3: ***A. stephensi*** is able to transmit the malaria parasite (picture http://en.wikipedia.org).

In children, malaria causes various clinical symptoms such as cerebral malaria, severe anemia, severe respiratory distress, renal failure, hypoglycemia, and pulmonary edema, appearing alone or in combinations. After repeated infections with *P. falciparum*, individuals in malaria-endemic regions gradually develop semi-immunity resulting in protection from clinical symptoms in adults (Marsh *et al.*, 1989; Bull *et al.*, 1998). Despite this semi-immunity, women become highly susceptible to

the disease again when they get pregnant, especially in first- or second-time pregnancies. Malaria substantially contributes to maternal death, stillbirth, and miscarriage, as well as to complications like maternal anemia and low birth weight babies (reviewed in Rogerson *et al.*, 2007).

1.2 The malaria pathogen *Plasmodium falciparum*

1.2.1 Life cycle

P. falciparum parasites exhibit a complex life cycle consisting of an asexual and a sexual phase (Fig. 1.4). The life cycle of the parasite switches between the human host and the female anopheline mosquito.

The infection begins with a bite by an infected female *Anopheles* mosquito. Together with the insect's saliva, *P. falciparum* sporozoites are injected into the blood system of the human host. Upon injection, they reach bigger blood vessels and are transported to the liver within minutes (Rosenberg *et al.*, 1990). Here sporozoites first transmigrate through Kupffer cells and several hepatocytes before they finally infect a hepatocyte (Mota *et al.*, 2001; Pradel and Frevert, 2001) and build up a parasitophorous vacuole (PV) (reviewed in Mota and Rodriguez, 2001). In *P. vivax* or *P. ovale* the sporozoites can remain dormant as hypnozoites in the liver or they develop into thousands of merozoites such as *P. falciparum* does. Budding of parasite vesicles, called merosomes, release merozoites which invade erythrocytes and then replicate (reviewed in Sturm and Heussler, 2007). To produce more merozoites the parasite will go through different morphological changes over a 48 h period. These stages of development are known as rings, trophozoites, schizonts, and merozoites (Fig. 1.5). The intra-erythrocytic stages of the life cycle are responsible for the pathogenesis of malaria. Each infected erythrocyte is able to produce up to 16-32 new merozoites which are released from the infected cell. Schizonts are stimulated to release merozoites which in turn develop into the first sexual precursor cells, named male and female gametocytes (Bruce *et al.*, 1990; reviewed in Hill, 2006). They are generated from merozoites in a process called gametocytogenesis. These transmissive stages develop into female macrogametes and male microgametes after ingestion by a blood-feeding mosquito. The male microgametocyte can release eight motile microgametes that fertilize a macrogamete which in turn forms a zygote. Cell fusion is followed by nuclear fusion and meiosis, and within 3 h the zygote becomes tetraploid (Janse *et al.*, 1986 a; reviewed in Kuehn and Pradel, 2010). This cell builds up a motile ookinete that

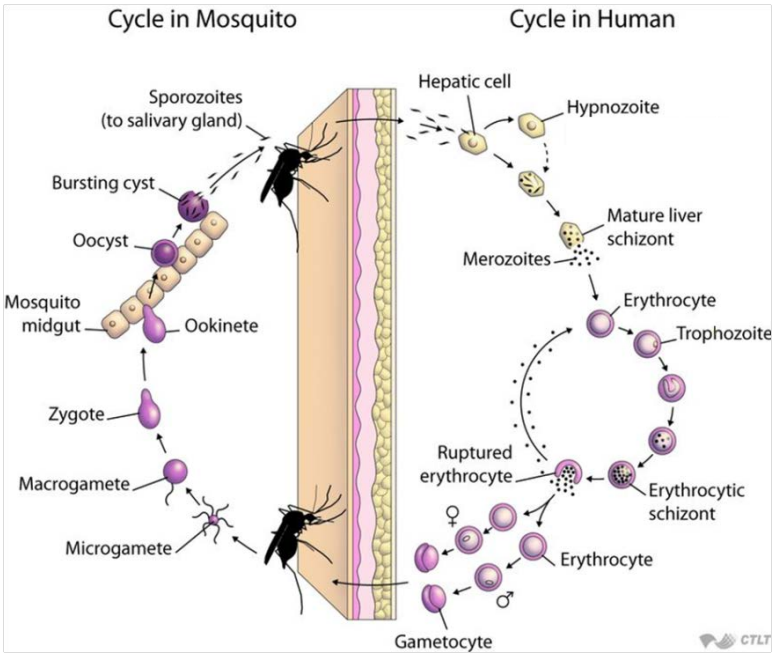


Figure 1.4: **Life cycle of *P. falciparum*.** The female *Anopheles* mosquito transmits sporozoites of the malarial parasite with the bite into the human host. After a period of maturation in the liver, merozoites are released into the bloodstream. These invade red blood cells as part of the asexual cycle. Some develop into first sexual stages, the male and female gametocytes. During a subsequent blood meal, a mosquito ingests gametocytes into its midgut, leading to macro- (female) and microgametes (male), which, after fertilization and zygote formation, produce an ookinete that penetrates the mosquito midgut wall and generates oocysts containing sporozoites. They transit to the salivary gland of the mosquito, where they are released into the blood stream of the next human host, during the next bite of the mosquito (from http://ocw.jhsph.edu /imageLibrary/).

penetrates the mosquito midgut wall and differentiates into an oocyst. Tetraploidy persists throughout the ookinete stage until sporozoites become haploid again (Janse *et al.*, 1986 a; reviewed in Kuehn and Pradel, 2010). When mature, this gives rise to a number of sporozoites capable of migrating into the salivary glands (Lobo and Kumar, 1998; reviewed in Ghosh and Jacobs-Lorena, 2009), from which they are discharged into the next human host during the blood meal.

Figure 1.5: **Schematic view of *P. falciparum* blood stages.** *P. falciparum* blood stages drawn from microscopic observation of thin blood smears: The maturation starts with a ring form in an erythrocyte which develops into a trophozoite and matures to a schizont. Each schizont releases merozoites which invade new erythrocytes to develop in the first sexual stages, the male or female gametocytes (modified from http://www.rph.wa. gov.au).

1.2.2 *Plasmodium falciparum* sexual stages

Gametocytes are responsible for the transmission of the malaria parasite to the mosquito vector (reviewed in Alano, 2007). Their vitality is of high importance for the maintenance of the *Plasmodium* population. Gametocytes exist as male and female parasites, referred to as micro- and macrogametocytes, respectively. Released merozoites from one schizont always develop to gametocytes of the same sex, indicating that sex determination occurs in the developing trophozoite of the preceding generation (Bruce *et al.* 1990; Silvestrini *et al.* 2000; Smith *et al.* 2000; reviewed in Paul *et al.,* 2002). After the invasion of the sexually predetermined merozoites, gametocytogenesis takes about ten days (Fivelman *et al.*, 2007). Both gametocyte sexes contain an apicoplast (see Fig. 1.6; Okamoto *et al.*, 2008). Male gametocytes show in comparison to females a reduction in cytoplasmic ribosome density, which correlates with the appearance of a nucleolus only in female gametocytes (Sinden, 1982).

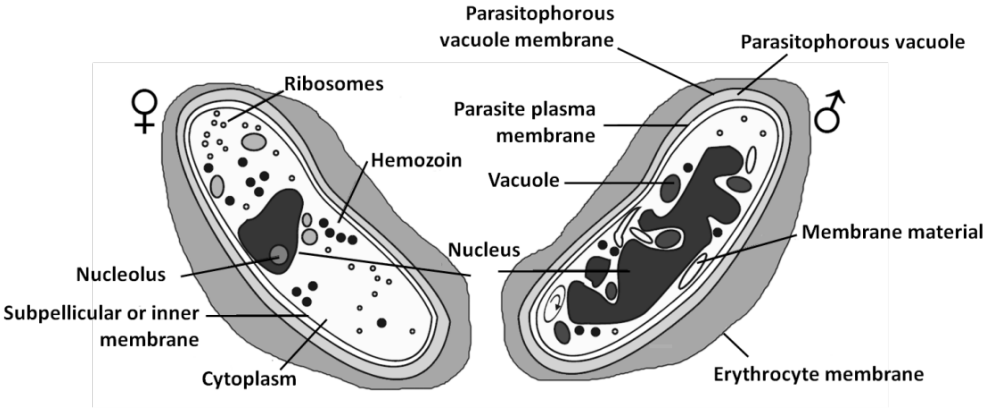


Figure 1.6: **Female and male gametocyte of *P. falciparum*.** Macro- and microgametocytes show morphologically different nuclei distributions. While female parasites have a small nucleus located in the middle of the parasite, the nucleus of male parasites is more widely distributed throughout the parasite. Both include four membranes; the erythrocyte membrane, the parasitophorous vacuole membrane, the parasite plasma membrane, and the subpellicular or inner membrane. The space between the parasitophorous vacuole membrane and the plasmalemma is named parasitophorous vacuole. Furthermore, gametocytes include cytoplasm with ribosomes, hemozoin, vacuoles, and membrane material (modified from http://www.gigers.com).

Differentiation of gametocytes is divided into five (I-V) major phenotypically distinct stages (see Fig. 1.7; Hawking *et al.*, 1971; Carter and Miller, 1979). Trophozoites and stage I gametocytes have a similar shape and are therefore difficult to distinguish. In stage II, the parasite becomes slightly elongated, appearing crescent-shaped. The hemoglobin of the erythrocyte is almost completely metabolized in stage III parasites

(see Fig. 1.7; Carter and Miller, 1979). In this stage the parasite features characteristic rounded ends. It continues to grow in length and reaches its maximum length in stage IV, where the parasite exhibits pointed ends along a straight axis. Stage V gametocytes exhibit again rounded ends. From this point onwards, the sex of mature stage V gametocytes can be distinguished. The cytoplasm of macrogametocytes stains blue, while male microgametocytes appear pink. The hemozoin crystals of female gametocytes accumulate mostly in the center of the parasite while the pigment of males is rather scattered. The gender ratio of gametocytes is female-biased with one mature male for about five mature female gametocytes (reviewed in Paul *et al.*, 2002 and Kuehn and Pradel, 2010). After further two or three days of circulation, gametocytes become infectious to mosquitoes, and can survive up to 21 days in the peripheral blood (Smalley and Sinden, 1977; Lensen *et al.*, 1999; reviewed in Talman *et al.*, 2004). Once in the midgut of the mosquito, the drop in temperature and the mosquito-derived molecule xanturenic acid (XA) trigger gametogenesis within a few minutes (Billker *et al.*, 1997; Billker *et al.*, 1998; Garcia *et al.*, 1998). The ingested gametocytes then lose their protecting erythrocyte membrane (EM) and transform into extracellular male microgametes and female macrogametes (reviewed in Kuehn and Pradel, 2010). Each macrogametocyte produces a single spherical extracellular non-motile gamete. After three rounds of nuclear division, the microgametocytes usually remain trapped within the erythrocyte plasma membrane until the eight haploid motile microgametes are extruded to fertilize a macrogamete (reviewed in Sinden, 1983; Aikawa *et al.*, 1984). During this time, the male gametocytes undergo a process of DNA replication and nuclear segregation, progressing from an approximately haploid genome to an octaploid genome with the formation and migration of eight nuclei into the extruding gametes (Janse *et al.*, 1986 a;

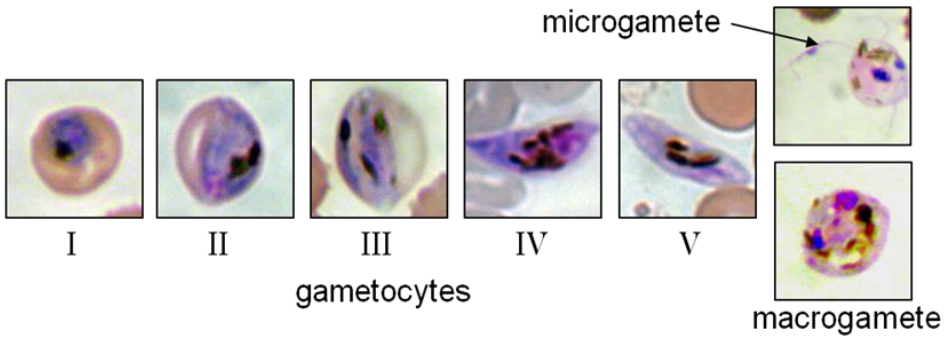


Figure 1.7: **Gametocyte stages and gametes of *P. falciparum*.** Giemsa stains of the five developmental stages of macro- and microgametocytes. After the activation in the mosquito midgut, male gametocytes form a spherical cell with eight motile flagella, called microgametes which are released to fertilize spherical immobile macrogametes.

Janse *et al.*, 1986 b). This process of male parasites is referred as 'exflagellation' and is completed by approximately 15-30 min after the blood meal. Fertilization of female gametes by free-swimming male gametes is completed within 30 min (Templeton *et al.*, 1998).

During gametogenesis, gametocytes exit the EM by an inside-out type of egress. The parasitophorous vacuole membrane (PVM) ruptures at multiple sites within less than a minute following activation and the EM ruptures at a single breaking point approximately 15 min post activation (Sologub *et al.*, 2011). A point of particular interest to the pathology and transmission of severe malaria is that gametes and zygotes are the only stages within the parasites life cycle that are able to survive outside a host cell for more than one day. In the mosquitoes midgut gametes and zygotes are compromised by factors found in the blood meal, including midgut bacteria, digestive enzymes, and components of the human immune system. This exposure results in an approximate 1000-fold loss of parasite abundance during transmission to the mosquito, and the malaria transmission stages are considered bottleneck stages of the parasite life cycle (Fig. 1.8; Vaughan *et al.*, 1994; Kuehn and Pradel, 2010).

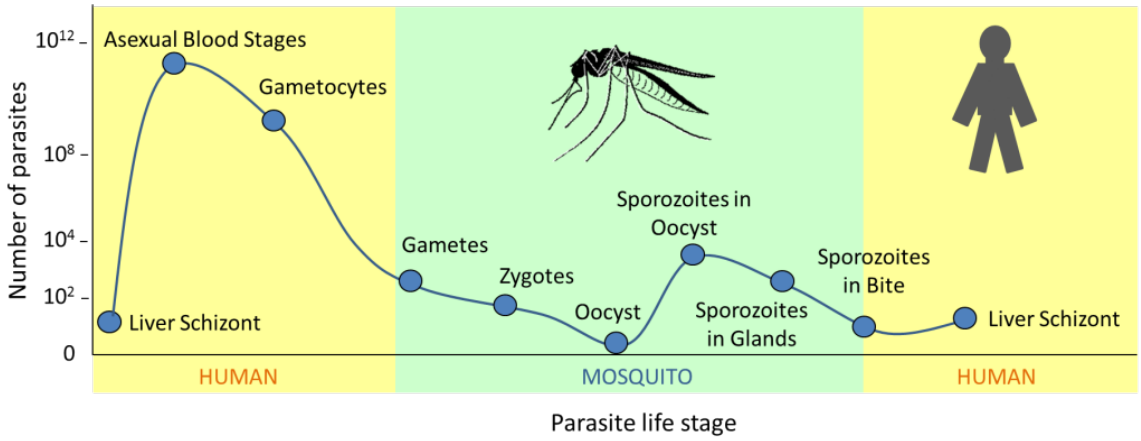


Figure 1.8: **Population ranges of the malaria parasite.** From about trillions of parasites in the human blood, the number of parasites drops below five ookinetes in the mosquito midgut (modified from Vogel, 2010).

1.3 Transmission blocking vaccine

As has been pointed out in the previous chapter the sexual phase *P. falciparum* parasites are highly vulnerable against external influences. Transmission blocking vaccines (TBVs) are aimed at blocking malaria transmission by interrupting the parasite life cycle in the mosquito. These vaccines do not directly protect vaccinated individuals

from infection; however, they contribute to elimination of the disease by lowering the parasite transmission efficiency. TBVs elicit antibodies against surface antigens of sexual- and mosquito-stage parasites and, thus, arrest subsequent development of parasite in the mosquito midgut (Fig. 1.9; reviewed in Carter *et al.*, 2000). Antigens specifically expressed by zygotes and ookinetes in the mosquito midgut, referred to as post-fertilization target antigens, have been shown to be effective for inducing transmission-blocking immunity (Kaslow *et al.*, 1988; Hisaeda *et al.*, 2000; Wu *et al.*, 2006). Zygote or ookinete surface proteins, which do not present antigens in the human host, may be boostable TBV candidates. Surprisingly some gametocyte proteins induce antibody response in about 40 % of patients with natural malaria infection (Ong *et al.*, 1990; Riley *et al.*, 1994). It is assumed that gametocyte proteins are not exposed to the human immune response because they are covered by the EM. During degradation of erythrocytes, which also envelope mature gametocytes, surface proteins of gametocytes may be presented to the human immune system. The advantage of most TBV candidate antigens may be that these proteins have never been previously under immune selection and therefore are potentially highly immunogenic.

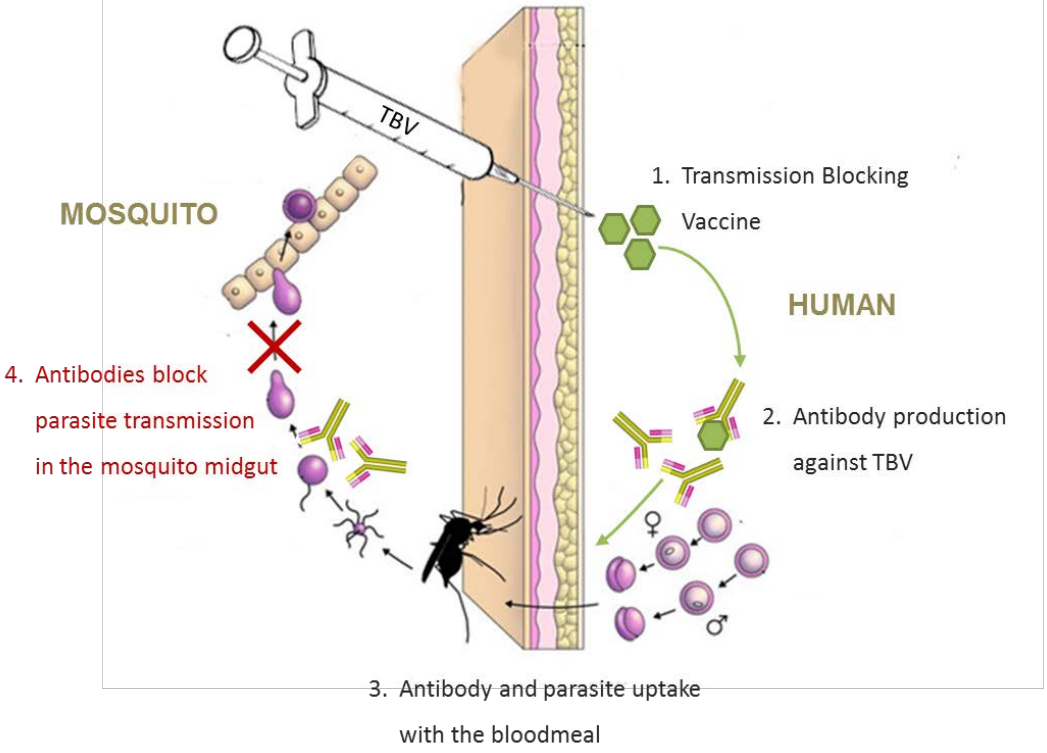


Figure 1.9: **Transmission blocking vaccine.** Vaccine antigens of sexual stage parasites were injected into the human host and antibodies were generated. Parasites take up malaria parasites and antibodies with the blood meal. The sexual stage-specific antibodies block parasite transmission in the mosquito and therefore malaria infection of the next human host (modified from http://ocw.jhsph.edu/imageLibrary/).

TBVs target a very sensitive phase of the parasites life cycle and are therefore a promising perspective to reduce the spread of malaria. Potentially this vaccine is able to eliminate the disease in the future (reviewed in Kaslow, 1997).

The aim of TBVs is to exterminate malaria parasites with an economical, stable formulated and easy administrable vaccine. It is unlikely that a single antigen subunit vaccine will be sufficient; an antigen-cocktail is more promising. To receive maximum distribution transmission-blocking vaccines will be combined with other vaccines or control modalities (reviewed in Kaslow, 1997).

1.4. Sexual stage-specific proteins of the malaria parasite

A series of potential transmission-blocking vaccine candidates have been identified over the last two decades (reviewed in Pradel, 2007). Of particular note are surface proteins of the sexual stages of the malaria parasite. The first of such vaccine candidates was Pfs25. This 25 kDa-protein is localized on the surface of gamete, zygote and ookinete stages of *P. falciparum* and consists of four tandem epidermal growth factor (EGF) domains and an GPI-anchor (Kaslow *et al.*, 1988). The potential for immunization against Pfs25 has been tested by generation of murine antibodies generated against *Vaccinia virus* infected mammalian cells (Kaslow *et al.*, 1991) or recombinant Pfs25 from yeast (Barr *et al.*, 1991). When 25 µg/ml of Pfs25 monoclonal antibodies, developed from the *Vaccina* system, were added to the mosquito's blood meal, the infectivity of *A. freeborni* with *P. falciparum* was reduced to 40 %. Oocyst viability was almost eliminated when the concentration of monoclonal antibodies was increased to 200 µg/ml and transmission blocking activity was reached when polyclonal antibodies were used (Kaslow *et al.*, 1991; reviewed in Coutinho-Abreu and Ramalho-Ortigao, 2010). A few years later, a phase I (human safety assessment) trial of *Pichia pastoris*-expressed Pfs25 antigen mixed with an adjuvant was carried out. The anti-Pfs25 human serum inhibited *P. falciparum* infectivity in *A. stephensi* by more than 90 %. Nevertheless, the local and systemic reactogenicity in human volunteers prevented the adjuvant (Montanide ISA 51) to be used in combination with Pfs25 antigen (Wu *et al.*, 2008). To increase transmission-blocking activity against *P. falciparum* a second zygote stage expressed small 28 kDa-protein named Pfs28 was included. Pfs25 and Pfs28 were produced as a unique chimeric protein in *Saccharomyces cerevisiae*, the 25-28c recombinant protein. Vaccination with 25-28c

led to complete arrest of oocyst development, using a lower dose for a longer of time, than vaccination with either Pfs25 or Pfs28 alone or a combination of both (Duffy and Kaslow, 1997; Gozar *et al.* 1998; Coutinho-Abreu and Ramalho-Ortigao, 2010). Latest studies focus on adenovirus-vectored *Pv*s25 (Miyata *et al.*, 2011) and plant-produced sexual stage protein Pfs25 (Farrance *et al.*, 2011 a) anticipate great success. Particularly antibodies produced in plants block transmission to the next human host completely.

Two further potential TBV candidates to control spread of *P. falciparum* are Pfs48/45 and Pfs230 (reviewed in Pradel, 2007). The secreted Pfs230 binds to the GPI-anchored Pfs48/45 protein on the parasite's surface of gametocytes and gametes (Kumar, 1987; Kumar and Wizel, 1992). Pfs48/45 gene disrupted gametes do not retain Pfs48/45 or Pfs230 on its surface (Eksi *et al.*, 2006). Antibodies against Pfs230 inhibit exflagellation and blocked transition of sporozoites from the mosquito midgut to the salivary glands, in *A. freeborni*. However, the blocking activity of monoclonal anti-Pfs230 can only be verified in presence of an active complement system (Quakyi *et al.*, 1987; Read *et al.*, 1994; Healer *et al.*, 1997; Eksi *et al.*, 2006). The *pfs48/45* gene encodes a protein that appears as a double band under non-reducing conditions (Milek *et al.*, 2000). This protein is localized on *P. falciparum* gametocyte and gamete surfaces and has a central role in male gamete fertility. Antibodies against Pfs48/45 prevent zygote development and therefore transmission (van Dijk *et al.*, 2001). Moreover, anti-Pfs230 and Pfs48/45 antibodies are raised rapidly after exposure to gametocytes in about 22-28 % of malaria patients and substantiate transmission-reducing activity of naturally infected humans (Bousema *et al.*, 2010; Ouédraogo *et al.*, 2011). Like described for Pfs25, also plant-produced antibodies against Pfs230, block malaria transmission to the mosquito (Farrance *et al.*, 2011 b). The Pfs48/45 paralogue, Pfs47, found specifically in female gametocytes and gametes, has no known essential function in female fertility, as revealed by research conducted by disruption of *pfs47* (van Schaijk *et al.*, 2006). A protein that is localized in the osmiophilic bodies of female gametocytes was named Pfg377 (Alano *et al.*, 1995; Severini *et al.*, 1999). Gene-disruption of *pfg377* concludes in the lacking of osmiophilic bodies in female gametocytes which are significantly less efficient in their emergence from the erythrocytes upon induction of gametogenesis and an almost complete blockade of infection in mosquitos (de Koning-Ward *et al.*, 2008). PfPeg3 or alleged Pfmdv-1 is a small protein expressed in gametocytes (Silvestrini *et al.*, 2005; Furuya *et al.*, 2005). The disruption of the *P. falciparum* gene *pfpeg3/pfmdv-1* linked to early arrest in male gametocytogenesis (Furuya *et al.*, 2005).

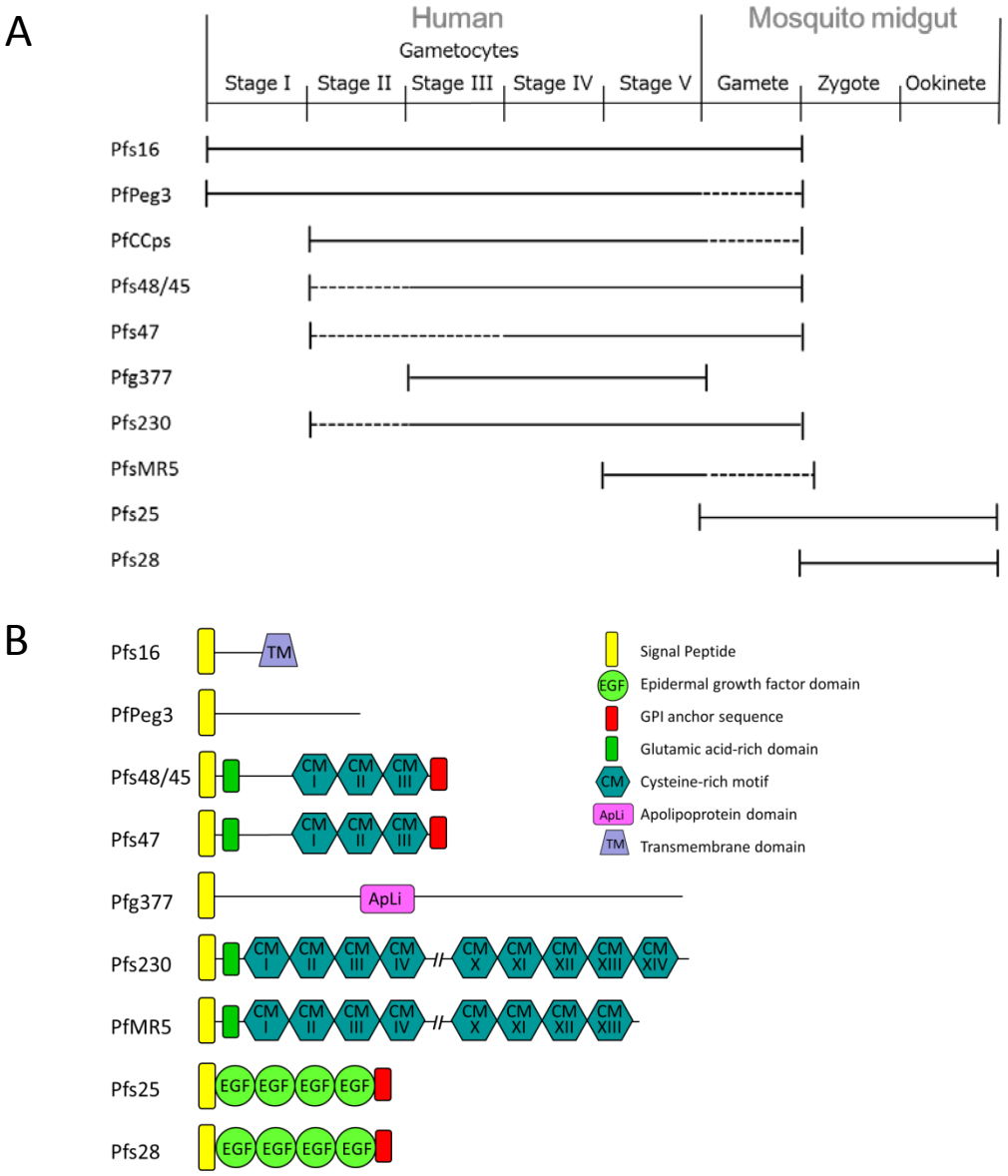


Figure 1.10: **A selection of sexual stage-specific proteins of *P. falciparum*. A.** Overview depicting a small assortment of proteins expressed during the sexual phase. Full lines represent high protein abundance, dotted lines low protein abundance. Proteins are sorted according to their time of occurrence during maturation. **B.** Domain structure of select *P. falciparum* sexual and mosquito stage proteins (modified from Pradel, 2007*)*.

Aside from that, it is important for female gametocyte activation (Lal *et al.*, 2009). One of the earliest known gene products expressed at the onset of gametocytogenesis is the integral PVM protein Pfs16 (Moelans *et al.*, 1991). Gene disruption is followed by a reduced gametocyte production and an impaired ability of male gametocytes to exflagellate (Kongkasuriyachai *et al.*, 2004). In recent studies, Pfs16 was used for studying the trafficking of material from the parasite across the PV space to the PVM and it was described, that during emergence this protein concentrates at the poles of

the parasite (Eksi and Williamson, 2011). One protein, which is expressed only in male stage V gametocytes, is the Pfs230 paralogue PfsMR5 (Eksi *et al.*, 2006).

An overview of the domain structure and the expression stages of all mentioned sexual stage proteins is shown in Figure 1.10. Within this overview of sexual stage proteins, orthologs in all *Plasmodium* species were found for Pfg377 and PfPeg3/Pfmdv-1. Pfs16 has orthologs in *P. knowlesi* and *P. vivax.* The members of the PfCCp protein family (detailed description in section 1.5) hold additionally to all *Plasmodium* species orthologs in *Theileria*, *Cryptosporidium, Toxoplasma and Babesia* (Templeton *et al.*, 2004).

1.5 PfCCp multi-adhesion domain proteins

Among the above described sexual stage proteins a highly conserved family of six secreted proteins has been identified in *P. falciparum* (Pradel *et al.*, 2004). They comprise multiple adhesive domains (Lasonder *et al.*, 2002; reviewed in Dessens *et al.*, 2004; Pradel *et al.*, 2004; Templeton *et al.*, 2004) and five of these proteins possess common Limulus coagulation factor C (LCCL) domains. They are termed PfCCp1 through PfCCp5, whereas a sixth protein, PfFNPA, lacks this domain but shares architectural features. This suggests an evolutionary relationship with PfCCp5 and provides evidence for its inclusion as a member of the multi-adhesion domain protein family. The PfCCp proteins are known as *Pb*LAP proteins in the rodent malaria parasite *P. berghei* and have orthologs in other apicomplexan parasites like *Cryptosporidium parvum* and *Toxoplasma gondii,* indicating an evolutionary conserved function across the apicomplexan clade (reviewed in Dessens *et al.*, 2004; Templeton *et al.*, 2004; Trueman *et al.*, 2004). The six PfCCp proteins are expressed during gametocytogenesis of *P. falciparum* and localize within the gametocyte PV (see Fig. 1.11; Pradel *et al.*, 2004; Pradel *et al.*, 2006; Scholz *et al.*, 2008). During gamete emergence PfCCp proteins are associated with the surface of macrogametes, but expression decreases within a day (Pradel *et al.*, 2004; Scholz *et al.*, 2008). Gene disruptions of *pfccp2* or *pfccp3* result in a complete blockage of infection in mosquitos, showing that these proteins are essential for the parasite development in the vector (Pradel *et al.*, 2004). Gene disruption of *pfccp4*, on the other hand reveals a reduced number of oocysts, but no blockage in mosquito transition (Scholz *et al.*, 2008). Antibodies directed against select PfCCp adhesion domains induce a reduction of exflagellation in the presence of an active complement system, marking the proteins as potential candidates as

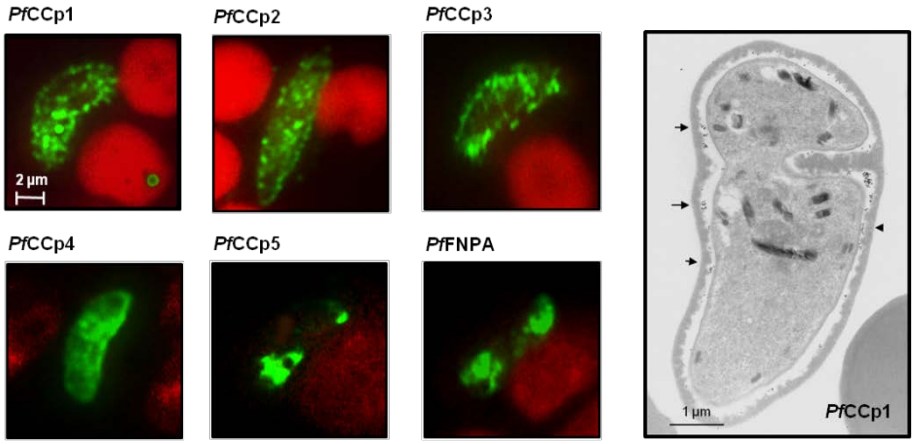


Figure 1.11: **Expression of *Pf*CCp proteins in gametocytes.** Expression patterns in mature gametocytes as determined by IFA. *Pf*CCp1 through *Pf*CCp3 show a punctuate pattern in mature gametocytes, whereas *Pf*CCp4 is homogenously expressed. *Pf*CCp5 and *Pf*FNPA proteins are predominantly localized at the poles of the gametocytes. *Pf*CCp expression is shown in green, while erythrocytes are highlighted in red. Immunoelectron microscopic shows *Pf*CCp1 localization in the PV in association with the PPM, labelled with anti-*Pf*CCp1 primary antibody and immunogold (Pradel *et al.*, 2004; Scholz *et al.*, 2008).

subunits for TBV (Scholz *et al.*, 2008). PfCCp1, PfCCp2, and PfCCp3 co-localize within the PV of mature gametocytes (Pradel *et al.*, 2006). It has been shown that PfCCp1, PfCCp2, PfCCp3, PfCCp4, or PfFNPA knockout gametocytes exhibit reduced abundance or an absence of other PfCCp proteins. This was observed only at protein and not on transcript level (Pradel *et al.*, 2006; Simon *et al.*, 2009).

Adhesion domains of PfCCp proteins

PfCCp proteins are composed of various widely conserved adhesion domains and contain a signal peptide (Fig. 1.12). The ricin domain of PfCCp1 and PfCCp2 is a carbohydrate-binding domain which is widely distributed in bacteria, fungi, animals, and plants. It is part of several carbohydrate and glycoprotein interacting proteins. The widespread discoidin domain can be found in eukaryotes and bacteria and is able to bind sugars and lipid head groups (Templeton *et al.*, 2004). The neurexin-collagen (NEC) domain consists of a deep cleft for ligand binding and exists in collagens, fibrinogen family proteins, and neurexins. The name for PfCCp proteins is derived from the 'LCCL' or '*Limulus* coagulation factor' domain. The LCCL domain was first discovered in the horseshoe crab (*Limulus*) and is composed of six conserved cysteine residues which are found in all PfCCp proteins, except of PfFNPA. It is thought to be an autonomously folding domain that has been found in various multi-domain proteins

(Trexler *et al.*, 2000). Some proteins which contain a LCCL domain, like the LPS endotoxin-sensitive trypsin type serine protease, are known to protect the organism from bacterial infections (Liepinsh *et al.*, 2001). The levanase associated lectin domain is found predominantly in bacterial secreted levanases and glucosidases, whereas the ApicA domain seems to be apicomplexa-specific. The LH2 or PLAT domain (Lipoxygenase homology) of PfCCp3 is found in a variety of membrane bound lipid associated proteins in plants, animals, and bacteria. It is thought to mediate membrane attachment via other protein-binding partners (Marchler-Bauer *et al.*, 2011). The scavenger receptor domains are disulfide rich extracellular domains and contain six conserved cysteines. They are found in several extracellular receptors and may be involved in protein-protein interactions. The single pentraxin domain in PfCCp3 is characterized by calcium dependent ligand binding (Emsley *et al.*, 1994) and proteins of the pentraxin family are involved in acute immunological responses (reviewed in Gewurz *et al.*, 1995). In contrast the fibronection type 2 domain of PfCCp5 and PfFNPA is a collagen binding protein domain that binds to cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin (Dean, *et al.*, 1987). The anthrax protective antigen domain (or PA14 domain) exists in several bacterial proteins such as the *Clostridium botulinum* C2 toxin and β-glucosidases. This domain is also present in other glycosidases, glycosyltransferases, proteases, amidases, yeast adhesins, and bacterial toxins, including anthrax protective antigen (PA). These domains probably mediate calcium dependent interactions (Rigden *et al.*, 2004; Templeton *et al.*, 2004).

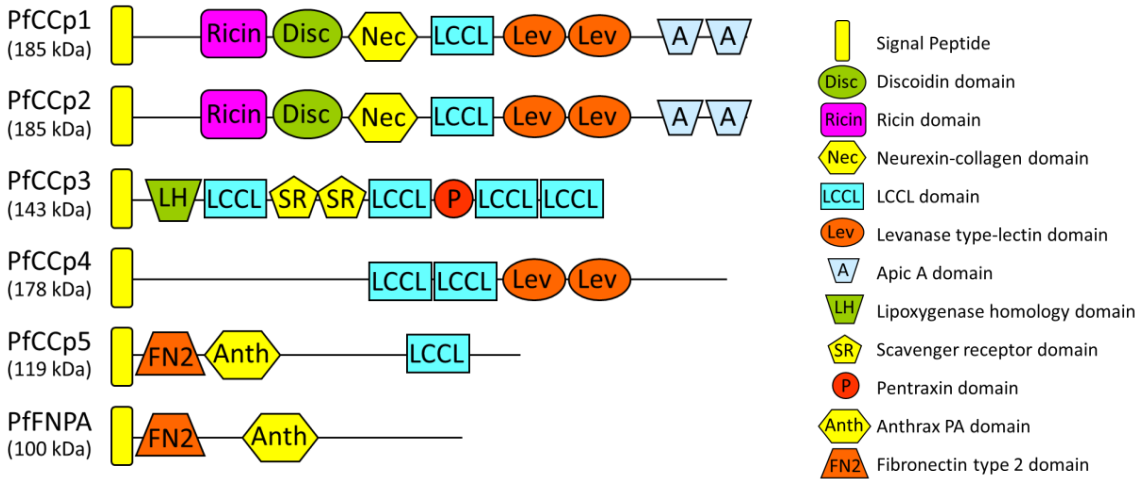


Figure 1.12: **Schematic of the domain structure of the *Pf*CCp multi-adhesion domain protein family.** The six *Pf*CCp proteins contain a variety of adhesion domains and all except of *Pf*FNPA contain the eponymous LCCL domain.

1.6 The malaria parasite and the human complement in the mosquito midgut

1.6.1 Sexual reproduction of malaria parasites in the mosquito midgut

Malaria parasites are taken up by the mosquito in form of sexual precursor cells, the intraerythrocytic gametocytes (reviewed in Pradel, 2007 and Kuehn and Pradel, 2010). After the ingestion into the mosquito's midgut the malaria parasite has not only to survive, it has to reproduce sexually in the aggressive environment of the mosquito midgut. Triggered by external stimuli in the mosquito midgut, gametocytes egress from the enveloping host erythrocyte and form gametes. During this process the surrounding and protecting parasite membranes rupture (Sologub *et al.*, 2011). Within approximately 20 min, fertile gametes have formed and fertilization occurs within one hour post-blood meal. The mosquito midgut contains digestive enzymes, midgut bacteria and furthermore, antibodies, immune cells, and human complement factors which were taken up together with the blood meal (see Fig. 1.13). Previous studies on *P. berghei* describe that during the first three hours of development in culture, 30-50 % of mosquito midgut stages survive complement exposure. Subsequently, parasites become increasingly sensitive to complement mediated lysis (Margos *et al.*, 2001). Initial studies on complement effects on the infectivity of malaria parasites were conducted with *P. gallinaceum*, the malaria parasite which infects birds. The ability of gametocytes to infect mosquitoes in the presence of native human serum has been previously been attributed to proteases that inactivate the human complement system before the gametes and zygotes emerge as extracellular parasites in the blood meal (Grotendorst *et al.*, 1986).

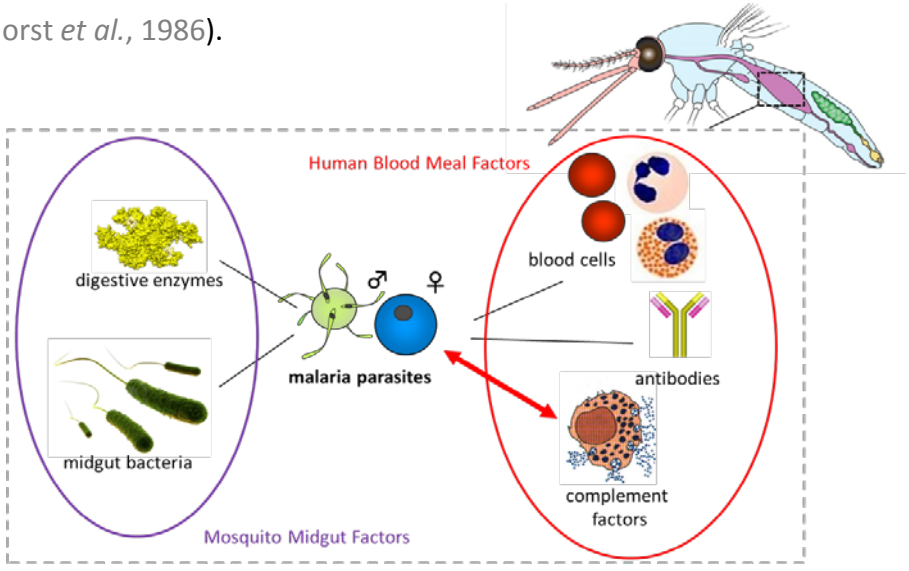


Figure 1.13: **The malaria parasite in the mosquito midgut** is surrounded by mosquito midgut factors and human blood meal contents.

1.6.2 The human complement system

The human complement system is part of the innate immune system. It contributes to the nonspecific, humoral host defense, helps to eliminate cellular antigens (e.g. bacteria), and leads through several cascade-like activation steps resulting in the lysis of the target cell. In terms of developmental physiology, the complement system is the oldest barrier against infections. The human complement system removes immune complexes and is a lytic system for the elimination of pathogens. Essentially, it can be initiated via three different activation cascades, the classical, the alternative, and the lectin pathway. The alternative pathway (AP) is a constitutive process activated by biological surfaces (reviewed in Zipfel and Skerka, 2009). The classical pathway is triggered by antibodies bound to the target antigen (Law and Reid, 1995), initiated for example by viruses or gram-negative bacteria. The lectin pathway is homologous to

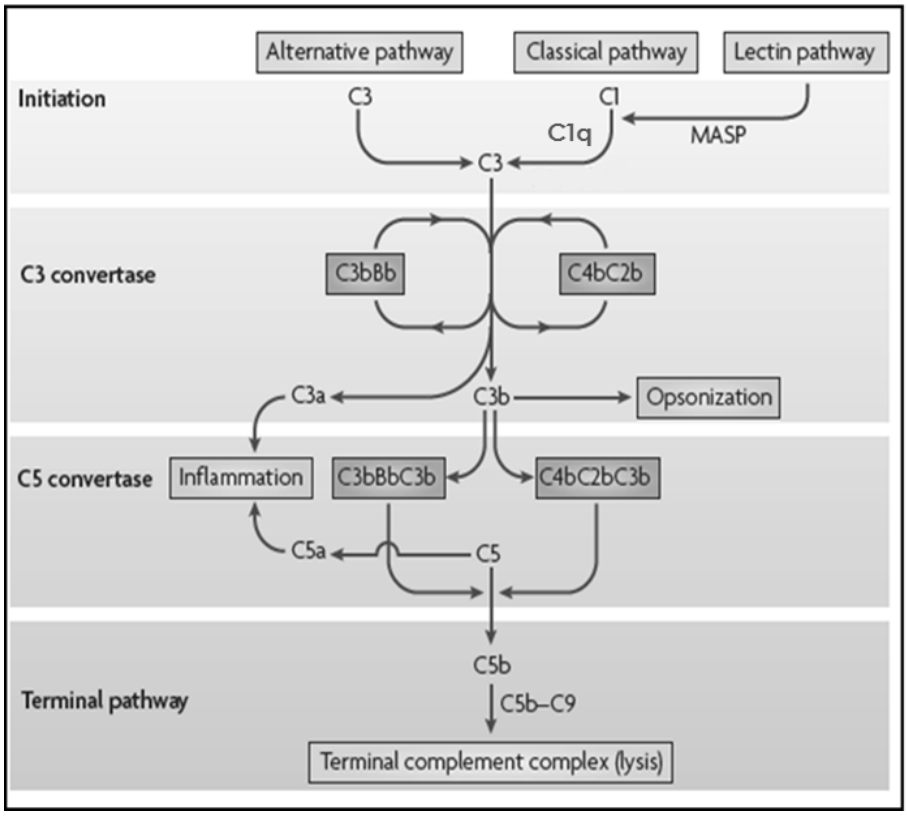


Figure 1.14: **Activation pathways of the human complement system.** Complement is initiated by three major pathways. The AP is spontaneously and continuously activated. The CP is induced when antibodies bind to their corresponding antigen and the lectin pathway is triggered by the binding of mannan binding to mannose residues on the surface of microorganisms, which activate MBL-associated serine proteases (MASPs) which cleave C4 and C2 like in the classical pathway. Complement activation occurs in a sequential manner. Enzymes termed C3 and C5 convertases cleave pathway products and lead to opsonization and lysis of the target surface (modified from the review Zipfel and Skerka, 2009).

the classical pathway, but with the opsonin, mannose-binding lectin (MBL) and is enabled by carbohydrates on microbial surfaces (reviewed in Fujita, 2002 and Degn *et al.*, 2007).

The first step of the activation of the classical pathway is the cleavage of C1. This leads to a deposition of C1q on the surface of the pathogen (Meri *et al.*, 2002 a). Activation of the classical pathway results in assembly of the first enzyme of the cascade, C3 convertase. The protein responsible varies between the AP, C3bBb, and for the classical and lectin pathways, C4bC2b (Fig. 1.15; reviewed in Zipfel and Skerka, 2009). These enzymes split the central complement component C3 and form the anaphylactic peptide C3a and the opsonin C3b which can be deposited onto any close surface. This activation step is followed by a strengthening reaction that generates more C3 convertases and deposits further C3b at the local site. C3b then becomes inactivated and sequentially degraded. These resulting proteins mediate further effector functions for opsonization. If activation is under way a new enzyme, the C5 convertase (C3bBbC3b in the AP and C4bC2bC3b in the classical and lectin pathways), is generated. C5 convertase cleaves C5 releasing C5b which initiates the terminal pathway together with the complement factors C6 to C9. This arrangement of proteins is termed the terminal complement complex (TCC) or membrane attack complex (MAC). It forms a pore in the target cell membrane and causes lysis (reviewed in Zipfel and Skerka, 2009).

1.6.3 Regulation of the alternative pathway of complement

The APC is one of three complement pathways that opsonize and kill pathogens. It is antibody-independent, is activated by spontaneous hydrolysis of C3, and is amplified by C3b deposition on the surface of pathogens (Delvaeye *et al.*, 2009). C3b attaches covalently to target surfaces to amplify complement response, labels cells for phagocytosis and stimulates the adaptive immune response. Cleavage of C3 (186 kDa) into C3b (177 kDa) and C3a (9 kDa) is the central step in the complement activation cascade. C3b covalently attaches to pathogenic, or apoptotic, target surfaces and thereby prefaces several biological processes. C3b provides a molecular platform for the formation of convertase complexes. Binding of pro-enzyme factor B to C3b and subsequent cleavage of factor B by factor D yields the short-lived C3bBb complex which converts C3 into C3b and C3a, thereby amplifying the complement response and

forming the C3b2Bb complex that cleaves C5 to initiate the formation of large, membrane inserted lytic pores, the MAC (Janssen *et al.*, 2006).

The formation of C3b is a central step that requires tight regulation. Host cells express numerous cell surface and soluble regulators that interfere with the C3bBb complex formation or support the proteolytic degradation of C3b into iC3b, the inactive form of C3b. Regulators of complement are the soluble Factor H (FH), the FH-like protein (FHL-1) or the Complement Receptor (CR1), which dissociate the C3bBb complex and act as co-factors for the proteolysis of C3b into iC3b, which is mediated by the plasma protease Factor I (FI). Further APC activation is thereby inhibited (Fig. 1.15; Janssen *et al.*, 2006).

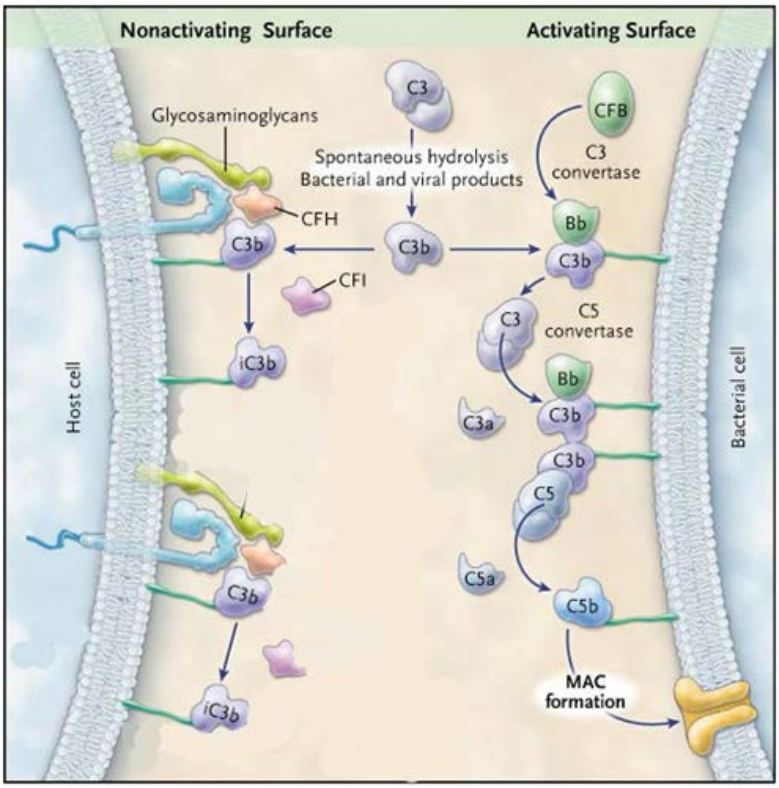


Figure 1.15: **Alternative Pathway of Complement, Activation, and Regulation.**
APC activation on the right side: On pathogen surfaces, that lack complement regulators, C3b is deposited to bind factor B (CFB) to form the C3 convertase of the AP, an enzyme complex (C3bBb) that cleaves additional C3 molecules. C3b also takes part in the emergence of the C5 convertase (C3b2Bb), which by cleaving C5, releases C5a, an anaphylatoxin, and C5b, which initializes assembly of the membrane attack complex (MAC).
No activation of APC on the left side: In host cells, several membrane-anchored and fluid-phase regulators control this cascade. For example complement FH in the fluid phase binds to cell-surface glycosaminoglycans and to C3b and act as a cofactor for FI which mediates the cleavage of C3b to iC3b. This decreases downstream activation of C3 and C5, thereby shielding the pathogens or host cell membrane (modified from Delvaeye *et al.*, 2009).

1.6.4 Properties of complement factor C3 and Factor H

The alternative complement pathway is able to distinguish human cells and tissues from a wide variety of potential pathogens. To avoid complement activation from targeting the host cell, there are several different kinds of regulatory proteins that interrupt the complement activation process. The primary mode of deactivation of the APC is the proteolytic cleavage of C3b by the protease FI, resulting in an inactive form of C3b, the iC3b. Several proteins such as cell surface CR1, C4bp and FH can act as cofactor for FI, and thereby operate as regulators for the APC (reviewed in Zipfel and Skerka, 2009; Serruto *et al.*, 2010). The complement regulator FH has cofactor activity for the FI-mediated cleavage of C3b and is an antagonist of C3b interactions with factor B and Bb (Whaley and Ruddy, 1976). To survive in the human host some pathogens have developed different strategies to escape the APC response. Some bind soluble complement factor regulators, such as FH, on their cell surface resulting in inactivation of the AP protecting the pathogen from the host's immune attack. High affinity of FH to C3b is favored by cell surface components present such as sialic acid residues on glycophorin A and glycosaminoglycans in bacteria, and leads to inhibition of complement activation (Jokiranta *et al.*, 1996; Schmidt *et al.*, 2008). Cleavage of C3 into C3a and C3b is the central step in the complement activation cascade (Janssen *et al.*, 2006). Detection of α'_1 and α'_2 fragments indicates inactivation of C3b (Fig. 1.16; Riley-Vargas *et al.*, 2005). The inactivated form of C3b is called iC3b.

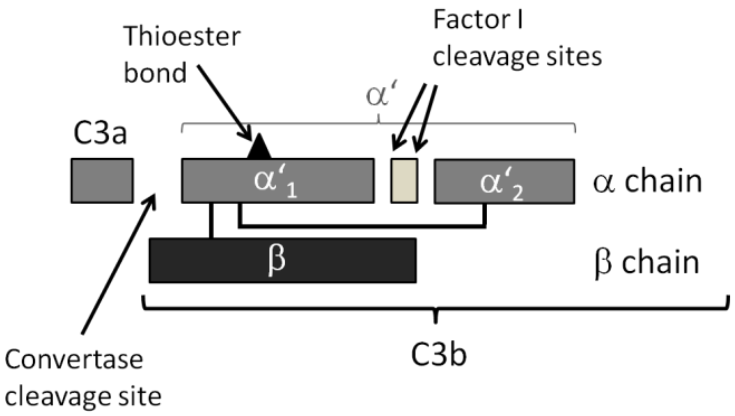


Figure 1.16: **Cleavage sides of Complement factor C3**. C3 (180 kDa) is composed of two chains, α (109 kDa) and β (75 kDa). Upon activation, C3a (9 kDa) is released, leaving the truncated α' chain (101 kDa) of C3b. FI cleaves the α' chain at two points, creating the fragments α'_1 (67 KDa) and α'_2 (40 kDa) (composed of Barilla-LaBarca *et al.*, 2002; Riley-Vargas *et al.*, 2005 and http://www.uniprot.org/uniprot/P01024).

The complement FH protein controls this cleavage and is one of the central regulators of the complement cascade. It is a soluble plasma protein, secreted primarily from the liver and composed of 20 homologous short consensus repeat (SCR) domains (reviewed in Rodriguez de Cordoba *et al.*, 2004). These domains are characterized by four invariant cysteines and many highly conserved amino acids folded in compact units. FH belongs to the FH-family-proteins which represent a group of seven human multidomain, multifunctional secreted proteins. The two most prominent regulating proteins of this family are FH and FHL-1 (reviewed in Zipfel *et al.*, 2008). FHL-1 is an alternative splicing product of FH and comprises seven SCR domains (reviewed in Józsi and Zipfel, 2008). FH and FHL-1 control the activity of the APC. Host cells utilize these soluble immune-regulators to manage complement inactivation directly at their surfaces. Three binding sites have been mapped for C3b and heparin on FH (Fig. 1.17). The first seven SCR domains of FHL-1 are identical to FH with same binding sides. The first four domains are in charge for complement regulation. FH (155 kDa) is present in the human plasma at a concentration of approximately 500 µg/ml, while the plasma concentration of FHL-1 (43 kDa; here running at a molecular weight of ~37 kDa) is 10-50 µg/ml (reviewed in Zipfel *et al.*, 2002). Another protein from the human FH protein family called CFHR-1 can also be found in plasma. It occurs in in two forms, CFHR-1α and CFHR-1β with molecular weights of 37 and 43 kDa (Fig. 1.17; Estaller *et al.*, 1991; Skerka *et al.*, 1991; reviewed in Zipfel *et al.*, 2002).

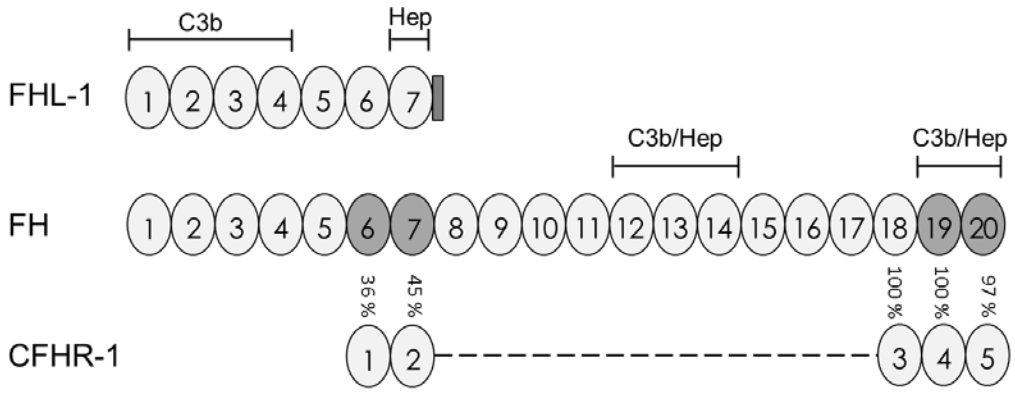


Figure 1.17: **Diagram of FH, FHL-1, and CFHR-1 structure including binding domains**. FH is composed of twenty SCR domains, FHL-1 is identical in sequence with the seven N-terminal SCRs of Factor h and includes at its C-terminal end an extension of four amino acids. The first four SCR domains of FH and FHL-1 have cofactor activity by binding C3b and three further binding sites have been mapped for C3b and heparin. Sequence alignment of CFHR-1 with FH reveals two conserved regions, which are marked in grey in the FH molecule (SCRs 6–7 and 18–20) (modified from Zipfel *et al.*, 2002).

1.6.6 Pathogens bind Factor H for complement inactivation

The human complement is a first line of defense against microbial infections. It is an important part of innate immunity. One of the major features of complement is the destruction of invading microbes (reviewed in Zipfel *et al.*, 2006). Thus, in order to achieve an infection and to become a pathogen, a microbe has to survive this immune defense and inactivate complement attack (Lachmann, 2002). This protection is thought to be mediated by regulatory proteins like FH or FHL-1 of the complement, which mark the foreign cells, and prevent complement mediated lysis, phagocytosis and opsonization (Kraiczy and Würzner, 2006; reviewed in Lambris *et al.*, 2008 and Zipfel *et al.*, 2008). Pathogenic microorganisms use this strategy and express surface proteins which imitate the host surface proteins and are able to bind FH and/or FHL-1 (reviewed in Zipfel *et al.*, 2002). Numerous proteins which bind complement regulatory factors are identified and called immune escape pathogen surface proteins (PSPs) (reviewed in Zipfel *et al.*, 2008). Surface proteins of the outer membrane of pathogens bind FH, recruite FI to cleave C3b on the pathogens surface, and thereby inactivate the APC (Fig. 1.18).

This mode of complement evasion has been initially shown with gram-positive bacteria such as *Streptococcus pyogenes* (Horstmann *et al.*, 1987). It has been shown that the M protein, a surface protein of Group A *S. pyogenes,* binds to the seventh SCR module of human complement FH (Blackmore *et al.*, 1998). Similarly gram-negative bacteria such as *Borrelia burgdorferi* bind FH and FHL-1 (Hellwage *et al.*, 2001; Kraiczy *et al.*, 2004; Bhide *et al.*, 2009) and *Bordetella pertussis* binds FH exclusively (Amdahl *et al.*, 2011) to evade the attack of the host immune system. PspC, a pneumococcal surface

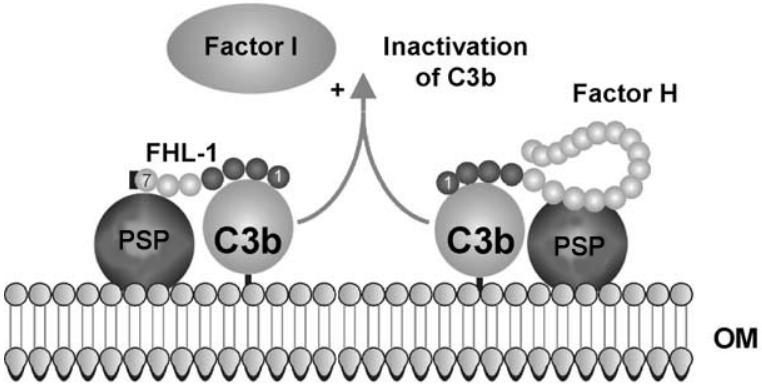


Figure 1.18: **Structure and model of two evasion mechanisms utilized by human pathogens**. Restriction possibilities of the alternative complement activation upon binding of FH or FHL-1 to pathogen surface proteins (PSP) on the outer membrane (OM) (modified from the review Kraiczy and Würzner, 2006).

protein of *S. pneumoniae* (Dave *et al.*, 2001; Duthy *et al.*, 2002) and anaerobic bacteria (Friberg *et al.*, 2008) bind the complement regulator FH to escape APC. Moreover, yeast-like fungi such as *Candida albicans* bind the two central complement regulators, FH and FHL-1, from normal human serum (Meri *et al.*, 2002 b). In 1988 FH binding of a parasite was observed for the first time. It was discovered that FH binds to *Toxocara canis* and *Schistosoma mansoni*, but no effect on complement inactivation was proven. The ability of microfilariae of *Onchocerca vulvulus* to utilize FH in complement regulation was later detected (Meri *et al.*, 2002 a), and this is so far the first known parasite which binds FH for complement evasion.

1.6 Aim of the study

Gametocyte development and gamete formation are accompanied by the coordinated expression of numerous sexual stage proteins, including the EGF domain-containing protein Pfs25, the cysteine motif-rich proteins Pfs230 and Pfs48/45, as well as the PfCCp multi-adhesion domain proteins. The majority of these proteins contain a variety of adhesion domains and are associated with the parasite surface, but expression usually ceases during fertilization. Preliminary data lead to the assumption that selected sexual stage adhesion proteins might form protein complexes on the surface of malaria gametocytes, which are taken up by the blood-feeding mosquito. The bloodmeal includes active components of the human complement system. In the mosquito midgut, the parasite egresses from the enveloping erythrocyte and thereby comes in contact with the human complement system.

The key aspect of this doctoral thesis was to scrutinize molecular interactions of the malaria parasite *P. falciparum* during the sexual reproduction in the mosquito midgut. Sexual stage surface proteins play an essential role during the sexual phase of the parasite in the mosquito midgut. In recent studies it was shown that the six PfCCp proteins are co-dependently expressed, resulting in loss of all PfCCp members, when one protein was knocked-out. Furthermore, previous co-localization and binding studies indicate interactions between Pfs230 and PfCCp4 in the PV of gametocytes. These data give sufficient evidence to presume that more surface associated proteins of the malaria parasite bind to each other.

The first aim of my doctoral thesis was to gain deeper insights into potential protein interactions between sexual stage proteins in the PV of gametocytes and on the

surface of newly emerged macrogametes. Protein complex formations need to be investigated in order to evaluate their function and their potential as TBV candidates. Extensive interaction studies on endogenous proteins using parasite lysates in Co-immunoprecipitation assays were planned in order to investigate interactions between well-known sexual stage proteins. Specific proteins of the precipitated protein complex should be identified by Western blot analysis using antibodies against the appropriate protein or via mass spectrometry. In order to elucidate direct interactions between the PfCCp proteins Co-elution binding assays with recombinant PfCCp proteins should be performed. Furthermore, the potential processing of PfCCp proteins during gametogenesis was to be examined.

The second aim of this work concentrates on interactions between the malaria parasite and the human complement system in the mosquito midgut. Up to date it is not known how sexual stages of malaria parasites are able to escape from the attack of the human complement system in the mosquito midgut. Some pathogens are known to bind human complement regulator proteins to avoid complement-mediated lysis. If *P. falciparum* also utilizes this strategy to survive in the mosquito midgut was aimed to be addressed in this thesis. In addition, the activity of the complement system after the uptake of the blood meal in the mosquito midgut should be determined. The possible protection mechanism of the malaria parasite to bind the human complement regulator FH should be characterized in order to prove following complement inactivation. Subsequent studies aimed at the identification and characterization of the receptor protein of FH on the parasite's surface, which might be a promising new TBV candidate.

2 Materials and Methods

2.1 Materials

2.1.1 Instruments

Table 2.1: Instruments used and manufacturer

Instrument	Company
AccuJet® pro	Brand, Wertheim
Amicon® Ultra-4, Ultra-10 filter units	Millipore, Schwalbach
Balances 440-47N and 440-33	Kern & Sohn GmbH, Balingen-Frommern
Binocular FOMI B 50	Zeiss, Oberkochen
Bunsen burner Gasi	Schütt, Göttingen
Cell culture flask 25 cm^2, 75 cm^2	Becton Dickinson, Falcon, Heidelberg
Centrifuge Megafuge	Heraeus, Hanau
Chromatography column PolyPrep®	Bio-Rad, München
Confocal fluorescence microscope	Zeiss, Oberkochen
Electrophoresis chamber Mini-Protean 3	Bio-Rad, München
French® Press FA078	Heinemann, Schwäbisch Gmünd
Gel drying apparatus 14 x 14 cm	Roth, Karlsruhe
Heat block Bio TBD-100, TBD-120	Lab-4you, Berlin
Hot plate OTS 40	Medite, Burgdorf
Incubator HERA cell	Heraeus, Hanau
Incubator Model 100-800	Memmert, Schwabach
Light microscope Leica DMLS	Leica, Solms
Light microscope Leitz Laborlux 11	Leitz, Wetzlar
Measuring cylinder	Roth, Karlsruhe
Microscope camera AxioCam	Zeiss, Oberkochen
Mini-Rocker MR1	Lab-4you, Berlin
Mosquito Incubator Model 2015	VWR, West Chester, USA

pH-Meter InoLab	WTW, Weilheim
Pipettes	Eppendorf, Hamburg
Rotator-Mixer	Labinco, DG Breda, Niederlande
Shaker SM 30 control	Edmund Bühler GmbH, Tübingen
Sterile bench HERAsafe	Heraeus, Hanau
Sterile filter Steritop™ 0,22 µm	Millipore, Schwalbach
Syringe filters	Roth, Karlsruhe
Tubes Sterican	Braun, Melsungen
Centrifuge Beckmann J2-HC	Beckmann, München
Vacuum Pump Laboport	KNF, Freiburg
Vortexer Power Mix Model L46	Labinco, Breda, Netherlands
Water bath Hecht 3185 WTE	Karl Hecht KG, Sondheim
Western blot apparatus Mini-Trans-Blot	Bio-Rad, München

2.1.2 Chemicals and consumables

Chemicals:

- AppliChem, Darmstadt
- ATCC, Manassas, USA
- Dianova, Hamburg
- GE Healthcare/Amersham Bioscience, München
- Invitrogen/Gibco/MolecularProbes, Karlsruhe
- Merck/Novagen/Calbiochem, Darmstadt
- Pharmacia/Pfizer, Wien
- Roth, Karlsruhe
- Santa Cruz Biotechnology, Heidelberg
- Sigma/Fluka, Taufkirchen
- TecoMedical Group/Quidel, Bünde
- WAK Chemie, Steinbach

Consumables:

- BD Falcon, Heidelberg
- Bio-Rad, München
- Greiner, Flacht
- Hartenstein, Würzburg
- Millipore, Schwalbach
- Noras, Höchberg
- Provac, Austria-Winkel
- Roth, Karlsruhe
- Sarstedt, Nürnbrecht

Miscellaneous

- Cell culture grade A^+ erythrocytes and serum was purchased from Bayerisches Rotes Kreuz (BRK), Würzburg.
- Six week old female NMRI mice for immunization were obtained from Charles River Laboratories, Sulzfeld for immunization.
- 50 l cylinders of gas (5 % O_2, 5 % CO_2, in 90 % N_2) were purchased from Tyczka Industriegase, Würzburg, for use in cell culture procedures.

2.1.3 Kits

- QiAprep Spin Miniprep Kit (250); Qiagen, Hilden

- C3a Plus EIA Kit MicroVue Complement, Quidel, TecoMedical Group, Bünde

2.1.4 Buffers and solutions

Table 2.2: Buffers and solutions

Designation	**Ingredients**
10 x PBS	80 g NaCl 2 g KCl 11.5 g Na_2HPO_4 2 g KH_2PO_4 H_2O_{bidest} *ad* 1000 ml pH 7.4
PBS-Mix	PBS pH 7.4 0.5 % Saponin
PBS-Mix/NP40	PBS pH 7.4 0.5 % Saponin 0.5 % NP40

2 x Sample buffer	2.5 ml 500 mM Tris-HCl. pH 6.8 2.0 ml Glycerin 4.0 ml 10 % SDS 0.5 ml 0.1 % Bromphenol blue H_2O_{bidest} *ad* 10 ml
10 x PAGE Running buffer	29 g Tris 144 g Glycerin 10 g SDS H_2O_{bidest} *ad* 1000 ml
10 x TBS	12.1 g Tris 87.3 g NaCl H_2O_{bidest} *ad* 1000 ml pH 7.5
Transfer buffer	3.03 g Tris 14.4 g Glycerin 200 ml Methanol H_2O_{bidest} *ad* 1000 ml
Blocking solution	50 ml 1 x TBS 0.5 g BSA (Fraction V Albumin) 2.5 g Milk powder
TBS milk	3 % Milk powder in 1 x TBS
Equilibration buffer	12.1 g Tris 5.8 g NaCl 10.2 g $MgCl_2$ H_2O_{bidest} *ad* 1000 ml pH 9.5
Stop buffer	1.2 g Tris 0.4 g EDTA H_2O_{bidest} *ad* 1000 ml pH 8
1000 x Ampicillin	100 mg/ml in H_2O_{bidest}
1000 x Kanamycin	50 mg/ml in H_2O_{bidest}
1000 x Gentamycin	50 mg/ml in H_2O_{bidest}
1000 x Hypoxanthine	0.05 g/ml in NaOH/H_2O_{bidest}
1mM Xanthurenic acid	0.05 g Xanthurenic acid 1 ml 0.5 M NH_4OH 243 ml H_2O_{bidest}
Lysis buffer for MBP purification	50mM Tris pH 8 1mM EDTA 100mM NaCl in H_2O_{bidest}
1 x TE (Tris-EDTA) buffer	10 mM Tris pH 8 1 mM EDTA pH 8 in H_2O_{bidest}
Column buffer for MBP purification	50 mM Tris pH 8 1 mM EDTA 100 mM NaCl in H_2O_{bidest}
Elution buffer for MBP purification	50 mM Tris pH 8 1 mM EDTA 100 mM NaCl 10 mM Maltose in H_2O_{bidest}

Lysis buffer for 6-His and GST purification	50 mM Tris pH 8 350 mM NaCl 10 % Glycerin 1 mM β-Mercaptoethanol 0.2 % IGEPAL 10 mM Imidazol in H_2O_{bidest}
Column buffer 3 for 6-His purification	50 mM Tris pH 8 1 mM β-Mercaptoethanol 350 mM NaCl 10 % Glycerin in H_2O_{bidest}
Elution buffer for 6-His purification	50 mM Tris pH 8 1 mM β-Mercaptoethanol 350 mM NaCl 10 % Glycerin 250 mM Imidazol in H_2O_{bidest}
Elution buffer for GST purification	50 mM Tris pH 8 10 mM reduced glutathione in H_2O_{bidest}
Lysis buffer inclusion bodies	50 mM Tris pH 8 0.25 % Sucrose (w/v) 1 mM EDTA in H_2O_{bidest}
Detergent buffer inclusion bodies	20 mM Tris/HCl pH 7.5 2 mM EDTA pH 8 200 mM NaCl 1 % Deoxycholicacid 1 % NP-40 in H_2O_{bidest}
Washing buffer inclusion bodies	0.5 % Triton X-100 1 mM EDTA pH 8 in H_2O_{bidest}
IFA incubation solution	1 x PBS pH 7.4 0.5 % BSA 0.01 % Saponin in H_2O_{bidest}
SAX (gametogenesis activation solution)	100 µM Xanturenic acid 1.67 mg/ml Glucose 8 mg/ml NaCl 1 mg/ml Tris pH 8,2 in H_2O_{bidest}

2.1.5 Medium and agar plates

Table 2.3: Cultivation media and agar plates

Designation	Ingredients
LB agar	10 g Tryptone 5 g Yeast extract 5 g NaCl 15 g Agar H_2O_{bidest} *ad* 1000 ml
LB	10 g Tryptone 5 g Yeast extract 5 g NaCl H_2O_{bidest} *ad* 1000 ml

LB agar for MBP tagged protein expression	10 g Tryptone 5 g Yeast extract 5 g NaCl 1 g Glucose H_2O_{bidest} *ad* 1000 ml
SOC medium	20 g Tryptone 5 g Yeast extract 0,5 g NaCl 10 ml 0,25 M KCl 5 ml 2 M $MgCl_2$ 20 ml 1 M Glucose H_2O_{bidest} *ad* 1000 ml
RPMI incomplete	10,43 g RPMI-1640 powder 5,94 g Hepes 0,05 g Hypoxanthine H_2O_{bidest} *ad* 1000 ml
RPMI complete	500 ml RPMI 1640 + 25 mM Hepes + L-Glutamine + Sodium bicarbonate 50 ml inactivated human A^+ serum 550 µl 1000 x Hypoxanthine (550 µl 1000 x Gentamycin)
Glycerolyte 57 solution	26,66 g Sodium lactate 584 mg NaH_2PO_4 x 2 H_2O 2344 mg Na_2HPO_4 x 7 H_2O 570 mg Glycerol 300 mg Potassium chloride H_2O_{bidest} *ad* 1000 ml pH 6.8
Giemsa buffer	7 g KH_2PO_4 10 g Na_2HPO_4 H_2O_{bidest} *ad* 1000 ml pH 7.2

2.1.6 Parasite and bacterial cell lines

Cell lines of *Escherichia coli (E. coli) and Staphylococcus aureus*

- *E. coli*-protein expression cell line: BL21-CodonPlus®-(DE3)-RIL, Stratagene.
- *E. coli*-transformation cell line: Nova blue-Competent-Cells, Stratagene.
- *S. aureus* cell line: Newman; kindly provided by AG Ohlsen, Institute for Molecular Infection Biology, Würzburg.

Cell lines of *P. falciparum*

Plasmodium falciparum WT NF54 strain is a gametocyte-producing strain. It is chloroquine sensitive and was isolated in 1982 from West Africa (Ponnudurai *et al.,* 1982).

2.1.7 Plasmids

pGEX-4T1

This high-copy protein expression vector contains an Ampicillin resistance and a Glutathione-S-transferase (GST) tag (Amersham Biosciences, Freiburg).

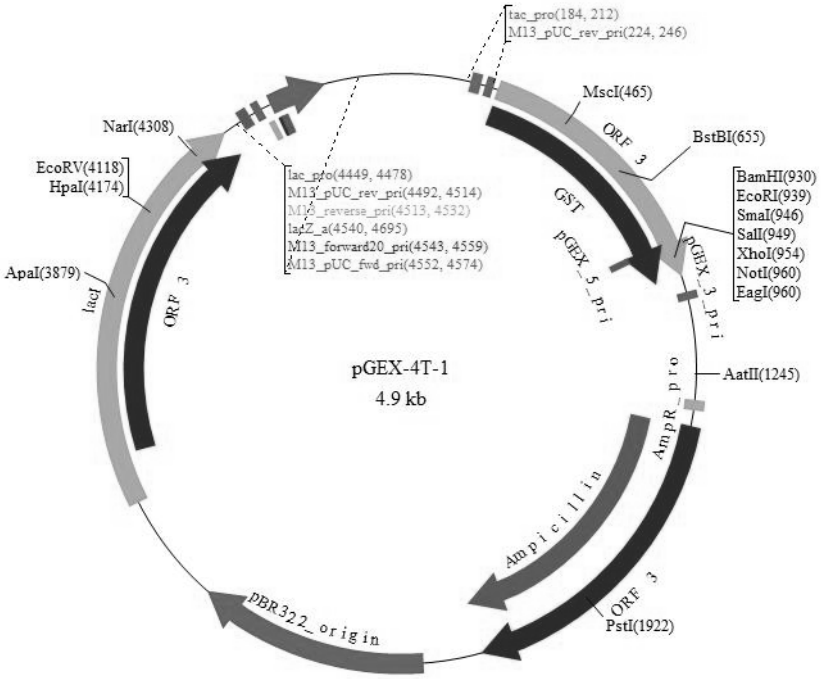


Figure 2.1: **pGEX-4T-1 expression vector** represented with restrictions sites and ampicillin resistance (http://www.biovisualtech.com/).

pSUMO/pSMT3

Low-copy-T7-Expression vector encodes a kanamycin resistance, N-terminal 6-His-peptid, and a SUMO chaperon protein sequence; based on the plasmid pET28b (Novagen, Merk; Darmstadt) and kindly provided by Chris Lima (New York, USA).

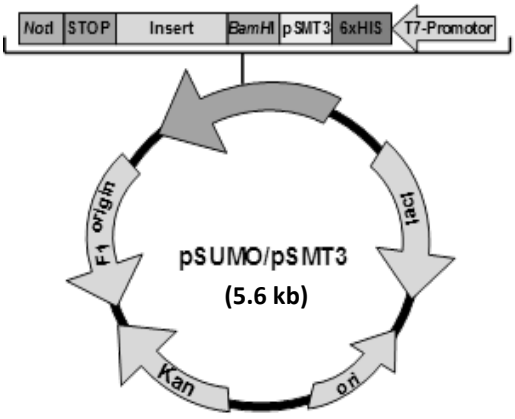


Figure 2.2: **pSUMO/pSMT3 expression vector** represented with restrictions sites and kanamycin antibiotic resistance (kindly provided by Chris Lima, New York).

pIH902/pMAL-c

The pIH902 expression vector is the precursor of the later designed pMAL-c and its derivates (Maina *et al.,* 1988; New England Biolabs, Frankfurt). It encodes a MBP-tag, a 6-His-tag, a *lacZα*-gen for blue/white selection, and an ampicillin resistance (kindly provided by Kim Williamson, Chicago).

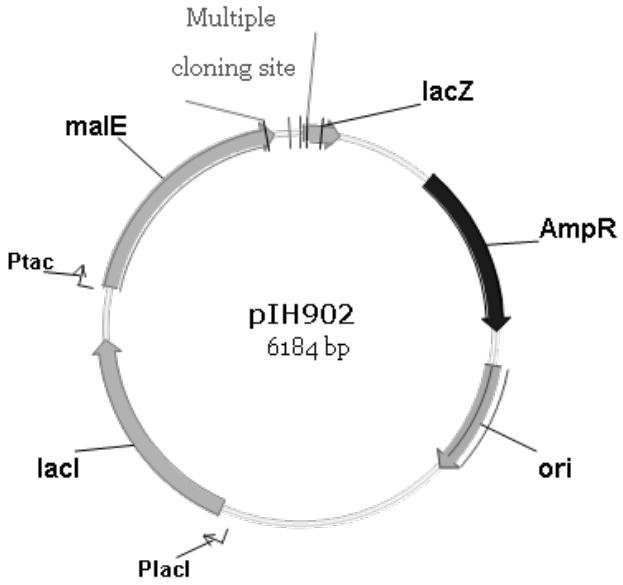


Figure 2.3: **pIH902 expression vector** represented with restrictions sites (vector map designed by Andrea Kühn).

2.1.8 Protein ladders

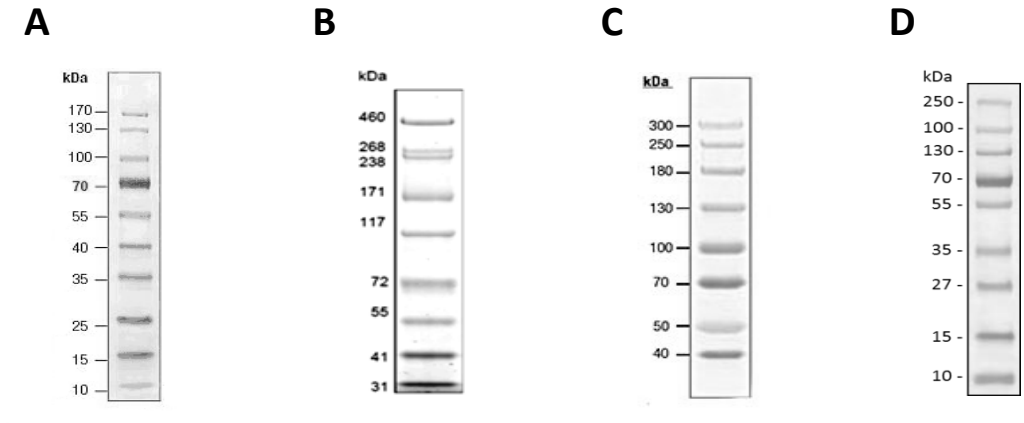


Figure 2.4: **Protein Ladders. A.** Page-Ruler^{TM}-prestained-Protein standard (Thermo Scientific/ Fermentas, Schwerte). **B.** HiMark™- Prestained-Protein standard (Invitrogen, Karlsruhe). **C.** Spectra™ Multicolor High Range Protein Ladder (Thermo Scientific/Fermentas, Schwerte). **D.** PageRuler™ Prestained Protein Ladder Plus (Thermo Scientific/Fermentas, Schwerte) (www.fermentas.de and www.invitrogen.com).

2.1.9 Antibodies and proteins

Table 2.4: Antibodies and proteins

Antibody	Animal	Dilution Western blot IFA		Source
Primary Antibodies				
Anti-PfCCp1/1rp1 pAb	Mouse	1:200	1:100	AG Pradel , Würzburg
Anti-PfCCp1/1rp6 pAb				
Anti-PfCCp2/2rp3 pAb	Mouse	1:200	1:100	AG Pradel , Würzburg
Anti-PfCCp3/SR pAb	Mouse	1:50	1:50	T. J. Templeton , New York
Anti-PfCCp3/3rp3 pAb	Mouse	1:100	1:100	AG Pradel , Würzburg
Anti-PfCCp4/4rp1 pAb	Mouse	1:100	1:50	AG Pradel , Würzburg
Anti-PfCCp5/5rp4 pAb	Mouse	1:200	1:100	AG Pradel , Würzburg
Anti-PfFNPA/rp1 pAb	Mouse	1:100	1:50	AG Pradel , Würzburg
Anti-Pfs230 pAb	Rabbit	1:100	1:200	Biogenes, Berlin
Anti-Pfs230 pAb	Mouse	1:500	1:400	K. Williamson, Chicago
Anti-Pfs48/45 pAb	Mouse	1:100	-	K. Williamson, Chicago
Anti-Pfs16 pAb	Mouse	1:500	-	K. Williamson, Chicago
Anti-PfMR5 pAb	Mouse	1:50	1:100	AG Pradel , Würzburg
Anti-Pf39 pAb	Mouse	1:500	-	AG Pradel , Würzburg
Anti-PfPeg3 pAb	Rat	1:100	1:100	P. Alano, Rom, Italy
Anti-PfActinII pAb	Mouse	1:200	-	AG Pradel , Würzburg
Anti-Proteasom SUα5 pAb	Mouse		1:50	AG Pradel , Würzburg
Anti-PF14_0412 (WD40 protein)	Mouse		1:50	AG Pradel , Würzburg
Anti-GST mAb	Goat	1:4000	-	GE Healthcare, Solingen
Anti-His mAb	Mouse	1:5000	-	Amersham/Pharmacia, Dübendorf

Anti-Pfs25 MRA-38 mAb	Rabbit	1:500	1:1000	ATCC/MR4, Manassas, USA
Anti-Pfs28 MRA-18 pAb	Rabbit		1:100	ATCC/MR4, Manassas, USA
Anti-Pfs230 MRA-27 mAb	Mouse			ATCC/MR4, Manassas, USA
Neutral mouse serum	Mouse	1:100	1:100	AG Pradel , Würzburg
Neutral goat serum	Goat	1:100	1:100	Sigma, Taufkirchen
Anti-FH pAb (against SCR1-20)	Goat	1:800	1:100	Calbiochem/Merck Darmstadt
Anti-FH mAb clone 131X (against SCR8-15)	Mouse	1:400	1:1 in TBA and GIA	Quidel, San Diego
Anti-FH pAb (against SCR1-4)	Rabbit	1:800		Santa Cruz Biotech-nology, Heidelberg
Anti-FH mAb (against SCR18-20)	Mouse	1:200		Enzo Life Sciences, Lörrach
Anti-C1q polyclonal	Goat	1:4000	1:1000	Quidel, San Diego
Anti-C3 polyclonal	Goat	1:1000	1:200	Quidel, San Diego
Secondary Antibodies				
Anti-Mouse IgG Alexa- 488	Goat	-	1:1000	Invitrogen, Karlsruhe
Anti-Mouse IgG Alexa-594	Goat	-	1:1000	Invitrogen, Karlsruhe
Anti-Rabbit IgG Alexa-488	Goat	-	1:1000	Invitrogen, Karlsruhe
Anti-Rabbit IgG Alexa-594	Goat	-	1:1000	Invitrogen, Karlsruhe
Anti-Goat IgG Alexa-488	Chicken		1:1000	Invitrogen, Karlsruhe
Anti-Mouse IgG alkaline phosphatase	Goat	1:6000	-	Sigma, Taufkirchen
Anti-Goat IgG alkaline phosphatase	Rabitt	1:7000	-	Sigma, Taufkirchen
Anti-Rat IgG alkaline phosphatase	Goat	1:5000	-	Sigma, Taufkirchen
Anti-Rabbit IgG alkaline phosphatase	Goat	1:5000	-	Sigma, Taufkirchen

Proteins		
purified human FH	Human Serum	Calbiochem/Merck Darmstadt
FHR-1 recombinant peptide	Insect Cells, Baculovirus expression vector system (BEVS)	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 1-4	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 1-7	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 8-11	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 11-15	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 15-18	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 15-19	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 8-20	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena
FH recombinant peptide SCR domain 19/20	Insect Cells, BEVS	C. Skerka, P.F. Zipfel, Jena

2.1.10 Protein identification numbers (PlasmoDB and Uniprot)

Table 2.5: Identification numbers of plasmodial and human proteins

Protein	Identification number
Plasmodial Proteins (PlasmoDB)	
Pf39	PF11_0098
PfActinII	PF14_0124
PfCCp1	PF14_0723
PfCCp2	PF14_0532
PfCCp3	PF14_0067
PfCCp4	PFI0185w
PfCCp5	PFA0445w
PfFNPA	PF14_0491
PfPeg3	PFL0795c

PfMR5	PFB0400w
Pfs230	PFB0405w
Pfs25	PF10_0303
Pfg377	PFL2405c
PfGAP50	PFI0880c
Pfalpha subunit (α-SU) type 5	PF07_0112
Human Complement Proteins (Uniprot)	
Human Complement Factor C3	P01024
Human Complement Factor H	P08603
Human Complement Factor I	P05156

2.1.11 Bioinformatic Sources and Computer Programs

- Clustal W sequence alignment
- Mascot MS Ion search
- OrthoMCL DB
- ExPASy Proteomics Server
- The NCBI Structure Group
- Wellcome Trust Sanger Institute Search Pfam
- SMART / EMBL Heidelberg
- EMBL-EBI Toolbox
- NCBI/ BLAST/ blastp suite
- Adobe® Acrobat x Pro
- Adobe® Photoshop CS
- Microsoft® Excel 2010
- Microsoft® Word 2010
- Microsoft® Powerpoint 2010
- ImageJ 1.44p

2.2 Methods

2.2.1 Cell biology

2.2.1.1 Cultivation and storage of bacteria

Bacteria were grown in LB with antibiotic pressure, dependent on selection conditions, in a shaking incubator at 37°C with 180-220 rpm. Cells were stored temporarily at 4°C, on an LB agar plate or in LB for a few days under selective pressure. Bacteria used as protein expression systems were not stored. These cells were transformed with corresponding plasmids directly before growth to ensure optimal protein expression. Long-term storage of bacterial cells was performed by resuspension in 50 % glycerol/LB at -80°C.

2.2.1.2 Transformation of competent bacterial cells

Chemically competent *E. coli* cells were transformed by heat shock. Cells were slowly thawed on ice and about 100 ng of plasmid DNA was added. After incubation for 30 min on ice, the bacteria were subjected to a heat shock at 42°C in a water bath for 30 sec. After a 2 min incubation period on ice, 250 µl of SOC medium was added and incubated at 37°C for 1 h. The cells were pelleted, resuspended in 50 µl medium, and plated on LB agar plates containing an appropriate selective antibiotic. The plates were incubated overnight at 37°C.

2.2.1.3 Culture of *Plasmodium falciparum*

Cultivation of *Plasmodium falciparum*

P. falciparum was cultured at 5 % hematocrit in 25 cm^2 or 75 cm^2 tissue culture flasks in a volume of 5 or 20 ml, respectively (Fig. 2.5). RPMI complete was used as culture medium. The parasites were propagated in erythrocytes of blood group A^+ in a gas environment of 5 % O_2, 5 % CO_2, and 90 % N_2 at 37°C. Culture medium was changed daily by gently tilting the flask and aspirating the medium with a glass Pasteur pipette (Fig. 2.5). Fresh medium, warmed to 37°C, was added to the final volume. Culture flasks were gassed for 20 sec, sealed tightly, and incubated at 37°C. Once a parasitemia above 2 % was reached, the culture was passaged. The process involved dilution of parasites in the medium to roughly 1 % parasitemia and addition of human A^+ erythrocytes to a final hematocrit of 5 %.

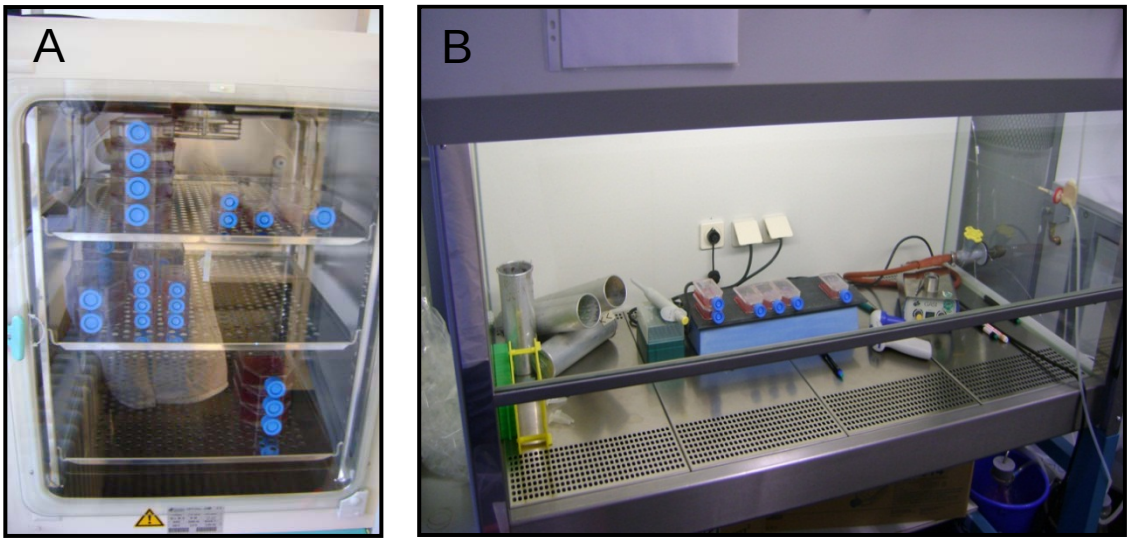


Figure 2.5: **Malaria parasite culturing. A.** Incubator at 37°C for *Plasmodium falciparum* parasites in 25 cm^2 and 75 cm^2 tissue culture flasks. **B.** Clean bench with hot plate for culture feeding.

For gametocyte maturation, a culture of asexual NF54 parasites was passaged until 1 % parasitemia and cultivated in a 75 cm^2 culture flask for about 10-20 days. Stage progression was monitored microscopically using Giemsa-stained slides. When uniform gametocyte stages were needed synchronous ring-stage parasites were cultured for 10-20 days in parasite media plus 62.5 mM N-acetyl glucosamine to inhibit merozoite invasion and thus asexual replication.

Freezing and thawing of *P. falciparum* cultures

Parasitemia of 3-4 % was attained, preferably containing high percentage of ring stages, the culture was centrifuged at 1000 x g for 5 min and the supernatant was discarded. The pellet was resuspended in 5 volumes of glycerolyte 57 solution and stored at -80°C or in liquid nitrogen until further required. To thaw and recultivate *P. falciparum*, a vial with 1 ml infected red blood cells was slowly thawed and transferred into a 15 ml falcon. 200 µl of 12 % NaCl solution was slowly added while swirling the tube. After a 2 min incubation period at RT, 10 ml 1.6 % NaCl per ml red blood cells suspension was added dropwise. The samples were centrifuged at 3000 x g for 5 min at room temperature (RT) and the supernatant was discarded. 10 ml of 0.2 % Dextrose/0.9 % NaCl solution was added drop by drop and centrifuged again at 3000 x g for 5 min. The cells were washed once with medium and cultivated as described above. The pellet was resuspended in 5 ml RPMI complete medium to 5 % hematocrit.

2.2.1.4 Determination of parasitemia and gametocytemia

Parasitemia

Parasite parasitemia of asexual cultures was determined every second day by preparation of a blood smear. 100 µl of the parasite culture was transferred into a 1.5 ml tube and centrifuged at 3,400 x g for 1 min. The supernatant was discarded and the pellet was resuspended in an equal volume of RPMI complete. A smear of the resuspended pellet was prepared on a glass slide (Fig. 2.6). After drying, the cells were fixed with methanol and dried again. Subsequently the slide was incubated for 15-20 min with Giemsa stain solution (1:20 dilution with Giemsa buffer), this followed by rinsing the slide with H_2O_{bidest} and air-drying. Then the blood smear was analyzed with a microscope at 1000 x magnification in oil immersion. The number of infected erythrocytes and of uninfected erythrocytes was counted in five fields containing more than 100 erythrocytes per field.

The mean percentage was calculated using the following formula:

$$\text{Parasitemia [\%]} = \frac{\text{\# Infected erythrocytes}}{\text{\# Infected erythrocytes + \#Uninfected erythrocytes}} \times 100$$

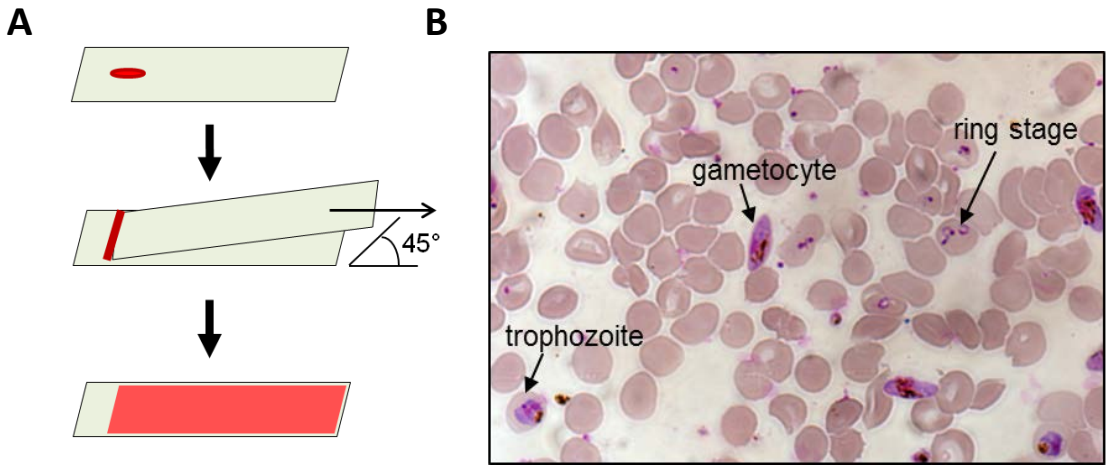


Figure 2.6: **Preparation of a blood smear. A.** Illustration of a culture blood smear for determination of parasitemia. One drop of culture is distributed over a glass slide with another slide in an angle of about 45°. **B.** Picture of a mixed *P. falciparum* culture after Giemsa staining showing ring, trophozoite and gametocyte stages.

Gametocytemia

The gametocytemia was calculated by counting erythrocytes in a Neubauer Hemacytometer (Fig. 2.7). A volume of 10 µl of diluted sample was placed into the chamber and the number of parasites in 80 small squares was counted. Then the number of cells in one µl was calculated using the formula below:

$$\frac{A \times B}{C \times D} = \text{Erythrocytes or gametocytes / µl}$$

A = number of cells in 80 small red marked squares

B = dilution of the cell solution

C = 80 (number of counted squares)

D = 0.00025 µl (Volume in one square)

Gametocytemia was calculated using the same formula as for parasitemia; however, instead of infected erythrocytes the number of gametocytes counted was substituted. With the same approach it was also possible to determine the ratio of erythrocytes to gametocytes (used for FH-binding experiments).

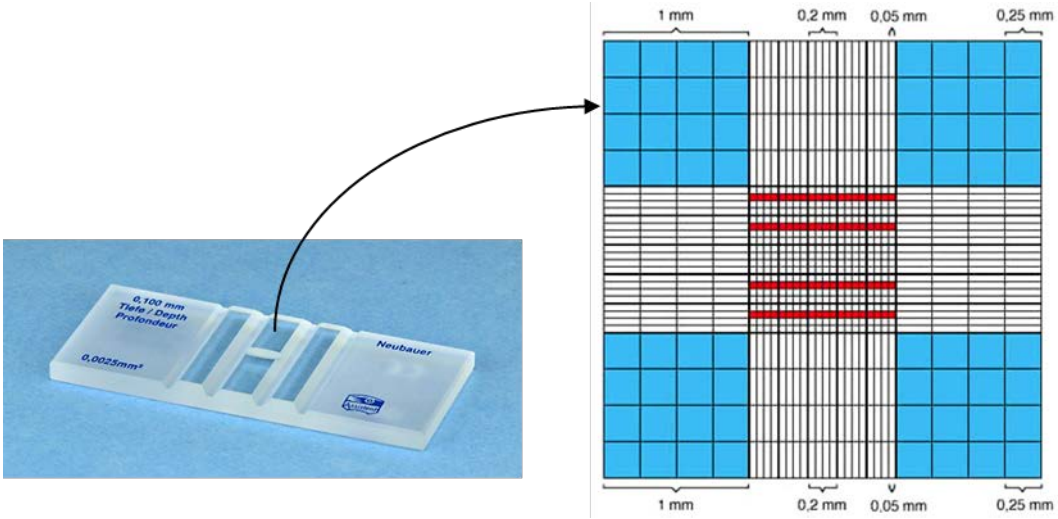


Figure 2.7: **Neubauer counting chamber** with cover slip and schematic representation of the counting grid. Red = erythrocyte and gametocyte counting areas (https://m3e.meduniwien.ac.at/).

2.2.1.5 Induction of gametogenesis

A volume of 100-300 µl mature gametocyte culture was centrifuged at 3,400 x g for 30 sec. The supernatant was discarded and the cells were gently resuspended with 20 µl of appropriate gametogenesis inducing medium at RT.

In vitro gametogenesis induction media:

- SAX-medium
- NHS (neutral human serum)
- HIS (heat inactivated human serum)

The sample was incubated for 15 min, transferred to a glass slide, and gently covered with a coverslip. Cells were then observed at 400 x magnification with a light microscope and the number of exflagellation centers was counted to determine the quality and maturity level of the culture (Fig. 2.8).

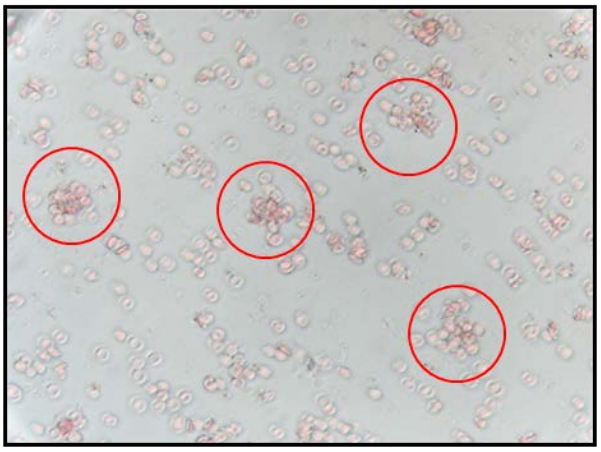


Figure 2.8: **Image of exflagellation centers.** The picture was taken using a 400 x fold magnification with a light microscope. Red circles indicate exflagellation centers.

2.2.1.6 Gametogenesis Inhibition Assay (GIA)

Anti-mouse mAb-FH (131X) was preincubated at a ratio of 1:1 with 10 µl NHS for 15 min at 37°C. 300 µl of a mature gametocyte culture was taken, centrifuged at 3,400 x g for 30 sec and the supernatant was discarded. The cell pellet was resuspended with the sera-antibody-mixture and 2 µl of XA (1 mM). Formation of microgametes was counted after 15 min using a 400 x magnification in a light microscope. The number microgametocytes in thirty optical fields were counted in triplicate. For the determination of macrogametes, the same culture was taken and coated on a slide. Macrogametes were labeled with Pfs25 antibody by IFA (see method 2.2.1.8) and erythrocytes were counterstained with Evans Blue (Sigma). The confocal microscope was then used to observe the amount of macrogametes in thirty optical fields in triplicate. The same method was applied after 20 hours. Zygotes were coated and counted by the use of IFA with an anti-Pfs28 antibody. HIS serum and PBS were used in place of the antibody solution as a negative control. Data was evaluated with a Students t-test for equal sample sizes and equal variance.

$$S_{X_1X_2} = \sqrt{\frac{1}{2}(S_{X_1}^2 + S_{X_2}^2)} \qquad t = \frac{\bar{X}_1 - \bar{X}_2}{S_{X_1X_2} \cdot \sqrt{\frac{2}{n}}}$$

$S_{X_1X_2}$ - standard deviation

X_1 - mean of group one

X_2 - mean of group two

n - sample size

2.2.1.7 Gametocyte purification

Gametocytes were grown until desired stage was obtained and purified using a percoll gradient (Kariuki *et al.,* 1998). All centrifugations and incubation steps were carried out at 37°C to avoid gametocyte activation. Gametocyte cultures were pelleted at 1800 x g for 5 min and washed once with 10 ml RPMI incomplete. The pellet was resuspended in 2 ml RPMI incomplete and coated thoroughly on a gradient containing 2 ml percoll layers of 80, 65, 50, and 35 %. After 10 min centrifugation at 1,300 x g the second interphase (counted from the top) was extracted and washed once with RPMI incomplete for gametocyte purification. When gametocytes should be activated after purification, the last washing step was performed using RPMI complete. The purity of the final pellet was evaluated with a Neubauer Hemacytometer (section 2.2.1.4).

2.2.1.8 Indirect Immunofluorescence Assay (IFA)

Parasite preparations for immunofluorescence microscopy included sexual stages, such as unactivated gametocytes (Gc), activated gametocytes (aGc), gametes (Gm), and zygotes of the *P. falciparum* NF54 isolate, and erythrocytes. Mature gametocytes were activated with different media (section 2.2.1.6) according to the experimental design. Preparations were air dried on IFA slides and fixed for 10 min in -80°C methanol. Blocking of non-specific binding was undertaken by fixation of cells which were incubated in 0.5 % BSA and 1 % neutral goat serum in PBS for 30 min each. When anti-goat sera were used for first antibody labeling, cells were blocked with 3 % BSA without neutral goat serum. Preparations were then incubated for 1.5 h at 37°C with an antibody against antigens specific to the suitable life stage in PBS. Binding of primary antibody was visualized using fluorescence-conjugated goat anti-mouse or goat anti-rabbit (Alexa Fluor 488 or Alexa Fluor 594) antibodies diluted 1:1000 in PBS for 1 h at 37°C (Fig. 2.9). For double-labeling experiments, specimens were consecutively incubated with the respective first antibody (sexual stage antibody) followed by Alexa Fluor 488-conjugated secondary antibody (green), before incubation with the respective second antibody followed by Alexa Fluor 594-conjugated secondary antibody (red). 0.05 % Evans Blue solution (Sigma) diluted in 1 x PBS was added to each well for 20 s as a red blood cell counterstain. Between all steps cells were washed twice with PBS. The slides were embedded with antifading medium MOWIOL (Citi Flour LTD, London), covered with a coverslip, and hermetically sealed with nail polish. Labelled specimens were examined with a Zeiss Axiolab microscope in conjunction with an Axiocam camera. Digital images were processed using Adobe Photoshop CS software.

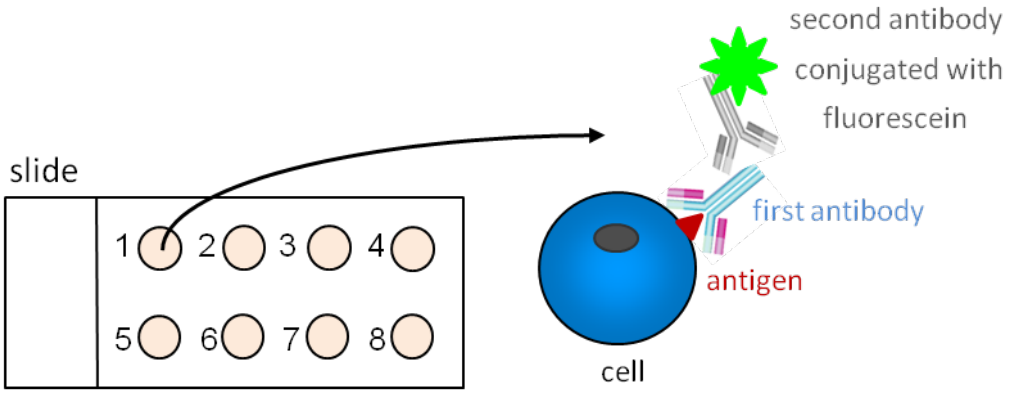


Figure 2.9: **Indirect Immunoflourescence.** The fluorescent second antibody binds the first antibody which is specific for the protein of interest.

2.2.2 Protein biochemical methods

2.2.2.1 Expression of recombinant proteins

E. coli BL21-CodonPlus®-(DE3)-RIL bacteria cells were transformed with a vector construct (section 2.2.1.3) and plated onto agar plates containing either 0.1 mg/ml kanamycin or ampicillin. The plates were incubated at 37°C overnight. The following day single colonies were picked and precultured with LB medium containing 0.1 mg/ml of the appropriate antibiotic overnight. The cells were then incubated in an Erlenmeyer flask on a shaking platform at 37°C overnight. Cultures were seeded at 1 % v/v of preculture with LB/antibiotic the following morning. Cells were grown at 37°C until an OD_{600} of 0.5 was reached to ensure the cells had entered the log phase of growth. The induction of protein expression was enabled by the artificial substrate IPTG (Isopropyl-β-D-thiogalactoside) at a concentration of 0.75 mM. After 5 h induction at RT, cultures were centrifuged at 5,000 x g at 4°C to obtain bacterial pellets. These were either stored at -20°C until use or lysed to purify the recombinant protein.

2.2.2.2 Purification of recombinant proteins

During the work with proteins, the samples were kept on ice to avoid loss of stability. Samples were kept in aliquots, and successive thawing was avoided in order to minimize protein denaturation.

Inclusion Bodies

A bacterial pellet from 1.5 l culture was resuspended in inclusion body lysis buffer after IPTG induction (section 2.2.2.1). 200 mg lysozyme was added and the suspension was incubated on ice for 10 min. The sample was sonicated (50 % intensity, 50 % duty cycle) on ice for 10 min and the lysate was resuspended in 200 ml detergent buffer followed by centrifugation at 5,000 x g for 10 min at 4°C. The pellet was resuspended in 250 ml washing buffer and centrifuged at 5,000 x g for 10 min several times, until a discrete pellet has formed. The resulting pellet of inclusion bodies was thoroughly washed in 250 ml of 70 % ethanol and resuspended in 2-5 ml of sterile PBS. The pellet was sonicated until the protein solution was able to pass through a 23 G needle for subsequent immunization of mice. Protein concentration was estimated by comparison of stained SDS-gels showing a dilution series of BSA proteins of known concentration. Inclusion body proteins were stored at -20°C.

GST and 6-His-tag purification

Pellets of *E. coli* BL21-CodonPlus®-(DE3)-RIL were resuspended in 6-His/GST lysis buffer and incubated for 1 h at 4°C on a shaker. Cell lysis was performed using a French Press in three stages followed by DNA degradation by pulse sonication for 2 min at 50 % intensity and 50 cycles. The resulting sample was centrifuged at 30,000 x g at 4°C. The supernatant was collected and filtered with a 0.22 µm syringe filter. Meanwhile, 500 µl glutathione-sepharose beads for GST purification or 50 % Ni-resin were washed three times with 1 x PBS and added to the supernatant followed by overnight incubation at 4°C on a rotating mixer. The solution was loaded onto a PolyPrep® column whereby unbound proteins passed through the matrix. However, proteins selectively bound to GST-sepharose or Ni-resin were retained. The column was washed three times with PBS for GST-purification. For 6-His-purification the column was washed two times with wash buffer 3 for and one time with wash buffer 5. Finally, the proteins were eluted in three or more fractions with 500 µl of the appropriate elution buffer. Fractions were stored at -20°C and protein concentration was determined by SDS-PAGE (section 2.2.2.3 and 2.2.2.4).

MBP-tag purification

For purification of MBP (maltose binding protein)-tagged recombinant proteins, the bacterial cells were grown in LB medium containing antibiotic and 0.1 % sterile glucose. The PBS-washed pellet was resuspended in MBP lysis buffer including lysozyme and 1 mM PMSF protease inhibitor and incubated on ice for 20 min. $MgCl_2$ and NaCl were added and the bacteria were incubated on a rotating mixer for 1 h at 4°C to induce cell lysis. The cell mixture was centrifuged for 30 min at 30,000 x g. During the centrifugation step 1 ml of the amylase resin was washed for three times in TE buffer with centrifugation at 2,000 x g for 5 min and added to the supernatant of the centrifuged cell solution. The mixture was rotated over night at 4°C. The beads were washed three times with column buffer and eluted afterwards eight times with 500 µl elution buffer containing maltose. Protein concentration of fractions were analyzed via SDS page and stored at -20°C.

2.2.2.3 SDS polyacrylamide gel electrophoresis (PAGE)

For the separation of proteins by SDS-PAGE, 8 %, 10 %, 12 % or 15 % polyacrylamide gels were prepared. The composition of stacking and resolving gels are given below. Samples were prepared in 2 x protein loading buffer, heated for 5 min at 95°C and

Table 2.6: Composition of different SDS-Gels

	Resolving Gel				Stacking Gel
	8 %	**10 %**	**12 %**	**15 %**	**5 %**
H_2O_{bidest}	2.3 ml	1.9 ml	1.6 ml	1.1 ml	2.4 ml
30 % Acrylamide	1.3 ml	1.7 ml	2.0 ml	2.5 ml	0.6 ml
1.5 M Tris pH 8.8	1.3 ml	1.3 ml	1.3 ml	1.3 ml	-
0.5 M Tris pH 6.8	-	-	-	-	1.0 ml
10 % SDS	50 µl	50 µl	50 µl	50 µl	12 µl
10 % APS	50 µl	50 µl	50 µl	50 µl	12 µl
TEMED	3 µl	2 µl	2 µl	2 µl	4 µl

cooled down for 2 min on ice. The proteins were separated in 1 x SDS-PAGE running buffer for approximately 20 min at 60 V until the dye front had passed through the stacking gel and entered the resolving gel. The voltage was then switched to 120 V. Estimation of protein sizes was done by comparison to the size standards given in section 2.1.8. The gel was then either used for Western blotting (section 2.2.2.5) or stained (section 2.2.2.4).

2.2.2.4 SDS gel staining, measurement of the protein concentration and conservation

After SDS-PAGE gels were washed three times for 5 min with distilled water and stained for a minimum of one hour with GelCode®-Blue-Stain (PIERCE, Thermo Fisher, Rockford, USA) on a rocker. Once the desired staining was achieved, the gel was washed with water for about 2 h. For silver staining the SilverSNAP Stain Kit for Mass Spectrometry (PIERCE, Thermo Fisher, Rockford, USA) was used and performed according to the manufacturers protocol. The approximate protein concentrations were estimated by comparing band intensity with protein bands of known BSA concentration. For long term gel preservation, the gel was incubated with a solution of 10 % glycerol and 20 % ethanol for about 30 min and then dried between two cellophane sheets in a gel drying frame for one day at RT.

2.2.2.5 Western blot analysis

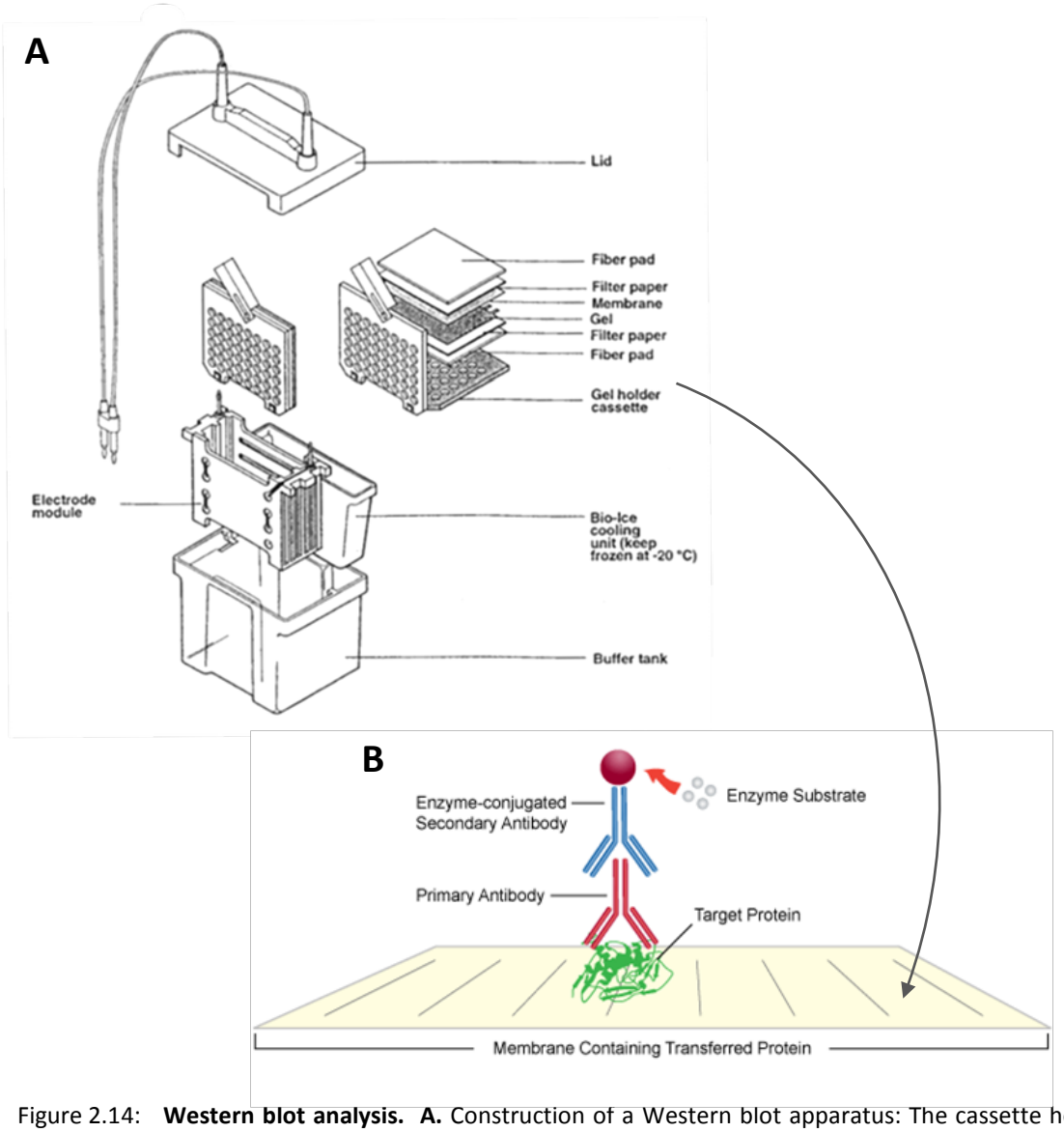


Figure 2.14: **Western blot analysis. A.** Construction of a Western blot apparatus: The cassette holds the gel and membrane between buffer-saturated filter paper and fiber pads. The cassette is inserted vertically in the transfer buffer-filled tank between the positive and negative electrodes. **B.** Illustration of protein detection on a nitrocellulose membrane. (modified from http://www.leinco.com/).

Proteins were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, München) using the Mini-Trans-Blot-Apparatus from Bio-Rad according to the manufacturers' instructions using transfer buffer (Fig. 2.14). The transfer was performed for 2 h at 25 V or overnight at 15 V. In some cases efficient protein transfer was controlled by staining the nitrocellulose membrane with Ponceau S solution for 1 min at RT and extensive rinsing with H_2O_{bidest}. The membranes were then incubated in 1 x TBS to wash off excess staining solution. Blocking of free protein binding sites was performed with 0.5 % BSA/5 % milk in TBS for 1 h min at RT

on a rocker. Primary antibodies were diluted in 3 % TBS/milk and incubated for 2 h at RT or overnight at 4°C on a rocker. After washing for 10 min at RT, one time with 3 % TBS/milk, twice with 3 % TBS/milk/0.1 %Tween and once again with 3 % TBS/milk, the membranes were incubated with the corresponding alkaline phosphatase-conjugated secondary antibody in 3 % TBS/milk for 1 h at RT. To remove unbound antibody, further washes were performed with TBS and TBS/0.1 % Tween followed by addition of equilibration buffer for 3 min. Nitrocellulose membrane was developed in a solution of nitroblue tetrazolium chloride (NBT) and 4-chlor-3-indoxylphosphate (BCIP) for 1-10 min and the reaction was stopped with stop buffer.

2.2.2.6 Interaction studies

Affinity co-elution binding assay

Bead-bound GST-fusion proteins (section 2.2.2.2/GST-tag purification) were transferred to a PolyPrep® column and washed two times with 10 ml PBS. Afterwards the prey-6-His protein sample was prepared similarly to the procedure described in section 2.2.2.2/6-His-tag purification, but without the addition of the Ni-resin, and was applied to the column. The column was washed three to four times with PBS to remove unbound proteins. Bound proteins were eluted three times from the column with 500 µl GST elution buffer. Finally, the obtained sample was analyzed by Western blotting for the presence of both, bait and prey fusion proteins (Fig. 2.11).

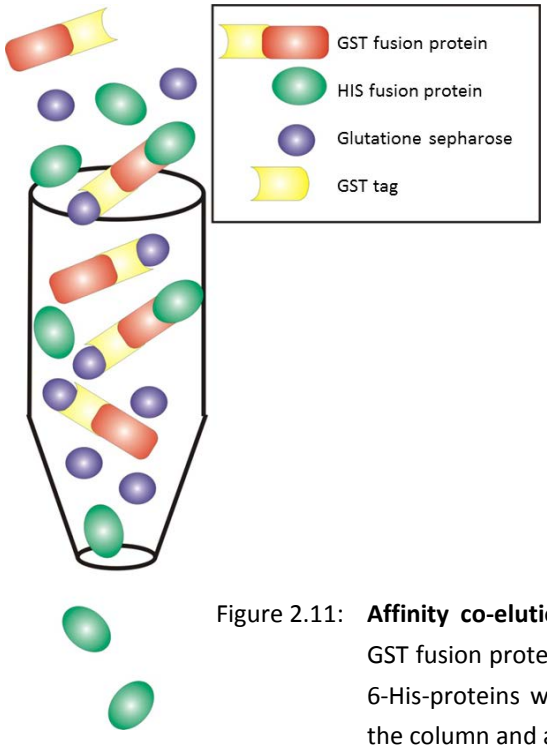


Figure 2.11: **Affinity co-elution binding column.** Glutathione sepharose bound GST fusion proteins were transferred to a column. After washing the 6-His-proteins were added. The protein samples were eluted from the column and analyzed via Western blot.

Co-immunoprecipitation (Co-IP)

Parasite pellets, obtained from wild-type NF54 strain were resuspended in 200 µl PBS-Mix or PBS-Mix/NP40 and sonicated with 50 % amplitude and 50 cycles followed by centrifugation at 13,000 x g and 4°C for 1 min. The supernatant was prepurified by consecutive incubation with 5 % v/v preimmune mouse sera and 20 µl of protein G-beads (Santa Cruz Biotechnology, Heidelberg) for 30 min each at 4°C. After centrifugation at 3,500 x g for 5 min, the supernatant was incubated for 1 h at 4°C with the respective bait antibody. A volume of 20 µl of protein G-beads was added and incubated for another hour or overnight at 4°C. The beads were centrifuged at 3,500 x g, washed five times with 1 ml cooled PBS, and mixed with an equal volume of loading buffer and loaded onto a SDS gel. Precipitated proteins were identified via Western blot analysis or silver staining and subsequent protein bands identification by mass spectrometry.

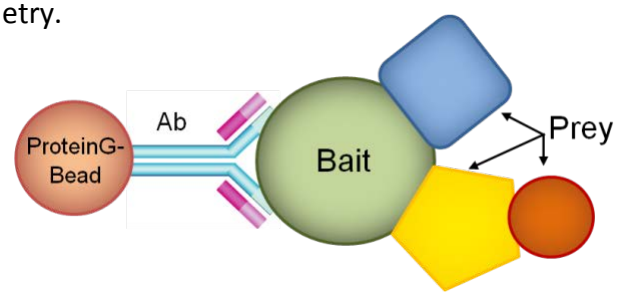


Figure 2.12: **Co-immunoprecipitation** works by selecting an antibody (Ab) that targets a known protein (bait). This pulls the entire protein complex (prey) out of solution and thereby it is possible to identify members of the complex.

2.2.2.7 Immunization of mice and generation of antibodies

Specific immune sera were generated by the immunization of six week old female NMRI mice. Before immunization the purified proteins were transferred, using Millipore Amicon filters, in sterile PBS / 5 % Glycerin. 100 µg of recombinant proteins, GST, 6-His, MBP tagged or inclusion bodies, were dissolved in 200 µl sterile PBS and emulsified in 200 µl Freund's incomplete adjuvant. The boost followed after four weeks with 50 µg of inclusion bodies or 100 µg of the purified recombinant protein and Freud's adjuvant. Ten days later mice were anesthetized by intraperitoneal injection with a mixture of ketamine and xylazine according to the manufacturer´s protocol (Sigma-Aldrich), and immune sera were collected via heart puncture. Sera from non-immunized mice served as a control for antibody reagent studies.

2.2.2.8 Preparation and handling of human serum

Blood samples were drawn using standard phlebotomy techniques from healthy donors that never had contact with malarial parasites before. The blood was left to coagulate for 15-20 min and was centrifuged for 10 min at 3.000 x g. The sera were removed, aliquoted, and frozen at -80°C. Frozen specimens were thawed rapidly at 37°C and transferred immediately to ice to prevent complement activation. Samples were kept on ice for no longer than two hours. Specimens were not thawed at RT or on ice as this can lead to C3 activation and affect results. Only one freeze/thaw cycle may be performed without affecting the samples. For complement activation assays the sera were not frozen and stored at 4°C for no longer than 10 days to avoid minor complement activation (Mollnes *et al.,* 1988) otherwise they were used immediately after blood withdrawal. For complement inactivation the sera was vacuum filtered and heat inactivated for 50 min in water bath at 55°C and stored at -20°C.

2.2.2.9 Determination of complement activity

To survey the activity of the complement system in the mosquito midgut from the uptake of the blood until 20 hours later the C3a Plus EIA Microvue Kit (Quidel, San Diego, USA) was used. It represents an enzyme immunoassay for the quantitation of the C3a fragment of the complement protein C3 in human sera and provides information on the activity of the innate complement system.

For this assay a mosquito feed was performed using fresh human serum (see method 2.2.2.8) and fresh erythrocytes. The erythrocytes were mixed with human serum to a ratio of 1:1 and fed immediately to the mosquitoes (2.2.3.2). The mosquito midguts were dissected at different time points to investigate complement activation during a time of 20 h (2.2.3.3). Two midguts were dissected at different time points in triplicate, and frozen in 10 µl PBS at -80°C immediately. For the measurement the midguts were resuspended with PBS, solid cellular constituents were centrifuged down, and 1 µl of the supernatant, including the soluble C3a, was mixed with 99 µl Specimen Dilutent Buffer. To evaluate that 1 µl is the optimal volume to obtain sustainable ELISA values between 0 and 3 (extinction 450 nm) different volumina of midgut mixtures were tested previously. In other words 1 µl complies 0.2 midguts and was the optimal amount of midgut serum contents to evaluate the complement activity in the mosquito midgut using the C3a Plus Kit. All following steps were performed according to the manufacturer´s protocol.

2.2.2.10 Co-Factor Activity Assay

Cofactor activity of FH on microbial cell surfaces was measured by the FI-mediated conversion of C3b to iC3b. Cells were incubated with an excess of purified FH for 30 min at RT. After washing, bound complement regulators were incubated with a molar excess of purified C3b (Calbiochem) and purified FI (Sigma) for 30 min at RT. After a last washing step iC3b generation was quantified via Western blot analyses of the treated samples using a polyclonal anti-goat C3 antibody.

2.2.2.11 Cell treatment for investigation of cell binding complement factors

Malaria parasites were cultured in RPMI complete medium containing 10 % HIS. To take off cell culture bound complement factors cells were washed twice with PBS at 37°C (1 min 3,400 x g). Then they were treated according to the type of experiment and washed once again with PBS. For Gc all following steps were carried out at 37°C. After induction of gametogenesis aGc were treated at RT. Erythrocytes were lysed with 1 ml 0.02 % Saponin/RPMI incomplete for 5 min at RT, centrifuged at 3,400 x g for 2 min and the remaining parasites were washed once again with PBS (3,400 x g 2 min).

2.2.3 Mosquito rearing and feeding techniques

2.2.3.1 Rearing of *Anopheles stephensi* mosquitoes

Each stock colony of *A. stephensi* mosquitoes was kept in a gauze-covered, wire-framed cage at 28°C and 80 % relative humidity. Adult female mosquitoes were fed once a week on uninfected rodent blood for 20 min with an anesthetized mouse. Eggs were laid by blood-fed female mosquitoes two to three days after a blood meal in small bowl filled with 1 ‰ NaCl water. The eggs were transferred into a vat with 1 ‰ NaCl water (Fig. 2.13). The eggs began to hatch over two days. The larvae pupated after six to eight days and adults emerged between days nine and eleven. Floating dry cat food was used as nutrition for larvae and replaced every day. Pupae were collected daily and placed in a small bowl with water in a cage. Adult mosquitoes emerged two to three days post pupation. A cotton wool pad soaked with 5 % glucose (Sigma) and 0.05 % p-aminobenzoic acid (PABA; Sigma) was placed on the cage within reach of mosquitoes and was replaced daily.

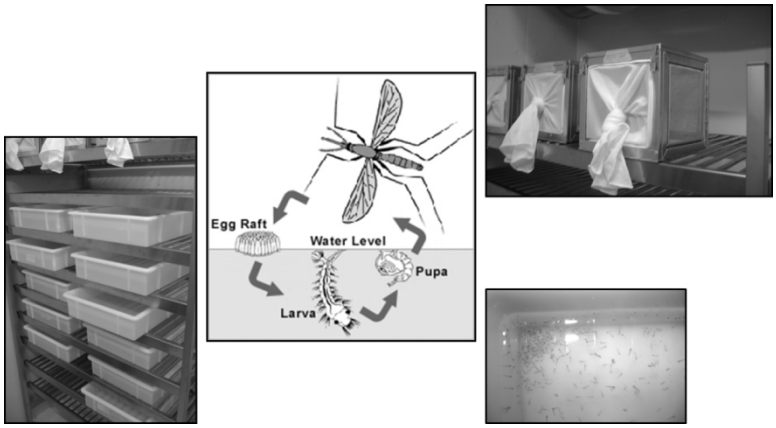


Figure 2.13: **Rearing of mosquitoes.** Adult mosquitoes were kept in cages. The eggs were transferred into plastic baskets were they grow to larvae until pupae stages. Then eclosion of pupae occurs in the cages.

2.2.3.2 *Ex vivo* feeding of mosquitoes

Mosquitoes to be used for Transmission Blocking Assays (TBA) were fed with a solution of 5 % glucose, 0.05 % PABA and 0.04 mg/ml gentamycin. On the day before the feed a mosquito container was prepared (500 ml Häagen-Dazs-cup) and 20-60 of four to six day-old virgin female mosquitoes were placed into the tub with the aid of a pooter (aspiration gun). A warm bottle was used to attract female mosquitoes to the front of the cage for collection with the pooter. Up to 20 mosquitoes were caught in the pooter during the transfer procedure. One day prior to feeding the cotton wool pad was removed to force maximum starvation. A Parafilm® M was placed on the blood feeder and the whole system was warmed up (Fig. 2.14). The following procedures were performed entirely at 37°C and as rapid as safe handling would permit. Good

Table 2.7: Ingredients of different mosquito feeds, incubated at 37°C prior to the feed.

	purified gametocytes	**fresh A^+ erythrocytes**	**A^+ serum**	**anti-FH or PBS**
Feed with parasites	1 vol.	1 vol.	2 vol.	-
Feed without parasites	-	1 vol.	1 vol.	-
Transmission Blocking	1 vol.	1 vol.	1 vol.	1 vol.

microbiological practice was observed in order to minimize the possibility of gametocytes committing to activation before the blood meal was taken up by the mosquito. The feeding-mix was transferred into the glass-feeder and placed within reachable distance for the mosquitos in the tub (Fig. 2.14). The light was turned off for 20 min, and the insectary was left to avoid disturbing the feeding mosquitoes. Then the mosquitoes were sealed safely in the S3**-Insectary. Subsequent to a blood feed, the mosquitoes were put on a 5 % glucose (without PABA) diet.

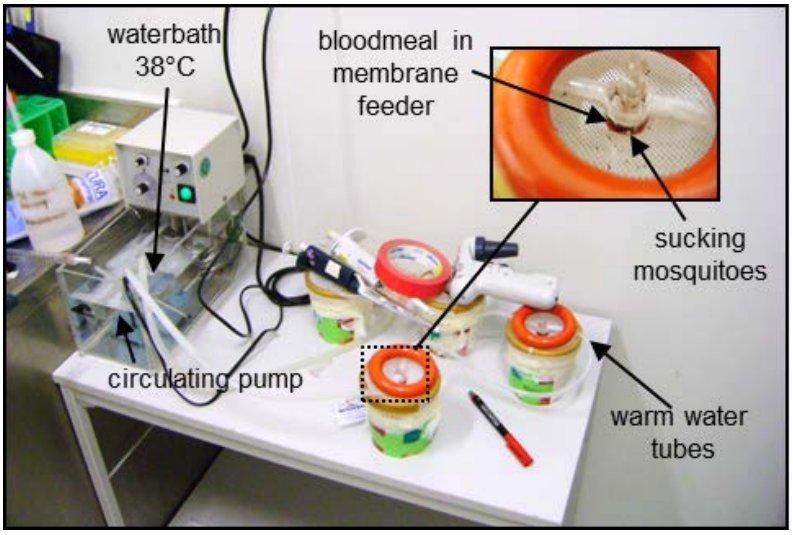


Figure 2.14: **System of a mosquito membrane feed.** Adult female mosquitoes were transferred in cups and fed with the bloodmeal. A circulation water bath connects the glass feeders with rubber tubing in a series.

2.2.3.3 Dissection of mosquito midguts

Blood fed mosquito midguts were dissected at different time points, from 5 min until 24 hours or 8 days after the feed, in case of oocyst counting in TBAs. The mosquitoes were removed from the tub via the pooter and cooled on ice for 3 min. The paralyzed mosquitoes were kept on ice in a closed petri dish to avoid escape. Individual mosquitoes were removed and mounted on glass microscope slides onto a drop of PBS. The mosquito midgut was dissected under a binocular light microscope using a 40 × objective (total magnification 400 ×). Midguts were extricated by holding the anterior of the abdomen with tweezers whilst at the same time a second pair of tweezers were used to gently pull on the apex of the abdomen until the gut and malpighian tubule were exposed (Fig. 2.15). Then the midgut was placed in PBS on ice and immediately stored at -80°C, in order to avoid further complement or enzyme activation. For TBAs berried mosquitos were dissected and the midgut oocysts were stained with 0.2 % mercurochrome/PBS for 10 min. Oocysts were counted at 400 x fold magnification.

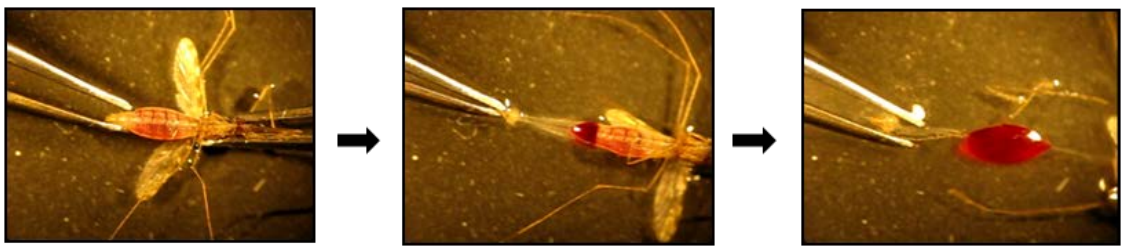


Figure 2.15: **Dissection of mosquito midguts.** Paralyzed blood-fed female mosquitoes were transferred on a glass slide and the midgut was extricated (photo by Matthias Scheuermeyer).

3 Results

3.1 Sexual stage proteins of the malaria parasite

3.1.1 Orthologs and adhesion domains of PfCCp proteins

Orthologs of the CCp proteins

After the completion of the *Plasmodium falciparum* genome sequences in 2002 (Gardner *et al.,* 2002) the genome was screened for proteins with extracellular adhesive domains. A new protein family suggesting involvement in parasite-parasite or parasite-host interactions called PfCCp multi-domain adhesion proteins was discovered (Pradel *et al.,* 2004; section 1.5). Orthologs of several PfCCp proteins were identified in different apicomplexan parasites. Within the last years the range of development of techniques in the field of bioinformatics have increased and re-annotation of PfCCp protein sequences were performed in this work. The investigations were carried out by bioinformatical screenings using the OrthoMCL database and the NCBI Structure Group. Up until now orthologs of all CCp proteins were found within the genus *Plasmodium,* in *P. falciparum, P. vivax, P. yoelii, P. knowlesi, P. chabaudi* and *P. berghei,* thus in all so far sequenced *Plasmodium* species. This genome survey study revealed that all CCp proteins, except of CCp4, were highly conserved throughout the apicomplexan clade, including *Toxoplasma gondii*, *Cryptosporidium muris, C. hominis, C. parvum, Theileria annulata, Th. parva, Babesia bovis, B. divergens* and *Neospora caninum* (Tab. 3.1). The number of identified orthologous CCp proteins increased with the rise of sequenced apicomplexan genomes during the last decade. For CCp1, CCp2 and CCp3 orthologous protein sequences were firstly discovered in *Toxoplasma gondii, Cryptosporidium muris, C. hominis, C. parvum, Theileria annulata* and *Th. parva* and for FNPA only in *C. parvum* (Pradel *et al.,* 2004; Templeton *et al.,* 2004). Orthologs of CCp proteins in *Babesia bovis, B. divergens* (Becker *et al.,* 2010) and *Neospora caninum* were found after genome sequence decoding of the respective parasites (Tab. 3.1). The occurrence of CCp5 and FNPA in *Cryptosporidium hominis, C. parvum, C. muris, Theileria annulata, Th. parva, Babesia bovis* and *Neospora caninum* was also proven in this study. An outstanding CCp protein, without orthologs in other species, is CCp4. It can only be found in *Plasmodium* (Tab. 3.1).

Table 3.1: Orthologs of CCp proteins in genera except *Plasmodium* (e-value < 10^{-10}, Query coverage > 30 %), the species were listed according to its date of identification as a CCp ortholog. *(Pradel *et al.,* 2004; Templeton *et al.,* 2004); **(Becker *et al.,* 2010)

CCp protein	Discovery Date	Species
CCp1	2004 by Pradel/Templeton*	*Toxoplasma gondii* *Cryptosporidium muris, C. hominis, C. parvum* *Theileria annulata, Th. parva*
	January 2010 by Becker**	*Babesia bovis, B. divergens*
	June 2011	*Neospora caninum*
CCp2	2004 by Pradel/Templeton*	*Toxoplasma gondii* *Cryptosporidium muris C. hominis, C. parvum* *Theileria annulata, Theileria parva*
	January 2010 by Becker**	*Babesia bovis*
	June 2011	*Neospora caninum*
CCp3	2004 by Pradel/Templeton*	*Toxoplasma gondii* *Cryptosporidium muris, C. parvum, C. hominis* *Theileria annulata*
	January 2010 by Becker**	*Babesia bovis*
	June 2011	*Neospora caninum* *Mus musculus*
CCp4		-
CCp5	2004 by Pradel/Templeton*	-
	January 2010	*Toxoplasma gondii*
	June 2011	*Cryptosporidium hominis, C. parvum, C. muris* *Theileria annulata, Th. parva* *Babesia bovis* *Neospora caninum*
FNPA	2004 by Pradel/Templeton*	*Cryptosporidium parvum*
	January 2010	-
	June 2011	*Toxoplasma gondii* *Cryptosporidium muris, C. parvum, C. hominis* *Theileria parva, Th. annulata* *Babesia bovis* *Neospora caninum*

3.1.2 Modules of the PfCCp proteins

Technological progress was also made in identifying domains in proteins using bioinformatic screenings. Re-annotation of PfCCp protein sequences revealed several additional adhesion domains. One Discoidin domain at the C-terminus of PfCCp1 and PfCCp2, a Ricin and an Anthrax Domain at the C-terminus of PfCCp4 and one additional Discoidin domain in PfCCp5, which is located between the Anthrax PA N-terminal domain and the LCCL domain, were discovered. All newly identified domains were surrounded by a dotted border (Fig. 3.1). For this study the results of five different databases were compared: ExPASy Proteomics Server, the NCBI Structure Group, Wellcome Trust Sanger Institute Search Pfam, SMART from EMBL Heidelberg, and EMBL-EBI Toolbox.

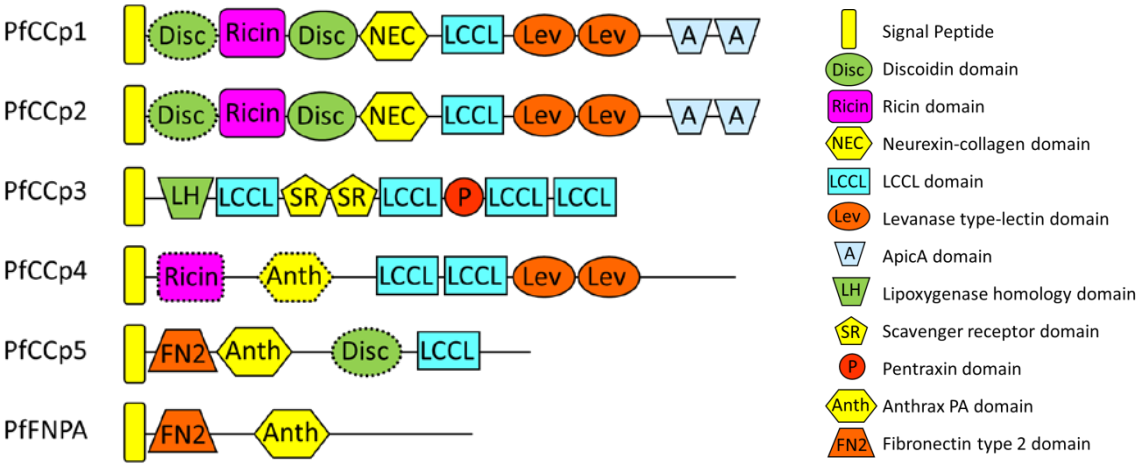


Figure 3.1: **Schematic of the domain structure of PfCCp proteins.** The overview depicts the six members of the PfCCp multi-domain adhesion protein family. Encircled black-dotted domains indicate newly identified motifs.

3.1.3 Molecular interactions between sexual stage proteins of *Plasmodium falciparum*

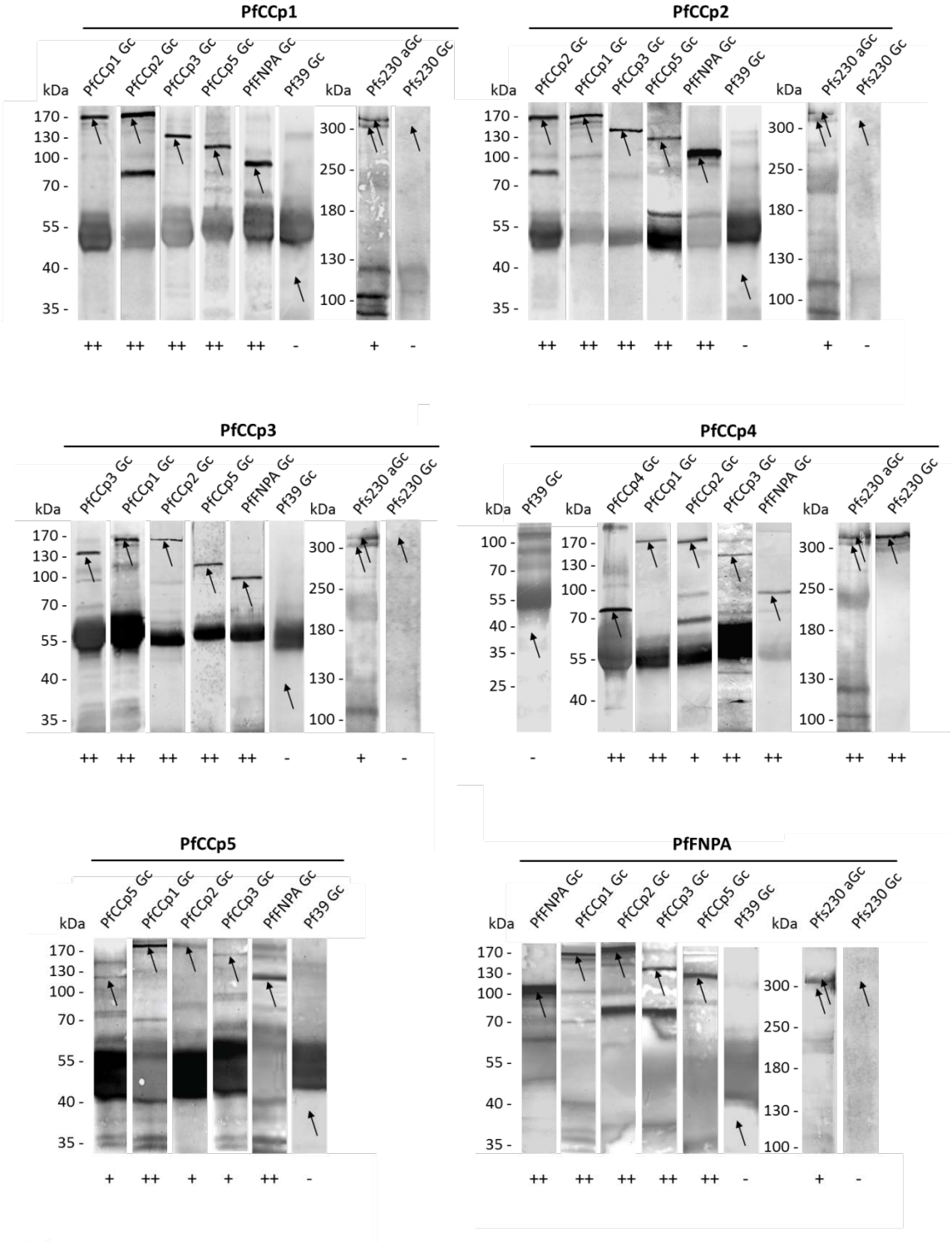
Recent studies revealed that the six PfCCp proteins were expressed in a co-dependent manner and that the absence of one protein results in complete or partial loss of all other protein family members (Pradel *et al.,* 2006; Simon *et al.,* 2009). This led to the assumption that these proteins may interact and form a multi-protein complex (MPC) in the PV of the parasite. Potential interactions between selections of sexual stage proteins were discovered in the following studies.

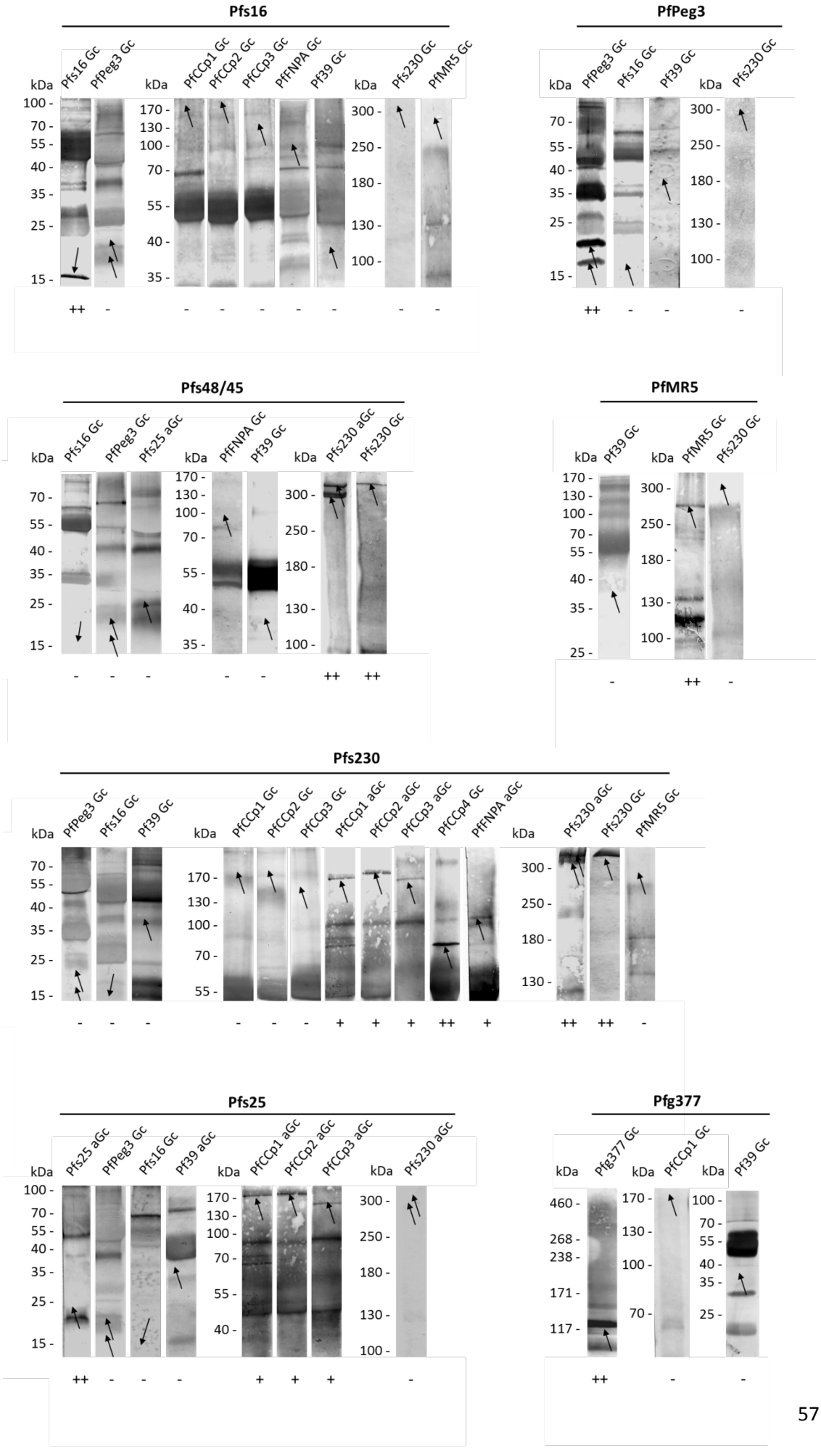
3.1.3.1 Co-immunoprecipitation studies in gametocytes and activated gametocytes

In order to prove, if protein-protein interactions take place between sexual stage proteins in the PV and on the surface of newly emerged macrogametes, an extensive set of experiments was designed. 14 sexual stage-specific proteins were included into the study, namely PfCCp1 to PfCCp5, PfFNPA, Pfs230, Pfs48/45, Pfs25, Pfs16, PfPEG3, PfMR5, and Pfg377. Pf39 was used as control protein. Pf39 encodes a protein localized to the endoplasmic reticulum (Templeton *et al.,* 1997). The investigations were performed as Co-IP assays (section 2.2.2.6.) using gametocyte (Gc) or activated gametocyte (aGc) lysate.

The precipitation studies revealed numerous protein-protein interactions between the distinct PfCCp proteins and of selected PfCCp proteins with Pfs230 and Pfs25. Prominent protein bands for PfCCp1, PfCCp2, PfCCp3, PfCCp5, PfFNPA and Pfs230 were detected by Western blot analysis of gametocyte lysates or activated gametocyte lysate, when these were individually precipitated with antibodies directed against one of these proteins (Fig. 3.2). In detail, PfCCp1, PfCCp2, PfCCp3, and PfFNPA showed binding to Pfs230 in lysates of activated gametocytes. By comparison PfCCp4 bound to Pfs230 in lysates of non-activated gametocytes and activated gametocytes. The detection of PfCCp4 binding to PfCCp1, PfCCp2, PfCCp3, PfFNPA and Pfs48/45 was shown by Sabrina Scholz (Scholz, 2007). The interactions between PfFNPA and PfCCp1, PfCCp2, PfCCp3, and PfCCp5 were demonstrated by Marie-Adrienne Dude using PfFNPA as bait (Dude, 2010). Additional interactions of Pfs25 with PfCCp1, PfCCp2 and PfCCp3 were detected in activated gametocyte lysate using Pfs25 as bait. Single Western blots including controls of Pfg377 and PfPeg3 were done by Andrea Kuehn (Kuehn, 2007). The plus (+) and minus (-) below the distinct Western blot analysis signify positive or negative protein-protein interaction. In the case of a double-plus (++), a very strong interaction was detected, like it was the case for all interactions within the PfCCp family and between Pfs230 and PfCCp4. The precipitated proteins migrated at the expected molecular masses of 185 kDa (PfCCp1 and PfCCp2), 150 kDa (PfCCp3), 125 kDa (PfCCp5), 100 kDa (PfFNPA). A double band for Pfs230 at 300 kDa and 360 kDa was detected in activated gametocyte lysates. In the case of non-activated gametocyte lysates Pfs230 appeared as a single band at 360 kDa. For PfCCp4 the full-length band at 178 kDa was not verifiable, instead a strong band at about 75 kDa displayed PfCCp4. All further proteins included in this study, like Pfs16 (16 kDa), PfPeg3 (25 kDa) and PfMR5 (290 kDa) revealed no binding to any of the other proteins.

The anti-rat PfPeg3 antibody detected the protein at the molecular weights of about 18 and 20 kDa. No protein bands of sexual stage proteins, included in this study, were detectable when lysates were precipitated with antibodies against Pf39 (39 kDa) or vice versa. All experiments were repeated two to three times including the control approach with Pf39. The heavy chain of the precipitating antibody was detected at 55 kDa and the light chain at 30 kDa. A schematic depicting the identified protein-protein interactions is shown in Figure 3.2.




Pfs16
Pfs16 Gc
PfPeg3 Gc
PfCCp1 Gc
PfCCp2 Gc
PfCCp3 Gc
PfFNPA Gc
Pf39 Gc
Pfs230 Gc
PfMR5 Gc
kDa
100 -
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130 -
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PfPeg3
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Pfs16 Gc
Pf39 Gc
Pfs230 Gc
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PfPeg3 Gc
Pfs25 aGc
PfFNPA Gc
Pf39 Gc
Pfs230 aGc
Pfs230 Gc
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PfCCp1 Gc
Pf39 Gc
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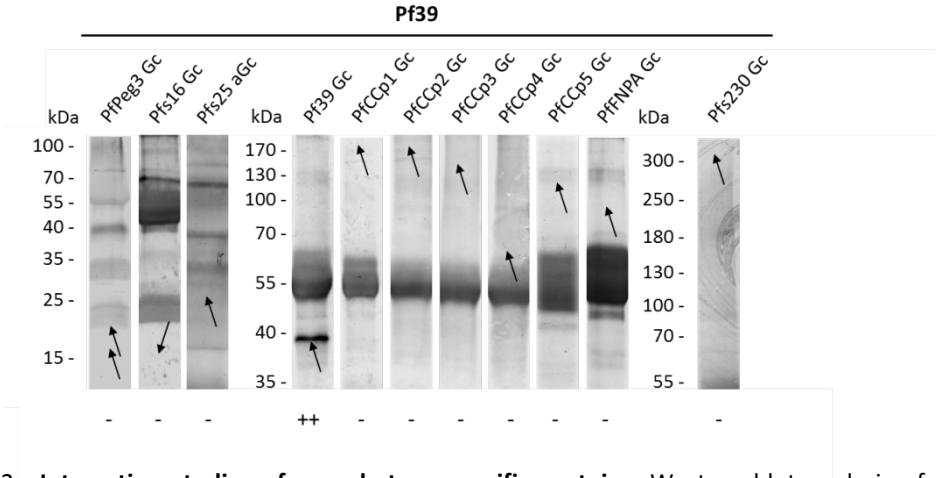


Figure 3.2: **Interaction studies of sexual stage specific proteins.** Western blot analysis of performed Co-IPs sorted by bait proteins (indicated above the horizontal line). The detection of potential prey proteins reflected the Western blot below. Interacting protein pairs were marked with a plus (+), positive controls and strong interactions with double plus (++), and negative controls and not interacting proteins with a minus sign (-). All Co-IPs were performed with gametocyte lysate (Gc) or activated gametocyte lysates (aGc), which is indicated after the prey protein. Interactions were determined using mouse antibodies against PfCCp1, PfCCp2, PfCCp3, PfCCp4, PfCCp5, PfFNPA, Pfs16, Pfs48/45, PfMR5, Pfs230, PfMR5 and Pf39. PfPeg3 and Pfg377 antibodies were derived from rats and kindly provided by Pietro Alano (Rome, Italy). Pfs25 antibodies were generated in rabbits. Protein bands migrate at molecular masses of 185 kDa (PfCCp1, PfCCp2), 143 kDa (PfCCp3), 75 kDa (PfCCp4), 125 kDa (PfCCp5), 100 kDa (PfFNPA), and double band at 360 kDa and 300 kDa (Pfs230) in activated gametocyte lysate, and a single band at 360 kDa for Pfs230 in gametocytes, 290 kDa (PfMR5), 16 kDa (Pfs16), 24 kDa (Pfs25) and a double band at 18 kDa and 20 kDa (PfPeg3). Mouse antibodies against PfCCp4 detected a 75 kDa band instead of the full-length protein. Decisive protein bands were indicated with arrows. Antibodies directed against the endoplasmic reticulum-specific protein Pf39 (39 kDa) were used as negative control and revealed no binding in all cases. Protein bands at 55 kDa for the heavy chain and 30 kDa for the light chain of the antibody were depicted. All PfCCp proteins bind strongly to other PfCCps. Pfs25 interacts with PfCCp1, PfCCp2, and PfCCp3 after activation of gametocytes. Equally Pfs230 binds to PfCCp1, PfCCp2, PfCCp3, and PfFNPA only in activated gametocytes. PfCCp4 interacts with Pfs230 in gametocytes and activated gametocytes. The proteins Pfs16, PfPeg3, PfMR5, and Pfg377 did not interact with any proteins used in this study.

All members of the PfCCp protein family were identified to be part of one protein complex. In addition Pfs230 and Pfs25 were part of this MPC in aGc. This protein assembly is connected with the parasite's plasma membrane by Pfs230 and its membrane-bound binding partner Pfs48/45. Furthermore PfCCp1 through PfCCp3 revealed binding with the membrane associated Pfs25 protein. Therefore the complex is once more connected to the parasites plasma membrane. No binding properties were attributed to the proteins Pfs16, PfPEG3, PfMR5 and Pfg377 in this study (Fig. 3.2).

3.1.3.2 Co-elution binding assays with recombinant PfCCp proteins

To investigate the molecular interactions between the PfCCp proteins in more detail, affinity chromatography co-elution binding assays were performed (section 2.2.2.6). Recombinant proteins corresponding to distinct adhesion domains were expressed for each of the six PfCCp proteins. Recombinant PfCCp3 adhesion domains, fused to a GST-tag, were immobilized to glutathione sepharose and used as main baits. Other recombinant PfCCp adhesion domains, fused to a 6-His/SUMO tag, functioned as preys and were incubated with the sepharose-bound PfCCp3 domains. Bound proteins were eluted after several washing steps. The eluted protein complexes were screened by Western blot analyses, in which the GST-tagged and 6-His/SUMO tagged proteins were detected via antibodies directed against the respective tags (see Fig. 3.3 for summary). Western blot assay representative of a positive interaction between the recombinant proteins PfCCp3rp3-GST and PfCCp1rp2-His6/SUMO gave an example for a co-elution binding assay between two proteins (PfCCp3rp4-GST and PfCCp2rp3-6-His/SUMO), which did not interact (Fig. 3.3 B). During each interaction experiment, a sample of the last washing step was investigated for the presence of prey or bait proteins and shown to be negative. In some cases, lower molecular mass protein bands of GST fusion proteins were detected in addition to the expected full size molecular mass recombinant protein. These protein bands are likely due to contamination of the recombinant protein with truncated recombinant products. In a set of negative controls, GST-tag alone was immobilized to sepharose, and the 6-His/SUMO tag alone was used as prey. In another negative control, GST-tagged Pf39 protein (termed Pf39rp1) was immobilized to sepharose and the PfCCp1rp1-6-His/SUMO protein was used as prey. In both control experiments, no interactions were detectable. In total, interactions between 33 combinations of recombinant PfCCp proteins were investigated. Of these pairs, 18 showed adhesive interaction, whereas 15 recombinant protein pairs did not interact in affinity chromatography co-elution binding assays (Fig. 3.3 A).

In an additional study binding events of distinct domains were analyzed. The number of binding events between domains of interacting proteins was compared with the counted number of domains which are part of recombinant proteins which did not interact (Tab. 3.2). Adhesion domains, which were predominantly involved in protein binding, comprise the Ricin, Nec, and SR domain (Fig. 3.4). Prominent was the

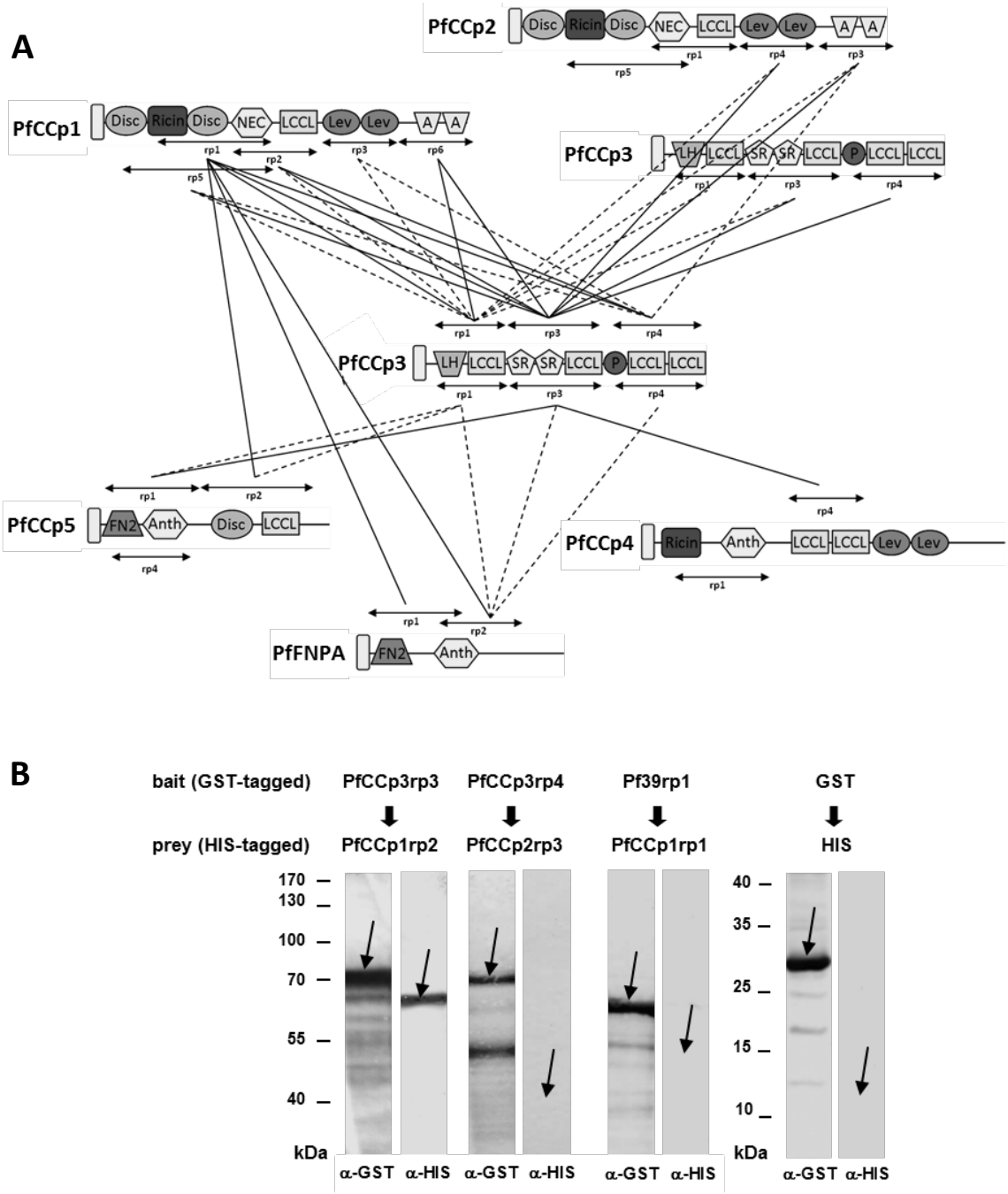


Figure 3.3: **PfCCp protein interactions through direct binding between distinct adhesion domains**.
A. Schematic overview of 33 implemented affinity chromatography co-elution binding assays. Tested recombinant proteins were indicated with lines below and above domains. Protein interaction (solid lines); no protein interaction (dotted lines). **B.** Examples of affinity chromatography co-elution binding assays using recombinant PfCCp proteins. Western blot analyses of eluted proteins, using anti-GST and anti-6-His antibodies, revealed an interaction between the two recombinant proteins PfCCp3rp3-GST and PfCCp1rp2-6-His and also showed a co-elution assay with two proteins (PfCCp3rp4-GST and PfCCp2rp3-6-His/SUMO) which did not interact. As negative control, sepharose-bound GST-tagged Pf39 protein (termed Pf39rp1) did not interact with PfCCp1rp1-6-His protein. In another negative control, column-bound GST-tag alone did not interact with 6-His/SUMO tag alone. The arrows either indicate the eluted recombinant protein or the approximate migration position expected for the eluted protein.

appearance of the SR domain in 22 interacting recombinant proteins and only four SR domain-containing proteins did not interact with other proteins (Tab. 3.2). Peptides comprising the LH2 or Levanase domains, predominantly showed a minor involvement in protein-protein-binding (Tab. 3.2/Fig. 3.4).

Table 3.2: Domains involved and not involved in interactions and the resulting difference; values < 1: not interacting domains dominate; values > 1: interacting domains dominate.

Domain	Interaction	no Interaction	Ratio
Discoidin	10	5	2
Ricin	8	2	4
Nec	10	3	3,33
LCCL	28	23	1,22
Levanase	2	6	0,33
ApicA	6	4	1,5
LH2	2	10	0,2
SR	22	4	5,5
Pentraxin	3	3	1
FN2	2	1	2
Anthrax	3	3	1

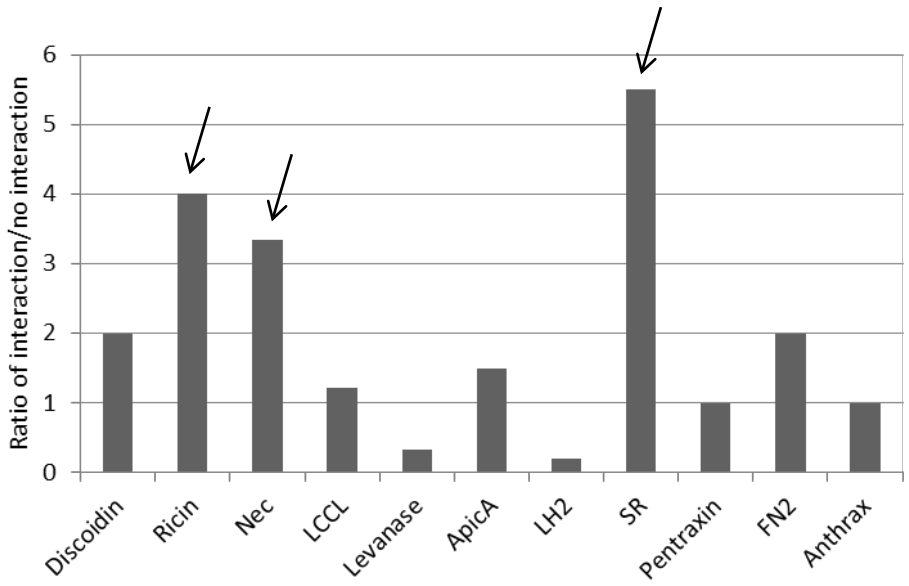


Figure 3.4: **Binding affinity of different adhesion modules / Diagram of Table 3.2.** The ratio of counted interactions and domains which are not involved in binding events was calculated. The Ricin, Nec, and SR domain (arrows) revealed highest ratios and thereby the most appropriate probability of all PfCCp domains to be involved in binding events.

3.1.3.3 Interaction studies of endogenous proteins with recombinant proteins

Additionally the ability of recombinant PfCCp domains to bind endogenous PfCCp was examined. In a first set of experiments, the capability of the recombinant PfCCp3rp3 to bind to the endogenous MPCs was examined. The protein complex, which was precipitated from gametocyte lysate via Co-IP using anti-PfCCp1 antibodies, was used as bait and incubated with purified recombinant PfCCp3rp3-GST protein. A prominent PfCCp3rp3 protein band was detected by Western blot analysis using anti-GST antibodies (Fig. 3.5 A). The MPC of gametocyte lysate was incubated with recombinant GST proteins alone and no interactions between the tag and the endogenous proteins was revealed (see arrow Fig. 3.5 A).

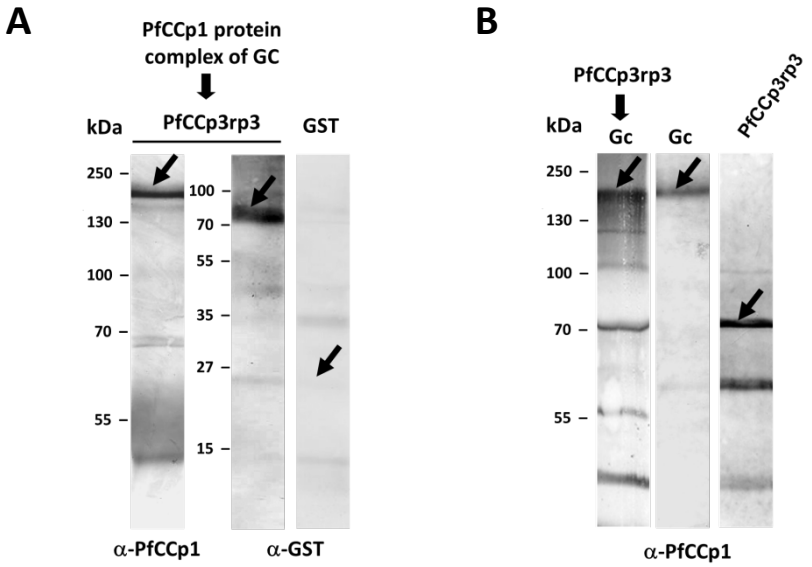


Figure 3.5: **Binding of endogenous PfCCp protein to recombinant protein A.** Double Co-IP assay on recombinant PfCCp protein: The PfCCp MPC was firstly precipitated from gametocyte lysate by anti-PfCCp1 antibodies and then used for co-precipitation of PfCCp3rp3 protein. Western blot analyses showed presence of endogenous PfCCp1 protein complex (left lane). Blotting with antibodies directed against the GST-tag revealed that the PfCCp1 complex had bound to PfCCp3rp3 (center lane). No interaction was detected when the MPC was incubated with GST alone (26 kDa, right lane). The arrows indicate the expected migration position of protein. **B.** Affinity chromatography co-elution binding assay on endogenous PfCCp protein. Western blot analysis indicate binding of endogenous PfCCp1 of gametocyte lysate (GC) to the column-bound PfCCp3rp3-GST (79 kDa; left lane), as detected with anti-PfCCp1 antibodies. The protein band for the co-eluted PfCCp1 migrates with the same molecular mass as PfCCp1 in gametocyte lysate (center lane). Additional protein bands that are visible in lane 1 likely represent bacterial proteins of the PfCCp3rp3-expressing bacterial strain, because the anti-PfCCp1 serum would be expected to recognize contaminant bacterial proteins that were carried through the immunization regimen. Similar protein bands were visualized by the anti-PfCCp1 serum, when the bacterially expressed PfCCp3rp3 was loaded to the gel directly (right lane).

In a vice versa approach affinity chromatography co-elution binding assays were performed using GST-tagged PfCCp3rp3 as bait protein which was incubated with gametocyte lysate. Western blot analysis on co-eluted samples using anti-PfCCp1 sera revealed binding between recombinant PfCCp3rp3 and endogenous PfCCp1 (Fig. 3.5 B). These results confirmed that recombinant PfCCp proteins, developed in *E. coli*, possessed the correct folding to bind to endogenous PfCCp proteins.

3.1.3.4 Identification of a further interaction partner of PfCCp proteins

In the preceding studies a MPC in the PV of malaria parasite sexual stages was identified. To figure out potential new interaction partners of this MPC Co-IPs were performed using PfCCp1 as bait in gametocyte lysate, activated gametocyte lysate, and erythrocyte lysate as a control approach. By silver staining and a following Mass Spectrometry analysis (kindly performed by Stefan Baumeister, Philipps-University Marburg) of a prominent protein band (arrow Fig. 3.6) one new potential interaction partner of PfCCp1 was identified in gametocytes. The same protein band was present when PfCCp1 was used as bait protein in Co-IPs in activated gametocytes (Fig. 3.6).

The identified protein with the gene ID PF14_0412 (PlasmoDB) exhibits two WD40 motifs and is a conserved *Plasmodium* protein (96 kDa) of unknown function. These

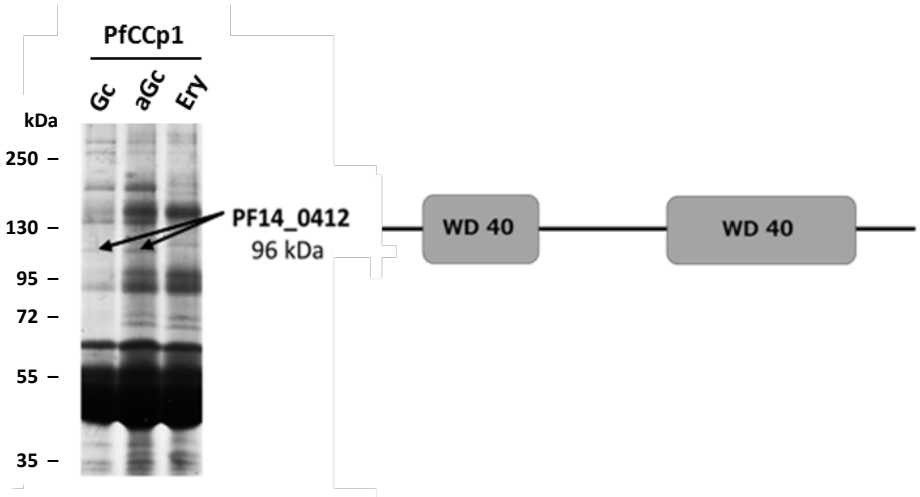


Figure 3.6: **Identification of an interaction partner of PfCCp1 via Mass Spectrometry.** With the use of PfCCp1 as bait in a Co-IP assay, it was possible to identify one new interaction partner of PfCCp1 in gametocyte lysate (Gc). The same prominent protein band was also detected in activated gametocyte lysate (aGc). Erythrocyte lysate (Ery) was utilized as control approach. A prominent protein band (arrow) in gametocyte lysate was analyzed via Mass Spectrometry (by Stefan Baumeister, Philipps-University Marburg). It was identified as a conserved *Plasmodium* protein of unknown function with the gene ID PF14_0412 (PlasmoDB). The identified protein contains two WD40 domains (e-value < 10^{-5} for domain prediction) and no transmembrane domain or signal peptide.

domains are found in a multitude of eukaryotic proteins involved in a variety of cellular processes. WD40 motifs act as a site for protein-protein interaction, and proteins containing WD40 repeats are known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins (reviewed in Smith *et al.,* 1999 and Li and Roberts, 2001). This protein may play an essential role in the assembly of the MPC in gametocytes and gametes. A recombinant protein construct for PF14_0412 was designed and cloned by Andrea Kuehn (Fig. 3.7 C). It was expressed as fusion protein with a MBP-tag using the pIH902 expression vector. Specific immune sera were generated by immunization of NMRI mice with recombinant protein (section 2.2.2.2 and 2.2.2.7). First IFAs confirmed expression in schizonts, gametocytes, and gametes (Fig. 3.7 A, kindly provided by Vanesa Ngongang). In Western blot analysis the α-PF14_0412 antibody detected a faint full-length protein band at 96 kDa and additional bands at 150 kDa, 70 kDa, and 36 kDa in schizonts.

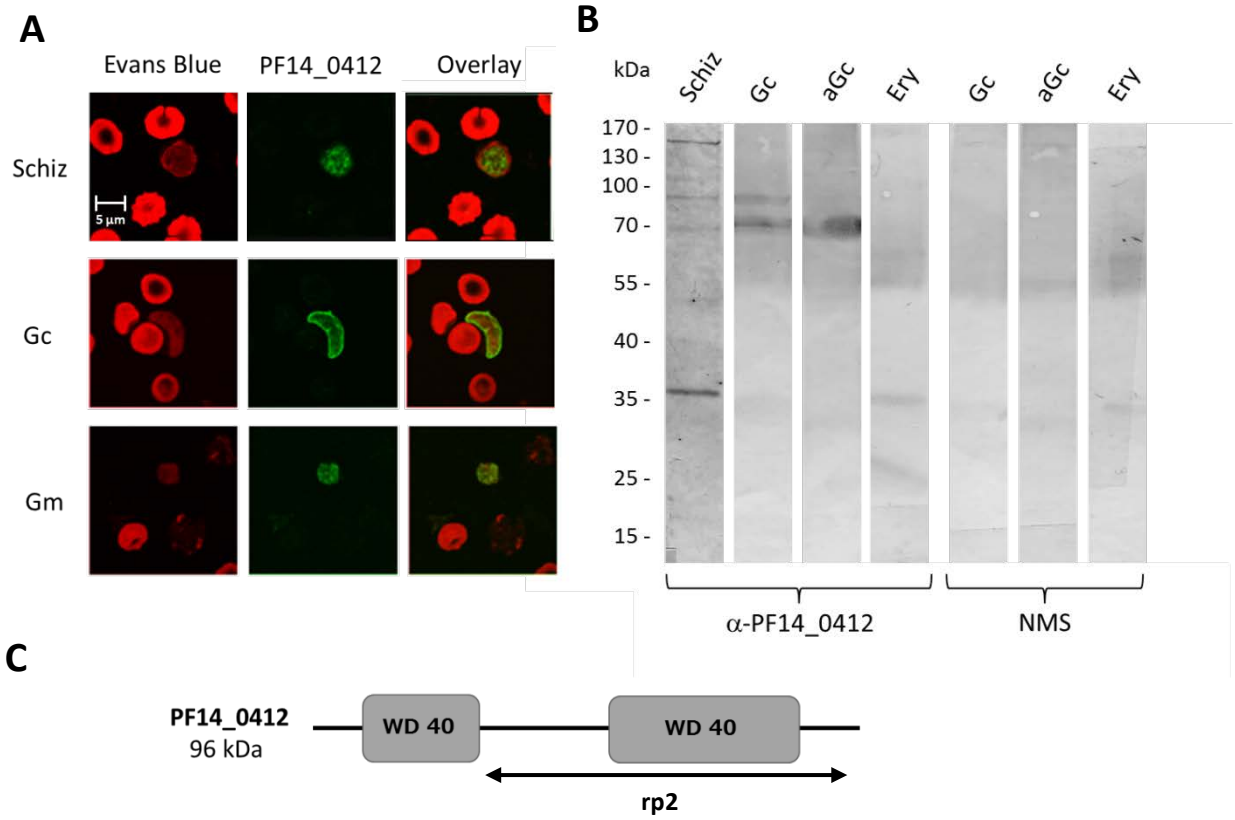


Figure 3.7: **Expression of the WD40 domain containing protein PF14_0412 in blood stage and sexual stage parasites. A.** Protein expression of schizont (Schiz), gametocyte (Gc), and gamete (Gm) stages using PF14_0412 mouse antiserum in IFAs. Erythrocytes were counterstained with Evans blue in red. **B.** Western blot analysis of schizont, gametocyte, activated gametocyte (aGc), and erythrocyte (Ery) lysates. Control approaches on erythrocyte, gametocyte, and activated gametocyte lysates with neutral mouse serum show unspecific binding of neutral mice sera (by Vanesa Ngongang). **C.** Localization of the PF14_0412 recombinant protein (rp2), the used antibodies are directed against. Animal specific neutral serum controls can be looked up in section 3.4.

In gametocytes the intensity of the full-length protein band and the 70 kDa band increased, which indicates protein procession. After activation the 96 kDa protein was not detectable anymore and the 70 kDa band came into the fore. This band might also be interpreted as double band in activated gametocytes and indicates potential procession of the 70 kDa protein after activation. In the erythrocyte control approach with α-PF14_0412 antibody incubation a faint protein band at 36 kDa was detected and indicates unspecific binding of the antibody to an erythrocyte protein in the schizont lysate either. When lysates of erythrocytes, Gc, or aGc were incubated with neutral mice serum a faint protein band running at 150 kDa was visible in all three control approaches and might match the 150 kDa band also in schizont lysates (Fig. 3.7; kindly provided by Vanesa Ngongang).

3.1.4 Processing of PfCCp proteins

The sexual stage protein Pfs230 is proteolytically processed during gamete formation in the mosquito midgut (Williamson *et al.,* 1996). To determine if PfCCp proteins become processed as well during activation, gametocyte and activated gametocyte lysates were analyzed for the presence of potential procession products (Fig. 3.8). Both antibodies against PfCCp1 (directed against the domains described in Fig. 3.8 B) detected the 185 kDa full length protein and additional bands at 70 kDa and 35 kDa, which did not appear after activation. Two strong bands were detectable for PfCCp2, 185 kDa and 80 kDa, but no difference between activated and non-activated lysates was observed. For PfCCp3 two different antibodies were used (Fig. 3.8 B) and both detected full-length protein at 143 kDa and a second band at 35 kDa. For both antibodies no 35 kDa band was visible after activation. The PfCCp4 antibody did not exhibit full length protein; instead it displayed strong bands at 70 kDa and 38 kDa. After activation the 70 kDa band became much weaker. The two anthrax domain containing proteins PfCCp5 and PfFNPA exhibited similar band pattern in gametocyte and activated gametocyte lysate (Fig. 3.8 A). PfCCp5 displayed the full-length protein at 125 kDa and additional, smaller bands due to an overload of protein or less dilution of antibody. PfFNPA antibodies displayed the full length protein (100 kDa) and a strong band about 150 kDa. The erythrocyte control approaches showed no protein bands after incubation with the different PfCCp antibodies used for this study (Fig. 3.8 A). To sum up, PfCCp1, PfCCp3, and PfCCp4 were processed in gametocytes and unbound peptides were released after activation, because surrounding membranes rupture.

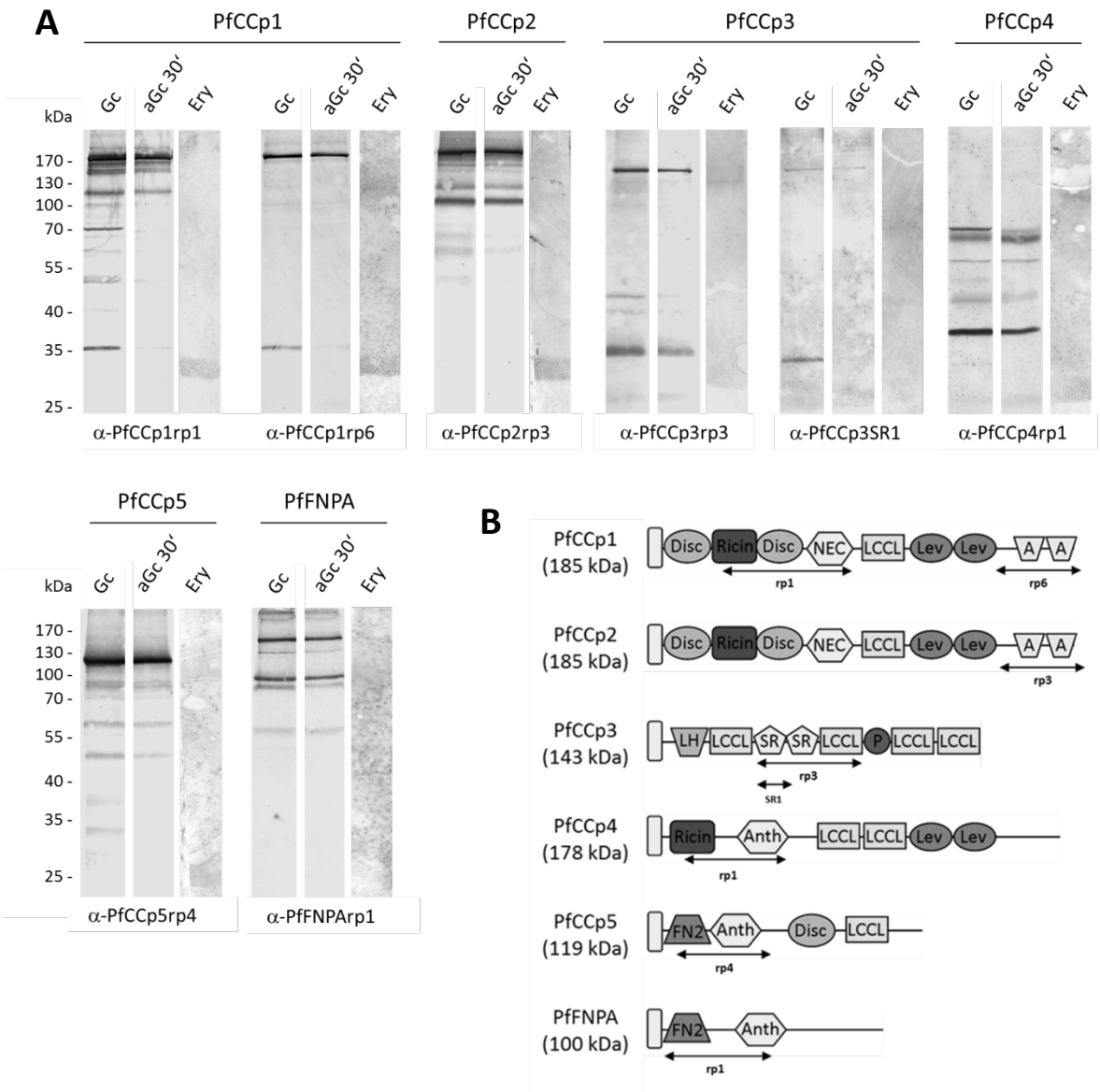


Figure 3.8: **Procession study of PfCCp proteins. A.** PfCCp expression in gametocyte (Gc), 30 min activated gametocyte (aGc 30'), and erythrocyte lysates. The same culture and amount of parasites activated with SAX medium was used for every lane. Mouse antibodies targeting one specific part of the appropriate PfCCp protein were used for detection. Protein bands migrate at the expected molecular masses of 185 kDa (PfCCp1, PfCCp2), 150 kDa (PfCCp3), 125 kDa (PfCCp5), and 100 kDa (PfFNPA). As an exception no full length protein of PfCCp4 was detectable at 178 kDa, but instead strong bands at 70 kDa and 38 kDa occurred. Two additional protein bands at a molecular mass of 70 kDa and 35 kDa were detected by the PfCCp1rp1 antibody, after activation both bands were not visualized any more. By using the PfCCp1rp6 antibody only the 35 kDa band appeared in gametocytes and was not detectable after activation. For PfCCp2 and PfCCp3 two strong bands emerged, but no significant difference between activated and non-activated gametocyte lysate was noticed. Except with an antibody which is targeted on the first SR1 domain one 30 kDa band disappeared after activation. For the PfCCp4 protein the 70 kDa band became much weaker after activation and for PfCCp5 and PfFNPA no difference between activated and non-activated gametocytes was detected. In the erythrocyte control approaches no proteins bands were detected by any PfCCp antibody used in this study. **B.** Target antigens of PfCCp proteins where the antibodies are directed against.

3.2 Human complement in the mosquito midgut

3.2.1 Activity of the complement system in the mosquito midgut

To survey the activity of the complement system in the mosquito midgut the C3a Plus EIA Microvue Kit (see method 2.2.2.9) was used. Female *Anopheles stephensi* mosquitoes were fed on human blood for 5 or 20 min and the engorged midguts were subsequently dissected at eight different time points between 5 min and 20 h post-feeding. The activity of the APC in the blood meal was investigated by determining the amount of C3a, which forms after the spontaneous hydrolysis of C3, into C3a and C3b and which is a marker for complement activation. The amount of C3a was measured by ELISA, and the OD_{450} of the color reaction in the blood meal samples was compared to the one of PBS control, which was set to 1. Two midguts per time point were investigated and each setting was measured in triplicate. In standard applications an exact amount of serum or plasma was used to examine the concentration of C3a in ng/ml using the Kit. In this case the amount of C3a in one-fifth of one midgut was measured. This roughly corresponded to the suggested volume of serum.

Table 3.3: Values of the ELISA-reader OD_{450}; each sample containing two midguts was measured in triplicate at different time points after the feed.

Time after the feed	Value 1	Value 2	Value 3	Mean	Standard deviation
control	1,00	1,00	1,00	1,00	±0,00
5 min	1,19	1,82	1,71	1,57	±0,34
20 min	2,72	2,34	2,27	2,44	±0,24
40 min	2,45	2,29	1,81	2,18	±0,34
1 h	2,12	1,77	1,67	1,85	±0,24
3 h	0,98	1,33	1,62	1,31	±0,32
6 h	0,89	1,38	1,01	1,09	±0,26
15 h	0,96	0,94	1,09	1,00	±0,08
20 h	1,02	0,98	0,92	0,97	±0,05

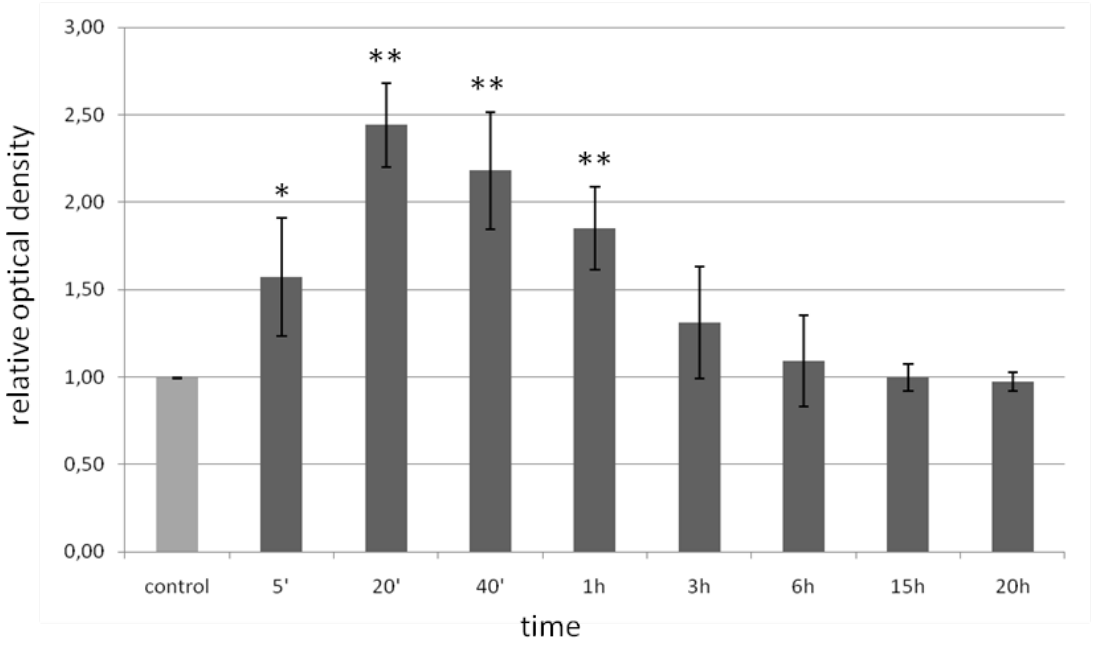


Figure 3.9: **Activity of the human complement system in the mosquito midgut.** The amount of C3a was measured by ELISA. The OD_{450} of the PBS control was set to 1. Mosquitoes were fed on fresh human blood and two mosquito midguts per time point were analyzed. The experiments were performed in triplicate. */**, significant increase in C3a compared to the control approach (*p < 0.05, **p < 0.01; student´s t-test).

The ELISA showed a significant increase in the amount of C3a in blood meal samples at 5 min until 1 h post-blood meal compared to the PBS control (Tab. 3.3; Fig. 3.9). These data indicate that the human APC is active in the blood meal during the first hour post-feeding, while subsequently the complement activity decreases rapidly.

3.2.2 Examination of classical pathway activation

The first component of the classical complement pathway is the complement protein C1. It is composed of three subunits, the subcomponent C1q (460 kDa) and the proenzymes C1r and C1s. The interaction of the C1q subcomponent with the Fc regions of IgG or IgM antibodies present in immune complexes or polyanionic structures, such as lipid A of gram-negative bacteria or certain viruses, efficiently initiate the classical pathway of complement (reviewed in Loos and Colomb, 1993). C1q binds directly to the pathogen surface. Anti-C1q antibodies were used to detect activation of classical complement pathway. Deposition of C1q to the surface of pathogens was assayed by Western blot analysis.

Mature gametocyte cultures were treated as described in 2.2.2.11, incubated in NHS either in the presence or absence of mAbs against Pfs230 and loaded under reducing conditions onto a 12 % SDS gel. It was previously shown that antibodies against Pfs230

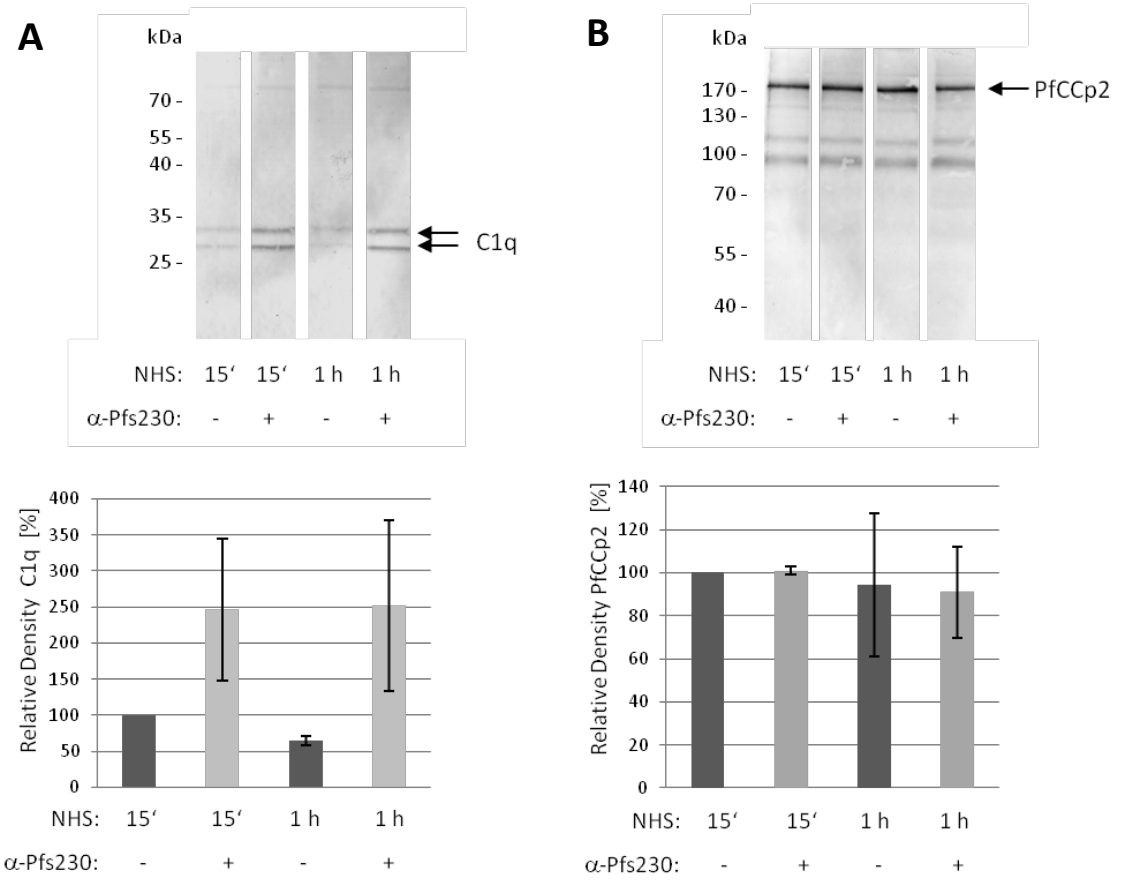


Figure 3.10: **Binding of C1q to sexual stage parasites in the presence of antibodies. A.** Gametocytes were activated with NHS in the absence or presence of anti-Pfs230 mAbs. Parasite pellets were harvested at 15 min and 1 h post activation and subjected to SDS-PAGE and Western blotting, using anti-C1q antibodies to detect the C1q protein (doublet at 26 kDa; top). The signal strength for the C1q protein band (arrows) was measured using the ImageJ program (bottom). The experiments were performed in duplicate and the C1q signal in gametocyte samples after 15 min activation was set to 100 %. **B.** For loading control of the C1q experiment, the same approach of activated gametocytes were subjected to Western blot analyses, as described above and immunoblotted with polyclonal anti-PfCCp2 antisera to visualize PfCCp2 (185 kDa; top). The signal strength for the PfCCp2 protein bands (arrow) was measured using the ImageJ program (bottom).

result in complement-mediated lysis of gametes during sexual reproduction (Quakyi *et al.,* 1987; Read *et al.,* 1994; Healer *et al.,* 1997). The C1q complex (C1r and C1s) was detected as a double band via Western blot analysis using a polyclonal C1q goat antibody (Fig. 3.10 A). The Western blot analysis revealed only a minor binding of C1q to the activated gametocytes, with C1q running as a protein doublet with molecular weights of 26 kDa and 30 kDa, which resemble C1q processing products. The binding of C1q to the sexual stage parasites, however, was increased in the presence of anti-Pfs230 mAbs, as indicated by an increase in the signal strength for C1q in samples of gametocytes that were activated in the presence of anti-Pfs230 mAbs (Fig. 3.10 A).

The experiment was performed in duplicate and the signal strength for the C1q protein bands was measured using the ImageJ program. Immunoblotting with antibodies against PfCCp2was used as a loading control (Fig. 3.10 B).

3.2.3 Binding of human complement factor C3 to malaria parasites

Immunofluorescence assays of C3b-binding to the malaria parasite

Malaria parasites lose their protective EM about 15 min after activation (Sologub *et al.*, 2011) in the mosquito midgut and expose their cell surface to the human complement system, which is taken up together with parasites during the bloodmeal of the mosquito. The APC becomes activated by pathogenic membrane surfaces. Binding of C3b to these surfaces results in AP activation and lyses the pathogenic cell. Binding of human complement regulators such as FH or FHL-1 can regulate inactivation of the APC. Complement inactivation on the cell surface can be observed by detection of C3 procession peptides as explained in sections 1.6.2 and 1.6.3. The presence of C3b

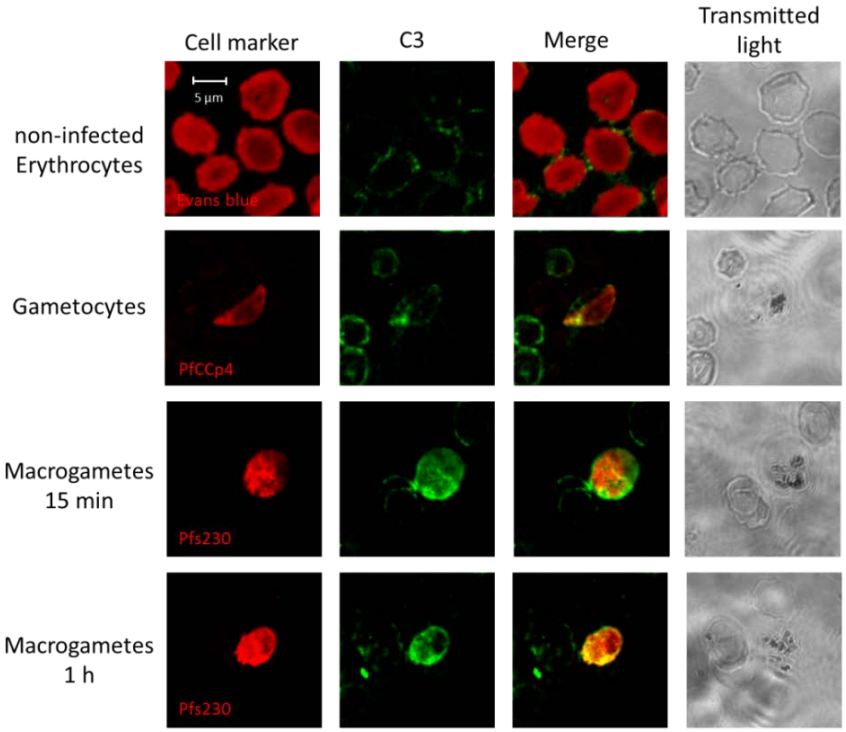


Figure 3.11: **Indirect Immunofluorescence Assay of C3-binding to different sexual stages of malaria parasites.** Gametocytes were activated with NHS and subjected to indirect immunofluorescence assays. Non-infected erythrocytes and non-activated gametocytes were used for negative controls. The assay showed strong C3 binding to activated gametocytes (15 min) and gametes (1 h). Parasites were labelled in green with a goat anti-human C3 antibody and counter-labeled using the appropriate sexual stage mouse antibody. The erythrocyte control was counterstained with Evans Blue (red). Animal specific neutral serum controls can be looked up in section 3.4.

was investigated on the surface of the activated gametocytes and gametes using indirect immunofluorescence assays. On the one hand C3b was labeled using anti-human C3 goat antisera, on the other hand activated gametocytes and macrogametes were highlighted with anti-Pfs230 antibodies. Non-infected erythrocytes were stained with Evans Blue and non-activated gametocytes, labeled with antibodies against PfCCp4, were used for negative controls. The erythrocytes and the non-activated intraerythrocytic gametocytes exhibited a fine C3b labeling on their surfaces. Emerging macrogametes, though, showed an intense C3b-labeling, when being examined between 15 min and 1 h post-feeding (Fig. 3.11).

Western blot assays of C3b-binding to the malaria parasite

Due to the fact that malaria parasites are able to survive in the mosquito midgut the following experiment examined, if C3b becomes inactivated on the surface of malaria parasites after activation. Therefore C3 binding experiments were repeated and the NHS treated parasites were further examined via Western blot analyses to evaluate C3 deposition using polyclonal goat anti-human C3 antibody. Inactivation of C3b can be proven by the detection of the procession products α'_1 (67 kDa) and α'_2 (40 kDa) of the α' chain (Fig. 3.12 B). *S. aureus* was used as positive control. This pathogen was recently found to bind FH and Factor H related protein 1 (CFHR-1) and thereby C3b inactivation on the bacterial cell surface was proven (Haupt *et al.,* 2008). For Western blot analysis mature stage V gametocytes were treated as described in 2.2.2.11, activated with 20 % NHS/SAX for different time periods and transferred under reducing conditions onto a 12 % SDS gel. Samples were taken at 15 min and 1 h post activation. For negative control, gametocytes were kept in NHS at 37°C for 15 min; and for positive control, *S. aureus* were kept in NHS for 1 h at 37°C. Similar numbers of gametocytes from the same culture were used for each setting. Gametocyte lysates were subjected to SDS-PAGE, followed by Western blotting, and probed with anti-C3-antibodies. Purified C3b was loaded on the gel in order to highlight the unprocessed α´ chain and the β chain. The Western blots showed α'_1 and α'_2 peptides in activated gametocytes. Signals were more intense than in the unactivated gametocyte control (Fig. 3.12 A). Further, the signals for α'_1 and α'_2 slightly increased at 1 h post activation. This procession indicates inactivation of C3b into the inactivated form iC3b. An additional weak protein band was observed directly above the α´ signal, which originated from the α chain of non-hydrolysed C3. Very intense protein bands for peptides α'_1 and α'_2 were detected in the *S. aureus* positive control (Fig. 3.12 A).

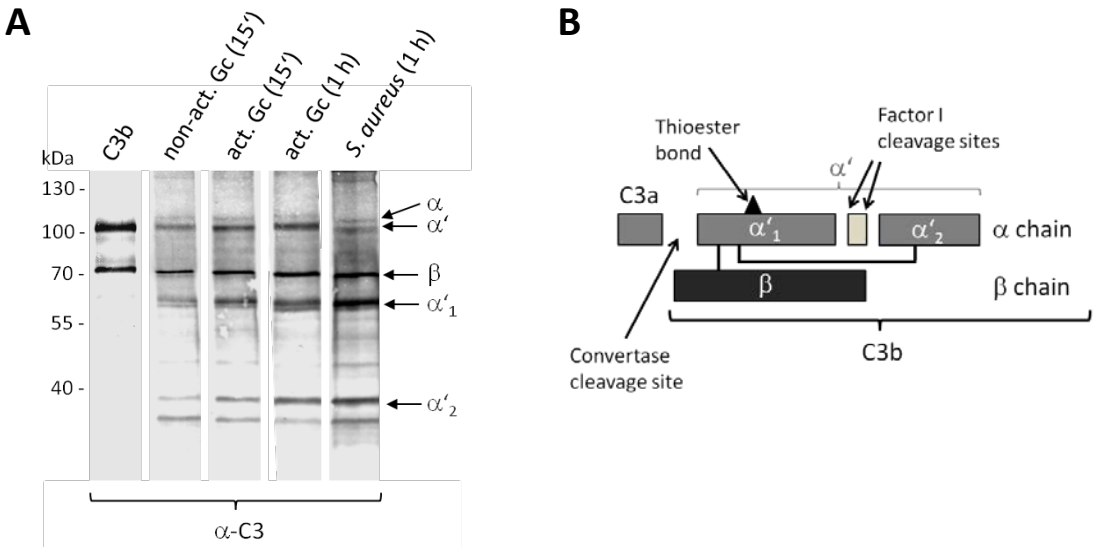


Figure 3.12: **C3b inactivation on the surface of activated gametocytes. A.** Gametocytes (Gc) were activated with NHS at RT (see 2.2.2.11). The parasite pellet was harvested at 15 min and 1 h post activation and subjected to SDS-PAGE and Western blotting, using anti-C3 antisera to visualize the C3b processing products. For negative control, gametocytes were kept in NHS at 37°C for 15 min; and for positive control, S. aureus strain Newman was kept in NHS for 1 h at 37°C. Purified C3b was loaded on the gel in order to highlight the unprocessed α´ chain and the β chain. **B.** Schematic depicting the composition of C3b. The cleavage sites are indicated (modified from Riley-Vargas *et al.,* 2005). Peptide sizes: C3 (180 kDa), α (109 kDa), β (75 kDa), C3a (9 kDa), α' chain (101 kDa), α'_1 (67 KDa) and α'_2 (40 kDa).

3.2.4 Impact of the active human complement on parasite gametogenesis

The studies above revealed that the complement system is active for about 1 h in the mosquito midgut and that C3b was inactivated on the surface of activated gametoocytes. The following experiments investigated the influence of an active complement system, in comparison to an inactivated one, on the gametogenesis of malaria parasites using gametogenesis inhibition assays (GIA). Furthermore the effect of antibodies against FH on the gametogenesis in the presence or absence of active complement was analyzed. Similarly the impact of antibodies against FH on the infection rate of parasite-fed mosquitoes was evaluated in Transmission Blocking Assays (TBA).

Gametogenesis Inhibition Assays

About 15 min after the activation of mature gametocytes male and female gametes develop. During the following hour, fertilization occurs and a zygote is formed. After showing that the human complement is active in the mosquito midgut for approximately 1 h, the effect of the APC on the fertilization of the parasite was

investigated. Mature gametocyte cultures were activated in vitro at RT in the presence of NHS or HIS. The number of exflagellation centers, as an indicator for activated microgametocytes, was counted by light microscopy 15 min after activation. The numbers of macrogametes and zygotes were determined by labeling the cells with antibodies against Pfs25 and Pfs28 in immunofluorescence assays at 20 min and 20 h post activation, respectively (section 2.2.1.8). Then the number of macrogametes or zygotes in thirty fields of vision was counted under the fluorescence microscope. Each experiment was performed in triplicate, and the number of sexual stage parasites in the NHS-treated samples was set to 100 %.

Table 3.4: Influence of an active complement system and FH-antibodies on the gametogenesis.

Micro-gametes	**1. Count [%]**	**2. Count [%]**	**3. Count [%]**	**Mean [%]**	**Standard deviation**	**t-value**	**Signifi-cance**
NHS + PBS	84,6	115,4	100,0	100,0	15,4		
NHS + α-FH	97,4	76,9	89,7	88,0	10,4	1,12	no
HIS + PBS	120,5	151,3	112,8	128,2	20,4	1,9	> 90 %
HIS + α-FH	146,2	128,2	92,3	122,2	27,4	1,22	no
Macro-gametes	**1. Count [%]**	**2. Count [%]**	**3. Count [%]**	**Mean [%]**	**Standard deviation**	**t-value**	**Signifi-cance**
NHS + PBS	80,0	114,9	105,2	100,0	18,0		
NHS + α-FH	75,9	63,6	58,6	66,0	8,9	2,93	> 95 %
HIS + PBS	145,0	174,0	108,1	142,4	33,0	1,95	> 90 %
HIS + α-FH	182,2	117,8	161,1	153,7	32,9	2,48	> 90 %
Zygotes	**1. Count [%]**	**2. Count [%]**	**3. Count [%]**	**Mean [%]**	**Standard deviation**	**t-value**	**Signifi-cance**
NHS + PBS	60,9	132,5	106,6	100,0	36,3		
NHS + α-FH	31,0	43,4	42,3	38,9	6,9	2,86	> 90 %
HIS + PBS	136,5	163,0	154,5	151,3	13,5	2,29	> 90 %
HIS + α-FH	133,7	156,8	189,5	160,0	28,1	2,26	> 90 %

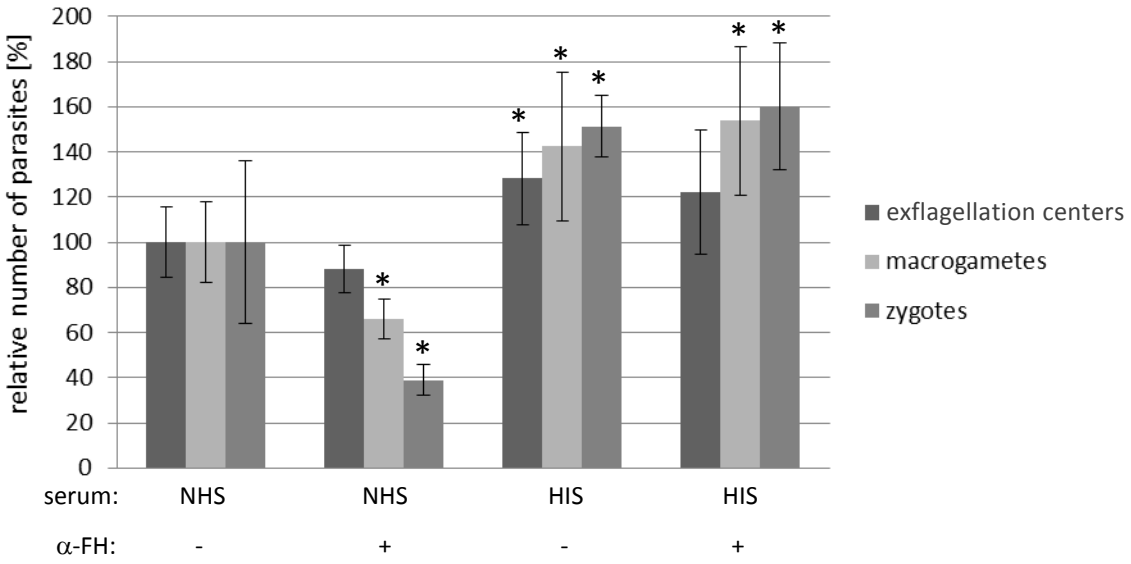


Figure 3.13: **Impact of active human complement with parasite gametogenesis.** Gametocytes were activated with NHS or HIS in the absence or presence of anti-FH mAb 131X, and the numbers of exflagellation centers was determined after 15 min, macrogametes after 20 min, and zygotes 20 h post activation, microscopically. The numbers of NHS-activated cells was set to 100 %. The gametogenesis of parasites is about 20-40 % higher in HIS comparable to parasites which have to develop in NHS. The monoclonal anti-FH antibody showed an inhibitory effect of on the gametogenesis of malaria parasites. The addition of the anti-FH antibody only displayed an inhibitory effect on macrogamete and zygote formation in NHS. The tested antibody showed no influence on the gamete formation in HIS (control). *, significant increase in the number of counted parasites (*$p < 0.05$; student´s t-test).

The *in vitro* assays showed that in HIS-treated activated gametocyte cultures, the numbers of exflagellation centers, macrogametes and zygotes were significantly higher than in cultures that were activated in the presence of NHS (Fig. 3.13). These data indicate that active human complement interferes with gametogenesis, reducing the number of gametes and zygotes.

Transmission Blocking Assay (TBA)

To evaluate the impact of FH antibodies on the infection rate of mosquitoes *ex vivo* TBAs were performed (section 2.2.3.2). Female *A. stephensi* mosquitoes were fed on mature gametocyte cultures in the presence of NHS for 20 min. In one experimental set up, anti-FH mAb 131X was added in a dilution of 1:4 to the gametocyte culture. Mosquitoes with engorged midguts were collected. The midguts were dissected 10 days post-feeding, and the numbers of oocysts were counted for each midgut.

Table 3.5: Transmission blocking activity of anti-FH antibodies.

TBA1				TBA2		
Midgut no.	PBS control	with α-FH		**Midgut no.**	PBS control	with α-FH
1	0	0		**1**	0	0
2	1	0		**2**	0	0
3	4	0		**3**	0	0
4	0	0		**4**	1	0
5	0	0		**5**	0	0
6	6	0		**6**	0	0
7	3	0		**7**	1	0
8	1	0		**8**	1	0
9	1	0		**9**	0	0
10	0	0		**10**	0	
11	3	0		**11**	5	
12	2	0		**12**	0	
13	16	0		**13**	3	
14	1	0		**14**	4	
sum	**∑ 38**	**∑ 0**		**15**	2	
				16	1	
				17	0	
				18	1	
				sum	**∑ 19**	**∑ 0**

		number of infected/total	**number of oocysts/midgut; range**	**Infection rate**	**Inhibition**
TBA1	PBS	10/14	4±4.4; 1-16	71 %	
	α-FH	0/14	0; not applicable	0 %	100 %
TBA2	PBS	9/18	2±1.5, 1-5	50 %	
	α-FH	0/9	0; not applicable	0 %	100 %

Two independent experiments were performed. While in NHS mosquitoes exhibited infection rates of 71 % and 50 %, no infected mosquito was observed, when the anti-FH mAbs were added to the cultures prior to the blood meal (Tab. 3.5). Thus, the TBA results demonstrate a 100 % inhibition of parasite transmission to the mosquito by antibody-mediated inactivation of FH in the blood meal and suggest complement-mediated clearance of parasites.

The *in vitro* and *ex vivo* experiments demonstrate that antibodies against FH inhibited the sexual development of *Plasmodium falciparum.* The following investigations will examine if FH binds to the malaria parasite and if it is responsible for the inactivation of C3b on the parasite's surface.

3.2.5 Human FH in the mosquito midgut

According to previous studies, the bloodmeal of the mosquito is digested within 60 h, the latest point of digestion enzyme activity (Billingsley *et al.,* 1991). The decay of activity and thus digestion of FH in the mosquito midgut in the time frame of 0 min to 20 h should be examined. After the last time point all parasites which developed into ookinetes should have traversed the midgut wall to form an oocyst. To examine the potential procession or digestion of FH under endogenous conditions it was tested *in vivo* how long FH is stable in the mosquito midgut following a blood meal.

Female *A. stephensi* mosquitoes were fed on human blood and the midguts were dissected at eight different time points between 0 min and 20 h post-feeding (section 2.2.3.2). Blood meal samples were subjected to native gel electrophoresis and immunoblotted with anti-FH-SCR1-20 antisera. The Western blot detected the 155 kDa full-length FH and revealed that FH was stable in the mosquito midgut for approximately 6 h (Fig. 3.14 B). Over time a degradation product with a molecular weight of approximately 110 kDa accumulated in the samples. At 15 h post-feeding, the amount of FH decreased in the samples, probably due to degradation, and at 20 h post-feeding, FH was no longer detected in the blood meal. Similarly, FHL-1 was detected for approximately 6 h post-feeding. Interestingly, no protein bands for CFHR-1α and CFHR-1β were detectable. In contrast, the three regulatory proteins, FH, FHL-1, and CFHR-1, were present in the NHS sample (Fig. 3.14 A), suggesting that CFHR-1 is immediately absorbed or degraded in the mosquito midgut. The experiment was also performed using mosquitoes fed with mature *P. falciparum* gametocyte cultures in the presence of NHS via glass feeders, and a similar time-line for FH degradation was observed (Fig. 3.14 C). The combined data indicate that FH and FHL-1 are stable in the mosquito midgut for approximately 6 h, thus for a longer time period than the APC was shown to be active.

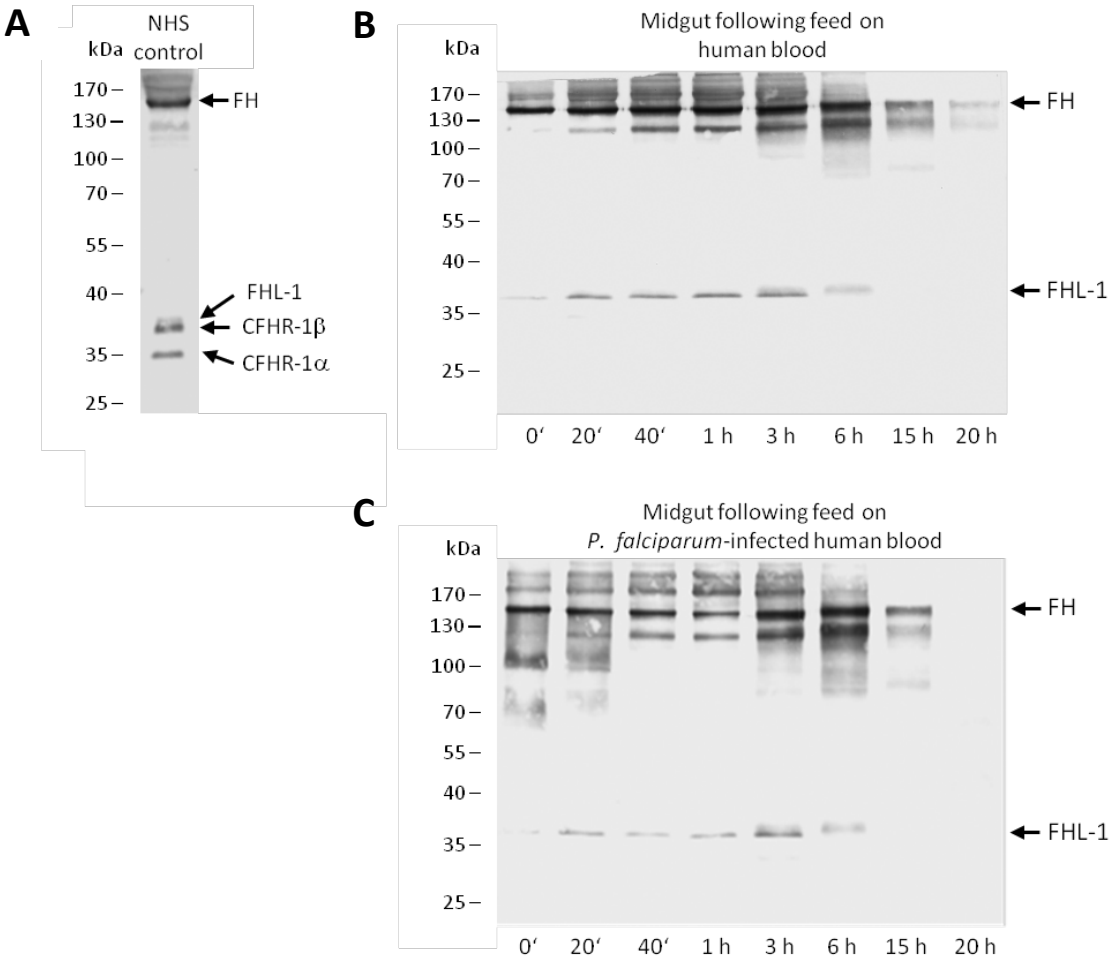


Figure 3.14: **Degradation of FH in the mosquito midgut. A.** Immunoblotting of NHS was used for positive control, subjected to native gel electrophoresis and immunoblotted with anti-FH-SCR1-20 antiserum. The full-length FH (155 kDa), FHL-1 (37 kDa), CFHR-1α (34 kDa), and CFHR-1β (36 kDa) were detected. **B.** Female *A. stephensi* mosquitoes were fed on human blood and the midguts were dissected at eight different time points between 5 min and 20 h post-feeding. Blood meal samples were subjected to native gel electrophoresis and immunoblotted with anti-FH-SCR1-20 antiserum. The full-length FH (155 kDa), a FH degradation product (110 kDa), and FHL-1 (37 kDa) were detected. **C.** The same experimental setup was conducted using mature gametocytes cultures mixed with human blood and resulted in a similar band pattern.

3.2.6 Binding studies of FH and FH family proteins to malaria parasites

3.2.6.1 Indirect Immunofluorescence Assay of FH binding

The malaria parasite can survive in the mosquito midgut, though it loses its protective EM during gametogenesis. The complement system of the human host is active for about 1 h after ingestion. The *in vitro* assays and the *ex vivo* TBVs indicated that FH has a protective function for the emerging gametes following activation, preventing these from complement-induced lysis. In subsequent studies was therefore investigated, if the extracellular sexual stage parasites are able to bind FH. First, mature gametocytes

were activated with 20 % NHS/SAX at RT and washed once with RPMI incomplete. Samples were taken at five different time points between 15 min and 6 h post activation and subjected to indirect immunofluorescence assays (section 2.2.1.8). On the one hand FH on the sexual stage parasite surface was labeled with polyclonal anti-FH-SCR1-20 antiserum that recognizes the whole FH molecule, FHL-1, and CFHR-1, on the other hand macrogametes and zygotes were labeled, using antibodies against the stage-specific marker proteins Pfs230 and Pfs25, respectively. Non-infected erythrocytes were counterstained with Evans Blue and non-activated gametocytes

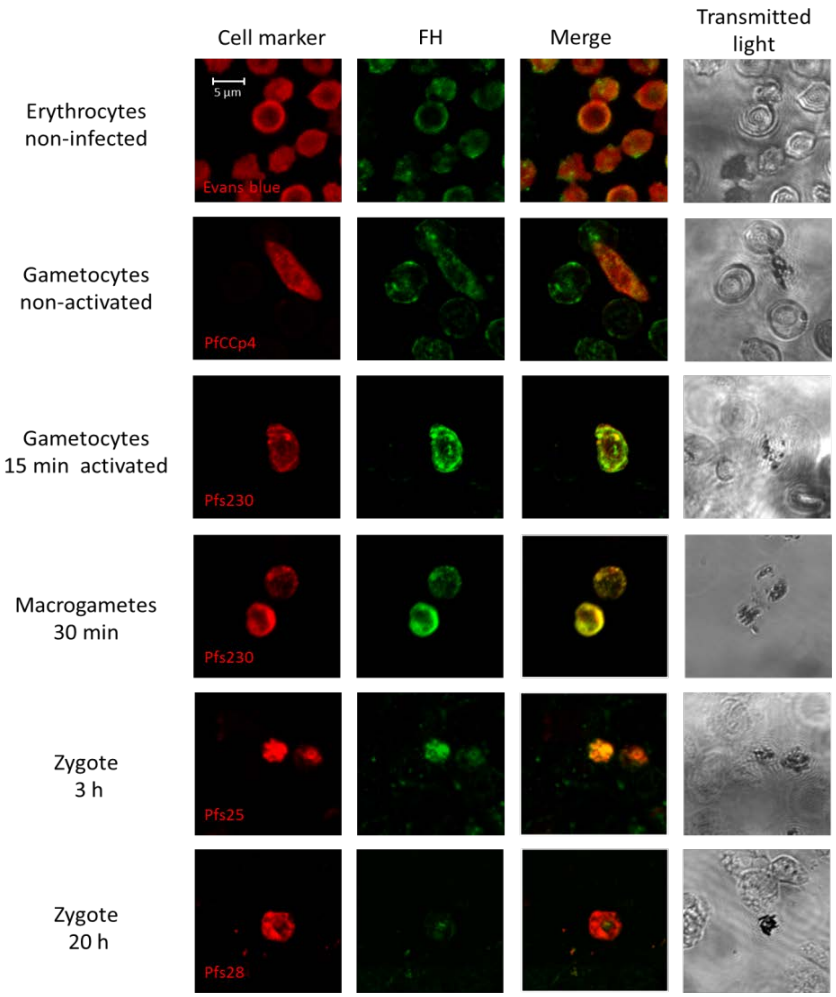


Figure 3.15: **Binding of FH to sexual stage parasites.** Gametocytes were activated with NHS and subjected to indirect immunofluorescence assays. Non-infected erythrocytes and non-activated gametocytes were used for negative controls. FH was labeled with polyclonal anti-FH-SCR1-20 antiserum (green), the sexual stage parasites were labeled with antibodies against the respective marker proteins (PfCCp4, gametocytes; Pfs230, macrogametes; Pfs25, young zygotes; Pfs28, old zygotes; red), non-infected erythrocytes were labeled with Evans Blue (Cell marker, red). FH displayed weak binding to erythrocytes and gametocytes. The strongest binding was verified for activated gametocytes 30 min post activation. At later time points the amount of FH, which binds to gametes or zygotes decreases. Animal specific neutral serum controls can be looked up in section 3.4.

were co-labeled with antibodies against PfCCp4 and used for negative controls (Fig. 3.15). The erythrocytes and the non-activated intraerythrocytic gametocytes exhibited a low FH labeling on their surfaces, which presumably is due to the permanent binding of FH to body cells. Emerging macrogametes and young zygotes, though, showed an enhanced labeling for FH on their surfaces, when being investigated at time points of 15 min to 3 h post activation. FH labeling decreased on zygotes after 20 h, which were labeled with antibodies against Pfs28 (Fig. 3.15).

3.2.6.2 Serum Absorbance Assay with malaria parasites

A serum absorbance assay was performed by Western blotting to examine if FH binds directly to activated gametocytes. Gametogenesis was induced in vitro at RT either by NHS or by SAX, which was supplemented with purified FH at concentrations found in plasma. A concentration of 0,5 mg/ml was used. Cells were treated like described in section 2.2.2.11 without saponin treatment, in order to compare protein binding to erythrocytes and parasites. Samples were taken at six different time points between 10 min and 6 h after activation, subjected to native gel electrophoresis and blotted with antibodies against FH-SCR1-20. The labeling intensity of the FH-positive bands, which showed a mobility of expected 155 kDa, was compared to the FH bands of

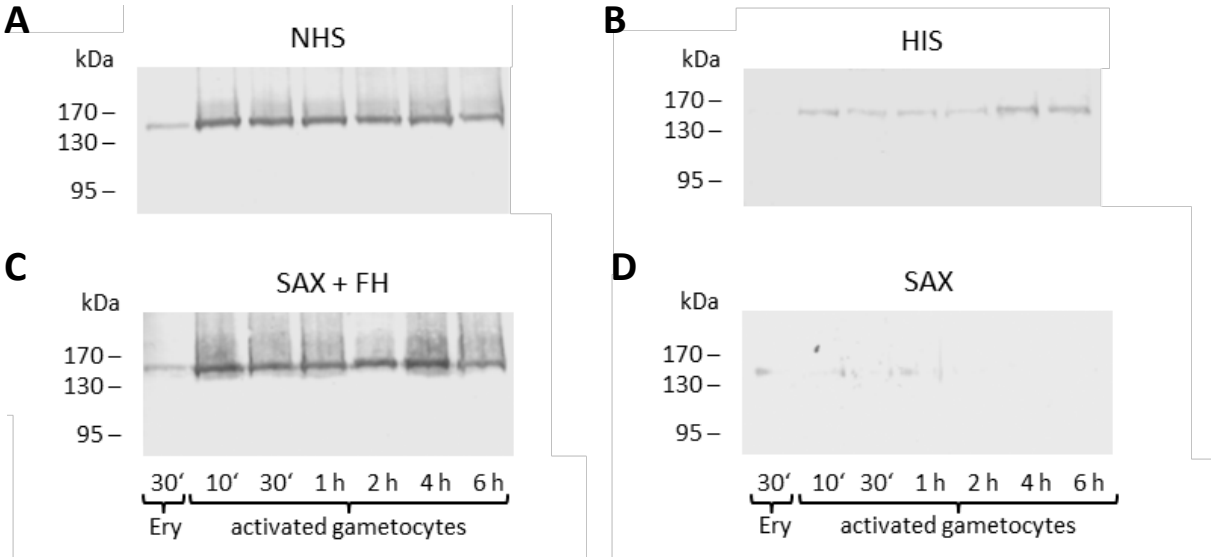


Figure 3.16: **Serum Absorbance Assays indicated that FH binds directly to activated gametocytes.** Gametocytes were activated with NHS or HIS or with SAX in the absence or presence of FH. The parasite pellet was harvested at six different time points post activation and subjected to native gel electrophoresis and Western blotting, using polyclonal anti-FH-SCR1-20 antibody in order to visualize bound FH (155 kDa). Non-infected erythrocytes (Ery) were used for negative control to indicate FH-binding to body cells.

equally treated non-infected erythrocytes, which were used for negative controls. For all time points, prominent FH bands of approximately 155 kDa were detected in the gametocyte samples that were activated by NHS (Fig. 3.16 A) or by SAX supplemented with purified FH (Fig. 3.16 C). The FH bands of these samples were more intense then the respective bands of the erythrocyte control. When gametocytes were activated with HIS, the intensity of the FH binding was strongly reduced (Fig. 3.16 B). In gametocytes activated with SAX, thus without any serum in the activation medium, FH labeling was very low (Fig. 3.16 D) and presumably resulted from remnants that had bound to the erythrocyte surface of the intraerythrocytic gametocytes during culturing. The combined data show that FH strongly binds directly to the surface of the in vitro cultivated gametes and young zygotes of *P. falciparum* for a time period of at least 6 h.

3.2.6.3 Binding studies of FH family proteins to malaria parasites by Western blotting

Different proteins which belong to the FH family can have regulatory functions for the APC. FH and FHL-1 are the most well characterized proteins which bind microbes like *Borrellia* as a complement evasion strategy (Bhide *et al.,* 2009). Other bacteria, for instance *Staphylococcus aureus,* bind FH and CFHR-1 to escape from the attack of the human APC (Haupt *et al.,* 2008). The following study investigated if FH, FHL-1 or CFHR-1 are recruited by *Plasmodium* parasites. Initially the presence of the two regulatory proteins, FH and FHL-1 in NHS was tested by Western blotting, using anti-FH antiserum. A protein band corresponding to the FHL-1 protein (expected molecular weight of 42 kDa, here running at a molecular weight of ~37 kDa), the two differently glycosylated chains of CFHR-1, termed CFHR-1α and CFHR-1β (expected molecular weights of 37 and 43 kDa, here running at two prominent protein bands of 34 and 36 kDa), and additionally the 155 kDa protein band of FH was detected (Fig. 3.17). Gametocytes were activated in vitro in NHS at RT and samples were taken at seven different time points between 30 min and 20 h after activation. Samples were subjected to Western blot analysis, and revealed a prominent FH signal as well as a faint protein band for FHL-1 for all time points. The intensity of the FH and FHL-1 bands stayed constant for 20 h. As a positive control, we used *Staphylococcus aureus* strain Newman, which was incubated with NHS at 37°C for 1 h and the lysates were blotted with anti-FH antibodies. Protein bands for FH, CFHR-1α, and CFHR-1β were detected (Fig. 3.17).

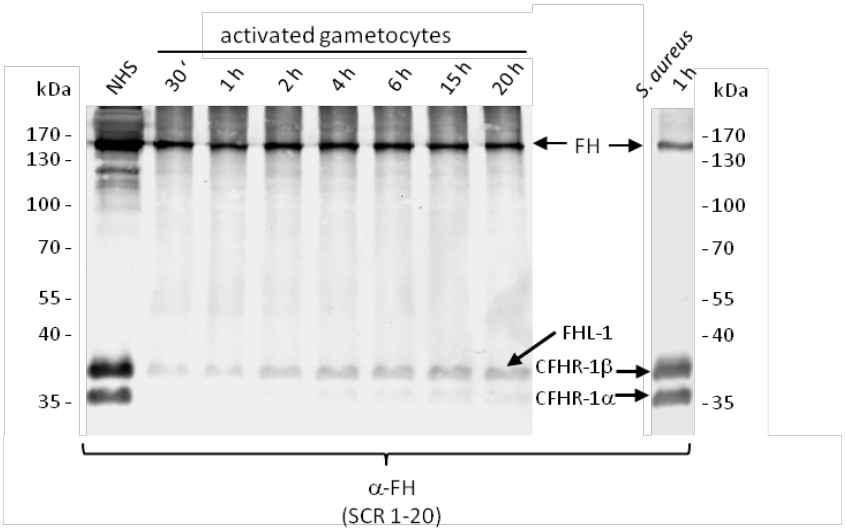
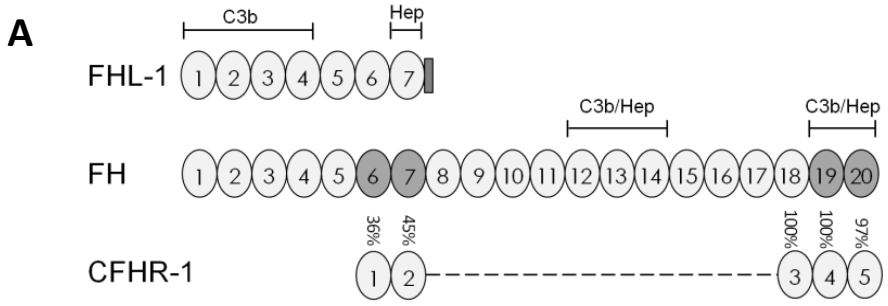


Figure 3.17: **Time study of FH and FHL-1 binding to sexual stage parasites.** Gametocytes were activated with NHS. The parasite pellet was harvested at seven different time points after activation and subjected to native gel-electrophoresis and Western blotting, using polyclonal anti-FH-SCR1-20 antibody. NHS was used for positive control. Immunoblotting visualized bound FH (155 kDa) and FHL-1 (37 kDa), but no binding of CFHR-1α (34 kDa) and CFHR-1β (36 kDa) to sexual stage parasites.

To confirm binding of FH and FHL-1 to the sexual stage parasites, similar binding assays were performed, using antibodies directed against different SCR domains of FH and FHL-1. While the mAb anti-FH-SCR18-20 antibody only detects FH and CFHR proteins, the mAb anti-FH-SCR1-4 antibody is able to detect FH and FHL-1, but not CFHR-1. Furthermore, mAb 131X is able to detect FH, but not the FH-related proteins. For the molecular architecture of FH, see Fig. 3.18 A. These antibodies were used for probing the lysates of activated gametocytes (15 min and 1 h). The polyclonal antiserum anti-FH-SCR1-20 was used as a positive control. In all cases FH was detected (Fig. 3.18 B). Furthermore, FHL-1 was detected binding to the parasite surface, when lysates were immunoblotted with the anti-FH-SCR1-4 and anti-FH-SCR1-20 antibodies. These data underline the binding of both FH and FHL-1 to the sexual stage surface and confirmed that malaria parasites did not bind CFHR proteins (Fig. 3.18 B).



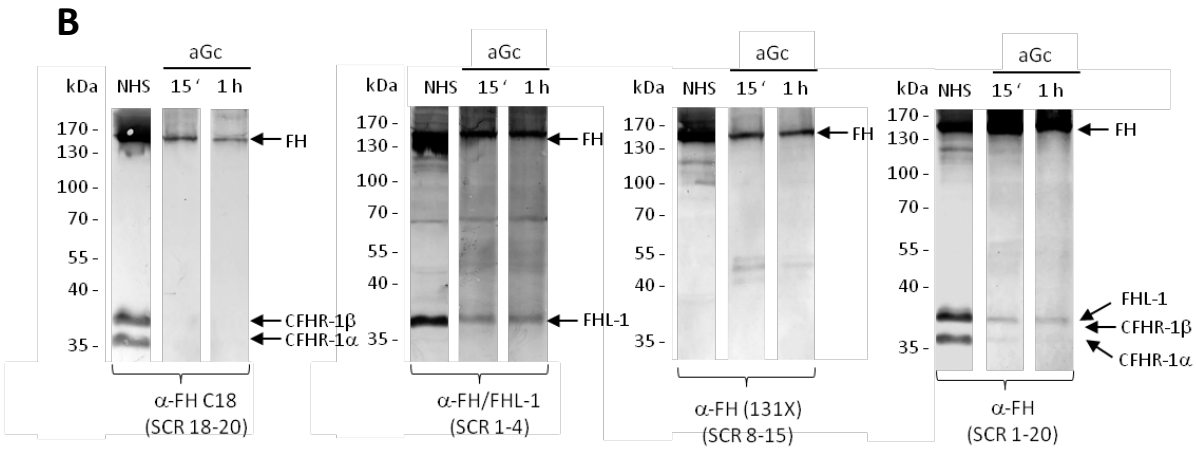


Figure 3.18: **Binding studies of FH and FHL-1 using antibodies directed against different FH domains.** **A.** Schematic depicting of the SCR domain structures of FH, FHL-1, and CFHR-1. The binding sites for C3b and heparin (Hep) are indicated (modified from Zipfel *et al.,* 2002). **B.** Gametocytes were activated with NHS. Parasite pellets were harvested at 15 min and 1 h and subjected to gel electrophoresis and Western blotting, using mAbs anti-FH-SCR18-20 and anti-FH-SCR8-15 (clone 131X), and polyclonal antibodies anti-FH-SCR1-4 and anti-SCR1-20 to detect binding of FH (155 kDa), FHL-1 (37 kDa), CFHR-1α (34 kDa), and CFHR-1β (36 kDa).

3.2.6.4 Peptide Binding Assay of recombinant FH-proteins on activated gametocytes

Previous studies revealed binding of FH and FHL-1 to activated gametocytes. FH is composed of 20 SCR modules (Fig. 3.19 A), which have previously been studied extensively, and for which different regions for the binding of C3b and heparin were identified (Kühn *et al.,* 1995; Haupt *et al.,* 2007; Kunert *et al.,* 2007; Skerka *et al.,* 2007; Mihlan *et al.,* 2009, 2011; Reuter *et al.,* 2010; Weismann *et al.,* 2011; Lauer et al. 2011). In order to determine which of the SCR modules were primarily involved in surface binding of the sexual stage parasites, eight peptides, comprising different SCR modules, were investigated for binding. One of the peptides, comprising SCR modules 1-7, resembled FHL-1. Additionally, a recombinant CFHR-1 molecule was tested (peptides were kindly provided by Christine Skerka and Peter F. Zipfel, Jena; overview in Fig. 3.19 B). Initially the purified fragments were loaded onto a gel in order to determine the molecular mobilities at which the respective proteins were running (Fig. 3.19 C). Subsequently, gametocytes were activated in vitro at RT for 45 min, using SAX medium, which was supplemented with a 100 ng/µl concentration of one of the recombinant FH peptides per sample (section 2.2.2.11). The gametocytes were thoroughly washed and the respective lysates were then subjected to native gel electrophoresis and blotted with anti-FH-SCR1-20 antisera in order to detect the

peptides. A protein band running at a molecular weight of approximately 37 kDa was detected, which resembled the protein SCR modules 1-7 (Fig. 3.19 D). For no other of the recombinant FH fragments, protein bands were detected including a FH fragment which was composed of SCR modules 1-4. Peptide SCR1-7 was again tested in a concentration of 10 ng/µl, and a prominent protein band was detected (Fig. 3.19 D, right lane). The combined data suggest that SCR modules 5-7 are involved in binding of FH to the surface of sexual stage parasites.

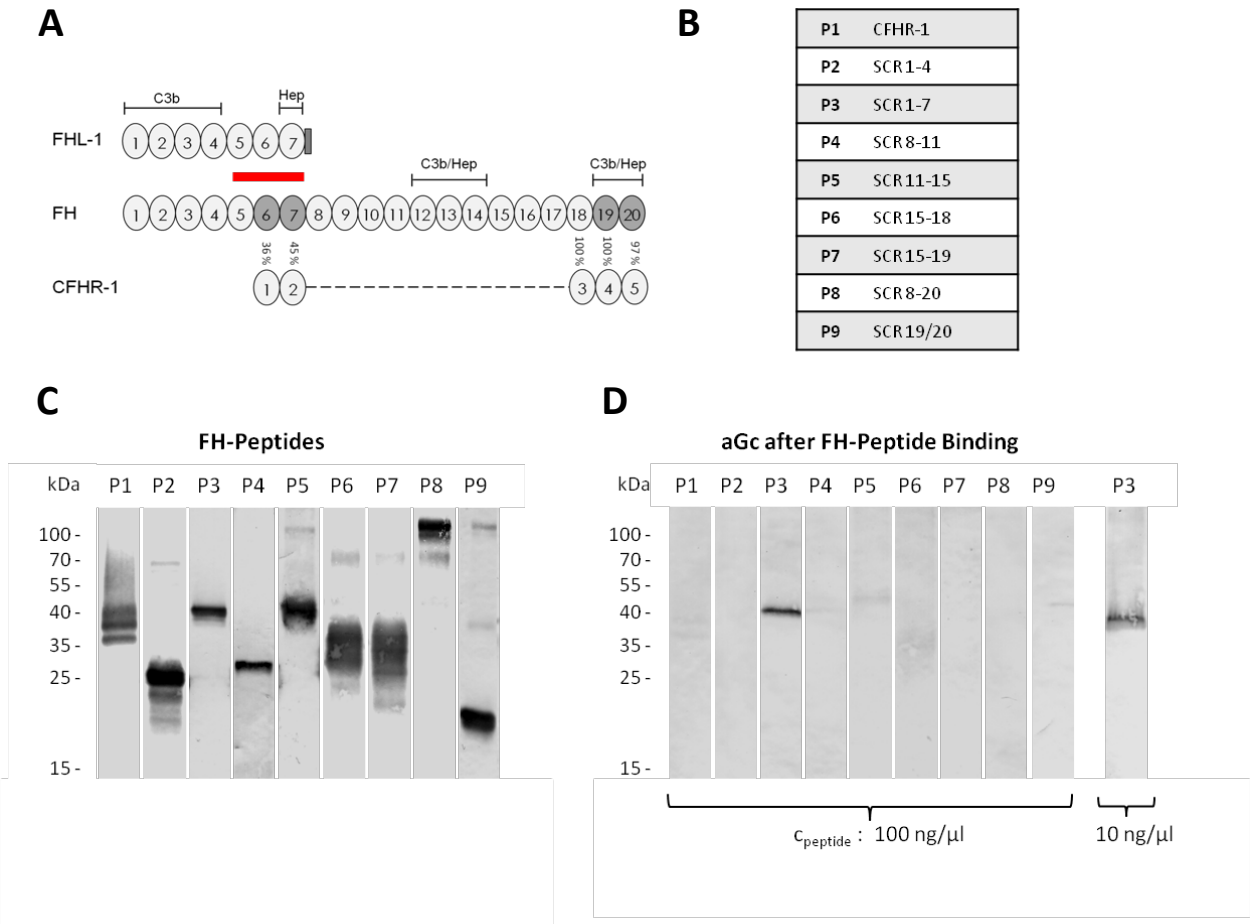


Figure 3.19: **Peptide binding assay with recombinant SCR domains. A.** Schematic overview of three FH family proteins; FHL-1, FH, and CFHR-1. The bar highlights domains of FH and FHL-1 for binding to aGc. **B.** Description of FH peptides with SCR domains. **C.** FH peptides alone were detected by Western blotting with a polyclonal anti-FH-SCR1-20. **D.** Binding of peptide SCR1-7 to sexual stage parasites. Gametocytes were activated with SAX medium in the presence of FH fragments at a concentration of 100 ng/µl (and of 10 ng/µl in the case of SCR1-7; right). The parasite pellet was harvested at 45 min after activation and subjected to native gel-electrophoresis and Western blotting, using polyclonal anti-FH-SCR1-20 antiserum. The binding of fragment SCR1-7 (= FHL-1; ~40 kDa) to sexual stage parasites was detected, while other recombinant SCR fragments as well as recombinant CFHR-1 did not bind.

3.2.7 Cofactor activity of FH on the surface of aGc

The Cofactor activity assay of FH should demonstrate if surface-bound C3b is inactivated by FH. The method was described the first time in Giannakis *et al.,* 2002. During C3b inactivation the α'-chain of C3 becomes cleaved into α'_1 and α'_2. This would indicate that APC inactivation is dependent on FH binding. In the absence of NHS, gametocytes were activated in vitro with SAX medium in the presence or absence of purified FH. After 30 min, gametocytes were thoroughly washed, and purified C3b and factor I were added to the activated cultures, which were incubated for another 30 min at RT (section 2.2.2.10). Subsequently, gametocyte lysates were subjected to SDS-PAGE, followed by Western blotting, and probed with anti-C3-antisera. In samples of activated gametocytes, to which FH was added during activation in order to inactivate C3b, the α´ chain was processed by factor I, and the peptides α'_1 and α'_2 (with molecular weights of 67 and 40 kDa) appeared in addition to the protein bands of α´ and β, which had mobilities of 101 and 75 kDa, respectively (Fig. 3.20). In samples without FH, however, the α´ chain was not cleaved to α'_1 and α'_2 by factor I (Fig. 3.20).

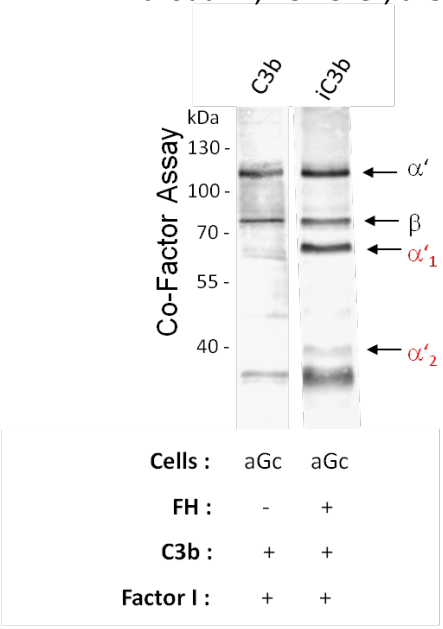


Figure 3.20: **FH-mediated processing of C3b by factor I.** Gametocytes were activated with SAX medium in the absence of NHS and in the presence or absence of purified FH. After 30 min activation time purified C3b and factor I were added and incubated for another 30 min at RT. After washing the gametocytes were subjected to SDS-PAGE and Western blotting, using anti-C3 antibodies, to visulize bound C3b products α´ (101 kDa) and β (75 kDa), and the processing products α'_1 (67 kDa) and α'_2 (40 kDa).

3.2.8 Influence of heparin on C3b and FH binding

Several C3b binding sites were previously identified within FH, namely for SCR1-4, SCR12-14 and SCR19-20 (see Fig. 3.19 A; Kühn *et al.,* 1995; Jokiranta *et al.,* 1996, Jokiranta *et al.,* 2000). For the latter two C3b-binding sites, and for module SCR7, also heparin binding affinity were described (Kühn *et al.,* 1995; Blackmore *et al.,* 1998; Prodinger *et al.,* 1998; Weismann *et al.,* 2011). To confirm involvement of the SCR7 domain in gametocyte attachment, competition studies were performed, where

gametocytes were activated for 15 min and 1 h in NHS in the presence or absence of heparin (5 mg/ml). Subsequently, gametocyte lysates were subjected to SDS-PAGE, followed by Western blot analysis, and probed with anti-C3 antisera. In samples from gametocytes activated in the presence of heparin, C3b was less degraded, as detected by the reduced presence of cleavage products α'_1 and α'_2, when compared to samples from gametocyte that were activated in the absence of heparin (Fig. 3.21 A, top). The experiment was performed in triplicate and the signal strength for the α'_1 protein bands was measured using the ImageJ program. The quantification revealed a 65 % reduction in the signal strength for α'_1 in samples that were treated with heparin (Fig.3.21 A, bottom). Because the binding of heparin to FH would consequently cause a

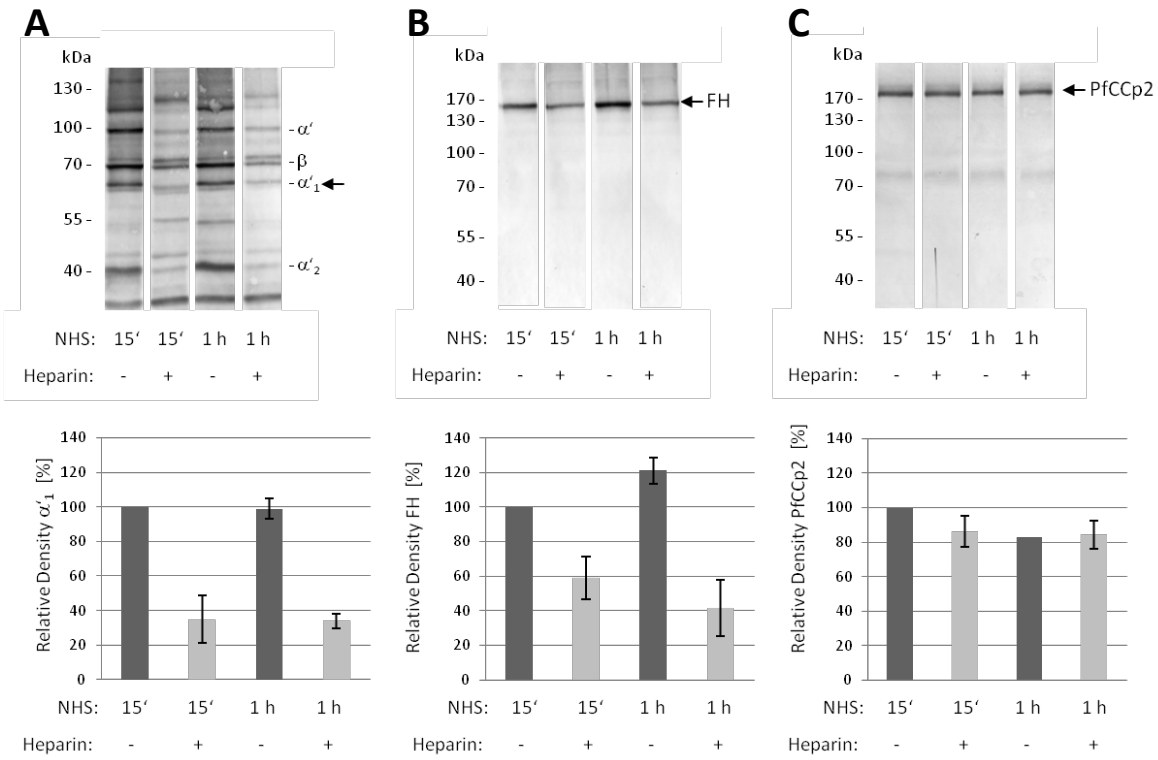


Figure 3.21: **Influence of heparin on C3b and FH binding. A.** Gametocytes were activated with NHS in the presence or absence of heparin. Parasite pellets were harvested after 15 min and 1 h activation time and subjected to SDS-PAGE and Western blotting, using anti-C3-antibodies to visualize the C3b processing products (top). The signal strength for the α'_1 protein bands (arrow) was measured in gametocyte samples after 15 min and set to 100 % (bottom). **B.** FH-binding to the sexual stage surface in the presence of heparin. Gametocytes were treated like described above and subjected to gel-electrophoresis and Western blotting, using anti-FH-SCR1-20 antiserum (top). The signal strength for the FH protein bands (arrow) was measured after 15 min and set to 100 %. **C.** Loading control: Gametocytes were treated like described above and subjected to SDS-PAGE and Western blotting, using anti-PfCCp2 antisera to detect PfCCp2 (185 kDa; top). The signal strength of all proteins was measured using the ImageJ program and all experiments were performed in triplicate.

reduction in the binding of FH to the surface, we further investigated the binding of FH to activated gametocytes, using the above described experimental setting. When the lysates of gametocytes activated in the presence of heparin were probed for FH binding using anti-FH-SCR1-20 antisera in Western blot analysis, a weaker FH signal was observed compared to samples of gametocytes activated in the absence of heparin (Fig. 3.21 B, top). Signal strength quantification revealed a reduced FH binding to the surface of the sexual stage parasites by 41 % (after 15 min activation) and 66 % (after 1 h activation), when these were preincubated with heparin (Fig 3.21 B, bottom). Immunoblotting with antisera against the sexual-stage specific protein PfCCp2 was used for loading control (Fig. 3.21 C). The combined data suggest that FH binds to the parasite surface via its heparin sites and mediates C3b inactivation.

3.3 Interaction partner of FH on the surface of activated gametocytes

3.3.1 Identification of the FH receptor protein

After demonstrating that sexual stage parasites bind FH on their surface in order to inactivate C3b, the responsible plasmodial receptor for FH should be identified. Therefore gametocytes were activated in the presence of NHS for 30 min and Co-IPs were performed (section 2.2.2.6), using anti-FH mAb 131X bound to protein G beads. Co-IPs performed with lysates of asexual blood stage parasites using the same setting were used for negative control. The precipitated proteins were separated by SDS-PAGE and visualized via silver staining. Band patterns were compared between samples of precipitated activated gametocyte and asexual parasite proteins. Thick bands running at 55 kDa and 30 kDa displayed the heavy and the light chain of the bait antibodies. A dominant protein band of the activated gametocyte sample, and not present in the asexual lysate precipitate, was subjected to mass spectrometric analysis (Fig. 3.22 A, left). It was running with a molecular weight of approximately 45 kDa. The detected peptides were used for MASCOT searches to identify full-length proteins. Mass spectrometry (kindly performed by Edwin Lasonder; Nijmegen Proteomics Facility) identified the 45 kDa protein as the glideosome-associated protein PfGAP50 (PlasmoDB Gene-ID: PFI0880c). Annotation of the protein sequence predicted an N-terminal signal peptide with a transmembrane domain and a C-terminal transmembrane domain. Annotation further indicated a metallo-dependent phosphatase domain (Fig. 3.22 B). PfGAP50 was originally assigned to the actin-myosin motor complex of the parasite invasive stages (reviewed in Baum *et al.,* 2006; Sanders

et al., 2007; Yeoman *et al.,* 2011) and was also described as a peripheral phosphatase in the *P. falciparum* blood stage parasites (Müller *et al.,* 2010). For further investigations polyclonal mouse antisera against the recombinant expressed PfGAP50 protein were generated by Matthias Scheuermeyer. When the antisera were used to probe the above precipitated protein samples via Western blot analysis, a similar protein band at 45 kDa was detected in the mAb 131X-precipitated gametocyte protein sample (running right below the broad protein band of the heavy chain of the precipitating antibody; Fig. 3.22 A), but not in the precipitated asexual blood stage protein sample.

PfGAP50 expression in non-activated and activated gametocytes was also investigated via Western blot analysis. Lysates of asexual blood stage schizonts (kindly provided by

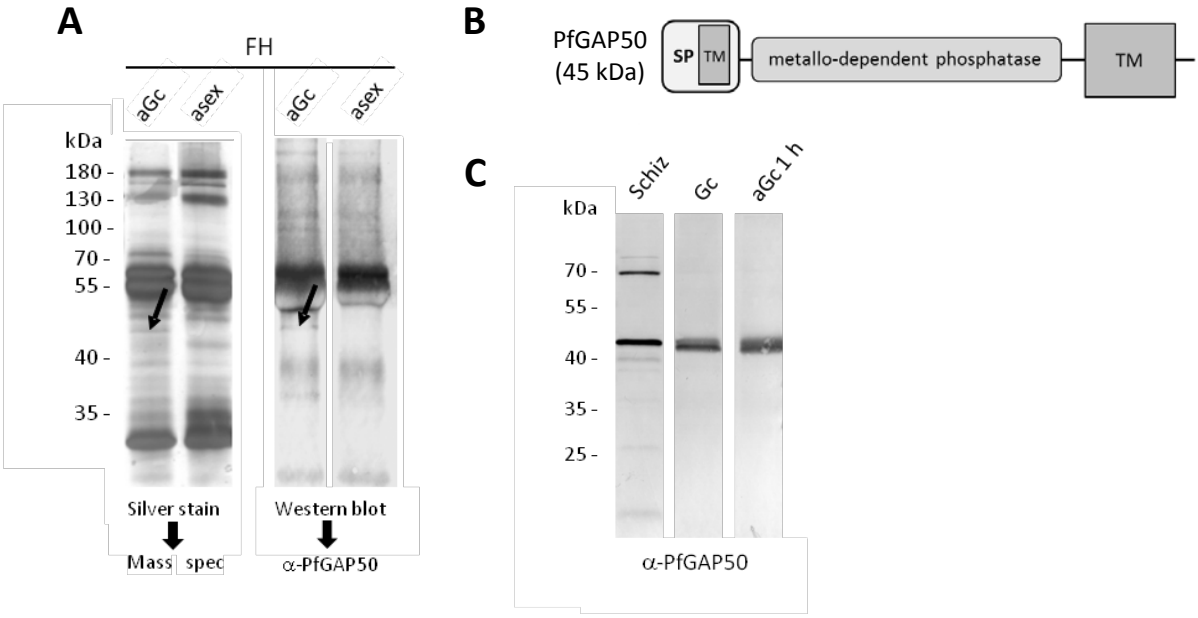


Figure 3.22: **Interaction of FH with the transmembrane protein PfGAP50. A.** Mass spectrometric identification of PfGAP50 as FH-binding protein. Proteins of gametocytes at 30 min post activation with NHS and of NHS-incubated mixed asexual cultures, which were co-immunoprecipitated with anti-FH mAb 131X, were subjected to SDS-PAGE and visualized via silver staining (left). A 45 kDa protein of the gametocyte sample was picked, subjected to mass spectrometric analysis and identified as PfGAP50. The co-immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting, using polyclonal anti-PfGAP50 antisera, which detected PfGAP50 in the activated gametocyte sample (right). **B.** Schematic depicting the domain architecture of PfGAP50. SP, signal peptide; TM, transmembrane domain. **C.** Expression of PfGAP50 in blood stage parasites and gametocytes. Lysates of synchronized blood stage schizonts, of mature non-activated gametocytes (Gc) and of gametocytes after 1 h activation were subjected to SDS-PAGE and Western blotting. Anti-PfGAP50 antisera were used to detect the respective protein, which was running at molecular weights of 70 kDa and 45 kDa in the schizonts lysate, and as a 45/42 kDa doublet in the gametocyte lysates.

Ludmilla Sologub), mature non-activated gametocytes and activated gametocytes (1 h post activation) were subjected to SDS-PAGE and blotted with the anti-PfGAP50 antisera. In lysates of schizonts, a protein band at the estimated molecular weight of 45 kDa and a second band at ~70 kDa was detected (Fig. 3.22 C). In the lysates of mature gametocytes and particularly of activated gametocytes, on the other hand, a protein doublet running at approximately 45 kDa and 42 kDa, and no 70 kDa band, was identified (Fig. 3.22 C), indicating that in these stages a processed form of PfGAP50 is present.

3.3.2 Expression and localization studies of PfGAP50 in sexual stage parasites

PfGAP50 is predicted to be localized in the inner membrane complex (IMC) of merozoites (reviewed in Baum *et al.,* 2006; Sanders *et al.,* 2007; Yeoman *et al.,* 2011). In the following studies it should be proven by IFAs how long PfGAP50 is expressed in the sexual stages of the malaria parasite and if PfGAP50 is surface associated after gametogenesis induction.

Expression pattern of PfGAP50

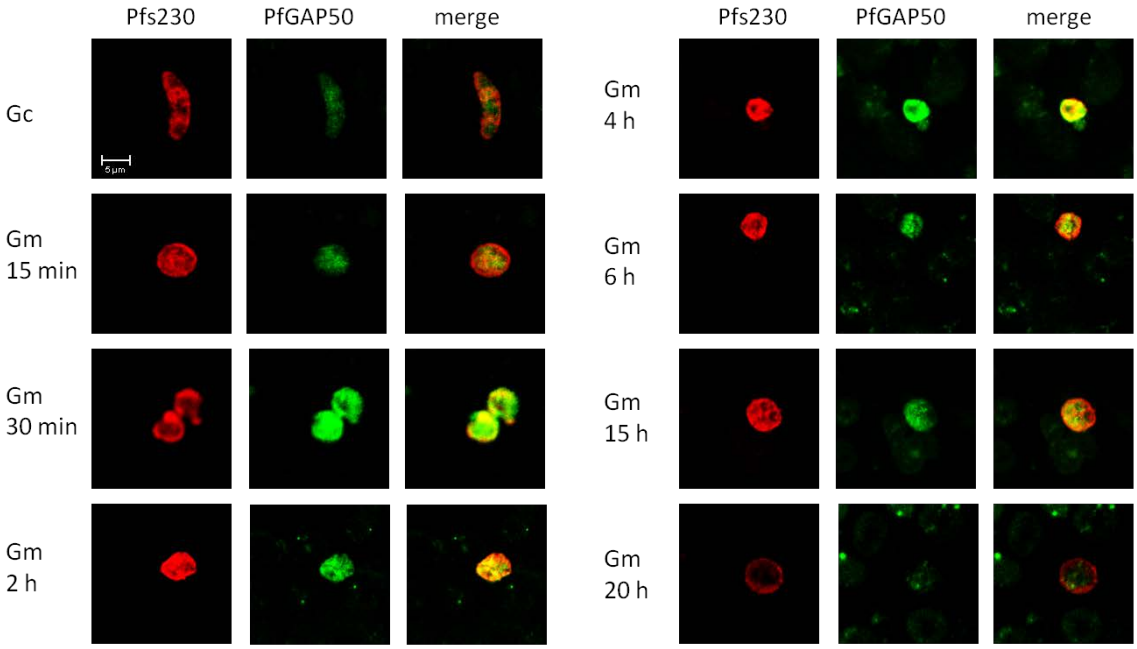


Figure 3.23: **Expression of PfGAP50 in sexual stage parasites.** Gametocytes were activated with NHS. Samples were taken at five different time points post activation. and prepared for indirect immunofluorescence assay. Specimens were saponin-permeabilized. Non-activated gametocytes were used for positive control. The sexual stage parasites were labeled with antibodies against Pfs230 (red) and against PfGAP50 (green). Animal specific neutral serum controls can be looked up in section 3.4.

After activation gametocyte cultures were coated on Teflon slides at different time points. The samples were methanol-fixed and permeabilized. PfGAP50 was labeled using the respective antisera, and the activated gametocytes and macrogametes were labeled with anti-Pfs230 antisera. PfGAP50 was detectable in activated gametocytes and macrogametes for more than 6 h (Fig. 3.24), thus PfGAP50 was present in the sexual stages for as long as FH binding to the parasite surface was demonstrated. Similarly the PfGAP50 protein was present in non-activated gametocytes and in gametocytes 15 min post activation, but showed lower protein expression than in macrogametes.

Surface association study of PfGAP50

Subsequently was investigated, if PfGAP50 is present on the surface of the emerging gametes and thus accessible to FH. Gametocyte cultures were activated and fixed with methanol for 5 min. Immunofluorescence assays were performed in the absence of saponin to make sure that only the extracellular proteins would be detected. When

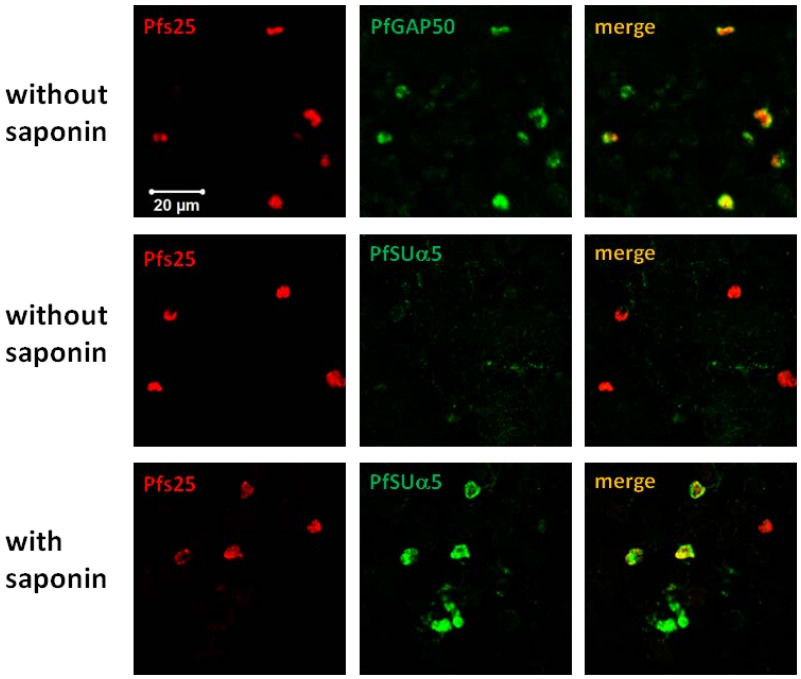


Figure 3.24: **Surface associated expression of PfGAP50 in activated gametocytes.** Mature gametocytes were activated, fixed with methanol for 5 min and were subjected to indirect immunofluorescence assays without saponin-permeabilization 30 min post activation. PfGAP50 was labeled with polyclonal anti-PfGAP50 antisera (green), macrogametes were highlighted by Pfs25-labeling (red). For negative control, specimens were incubated with antisera against the intracellular proteasome subunit SU α5, and no labeling was detected. Contrary, SU α5 labeling was detected, when the specimens were saponin-permeabilized before antisera incubation (green). Animal specific neutral serum controls can be looked up in section 3.4.

activated gametocyte cultures were fixed with methanol in the absence of saponin, the emerged Pfs25-positive macrogametes were labeled for PfGAP50 (Fig. 3.25, top). As a negative control, indirect immunofluorescence assays were performed, using an antiserum directed against the intracellular protein proteasome subunit alpha 5 (SU α5), a component of the plasmodial 26S proteasome (Aminake *et al.,* 2011). No labeling of the Pfs25-positive macrogametes was detected (Fig. 3.25, center). When the cultures were permeabilized with saponin before incubation with the anti-SU α5 antibody, on the other hand, the Pfs25-positive macrogametes showed a SU α5 signal (Fig. 3.25, bottom). The data indicate that PfGAP50 is relocated to the parasite surface during gametocyte activation and subsequently present on the surface of the emerged macrogametes for several hours.

3.3.3 Interaction studies with recombinant PfGAP50 proteins and FH peptides

Finally it has to be investigated, if PfGAP50 is able to directly bind FH and FHL-1. Recombinant PfGAP50 was immobilized to a microtiter plate and incubated with recombinant FH (SCR1-20) or FHL-1 (SCR1-7), which were applied at concentrations of 2.5 µg/ml, 5 µg/ml, or 10 µg/ml. The binding intensity was colorimetrically evaluated in

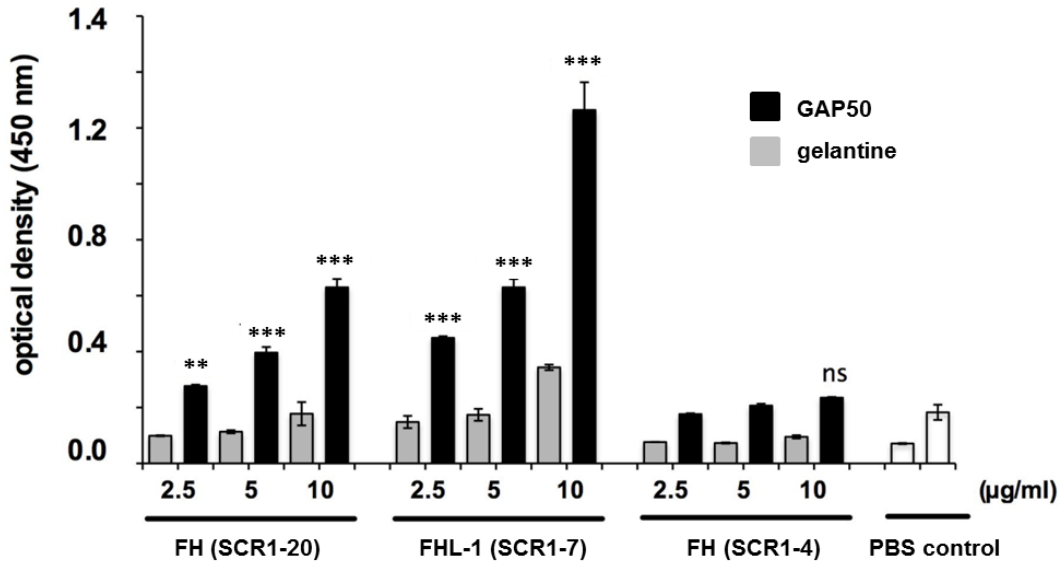


Figure 3.25: **Dose-dependent binding of FH and FHL-1 to PfGAP50.** Recombinant immobilized PfGAP50 was incubated with recombinant SCR1-20 (=FH), SCR1-7 (=FHL-1) and FH-SCR1-4 at concentrations of 2.5 µg/ml, 5 µg/ml, or 10 µg/ml. FH peptide binding was measured colorimetrically at OD_{450}. Immobilized gelantine and PBS alone were used for negative controls; **p < 0.01, *** p < 0.001, ns-no significance, related to the PBS control (from Christine Skerka, Jena).

triplicate. FH as well as FHL-1 bound to PfGAP50 and these effects were dose-dependent (Fig. 3.23). The fragment representing SCR1-4 did not bind to recombinant PfGAP50, confirming that binding of FH to the parasite is preferentially mediated by SCR modules 5-7. Unspecific binding can be excluded, as the FH peptides did not bind to gelantine-coated wells. PBS was used for a background color control (kindly performed in the laboratory of Christine Skerka and Peter F. Zipfel, Leibniz-Institut, Jena).

3.4 IFA controls of neutral animal sera

All antisera used in IFAs in this thesis were polyclonal. To exclude unspecific binding of the polyclonal antibodies control IFAs using different neutral animal sera and sera against the GST-tag alone were performed and summarized in one figure (Figure 3.26). The parasites were counter labeled with antibodies against Pfs230. Incubation with neutral sera revealed no labeling of the parasites. However, rabbit antiserum gave a very faint background labeling.

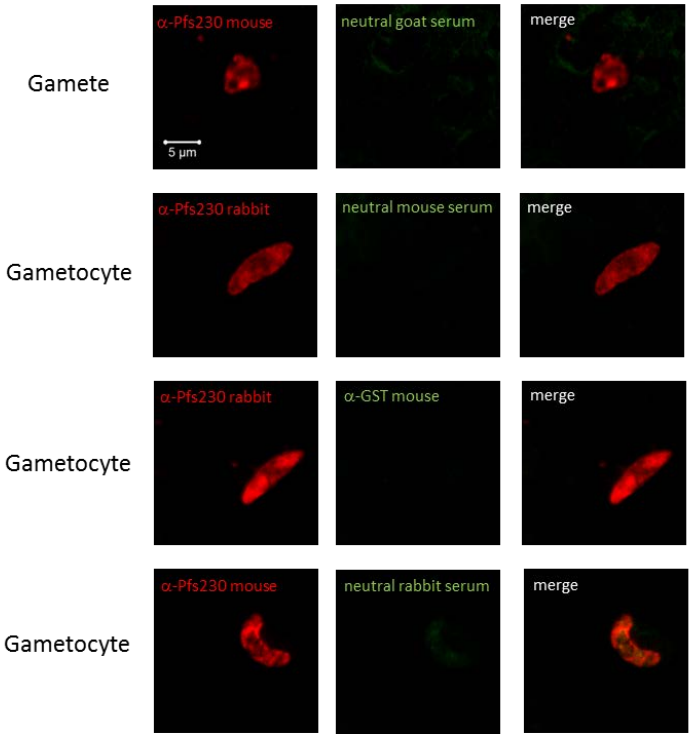


Figure 3.26: **Control IFAs with neutral animal sera.** Sexual stage malaria parasites were counter labeled with rabbit or mouse anti-Pfs230 serum. Neutral goat, mouse, and rabbit sera were used for second antibody labeling and showed no binding, except of a faint background labeling of neutral rabbit antiserum. Furthermore antibodies against the GST-tag alone revealed no binding to malaria parasites.

4 Discussion

The sexual phase of the malaria pathogen begins with the differentiation of gametocytes in the human host and continues after the blood meal in the midgut of the mosquito vector. Gametogenesis, the formation of gametes, is triggered by environmental factors of the mosquito midgut. Subsequently, fertilization takes place, and the resulting zygote transforms into the infective ookinete, which leaves the midgut lumen to form an oocyst between the epithelium and the basal lamina of the midgut wall (reviewed in Pradel, 2007 and Kuehn and Pradel, 2010). The malaria sexual phase represents a crucial step in the spread of the disease, but to date the molecular details of parasite interactions with midgut contents during fertilization remain largely unknown. The primary focus of this doctoral thesis was to study the molecular interplay of *P. falciparum* sexual stage proteins during reproduction in the mosquito midgut. Initial investigations aroused interest in the details of intermolecular binding between surface associated adhesion proteins in gametocytes and during gametogenesis in the mosquito midgut. Follow-up studies addressed the interaction between midgut parasites and the human complement system during sexual reproduction in the mosquito midgut.

4.1 Sexual stage surface proteins of the malaria parasite

Orthologs of CCp proteins

A gene related to a second gene by descent from a common ancestral DNA sequence is termed homolog. This term may apply to the relationship between genes separated by speciation (ortholog), or to the relationship between genes originating via genetic duplication (paralog) (Fitch, 1970). Deciphering of an increasing number of completed parasite genome sequences were completed during the last two decades. The raising bioinformatic possibilities discovered a growing number of orthologous genes. To explore orthologs of PfCCp proteins, two independent databases were scanned by bioinformatical screenings. The OrthoMCL database was established 2005 and the number of sequenced apicomplexan parasite genomes was doubled since this time (Chen *et al.,* 2006). A recent study identified the CCp family members in the early branching apicomplexan class of gregarines, *Ascogregarina taiwanensis* (Templeton *et al.,* 2010). The re-annotation work of CCp orthologs in this study did not support those findings, most probably due to the fact that the databases used in this study did not

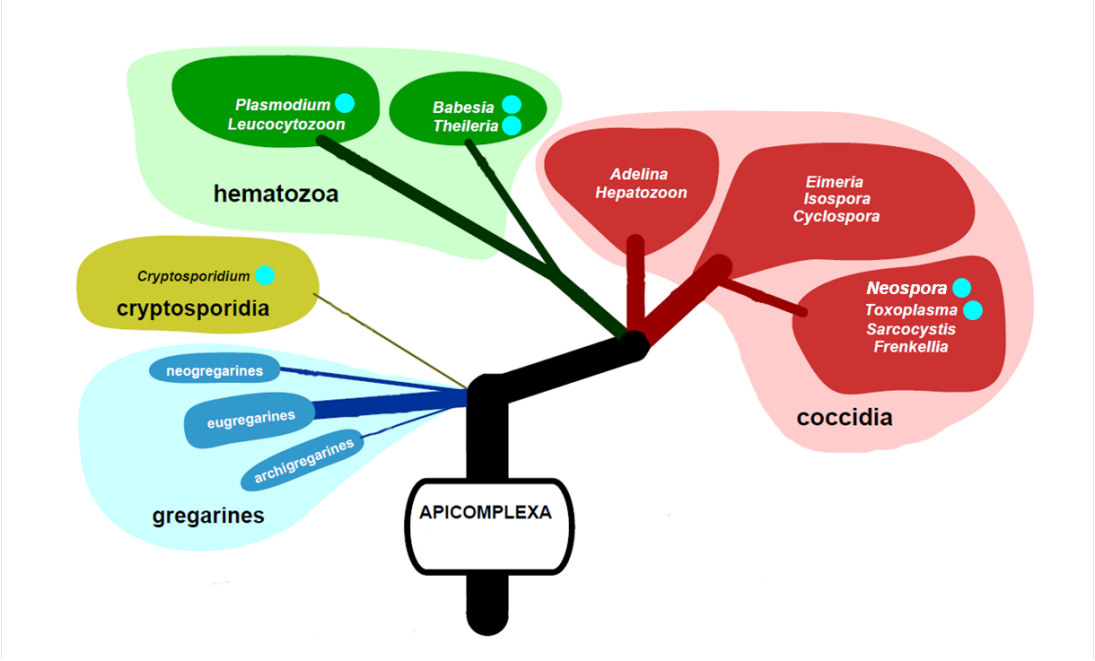


Figure 4.1: **Hypothetical tree of Apicomplexa**. Four principal apicomplexan subclasses were depicted: gregarines, cryptosporidia, hematozoa and coccidia. Occurrence of orthologous CCp proteins was highlighted with a turquoise dot (modified from J. Slapeta http://tolweb.org/Apicomplexa/2446).

provide the gregarines genome sequence. The presence of *Mus musculus,* which seemingly comprises the CCp3 protein as a homologue can be described as exception, but also indicates the close relationship of the Scavenger receptor domain and the Pentraxin domain to vertebrate proteins. The overview of deciphered apicomplexan genomes of the OrthoMCL data base suggested that CCp homologues occur in all Apicomplexa, because they are found in all genomes sequenced so far. Similarly, the description of classification of apicomplexa is not consistent. Some sources create a new class for *Cryptosporidium* or combine Haemosporida and Piroplasmida in one group named Hematozoa (Fig. 4.1). In order to gain a schematic overview of identified orthologous CCp proteins a hypothetical tree of apicomplexa was developed. The apicomplexan genera containing CCp proteins were labelled with a turquoise dot (Fig. 4.1).

For most of the CCp orthologs, sites of expression in the respective parasite species are yet unknown. Studies on Cryptosporidia and *Plasmodium berghei* revealed more detailed information on the CCp localization in these parasites. It is known that the expression of CpCCp1 of *C. parvum* (also referred to as Cpa135) starts in oocyst-sporozoites and increases rapidly after excystation (Snelling *et al.,* 2006). In sporozoites, CpCCp1/Cpa135 is seemingly stored in the micronemes and is excreted upon host cell invasion. Subsequently, the protein localizes to the PVM. With the

formation of merozoites, new transcript of CpCCp1/Cpa135 is detectable (Tosini *et al.,* 2004). Whereas characterization studies on CCp proteins in *Babesia* are still at the very beginning. BpCCp1, BpCCp2, and BpCCp3 were identified as markers for babesian sexual stages. So far it was not possible to differentiate between asexual and sexual stages of Babesia morphologically. Antibodies directed against BpCCp proteins offer new opportunities to study the sexual development of the babesian parasite (*Becker et al.,* 2010). Like the degrees of relatedness the characteristics of CCp proteins in the rodent malaria parasite *Plasmodium berghei* are much more similar to the human pathogen than to other apicomplexa. CCp proteins are mainly expressed in female gametocytes (Khan *et al.,* 2005; Raine *et al.,* 2007). They are localized in the parasitophorous vacuole of gametocytes and attached to the parasites membrane of macrogametes after activation for about 1 h. Studies on GFP fusions of PbCCp3/LAP1 (also termed PbSR), PbCCp1/LAP2, and PbCCp5/LAP3 revealed accumulation of these proteins in crystalloids. These organelles are formed in the ookinete and persist until the early oocyst stage (Carter *et al.,* 2008; Saeed *et al.,* 2010). Crystalloids are transient structures whose presence is restricted to the mosquito-specific ookinete and young oocyst stages of the parasite. The recent discoveries point to a potential important role in protein trafficking and sporozoite transmission that could be exploited as new targets for control of malaria transmission (Dessens *et al.,* 2011). Similar observations were made in ookinete stages by IFA of *Plasmodium falciparum.* Strong single points of PfCCp proteins were detected with antibodies against the appropriate CCp protein in ookinetes (Bachelor Thesis of Vanesa Ngongang, 2011).

The PfCCp proteins are comprised of multiple adhesive domains, whose remarkable architectures are conserved throughout the apicomplexan clade (Templeton *et al.,* 2004). The complex structure of these proteins suggests playing a role in protein, lipid, and carbohydrate interactions (Templeton *et al.,* 2004; reviewed in Pradel *et al.*, 2007). To benefit from progress in bioinformatic methods a domain search for PfCCp proteins was repeated and revealed five additional domains in the PfCCp protein family (reviewed in Kuehn *et al.,* 2010). These findings filled gaps of so far not described CCp protein regions. Domain search was conducted via seven independent sequence analyzing programs and was confirmed by the PlasmoDB domain overview of each protein.

To further understand the biological role of PfCCp proteins structural analyses are required. Experimental structure prediction methods can rely on detectable similarity

spanning of known structures or they predict the structure from sequence alone (Baker and Sali, 2001). Potentially these findings will give further understanding of the PfCCp function during the sexual development of malaria parasites.

Interactions between sexual stage proteins in *Plasmodium falciparum*

First studies on the PfCCp proteins reveal their localization in the PV of predominantly female gametocytes. Protein distribution occurs in a punctuated pattern for PfCCp1, PfCCp2 and PfCCp3 and mainly at the poles of gametocytes for PfCCp5 and PfFNPA. Further, PfCCp4 is spread homogenously (Delrieu *et al.,* 2002; Pradel *et al.,* 2004 and 2006; Scholz *et al.,* 2008). During gametogenesis PfCCp proteins appear to be partly released and later locate on the surface of newly emerged macrogametes (Pradel *et al.,* 2004; Simon *et al.,* 2009). Similarly it was shown that PfCCp2 and PfCCp3 knock-out parasites are not able to migrate to salivary glands (Pradel *et al.,* 2004). This allows assigning these proteins to the group of potential TBV candidates. Furthermore it was recently reported that PfCCp1, PfCCp2, and PfCCp3 proteins are co-dependently expressed and that the abrogation of PfCCp3 in gene-disruptant gametocytes leads to the lack of PfCCp1 and PfCCp2 (Pradel *et al.,* 2006). Studies verify that all PfCCp proteins are co-dependently expressed and that the lack of one of the proteins leads to partial or complete loss of the other family members (Simon *et al.,* 2009). This guides to the hypothesis that PfCCp proteins interact with each other to form a MPC. In the present study this hypothesis is addressed. Molecular interactions between the six members of the PfCCp protein family were investigated and confirmed. It was subsequently revealed that the six endogenous PfCCp proteins interact by forming protein complexes that were co-precipitated using a variety of PfCCp antibodies. Protein interactions of recombinant PfCCp proteins were investigated by direct protein binding of distinct adhesion domains using bacterially expressed recombinant proteins. These results suggest direct protein-protein interactions between these sexual-stage proteins. Binding capacity was also revealed between the PfCCp protein complex and the surface associated protein Pfs230 and the GPI-anchored protein Pfs25. Binding studies revealed that PfCCp proteins showed binding capacity only to Pfs230 and Pfs25 only after activation of gametocytes. One might hypothesize that PfCCp proteins have to be tightened to the plasma membrane of the parasite after the rupture of the PVM during activation. This is the first time that such complexes involving interactions of multiple adhesive proteins are described for the sexual stages of malaria parasites.

Other protein complexes have been identified in asexual parasites, for example on the merozoite surface (Kauth *et al.,* 2003; Blackman *et al.,* 1991; Ranjan *et al.,* 2011) or in the rhoptries of mature schizonts (Cooper *et al.,* 1988).

During the last two decades a substantial number of proteins expressed in the sexual and mosquito stages of malaria parasites have been identified (reviewed in Pradel, 2007). The majority of these sexual stage proteins are initially expressed in the PV during gametocyte differentiation, and some are later exposed on the surface of emerged gametes and fertilized zygotes. Surface-associated proteins include Pfs25 and Pfs28, Pfs48/45, Pfs230, as well as the six PfCCp proteins (reviewed in Pradel, 2007). Despite ongoing characterizations for most of the identified proteins, the functional basis for their expression within the PV of gametocytes, their exposure during emergence, and the role of the numerous adhesive motifs of these proteins remain

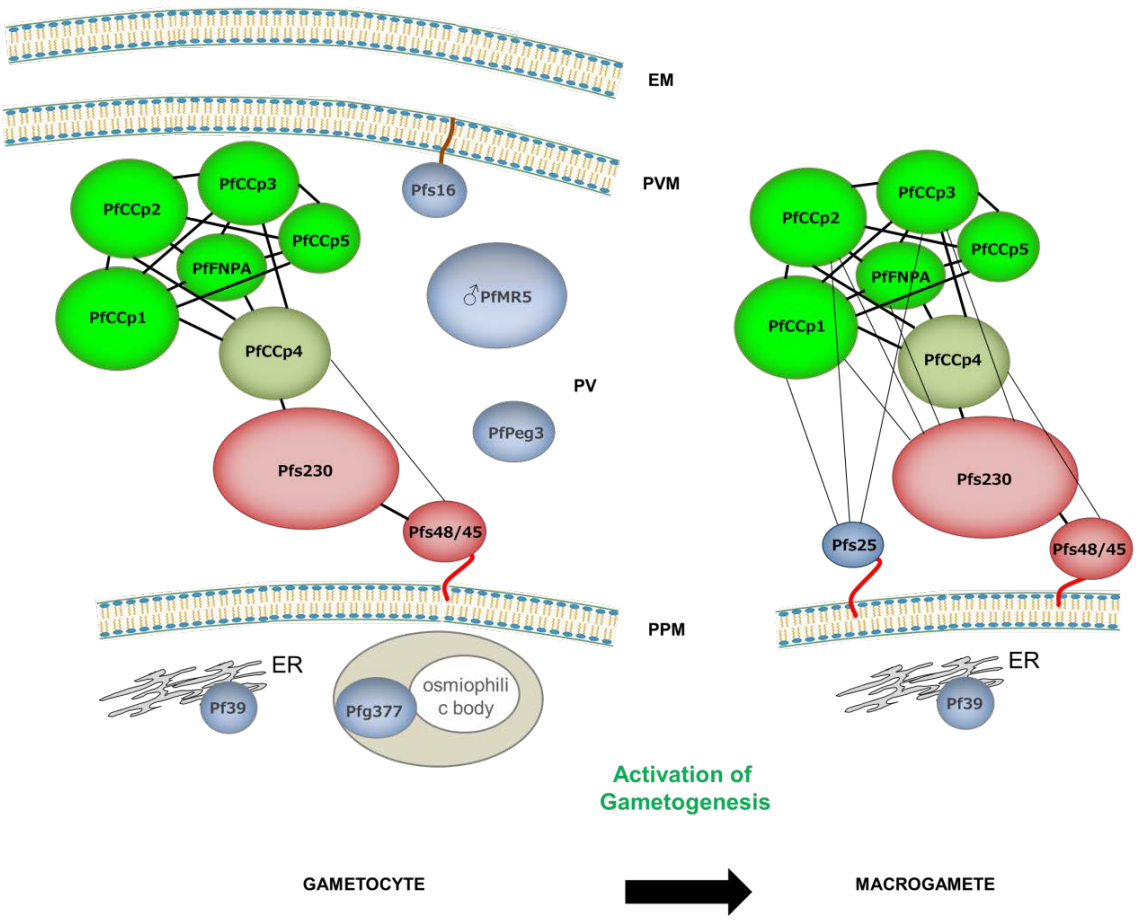


Figure 4.2: **Schematic overview of interacting proteins in the parasitophorous vacuole of gametocytes and on the surface of macrogametes.** This picture represents all proteins included in the study and their potential localization. Thick lines indicate strong interactions, thin lines standard interactions. PPM - parasite plasma membrane; PV - parasitophorous vacuole; PVM -parasitophorous vacuole membrane.

unclear. The PfCCp protein family comprises prominent members of sexual stage proteins with their expression lasting for about ten days (Scholz *et al.,* 2008). Similarly it was demonstrated that the TBV candidate Pfs230 is expressed in male and female sexual parasites in association with the plasmalemma (Quakyi *et al.,* 1987; Williamson *et al.,* 1995). It mediates contact with erythrocytes and as a consequence is responsible for the formation of exflagellation centers (Eksi *et al.,* 2006). During gametogenesis the protein becomes processed proteolytically and peptides are released into the surrounding medium (Williamson *et al.,* 1996). Interestingly recent studies revealed that Pfs230 co-localizes with PfCCp4 in gametocytes and an interaction of both proteins via Co-IPs has been proven (Scholz *et al.,* 2008).

The above described findings lead to setting up an organization of an extended set of interaction studies between sexual stage proteins. This resulted in the supposition that all PfCCp proteins form a MPC inside the PV of gametocytes and on the surface of newly emerged gametes. The complex is tightend by Pfs25, Pfs230 and Pfs48/45 to the PM only in activated gametocytes (Fig. 4.2; reviewed in Kuehn *et al.,* 2010). All experiments were implemented with endogenous proteins and protein complexes were co-precipitated using a variety of antibodies against sexual stage proteins. It is striking that interactions between PfCCp proteins seem to be very close or firm, as compared to interactions between Pfs230 and the PfCCp proteins, because bands of interacting PfCCp proteins are remarkably strong.

However, the investigated proteins form an extensive MPC, whereas the sequence of interacting proteins remains unclear. Furthermore, the distance between the PPM and the PVM seems to be closer in *pfs230* mutant parasites, than in the wildtype (unpublished observations of Gabriele Pradel). This emphasizes that Pfs230 stabilizes together with the PfCCp proteins the space between the PPM and the PVM in gametocytes. Interactions between the PfCCp proteins (except of PfCCp4) and Pfs230 could only be proven after activation of gametocytes (Fig. 4.2). This confirms the hypothesis that Pfs230 tightens the PfCCp proteins on the surface of newly emerged macrogametes. In addition recent expression studies of *pfs230* knockout parasites indicate that after activation PfCCp proteins are not detectable on the surface of macrogametes any more. However, it was proven that PfCCp proteins are expressed in *pfs230* knockout gametocytes. Further investigations on activated gametocytes of *pfs230* mutant parasites are under investigation.

Proteins like Pfs16 and Pfg377 showed no binding to the discovered complex, which can be explained by the fact that they are located in other cell compartments. Pfg377 is associated with osmiophilic bodies in *Plasmodium falciparum.* These organelles lie beneath the subpellicular membrane of gametocytes, and release their contents, including Pfg377, into the PV after activation (de Koning-Ward *et al.,* 2008). Pfs16 is described as gametocyte-specific protein, which exhibits one transmembrane domain and is integrated in the PVM (Baker *et al.,* 1994), whereas the discovered MPC is associated with the PPM. Moreover a recent study revealed pole-oriented distribution of Pfs16 after the onset of gametogenesis, which is not corresponding with the distribution of the proteins belonging to the protein complex discovered in this thesis (Eksi and Williamson, 2011). Not many protein complexes are identified in *Plasmodium falciparum* beside one well-characterized complex involved in merozoites attachment is constituted by the PfMSP (merozoite surface protein) group (Blackman *et al.,* 1991). The majority of these peripheral proteins is secreted into the PV of schizonts and subsequently binds to the surface of developing merozoites via interaction with GPI-anchored proteins such as PfMSP1 (Blackman *et al.,* 1991). This adhesive MSP-based protein complex, which consists of multiple secreted proteins assembling around a GPI-anchored EGF-domain protein (like Pfs25), shows superficial similarities with the here discussed cell surface-associated PfCCp MPC (Kuehn *et al.,* 2010).

The molecular interactions between the PfCCp proteins were investigated in more detail with affinity chromatography co-elution binding assays. An extensive range of recombinant PfCCp proteins was available and direct interaction studies of a selection of PfCCp domains were carried out. These investigations showed that PfCCp proteins directly interact with each other. The domain which is most frequently part of interacting proteins is the Scavenger Receptor domain. These investigations are not fully quantitative, because domains did not appear in the same number, and were not used with the same degree. Of course domains of the CCp3 proteins have more chances to attract attention because they were used as main bait in most of the interaction studies. Furthermore working with recombinant proteins expressed in *E. coli* always includes the risk of misfolding. Beside that *E. coli* is not able to glycosylate the proteins which are originally from eukaryotes.

To prove if endogenous plasmodial proteins also bind recombinant proteins and vice versa, additional binding studies were performed. Results revealed that recombinant proteins produced by *E. coli* interact with endogenous proteins from parasite lysates.

In connection with the identified protein-protein interactions it is very likely that further so far uncharacterized proteins are part of the discovered MPC. To investigate this question the protein complex was precipitated using PfCCp1 as bait from gametocyte lysate. Mass spectrometry revealed one potential new member of the protein complex. A conserved, so far uncharacterized protein with the geneID PF14_0412 was identified. This 96 kDa protein contains two WD40 domains and no signal peptide or transmembrane domains. Proteins containing WD40 repeats often serve as platforms for the assembly of protein complexes or mediate the interplay of proteins (reviewed in Smith *et al.,* 1999 and Li and Roberts, 2001). Further characterization of this protein is now under investigation. Antibodies against PF14_0412 were generated in mice. IFAs revealed protein expression in asexual parasites and gametocytes. In gametocytes the WD40 protein is located to the surface in a punctuated distribution, very similar to the expression pattern of PfCCp1-3. Therefore first results on the characterization of the WD40 protein emphasize that it is possibly involved in the protein complex formation in gametocytes. Preparation of PF14_0412 knock-out parasites and further investigation of protein expression and localization are currently under investigation by Andrea Kuehn and Vanesa Ngongang. Another potential function of this protein may be protein trafficking like it was assumed for a WD40-containing protein in asexual parasites (Adisa *et al.,* 2001). Similarly an association with the myosin-motor-complex is not precluded. Myosin tails (MyoF) contain four to six WD40 repeats that have been implicated in diverse functions like signal transduction and transcriptional regulation (Foth *et al.,* 2006). The PfMyoF-myosin is expressed steadily throughout development and maturation and hence also in gametocytes (Chaparro-Olaya *et al.,* 2005). Actin is the most abundant protein found in eukaryotic cells and has multiple binding sites for myosin. Recent findings suggest interaction of Pfs230 and PfActin II (unpublished observations by Andrea Kühn). Furthermore, Actin II is not detectable in *pfs230*-knockout parasites, which indicates co-dependent expression of the two proteins. Actin II is described as a sexual stage-specific protein (Wesseling *et al.,* 1988 and 1989) and disruption of *actin II* in *P. berghei* resulted in an inhibition of male gametogenesis (Deligianni *et al.,* 2011). WD40-repeats have also been identified in actin-interacting proteins (Voegtli *et al.,* 2003; Mohri *et al.,* 2004). The connection between the WD40-containing protein PF14_0412, Pfs230, and PfActin II has to be investigated. Furthermore its potential responsibility in the assembly of the newly identified MPC on the surface of activated gametocytes has to be explored.

Procession of PfCCps

After the uptake by the mosquito, both male and female gametocytes round up and escape from the enveloping membranes about 15 min post activation. The EM and the PVM therefore rupture within minutes after activation (Sologub *et al.,* 2011) and unbound proteins of the PV are set free. For PfCCp1, PfCCp2, and PfCCp3, a partial release of protein during egress from the erythrocyte was reported previously, and the proteins later relocate surrounding exflagellation centers (Pradel *et al.,* 2004). These findings correspond to the missing protein bands in some activated gametocyte lysates in comparison to non-activated gametocytes of the processing studies in this work. Processed, unbound PfCCp proteins might be released after activation because surrounding membranes (EM and PVM) rupture. Western blot analysis of PfCCp proteins in gametocyte and activated gametocyte lysates display additional protein bands beside the full length protein. Protease inhibitor was added directly in front of freezing the gametocyte or activated gametocyte lysate to avoid subsequent protein degradation. It is very likely that the additional bands are processed proteins. Despite this, an unspecific binding of antibodies or an overload of proteins might also lead to additional protein bands. The same amount of protein lysate was used for every lane. Since proteins differ in their expression level an overload of proteins might lead to unspecific protein bands. Processing of surface proteins in the malaria parasite was also described for the merozoites surface protein-1 (MSP1) (Blackman and Holder, 1992) and for the Pfs230 protein (Williamson *et al.,* 1996). But in comparison to the Pfs230 protein processing, which occurs after activation, PfCCp proteins are cleaved prior to activation and released when the surrounding membranes rupture during gametogenesis.

Hypothesis of the MPC function

PfCCp proteins form together with Pfs25, Pfs230, and Pfs48/45 an extensive protein complex which covers the surface of macrogametes, but functional details of this protein envelope are hitherto unknown. The expression of the PfCCp proteins starts during the transition from stage II to stage III gametocytes (Pradel *et al.,* 2004). During gametocyte maturation some proteins become processed and protein complexes containing all PfCCp proteins are formed. After activation female gametocytes round up and lose the EM and PVM. Processed unbound PfCCp peptides are released and the PfCCp complexes bind to the surface associated Pfs230 and Pfs25 and form an

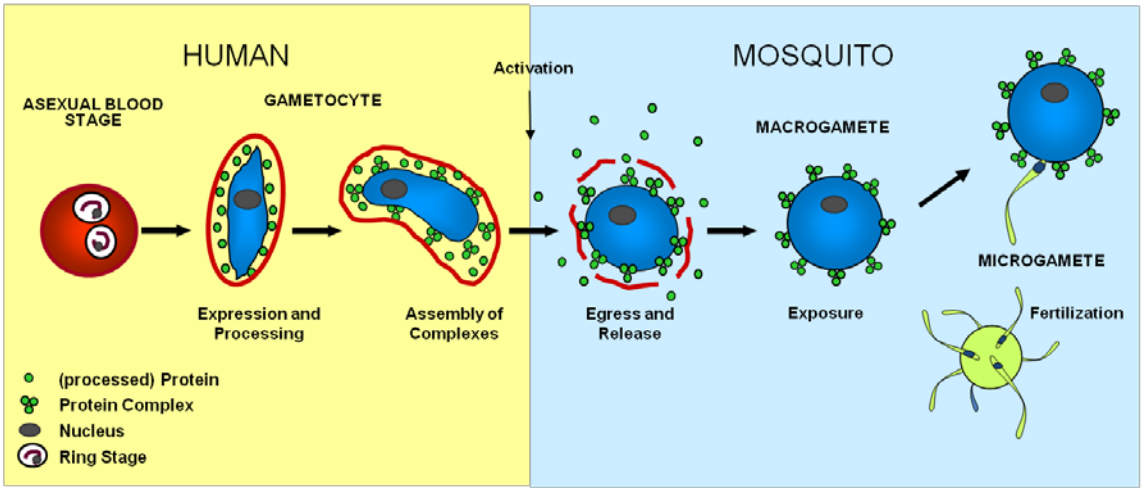


Figure 4.3: **Working Hypothesis: MPC formation of sexual stage parasites.** During gametocyte development, adhesion proteins are expressed in the PV, become processed and assemble to MPCs. During gamete emergence processed peptides were released and the complexes are exposed to the macrogamete's surface.

extensive protein complex on the surface of macrogametes. In addition, it was found that macrogametes form filamentous cell-to-cell connections alleged "nanotubes" (Rupp *et al.,* 2011). These filaments contain Pfs230 and Pfs25 proteins. This facilitates the hypothesis that these proteins support interactions between macrogametes. Furthermore the adhesive properties of the PfCCp multi-protein complexes might form a protective shield that represents a barrier between the newly exposed gametes and the aggressive environment of the mosquito midgut. The malaria parasite's surface might be affected by human blood meal factors which are taken up with the blood meal. Furthermore midgut bacteria or digestive enzymes can attack the surface of the newly emerged macrogametes, which might need time to stabilize their surface membrane after gametogenesis.

4.2 The human complement system and malaria parasites in the mosquito midgut

The binding of complement regulators by microbes is a means to mimic host cell surfaces and represents a mechanism of complement evasion that is used by a broad range of pathogens, including gram-positive and gram-negative bacteria, viruses, fungi and parasitic worms like *Echinococcus granulosis* and *Onchocerca volvulus*. Each of these pathogens bind soluble regulator proteins, like FH, FHL-1, CFHR-1, or the C4-binding protein C4BP on their surface, thereby inactivating C3b or C4b. During recent years, several types of microbial complement-binding receptor proteins have

been identified and functionally characterized. Among others, this highly diverse group of proteins includes the streptococcal M proteins, the pneumococcal PspC proteins, and the complement regulator-acquiring surface proteins CRASP1-5 of *Borrelia burgdorferi* and *Candida albicans*. Each of these complement-binding receptors is able to interact with multiple soluble host proteins and complement regulators (reviewed in Zipfel *et al.,* 2007). Despite the growing knowledge on complement evasion mechanisms of human pathogens, surprisingly few data were hitherto obtained on the complement evasion strategies of protozoan parasites. In the late 1980s APC evasion of *Trypanosoma cruzi* was investigated and discovered that the infective metacyclic form accumulates inactivated C3b on its surface (Joiner *et al.,* 1986). The authors proposed that C3b inactivation was not due to FH binding, but to inefficient binding of the amplification component, factor B. At the same time APC evasion of sexual stages of *P. gallinaceum,* after emerging from the enveloping red blood cell, was examined (Grotendorst *et al.*, 1986). Young zygotes were shown to have a protection mechanism against APC lysis, but the protection was lost at 6-8 h post-blood meal. The authors speculate that a protease derived from the mosquito midgut is responsible for APC inactivation (Grotendorst *et al.,* 1986 and 1987). Several years later, APC activity in the mosquito midgut during infection with *P. berghei* was investigated (Margos *et al.,* 2001). Again, the sexual stages were shown to be protected from complement-induced lysis for approximately 3 h. The authors hypothesized that the sexual stages ingest the GPI-anchored protectin CD59 and subsequently present the receptor on their surface, like it had been proposed for *Escherichia coli* and *Helicobacter pylori* (Rautemaa *et al.,* 1998; Mihlan *et al.,* 2011). However, no CD59 was detected in the zygotes (Margos *et al.,* 2001).

We now identified the protective component which enables APC evasion of malaria parasites in the blood meal during sexual reproduction as human FH. Emerging gametes and young zygotes bind FH, and to a lower extend FHL-1, on their surface. This results in decreased parasite lysis by the human complement. In consequence, surface-bound C3b becomes inactivated and processed by cofactor FH and factor I. Furthermore, FH becomes slowly degraded in the midgut during a period of 6 h, while the human APC of the blood meal is active for about 1 h. Thus APC activity ceases before FH is being degraded. This explains previous finding that malaria parasites are protected for approximately 3 h, but that they lose the protection after 6-8 h (Grotendorst *et al.,* 1986 and 1987; Margos *et al.,* 2001).

Activity of the human complement system

In this thesis serum from uninfected, healthy humans was taken to analyze complement activation after the uptake of the human blood into the mosquito midgut. These investigations show that complement activation becomes initiated within minutes, is most active during the first 20 min post activation, and that activity persists up to 1 h. The resulting ELISA data cannot be compared with the presented standard curve in the user's manual because a mixture of the whole content of mosquito midguts was measured and not only serum, like it was prescribed in the instructions of the C3a Plus EIA Microvue Kit. The small deviations of C3a measurements from different mosquito midgut lysates point out that the used method reveals plausible results. Almost the same volume of serum out of one midgut was examined, because no protein reduction and thereby volume reduction by digestion enzymes of the bloodmeal occurs within the first 4 h after the blood meal, where only osmoregulatory processes proceed. The synthesis of first secretory material, including digestive enzymes, starts 4-8 h after the bloodmeal and digestion enzymes at the earliest 8 h after feeding (Houk and Hardy, 1982; Jahan *et al.,* 1999). This ensures that reduction of C3a occurs not due to digestion of midgut contents. The results demonstrate that the complement system is active in the midgut for a period of 3 h post infection. It was recently shown that during gametogenesis the parasites remain in the membrane of the host erythrocyte for approximately 15 min, before the membrane ruptures and releases the newly formed gametes (Sologub *et al.,* 2011). The prolonged stay of the emerging gametes in the remnants of the host cell is probably a strategy to hide from the APC during its most active phase. Subsequently, the gametes have to rapidly bind FH to be protected from complement-mediated lysis. The gametogenesis inhibition assays pursued in this study indicate that about 1/3 of the emerging gametes are lysed by the complement. These might in part represent parasites that did not bind FH effectively or fast enough for protection.

Classical pathway activation was examined either in the presence or absence of monoclonal antibodies against Pfs230 and the protein band strength of C1q was measured. C1q bound strongly to activated gametocytes only in the presence of antibodies, which strengthens the potential of Pfs230 as a TBV candidate. In the absence of Pfs230 antibody classical pathway activation was very weak. Therefore, classical pathway activation can be neglected, when mosquitoes were fed on blood of naïve individuals. But measuring classical complement activation with sera of

uninfected humans did not match with natural conditions in some cases. Studies reveal antibody production, for example against Pfs230 and Pfs48/45 in 15-20 % of infected humans (Bousema *et al.,* 2010). These findings suggest that in several malaria patients proteins from the PPM within the PV were exposed to the human's immune system and presented antigens for antibody production. Erythrocytes and gametocytes were degraded in the spleen or by hemolysis and thereby gametocyte proteins were released. Beside that, mature gametocytes die after several days and parasite proteins were set free in the human host. Gametocytes normally sequester away from the peripheral circulation (Day *et al.,* 1998; Rogers *et al.,* 1996), thereby avoiding clearance by the spleen. It has been suggested that stage I-II gametocytes adhere to CD36, before undergoing a switch in stage III / IV that permits sequestration in the bone marrow, while the mature stage V gametocytes lose the ability to cytoadhere (Rogers *et al.,* 2000). However, a recent study does not support this receptor-mediated binding of host cells to gametocytes. The authors support a more efficient cell adhesion of asexual parasites to bone-marrow derived endothelial cells, than immature gametocytes (Silvestrini *et al.,* 2012). Therefore, gametocytes die or can become degraded in the spleen and surface proteins like Pfs230 were presented to the immune system and might induce antibody production in infected humans.

The central component of the complement system is the human complement factor C3. Activation of C3 follows from proteolytic cleavage of the C3 molecule into two biologically active fragments C3b and C3a and depicts complement activation (reviewed in Liszewski *et al.,* 1996). Initial experiments via IFA display that C3b binds extensively to activated gametocytes and only a very faint binding to erythrocytes and Gc was found (Fig. 3.11). To gain deeper insight into the inactivation status of C3 on the surface of activated gametocytes C3 binding assays were carried out and C3 deposition on the parasite's surface was detected via Western blot analysis. This displays an inactivation of C3b which is indicated by the detection of the α'_1 and α'_2 peptide of the α'-chain of C3 (Pangburn *et al.,* 1977; Barilla-LaBarca *et al.,* 2002; Riley-Vargas *et al.,* 2005). Different factors are known to regulate the activity of the human complement system and thereby the cleavage of C3b. Proteins such as CR1, the membrane cofactor protein MCP, and the decay-accelerating factor DAF are known to be inhibitors of complement activation (Meri and Jarva, 2001). Inactivation of the APC on the surface of pathogens often occurs by binding of the complement regulator FH and/or FHL-1 (Dave *et al.,* 2001; Areschoug *et al.,* 2002; Bhide *et al.,* 2009, Amdahl *et*

al., 2011). Due to these findings it is assumed that malaria parasites bind regulatory factors like FH as well. Therefore the following experiments focused on FH characterization in the mosquito midgut and its binding capacity to different cell types present in the mosquito's blood meal.

Impact of the active human complement on the parasite gametogenesis

To study the influence of active complement on the formation of gametes gametogenesis inhibition assays were performed. The numbers of male parasites were evaluated by exflagellation assays. With this method it is possible to count the formation of male gametes. Exflagellation was compared in samples with HIS and NHS. The results reveal a 20 % lower exflagellation rate of male gametes activated in NHS in comparison to HIS activated male parasites. Therefore it can be expected that about 40 % of female parasites become lysed after the uptake into the mosquito's midgut. But remaining 60 % of parasites are able to survive. The reason for the loss of parasites may be due to the short time parasites have time to prepare protective measures. Similarly, in NHS-treated gametocyte cultures, the presence of anti-FH antibodies significantly reduces the number of macrogametes and zygotes and further reduces the number of exflagellation centers compared to NHS-treated controls. Besides, anti-FH antibodies are able to block transmission of *Plasmodium* to the mosquitoes, when taken up with the infected blood meal. The results reveal that FH-binding to the sexual stage surface is essential for parasite survival in the midgut.

To investigate the time period FH, related proteins like FHL-1, or CFHR-1 are present in the mosquito midgut, contents were analyzed via Western blot analysis for their existence of these proteins. Full length FH was detected up to 15 h post feeding. First procession products were observed at 40 min post-feeding. The protein pattern of infected midguts is not substantially different from uninfected ones. It was proven that FH and FHL-1 are present for about 6 h in the mosquito's midgut, but the α and β peptides of CFHR-1 are not detectable at any stage. Possibly CFHR-1 is degraded by the mosquitoes' anticoagulants or an anti-complement protein. Only in blood-feeding arthropods anticoagulants have been found (reviewed in Ribeiro, 1987 and Ribeiro and Francischetti, 2003) and influence the consistency of the blood meal. The tick *Ixodes scapularis* owns an anti-complement protein, which is injected during the bite and reduces human complement activation (Valenzuela *et al.,* 2000) and also *Anopheles*

mosquitoes have similar mechanisms to reduce complement activity in the mosquito midgut (Francischetti *et al.,* 2002).

The following studies focused on the binding of FH and related proteins to different cell types present in the bloodmeal of the mosquito. First IFAs revealed faint binding to erythrocytes and gametocytes. FH binding of sexual parasites increases considerably after activation and diminished after 3 h. The used FH antibody detects FH, FHL-1, and CFHR-1. To gain a deeper insight into the binding capacity of these proteins to malaria parasites *in vitro* binding studies were performed. This reveals that FH and FHL-1, but not CFHR-1 bound to activated gametocytes. With purified FH it was proven that FH binds directly to activated gametocytes. FH binding to parasites incubated in HIS remained very faint probably due to misfolding of FH during heat inactivation. That FH of HIS loses cell binding capacity after heat inactivation had previously been proven (Ollert *et al.,* 1995). For all experiments it was indispensable to wash cells thoroughly because FH residues from cell culture incubation were always detectable, although the serum in cell culture medium was heat inactivated.

Further investigations focused on the identification of specific binding domains of FH to the gametes surface. FH binding is mediated by SCR modules 5-7. The modules are also present in FHL-1, which is an alternative splicing product of FH and identical with the first seven SCR modules of FH. It is likely that only the SCR domains 6 and 7 bind to the parasite surface, like it was described for *Candida albicans* (Meri *et al.,* 2002 b). In fact, binding of both FH and FHL-1 to the activated gametocytes as was detected. Because FHL-1 is present in the blood in a much lower concentration than FH (with a plasma concentration of 30 µg/ml for FHL-1 versus 500 µg/ml for FH; reviewed in Zipfel and Skerka, 1999 and 2009; Rodriguez de Cordoba *et al.,* 2004), the predominant protein that bound to the surface of sexual stage parasites was FH. It is therefore presumed that malaria parasites preferentially co-opt FH for protection against the human APC.

To ensure that FH is the decisive protein which is responsible for the inactivation of C3b on the surface of macrogametes, a Co-Factor activity assay was performed. Instead of NHS purified C3b, factor I and FH were incubated with gametocytes during activation. Distinct bands for the α' cleavage were detectable only in the presence of FH, which demonstrated that FH is the decisive factor for C3b inactivation on the surface of activated gametocytes.

First FH binding of parasites was discovered in 1988 and demonstrated in *Toxocara canis* and *Schistosoma mansoni* (Kennedy *et al.,* 1988). However, this did not correlate with the functional inhibition of C3b on the surface of these parasites. Thus it was shown that binding of FH does not necessarily inhibit the alternative pathway (Joiner *et al.,* 1986; Kennedy *et al.,* 1988). Bovine FH was discovered to bind to *Echinococcus granulosus* cyst walls (Diaz *et al.,* 1997) and finally this was the first indicator that FH inhibits complement activation to protect parasites. In studies on microfilariae of *Onchocerca volvulus* FH binding was discovered and the Co-Factor Assay revealed the first time that human FH is responsible for inactivation of C3b into iC3b on a parasites surface (Meri *et al.,* 2002 a). Immune evasion strategy can also be described as molecular mimicry. Host cell surfaces are imitated by parasites. One example in *Plasmodium* are the PfEMP1 variants, which are identical to parts of the heparin-binding domain in the immunosuppressive human serum protein vitronectin (Ludin *et al.,* 2011). Vitronectin promotes cell adhesion, spreading, and inhibits membrane-damaging. It binds to bacterial surfaces and is known to protect from membrane-attack-complex-mediated lysis by the complement system (Singh *et al.,* 2010).

FH comprises four C3b and three heparin binding domains, which overlap partly (Kühn *et al.,* 1995; Haupt *et al.,* 2007; Kunert *et al.,* 2007; Skerka *et al.,* 2007; Mihlan *et al.,* 2009, 2011; Reuter *et al.,* 2010; Lauer et al. 2011; Weismann *et al.,* 2011). SCR7 is one of the heparin binding sites and was shown to be part of the binding domains which bind to the surface of the malaria parasite. In order to confirm involvement of the SCR7 domain in gametocyte attachment, competition studies were performed. In the presence of heparin C3b binding was strongly reduced, because two of the three C3b binding sites were blocked by heparin. Moreover, C3b was less degraded, as detected by the reduced presence of cleavage products α'_1 and α'_2, when compared to samples activated without heparin. This indicates that heparin competed with FH binding to the gametocytes, resulting in less inactivation of C3b. Similarly, FH showed a weaker binding to activated gametocytes when activated with heparin treated serum. Due to the fact that it is not possible to block FH binding completely, it is assumed that SCR5 and/or SCR6 play also an important role in FH binding. In addition, it is possible that FH shares a second binding site for attachment of malaria parasites and incorrect folding of recombinant proteins can never be excluded.

FH binding of a plasmodial cell surface protein

In search of the FH binding protein on the surface of activated gametocytes, Co-IPs were performed using FH as bait protein. Out of the precipitated protein complex one 45 kDa protein came to the fore in silver stained SDS-gels and was identified as PfGAP50. It was originally assigned to the actin-myosin motor complex of the invasive stages of *P. falciparum* and *Toxoplasma gondii*, where it forms a protein complex with GAP45 and myosin A (Fig. 4.5; reviewed in Baum *et al.,* 2006; Sanders *et al.,* 2007; Frénal *et al.,* 2010). Further orthologs of GAP50 exist in *Plasmodium vivax, P. yoelii, P. knowlesi, P. chabaudi* and *P. berghei*, in *Babesia bovis, Neospora caninum, Toxoplasma gondii, Theileria annulata, Eimeria tenella*, *Cryptosporidium parvum, C. muris,* and *C. hominis* (reviewed in Baum *et al.,* 2006). Full-length PfGAP50 is located in the endoplasmic reticulum in the early blood stages of *P. falciparum* and then redistributes to the surface of daughter merozoites during schizogony (Yeoman *et al.,* 2011). The crystal structure of PfGAP50 was recently solved and the structure shows an αββα fold (Bosch *et al.,* 2012). Interestingly, GAP50 exhibits a metallo-dependent phosphatase domain and was recently described as an active phosphatase in the *P. falciparum* blood stage parasites (Müller *et al.,* 2010). The authors show that PfGAP50 enters the secretory pathway to the parasite periphery and is subsequently engulfed into the food vacuole. In accordance with previous findings PfGAP50 was found predominantly intracellular in merozoites. These cells exhibit an IMC, which plays a fundamental role in reinforcement and the motility of the invasive plasmodial parasite stages. Similarly,

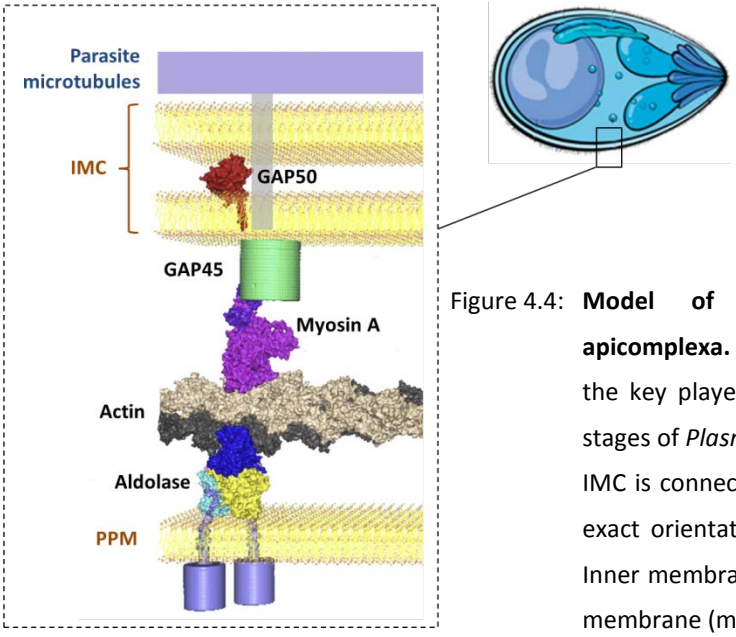


Figure 4.4: **Model of the invasion machinery of apicomplexa.** The schematic drawing represents the key players of the motor complex of motile stages of *Plasmodium* and other apicomplexa. The IMC is connected to the actin-myosin motor. The exact orientation of PfGAP50 is unknown. IMC - Inner membrane complex; PPM - Parasite plasma membrane (modified from Bosch *et al.,* 2012).

the IMC is an essential compartment for the development of the sexual stages of *Plasmodium* and seems to drive morphological changes during gametocytogenesis (Kono *et al.,* 2012). In asexual and gametocyte stages, the main part of PfGAP50 is in the IMC. During gametocyte maturation PfGAP45 disassociates from PfGAP50, and PfGAP50 directs towards the periphery of the parasite (Dearnley *et al.,* 2012). After gametocyte activation and following disintegration of the IMC during gametogenesis (Sologub *et al.,* 2011), though, PfGAP50 can be found on the macrogamete surface. During this emerging from the host-cell the IMC of gametocytes disintegrates and PfGAP50 turns up to the surface. In schizonts PfGAP50 displayed a 70 kDa and a full-length 46 kDa band, the 70 kDa band might be a dimer, or occurred due to unspecific binding of the antibody. In mature and activated gametocytes a protein band doublet was detected, indicating that beside the full-length protein a processed version of PfGAP50 is present in these stages. Bioinformatic analyses predict two transmembrane domains, one at the C-terminal end and one inside the N-terminal signal peptide. The predicted N-terminal transmembrane helix is in fact part of the signal peptide sequence, which is processed and cleaved of (Müller *et al.,* 2010; Yeoman *et al.,* 2011; Bosch *et al.,* 2012) during maturation of the protein. The conserved surface of PfGAP50, 80 % identity within apicomplexa (reviewed in Anantharaman *et al.,* 2007), suggests important transient interaction partners (Bosch *et al.,* 2012). The C-terminal transmembrane helix remains intact and serves as anchor to the IMC (Bosch *et al.,* 2012). Interestingly, Yeoman et al., 2011 reported that a mutant of *pfgap50,* lacking the C-terminal membrane anchor, is not directed to the IMC, but to the parasitophorous vacuole. One can hypothesize that the C-terminal peptide might be cleaved off during gametocyte activation, thereby removing the C-terminal transmembrane domain, which results in the re-localization of PfGAP50 to the parasite surface. There PfGAP50 might be associated to the plasma membrane by an unknown protein of the plasma membrane. Another possibility would be that the membranes of the IMC form loops, which relocate GAP50 to the surface of the parasite. Dearnley *et al.,* 2012 describes kind of looping extensions of the IMC in stage III gametocytes. Potentially a similar phenomenon occurs during gametogenesis, whereby the IMC disintegrates, like described in Sologub *et al.,* 2011. Thereby GAP50 is navigated to the surface and fuses with the PPM. Once PfGAP50 is exposed on the surface of the newly formed macrogametes, it functions as a FH and FHL-1 receptor (Fig. 4.5).

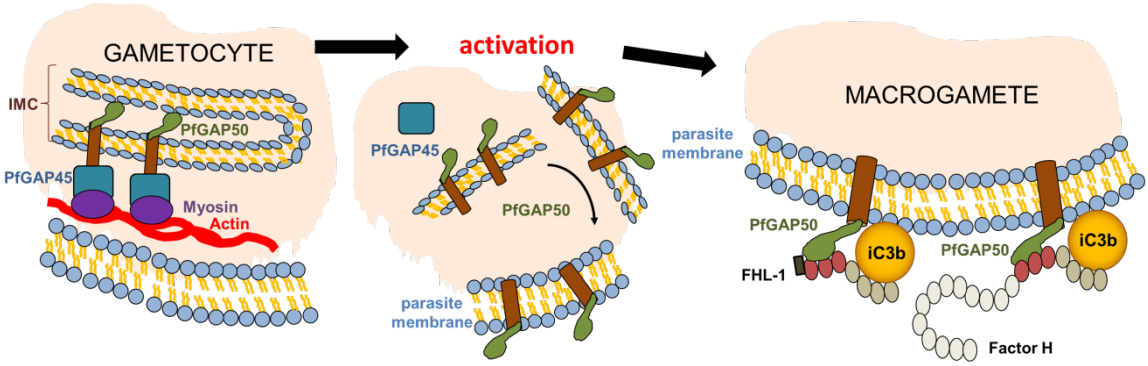


Figure 4.5: **Working Hypothesis: Navigation of PfGAP50 to the surface of macrogametes during gametogenesis in order to bind FH and FHL-1.** PfGAP50 is a transmembrane protein of the IMC in merozoites and gametocytes. During activation the IMC disintegrates and PfGAP50 is navigated to the surface of newly emerged macrogametes, potentially by loop formation of the IMC. After activation the human complement regulators FH and FHL-1 bind to C3b on the surface of *Plasmodium falciparum* via the membrane bound receptor PfGAP50. Thereby C3b becomes inactivated and the parasite is protected against the attack of the human complement system.

This doctoral thesis provides new insights into molecular interactions of the malaria parasite in the mosquito midgut. Extensive protein complexes were formed in the PV of gametocytes and coat the parasites surface after activation in the mosquito midgut. All proteins which are components of this protein complex are potential TBV candidates and probably most effective when the vaccine will be administered as a protein cocktail. Moreover it was elucidated how the parasite is able to escape from the attack of the human complement system in the mosquito midgut. Thereby the receptor protein on the surface of newly emerged gametes, which binds regulatory human complement factors, has been identified and promises to become a potential TBV candidate as well.

These recent findings elucidate several details about the sexual reproduction of the malaria parasite in the mosquito midgut and thereby offer additional opportunities to block the development of the parasite during this sensitive part of the life cycle. It is still desirable that the spread of the disease can be reduced enormously within the next years.

5 Future perspectives

In *P. falciparum* gametocyte development and gamete formation is accompanied by the co-ordinated expression of numerous sexual stage proteins, including the multi-adhesion domain PfCCp proteins, the EGF domain-containing protein Pfs25 and the cysteine motif-rich proteins Pfs230 and Pfs48/45. After activation, these proteins form complexes, which bind to the surface of newly emerged macrogametes. These proteins are potential candidates for components of a transmission blocking vaccine and future research continuously focuses on investigating the function of these proteins during the sexual development of the malaria parasite. Determining the role of PfCCp proteins is currently under investigation by studying PfCCp knock-out parasites. To pinpoint the phenotype of the different PfCCp mutants its survival through the transmission stages of the parasite in the mosquito vector will be studied by membrane feeding of mosquitoes with the mutant parasite line. Similarly the newly discovered WD40 domain-containing protein (PF14_0412) has to be further characterized. It may be an essential factor for protein complex assembly. Beside that the potential of PF14_0412 to bind PfActin II will be examined. The generation of a knock-out parasite line is currently in progress and antibodies against this WD40-protein were generated for further characterization of this protein. Localization, function and the potential connection of the WD40-protein to the multi-protein complex will be examined by IFAs and Co-IPs on parasite lysates. Despite this, the identification by Co-IPs of further proteins, which are members of the newly identified protein complex in gametocytes and on macrogametes, is included in future projects.

First studies on interactions between the malaria parasite and blood meal factors in the mosquito midgut revealed that the human complement regulators FH and FHL-1 bind to the surface of *P. falciparum* macrogametes to protect these cells against the attack of the human complement system. The receptor of FH and FHL-1 was identified as PfGAP50 and described to be a glideosome-associated protein of the inner membrane complex. Protein localization studies revealed surface associated localization of PfGAP50 on macrogametes. Moreover, the way how this protein is transported from the inner membrane complex to the surface of macrogametes will be examined by IFAs or by Immunoelectron microscopy. Approaches in the future would be blocking the complement binding receptors of malaria parasites by peptides

identical to selected SCR domains of FH. Such inhibitory peptides could be introduced to the vector by transgenic midgut bacteria, thus independent from the human.

In summary, research focus of future work will lie in understanding the molecular and cellular mechanisms of reproduction in the malaria parasite *P. falciparum* in the mosquito midgut. Proteins of the newly identified protein complex and PfGAP50 are potential candidates for Transmission Blocking Vaccines. Sexual stages of malaria parasites represent the least understood stages of the parasite's life cycle. Gaining insight into these mechanisms increases the probability to find effective candidates for eradication of the disease in the future.

6 Summary

The sexual phase of *Plasmodium falciparum* begins with the differentiation of intraerythrocytic sexual stages, termed gametocytes, in the human host. Mature gametocytes circulate in the peripheral blood and are taken up by the mosquito during the blood meal. These stages are essential for the spread of the malaria disease and form gametes in the mosquito midgut within minutes. A highly conserved family of six secreted proteins has been identified in *Plasmodium falciparum*. They comprise multiple adhesive domains and are termed PfCCp1 through PfCCp5, and PfFNPA. It was revealed in this work that PfCCp multi-domain adhesion proteins form protein complexes in gametocytes and on the surface of newly emerged macrogametes by adhesion domain-mediated binding. Co-Immunoprecipitation assays with activated gametocyte lysates show interactions between PfCCp proteins and indicate surface association via Pfs230 and Pfs25. Pfs230 is connected with the plasma membrane of the parasite by its interaction partner Pfs48/45. This protein is linked to the plasma membrane by a GPI anchor and presumably retains the multi-protein complex on the surface of newly emerged macrogametes in the mosquito midgut. A WD40 domain-containing protein was identified to be part of this protein complex. It might serve as platform for the assembly of the multi-protein complex or mediate the interplay among proteins, as suggested from known functions of the WD40 domain repeats.

During egress from the host erythrocyte, the emerging gametes become vulnerable to factors of the human complement, which is taken up with the blood meal. In this thesis it was found that the complement system is active for about one hour post feeding. Macrogametes defend against complement-mediated lysis by co-opting the human complement regulators Factor H and FHL-1 from the blood-meal. These serum proteins bind via its SCR domains 5-7 to the surface of macrogametes. Once bound, they trigger complement inactivation of the alternative pathway, which prevents induction of complement lysis on the surface of the malaria parasite. Antibodies against Factor H are able to impair the sexual development *in vitro* and are able to block transmission to the mosquito. Interaction studies on endogenous proteins and immobilized recombinant proteins revealed the PfGAP50 protein as binding partner of Factor H and FHL-1. This protein was hitherto described as a glideosome-associated protein in invasive parasite stages, but has not yet been characterized in gametes. First

localization studies indicate a relocation of PfGAP50 from the inner membrane complex to the surface of macrogametes.

Malaria still persists as one of the deadliest infectious diseases worldwide. Investigations on the essential transmissive stages, gametocytes and gametes of *Plasmodium falciparum*, stood in the background of research for a long time. This work deciphered details on protein interactions on the surface of the malaria parasite and provides first information about coactions between the parasite and the human complement in the mosquito midgut.

7 Zusammenfassung

Die Sexualphase von *Plasmodium falciparum* beginnt mit der Ausbildung von intraerythrozytären Sexualstadien, sogenannten Gametozyten, im menschlichen Wirt. Reife Gametozyten zirkulieren im peripheren Blut und werden während der Blutmahlzeit von der Mücke aufgenommen. Dieses Parasitenstadium ist ausschlaggebend für die Verbreitung von Malaria und bildet im Mückendarm innerhalb von Minuten Gameten. In *Plasmodium falciparum* wurde eine hochkonservierte Familie bestehend aus sechs sekretierten Proteinen entdeckt. Diese bestehen aus verschiedenen Adhäsionsdomänen und werden PfCCp1 bis PfCCp5 und PfFNPA genannt. In dieser Arbeit wurde gezeigt, dass PfCCp Multiadhäsionsproteine Komplexe in Gametozyten und auf der Oberfläche von jungen Makrogameten mittels domänenvermittelter Bindungen bilden. Ko-Immunpräzipitationen mit Lysat aus aktivierten Gametozyten zeigten oberflächenvermittelte Interaktionen der PfCCp Proteine durch Pfs230 und Pfs25. Pfs230 ist mit seinen Interaktionspartner Pfs48/45 durch einen GPI-Anker mit der Plasmamembran des Parasiten verbunden. Der Multi-Proteinkomplex wird somit auf der Oberfläche von jungen weiblichen Gameten festgehalten. Zudem wurde in dem neu identifizierten Proteinkomplex ein Protein entschlüsselt welches WD40-Domänen aufweist. Bereits bekannte Funktionen von sich wiederholenden WD40-Domänen lassen vermuten, dass dieses Protein möglicherweise als Plattform für den Zusammenbau des Proteinkomplexes dient oder das Wechselspiel zwischen Proteinen vermittelt.

Während des Ausbruchs aus der Wirtszelle, dem Erythrozyten, werden Gameten angreifbar für Faktoren des humanen Komplements, welches mit der Blutmahlzeit in den Mückendarm aufgenommen wird. In dieser Arbeit wurde ermittelt, dass das Komplementsystem nach der Blutmahlzeit etwa eine Stunde lang im Mückendarm aktiv ist. Durch die Bindung der Regulatoren Faktor H und FHL-1 des menschlichen Komplementsystems aus der Blutmahlzeit, schützen sich Makrogameten gegen eine komplementvermittelte Lyse. Diese Serumproteine binden mittels ihrer SCR-Domänen 5-7 an die Oberfläche von Makrogameten und vermitteln damit die Inaktivierung des alternativen Komplementweges. Dadurch schützen sie sich vor der komplementinduzierten Lyse auf der Oberfläche des Parasiten. Antikörper gegen

Faktor H vermindern die sexuelle Entwicklung *in vitro* und können die Weiterentwicklung des Erregers in der Mücke blockieren. Interaktionsstudien mit endogenen Proteinen und immoblilisierten rekombinanten Proteinen offenbarten PfGAP50 als Bindungspartner von Faktor H und FHL-1. PfGAP50 wurde bislang einem Motorkomplex zugeschrieben, welcher für die Parasitenbewegung von invasiven Stadien zuständig ist. Es wurde jedoch bis heute nicht in Gameten charakterisiert. Erste Lokalisationsstudien weisen auf eine Relokalisierung von PfGAP50 vom inneren Membrankomplex zur Oberfläche von Makrogameten hin.

Malaria ist weiterhin eine der tödlichsten Infektionskrankheiten weltweit. Die Erforschung dieser für die Übertragung essentiellen Stadien, den Gametozyten und Gameten von *Plasmodium falciparum*, stand lange im Hintergrund der Forschung. Diese Arbeit entschlüsselt Details über Proteininteraktionen auf der Oberfläche des Malariaparasiten und beschreibt das Zusammenwirken des Parasiten mit dem menschlichen Komplementsystem im Darm der Mücke.

8 References

Abu-Raddad LJ, Patnaik P, Kublin JG.
Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa.
Science. **2006** Dec 8; 314(5805):1603-6. Erratum in: Science. 2007 Feb 2; 315(5812):598.

Adisa A, Albano FR, Reeder J, Foley M, Tilley L.
Evidence for a role for a *Plasmodium falciparum* homologue of Sec31p in the export of proteins to the surface of malaria parasite-infected erythrocytes.
J Cell Sci. **2001** Sep; 114(Pt 18):3377-86.

Aikawa M, Carter R, Ito Y, Nijhout MM.
New observations on gametogenesis, fertilization, and zygote transformation in *Plasmodium gallinaceum.*
J Protozool. **1984** Aug; 31(3):403-13.

Alano P.
Plasmodium falciparum gametocytes: still many secrets of a hidden life.
Mol Microbiol. **2007** Oct; 66(2):291-302. Epub 2007 Sep 3. **Review.**

Alano P, Read D, Bruce M, Aikawa M, Kaido T, Tegoshi T, Bhatti S, Smith DK, Luo C, Hansra S, Carter R, Elliott JF.
COS cell expression cloning of *Pf*g377, a *Plasmodium falciparum* gametocyte antigen associated with osmiophilic bodies.
Mol Biochem Parasitol. **1995** Nov; 74(2):143-56.

Amdahl H, Jarva H, Haanperä M, Mertsola J, He Q, Jokiranta TS, Meri S.
Interactions between Bordetella pertussis and the complement inhibitor factor H.
Mol Immunol. **2011** Jan; 48(4):697-705. Epub 2010 Dec 16.

Aminake MN, Schoof S, Sologub L, Leubner M, Kirschner M, Arndt HD, Pradel G.
Thiostrepton and derivatives exhibit antimalarial and gametocytocidal activity by dually targeting parasite proteasome and apicoplast.
Antimicrob Agents Chemother. **2011** Apr; 55(4):1338-48. Epub 2011 Jan 18.

Amino R, Thiberge S, Shorte S, Frischknecht F, Ménard R.
Quantitative imaging of *Plasmodium* sporozoites in the mammalian host.
C R Biol. **2006** Nov; 329(11):858-62. Epub 2006 Aug 17.

Anantharaman V, Iyer LM, Aravind L.
Comparative genomics of protists: new insights into the evolution of eukaryotic signal transduction and gene regulation.
Annu Rev Microbiol. **2007**; 61:453-75. **Review.**

Areschoug T, Stålhammar-Carlemalm M, Karlsson I, Lindahl G.
Streptococcal beta protein has separate binding sites for human factor H and IgA-Fc.
J Biol Chem. **2002** Apr 12; 277(15):12642-8. Epub 2002 Jan 25.

Baker D, Sali A.
Protein structure prediction and structural genomics.
Science. **2001** Oct 5; 294(5540):93-6.

Baker DA, Daramola O, McCrossan MV, Harmer J, Targett GA.
Subcellular localization of Pfs16, a *Plasmodium falciparum* gametocyte antigen.
Parasitology. **1994** Feb; 108 (Pt 2):129-37.

Barilla-LaBarca ML, Liszewski MK, Lambris JD, Hourcade D, Atkinson JP.
Role of membrane cofactor protein (CD46) in regulation of C4b and C3b deposited on cells.
J Immunol. **2002** Jun 15; 168(12):6298-304.

Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC.
Recombinant *Pfs*25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals.
J Exp Med. **1991** Nov 1; 174(5):1203-8.

Baum J, Papenfuss AT, Baum B, Speed TP, Cowman AF.
Regulation of apicomplexan actin-based motility.
Nat Rev Microbiol**. 2006** Aug; 4(8):621-8. **Review**

Becker CA, Malandrin L, Depoix D, Larcher T, David PH, Chauvin A, Bischoff E, Bonnet S.
Identification of three CCp genes in Babesia divergens: novel markers for sexual stages parasites.
Mol Biochem Parasitol. **2010** Nov; 174(1):36-43. Epub 2010 Jul 21.

Bhide MR, Escudero R, Camafeita E, Gil H, Jado I, Anda P.
Complement factor H binding by different Lyme disease and relapsing fever *Borrelia* in animals and human.
BMC Res Notes. **2009** Jul 15; 2:134.

Billingsley PF, Hecker H.
Blood digestion in the mosquito, Anopheles stephensi Liston (Diptera: Culicidae): activity and distribution of trypsin, aminopeptidase, and alpha-glucosidase in the midgut.
J Med Entomol. **1991** Nov; 28(6):865-71.

Billker O, Shaw MK, Margos G, Sinden RE.
The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghei* in vitro.
Parasitology. **1997** Jul; 115 (Pt 1):1-7.

Billker O, Lindo V, Panico M, Etienne AE, Paxton T, Dell A, Rogers M, Sinden RE, Morris HR.
Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito.
Nature. **1998** Mar 19; 392(6673):289-92.

Blackman MJ, Ling IT, Nicholls SC, Holder AA.
Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains.
Mol Biochem Parasitol. **1991** Nov; 49(1):29-33.

Blackman MJ, Holder AA.
Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1.
Mol Biochem Parasitol. **1992** Feb; 50(2):307-15.

Blackmore TK, Fischetti VA, Sadlon TA, Ward HM, Gordon DL.
M protein of the group A Streptococcus binds to the seventh short consensus repeat of human complement factor H.
Infect Immun. **1998** Apr; 66(4):1427-31.

Bosch J, Paige MH, Vaidya A, Bergman L, Hol WG.
Crystal structure of GAP50, the anchor of the invasion machinery in the inner membrane complex of *Plasmodium falciparum*.
J Struct Biol. **2012** Feb 22. [Epub ahead of print]

Bousema T, Roeffen W, Meijerink H, Mwerinde H, Mwakalinga S, van Gemert GJ, van de Vegte-Bolmer M, Mosha F, Targett G, Riley EM, Sauerwein R, Drakeley C.
The dynamics of naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens *Pf*s230 & *Pf*s48/45 in a low endemic area in Tanzania.
PLoS One. **2010** Nov 29; 5(11):e14114.

Bruce MC, Alano P, Duthie S, Carter R.
Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development.
Parasitology. **1990** Apr; 100 Pt 2:191-200.

Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K.
Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria.
Nat Med. **1998** Mar; 4(3):358-60.

Carter R, Mendis KN, Miller LH, Molineaux L, Saul A.
Malaria transmission-blocking vaccines--how can their development be supported?
Nat Med. **2000** Mar; 6(3):241-4. **Review**.

Carter R, Miller LH
Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture.
Bull World Health Organ. **1979**; 57 Suppl 1:37-52.

Carter V, Shimizu S, Arai M, Dessens JT.
*Pb*SR is synthesized in macrogametocytes and involved in formation of the malaria crystalloids.
Mol Microbiol. **2008** Jun; 68(6):1560-9. Epub 2008 Apr 29.

Chaparro-Olaya J, Margos G, Coles DJ, Dluzewski AR, Mitchell GH, Wasserman MM, Pinder JC.
Plasmodium falciparum myosins: transcription and translation during asexual parasite development.
Cell Motil Cytoskeleton. **2005** Apr; 60(4):200-13.

Chen F, Mackey AJ, Stoeckert CJ Jr, Roos DS.
OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups.
Nucleic Acids Res. **2006** Jan 1; 34(Database issue):D363-8.

Cooper JA, Ingram LT, Bushell GR, Fardoulys CA, Stenzel D, Schofield L, Saul AJ.
The 140/130/105 kilodalton protein complex in the rhoptries of Plasmodium falciparum consists of discrete polypeptides.
Mol Biochem Parasitol. **1988** Jun; 29(2-3):251-60.

Coutinho-Abreu IV, Ramalho-Ortigao M.
Transmission blocking vaccines to control insect-borne diseases: a review.
Mem Inst Oswaldo Cruz. **2010** Feb; 105(1):1-12. **Review**.

Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B.
Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening.
Clin Infect Dis. **2008** Jan 15; 46(2):165-71.

Dave S, Brooks-Walter A, Pangburn MK, McDaniel LS.
PspC, a pneumococcal surface protein, binds human factor H.
Infect Immun. **2001** May; 69(5):3435-7.

Day KP, Hayward RE, Smith D, Culvenor JG.
CD36-dependent adhesion and knob expression of the transmission stages of *Plasmodium falciparum* is stage specific.
Mol Biochem Parasitol. **1998** Jun 1; 93(2):167-77.

Dean DC, Bowlus CL, Bourgeois S.
Cloning and analysis of the promotor region of the human fibronectin gene.
Proc Natl Acad Sci U S A. **1987** Apr; 84(7):1876-80.

Dearnley MK, Yeoman JA, Hanssen E, Kenny S, Turnbull L, Whitchurch CB, Tilley L, Dixon MW.
Origin, composition, organization and function of the inner membrane complex of *Plasmodium falciparum* gametocytes.
J Cell Sci. **2012** Feb 10. [Epub ahead of print]

Degn SE, Thiel S, Jensenius JC.
New perspectives on mannan-binding lectin-mediated complement activation.
Immunobiology. **2007**; 212(4-5):301-11. Epub 2007 Jan 22. **Review.**

Deligianni E, Morgan RN, Bertuccini L, Kooij TW, Laforge A, Nahar C, Poulakakis N, Schüler H, Louis C, Matuschewski K, Siden-Kiamos I.
Critical role for a stage-specific actin in male exflagellation of the malaria parasite.
Cell Microbiol. **2011** Nov; 13(11):1714-30. doi: 10.1111/j.1462-5822.2011.01652.x. Epub 2011 Aug 11.

Delrieu I, Waller CC, Mota MM, Grainger M, Langhorne J, Holder AA.
*P*SLAP, a protein with multiple adhesive motifs, is expressed in *Plasmodium falciparum* gametocytes.
Mol Biochem Parasitol. **2002** Apr 30; 121(1):11-20.

Delvaeye M, Noris M, De Vriese A, Esmon CT, Esmon NL, Ferrell G, Del-Favero J, Plaisance S, Claes B, Lambrechts D, Zoja C, Remuzzi G, Conway EM.
Thrombomodulin mutations in atypical hemolytic-uremic syndrome.
N Engl J Med. **2009** Jul 23; 361(4):345-57.

Dessens JT, Sinden RE, Claudianos C.
LCCL proteins of apicomplexan parasites.
Trends Parasitol. **2004** Mar; 20(3):102-8. **Review**.

Dessens JT, Saeed S, Tremp AZ, Carter V.
Malaria crystalloids: specialized structures for parasite transmission?
Trends Parasitol. **2011** Mar; 27(3):106-10. Epub 2011 Jan 13.

Díaz A, Ferreira A, Sim RB.
Complement evasion by E*chinococcus granulosus*: sequestration of host factor H in the hydatid cyst wall.
J Immunol. **1997** Apr 15; 158(8):3779-86.

Dude MA Dissertation
The expression of the multi-adhesiondomain containing proteins PfCCp5 and PfFNPA during the life cycle of *Plasmopdium falciparum* and cysteine proetease inhibitors as putative agents against malaria **2010**

Duffy PE, Kaslow DC.
A novel malaria protein, *Pfs*28, and *Pfs*25 are genetically linked and synergistic as *falciparum* malaria transmission-blocking vaccines.
Infect Immun. **1997** Mar; 65(3):1109-13.

Duthy TG, Ormsby RJ, Giannakis E, Ogunniyi AD, Stroeher UH, Paton JC, Gordon DL.
The human complement regulator factor H binds pneumococcal surface protein PspC via short consensus repeats 13 to 15.
Infect Immun. **2002** Oct; 70(10):5604-11.

Eksi S, Czesny B, van Gemert GJ, Sauerwein RW, Eling W, Williamson KC.
Malaria transmission-blocking antigen, *Pfs*230, mediates human red blood cell binding to exflagellating male parasites and oocyst production.
Mol Microbiol. **2006** Aug; 61(4):991-8.

Eksi S, Williamson KC.
Protein targeting to the Parasitophorous vacuole membrane *of Plasmodium falciparum*.
Eukaryot Cell. **2011** Jun; 10(6):744-52. Epub 2011 Apr 15.

Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, Blundell TL, Pepys MB, Wood SP.
Structure of pentameric human serum amyloid P component.
Nature. **1994** Jan 27; 367(6461):338-45.

Estaller C, Schwaeble W, Dierich M, Weiss EH.
Human complement factor H: two factor H proteins are derived from alternatively spliced transcripts.
Eur J Immunol. **1991** Mar; 21(3):799-802.

Farrance CE, Chichester JA, Musiychuk K, Shamloul M, Rhee A, Manceva SD, Jones RM, Mamedov T, Sharma S, Mett V, Streatfield SJ, Roeffen W, van de Vegte-Bolmer M, Sauerwein RW, Wu Y, Muratova O, Miller L, Duffy P, Sinden R, Yusibov V.
Antibodies to plant-produced *Plasmodium falciparum* sexual stage protein Pfs25 exhibit transmission blocking activity.
Hum Vaccin. 2011 Jan-Feb; 7 Suppl:191-8. Epub 2011 Jan 1. **(a)**

Farrance CE, Rhee A, Jones RM, Musiychuk K, Shamloul M, Sharma S, Mett V, Chichester JA, Streatfield SJ, Roeffen W, van de Vegte-Bolmer M, Sauerwein RW, Tsuboi T, Muratova OV, Wu Y, Yusibov V.
A plant-produced Pfs230 vaccine candidate blocks transmission of *Plasmodium falciparum*.
Clin Vaccine Immunol. 2011 Aug; 18(8):1351-7. Epub 2011 Jun 29. **(b)**

Fitch WM.
Distinguishing homologous from analogous proteins.
Syst Zool. **1970** Jun; 19(2):99-113.

Fivelman QL, McRobert L, Sharp S, Taylor CJ, Saeed M, Swales CA, Sutherland CJ, Baker DA.
Improved synchronous production of *Plasmodium falciparum* gametocytes in vitro.
Mol Biochem Parasitol. **2007** Jul; 154(1):119-23. Epub 2007 Apr 20.

Foth BJ, Goedecke MC, Soldati D.
New insights into myosin evolution and classification.
Proc Natl Acad Sci U S A. **2006** Mar 7; 103(10):3681-6. Epub 2006 Feb 27.

Francischetti IM, Valenzuela JG, Pham VM, Garfield MK, Ribeiro JM.
Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector Anopheles gambiae.
J Exp Biol. **2002** Aug;205(Pt 16):2429-51.

Frénal K, Polonais V, Marq JB, Stratmann R, Limenitakis J, Soldati-Favre D.
Functional dissection of the apicomplexan glideosome molecular architecture.
Cell Host Microbe. **2010** Oct 21; 8(4):343-57.

Friberg N, Carlson P, Kentala E, Mattila PS, Kuusela P, Meri S, Jarva H.
Factor H binding as a complement evasion mechanism for an anaerobic pathogen, *Fusobacterium necrophorum.*
J Immunol. **2008** Dec 15; 181(12):8624-32.

Fujita T.
Evolution of the lectin-complement pathway and its role in innate immunity.
Nat Rev Immunol. **2002** May; 2(5):346-53. **Review.**

Furuya T, Mu J, Hayton K, Liu A, Duan J, Nkrumah L, Joy DA, Fidock DA, Fujioka H, Vaidya AB, Wellems TE, Su XZ.
Disruption of a *Plasmodium falciparum* gene linked to male sexual development causes early arrest in gametocytogenesis.
Proc Natl Acad Sci U S A. **2005** Nov 15; 102(46):16813-8. Epub 2005 Nov 7. Erratum in: Proc Natl Acad Sci U S A. 2006 Jan 10; 103(2):504.

Ganter M, Schüler H, Matuschewski K.
Vital role for the *Plasmodium* actin capping protein (CP) beta-subunit in motility of malaria sporozoites.
Mol Microbiol. **2009** Dec; 74(6):1356-67. Epub 2009 Aug 4.

Garcia GE, Wirtz RA, Barr JR, Woolfitt A, Rosenberg R.
Xanthurenic acid induces gametogenesis in *Plasmodium*, the malaria parasite.
J Biol Chem. **1998** May 15; 273(20):12003-5.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B.
Genome sequence of the human malaria parasite *Plasmodium falciparum*.
Nature. **2002** Oct 3; 419(6906):498-511.

Gewurz H, Zhang XH, Lint TF.
Structure and function of the pentraxins.
Curr Opin Immunol. **1995** Feb; 7(1):54-64. **Review**.

Ghosh AK, Jacobs-Lorena M.
Plasmodium sporozoite invasion of the mosquito salivary gland.
Curr Opin Microbiol. **2009** Aug; 12(4):394-400. Epub 2009 Jul 14. **Review**.

Giannakis E, Jokiranta TS, Ormsby RJ, Duthy TG, Male DA, Christiansen D, Fischetti VA, Bagley C, Loveland BE, Gordon DL.
Identification of the streptococcal M protein binding site on membrane cofactor protein (CD46).
J Immunol. **2002** May 1; 168(9):4585-92.

Gozar MM, Price VL, Kaslow DC.
Saccharomyces cerevisiae-secreted fusion proteins *Pfs*25 and *Pfs*28 elicit potent *Plasmodium falciparum* transmission-blocking antibodies in mice.
Infect Immun. **1998** Jan; 66(1):59-64.

Grotendorst CA, Carter R, Rosenberg R, Koontz LC.
Complement effects on the infectivity of *Plasmodium gallinaceum* to Aedes aegypti mosquitoes. I. Resistance of zygotes to the alternative pathway of complement.
J Immunol. **1986** Jun 1; 136(11):4270-4.

Grotendorst CA, Carter R.
Complement effects of the infectivity of *Plasmodium gallinaceum* to *Aedes aegypti* mosquitoes. II. Changes in sensitivity to complement-like factors during zygote development.
J Parasitol. **1987** Oct; 73(5):980-4.

Haupt K, Kraiczy P, Wallich R, Brade V, Skerka C, Zipfel PF.
Binding of human factor H-related protein 1 to serum-resistant Borrelia burgdorferi is mediated by borrelial complement regulator-acquiring surface proteins.
J Infect Dis. **2007** Jul 1; 196(1):124-33. Epub 2007 May 23.

Haupt K, Reuter M, van den Elsen J, Burman J, Hälbich S, Richter J, Skerka C, Zipfel PF.
The *Staphylococcus aureus* protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b.
PLoS Pathog. **2008** Dec; 4(12):e1000250. Epub 2008 Dec 26.

Hawking F, Wilson ME, Gammage K.
Evidence for cyclic development and short-lived maturity in the gametocytes *of Plasmodium falciparum.*
Trans R Soc Trop Med Hyg. **1971**; 65(5):549-59.

Hay SI, Cox J, Rogers DJ, Randolph SE, Stern DI, Shanks GD, Myers MF, Snow RW.
Climate change and the resurgence of malaria in the East African highlands.
Nature. **2002** Feb 21; 415(6874):905-9.

Healer J, McGuinness D, Hopcroft P, Haley S, Carter R, Riley E.
Complement-mediated lysis of *Plasmodium falciparum* gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs230.
Infect Immun. **1997** Aug; 65(8):3017-23.

Hellwage J, Meri T, Heikkilä T, Alitalo A, Panelius J, Lahdenne P, Seppälä IJ, Meri S.
The complement regulator factor H binds to the surface protein OspE of *Borrelia burgdorferi*.
J Biol Chem. **2001** Mar 16; 276(11):8427-35. Epub 2000 Dec 11.

Heussler V, Doerig C.
In vivo imaging enters parasitology.
Trends Parasitol. **2006** May; 22(5):192-5; discussion 195-6. Epub 2006 Mar 20. **Review**.

Hill AV.
Pre-erythrocytic malaria vaccines: towards greater efficacy.
Nat Rev Immunol. **2006** Jan; 6(1):21-32. **Review**.

Hisaeda H, Stowers AW, Tsuboi T, Collins WE, Sattabongkot JS, Suwanabun N, Torii M, Kaslow DC.
Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes.
Infect Immun. **2000** Dec; 68(12):6618-23.

Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA.
Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H.
Proc Natl Acad Sci U S A. **1988** Mar; 85(5):1657-61.

Houk EJ, Hardy JL.
Midgut cellular responses to bloodmeal digestion in the mosquito, Culex tarsalis coquillett.
Int J Insect Morphol and Embryol **1982**; 11 (2): 109-119

Jahan N, Docherty PT, Billingsley PF, Hurd H.
Blood digestion in the mosquito, *Anopheles stephensi*: the effects of *Plasmodium yoelii* nigeriensis on midgut enzyme activities.
Parasitology. **1999** Dec; 119 (Pt 6):535-41.

Janse CJ, van der Klooster PF, van der Kaay HJ, van der Ploeg M, Overdulve JP.
DNA synthesis in *Plasmodium berghei* during asexual and sexual development.
Mol Biochem Parasitol. **1986** Aug; 20(2):173-82. **(a)**

Janse CJ, Van der Klooster PF, Van der Kaay HJ, Van der Ploeg M, Overdulve JP.
Rapid repeated DNA replication during microgametogenesis and DNA synthesis in young zygotes of *Plasmodium berghei*.
Trans R Soc Trop Med Hyg. **1986**; 80(1):154-7. **(b)**

Janssen BJ, Christodoulidou A, McCarthy A, Lambris JD, Gros P.
Structure of C3b reveals conformational changes that underlie complement activity.
Nature. **2006** Nov 9; 444(7116):213-6. Epub 2006 Oct 15.

Joiner K, Sher A, Gaither T, Hammer C.
Evasion of alternative complement pathway by Trypanosoma cruzi results from inefficient binding of factor B.
Proc Natl Acad Sci U S A. **1986** Sep; 83(17):6593-7.

Jokiranta TS, Hellwage J, Koistinen V, Zipfel PF, Meri S.
Each of the three binding sites on complement factor H interacts with a distinct site on C3b.
J Biol Chem. **2000** Sep 8; 275(36):27657-62.

Jokiranta TS, Zipfel PF, Hakulinen J, Kühn S, Pangburn MK, Tamerius JD, Meri S.
Analysis of the recognition mechanism of the alternative pathway of complement by monoclonal anti-factor H antibodies: evidence for multiple interactions between H and surface bound C3b.
FEBS Lett. **1996** Sep 16; 393(2-3):297-302.

Józsi M, Zipfel PF.
Factor H family proteins and human diseases.
Trends Immunol. 2008 Aug;29(8):380-7. Epub 2008 Jul 2. **Review.**

Kariuki MM, Kiaira JK, Mulaa FK, Mwangi JK, Wasunna MK, Martin SK.
Plasmodium falciparum: purification of the various gametocyte developmental stages from in vitro-cultivated parasites.
Am J Trop Med Hyg. **1998** Oct; 59(4):505-8.

Kaslow DC.
Transmission-blocking vaccines: uses and current status of development.
Int J Parasitol. **1997** Feb; 27(2):183-9. **Review**.

Kaslow DC, Quakyi IA, Syin C, Raum MG, Keister DB, Coligan JE, McCutchan TF, Miller LH.
A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains.
Nature. **1988** May 5; 333(6168):74-6.

Kaslow DC, Isaacs SN, Quakyi IA, Gwadz RW, Moss B, Keister DB.Induction of *Plasmodium falciparum* transmission-blocking antibodies by recombinant V*accinia* virus.
Science. **1991** May 31; 252(5010):1310-3.

Kauth CW, Epp C, Bujard H, Lutz R.
The merozoite surface protein 1 complex of human malaria parasite Plasmodium falciparum: interactions and arrangements of subunits.
J Biol Chem. **2003** Jun 20;278(25):22257-64. Epub 2003 Mar 24.

Kennedy MW, Kuo YM.
The surfaces of the parasitic nematodes Trichinella spiralis and Toxocara canis differ in the binding of post-C3 components of human complement by the alternative pathway.
Parasite Immunol. **1988** Jul; 10(4):459-63.

Kessler S, Guerin PM.
Responses of *Anopheles gambiae*, *Anopheles stephensi, Aedes aegypti*, and *Culex pipiens* mosquitoes (Diptera: Culicidae) to cool and humid refugium conditions.
J Vector Ecol. **2008** Jun; 33(1):145-9.

Khan SM, Franke-Fayard B, Mair GR, Lasonder E, Janse CJ, Mann M, Waters AP.
Proteome analysis of separated male and female gametocytes reveals novel sex-specific *Plasmodium* biology.
Cell. **2005** Jun 3; 121(5):675-87.

Kongkasuriyachai D, Fujioka H, Kumar N.
Functional analysis of *Plasmodium falciparum* parasitophorous vacuole membrane protein (*Pf*s16) during gametocytogenesis and gametogenesis by targeted gene disruption.
Mol Biochem Parasitol. **2004** Feb; 133(2):275-85.

Koning-Ward de TF, Olivieri A, Bertuccini L, Hood A, Silvestrini F, Charvalias K, Berzosa Díaz P, Camarda G, McElwain TF, Papenfuss T, Healer J, Baldassarri L, Crabb BS, Alano P, Ranford-Cartwright LC.
The role of osmiophilic bodies and *Pf*g377 expression in female gametocyte emergence and mosquito infectivity in the human malaria parasite *Plasmodium falciparum*.
Mol Microbiol. **2008** Jan; 67(2):278-90. Epub 2007 Dec 11.

Kraiczy P, Würzner R.
Complement escape of human pathogenic bacteria by acquisition of complement regulators.
Mol Immunol. **2006** Jan; 43(1-2):31-44. **Review**.

Kraiczy P, Hellwage J, Skerka C, Becker H, Kirschfink M, Simon MM, Brade V, Zipfel PF, Wallich R.
Complement resistance of *Borrelia burgdorferi* correlates with the expression of *Bb*CRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins.
J Biol Chem. **2004** Jan 23; 279(4):2421-9. Epub 2003 Nov 7.

Kuehn A, Pradel G.
The coming-out of malaria gametocytes.
J Biomed Biotechnol. **2010**; 2010:976827. Epub 2010 Jan 5. **Review**.

Kühn S, Skerka C, Zipfel PF.
Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1.
J Immunol. **1995** Dec 15; 155(12):5663-70.

Kumar N.
Target antigens of malaria transmission blocking immunity exist as a stable membrane bound complex.
Parasite Immunol. **1987** May; 9(3):321-35.

Kumar N, Wizel B.
Further characterization of interactions between gamete surface antigens of *Plasmodium falciparum*.
Mol Biochem Parasitol. **1992** Jul; 53(1-2):113-20.

Kunert A, Losse J, Gruszin C, Hühn M, Kaendler K, Mikkat S, Volke D, Hoffmann R, Jokiranta TS, Seeberger H, Moellmann U, Hellwage J, Zipfel PF.
Immune evasion of the human pathogen Pseudomonas aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein.
J Immunol. **2007** Sep 1; 179(5):2979-88.

Lachmann PJ.
Microbial subversion of the immune response.
Proc Natl Acad Sci U S A. **2002** Jun 25; 99(13):8461-2. Epub 2002 Jun 19.

Lal K, Delves MJ, Bromley E, Wastling JM, Tomley FM, Sinden RE.
Plasmodium male development gene-1 (mdv-1) is important for female sexual development and identifies a polarised plasma membrane during zygote development.
Int J Parasitol. 2009 Jun; 39(7):755-61. Epub 2008 Dec 24.

Lambris JD, Ricklin D, Geisbrecht BV.
Complement evasion by human pathogens.
Nat Rev Microbiol. **2008** Feb; 6(2):132-42. **Review**.

Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, Sauerwein RW, Eling WM, Hall N, Waters AP, Stunnenberg HG, Mann M.
Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry.
Nature. **2002** Oct 3; 419(6906):537-42.

Lauer N, Mihlan M, Hartmann A, Schlötzer-Schrehardt U, Keilhauer C, Scholl HP, Charbel Issa P, Holz F, Weber BH, Skerka C, Zipfel PF.
Complement regulation at necrotic cell lesions is impaired by the age-related macular degeneration-associated factor-H His402 risk variant.
J Immunol. 2011 Oct 15; 187(8):4374-83. Epub **2011** Sep 19.

Law, S. K. A. and Reid, K.B.M.
Complement 2nd edition
Oxford Univ. Press **1995.** (ISBN 0199633568)

Lensen A, Bril A, van de Vegte M, van Gemert GJ, Eling W, Sauerwein R.
Plasmodium falciparum: infectivity of cultured, synchronized gametocytes to mosquitoes.
Exp Parasitol. **1999** Jan; 91(1):101-3.

Li D, Roberts R.
WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases.
Cell Mol Life Sci. **2001** Dec; 58(14):2085-97. **Review**.

Liepinsh E, Trexler M, Kaikkonen A, Weigelt J, Bányai L, Patthy L, Otting G.
NMR structure of the LCCL domain and implications for DFNA9 deafness disorder.
EMBO J. **2001** Oct 1; 20(19):5347-53.

Liszewski MK, Farries TC, Lublin DM, Rooney IA, Atkinson JP.
Control of the complement system.
Adv Immunol. **1996**; 61:201-83. **Review**.

Lobo CA, Kumar N.
Sexual differentiation and development in the malaria parasite.
Parasitol Today. **1998** Apr; 14(4):146-50.

Loos M, Colomb M.
C1, the first component of complement: structure-function-relationship of C1q and collectins (MBP, SP-A, SP-D, conglutinin), C1-esterases (C1r and C1s), and C1-inhibitor in health and disease.
Behring Inst Mitt. **1993** Dec; (93):1-5. **Review**.

Ludin P, Nilsson D, Mäser P.
Genome-wide identification of molecular mimicry candidates in parasites.
PLoS One. **2011** Mar 8; 6(3):e17546.

Maina CV, Riggs PD, Grandea AG 3rd, Slatko BE, Moran LS, Tagliamonte JA, McReynolds LA, Guan CD.
An Escherichia coli vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein.
Gene. **1988** Dec 30; 74(2):365-73.

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH.
CDD: a Conserved Domain Database for the functional annotation of proteins.
Nucleic Acids Res. **2011** Jan; 39(Database issue):D225-9. Epub 2010 Nov 24.

Margos G, Navarette S, Butcher G, Davies A, Willers C, Sinden RE, Lachmann PJ.
Interaction between host complement and mosquito-midgut-stage *Plasmodium berghei.*
Infect Immun. **2001** Aug; 69(8):5064-71.

Marsh K, Otoo L, Hayes RJ, Carson DC, Greenwood BM.
Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection.
Trans R Soc Trop Med Hyg. **1989** May-Jun; 83(3):293-303.

Meri S, Jarva H.
Complement Regulatory Proteins
Encyclopedia of Life Sciences/**2001** Nature Publishing group

Meri T, Jokiranta TS, Hellwage J, Bialonski A, Zipfel PF, Meri S.
Onchocerca volvulus microfilariae avoid complement attack by direct binding of factor H.
J Infect Dis**. 2002** Jun 15; 185(12):1786-93. Epub 2002 May 31. (a)

Meri T, Hartmann A, Lenk D, Eck R, Würzner R, Hellwage J, Meri S, Zipfel PF.
The yeast *Candida albicans* binds complement regulators factor H and FHL-1.
Infect Immun. **2002** Sep; 70(9):5185-92. (b)

Mihlan M, Stippa S, Józsi M, Zipfel PF.
Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H.
Cell Death Differ. **2009** Dec; 16(12):1630-40. Epub 2009 Aug 14.

Mihlan M, Blom AM, Kupreishvili K, Lauer N, Stelzner K, Bergström F, Niessen HW, Zipfel PF.
Monomeric C-reactive protein modulates classic complement activation on necrotic cells.
FASEB J. 2011 Dec; 25(12):4198-210. Epub **2011** Aug 19.

Milek RL, Stunnenberg HG, Konings RN.
Assembly and expression of a synthetic gene encoding the antigen *Pf*s48/45 of the human malaria parasite *Plasmodium falciparum* in yeast.
Vaccine. **2000** Jan 31; 18(14):1402-11.

Miyata T, Harakuni T, Sugawa H, Sattabongkot J, Kato A, Tachibana M, Torii M, Tsuboi T, Arakawa T.
Adenovirus-vectored *Plasmodium vivax* ookinete surface protein, Pvs25, as a potential transmission-blocking vaccine.
Vaccine. 2011 Mar 24; 29(15):2720-6. Epub 2011 Feb 11.

Moelans II, Klaassen CH, Kaslow DC, Konings RN, Schoenmakers JG.
Minimal variation in Pfs16, a novel protein located in the membrane of gametes and sporozoites of *Plasmodium falciparum*.
Mol Biochem Parasitol. **1991** Jun; 46(2):311-3.

Mohri K, Vorobiev S, Fedorov AA, Almo SC, Ono S.
Identification of functional residues on *Caenorhabditis elegans* actin-interacting protein 1 (UNC-78) for disassembly of actin depolymerizing factor/cofilin-bound actin filaments.
J Biol Chem. **2004** Jul 23; 279(30):31697-707. Epub 2004 May 18.

Mollnes TE, Garred P, Bergseth G.
Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation.
Clin Exp Immunol. **1988** Sep; 73(3):484-8.

Moore RB, Oborník M, Janouskovec J, Chrudimský T, Vancová M, Green DH, Wright SW, Davies NW, Bolch CJ, Heimann K, Slapeta J, Hoegh-Guldberg O, Logsdon JM, Carter DA.
A photosynthetic alveolate closely related to apicomplexan parasites.
Nature. **2008** Feb 21; 451(7181):959-63. Erratum in: Nature. 2008 Apr 17; 452(7189):900.

Mota MM, Rodriguez A.
Migration through host cells by apicomplexan parasites.
Microbes Infect. **2001** Nov; 3(13):1123-8. **Review**.

Mota MM, Pradel G, Vanderberg JP, Hafalla JC, Frevert U, Nussenzweig RS, Nussenzweig V, Rodríguez A.
Migration of Plasmodium sporozoites through cells before infection.
Science. 2001 Jan 5;291(5501):141-4.

Müller IB, Knöckel J, Eschbach ML, Bergmann B, Walter RD, Wrenger C.
Secretion of an acid phosphatase provides a possible mechanism to acquire host nutrients by *Plasmodium falciparum*.
Cell Microbiol. **2010** May 1; 12(5):677-91. Epub 2010 Jan 11.

Okamoto N, Spurck TP, Goodman CD, McFadden GI.
Apicoplast and mitochondrion in gametocytogenesis of *Plasmodium falciparum*.
Eukaryot Cell. **2009** Jan; 8(1):128-32. Epub 2008 Nov 7.

Ollert MW, David K, Bredehorst R, Vogel CW.
Classical complement pathway activation on nucleated cells. Role of factor H in the control of deposited C3b.
J Immunol. **1995** Nov 15; 155(10):4955-62.

Ong CS, Zhang KY, Eida SJ, Graves PM, Dow C, Looker M, Rogers NC, Chiodini PL, Targett GA.
The primary antibody response of malaria patients to *Plasmodium falciparum* sexual stage antigens which are potential transmission blocking vaccine candidates.
Parasite Immunol. **1990** Sep; 12(5):447-56.

Ouédraogo AL, Roeffen W, Luty AJ, de Vlas SJ, Nebie I, Ilboudo-Sanogo E, Cuzin-Ouattara N, Teleen K, Tiono AB, Sirima SB, Verhave JP, Bousema T, Sauerwein R.
Naturally acquired immune responses to *Plasmodium falciparum* sexual stage antigens Pfs48/45 and Pfs230 in an area of seasonal transmission.
Infect Immun. **2011** Dec; 79(12):4957-64. Epub 2011 Oct 3.

Pain A, Hertz-Fowler C.
Plasmodium genomics: latest milestone.
Nat Rev Microbiol. **2009** Mar; 7(3):180-1.

Pangburn MK, Schreiber RD, Müller-Eberhard HJ.
Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution.
J Exp Med. **1977** Jul 1; 146(1):257-70.

Pasvol G.
Protective hemoglobinopathies and *Plasmodium falciparum* transmission.
Nat Genet. **2010** Apr; 42(4):284-5.

Paul RE, Brey PT, Robert V.
Plasmodium sex determination and transmission to mosquitoes.
Trends Parasitol. **2002** Jan; 18(1):32-8. **Review**.

Ponnudurai T, Meuwissen JH, Leeuwenberg AD, Verhave JP, Lensen AH.
The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitoes.
Trans R Soc Trop Med Hyg. **1982**; 76(2):242-50.

Pradel G.
Proteins of the malaria parasite sexual stages: expression, function and potential for transmission blocking strategies.
Parasitology. **2007** Dec; 134(Pt.14):1911-29. Epub 2007 Aug 23. **Review**.

Pradel G, Frevert U.
Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion.
Hepatology. **2001** May; 33(5):1154-65.

Pradel G, Hayton K, Aravind L, Iyer LM, Abrahamsen MS, Bonawitz A, Mejia C, Templeton TJ.
A multidomain adhesion protein family expressed in *Plasmodium falciparum* is essential for transmission to the mosquito.
J Exp Med. **2004** Jun 7; 199(11):1533-44.

Pradel G, Wagner C, Mejia C, Templeton TJ.
Plasmodium falciparum: Co-dependent expression and co-localization of the *Pf*CCp multi-adhesion domain proteins.
Exp Parasitol. **2006** Apr; 112(4):263-8. Epub 2006 Jan 4.

Prodinger WM, Hellwage J, Spruth M, Dierich MP, Zipfel PF.
The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins.
Biochem J. **1998** Apr 1; 331 (Pt 1):41-7.

Quakyi IA, Carter R, Rener J, Kumar N, Good MF, Miller LH.
The 230-kDa gamete surface protein of *Plasmodium falciparum* is also a target for transmission-blocking antibodies.
J Immunol. **1987** Dec 15; 139(12):4213-7.

Raine JD, Ecker A, Mendoza J, Tewari R, Stanway RR, Sinden RE.
Female inheritance of malarial lap genes is essential for mosquito transmission.
PLoS Pathog. **2007** Mar; 3(3):e30.

Ranjan R, Chugh M, Kumar S, Singh S, Kanodia S, Hossain MJ, Korde R, Grover A, Dhawan S, Chauhan VS, Reddy VS, Mohmmed A, Malhotra P.
Proteome analysis reveals a large merozoite surface protein-1 associated complex on the *Plasmodium falciparum* merozoite surface.
J Proteome Res. **2011** Feb 4; 10(2):680-91. Epub 2010 Dec 22.

Rautemaa R, Jarvis GA, Marnila P, Meri S.
Acquired resistance of *Escherichia coli* to complement lysis by binding of glycophosphoinositol-anchored protectin (CD59).
Infect Immun. **1998** May; 66(5):1928-33.

Read D, Lensen AH, Begarnie S, Haley S, Raza A, Carter R.
Transmission-blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing.
Parasite Immunol. **1994** Oct; 16(10):511-9.

Reuter M, Caswell CC, Lukomski S, Zipfel PF.
Binding of the human complement regulators CFHR1 and factor H by streptococcal collagen-like protein 1 (Scl1) via their conserved C termini allows control of the complement cascade at multiple levels.
J Biol Chem. **2010** Dec 3; 285(49):38473-85. Epub 2010 Sep 20.

Ribeiro JM.
Role of saliva in blood-feeding by arthropods.
Annu Rev Entomol. **1987**; 32:463-78. **Review**.

Ribeiro JM, Francischetti IM.
Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives.
Annu Rev Entomol. **2003**; 48:73-88. Epub 2002 Jun 4. **Review.**

Rigden DJ, Mello LV, Galperin MY.
The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules.
Trends Biochem Sci. **2004** Jul; 29(7):335-9.

Riley EM, Bennett S, Jepson A, Hassan-King M, Whittle H, Olerup O, Carter R.
Human antibody responses to *Pfs*230, a sexual stage-specific surface antigen of *Plasmodium falciparum*: non-responsiveness is a stable phenotype but does not appear to be genetically regulated.
Parasite Immunol. **1994** Feb; 16(2):55-62.

Riley-Vargas RC, Lanzendorf S, Atkinson JP
Targeted and restricted complement activation on acrosome-reacted spermatozoa.
J Clin Invest. **2005** May; 115(5):1241-9. Epub 2005 Apr 21.

Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P.
The human complement factor H: functional roles, genetic variations and disease associations.
Mol Immunol. **2004** Jun; 41(4):355-67. **Review**.

Rogers NJ, Daramola O, Targett GA, Hall BS.
CD36 and intercellular adhesion molecule 1 mediate adhesion of developing *Plasmodium falciparum* gametocytes.
Infect Immun. **1996** Apr; 64(4):1480-3.

Rogers NJ, Hall BS, Obiero J, Targett GA, Sutherland CJ.
A model for sequestration of the transmission stages of *Plasmodium falciparum*: adhesion of gametocyte-infected erythrocytes to human bone marrow cells.
Infect Immun. **2000** Jun; 68(6):3455-62.

Rogerson SJ, Mwapasa V, Meshnick SR.
Malaria in pregnancy: linking immunity and pathogenesis to prevention.
Am J Trop Med Hyg. **2007** Dec; 77(6 Suppl):14-22. **Review**.

Rosenberg R, Wirtz RA, Schneider I, Burge R.
An estimation of the number of malaria sporozoites ejected by a feeding mosquito.
Trans R Soc Trop Med Hyg. **1990** Mar-Apr; 84(2):209-12.

Rupp I, Sologub L, Williamson KC, Scheuermayer M, Reininger L, Doerig C, Eksi S, Kombila DU, Frank M, Pradel G.
Malaria parasites form filamentous cell-to-cell connections during reproduction in the mosquito midgut.
Cell Res. **2011** Apr; 21(4):683-96. Epub 2010 Dec 21.

Sachs J, Malaney P.
The economic and social burden of malaria.
Nature. **2002** Feb 7; 415(6872):680-5. **Review.**

Saeed S, Carter V, Tremp AZ, Dessens JT.
Plasmodium berghei crystalloids contain multiple LCCL proteins.
Mol Biochem Parasitol. **2010** Mar; 170(1):49-53. Epub 2009 Nov 22.

Sanders PR, Cantin GT, Greenbaum DC, Gilson PR, Nebl T, Moritz RL, Yates JR 3rd, Hodder AN, Crabb BS.
Identification of protein complexes in detergent-resistant membranes of *Plasmodium falciparum* schizonts.
Mol Biochem Parasitol. **2007** Aug; 154(2):148-57. Epub 2007 Apr 27.

Schmidt CQ, Herbert AP, Kavanagh D, Gandy C, Fenton CJ, Blaum BS, Lyon M, Uhrín D, Barlow PN.
A new map of glycosaminoglycan and C3b binding sites on factor H.
J Immunol. **2008** Aug 15; 181(4):2610-9.

Scholz SM, Simon N, Lavazec C, Dude MA, Templeton TJ, Pradel G.
*Pf*CCp proteins of *Plasmodium falciparum*: gametocyte-specific expression and role in complement-mediated inhibition of exflagellation.
Int J Parasitol. **2008** Mar; 38(3-4):327-40. Epub 2007 Sep 20.

Scholz SM Dissertation
Analysis of cellular and molecular interactions of the PfCCp multi-domain adhesion proteins and functional characterization of PfCCp4 during the sexual phase of the malaria pathogen *Plasmodium falciparum* **2008**

Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JA.
Molecular mechanisms of complement evasion: learning from staphylococci and meningococci.
Nat Rev Microbiol. **2010** Jun; 8(6):393-9.

Severini C, Silvestrini F, Sannella A, Barca S, Gradoni L, Alano P.
The production of the osmiophilic body protein Pfg377 is associated with stage of maturation and sex in *Plasmodium falciparum* gametocytes.
Mol Biochem Parasitol. **1999** May 25; 100(2):247-52.

Silvestrini F, Alano P, Williams JL.
Commitment to the production of male and female gametocytes in the human malaria parasite *Plasmodium falciparum*.
Parasitology. **2000** Nov; 121 Pt 5:465-71.

Silvestrini F, Bozdech Z, Lanfrancotti A, Di Giulio E, Bultrini E, Picci L, Derisi JL, Pizzi E, Alano P.
Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*.
Mol Biochem Parasitol. **2005** Sep; 143(1):100-10.

Silvestrini F, Tibúrcio M, Bertuccini L, Alano P.
Differential Adhesive Properties of Sequestered Asexual and Sexual Stages of *Plasmodium falciparum* on Human Endothelial Cells Are Tissue Independent.
PLoS One. **2012**; 7(2):e31567. Epub 2012 Feb 21.

Simon N, Scholz SM, Moreira CK, Templeton TJ, Kuehn A, Dude MA, Pradel G.
Sexual stage adhesion proteins form multi-protein complexes in the malaria parasite *Plasmodium falciparum.*
J Biol Chem. **2009** May 22; 284(21):14537-46. Epub 2009 Mar 20.

Sinden RE.
Gametocytogenesis of *Plasmodium falciparum* in vitro: an electron microscopic study.
Parasitology. **1982** Feb; 84(1):1-11.

Sinden RE.
The cell biology of sexual development in *Plasmodium*.
Parasitology. **1983** Apr; 86 (Pt 4):7-28. **Review**.

Singh B, Su YC, Riesbeck K.
Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasion.
Mol Microbiol. **2010** Nov; 78(3):545-60. doi: 10.1111/j.1365-2958.2010.07373.x. Epub 2010 Sep 27. **Review**.

Skerka C, Lauer N, Weinberger AA, Keilhauer CN, Sühnel J, Smith R, Schlötzer-Schrehardt U, Fritsche L, Heinen S, Hartmann A, Weber BH, Zipfel PF.
Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration.
Mol Immunol. **2007** Jul; 44(13):3398-406. Epub 2007 Mar 30.

Skerka C, Horstmann RD, Zipfel PF.
Molecular cloning of a human serum protein structurally related to complement factor H.
J Biol Chem. **1991** Jun 25; 266(18):12015-20.

Smalley ME, Sinden RE.
Plasmodium falciparum gametocytes: their longevity and infectivity.
Parasitology. **1977** Feb; 74(1):1-8.

Smith TF, Gaitatzes C, Saxena K, Neer EJ.
The WD repeat: a common architecture for diverse functions.
Trends Biochem Sci. **1999** May; 24(5):181-5. **Review**.

Smith TG, Lourenço P, Carter R, Walliker D, Ranford-Cartwright LC.
Commitment to sexual differentiation in the human malaria parasite, *Plasmodium falciparum*.
Parasitology. **2000** Aug; 121 (Pt 2):127-33.

Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, Pozio E, Dooley JS, Lowery CJ.
Proteomics analysis and protein expression during sporozoite excystation of Cryptosporidium parvum (Coccidia, Apicomplexa).
Mol Cell Proteomics. **2007** Feb; 6(2):346-55. Epub 2006 Nov 23.

Sologub L, Kuehn A, Kern S, Przyborski J, Schillig R, Pradel G.
Malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito.
Cell Microbiol. **2011** Mar 3. doi: 10.1111/j.1462-5822.2011.01588.x. [Epub ahead of print]

Sturm A, Heussler V.
Live and let die: manipulation of host hepatocytes by exoerythrocytic Plasmodium parasites.
Med Microbiol Immunol. 2007 Sep;196(3):127-33. Epub 2007 Apr 5. **Review.**

Talman AM, Domarle O, McKenzie FE, Ariey F, Robert V.
Gametocytogenesis: the puberty of *Plasmodium falciparum*.
Malar J. **2004** Jul 14; 3:24. **Review**.

Templeton TJ, Fujioka H, Aikawa M, Parker KC, Kaslow DC.
Plasmodium falciparum Pfs40, renamed Pf39, is localized to an intracellular membrane-bound compartment and is not sexual stage-specific.
Mol Biochem Parasitol. **1997** Dec 1; 90(1):359-65.

Templeton TJ, Keister DB, Muratova O, Procter JL, Kaslow DC.
Adherence of erythrocytes during exflagellation of *Plasmodium falciparum* microgametes is dependent on erythrocyte surface sialic acid and glycophorins.
J Exp Med. **1998** May 18; 187(10):1599-609.

Templeton TJ, Iyer LM, Anantharaman V, Enomoto S, Abrahante JE, Subramanian GM, Hoffman SL, Abrahamsen MS, Aravind L.
Comparative analysis of apicomplexa and genomic diversity in eukaryotes.
Genome Res. **2004** Sep; 14(9):1686-95.

Templeton TJ, Enomoto S, Chen WJ, Huang CG, Lancto CA, Abrahamsen MS, Zhu G.
A genome-sequence survey for *Ascogregarina taiwanensis* supports evolutionary affiliation but metabolic diversity between a Gregarine and *Cryptosporidium*.
Mol Biol Evol. **2010** Feb; 27(2):235-48. Epub 2009 Sep 24.

Tosini F, Agnoli A, Mele R, Gomez Morales MA, Pozio E.
A new modular protein of *Cryptosporidium parvum*, with ricin B and LCCL domains, expressed in the sporozoite invasive stage.
Mol Biochem Parasitol. **2004** Mar; 134(1):137-47.

Trexler M, Bányai L, Patthy L.
The LCCL module.
Eur J Biochem. **2000** Sep; 267(18):5751-7.

Trueman HE, Raine JD, Florens L, Dessens JT, Mendoza J, Johnson J, Waller CC, Delrieu I, Holders AA, Langhorne J, Carucci DJ, Yates JR 3rd, Sinden RE.
Functional characterization of an LCCL-lectin domain containing protein family in *Plasmodium berghei.*
J Parasitol. **2004** Oct; 90(5):1062-71.

Valenzuela JG, Charlab R, Mather TN, Ribeiro JM.
Purification, cloning, and expression of a novel salivary anticomplement protein from the tick, Ixodes scapularis.
J Biol Chem. **2000** Jun 23; 275(25):18717-23.

van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, Stunnenberg HG, van Gemert GJ, Sauerwein RW, Eling W.
A central role for *P*48/45 in malaria parasite male gamete fertility.
Cell. **2001** Jan 12; 104(1):153-64.

van Schaijk BC, van Dijk MR, van de Vegte-Bolmer M, van Gemert GJ, van Dooren MW, Eksi S, Roeffen WF, Janse CJ, Waters AP, Sauerwein RW.
*Pfs*47, paralog of the male fertility factor *Pfs*48/45, is a female specific surface protein in *Plasmodium falciparum*.
Mol Biochem Parasitol. **2006** Oct; 149(2):216-22. Epub 2006 Jun 19.

Vaughan JA, Hensley L, Beier JC.
Sporogonic development of *Plasmodium yoelii* in five anopheline species.
J Parasitol. **1994** Oct; 80(5):674-81.

Voegtli WC, Madrona AY, Wilson DK.
The structure of Aip1p, a WD repeat protein that regulates Cofilin-mediated actin depolymerization.
J Biol Chem. **2003** Sep 5; 278(36):34373-9. Epub 2003 Jun 14.

Vogel G.
The 'do unto others' malaria vaccine.
Science. **2010** May 14; 328(5980):847-8.

Weismann D, Hartvigsen K, Lauer N, Bennett KL, Scholl HP, Charbel Issa P, Cano M, Brandstätter H, Tsimikas S, Skerka C, Superti-Furga G, Handa JT, Zipfel PF, Witztum JL, Binder CJ.
Complement factor H binds malondialdehyde epitopes and protects from oxidative stress.
Nature. **2011** Oct 5; 478(7367):76-81. doi: 10.1038/nature10449.

Wesseling JG, Smits MA, Schoenmakers JG.
Extremely diverged actin proteins in *Plasmodium falciparum*.
Mol Biochem Parasitol. **1988** Aug; 30(2):143-53.

Wesseling JG, Snijders PJ, van Someren P, Jansen J, Smits MA, Schoenmakers JG.
Stage-specific expression and genomic organization of the actin genes of the malaria parasite *Plasmodium falciparum*.
Mol Biochem Parasitol. **1989** Jun 15; 35(2):167-76.

Whaley K, Ruddy S.
Modulation of the alternative complement pathways by beta 1 H globulin.
J Exp Med. **1976** Nov 2; 144(5):1147-63.

WHO 2010
Global report on antimalarial drug efficacy and drug resistance: 2000-2010.
WHO Library Cataloguing-in-Publication Data

WHO. World Malaria Report; **2011**.
http://www.who.int/malaria/world_malaria_report_2011/en/

Williamson KC, Keister DB, Muratova O, Kaslow DC.
Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes.
Mol Biochem Parasitol. **1995** Dec; 75(1):33-42.

Williamson KC, Fujioka H, Aikawa M, Kaslow DC.
Stage-specific processing of *Pfs*230, a *Plasmodium falciparum* transmission-blocking vaccine candidate.
Mol Biochem Parasitol. **1996** Jun; 78(1-2):161-9.

Wu Y, Przysiecki C, Flanagan E, Bello-Irizarry SN, Ionescu R, Muratova O, Dobrescu G, Lambert L, Keister D, Rippeon Y, Long CA, Shi L, Caulfield M, Shaw A, Saul A, Shiver J, Miller LH.
Sustained high-titer antibody responses induced by conjugating a malarial vaccine candidate to outer-membrane protein complex.
Proc Natl Acad Sci U S A. **2006** Nov 28; 103(48):18243-8. Epub 2006 Nov 16.

Wu Y, Ellis RD, Shaffer D, Fontes E, Malkin EM, Mahanty S, Fay MP, Narum D, Rausch K, Miles AP, Aebig J, Orcutt A, Muratova O, Song G, Lambert L, Zhu D, Miura K, Long C, Saul A, Miller LH, Durbin AP.
Phase 1 trial of malaria transmission blocking vaccine candidates *Pfs*25 and Pvs25 formulated with montanide ISA 51.
PLoS One. **2008** Jul 9; 3(7):e2636.

Yeoman JA, Hanssen E, Maier AG, Klonis N, Maco B, Baum J, Turnbull L, Whitchurch CB, Dixon MW, Tilley L.
Tracking Glideosome-associated protein 50 reveals the development and organization of the inner membrane complex of *Plasmodium falciparum*.
Eukaryot Cell. **2011** Apr; 10(4):556-64. Epub 2011 Jan 14.

Zipfel PF, Skerka C.
FHL-1/reconectin: a human complement and immune regulator with cell-adhesive function.
Immunol Today. **1999** Mar; 20(3):135-40. **Review.**

Zipfel PF, Skerka C, Hellwage J, Jokiranta ST, Meri S, Brade V, Kraiczy P, Noris M, Remuzzi G.
Factor H family proteins: on complement, microbes and human diseases.
Biochem Soc Trans. **2002** Nov; 30(Pt 6):971-8. **Review**.

Zipfel PF, Heinen S, Józsi M, Skerka C.
Complement and diseases: defective alternative pathway control results in kidney and eye diseases.
Mol Immunol. **2006** Jan; 43(1-2):97-106. **Review**.

Zipfel PF, Würzner R, Skerka C.
Complement evasion of pathogens: common strategies are shared by diverse organisms.
Mol Immunol. **2007** Sep; 44(16):3850-7. **Review.**

Zipfel PF, Hallström T, Hammerschmidt S, Skerka C.
The complement fitness factor H: role in human diseases and for immune escape of pathogens, like pneumococci.
Vaccine. **2008** Dec 30; 26 Suppl 8:I67-74. **Review**.

Zipfel PF, Skerka C.
Complement regulators and inhibitory proteins.
Nat Rev Immunol. **2009** Oct; 9(10):729-40. Epub 2009 Sep 4. **Review.**

9 Appendix

9.1 Abbreviations

%	Percent
‘	Minutes
°C	Degree Celsius
AG	Workgroup (Arbeitsgruppe)
aGc	Activated gametocytes
AP	Alternative pathway
APC	Alternative pathway of complement
APS	Ammonium peroxide sulphate
B	*Babesia*
***Baculovirus* expression vector system**	BEVS
BCIP	5-Bromo-4-chloro-3-indoxylphosphate
BSA	Bovine serum albumin
c	Concentration
C	*Cryptosporidium*
CO_2	Carbon dioxide
Co-IP	Co-Immunoprecipitation
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamide tetracetic acid
EM	Erythrocyte membrane
ER	Endoplasmic reticulum
et al.	*Et altera*
Fig.	Figure
g	Gram
Gc	(unactivated) gametocytes
GIA	Gametogenesis inhibition assay
Gm	Gamete
GST	Glutathione-S-transferase
h	Hour
H_2O_{bidest}	Double distilled water
HIS	Heat inactivated human serum
HIV	Human immunodeficiency virus
IFA	Indirect immunofluorescence assay
IMC	Inner membrane complex
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kDa	Kilodalton
KO	Knock-out
l	Liter
IMC	Inner membrane complex
LB	Lysogeny broth
LCCL	Limulus coagulation factor C
M	Molar
m	Milli
mAb	Monoclonal antibody
MAC	Membrane attack complex

MBP	Myelin basic protein
MeOH	Methanol
min	Minutes
MPC	Multi protein complexes
n	Nano
NaCl	Sodium chloride
NBT	Nitroblue tetrazoliumchloride
NGS	Neutral goat serum
NHS	Neutral human serum
NMS	Neutral mouse serum
no.	Number
NP40	Nonidet P-40
OD	Optical density
P	*Plasmodium*
p	Page
pAb	Polyclonal antibody
PABA	p-aminobenzoic acid
PAGE	Polyacrylamide gel electrophoresis
Pb	*Plasmodium berghei*
PBS	Phosphate buffer saline
Pf	*Plasmodium falciparum*
PFA	Paraformaldehyde
Pv	*Plasmodium vivax*
PVM	Parasitophorous vacuole membrane
rp	Recombinant protein
rpm	Rounds per minute
RPMI	Roswell-Park-Memorial-Institute-Medium
RT	Room temperature
RT	Room temperature
S	*Staphylococcus*
SAX	Gametogenesis activation solution
SDS	Sodium dodecylsulfate
sec	Seconds
TBA	Transmission blocking assay
TBS	Tris buffered saline
TBSM	Milk powder in TBS
TBV	Transmission blocking vaccine
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Th	*Theileria*
Tris	Tris-(hydroxymethyl)-aminomethane
V	Volt
v/v	Volume/volume
w/v	Weight/volume
WHO	World health organization
WT	Wild type
x g	Gravitational force, g = 9.81 m/s^2
XA	Xanthurenic acid
µ	Micro
α	Anti

9.2 List of figures

9.3 List of tables

9.5 Publications and participation of conferences

Publications

Kuehn, A., **Simon N.,** Pradel, G. (2010) Family members stick together - Multi-protein complexes of the malaria parasite sexual stages. Medical Microbiology and Immunology. 199: 209-26. (Review)

Simon, N., Scholz, S.M., Moreira, C., Templeton, T.J., Kuehn, A., Dude, M.-A., Pradel, G. (2009). Sexual stage proteins form multi-protein complexes in the malaria parasite Plasmodium falciparum. Journal of Biological Chemistry, 284: 14537-14546.

Scholz, S.M., **Simon, N.,** Lavazec, C., Dude, M.-A., Templeton, T.J., Pradel., G. (2008). Malaria PfCCp multi-domain adhesion proteins are expressed during gametogenesis and select members support complement-mediated inhibition of exflagellation. International Journal for Parasitology 38: 327-340.

Publication in revision

Simon, N., Lasonder, E., Scheuermayer, M., Kuehn, A., Fischer, R., Zipfel, P.F., Skerka, C., and Pradel, G. (2012) Malaria parasites co-opt human factor H to protect from complement-mediated lysis in the mosquito midgut. Cell Host & Microbe, (in revision)