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Molecular and functional characterization of secretory organelles of the transmission stages of the malaria parasite *Plasmodium falciparum*

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Contents

Part I

Abstract

Plasmodium falciparum is responsible for the immense majority of malaria deaths. Plasmodium sexual stages, the gametocytes, are the only parasite forms capable of becoming gametes and propagate in mosquitoes, which in turn infect a new human host, completing the malaria life cycle.

Osmiophilic bodies (OBs) are membranous secretory organelles found only in Plasmodium gametocytes. They have been shown to play a role in gamete egress from the host erythrocyte and to be important for mosquito infectivity. Gametocytes defective for Pfg377, the only known protein localized to OBs of *P. falciparum*, are depleted from these organelles. This unique feature of the Pfg377 defective parasites was successfully exploited in this project to perform a comparative proteomic analysis between WT and stably disrupted *pfg377*KO gametocytes to identify candidate proteins as OB candidate resident proteins (OBCs).

Together with Pfg377 co-IP experiments and previous knowledge of protein components of OBs in related species, five OBCs were identified.

Localization of OBCs was performed either by generating antibodies to recombinant fragments of individual OBCs or by producing transgenic *P. falciparum* parasite lines where the gene of interest was C-terminally fused with the gene of the Green Fluorescent Protein. Immunofluorescence assay (IFA) analysis exploring co-localization of the OBC proteins with antibodies against Pfg377, in some instances complemented by immunoelectron microscopy observation, led to the identification of four OBCs, including two proteases, as components of the OBs of *P. falciparum*.

In order to functionally characterize the OBC encoding genes and to investigate a possible role in gamete egress, parasite lines were obtained where the respective coding sequence were disrupted. In order to improve current tools for the analysis of *P. falciparum* gametogenesis, a protocol to measure gamete egress was developed, based on the staining of the host erythrocyte membrane. Functional analysis of the mutant parasites using the new protocol revealed that the *pfg377*-disrupted lines and one other OBC-depleted line produced gametocytes with no obvious defect in egress, with the only exception of one KO line with a mild egress phenotype.

The data generated in this thesis revealed novel proteins localized in these organelles. The functional analysis showing no obvious gamete egress phenotype in gametocytes depleted of these organelles or of some of the OB resident proteins revised current hypotheses on the role of these organelles in the release of gametes from the surrounding erythrocyte membrane, opening the question of a different role of these organelles in later mosquito stages of the parasite.

State of the art

The global burden of malaria

Malaria is still a huge burden for human populations of tropical latitudes. It is estimated that approximately 200 million cases of this disease took place in 2013, among the more than 3 billion people at risk of contracting it. 90% of the cases were located in Africa, while 77% of the annual 600-700 thousand deaths correspond to children aged under 5 years, with pregnant women at higher risk as well. Poor communities are the most affected by malaria in endemic countries, reflecting the evitable nature of these deaths [1].

Malaria is caused by parasites of the protozoan Plasmodium genus, which are transmitted to the human host through the bite of an infected Anopheles mosquito. Currently, 5 species are known to cause malaria in humans: *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*. Almost all malaria deaths are caused by *P. falciparum*, which is prevalent in sub-Saharan Africa and present as well in South and Central America and South-East Asia and Oceania [2].

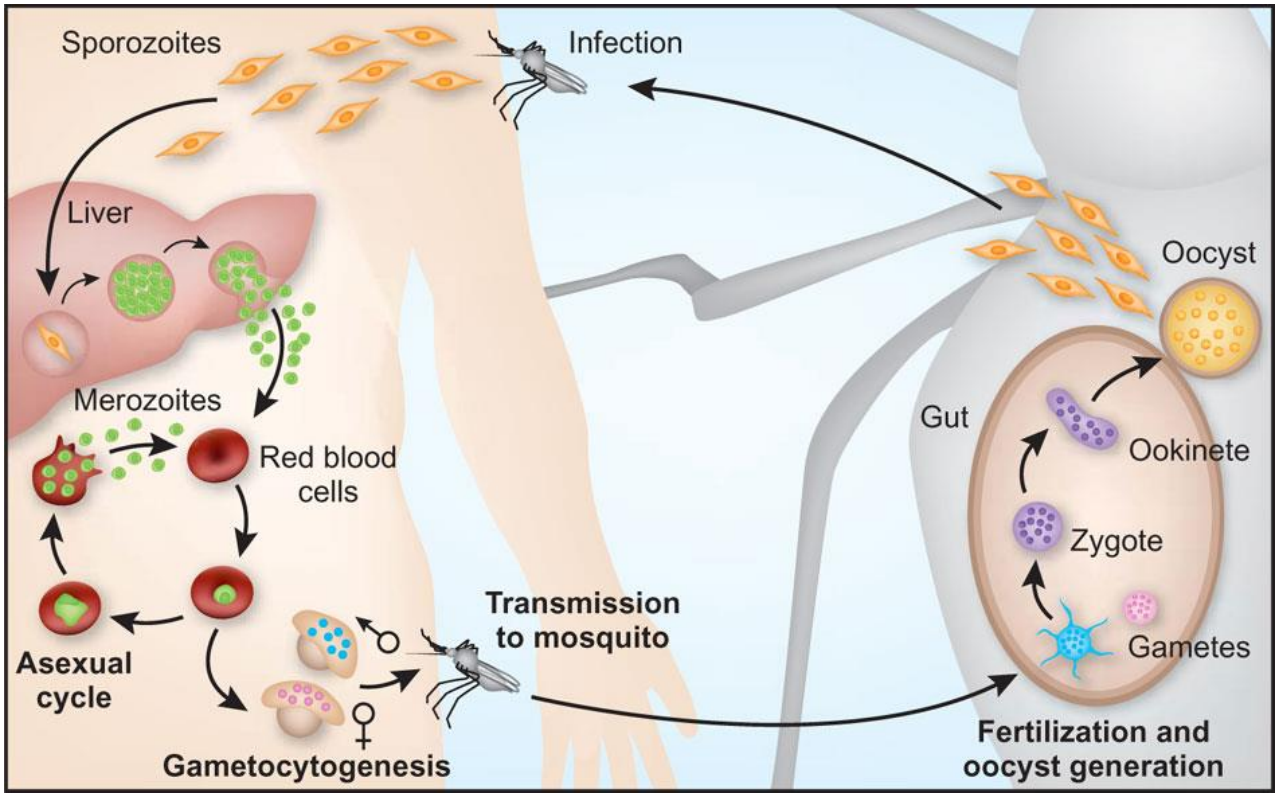
A century ago malaria was a global disease, with about three quarters of the world population at risk. The identification of the disease's vector led to initial efforts at vector control, which greatly reduced the geographic distribution of malaria through eradication of Anopheles mosquitoes in extended non-tropical areas [3]. Difficulties in vector control together with the rise of drug resistance in the parasite contributed to the persistence of malaria in the tropics through the twentieth century [4], although renewed efforts in the last years have greatly contributed to a reduction of over 47% of deaths from malaria in the first fourteen years of the twenty-first century.

Current strategies to fight malaria in the field are directed mainly at the reduction of infections through vector control, especially with the use of insecticide-treated mosquito nets (ITN), together with chemoprevention and case treatment [5]. Use of antimalarial drugs is vital for the survival of patients, keeping death rate under 0.01% in case of adequate treatment. In the last years, the most urgent problem jeopardizing malaria control efforts is the emergence and spread of drug resistance in Plasmodium [6]. A delayed sensitivity to artemisinin compounds, which have been introduced in recent years as essential components of first-line drug treatment of malaria, is emerging in South-East Asia. This phenomenon could greatly affect control of the disease, as happened previously with the spread of resistance to chloroquine and sulfadoxine-pyrimethamine antimalarials [6, 7].

The lack of any effective vaccine aggravates the global threat of malaria, together with vector resistance to currently used insecticides, which has been observed in gradually increasing areas of malaria transmission [8]. This configures a scenario in which gaining knowledge into the

mechanisms of disease and parasite transmission will be essential in making it possible to enter the perspective of malaria eradication.

Figure 1. Life cycle of *Plasmodium falciparum*. Adapted from [9].



Plasmodium falciparum life cycle and pathology

Female anopheline mosquitoes transmit malaria parasites during a blood feed by inoculating microscopic motile sporozoites into the skin, from where they enter the bloodstream and reach and invade hepatocytes. A single sporozoite can produce tens of thousands of daughter merozoites in 5.5-8 days, which burst from the hepatocyte and invade erythrocytes in the blood stream, commencing the asexual blood cycle of the parasite.

After erythrocyte invasion the parasite consumes the red blood cell (RBC) content, extensively remodeling its surface and structure, which allows the parasite to avoid spleen clearance and facilitates import of nutrients [10, 11]. Inside the host cell, in the first 24h of the asexual cycle, the parasite grows from the initial ring stage into a trophozoite. This stage sequesters to various organs by binding to the capillary endothelium, thus removing itself from peripheral circulation and avoiding passage through the spleen. This sequestration is achieved in the asexual parasites through the export of PfEMP1 (Erythrocyte Membrane Protein 1) to the surface of the infected erythrocyte.

PfEMP1 first localizes to specialized membranous structures induced by the parasite in the cytosol of the infected cell, called Maurer's clefts. Maurer's clefts are associated to the so called "knobs" in the surface of the infected erythrocyte, to which PfEMP1 and other proteins important for adhesion are targeted and eventually exposed [12, 13]. PfEMP1polymorphic proteins possess adhesive domains capable of interacting with specific molecules of the endothelium, making adherence and sequestration possible.

In the last part of the asexual cycle the parasite develops into a schizont, dividing into 6 to 20 new merozoites, which are released to the blood current after rupture of the RBC, roughly 48h post-infection. Each merozoite invades a new erythrocyte in which a new asexual cycle takes place.

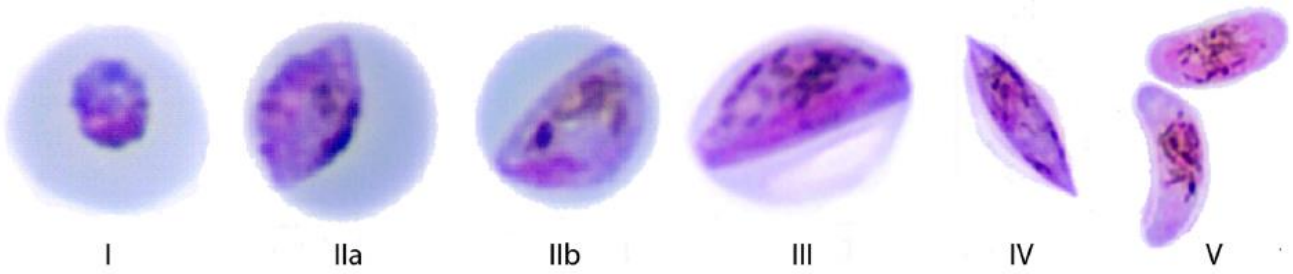
Pathology of malaria is derived from the effects of erythrocyte parasitisation and destruction and from the host reaction [14]. Its first symptoms, appearing 10 days to 4 weeks after the infected mosquito bite, include a vague absence of wellbeing, headache, fatigue, muscle aches and abdominal discomfort, which are followed by irregular fever. Synchronous rupture of schizonts release host and parasite cellular material into the blood, which activates monocytes and macrophages, inducing the characteristic cyclic fever of the malaria patient [15]. In a fraction of malaria cases, generalized seizures, associated specifically with *P. falciparum* malaria, might be followed by coma (cerebral malaria), due to high burden of sequestered infected erythrocytes in the brain. The placenta in pregnant women is another organ of parasite preferential sequestration, which is the cause of the elevated risks of suffering the disease during pregnancy. In these organs, sequestered parasites interfere with microcirculation, leading to loss of function of the affected tissues, and possibly to death[16]. This polymorphic protein is encoded by more than 50 *var* genes in the parasite's genome, of which only one is expressed at any time in one asexual cycle, leading to an antigenic variability that has great incidence in the escape of parasites from the immune response [17].

Symptoms of malaria can last for several weeks and, in *P. vivax* and *P. ovale* infections, parasites can remain dormant for months to years in hepatocytes in the form of hypnozoites [18, 19]. Acquired immunity develops over time and repeated infections, greatly reducing the symptoms and risks of the disease on healthy adults exposed to recurrent infection. This immunity however is reversible after several months without exposure to the parasite [20].

The pathogenic asexual parasites cannot achieve malaria parasite transmission to the mosquito. This is instead ensured by the parasite sexual blood stages, the gametocytes. In *P. falciparum*, after the rupture of the asexual schizont and the release of merozoites, some of the newly invading parasites exit proliferation and differentiate into sexual cells, the gametocytes. Sexually committed ring stages undergo gametocytogenesis, in which 10 to 12 days of maturation produce a mature male or female

gametocyte. This developmental process is classified in five morphological stages (I to V) in which the shape of the host and parasite cells change characteristically, from spherical to the enlarged elongated falciform shape of stage II to V gametocytes, after which the species is named. At stage I of this process the parasite sequesters to internal organs, through mechanism which are different from those of the asexual parasite, and which could rely on mechanical sequestration or/and on adhesive binding through yet undefined specific molecules [21, 22]. The sequestration niche of developing gametocytes is different from that of the asexual parasite cell, with recent work describing the bone marrow as a preferential sequestration site [23, 24]. The mechanical properties of gametocyte-infected RBCs change in the course of maturation. Stages I through IV are less deformable than uninfected erythrocytes, a property which could contribute to the maintenance of sequestration of immature gametocytes. When gametocytes mature, they become more deformable, and this may contribute to their return to peripheral blood and the ability to circulate through the spleen for several days, ready to be uptaken by a female Anopheles mosquito biting the infected person [21].

Figure 2. Developmental stages of *P. falciparum* gametocytes. Adapted from [25].



Once gametocyte-infected RBCs are engorged in the mosquito midgut, gametogenesis takes place [26, 27]. The transformation of gametocytes into gametes begins with the ‘rounding-up’ of the elongated gametocyte, which then disrupts the parasitophorous vacuole and the surrounding RBC membrane in order to egress from the host cell. In the extracellular environment, one male gametocyte produces 8 flagellated microgametes after three fast rounds of mitosis, while the female gametocyte transforms into a single spherical macrogamete, which is fertilized by a male microgamete, forming a zygote. From the zygote, a motile ookinete develops, which then traverses the midgut cell wall to enter the mosquito hemocel where it further transforms into an oocyst. Several thousand sporozoites are produced in one oocyst, which, after its rupture, migrate to the salivary glands of the mosquito, ready to be ejected with the next mosquito bite, thus infecting another human host and starting a new parasite life cycle.

Plasmodium falciparum gametocytes

It is not yet clear how and when asexual parasites become committed to the sexual path of differentiation. Environmental factors negatively affecting asexual development, like immune response from the host, drug treatment or the presence of other parasites in the host, increase the conversion rate from the asexual cycle. Gametocytogenesis is commonly induced in vitro by letting *P. falciparum* cultures to grow to high parasitaemia. Commitment takes places at an undetermined step of the asexual cycle previous to the appearance of gametocytes, as it has been suggested by experiments showing that gametocytes derived from a single schizont are all male or all female [28]. Recent work has identified a role in sexual differentiation for one conserved member of the Plasmodium Apetala 2 family of DNA binding proteins (AP2), first characterized in plants, and named after the phenotype observed in plants lacking the first studied protein of this family). In *P. falciparum* and in *P. berghei* the AP2G protein was shown to act as a key transcription regulator active at the onset of gametocytogenesis, with an essential role in triggering transcriptional activation of a set of gametocyte-specific genes in the early stages of this process [29–31], some of which encode proteins which are currently used as markers of early gametocytes, including Pfs16 and Pfg27.

The Plasmodium gametocyte is a cell specialized to mature into gametes, the cells accomplishing the transition between the human and the mosquito host. Several changes occur in the formation of these parasite stages [32], including the expression of hundreds of gametocyte-specific proteins [33], as well as major rearrangements of subcellular structures. Alike asexual stages of *P. falciparum*, the gametocyte is surrounded by the parasite plasma membrane (PPM), the parasitophorous vacuole membrane (PVM) and the erythrocyte membrane (EM). In both stages the PVM originates from the invagination of the EM at the point of invasion of the parasite into the host cell. The PVM is modified by the release of parasite proteins shortly after invasion, and plays an essential role in the interactions between host cell and parasite. In gametocyte development, a pellicular complex appears from early gametocytogenesis under the PPM, comprising a flattened bilamellar membranous structure, the subpellicular membrane (SPM), and an underlying subpellicular microtubule array. This complex structure has been recently shown to be molecularly similar to the Inner Membrane Complex (IMC) described in other parasite stages, where it has a role in motility and invasion. In contrast, in gametocytes the SPM/IMC is proposed to be implicated in the changes in shape of the gametocyte during gametocytogenesis [34, 35].

The intracellular development of gametocytes is marked by the production of specific elongated organelles surrounded by a single membrane, whose electrondense appearance in osmium stained

ultrastructural sections led to name them osmiophilic bodies. These organelles are similar to other electron dense organelles seen in other parasite stages (see below). They appear in sexual differentiation from stage III of gametocytogenesis in the periphery of the cell, underneath the pellicular complex, and accumulate preferentially in female gametocytes. The fact that these organelles are not found in the extracellular gametes led to think that their discharge and disappearance was functionally linked to egress.

Gamete egress and transmission

Mature gametocytes are the only Plasmodium cells capable of transmitting the parasite to the mosquito. When the parasite enters the mosquito midgut after it bites an infected person, gametogenesis, the development of a gametocyte into a gamete, is triggered by several factors, of whichsuch as the presence of xanthurenic acid (XA) and a drop in temperature. Once activated, the parasite becomes spherical and a complex process begins in which multiple the membranous layers surrounding it are disrupted prior to the egress of the gamete from the host cell.

An important regulatory aspect of gametogenesis in the mosquito midgut is that a large group of translationally repressed RNAs stored in female gametocytes now escape this mode of negative regulation, leading to production of key proteins such as the gamete and zygote surface proteins Pfs25 and Pfs28 [36].

XA is an important regulator of gamete activation. This molecule is produced in the mosquito as a byproduct of eye pigment metabolism and is excreted to the insect midgut, where it interacts with ingested gametocytes. The mechanism by which XA triggers gametogenesis is unknown, although it is likely to interact with one or more of the several signaling pathways known to be involved in early phases of gamete activation. Cyclic GMP (cGMP), produced by guanylyl cyclase (GC) and regulated through hydrolysis by phosphodiesterase (PDE), has been shown to be necessary for rounding up and egress [37]. cGMP typically regulates the activity of cGMP-mediated protein kinase (PKG), a master regulator of protein activity which is refractory to deletion in Plasmodium. The addition of a highly specific inhibitor of coccidian PKG to gametocytes inhibited rounding up in the sub-low micromolar range, while it did not affect the rounding up of transgenic parasites harboring an inhibitor resistant mutated PKG. Interestingly, egress was also affected in transgenic parasites, indicating the possibility of other kinases important for egress being inhibited [38].

Calcium signaling and phosphoinositide metabolism have also been proposed to be associated to gametogenesis. Phosphoinositide-specific phospholipase C (PLC) is necessary for the production of the secondary messenger IP_3, whose level increases after XA addition to the medium in *in vitro*

experiments. IP_3 in turn regulates calcium storage; its increase due to increased PLC activity after XA addition precedes the release of calcium from intracellular stores, causing an spike in its cellular concentration which is necessary for the egress of gametes from the host cell [38–40]. It has been proposed that cGMP control takes place upstream calcium signaling, making it the earliest known regulator of gametogenesis[41]. It is currently unknown which pathway activates GC in Plasmodium parasites. GC is upregulated in other organisms by a G protein couple receptor (GPCR), but the intriguing lack of orthologous genes of this family in malaria parasites constitutes a yet unanswered question.

Around 15 to 20 minutes after rounding up, both female and male parasites egress from the RBC.

OBs, which are much more abundant in female gametocytes, are often seen as juxtaposed under the PPM in the mature gametocytes and disappear within the first minutes postactivation, coevally with the rupture of the PVM. These organelles were observed to coalesce to points of the PVM at its rupture, before their content was released. After this process, the SPM further disorganizes, followed by the rupture of the EM is observed, after which the gamete egresses to the extracellular medium. Several protease inhibitors have been shown to block egress, suggesting that different proteases are involved in the rupture of these multiples membranes. The aspartic protease inhibitor EPNP, as well as the cysteine/serine protease inhibitor TLCK, considerably reduce SPM disintegration, while E64d, also a cysteine inhibitor, inhibits the rupture of PVM. Treatment with these inhibitors, as well as with the zinc metalloprotease inhibitor 1-10-PH, resulted in a high number of parasites surrounded by intact EM at the end of the experiment [27, 35].

Of the proteases which are involved in egress, PPLP2, a protein part of the membrane attack complex/perforin (MACPF) superfamily, has been identified as important for the egress of *P. falciparum* gametocytes. Disruption of the gene results in impaired egress from RBC, due to inability of the deficient mutants to rupture the EM after the successful disruption of the PVM [42].

In male gamete formation, it has been speculated that mechanical forces associated to flagellum beating could contribute to egress of the microgametes. However, the existence of a *P. berghei* mutant line whose gametocytes, defective in the production of the PbMDV1-Peg3 protein, show beating microgamete flagella trapped within the PVM and EM and unable to egress, suggest that this may not be the case [43, 44].

After detaching from the exflagellation center residual body, the male gamete encounters and adheres to a macrogamete, and fertilization begins by the fusion of the gamete plasma membranes, followed by the male nucleus entering the macrogamete cytoplasm and forming the zygote. Several proteins have been identified to play a role in fertilization. Antibodies against gamete surface proteins Pfs230

and Pfs45/48 were able to block fertilization, and their presence in the mosquito midgut in experimental infections of mosquitoes resulted in transmission-blocking activity.

Secretory organelles of apicomplexan parasites

Plasmodium parasites are part of the Apicomplexa phylum, named after the apical complex, an assemblage of structural and secretory elements at the apical point of the cell, specialized in invasion of the host cell. In the case of Plasmodium, the apical complex is apparent in the merozoites, in which it is essential for entering the host RBC. This complex can also be observed, with differences in its components, in the other invasive stages of the parasite: the ookinete and the sporozoite. The apical complex contains different types of secretory organelles such as micronemes, rhoptries and dense granules, which are described in some details for their morphological and possibly functional similarities with the gametocytes osmiophilic bodies.

Micronemes

Micronemes are small rod-shaped organelles with an approximate length of 100nm, restricted to the apical third of the parasite, in a variable number depending on the species. The organelle is surrounded by a typical bilayer membrane, and is electrondense due to its high protein content. All proteins directed to micronemes are synthetized with an N-terminal domain containing a conserved aminoacidic sequence, most have a transmembrane domain or are membrane-anchored through glycolipids, and several include one or more adhesive domains in their sequence.

Micronemes are derived from Golgi vesicles and can be found in merozoites, sporozoites and ookinetes, which are equipped with an apical complex to be able to invade RBCs, hepatocytes and mosquito midgut respectively. Stage-specific micronemal proteins have been identified in each of these cellular forms, indicating a specialized function of these organelles in each parasite stage.

In the case of merozoites, these parasite stages are released from schizonts into the bloodstream, where they contact a new RBC, to which an initial attachment is mediated by recognition of specific proteins of the erythrocyte and the parasite surface. After the initial attachment, the parasite reorients itself, resulting in its apical end being juxtaposed to the RBC membrane, after which invasion initiates. An increase in parasite intracellular calcium triggers the release of the micronemal content to the junction between the invading cell and the host. Here the released micronemal proteins are thought to connect external receptors to the submembranous acto-myosin motor that provides the power for parasite motility. This is important for the progression of host invasion, which is mediated by a moving junction that mechanically facilitates the invasion of the host cell. Proteins from the

merozoite surface and the micronemes mediating the anchoring of the parasite to the invaded cell are not incorporated into the vacuole that originates from the invagination of the EM during merozoite invasion. These proteins are instead cleaved by specific proteases to allow further advance of the junction. The junction finally fuses at the back of the newly invaded merozoite, generating the parasitophorous vacuole.

Several microneme proteins are known to be important for Plasmodium invasion into the host cell. The Merozoite Trombospondin-Related Anonymous Protein (MTRAP) is important for adhesion to the erythrocyte through interaction with aldolase, which acts as an acting binding protein of the moving junction; the Erythrocyte Binding Antigen 175 (EBA175) binds to sialic acid, which plays a role in the interaction between the cells; and Apical Membrane Antigen 1 (AMA1) has been shown to have a role in the moving junction [45]. Another microneme protein shown to be essential for the asexual cycle of *P. falciparum* is Subtilisin 2 (SUB2), a serine protease which is able to cleave AMA1, possibly playing a role in shedding this protein from the parasite surface as the moving junction progresses along the parasite surface during invasion [46–49].

In the case of ookinetes and of sporozoites, which respectively traverse the insect midgut wall and invade hepatocytes, proteins that are specific of the respective micronemes are described. Examples are the Secreted Ookinete Adhesive Protein (SOAP), the Circumsporozoite and TRAP Related Protein (CTRP) and, in the case of sporozoites, the Circumsporozoite Protein 1(CSP-1) and the Trombospondin-Related Anonymous Protein (TRAP), important for invasion and motility, with adhesive properties being common among them[50, 51].

Rhoptries

Rhoptries are the largest of the parasite secretory organelles contained in the apical complex. They have a club-like shape and, in the case of Plasmodium, two copies of these organelles are present in the merozoite, showing a markedly elongated shape of about 600nm. A basal bulbous portion and an apical duct-like portion (the rhoptry neck) can be observed in micrographies of these organelles, with different proteins associated to each portion. The rhoptries are originated in the Golgi complex and contain granular material as well as several transmembrane proteins, inserted in the bilayer membrane surrounding the organelle. As in the case of dense granules and micronemes, it is not clear how the cell is able to sort proteins to specific organelles or part of a given organelle during its biogenesis and maturation. The recognition of a specific sequence in the cytoplasmic tail of the transmembrane proteins is generally thought to be important in this process, but this domain is not found in all secretory organelles proteins, suggesting that additional mechanisms must exist. Indeed, in the specific case of Plasmodium parasites, several identified rhoptry-specific proteins do not

contain a transmembrane domain, indicating that they are directed to this organelle in a different, yet unknown, manner [52].

During the invasion of the RBC by the merozoite, after microneme proteins are secreted, the rhoptries collapse as they discharge their content. Proteins from the neck are generally involved in host adhesion before and during invasion. Reticulocite-binding homologs (Rh) proteins are important in attachment to the RBC, with Rh5 being refractory to genetic disruption, while RON (rhoptry neck) proteins 2,4 and 5 play a role both in cell attachment and in the formation of the tight junction by binding to the micronemal protein AMA1. Several rhoptry neck proteins have been termed "invasins" after their role in tight-junction mediated invasion, like Apical Sushi Protein (ASP). Finally, the function of other proteins localized to the neck remains unknown, as is the case for Pf34, AARP or RON6.

Proteins from the bulb portion of rhoptries generally have a role after parasite invasion. In *T. gondii*, many proteins of the rhoptry bulb are kinases, in some cases interacting with host signaling pathways. By contrast, no rhoptry bulb protein of Plasmodium is predicted to be a kinase; rhoptry bulb proteins in Plasmodium have generally no homologues in proteins outside the genus, suggesting a specific evolution of the Plasmodium organelles in the interaction with their host cell targets. In *P. falciparum* RhopH and RAP proteins form two well characterized protein complexes in the bulb. The function of RAP has been implicated in invasion since peptides against RAP1 inhibited RBC invasion *in vitro*. The RhopH complex is transferred to the host cell cytoplasm and plasma membrane post-invasion, and has been implicated in several processes downstream from invasion. RhopH1/clag9 has been related to cytoadherence of the infected RBC to the microvasculature, while RhopH1/clag3 influences permeability of the host cell [53, 54].

Interestingly, lipids have also been detected in rhoptries, which later are targeted to the PV, thus possibly modifying the properties of this compartment by a yet undefined mechanism [55].

Dense granules

Dense granules were identified as distinct organelles from rhoptries and micronemes only at a later time, in part because they have been mistaken in electron microscopy as transverse sections of rhoptries. Dense granules can vary in shape, size and number. In the case of *P. falciparum* they are spherical, with a diameter of about 80nm, and present a uniform electron-dense aspect in electron microscopy examination due to high content of proteins. As with micronemes and rhoptries, dense granules are surrounded by a single bilayer membrane. In contrast to the apical location of other secretory organelles, dense granules are localized more evenly in the parasite.

Dense granules are observed to persist for several minutes within the parasitophorous vacuole after the invasion of the RBC. Secretion from dense granule takes place in the lateral region of the parasite, and the released proteins associate with the host-derived PV and with the parasite-derived IMC.

Secretion from dense granules seems to take place in both a regulated and a constitutive pathway. A regulated burst of secretion after the formation of PV is observed during the first hours in after invasion, dependent on the increase of intracellular calcium. On the other hand, a constitutive pathway of dense granule secretion is supported by observation of a calcium independent secretion of specific proteins from the dense granules to the PPM.

Few proteins of the dense granules are described in *P. falciparum*. The Ring-infected Erythrocyte Surface Agent (RESA) protein is thought to cross the PVM to interact with the RBC cytoskeleton, while no clear function has been proposed for the Ring Membrane Antigen (RIMA), which is transferred to the parasite membrane in the ring stage [56]. Interestingly, RESA has been detected in the cytoplasm of the RBC after activation of gametogenesis, in a parasite cell where the only described electrondense organelles are OBs [57]. In other species, a higher number of proteins have been described in dense granules, as is the case of *T. gondii*, in which at least 16 dense GRAanule (GRA) proteins are known to localize to these organelles. Most of the above proteins have been shown to interact with the PV, where they contribute to the growth and maintenance of this important compartment and to regulate the interaction of the parasite with the host cell through this interface. Many of these proteins possess a transmembrane domain enabling their insertion in host membranes. Recent reports indicate possible roles of some of these proteins in controlling host cell effectors, like activation factors or GTPase proteins [58]. In the case of *T. gondii*, which invades nucleated cells, some GRA proteins have been observed to be secreted from dense granules and targeted to the host cell nucleus, where they modulate host transcriptional processes as well. This diverse functions and times of secretion of dense granule proteins prompt the question of whether there are several subpopulations of dense granules in Toxoplasma, and if this may be the case also for Plasmodium [49, 54, 59].

Other secretory organelles

Recent work described other electron dense organelles, differing from the above three types. In *P. falciparum* secretory granules have been recently described, named exonemes. Their diameter and morphology in transmission electron microscopy are similar to those of dense granules. Exonemes have been recently shown to contain the serine protease Subtilisin 1 (SUB1), which is able to cleave

the immature forms of several Serin-Rich Antigen (SERA) proteases localized in the PV and important for erythrocyte lysis resulting in the egress of merozoites in the asexual cycle [60, 61].

Last, another thread-like organelle termed mononeme has been identified in *P. falciparum*, which contains Rhomboid 1 (ROM1), a membrane associated protease capable of cleaving the microneme protein AMA1 *in vitro*, with which it forms a complex in the merozoite plasma membrane during its release from the RBC. This implies the secretion of the content of this organelle, present in one copy in the parasite, to the surface of the parasite through a yet undescribed mechanism [62].

Protein targeting to secretory organelles

Trafficking of proteins from the Golgi complex to the different secretory organelles is a complex process. It is not clear whether conserved domains are able to target proteins to specific destinations as no obvious common sequences as putative targeting domains are shared between proteins localized in any specific organelle.

Many of the proteins directed to secretory organelles are synthetized as immature forms and they undergo a maturation process. The prodomains of several micronemal proteins have been shown to be important for the correct targeting to these organelles in *T. gondii* [63, 64]. A similar situation was described for the Plasmodium rhoptry protein RAMA, which acts as escorter to recruit RAP1, RAP2 and RAP3 into rhoptries, with the prodomains of RAMA and RAP1 being essential for correct trafficking [65].

In mammals many organellar proteins depend on their cytoplasmic tails and transmembrane domain for correct targeting, which is ensured by their specific interaction with adaptor complexes. This mechanism has been shown to be conserved in *T. gondii* [66, 67]. In Plasmodium, organelle targeting of several plasmodial proteins have been shown to be independent from their cytoplasmic tail or their transmembrane domain, as in the case of EBA proteins and the microneme protein AMA1 [68]. In the case of EBA, a cysteine-rich region was found to be necessary for correct targeting.

Recently, a sortilin-like protein, SORTLR, which in other organisms regulates the trafficking of vesicles to various membranous organelles, has been functionally characterized in *T. gondii*. The SORTLR protein is important in directing proteins to their microneme or rhoptry destinations, by recruiting them into secretory vesicles and guiding them through the Golgi complex. This is achieved through interactions of the N-terminal tail of SORTLR with effectors of the trafficking machinery like the adaptor complex proteins adaptins or clathrins [69]. A SORTLR homologue exists in Plasmodium, suggesting that also this parasite makes use of this system to sort proteins to its secretory organelles.

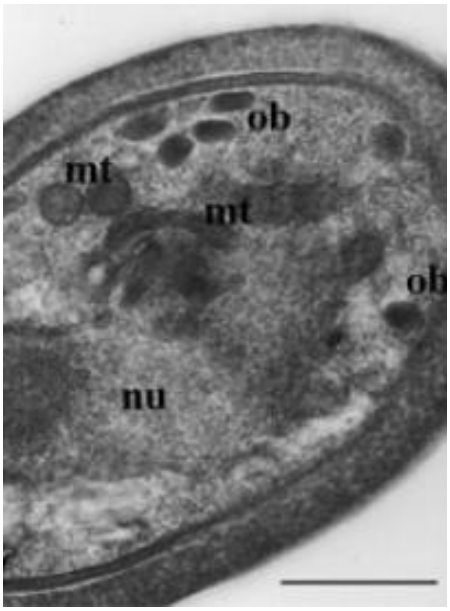
Osmiophilic bodies

OBs are secretory organelles found exclusively in Plasmodium gametocytes, which have been first identified in *P. falciparum* as electron-dense organelles in seminal electron microscopy studies of the parasite sexual stages [70]. OBs show a 200nm diameter and an ovoid shape and are surrounded by a single bilayer membrane. They are found predominantly in the periphery of the female gametocytes from stage III of gametocytogenesis [28].Electron microscopy studies occasionally described that OBs appear to be connected to the subpellicular membrane through a membranous duct [71].

Once gametogenesis is triggered, OBs can be observed to associate to regions of rupture of the PVM, forming assemblies of up to five OBs. During this process OB content is released and they are no longer detectable in the mature extracellular female gametes, which led to hypothesize a role for these organelles in the process of PVM and erythrocyte membrane rupture and gamete egress [72].

The only known protein specifically localized to the OBs in *P. falciparum* is Pfg377, with no homologues outside the Plasmodium genus. Pfg377 contains a signal peptide and an adjacent N-terminal region conserved in other Plasmodium species, which is sufficient to target the protein to the OBs [73, 74].

Figure 3. Osmiophilic bodies of *P. falciparum* gametocytes. Transmission electron micrograph of the apical part of a mature gametocyte. Scale bar 500nm. Adapted from [75].



Recently, the protein Pbg377, the Pfg377 homologue described in the gametocytes of the rodent malaria *Plasmodium berghei*, was also shown to reside in the OBs [43]. In *P. berghei*, other proteins have been described to localize in OBs. The Male development/Protein of early gametocytes (PbMDV-1/Peg3) protein was also shown to localize to the OBs in the murine parasite gametocytes. Immunoelectron microscopy localization studies in gametocytes of the human malaria parasites with antibodies against the *P. falciparum* homologous protein showed however localization to multiple parasite-derived membranes and not only in OBs [76, 77]. The last protein to be identified as a OB component is the Gametocyte Egress and Sporozoite Traversal protein PbGEST, which has been so far described only in the rodent malaria parasite [78].

In the above three cases, the genes coding for these proteins could be disrupted. As Plasmodium parasites are haploid in most of their life cycle, the possibility to generate and propagate parasites defective in one gene after disruption indicates that the function of that gene is not essential for asexual growth. The fact that the transcription profiles of the above genes indicated their sexual stage specific expression is consistent with the amenability of their genetic disruption [79].

Phenotypic analysis showed that the *pbGEST*KO parasites showed an aberrant gametogenesis, with male gamete exflagellation happening inside an intact host cell and fertilization greatly impaired. Analysis of the few parasites still able to fertilize and transform into ookinetes showed that the resulting sporozoites produced in the mosquito were unable to infect mice, due to a defect in their capacity to traverse host hepatocytes [78].

The gene encoding PfMDV-1/Peg3 has also been disrupted in both the rodent and the human parasites. The defective parasites lacking this protein showed that sexual development was blocked in early stage gametocytes, and that abnormal membrane structures were detectable in the residual gametocytes. The lack of this protein also caused a marked decrease in number of exflagellation centers and in mosquito infectivity by the few stage V gametocytes produced. In *P. berghei* parasites defective for the homologous protein no defect in gametocyte production was observed, but fertilization was greatly impaired in these parasites, due to a defect in egress from the PVM and the host erythrocyte in both male and female gametes [44].

*pfg377*KO parasites have also been produced and characterized both in *P. falciparum* and in *P. berghei*. In the human parasite, although gametocytes showed a normal morphology and sex ratio in Giemsa-stained smears of gametocyte cultures, an electron microscopy analysis showed a striking reduction in the number of OBs in the Pfg377 defective gametocytes in comparison with WT parasites. This suggests a critical role of Pfg377 in the biogenesis of OBs. When mutant gametocytes were examined for their ability to egress from the host RBC by immunofluorescence assay, a twofold

reduction was observed in the egress of female, but not of male, gametocytes compared to WT parasites. In addition, infectivity to the mosquito was greatly reduced in these parasites. Recently, a strain of *P. berghei* with a disrupted gene for Pbg377 has been produced. Analysis of gametocytes from the defective line showed that the size, but not the number, of OBs was reduced, and that female gamete egress was delayed but not impaired. The *pbg377*KO parasites were also examined for *in vitro* ookinete production, in which they showed no defect compared to WT parasites, although their infectivity to mosquito *in vivo* was not examined. In this work, interestingly, a new class of OBs was identified in male gametocytes (MOBs). These organelles were club-shaped and their morphology was not affected by the lack of Pbg377. PbMDV-1/Peg3 has been localized to these organelles [43].
Analysis of the structural features of the above three proteins from the rodent parasite revealed that all of them contain a signal peptide and a transmembrane domain, separated by 13 aminoacid residues, which share very little homology with the orthologous counterparts in the human parasite. No homology to other known protein domains has been found in these proteins, which prevents hypotheses about their function.

OBs are the only described secretory organelle in Plasmodium gametocytes. Although some of their resident proteins have been shown to be important for the egress of gametes from host RBCs, very little is known about the molecular mechanisms implicated in this process. Importantly, very few additional proteins have been identified in these organelles. Expanding our knowledge of the components of OBs and their role in gamete egress will result in a wider understanding of this process and could result in the discovery of new molecular targets to prevent malaria transmission to the mosquito vector, which will be an essential part of any global attempt to tackle this disease. The work for my thesis was consequently designed to fill this relevant gap in knowledge through the achievement of the following objectives.

Aim of the project

1) Development of a fast and simple protocol to measure gamete egress in *P. falciparum*.

2) Identification of novel proteins of the *P. falciparum* gametocyte osmiophilic bodies.

3) Functional characterization of the role of novel osmiophilic body proteins in gamete egress.

Main results

Development of a fast and simple protocol to measure gamete egress in *P. falciparum*

As the first step to study the role of OBs, we developed a protocol to easily measure the egress of gametes from their host RBC. A reliable quantitative assessment of egress is not straightforward, particularly in the case of the spherical female gametes, and so far previous methods were based either on analysis of Giemsa-stained smears or on cell surface staining with specific antibodies. Antibody-based protocols require parasite fixation followed by incubations with primary and secondary antibodies and several washing steps. These lengthy manipulations risk altering integrity of the sample, thus confounding the evaluation of egress. For this reason, a much faster and simpler protocol was developed to measure gamete egress, based on the labelling of gametocyte-infected erythrocytes prior to induction of gametogenesis.

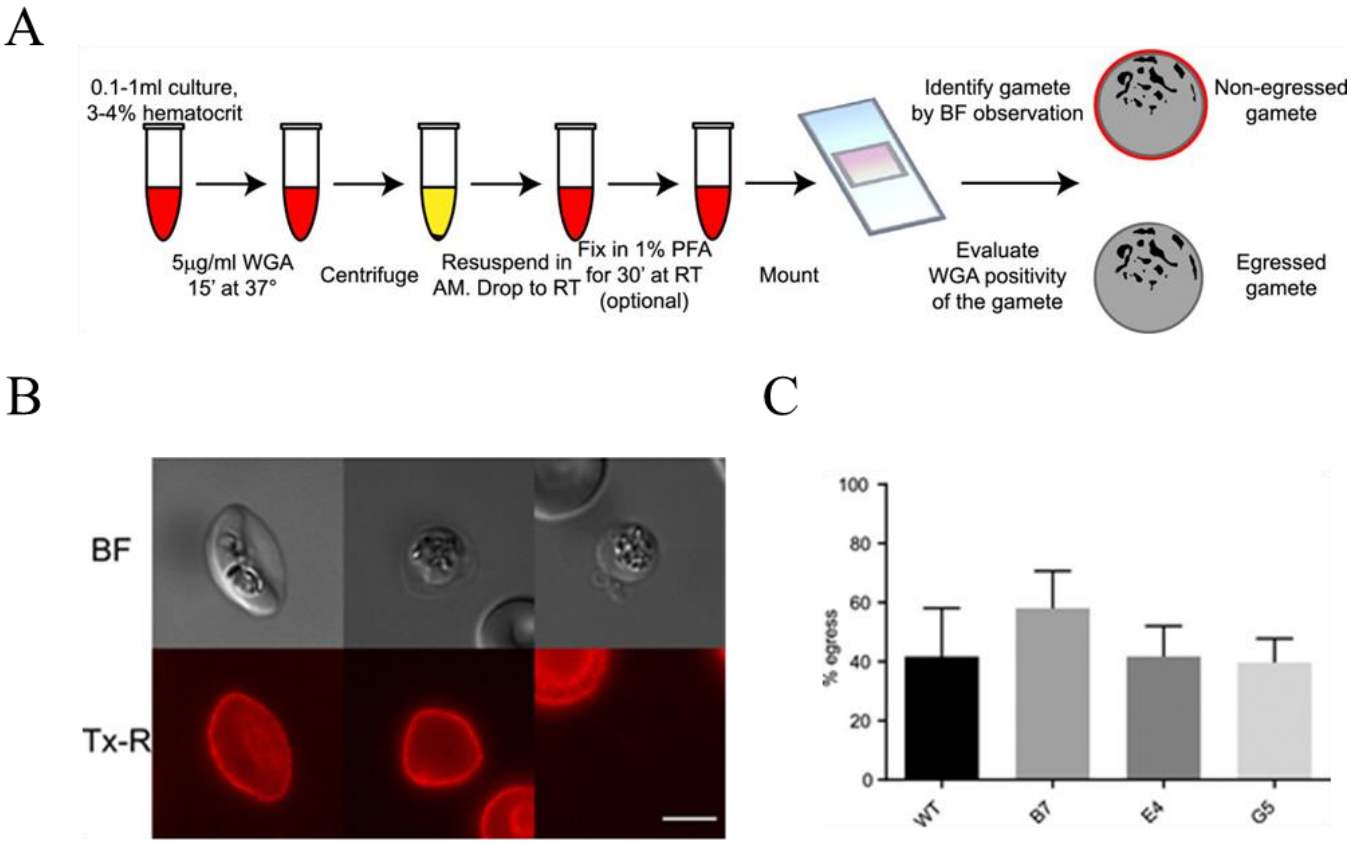
Wheat Germ Agglutinin (WGA) covalently linked to fluorescent dyes such as Texas Red or Alexa 488 was chosen to obtain mature gametocytes contained in erythrocytes stained by fluorofore-bond WGA. A 15-min incubation of a culture containing infected erythrocytes at 37°C with 5 μg/ml WGA was sufficient to obtain samples in which virtually all erythrocytes showed a clear and homogeneous fluorescent signal on their surface.

In order to measure egress, synchronous stage V gametocyte cultures were stained as described above and gametogenesis was induced. Inspection of the spherical parasites with appropriate filters enabled to clearly distinguish cells still surrounded by the fluorescent WGA signal, which were scored as intracellular gametes, from those devoid of any fluorescence, suggesting that they had shed the surrounding erythrocyte membrane. Egress efficiency and kinetics were similar to those found in previous publications [38, 75, 80]. Interestingly, gametes observed in the process of egressing the host RBC consistently did it through a single rupture point of the erythrocyte membrane. We also evaluated the specificity of WGA staining, finding that it does not decorate the surface of egressed gametes.

We then evaluated the possibility to fix these samples, observing that WGA-associated fluorescence can still be observed in paraformaldehyde-fixed samples, and that egressed gametes counts were very similar between live and fixed parasites.

These experiments show that gametocytes inside WGA-stained erythrocytes can undergo rounding-up and egress normally, and that parasites can be sampled and fixed and their egress efficiency evaluated (figure 4).

Figure 4. A: Flow chart of the wheat germ agglutinin-staining protocol. WGA: wheat germ agglutinin, AM: activation medium, RT: room temperature, PFA: paraformaldehyde, BF: bright field. **B**: Mature gametocyte (left panel), non-egressed gamete (centre), egressed gamete (right panel) stained with WGA-conjugated Texas Red. The Laveran's bib can be seen in the gametocyte as a brighter fluorescent area. Scale bar: 5 µm. BF: bright field. Tx-R: Texas Red. **C**: Egress efficiency of gametes from WT parasites and three *pfg377KO* clones after induction with pH 8.2 at room temperature. Error bar represents SD.



We then employed our newly developed protocol to evaluate the only *P. falciparum* mutant whose gamete egress efficiency has been reported to be deficient. In previous publications parasites lacking the OB protein Pfg377 showed a two-fold reduction in egress compared to WT parasites, when egress was measured through antibody-associated methods.

Egress efficiency in *pfg377*KO gametes was re-evaluated with the new protocol, comparing the three previously published clones of *pfg377*KO and their parental parasites [75], under the same activation conditions that had been used previously.

The previously reported two fold reduction in egress efficiency could not be reproduced for any of the *pfg377*KO clones. This discrepancy could be explained by the lack of Pfg377 diminishing the exposition of the selected antigen protein in mutant parasites, rather than actually impairing gamete egress.

Examination of *pfg377*KO gamete egress with the new protocol does not support the hypothesis of a major role of OBs in the ability of female gamete to disrupt and shed the erythrocyte membrane during egress. As however the Pfg377-defective parasites showed a dramatic defect in vector transmissibility [75], the above results rather propose that OB discharge and/or the associated Pfg377 mobilization play a functional role in subsequent steps leading to gamete fertilization or in further parasite development in the mosquito.

Identification of novel proteins of the *P. falciparum* gametocyte osmiophilic bodies

Generation of stable *pfg377*KO parasites

The previously characterized *pfg377*KO parasites occasionally presented reversion to the WT locus, albeit with very low occurrence. New *pfg377*KO parasite lines were produced in which this gene was stably disrupted by the gene replacement construct pHH-TK-Pfg377, irreversibly inserting a selection cassette disrupting the *pfg377* coding sequence. Parasite lines from two independent transfections were obtained and examined through diagnostic Southern blot analysis. These lines were then cloned by limiting dilution, and DNAs from the obtained clones of each independent transfection were analyzed trough PCR analysis, showing the expected products indicative of disruption of *pfg377* and the absence of the products associated to the WT locus in several clones. Clones *pfg377*KO 5-2 and 8-20, one from each of the independent transfections, were selected for further study.

Characterization of the new *pfg377*KO parasites

Parasite cultures of clones *pfg377*KO 5-2 and 8-20 showed no obvious difference with WT parasites in asexual growth rate, nor any loss in gametocyte production (which can result from the long term propagation needed during generation of *P. falciparum* mutants). Giemsa-stained smears of gametocyte cultures from these clones showed no differences in gametocyte morphology compared to WT, confirming what was observed in the characterization of the previously published *pfg377*KO strains [75].

Western blot (WB) and immunofluorescence analysis (IFA) were performed with antibodies against two different regions of Pfg377 to confirm the expected lack of this protein in the selected *pfg377*KO clones. WB analysis conducted on stage V gametocytes of clones 5-2 and 8-20 showed that none of the antibodies could detect a WT protein pattern in the *pfg377*KO clones. IFA conducted on WT parasites consistently showed the characteristic granular pattern associated to Pfg377, while no specific signal was detectable on stage V gametocytes of the *pfg377*KO parasites.

Together, these results showed that the Pfg377 protein was undetectable in mature gametocytes of the 5-2 and 8-20 clones, confirming that disruption of the *pfg377* locus was successful.

Phenotypic analysis of the *pfg377*KO parasites from clones 5-2 and 8-20

Electron microscopy observations on gametocytes from the previously published *pfg377KO* parasites showed a striking reduction in the number of OBs compared to WT gametocytes [75]. To determine whether the newly generated *pfg377KO* clones showed the same phenotype, mature gametocytes from the independent clones 5-2 and 8-20 were examined in transmission electron microcopy and compared to WT gametocytes. In contrast to the typical number and morphology of OBs observed in WT gametocytes, sections from *pfg377*KO gametocytes showed dramatically reduced number of OBs. The residual OBs in *pfg377*KO parasites were also smaller and more spherical than their WT counterparts, as confirmed by a significant decrease in the eccentricity of the sections of OBs from clones 5-2 and 8-20 observed in electron microscopy.

The efficiency of the *pfg377*KO parasite clones in egress from the erythrocyte at gametogenesis was measured using the newly established protocol described above. No significant differences between WT and *pfg377*KO parasites could be detected in our experiments. This confirms our results with the previously published *pfg377*KO gametocytes (described above), and it further supports the hypothesis that Pfg377 is not necessary for an efficient egress in *P. falciparum*.

Identification of new OB resident proteins through comparative label-free quantitative proteomic analysis of OB+ and OB- gametocytes

The dramatic depletion of OBs observed in the gametocytes of the *pfg377*KO parasites was exploited to investigate the protein composition of OBs with the aim of identifying novel proteins residing in these organelles. The approach undertaken was to perform a comparative label-free proteomic analysis of OB+ and OB- gametocytes, based on the assumption that proteins residing in OBs in WT parasites will be underrepresented in the OB-depleted KO parasites.

WT, *pfg377*KO 5-2 and *pfg377*KO 8-20 mature gametocytes were cultured and purified in parallel, and several quality control steps were performed before submitting the three samples to proteomic analysis, to ensure adequate comparability of the samples. This was repeated to obtain a total of three independent biological replicates, producing a total of nine different samples.

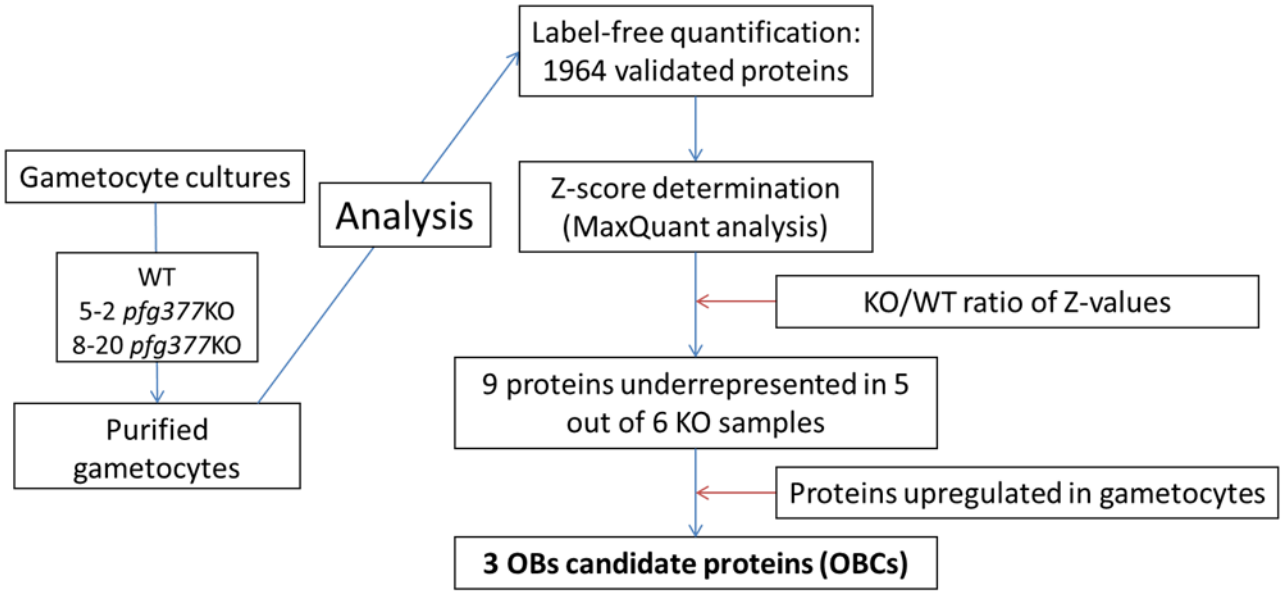
Label-free quantitative mass spectrometry identification of the proteins contained in each sample was performed, resulting in nine datasets. Only proteins detected in all samples (n=1964) were included in the MaxQuant quantitative determination to calculate the relative abundance of each protein in each sample, for which its Z score was calculated.

Cluster analysis of the nine different datasets using the calculated Z-values indicated that while in two out of three biological replicates the *pfg377*KO samples were more similar to each other than to the WT samples, the overall difference in the proteomic profiles between the three experiments was higher than that between proteomic profiles of the WT and the *pfg377*KO gametocyte samples.

The high variability among biological replicates therefore suggested to apply a stringent statistical threshold in the selection of proteins underrepresented in *pfg377*KO gametocytes.
Protein abundance of each of the 1964 selected proteins in each of the *pfg377*KO samples relative to its abundance in the correspondent WT sample was calculated, obtaining a total of six KO/WT ratios. It was assumed that proteins significantly underrepresented in KO samples may be considered as OB candidate proteins. Only proteins in which underrepresentation (KO/WT ratio) in at least five out of the six *pfg377*KO to WT gametocytes comparisons was significant were selected.
A total of only nine OB candidate proteins met this condition, of which one was Pfg377, as expected. An additional criterion was applied to select candidate gene products for the subsequent functional analysis. This was based on the fact that OBs appear in stage III gametocytes, from day four of sexual differentiation. For this reason we selected proteins upregulated in mature gametocytes according to previous transcriptional data [81]. Apart from Pfg377, three of the above proteins were overrepresented in mature gametocytes. This motivated the choice to concentrate on these proteins, PF3D7_0513700 (PfPSOP12), PF3D7_1247800 (Dipeptidyl aminopeptidase 2, PfDPAP2) and Pf3D7_1136900 (Subtilisin-like protease 2, PfSUB2) for further studies (figure 5).

Figure 5. A: Flowchart showing the process for the comparative proteomic determination of OBCs. B: List of selected OBCs. PlasmoDB Gene ID, Product Description and Selection method leading to inclusion in the list are shown. I: Comparative proteomics. II: Co-IP. III: Homologue similarity.

A



B

Gene ID	Gene product	Selection method
PF3D7_0513700	Secreted ookinete protein, putative (PSOP12)	I/II
PF3D7_1247800	Dipeptidyl peptidase 2, putative (DPAP2)	I
PF3D7_1136900	Subtilisin-like protease 2 (SUB2)	I
PF3D7_1214800	Unknown protein	II
PF3D7_1449000	PfGEST	III

Identification of proteins co-immunoprecipitated with Pfg377

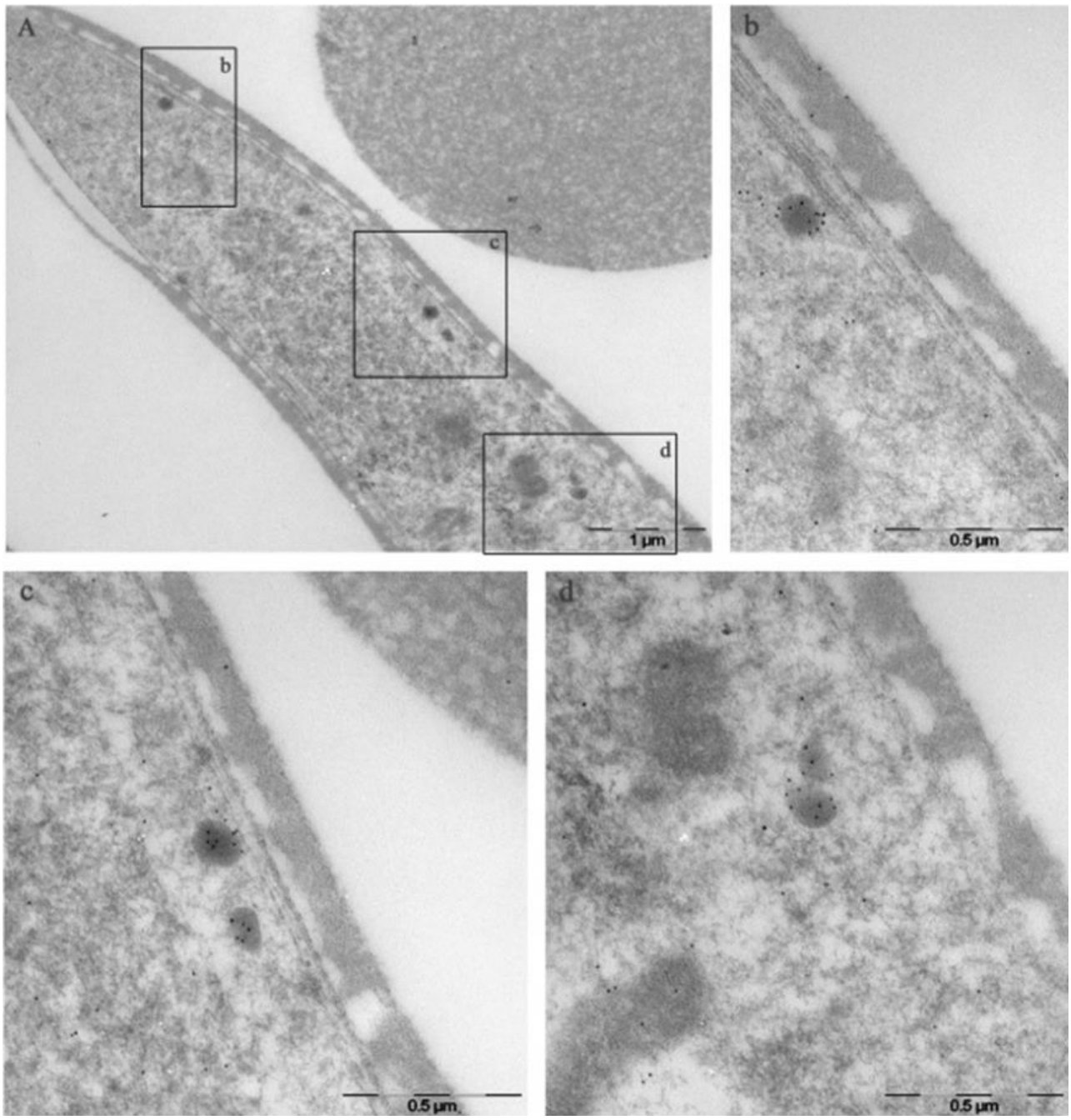
In another approach to identify OB resident proteins, a co-immunoprecipitation (co-IP) experiment was conducted using Pfg377 as bait. The absence of Pfg377 in KO gametocyte samples provided an optimal negative control in this experiment, allowing to perform parallel co-IP experiments with α-Pfg377KO antibodies on extracts from WT and *pfg377*KO gametocytes. The whole final co-IP eluates were analyzed by mass spectrometry, and the WT/KO ratio of the quantified proteins calculated. An arbitrary ratio of 100-fold presence in the WT over the KO sample was stablished as the threshold at which a protein would be considered a true Pfg377 interactor. Three proteins were overrepresented in WT samples over the threshold: Pfg377 itself, PSOP12, also identified in the above whole cell proteomic analysis, and protein PF3D7_1214800. PF3D7_1214800 was added to the list of potential OB localized proteins as OBCIp (OB Candidate Ip protein).

Characterization of the *Plasmodium falciparum* homologue of the *P. berghei* protein PbGEST

The PbGEST protein has been previously shown to localize to the OBs in *P. berghei* gametocytes [78]. We explored the localization of the *P. falciparum* homologue PfGEST, which has been previously detected in gametocytes [79, 81], as a possible OB resident protein. To investigate if PfGEST localizes to OBs, mouse polyclonal antibodies against the C terminal region of PfGEST were produced.

Immunoelectron microscopy was performed using α-PfGEST antibodies on samples of WT mature gametocytes. The results show localization of the antibodies to the OBs of gametocytes, indicating PfGEST resides in these organelles (figure 6).

Figure 6. Representative immunoelectron micrographs using mouse αPfGEST antibodies on WT gametocytes.



IFA analysis on WT stage V gametocytes using α-PfGEST antibodies showed a fluorescent granular pattern associated to the antibodies, and double IFA with the OB protein α-Pfg377 and α-PfGEST antibodies showed colocalization of the fluorescent patterns associated to the two antibodies, indicating that the targeted proteins colocalize, supporting the electron microscopy results.

Male gametocytes are not stained by Pfg377 in IFA experiments. This was used in double IFA experiments using antibodies against PfGEST and Pfg377 to identify male gametocytes and evaluate the presence of PfGEST-associated fluorescence in them. Results showed PfGEST-associated fluorescence in male gametocytes, suggesting this protein is expressed in gametocytes of both sexes. Additionally, IFA performed on gametocytes from the *pfg377*KO strain, while failing to show Pfg377-associated fluorescence, conserved the granular pattern of PfGEST-associated fluorescence.

Western blot analysis on WT mature gametocyte samples detected a band of a molecular weight in reasonable agreement with the expected size of PfGEST, while this band was absent in asexual samples. The PfGEST-associated band is also present in samples from *pfg377*KO gametocytes, in agreement with the results obtained in IFAs.

Taken together, the above experiments strongly indicate that PfGEST is expressed in gametocytes, where it localizes to the OBs of *P. falciparum*.

Functional characterization of Osmiophilic Body Candidate proteins (OBCs)

The results of the above work led to the identification of 4 OBC from the whole cell comparative proteomic analysis and the Pfg377 co-IP approaches. PfGEST was also included in the group of proteins selected for further study (figure 5B). Transcriptional and expression profiles found in previous publications [81, 82] were analyzed for the selected candidates. Proteomic data indicate prevailing presence of these proteins in mature gametocytes, compared to early gametocytes and asexual forms [81]. Transcription of all five candidates is upregulated in gametocytes stages, with a peak in mid to late gametocytogenesis, strongly resembling Pfg377 transcription profile [79]. Regarding protein architecture in the selected candidates, all five proteins include a signal peptide and a transmembrane domain in their predicted sequence, typically associated to proteins localized to membranous organelles.

Generation of transgenic OBC-GFP parasites

The first part of the characterization of the newly identified OBC gene product was to investigate their subcellular localization. To this aim 3 OBC-GFP lines were produced. TK-GFP-ROM3 (TG3), a new GFP-tagging vector, was developed and used to clone the 3' regions of the coding sequences for PSOP12, SUB2 and OBCIp in frame with the GFP coding sequence, producing plasmids pTG3-PSOP12, pTG3-SUB2 and pTG3-OBCIp. These plasmids were then used to produce the PSOP12-GFP, SUB2-GFP and OCBIp-GFP lines through single crossover homologous recombination, each with the endogenous copy of the respective target gene modified by the coding sequence for GFP at the 3' end of the gene. OBC-GFP parasites were obtained and correct integration of the plasmid evaluated through diagnostic PCR, obtaining PCR products indicative of the correct integration of the plasmid in the expected loci. These lines were then cloned by limiting dilution, and the resulting clones were again evaluated for the expected integration events, obtaining PCR products indicative of integration in clones from all three lines. Clones PSOP12-GFP1, SUB2-GFP1 and OBCIp-GFP1 were selected for further characterization.

Regarding the rest of OBCs, localization of PfGEST had been determined through the production of mouse multiclonal antibodies as described above, while a clone from a DPAP2-GFP tagged line was kindly provided by Dr. M. Klemba, Virginia Tech, Virginia, USA (unpublished work). This clone was named DPAP2-GFP1.

Characterization of OBC-GFP parasites

Mature gametocytes from PSOP12-GFP1, SUB2-GFP1, OBCIp-GFP1 and DPAP2-GFP1 clones were analyzed for GFP-associated fluorescence. Live SUB2-GFP1 and OBCIp-GFP1 gametocytes showed a granular, peripheral fluorescence pattern, which was less obvious in the case of DPAP2-GFP, while no clear signal could be observed in PSOP12-GFP parasites. Additionally, fluorescence was detected in live asexual SUB2-GFP schizonts, in agreement with the established presence of PfSUB2 in merozoite micronemes [83].

These results show that parasites from at least three of the four OBC-GFP parasites produce a functional GFP-tagged version of their respective OBC protein, and that the observed fluorescent pattern of such fusion proteins in gametocytes was possibly compatible with their localization in OBs.

This hypothesis was tested by IFA using two different α-GFP antibodies to mark the above OBC fusion proteins and antibody to the OB resident protein Pfg377. Results of the IFA with anti-GFP antibodies alone showed, in the case of SUB2-GFP, OBCIp-GFP and DPAP2-GFP gametocytes, a

granular GFP-associated fluorescence pattern reminiscent of that found with antibodies against Pfg377, while PSOP12-GFP1 gametocytes showed a more diffuse fluorescence pattern in the apices of gametocytes.

In double IFA where the α-Pfg377 antibodies were used in conjunction with α-GFP antibodies, it was possible to show colocalization of the fluorescence signals associated to anti-GFP antibodies with that of the anti-Pfg377 antiserum in gametocytes expressing the SUB2, OBCIp and the DPAP2 fusion proteins, indicating localization of the GFP-tagged proteins in the Pfg377-positive organelles.

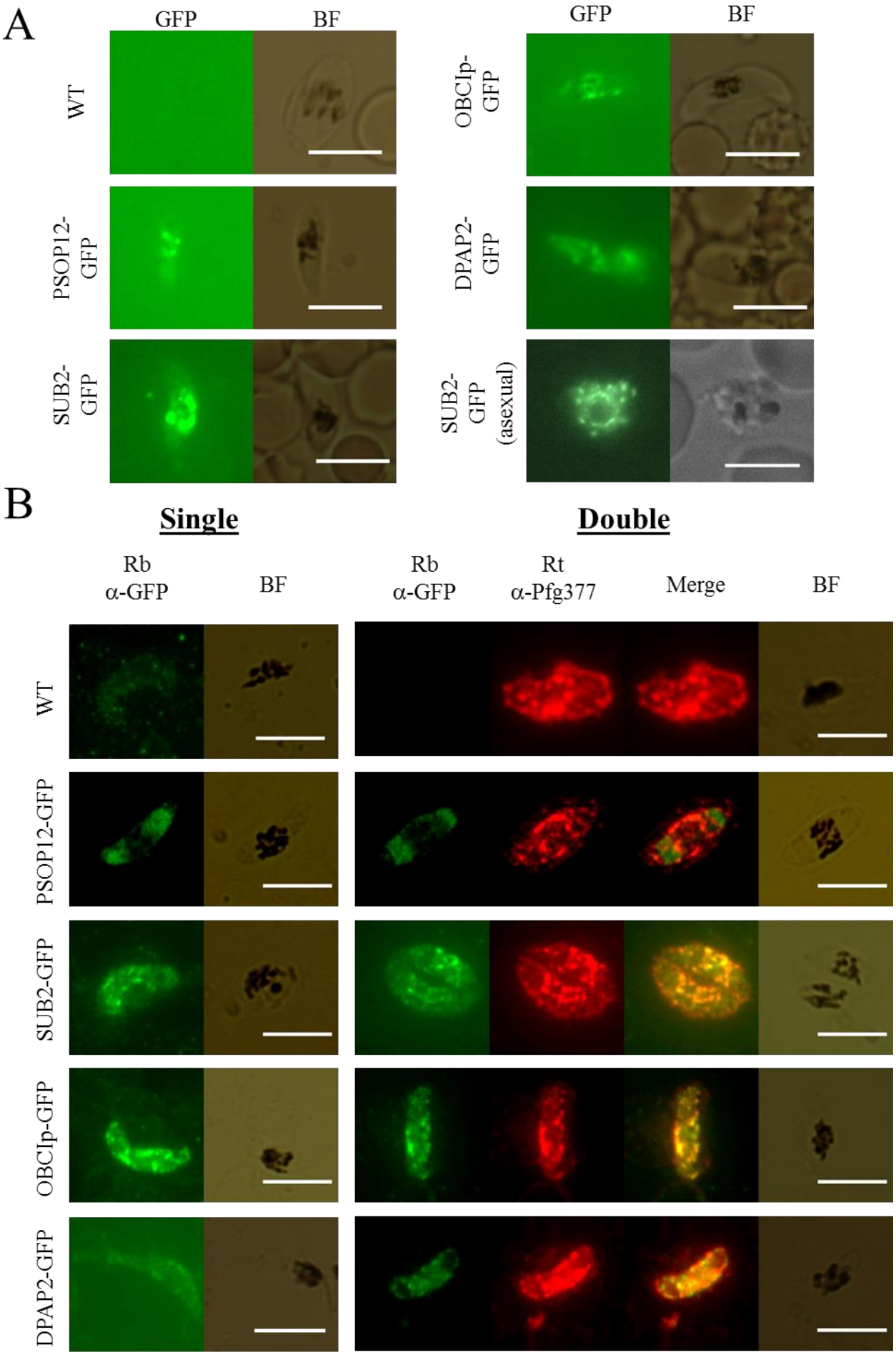
In contrast, in the case of PSOP12, the diffuse apical signal observed in single IFA with anti-GFP antibodies was observed again in the above double IFA, and it did not colocalize with the granular pattern associated to α-Pfg377 antibodies.

These results strongly suggest that the proteins PfSUB2, PfOBCIp and PfDPAP2 localize to the OBs in *P. falciparum* gametocytes (figure 7).

To investigate the presence of OBC proteins in male gametocytes, double IFA with α-Pfg377 and α-GFP antibodies was conducted on OBC-GFP gametocytes. Gametocytes negative for Pfg377-associated fluorescence, considered to be male gametocytes, were evaluated for the presence or absence of GFP-associated fluorescence. In the case of SUB2-GFP and OBCIp-GFP parasites, male gametocytes were negative for GFP-associated fluorescence. PSOP12-GFP male gametocytes showed the apically associated GFP fluorescence pattern described in female gametocytes, while for DPAP2-GFP not enough parasites have been examined so far to evaluate the presence or absence of the tagged protein in male gametocytes. These results support a possible sex-specific expression of SUB2 and OBCIp in gametocytes.

Taken together, our results show that the GFP-tagged SUB2, OBCIp and DPAP2 proteins localize to the OBs in female gametocytes.

Figure 7. GFP-associated fluorescence patterns of OBC-GFP gametocytes. **A**: Live parasites of OBC-GFP lines. OBC-GFP gametocytes (and schizonts in the case of asexual pararasites of the SUB2-GFP line) produce GFP-associated fluorescence when observed in vivo. **B**: IFA of OBC-GFP parasites using Rabbit α-GFP antibodies. Mature gametocytes from the tagged strains were fixed on acetone and incubated with the indicated antibodies. Fluorescein and rhodamine were used as the secondary antibodies for α-GFP and α-Pfg377 antibodies respectively. Left panel: single α-GFP IFA. Right panel: Double α-GFP/α-Pfg377 IFA. WT: Wild Type parasites. BF: Bright field. Rb: Rabbit antibodies. Rt: Rat antibodies. Scale bar: 5μm.


A
GFP
BF
WT
PSOP12-GFP
SUB2-GFP
OBCIp-GFP
DPAP2-GFP
SUB2-GFP (asexual)
B
Single
Double
Rb α-GFP
BF
Rb α-GFP
Rt α-Pfg377
Merge
BF
WT
PSOP12-GFP
SUB2-GFP
OBCIp-GFP
DPAP2-GFP

Functional characterization of the role in gamete egress of the newly identified osmiophilic body proteins

Generation of OBC-defective *P. falciparum* lines

Genes coding for OBC proteins were then selected to be disrupted through double crossover recombination, using plasmid pCC1 [84]. Plasmids pCC1-GEST-KO, pCC1-PSOP12-KO and pCC1-OBCIp-KO were constructed and transfected into parasites, to generate the GEST-KO, PSOP12-KO and OBCIp-KO strains through double crossover recombination. DPAP2-KO1, a clone from a published DPAP2KO strain, was kindly provided by Kim Williamson, Loyola University, Chicago, USA [85]. In the case of SUB2, disruption of the gene has been unsuccessful previously [86], which supports the notion that the protein is essential for the asexual cycle, and therefore a plasmid to generate SUB2KO parasites was not produced.

Parasite clones GEST-KO1, PSOP12-KO1 and OBCIp-KO1 were obtained for each strain, and the structure of the target gene was evaluated through diagnostic PCR. Genomic DNAs from PSOP12-KO1 and OBCIp-KO1 were able to produce PCR products indicative of double crossover recombination in their respective loci. DNA from OBCIp-KO1 was not able to produce the product associated to the WT locus, while minimal amount of this product was produced in samples from PSOP12-KO1 DNA, indicating a small population of parasites containing this unmodified locus in the culture. DNA from GEST-KO1 clone was able to generate the PCR product associated to 5' integration of the plasmid through homologous recombination, but failed to generate the product associated to 3' integration and consistently generated the product associated to *pfGEST* WT locus. Several more GEST-KO clones produced the same results. IFA was conducted on gametocytes from the GEST-KO1 clone with a-GEST antibodies, showing that they produce a GEST-associated pattern indistinguishable from that observed in WT gametocytes, and therefore GEST-KO parasites were discarded for further study.

Parasite clones PSOP12-KO1, OBCIp-KO1 and DPAP-KO1 were selected for further characterization.

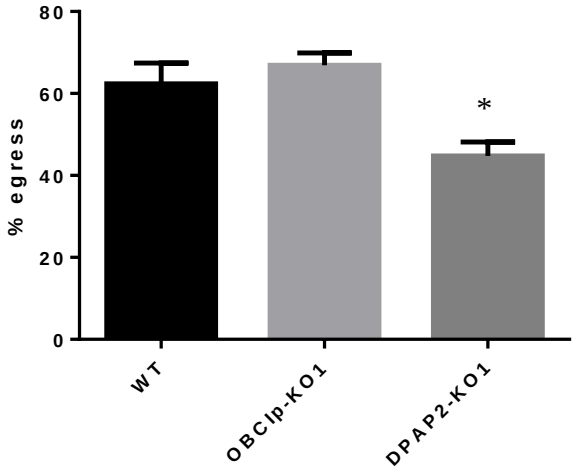
Characterization of the egress phenotype of OBC-KO gametocytes

Although our results indicate that *pfg377*KO parasites can egress efficiently from the host RBC, the discharge of the OB content during gametogenesis and the important role of two proteins present in the OBs of *P. berghei* in the efficiency of this process [44, 78] suggest that these organelles or their resident proteins may still play some role in gamete egress.

To further investigate this point, we evaluated the egress efficiency of gametocytes of the above OBC-KO clones. In this work we observed that parasites of the OBCIp-KO1 and DPAP2-KO1 clones produced mature gametocytes, while the PSOP12-KO1 clone did not produce gametocytes in repeated attempts of induction of sexual development.

Gamete egress efficiency was measured with the new protocol described above in the above two defective parasite clones able to produce gametocytes [87]. No difference in egress efficiency was found in OBCIp-KO parasites compared to WT parasites, while DPAP2-KO gametocytes showed a slightly reduced egress efficiency (from 60% to 45%) compared to that of WT gametes, which was found to be statistically significant (figure 8).

Figure 8. Egress efficiency of OBC-KO gametocytes. Egress is given as % of WGA negative spherical gametes over total spherical gametes. WT and OBC-KO mature gametocytes were activated in presence of XA and their egress efficiency evaluated in 4 replicate experiments. Error bar: SD. n=200. ∗: Significant egress deficiency compared to WT gametes.



Discussion and future prospects

In my thesis, I have aimed to gain understanding of the processes involved in *P. falciparum* gamete egress from its erythrocyte host and in the subsequent events of gamete fertilization and mosquito infection. I approached this problem attempting to develop new tools to study it and to specifically dedicating my attention to osmiophilic bodies, organelles proposed to be a key organelle in gamete egress.

Development of a protocol to measure gamete egress

I have developed a fast and simple tool to evaluate gamete egress efficiency in Plasmodium, making it possible to study multiple samples in a short time. Previous methods used to measure gamete egress were lengthy and invasive, and relied on indirect methods based on antibody staining of either the gamete surface or of the erythrocyte surface [43, 75]. Partly for the lack of an easy and reliable method to measure egress of the female spherical gametes, numerous studies of proteins related with gametogenesis failed to measure egress and concentrated in the more obviously detectable process of male gamete exflagellation. As male gametocytes are a minority of the gametocytes in a culture due to the female-biased sex ratio of Plasmodiun sexual stages, this approach only indirectly evaluated the egress of a fraction of the total gametes of the sample. In contrast, in the protocol that I developed, the evaluation of the presence or absence of the host RBC through the signal emitted by a WGA-conjugated fluorophore that selectively binds to RBCs can be achieved in short times and constitutes a more straightforward measure of gamete egress, allowing evaluation of the egress of all gametes in the sample.

Pfg377 is not necessary for efficient gamete egress

To date, the few proteins known to specifically reside in OBs, largely in the rodent malaria parasite *P. berghei*, had been implicated in egress, consequently with mosquito infectivity being affected as well [43, 44, 75, 77, 78]. Of the few proteins localized to OBs, only Pfg377 had been identified in these organelles in *P. falciparum* gametocytes. The lack of OBs observed in *pfg377*KO gametocytes was proposed to be associated to a defect in gamete egress. However I could not detect any obvious defect in egress compared to WT parasites when I repeated these experiments with my newly developed protocol. This result was later confirmed in the analysis of egress efficiency conducted on the novel *pfg377*KO parasites described in this project, further supported by a minimal, if any, egress defect reported in *P. berghei* gametes defective for the homologue Pbg377 [43]. It is possible that the

previous report of decreased gamete egress efficiency in *pfg377*KO parasites was related to a bias in the method used to evaluate egress.

As the article describing the egress defect of the *pfg377*KO gametocytes also showed that these parasites failed to efficiently infect mosquitoes, such a mosquito infectivity phenotype needs to be re-evaluated as well.

Identification of new proteins of the OBs in *P. falciparum* gametocytes

The unique possibility to exploit OB-depleted *pfg377*KO gametocytes allowed us to design a comparative proteomics approach to identify new OB-resident proteins. Initial attempts to obtain purified OBs or a cellular fraction enriched for these organelles through gradient centrifugation using WT gametocyte samples were unsuccessful. This led us to choose a whole cell quantitative proteomic analysis to compare the proteomes of WT and OB-depleted gametocytes.

Our hypothesis was that proteins normally localized to OBs in WT gametocytes would be underrepresented in OB-depleted *pfg377*KO gametocytes. Based on what is known about protein trafficking to other secretory organelles of Plasmodium parasites [69], these proteins are expected to be included in vesicles originated in the Golgi apparatus. The lack of their target organelle, while not affecting the synthesis of such proteins, would result in their accumulation in the cytoplasm, making it possible that they are more exposed to degradation, resulting in a lower amount of these proteins in the OB-depleted mature gametocytes compared to WT parasites. This process would affect different proteins in a different way, depending on their folding and solubility properties, possibly associated to different degradation rates. This could result in only a subpopulation of OB proteins actually being underrepresented in *pfg377*KO parasites. All OBC selected through this comparative approach contain a transmembrane domain in their sequence; it is therefore possible that such proteins are more subject to degradation in absence of their target organelles, and therefore were more underrepresented in *pfg377*KO gametocytes and more easily detectable as OBCs.

In addition to the above ‘omics’ approach, I also undertook a co-IP approach using Pfg377 as bait in order to include candidate interactors of Pfg377 to the OBC list. This experiment resulted in identifying an additional candidate (OBCIp) to the list of OBCs from the above analysis, and also identified one of the proteins selected in the above comparative proteomics study (PfPSOP12).

The localization analysis conducted on some of the OBC candidate gene products largely confirmed that the above approaches were suited to identify proteins in these organelles as two of the three

candidates selected through this method (PfSUB2 and PfDPAP2) were indeed shown to reside in OBs.

The case of PSOP12 in contrast showed that the comparative proteomics method could also detect false OB candidates. More data will be necessary to establish the cause of this, as we have not been able to detect expression of PSOP12-GFP to a specific location in gametocytes in our experiments, and therefore we cannot exclude that the endogenous protein is directed to the OBs. On the other hand, there are several explanations for an absence of PfPSOP12-GFP in the OBs. Although our co-IP data support an interaction between the OB-resident Pfg377 protein and PSOP12, this interaction could occur at a later stage in parasite life cycle. Recent reports indicate that the homologue PbPSOP12 is located in the surface of *P. berghei* gametes [88]. Therefore, it will be important to evaluate the presence of PfPSOP12 in the surface of gametes.

Taking into account the co-IP and the comparative proteomics approaches, and the inclusion of the *P. falciparum* homologue of a *P. berghei* OB protein, we have shown that four of the five selected proteins localize to OBs in mature *P. falciparum* gametocytes, greatly expanding the number of proteins known to be targeted to these organelles.

Characteristics of the newly identified OB proteins

Of the newly identified OB proteins, PfGEST and PfOBCIp lack known functional domains, and therefore it is difficult to predict their possible function in the parasite. The gamete egress and sporozoite traversal defect in *pbGEST*KO parasites suggest that the role of PfGEST, localized to the OBs as well, may be similar to that of its homologue in *P. berghei*. In the case of PfOCBIp, there is no obvious way of inferring its possible function in the mosquito stages of the parasites.

PfSUB2 and PfDPAP2 are proteases. PfSUB2 is a metalloprotease essential in the asexual cycle, where it is necessary for the invasion of new RBCs by merozoites [89]. PfSUB2 has been observed previously in the ookinete stage, where it is secreted to the cytoplasm of invaded cells of the mosquito midgut [90]. This raises the question of whether PfSUB2 is stored in OBs and then discharged to the surface of the gamete for a later role in mosquito invasion.

PfDPAP2 is an aminopeptidase expressed only in sexual stages [81]. Based on the essential role of the homologue PfDPAP1 in hemoglobin degradation in the food vacuole and development of asexual parasites [91], *pfDPAP2*KO gametocytes were produced and its development was evaluated, but no defect was observed [85]. Additionally, *pbDPAP2*KO parasites showed normal development and

mosquito infectivity, although a compensatory effect was observed in these parasites, in the form of an increased transcription of *pbDPAP1*.

Our finding that PfDPAP2 is localized to secretory organelles likens it to the other homologue in the family, PfDPAP3. PfDPAP3 has a granular distribution in merozoites, possibly localizing to membranous organelles [92]. PfSUB1 is present in merozoite exonemes, and is a protease necessary for the egress of merozoites from its host RBC. It has been observed this PfDPAP3 is able to cleave PfSUB1, which fits with its observed granular distribution in merozoites. The parallelism between the location of these micronemal proteases and the newly found OBs proteases begs the question of whether DPAP2 may play a role in cleavage of SUB2 in OBs.

Our observation that DPAP2-defective gametes have a minor egress defect will have to be confirmed by further observations. The possible change in PfDPAP1 protein levels in *pfDPAP2*KO gametocytes will also be addressed, as a compensation mechanism could be hiding an important role of PfDPAP2 in gamete egress or later mosquito stages.

Reevaluating the role of OBs

Several proteins previously identified as OB components have been linked to a role in gamete egress [75, 77, 78]. This, together with the observed secretion of the content of these organelles during gametogenesis, led to the proposition of OBs as a key organelle in this process. In the only study quantifying the gamete egress of gametocytes in absence of an OB protein, the gamete egress defect found in *Pfg377*KO parasites was not as marked as the defect in mosquito infectivity. This was hypothesized to be the result of a relatively minor disruption in the precise process of gamete egress greatly impairing gamete efficiency to continue the infection into the mosquito [75].

In contrast with the previously proposed role of OBs in gamete egress, we have shown that a depletion of these organelles in *pfg377*KO parasites does not affect egress efficiency in *P. falciparum* gametes. This observation, coupled with the very marked decrease in mosquito infectivity in parasites lacking Pfg377 [75], suggests that components of OBs could have a role after egress in later mosquito stages. It is possible to propose that to have a later role in the parasite life cycle, proteins originally in the OBs of gametocytes could remain in the gamete surface after egress. PfPSOP12 is a clear candidate to be present in the gamete surface, as its *P. berghei* homologue has been found there after gametogenesis. PfSUB2 is found in the surface of merozoites previous to RBC invasion, which suggests that PfSUB2 expressed in gametocytes could also locate to the surface of the parasite once gametogenesis takes place. PfGEST is also expected to remain in the gamete surface, as suggested by

our WB data. It will be important to evaluate the presence of these and other OBC in gametes with the tools we have developed.

OBs have been traditionally linked to gamete egress. In the light of our results, we propose that components of the OBs could play a direct role in later mosquito stages, explaining their essential role in mosquito infectivity independently of their importance during gamete egress.

Future directions

Several questions remain unanswered and further experiments will be conducted to understand the role of the newly identified OB resident proteins. Main directions of these activities are:

- The expression and localization of OBCs during gametocyte development, in gametes and in early mosquito stages will be studied through additional WB, electron microscopy and IFA experiments.
- Further attempts to produce GEST-KO mutants will be done and a second clone from an independent transfection of the OBC-KO lines will be obtained. These new OBC-KO parasites will allow a more consistent evaluation of a possible gamete egress defect in these parasites.
- The phenotype of the PSOP12 mutant clone unable to produce gametocytes will be evaluated with additional independent clones to investigate a possible role of the PSOP12 expression in gametocyte production.
- A conditional *pfSUB2*-KO line will be generated, enabling to study the function of this important protein in asexual and sexual stages.
- Mosquito infectivity will be evaluated in *pfg377*KO and OBC-KO parasites, to confirm the published phenotype in the former and investigate any defect in infectivity in the later.

The results obtained in this thesis open new directions to explore the function of OBs. To date, few proteins have been detected in the surface of gametes, and several have proven to be interesting candidates for transmission-blocking antibodies [88, 93, 94]. Our finding of several new OB resident proteins that may stay in the surface of the gamete could increase this reduced list and provide new candidates for transmission-blocking vaccines in the long term.

Additionally, the substrates of the newly found OB resident proteases will be explored. PfSUB2 has also been evaluated as a target for protective antibodies [95], and its presence and possible role in sexual stages of *P. falciparum* could improve its suitability as a target, giving the possibility to tackle both asexual and parasite progression. It is important to establish the mechanisms of action of PfSUB2 and PfDPAP2 in the sexual stages, which will allow to better evaluate their possible use as antigens for transmission-blocking antibodies or as targets for specific inhibitory compounds.

Final conclusions

I have in this thesis aimed to understand the components and role of OBs, the only known secretory organelles in *P. falciparum* gametocytes. Taking advantage from the OB depletion of parasites defective in Pfg377, previously the only known protein specific of these organelles, I have identified four new component of OBs and evaluated their role in gametogenesis, also developing for this purpose a new protocol to facilitate the measurement of gamete egress in Plasmodium parasites. This work resulted in the unexpected finding that Pfg377-defective parasites and parasites defective in the OBC proteins identified in this work showed no defect in gamete egress, with only the DPAP2-defective gametes slightly impaired in their egress. Further experiments measuring gamete egress of several other identified *P. falciparum* OB resident proteins will be conducted as well. Most importantly, mosquito infectivity will be evaluated in the new OBC-KO parasites, in the case of Pfg377 to confirm the previously described defect, and in the case of the rest of OBC-KO parasites to uncover a possible role of the disrupted genes in mosquito phases after gamete egress.

Our identification of two proteases resident in the OBs, together with the predicted targeting to the surface of several proteins of these organelles, positions OBs as a possible key organelle not only in gamete egress but also in mosquito invasion processes.

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Part II

Published paper: A fast, non-invasive, quantitative staining protocol provides insights in *Plasmodium falciparum* gamete egress and in the role of osmiophilic bodies

Authors: Pablo Suárez Cortés, Francesco Silvestrini and Pietro Alano

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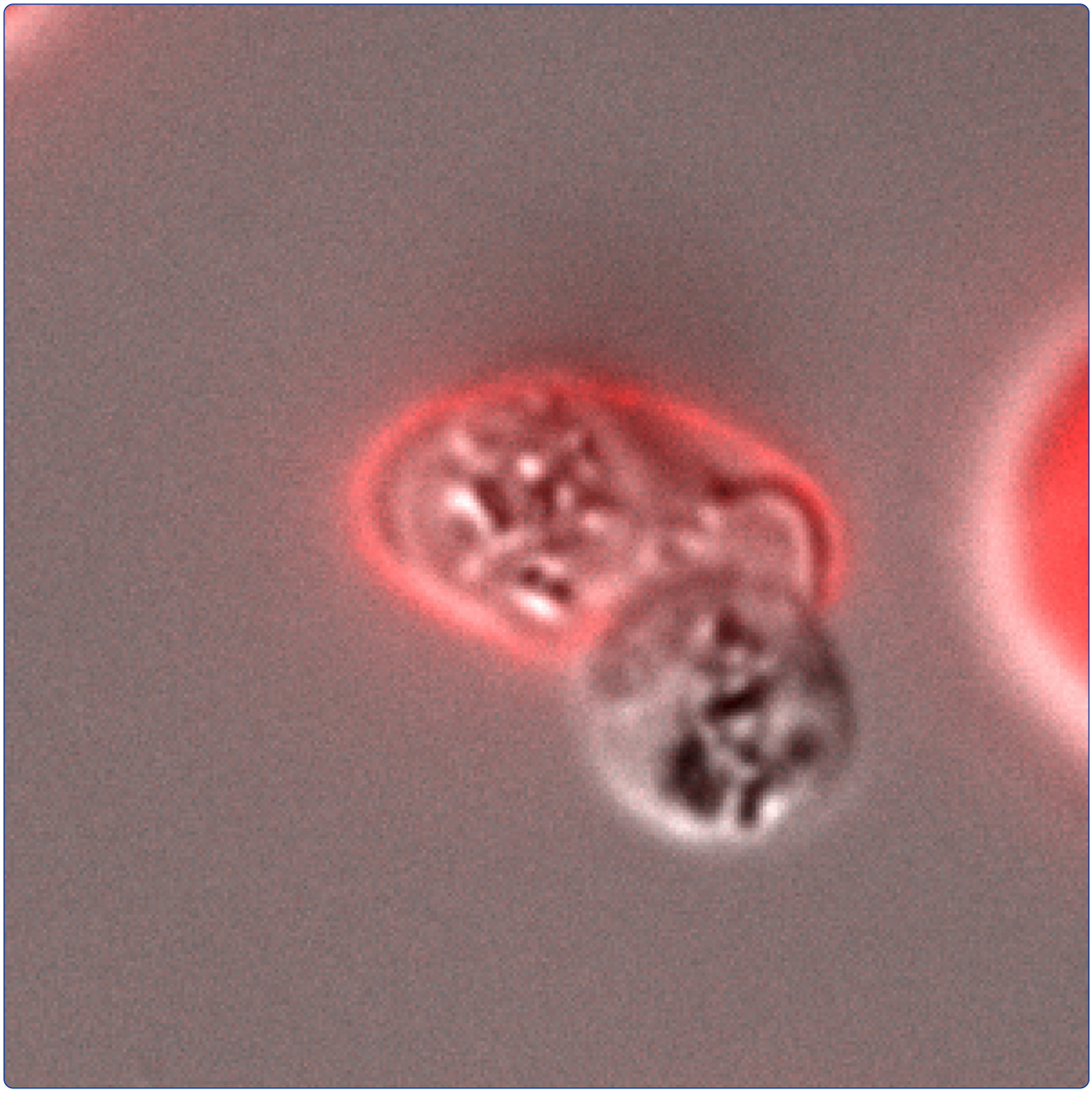

A fast, non-invasive, quantitative staining protocol provides insights in *Plasmodium falciparum* gamete egress and in the role of osmiophilic bodies

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METHODOLOGY Open Access

A fast, non-invasive, quantitative staining protocol provides insights in *Plasmodium falciparum* gamete egress and in the role of osmiophilic bodies

Pablo Suaréz-Cortés, Francesco Silvestrini and Pietro Alano*

Abstract

Background: Ability of *Plasmodium falciparum* gametocytes to become extracellular during gametogenesis in the mosquito midgut is a key step of the parasite life cycle. Reliable and quantitative measurement of the efficiency of gamete egress is currently constrained by the fact that this phenomenon is usually observed and quantified *in vitro* either by live microscopy, by statistically limited ultrastructural analysis or by surface antibody-based protocols which can interfere with this fast and complex cellular process.

Methods: A protocol was developed based on fluorescent wheat germ agglutinin (WGA) surface staining of erythrocytes containing mature *P. falciparum* gametocytes. After a single centrifugation step and within minutes from the induction of gametogenesis, the activated gametes can be inspected for presence or absence of the fluorescent WGA staining of the host erythrocyte membrane and scored respectively as intracellular or emerged from the erythrocyte.

Results: Gametogenesis and gamete egress from WGA surface stained, infected erythrocytes occur with normal kinetics and efficiencies. Quantitative measurements of gamete egress can be obtained in live and in paraformaldehyde-fixed cells, which validates this protocol as a suitable tool both for live imaging studies and for higher throughput applications. The protocol was used here to provide functional information on the ability of gametes to egress through a single exit point induced in the host red blood cell membrane, and to re-analyse the phenotype of Pfg377- and osmiophilic body-defective gametes, suggesting that such parasite components are not directly involved in disruption and shedding of the erythrocyte membrane in female gamete egress.

Conclusions: The development of a reliable, fast, non-invasive and quantitative protocol to finely describe and to measure efficiency of *P. falciparum* gamete egress is a significant improvement in the tools for functional studies on this key process of the parasite life cycle. This protocol can be used to investigate the molecular mechanisms underlying gamete egress and its adaptation to high throughput applications will enable identification of transmission blocking inhibitors.

Keywords: *Plasmodium falciparum*, Gametocytes, Gametogenesis, Parasite egress, Wheat germ agglutinin, Osmiophilic bodies, Mosquito transmission

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Background

The observation that inhibition of *Plasmodium falciparum* transmission has beneficial effects on incidence and morbidity of malaria [1] boosted a renewed interest in the biology of parasite sexual stages and in the design of *Plasmodium* transmission blocking strategies. Improvement of available methodologies to dissect and quantitatively monitor cellular mechanisms of parasite sexual differentiation becomes an essential activity to identify critical parasite targets and to assess the efficacy of anti-transmission interventions.

The transformation of the intracellular *P. falciparum* mature gametocytes into extracellular gametes is definitely one of the most critical points and a significant bottleneck in the parasite life cycle [2-4]. In this process, which occurs within minutes from the uptake of the blood meal in the mosquito gut and that can be readily reproduced *in vitro*, elongated mature stage V gametocytes of both sexes initially become round shaped within the infected erythrocyte ('rounding up'), then progressively disrupt the surrounding parasitophorous vacuole membrane (PVM) and the erythrocyte membrane to finally egress. During this process the male gametocyte undergoes a dramatic transformation leading to the production of eight haploid motile microgametes, whilst the female gamete retains its spherical shape after egress and can be fertilized by a male gamete to produce a zygote, which further transforms into an ookinete.

The molecular and cellular mechanisms responsible for gamete egress are comparatively less understood than those governing egress of merozoites at the burst of asexual schizonts. In *P. falciparum* treatment with protease inhibitors and ablation of specific parasite genes have been reported to block or inhibit this process in female gametes [5] and in gametes of both sexes [6,7]. In *Plasmodium berghei* the process was blocked in male gametes defective for PbActinII or a perforin-like protein [8,9] and in gametes of both sexes upon disruption of the *pbpeg3mdv1* or the *pbgest* genes [10,11]. Analysis of gamete egress in several studies however largely relied on optical microscopy of live or fixed parasites or on examination of a limited number of independent ultrastructural sections of induced gametes. A reliable quantitative assessment of egress is not straightforward, particularly in the case of the spherical female gametes, and so far relied either on analysis of Giemsa-stained smears or on using two protocols based on cell surface staining with specific antibodies. In one protocol, fixed and reacted spherical gametes are inspected for presence or absence of surface reactivity to antibodies against the red blood cell surface molecule Band3 and respectively scored as intracellular or egressed [6,12]. In the other method, fixed non-permeabilized gametes are analysed for surface reactivity to an antibody against the gametocyte/gamete surface antigen Pfs230, in this case with the positive cells scored as successfully egressed [5].

As both protocols require parasite fixation followed by incubations with primary and secondary antibodies and several washing steps, one concern was that such lengthy manipulations might alter integrity of the parasite and/or host cell membrane compartments, which are rapidly rearranged in gametogenesis, thus confounding the evaluation of egress. For this reason, a much faster and simpler protocol was developed to measure gamete egress, based on the labelling of infected erythrocytes prior to induction of gametogenesis. The protocol described here minimizes cell manipulation and experimental time prior to analysis and it is particularly suited to measure egress in female gametes, the most difficult to be unambiguously scored by optical microscopy. The new protocol was used here to closely follow disruption of the erythrocyte membrane in gamete egress and to further analyse the role in this process of osmiophilic bodies (OBs), the gametocyte secretory organelles abundant in female gametocytes.

Methods

Plasmodium falciparum parasites and cultures

Parasites from clones 3D7 and *pfg377KO* B7, E4 and G5 [5] were cultured in 0^+ human red blood cells at 5% haematocrit in RPMI 1640 plus hypoxanthine 50 μg/ml, HEPES 25 mM, 0.225% sodium bicarbonate and 10 mg/ml gentamicin, supplemented with 10% heat inactivated human serum. Parasites were kept at 37°C, in a 2% O_2, 5% CO_2 and 93% N_2 atmosphere. For gametocyte production, asynchronous parasites were grown to high parasitaemia (>8%) and culture medium was doubled at this point. The day after, medium was changed and N-acetylglucosamine 50 mM added. N-acetylglucosamine was maintained for three days until no asexual parasites were detected in the culture. Stage II gametocytes were detected 48 hours after the addition of N-acetylglucosamine while mature stage V appeared nine days after the start of the treatment.

Erythrocyte surface labelling and gamete egress measurement

Cultures of red blood cells (RBC) uninfected or infected with mature stage V gametocytes were incubated with 5 μg/ml WGA-Alexa Fluor conjugate (Life Technologies) at 37°C for 15 min. Gametogenesis was then induced by pelleting the samples at 1,000 g for 1 min and rapidly resuspending cells in the selected conditions. For xanthurenic acid (XA) induction, 20 μM of the compound was added to complete medium with serum. In the case of gametogenesis induced by an increase in pH, complete medium was adjusted to pH 8.2 with NaOH. After resuspending the samples, cells were incubated at 25°C for the desired time during which gametogenesis took place. At the various time points, gametes could be observed alive or fixed for 30 min

at room temperature in 1% paraformaldehyde. After either sedimentation for two hours at room temperature or centrifugation at 1,000 g for 1 min, stained cells were resuspended at 50% haematocrit in the same medium, mounted in a microscope slide under a sealed coverslip. In the analysis of live cells the sample was transferred to ice until observation to minimize metabolic activity in the parasites. Gametes were identified in bright field as round, pigmented objects with a diameter of about 5 μm and then evaluated for the presence or absence of WGA surface fluorescence, diagnostic respectively of the gamete intracellular or extracellular state. Percent of egress in the experiments described here was measured counting a minimum of 100 gametes per sample. A Leica DMRB microscope was used to visualize live samples. Images were acquired using a Leica DFC340 FX camera through a Leica PL FLUOTAR 40x objective. Filters used to detect Alexa 488 fluorescence were: EX: 515–560, EM: 590 long-pass filter. A Deltavision Elite microscope was used to visualize fixed samples. Images were acquired using a Coolspnap HQ2 CCD camera through an Olympus 100X UPlanSApo NA1.4 objective. Filters used to detect Texas Red fluorescence were: EX 575/25, EM 632/60. Images were processed using ImageJ 1.46r (NIH).

Results and discussion

Egress of *Plasmodium falciparum* gametes from fluorescently labelled erythrocytes

In order to restrict to a minimum the manipulation of the sexual stage parasites before and after gametogenesis, it was decided to obtain mature gametocytes contained in fluorescently stained erythrocytes. WGA covalently linked to fluorescent dyes such as Texas Red or Alexa 488 was chosen for this purpose. As it was previously reported that WGA staining increases adhesive properties and rigidity of RBCs [13], it was preliminarily established that using WGA concentration up to 5 μg/ml with volumes between 0.1 to 1 ml of blood at 3-4% haematocrit did not produce cell clumping. A 15-min incubation of uninfected and infected erythrocytes at 37°C with 5 μg/ml fluorescent WGA was sufficient to obtain cell samples in which virtually all erythrocytes showed a clear and homogeneous fluorescent signal on their surface (Figure 1A). The signal showed a virtually indistinguishable intensity on the surface of uninfected and infected RBCs, also in the case of erythrocytes deformed by the elongated mature gametocytes (Figure 1B, left panel).

In order to measure egress, synchronous stage V gametocyte cultures (routinely 2-3% gametocytaemia) were stained as described above. A 0.5 ml sample of the stained gametocyte culture was centrifuged for 1 min and the pelleted RBCs were immediately resuspended at 25°C in complete medium with 20 μM XA to trigger rounding up and gamete formation. Parasites were analysed between 5 and 15 min post activation, and spherical gametes were readily identified at all time points in bright field microscopy, as expected. Inspection of the spherical parasites with appropriate filters enabled to clearly distinguish cells still surrounded by the fluorescent WGA signal, which were scored as intracellular gametes (Figure 1B, central panel), from those devoid of any fluorescence, suggesting that they had shed the surrounding erythrocyte membrane (Figure 1B, right panel). A time course measuring the two classes of parasites indicated that gamete egress reaches a plateau within 10–15 min from induction (Figure 2A), showing comparable kinetics and efficiency as measured in previously published experiments [5,7,12]. Finally, in order to rule out that free WGA could artifactually decorate the surface of extracellular gametes, a gametocyte culture was divided in two aliquots, one stained with WGA and one unstained. Both were induced to gametogenesis and the latter sample was stained with WGA after 15 min. Counts from pre-stained and post-stained triplicate samples were very similar, respectively measuring a 61.3% (SD: 6,6) and a 60,3% (SD: 3,3) in egress efficiency. In both cases only uninfected RBCs and the residual intracellular gametes were surrounded by the fluorescent signal.

These experiments indicate that mature gametocytes inside fluorescent WGA-stained erythrocytes are able to

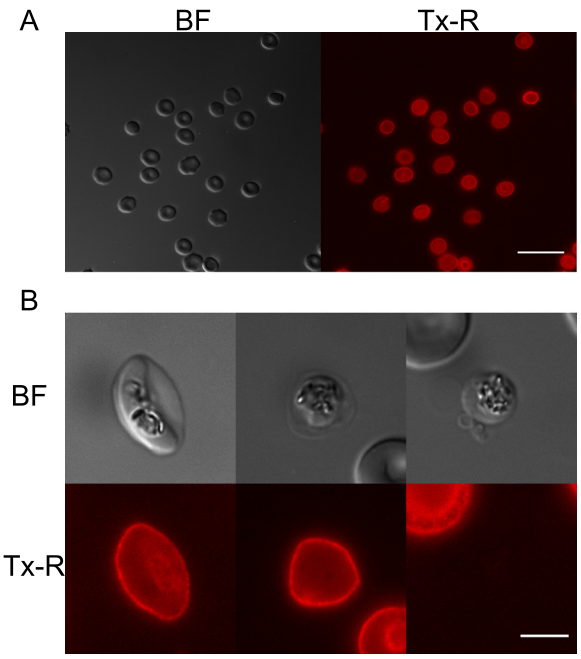

Figure 1 Wheat germ agglutinin-stained uninfected and infected red blood cells. A) Human uninfected RBCs stained by WGA-conjugated Texas Red observed in bright field and under UV light. Scale bar: 20 μm. **B)** Mature gametocyte (left panel), non-egressed gamete (centre), egressed gamete (right panel) stained with WGA-conjugated Texas Red. The Laveran's bib can be seen in the gametocyte as a brighter fluorescent area. Scale bar: 5 μm. BF: bright field. Tx-R: Texas Red.

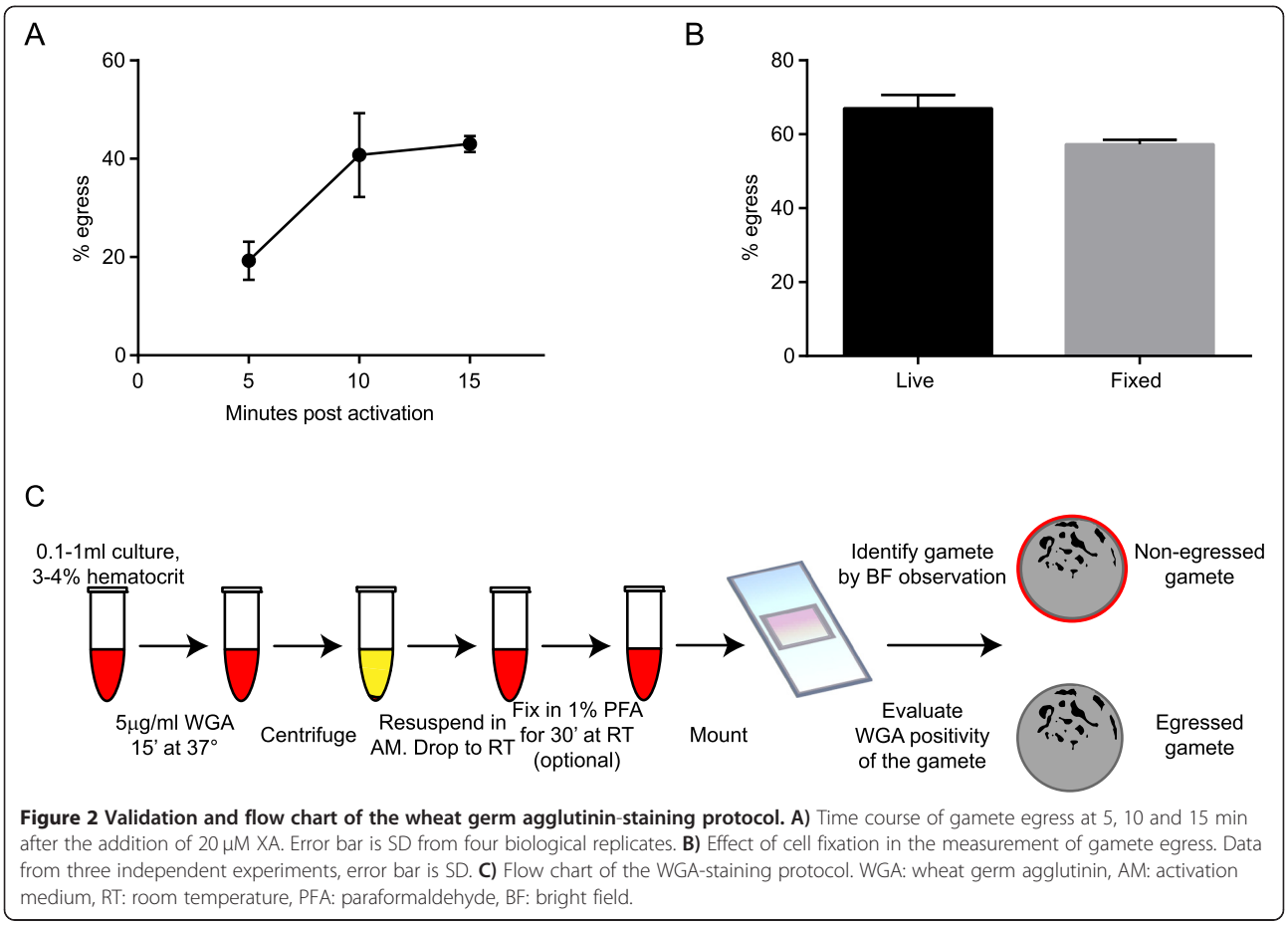

Figure 2 Validation and flow chart of the wheat germ agglutinin-staining protocol. A) Time course of gamete egress at 5, 10 and 15 min after the addition of 20 μM XA. Error bar is SD from four biological replicates. **B)** Effect of cell fixation in the measurement of gamete egress. Data from three independent experiments, error bar is SD. **C)** Flow chart of the WGA-staining protocol. WGA: wheat germ agglutinin, AM: activation medium, RT: room temperature, PFA: paraformaldehyde, BF: bright field.

undergo rounding up and to egress with the expected kinetics and efficiency, and that gametes can be sampled and inspected without further delay after induction to assess their intracellular or extracellular status.

Cell fixation does not interfere with gamete egress and ensures cell sample storage

The above experiments provide the basis for an easy, fast and minimally invasive protocol to visualize and measure gamete egress. This protocol is suitable for live imaging applications, time course experiments and determination of dose response effects of treatments or compounds on this process. In order to improve the throughput of the protocol with the possibility to store parasite samples for subsequent analysis, measurements were compared of gamete egress in parallel samples of gametes, sampled at 15 min from induction, treated or untreated with 1% paraformaldehyde for 30 min. Results showed that number of egressed gametes counted in the fixed parasite samples was slightly reduced compared to that measured in the unfixed cells, possibly suggesting that a small fraction of unfixed gametes manages to egress during the sample storage time (Figure 2B). These experiments therefore showed that a cell fixation step provides the important possibility to store samples for subsequent analysis and was for this reason introduced in the flow chart of the protocol (Figure 2C).

To evaluate performance of the newly established methodology, this protocol was applied in a series of experiments addressing distinct aspects of gamete egress.

Live imaging of erythrocyte disruption at gamete egress

The newly established protocol provides the opportunity to follow the events of gamete egress in live parasites. A few live microscopy descriptions exist of this process, although lack of specific markers constrained the unambiguous identification of the membranes involved. For instance, live observations in *Plasmodium gallinaceum* proposed that in female gamete egress, unlike in male exflagellation, the RBC membrane ruptures before the PVM [14], an observation which was not confirmed by subsequent ultrastructural work on female gametes, which supported instead an 'inside-out' egress mechanism [6,15-20]. As in some of these studies extracellular gametes were observed by ultrastructure [6,19] or optical microscopy [20] next to erythrocyte ghosts showing a single large opening, live observations were performed using the new protocol to investigate this point in more

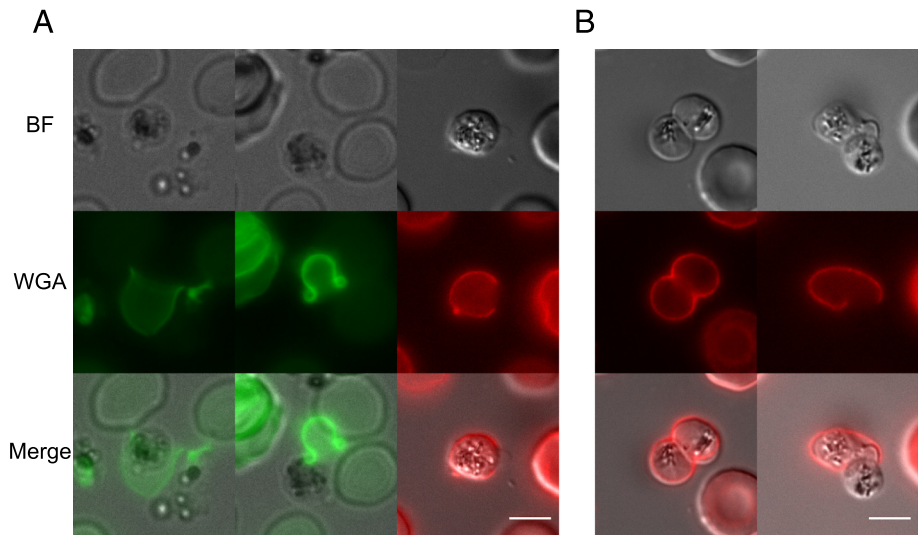

Figure 3 Red blood cell membrane disruption in gametes egress. A) Gamete egress observed on live (left and central panels, WGA-Alexa488 staining) and fixed (right panel, WGA-Texas Red staining) parasites. **B)** Two gametes within the same red blood cell. The host cell shows in one case an intact membrane (left panel) and a single opening at the start of gamete egress (right panel). Scale bars: 5 μm. BF: bright field. WGA: wheat germ agglutinin.

detail. In these experiments it was consistently observed that spherical gametes indeed gain access to the extracellular milieu through a single exit point in the infected erythrocyte membrane (Figure 3A), confirming this egress mechanism [6]. Amongst the at least 50 events observed, it was remarkable to see that also in the few cases of erythrocytes co-infected by two gametocytes, the egress of both spherical gametes was clearly occurring from a single opening induced in the RBC membrane (Figure 3B). These results highlight an intriguing similarity between the egress of gametes and that of merozoites, despite the different size of the two parasite stages. It is described that the burst of the asexual schizont is triggered by the egress of an individual merozoite through a single disruption point induced in the erythrocyte membrane [21]. In the gamete egress experiments erythrocyte ghosts could be occasionally detected whose morphology is intriguingly reminiscent of that of erythrocyte membranes 'curling' upon merozoite release (Figure 3A) [21], leading to speculate that mechanistic aspects of egress may be shared by asexual and sexual stages.

The new protocol enables live observations also of the comparatively less frequent event of male gamete egress. In preliminary observations on 30 male gamete activation events, in 25 cases the exflagellation centres were not associated with a WGA fluorescent signal, whereas in the remaining five cases some of the newly formed gametes were still transiently associated to the erythrocyte ghosts.

As a further improvement, the newly established protocol could be used in live imaging application with gametocytes in which the PVM is fluorescently labelled by specific reporter proteins [20]. This may provide the possibility to obtain a dynamic description of the disruption of

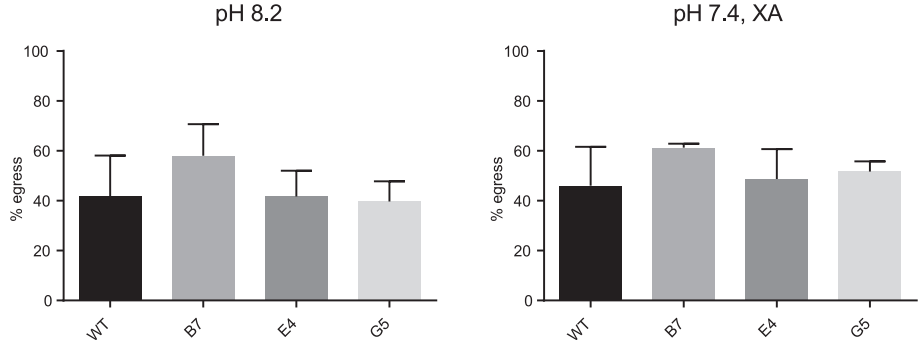

Figure 4 Egress efficiency in *wt* and *pfg377KO* gametes. Egress efficiency of gametes from *wt* and three *pfg377KO* clones after induction with pH 8.2 and pH 7.4 plus 20 μM XA, error bar is SD.

both membrane compartments surrounding the rounded up gamete. So far, a clear distinction between disruption of the two membranes has been chiefly achieved by examination of ultrastructural sections [7,9].

Egress of osmiophilic body-depleted female gametocytes

Given its suitability to reliably measure female gamete egress, the protocol was used to examine the only *P. falciparum* mutant whose gametogenesis was reported to be affected only in this sex. Pfg377 is the only protein described to specifically reside in electron dense organelles named osmiophilic bodies (OBs), which preferentially accumulate in female gametocytes [22,23]. Disruption of the *pfg377* gene results in female gametocytes with a dramatically reduced content of these organelles. Experiments analysing egress of the OB-depleted female gametocytes showed a two-fold reduction in egress efficiency at 15 min from induction in the mutant parasites compared to the parental line [5]. This suggested a possible, albeit non-essential, role of such organelles in this process, also supported by the observation that OBs are no longer detectable as soon as female gametes are formed.

In order to further analyse this point, the egress efficiency of female gametes was re-assessed with the new protocol in the three previously published *pfg377KO* clones, from two independent gene disruption experiments, and in their parental parasites [5]. Egress was measured at 15 min post induction in nine independent experiments, in six cases triggered by raise in pH only and in three by XA only. In all cases, the result was that the previously described two-fold reduction in egress efficiency could not be reproduced (Figure 4). This discrepancy could be explained as follows: in the previous report extracellular gametes were positively identified as fixed, non-permeabilized spherical parasites whose surface was stained by a monoclonal antibody against the gametocyte/gamete surface antigen Pfs230. As in fact was considered in that publication, it is conceivable that the depletion of OBs and/or absence of Pfg377 does not reduce efficiency of egress from the erythrocyte of the mutant gametes but rather it impairs the reactivity of their surface to the anti-Pfs230 antibody, leading to an underestimate of egress efficiency. Examination of *pfg377*KO gamete egress with the new protocol does not support the hypothesis of a major role of OBs in the ability of female gamete to disrupt and shed the erythrocyte membrane during egress. As however the Pfg377-defective parasites showed a dramatic defect in vector transmissibility [5], the above results rather propose that OB discharge and/or the associated Pfg377 mobilization play a functional role in subsequent steps leading to gamete fertilization or in further parasite development in the mosquito.

Conclusions

Studying the egress of *P. falciparum* gametes is important to understand the mechanisms of transmission of the malaria parasite to its mosquito vector. The protocol presented here provides a quantitative method for the fast and simple determination of egress of *Plasmodium* gametes from the infected RBCs. This method is applicable to *in vivo* imaging and, as gametes can be fixed and stored, to higher throughput analyses, such as the screening of compounds inhibiting this key process of the parasite life cycle.

Abbreviations

WGA: Wheat germ agglutinin; OBs: osmiophilic bodies; XA: xanthurenic acid; RBC: red blood cell; PVM: parasitophorous vacuole membrane.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

PSC designed and conducted the experiments and contributed to writing the manuscript; FS proposed and performed preliminary experiments; PA drafted and discussed the manuscript. All authors read and approve the final manuscripts.

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Part III

Manuscript in preparation: Molecular and functional characterization of proteins of the Osmiophilic Bodies of *Plasmodium falciparum*.

Background

Malaria is still a huge problem in tropical latitudes, mainly due to the lack of any effective vaccine against this disease and the appearance of drug resistance in the etiological agent Plasmodium. It is estimated that approximately 200 million cases of this disease took place in 2013, causing over half a million deaths [1].

The most lethal form of malaria is caused by the apicomplexan parasite *Plasmodium falciparum*, which is transmitted to the human host through the bite of an infected Anopheles mosquito [2]. Parasite transmission is achieved through the sexual stages of Plasmodium, the gametocytes, in a complex developmental process which is not fully understood at the molecular level. This configures a scenario in which gaining knowledge into the mechanisms of parasite transmission will be essential in the perspective of malaria eradication.

Gametocytes develop in the human host in the process of gametocytogenesis, which proceeds through five developmental stages for ten to twelve days, until gametocytes reach maturity and are ready to infect the mosquito [3]. Once gametocyte-infected erythrocytes are engorged into the mosquito midgut, a combination of physical and chemical signals triggers the formation of the male and female gametes. Gametogenesis begins with the rounding up of the parasites, after which the surrounding membranes break in an inside-out manner, with parasitophorous vacuole membrane (PVM) breaking first. The erythrocyte membrane (EM) breaks last, resulting in the egress of the gamete from the host cell. Subsequently, male gametes will divide into 8 flagellated microgametes, while female gametes remain as a single macrogamete that is fertilized by a microgamete, forming a zygote. The zygote is then transformed into an ookinete, which will traverse the mosquito midgut and will ensure progression of the life cycle into the insect [4–6].

Osmiophilic bodies (OBs) are organelles found only in Plasmodium gametocytes. They appear from stage III of gametocytogenesis, and are more abundant in female gametocytes. They are defined by a single bilayer membrane, and their content has an electrondense appearance in electron microscopy preparations, which is an indicator of high protein or lipid content. They are oval-shaped and localize to the periphery of the mature gametocyte, underneath the inner membrane complex (IMC). During gametogenesis, OBs disappear after rounding up and before gamete egress, releasing their content, which suggests that they could play a role in gamete egress from the host cell [7].

Pfg377 is the only *P. falciparum* protein known to localize specifically in the OBs [8]. Its homologue in *Plasmodium berghei*, a murine malaria model, is also localized to the OBs in this species. Two other proteins have been localized in the OBs of *P. berghei*: the Gamete Egress and Sporozoite Traversal (PbGEST) protein and the Male Development-1/protein of early gametocyte 3 (PbMDV-1/Peg3) protein. When the *P. falciparum* MDV-1/Peg3 homologue was studied, it was shown that this protein localized to several parasite-derived membranes, not being specific of OBs [9], while localization of PfGEST has not been characterized yet in the human parasite gametocytes. The gene *mdv-1/peg3* has been disrupted both in *P. berghei* and P. *falciparum*. In the human parasite, gametocytes lacking MDV-1/Peg3 were defective in egress from the host erythrocyte and ineffective in their ability to infect mosquitoes [9, 10]. Genetic disruption of the rodent counterpart *pbgest* resulted in a similar phenotype, with gametocytes not being able to efficiently egress from erythrocytes [11].
When the gene for Pfg377 was disrupted in *P. falciparum*, the *pfg377*KO phenotype was that the number of OBs in gametocytes was dramatically reduced compared to WT gametocytes. The lack of Pfg377 was also associated to a defect in egress and to a marked drop in parasite ability to infect mosquitoes. However our recent work [12] indicates that the observed defect in egress could be due to an artifact related to the measurement method [12, 13].

In this work, we aimed to identify and to functionally characterize novel proteins residing in the OBs of *P. falciparum*. For this we exploited the unique feature of Pfg377 defective parasites, depleted from OBs.

Material and Methods

Plasmodium falciparum culture

Parasites were cultured in 0+ human red blood cells at 5% haematocrit in RPMI 1640 plus hypoxanthine 50 μg/ml, HEPES 25 mM, 0.225% sodium bicarbonate and 10 mg/ml gentamicin, supplemented with 10% heat inactivated human serum. Parasites were kept at 37°C, in a 2% O, 5% CO2 and 93% N2 atmosphere. For gametocyte production, asynchronous parasites were grown to high parasitaemia (>8%) and culture medium was doubled at this point. The day after, medium was changed and N-acetylglucosamine 50 mM added. N-acetylglucosamine was maintained for three days until no asexual parasites were detected in the culture. Stage II gametocytes were detected 48 hours after the addition of N-acetylglucosamine while mature stage V appeared nine days after the start of the treatment. Percoll purification of gametocytes was performed as described [14]. Protocols used to thaw and freeze parasites are described elsewhere [15].

DNA extraction from parasites

Genomic DNA from asexual parasites for southern analysis as well as for PCR analysis was obtained using standard procedures [15]. Asexual parasites were pelleted at 2000g for 5 minutes and resuspended in 0.15% saponin solution to break up RBCs. The remaining parasites were pelleted at 10.000g for 1 minute and the pellet frozen for later processing. Genomic DNA was released by resuspending pellet in 1x PCR reaction buffer and boiling for 5 minutes. Supernatant containing genomic DNA was then stored at -20o and later used as template in PCR reactions. Alternatively, QIAamp Blood Mini Kit (Qiagen) was used to obtain Genomic DNA from saponinated parasites.

Production of transfectant parasites

Ring stage parasites at 2–3% parasitaemia were transfected by electroporation with 80–100 mg of transfection vectors using the following conditions: voltage: 0.31 kV, capacitance: 960 mF, resistance to infinity [16].Transfected parasites were then added to 5 ml of fresh media, 5% haematocrit. Parasites were cultures without drug selection for 24 hours and WR99210 5nM was then added. Medium was renewed every day during the first 4 days, and every two days after that. Fresh 1% haematocrit RBCs were added every 7 days until resistant parasites appeared, which happened from 14 days after transfection. To increase the population in which plasmid integration had happened, parasites were then cultured for a week with no drug pressure, thus eliminating the majority of episomal plasmids, and WR was added again to select for plasmids with integrated parasites. Stable

stocks of the resulting parasites were then frozen. In the case of KO plamids, negative selection was then performed adding 40 μM 5-fluorocytosine (5-FCT) to the medium [17]. Parasites resistant to both WR and 5FCT appeared in Giemsa smears after a week of culture, and stable frozen stocks of these cultures prepared. GFP tagged and KO lines were then cloned by limiting dilution [18].

Antibodies

Antibodies against Pfg377. Polyclonal serum produced in rat against B region of Pfg377 was described in previous work [8]. In the production of rabbit polyclonal against B region of Pfg377, the antigen described previously was used to immunize a rabbit (New Zealand). For immunization, 200 mg of recombinant protein in 1 ml of PBS and complete and incomplete Freund Adjuvant were injected subcutaneously on three subsequent occasions, followed by a final injection of 100 mg of the protein. Rabbit blood samples were naturally clotted at 37°C, and serum was obtained after centrifugation. Rat antibodies against A2 described in [8] were also used in immunofluorescence assays.

Antibodies against PfGEST. The C terminal region of *PfGEST* (PF3D7_1449000) was synthesized through PCR (Primers 15 and 16) and cloned into the GST expression vector pGEX-6P-1 vector (GE Healtcare). The recombinant GST-PfGEST^{Cterm} protein was expressed in *Escherichia coli* BL21 (DE3) following induction with 1mM IPTG. Harvested cells were lysed using BugBuster (Novagen) and recombinant protein was purified in a glutathione agarose column. 2 female BALB/c mice were immunized via intraperitoneal injection with 100ug of protein + Freund's adjuvant. After 4 weeks, 3 Boost injections of 50ug of protein + Freund' s adjuvant were given every 2 weeks. 2 weeks after the third boost, mice were bled and serum was obtained after natural clotting.

Antibodies against AMA-1. Rabbit α-AMA1 antibodies have been described previously [19].

Antibodies against GFP. Commercially available antibodies were used: Monoclonal mouse antibody 11814460001 (Roche) and polyclonal rabbit antibody A-6455 (Life Tehnologies).

Immunofluorescence assays

Immunofluorescence assays (IFAs) were performed on blood smears of parasites on glass slides. Slides were submerged in acetone for 1 minute and air-dried. Treatment with acetone is sufficient to permeabilize samples and therefore no additional permeabilization step was performed. After fixation and permeabilization, samples were blocked in a 3% BSA in PBS solution for 30 minutes, and then primary antibody diluted in 3% BSA in PBS was incubated for 1h. After PBS washing, secondary antibody conjugated to the correspondent fluorophore (rodamine or fluoresceine) and diluted in 3%

BSA in PBS was incubated for 1h, and washed with PBS. 5µL of SlowFade antifade reagent (Life Technologies) were added before sealing the sample.

An Olympus BX53 microscope connected to a Prior Lumen 200 fluorescence light source was used to visualize live and acetone-fixed samples. Images were acquired using a TUCSEN H Series camera and an Olympus UPlan FLN 100X objective. Filters used to detect GFP and fluorescein fluorescence were: EX: 450-490, EM: 520, dichroic mirror FT580. Filters used to detect rhodamine fluorescence were: EX: 450-490, EM: 520, dichroic mirror 510.

Immunoelectron microscopy

Stage V gametocytes were Percoll purified and processed for immunoelectron microscopy with slight modifications to the protocol described previously [20]. Briefly, samples were fixed overnight at 4°C with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer. Next, the suspension was gently washed in sodium cacodylate buffer, dehydrated in ethanol serial dilutions and embedded in LR White, medium-grade acrylic resin (London Resin Company, UK). The samples were polymerised in a 55°C oven for 72 h and ultrathin sections, collected on gold grids, were sunk in 100% ethanol for 3 min, immersed in Tris buffer 0.05 M (pH 10.0) in PCR tubes, and then kept at 99°C for 30min using a constant temperature box [21].

For immunostaining, the grids were floated on drops of PBS containing 0.1 M glycine for 10 min, washed with PBS, blocked with 5% normal goat serum/1% BSA in PBS for 30 min and incubated overnight at 4 °C with mouse polyclonal anti-PfGEST serum (1:50) in PBS/0.1% BSA/0,05% TWEEN20 buffer. After washing, the grids were incubated at room temperature for 1h with 10nm gold-conjugated goat anti-mouse IgG (Sigma Aldrich) diluted 1:50, rinsed in buffer followed by distilled water and air dried. Finally, the samples were stained with uranyl acetate 2% in H_20 and Reynolds lead citrate solution, and observed with a 208 Philips transmission electron microscope.

As controls, the sections were stained without any heating, without the primary antibody and with the diluted gold anti-mouse IgG or with the diluted mouse pre-immune serum in place of the first antibody.

Southern blot and Western blot

Southern blot was carried out using standard procedures. Genomic DNA was prepared from asexual parasites with QIAamp DNA Blood mini kit.

For Western blot, proteins from the indicated purified parasites were separated on SDS 4-12% PAGE and transferred to a nitrocellulose membrane, according to standard procedures [22]. Nitrocellulose blots were processed in TBS (50mM tris, pH 7.5, 150mM NaCl) using BSA 3% as blocking agent. Antibodies were used at the indicated dilutions. Secondary Horseradish peroxidase (HRP)-conjugated antibodies were used at 1:200 dilution. Bound antibodies were detected through reaction with SuperSignal West Pico Chemiluminiscent Substrate (Life technologies).

Measurement of OBs in gametocytes

Micrographs of mature gametocytes from WT and *Pfg377KO* strains were used to count the number of OBs found in the section. A morphometric analysis was conducted to calculate the eccentricity (e) of OBs in the WT and in the *pfg377*KO gametocytes, according to the formula $\sqrt{\quad}$—. Student's t test was performed with the eccentricity data of OBs from each strain to determine if they were significantly different from WT OBs.

Egress efficiency of *P. falciparum* gametocytes

Egress efficiency of gametes was measured as described previously [12]. Briefly, mature gametocyte cultures were stained with fluorophore-bound WGA, which binds to RBCs surface, and then gametogenesis was triggered by adding 20μM xanthurenic acid or by resuspension in complete medium at pH 8.2. After 15 minutes parasites were fixed in 2% PFA and mounted in microscope slides. Gametes were identified and the presence or absence of RBC was assessed depending on the presence or absence of the fluorescence surrounding the parasites. For *pfg377*KO parasites, 6 independent replicate experiments were done, counting 100 gametes in each case. For OBC-KO gametocytes, 4 independent replicate experiments were done, counting 200 gametes in each case. Error bar: SD. T-tests were performed on individual pairs of WT-KO parasites to determine whether they were significantly different in their egress efficiency.

Purification of gametocyte samples for proteomic analysis

Magnetic purification was used to purify samples for mass spectrometry, using CS columns in a MaxiMACS (Miltenyi Biotec) to retain the gametocytes while non-infected RBCs passed through the

column. Prior to purification, Giemsa smears were analyzed to ensure synchronicity of the samples and absence of asexual parasites or immature gametocytes. Purified parasites were counted using a cell counter chamber, ensuring that uninfected RBCs amounted to no more than 20% of the sample, and that spontaneously rounded up parasites were no more than 10% of the total parasites. $2x10^7$ parasites were pelleted and frozen for each sample. Gametocyte parasites were lysed in 4% SDS in PBS, 4X Laemmli Sample Buffer, and reduced with 50 mM DTT at 95 ^{0}C for 5 minutes prior to protein gel electrophoresis. Gel based fractionation of the proteins prior to tryptic digestion was conducted by gel electrophoresis using 10 % Criterion XT Bis-Tris precast gels (Biorad inc, UK) and XT-MES running buffer pH 6.4 . Gels were stained with Colloidal Coomassie Blue (Severn Biotech), and divided into 6 slices per lane. Gel slices were treated with 10 mM DTT and 55 mM iodoacetamide and digested by trypsin overnight at 37 ^{0}C as described previously [23]. Tryptic digests were acidified to a final concentration of 0.1% TFA and purified by STAGE tips [24].

Co-immunoprecipitation (co-IP) experiments

Previous to the experiment, antibodies against Pfg377 B fragment were crosslinked to agarose beads: Agarose beads (50μL) were washed 3 times with PBS 0.1% Tween-20 (Sigma-Aldrich) and resuspended in 100 mM Tris-HCl, 0.5 M NaCl (pH 8.0). Antibodies (20μL) were added and the mix incubated for 3h at room temperature. Beads were then washed (500g centrifugations for 5 minutes was performed to pellet beads throughout the experiment) three times with 200mM sodium borate pH9.0 to eliminate unbound antibody. Beads were then resuspended in 3 volumes of methylpimelimidate (DMP, the crosslinking agent) 20mM in 200mM borate pH9.0 and incubated for 90 minutes at room temperature in a shaker. Crosslinking solution was washed away and beads resuspended washed 3 times in TrisHCl 50mM pH7.5, and then incubated at room temperature for 2 hours, to terminate the crosslinking reaction. Non-crosslinked antibodies were removed by washing 3 times with 1mL of 0.58% v/v acetic acid 150mM NaCl. Beads were then washed 3 times in PBS and stored at 4° until experiment began. Correct crosslinking was evaluated through an acrylamide protein gel in which crosslinked beads showed to contain a protein of the expected length of the α-Pfg377B antibodies.

Samples containing $3x10^7$ mature synchronous gametocytes from WT and Pfg377KO 8-20 strains were isolated via magnetic columns, pelleted and frozen. Lysis occurred for 30 minutes at 4°C in lysis buffer (50mM Tris-HCl pH7.5, 150mM sodium chloride, 1% Nonidet P40 (Sigma-Aldrich), 0.5% sodium deoxycholate, Complete protease inhibitor). Debris was pelleted and supernatants were incubated for 3 hours at 4°C with non-crosslinked agarose beads to pre-clear the lysate. Beads were

pelleted and discarded, and pre-cleared supernatant was then incubated overnight at 4°C with crosslinked beads containing Pfg377 fragment B antibodies. Beads were then pelleted and washed three times in lysis buffer. Elution of the bound protein was achieved through addition of 2x Laemmli sample buffer (Life Sciences) to the beads and incubating the sample at 95°C for 5 minutes. Gel based fractionation of the proteins in the supernatants was conducted by gel electrophoresis in a 4%-12% Bis-Tris precast gel (Life Technologies) and MES-SDS buffer. The gel was stained with Colloidal Comassie Blue and a single slice containing each sample was excised. Gel slices were treated with 10 mM DTT and 55 mM iodoacetamide and digested by trypsin overnight at 37 ^{0}C as described previously [23]. Tryptic digests were acidified to a final concentration of 0.1% TFA and purified by STAGE tips [24].

Liquid chromatography tandem mass spectrometry

Ultimate 3000 UPLC (Thermo Fisher, Germany) connected to the Orbitrap Velos Pro mass spectrometer (Thermo Fisher, Germany) was used to perform tandem mass spectrometry experiments. Peptides were loaded on a 2 cm Acclaim™ PepMap™100 Nano-Trap Column (Thermo Fisher, Germany) and were separated by a 25 cm Acclaim™ PepMap™100 Nano LC column (Thermo Fisher, Germany) packed with 3 μm C18 beads. Sample run with a flow-rate of 300nl/min in a 120 min gradient of 95% buffer A/5% buffer B to 65% buffer A /35 % buffer B (buffer A: 0.5% acetic acid. Buffer B: 0.5% acetic acid in 100% acetonitrile). Peptides eluting from the column were electrosprayed into the mass spectrometer at 2.3 kV pray voltage. Orbitrap was used for data acquisition in a data-dependent mode automatically switching between MS and MS2. Orbitrap cell was used to acquire full-scan spectra of intact peptides (m/z 350-1500) with automated gain control accumulation value of 1.000.000 ion, with a resolution of 60.000. The ten most abundant ions were then sequentially isolated and fragmented in the linear ion trap, where dissociation was induced through collision, using an accumulation target value of 10.000, a normalized collision energy of 32% and a capillary temperature of 275°C. Dynamic exclusion of ions sequenced within the 45 previous seconds was applied. Unassigned charge states and singly charged ions were excluded from sequencing. For MS2 selection, a minimum of 10.000 counts was required.

Peptide identification and quantification by MaxQuant

Andromeda [25] search engine integrated in the MaxQuant (Version 1.3.0 [26]) was used to identify proteins. The protein database contained protein sequences from P. falciparum (http://plasmodb.org/common/downloads/release-9.1/Pfalciparum3D7/) and human IPI version 3.68

(ftp://ftp.ebi.ac.uk/pub/databases/IPI/) supplemented with frequently observed contaminants. Andromeda search parameters for protein identification were set to tolerance of 6 ppm for the parental peptide and 0.5 Da for fragmentation spectra and trypsin specificity allowing up to 2 miscleaved sites. Deamination of glutamine, oxidation of methionine, and asparagine and protein N-terminal acetylation were set as variable modifications, carboxyamidomethylation of cysteines was specified as a fixed modification. Minimal required peptide length was specified at 6 amino acids. MaxQuant was used as described [26] to perform internal mass calibration of measured ions and peptide validation by the target decoy approach. Peptides and proteins with a false discovery rate (FDR) lower than 1% were accepted.

Proteins were quantified by normalized summed peptide intensities [27, 28] computed in MaxQuant as label free quantification (LFQ). Perseus [29] was used to perform Statistical analysis and hierarchical clustering of protein LFQ replicates. Statistical significant differences in protein abundance between gametocytes samples of 3D7 wild type and *Pfg377*KO parasites were computed with the Perseus software (http://www.maxquant.org/). Data was loaded into Perseus, log2 transformed and then filtered requiring detection in at least 50 % of the samples. Missing LFQ data were imputed with intensities from the lower part of normal data distribution, with an imputation width of 0.3 and a shift of 0.8. LFQ samples between samples were normalised using Z-score transformation. Significantly changed abundance levels in proteins were calculated with a two-tailed t-test and with a relative expression test, correcting p-values for multiple testing using a permutation-based false discovery rate with a 0.05 threshold.

Validation of proteomic data

R (version 3.0.1) was used to perform a cluster analysis taking as samples each of the 27 readouts obtained (3 technical replicates from 3 samples from 3 biological replicates), using z-scores and relative expression values. A dendrogram based on Euclidean distances between values from the same protein across different samples was generated using the default settings of the heatmap.2 function from the gplots package.

Data from previous work [3] was used to establish a list of proteins expressed preferentially in asexual stages. The presence of these proteins in the Pfg377KO samples was compared to the presence in WT sample of the correspondent experiment, and the average of the values of this ratio across all 6 samples obtained. Only proteins for which a KO/WT ratio was obtained in all 6 cases were included. The KO/WT ratios of proteins previously found specific of female and male

gametocytes [30] were processed in an analogous way. The list of overrepresented proteins in each sex was provided by E. Lasonder (unpublished work).

Candidate proteins bioinformatic analysis

Predicted sequences of candidate proteins were analyzed for the presence of transmembrane domains with TMpred (Acess through ExPASy, and based on TMbase25) using default settings, while signal peptide was predicted using SignalP 4.1 on standard settings (except for OBCIp, for which sensitive settings and no N-terminal truncation value were set to detect signal peptide).

Plasmid construction

Generation of pHH-TK-Pfg377 plasmid

Two homology regions were amplified and cloned into pHH-TK (gently provided by A. Cowman) to produce plasmid pHH-TK-Pfg377, as follows. Primers 1-2 and 3-4 were used to PCR amplify fragments of *pfg377* using P. falciparum 3D7 genomic DNA as template, to produce the homology regions (HR) I and II, producing fragments of 917 and 1202 bp respectively.

HRI was purified and digested using SpeI and BglI restriction enzimes, purified again and inserted into PHH-TK [17], which had previously been linearized using SpeI and BglI, producing TK-HRI. Then the process was repeated inserting HRII into TK-HRI using the appropriate restriction enzimes, producing pHH-TK-Pfg377.

Generation of TG3 plasmid

Myc tag was generated by annealing of 2 oligonucleotides containing sites for SalI and XmaI restriction enzimes (primers 39-40). ROM3 (PF3D7_0828000) has been identified as very upregulated during gametocytogenesis [31]. Plasmid pASEX-GFP-GL3-3'UTR, containing a GFP coding sequence followed by the 3' UTR region of ROM3 (G. Siciliano unpublished work) was used to clone Myc tag, digesting it with SalI and XmaI and purifying the linearized plasmid. The resulting plasmid was named pMyc-GFP-GL3-3'UTR.

In parallel, an adaptor including restriction sites AgeI, AscI, SalI and NotI was built using primers 40-41, and was inserted in the NotI site of pHH-TK plasmid [17], generating plasmid TK-PL. Then, the portion of pMyc-GFP-GL3-3'UTR containing Myc, GFP and GL3 3'UTR was excised using XmaI-NotI, gel purified and inserted into SalI-NotI cut TK-PL, giving as a result TG3 (TK-GFP-GL3 3') plasmid.

Generation of GFP-tagging plasmids

The 3' coding regions of *PfPSOP12*, *PfSUB2* and *PfOBC12* were amplified, using primers 29-30, 31-32 and 37-38 respectively. PCR products were column purified and digested with the corresponding restriction enzymes, and purified again to clone them into TG3 after AgeI-SalI linearization and purification. Clones were obtained and amplified fragments were sequenced to discard mutations.

Generation of KO plasmids

pCC1 [32] plasmid, kindly provided by Alan Cowman, was used to generate disrupting plasmids for the selected genes. Two homology regions were cloned for *pfGEST* (primers 19-20 and 21-22), *pfPSOP12* (primers 25-26 and 27-28) and *pfOBCIp* (primers 33-34 and 35-36). PCR products corresponding to HRI were gel-purified and digested with the appropriate restriction enzimes, and inserted into linearized pCC1. The cloning process was repeated, introducing HRII fragments in the correspondent intermediate plasmids, and producing the definitive KO plasmid. Constructs were digested to ensure correct insertion of HR in the plasmid backbone.

List of primers

NUMBER	NAME	SEQUENCE
1	377.1.D	GGGACTAGTGTGTACCATTAATTTTTCTCAAACGTACC
2	377.1.R	GGGGAGATCTTTCTCTTCAGGAGTTAATTCCACAACG
3	377.2.D	GGGGAATTCAGAAGATTTTAGAGCACAGTTTAAAGGTGC
4	377.2.R	GGGCCATGGCATAAGATCCCTAATAACATCTGGTACGG
5	pfg377KOprobe-DIR	ATGGCAGTTATATTGAATATACATAATATTCGC
6	pfg377KOprobe-REV	ATATTTATGTGTATACTGTGAAAATGTTCTGAC
7	pfg377KO5'DIR	TACTTTTTGAAAATTTATCAAGCAC
8	pfg377KO5'REV	TGAAAGCTGTACTTTTATTACCTGC
9	pfg377KO3'DIR	GTGATGTTGTTCGTTTATCTGATCG
10	pfg377KO3'REV	CAGATACTGGAACTATTCTAGGAGC
11	PfCAM5'REV	AAAATGGTTAACAAAGAAGAAGCTC
12	PfHRP2DIR	TTACAATATGAACATAAAGTACAAC
13	377KO.RTPCR.A2-dir	AGAATATGAATTACCTAACGTTGTGG
14	377KO.RTPCR.A2-rev	ATAATTTAAAATCAAAAGCATGTACC
15	GEST Cterm Ab F	GGGGATCCTGGAAACAAAGAACATTAGAAGCTACTC
16	GEST Cterm Ab R	GGGCGGCCGCTATCTTGCTTATCCTTATCTAATTGTCC
17	GEST Nterm Ab F	GGGGATCCCAACTTCATTCTGTAAATTATGCTCCCTTG
18	GEST Nterm Ab R	GGGCGGCCGCCAAACTAAGAAGCTTTTCCTTTGCCTTTG
19	GEST KO 5' F	TGACCGCGGATTTAACATTGCTTCTACAAATAGCTAGC
20	GEST KO 5' R	GTACTAGTTTATAGGAAGAACTAATGCTATCAAGAGG
21	GEST KO 3' F	AGACCATGGGTAGAAGATGAATTAAATGCTCTTGG
22	GEST KO 3' R	AGACCTAGGATATAAGATTTTGAATTTATATTTAGTGTCG
23	GEST KO WT F CHECK	AGACCGGTCTATACAACTTCATTCTGTAAATTATGCTC

24	GEST KO WT K CHECK	AGAGTCGACTTGCTCTTCTAATTTTTGTATATCTTGC
25	PSOP12 KO 5' F	TGACCGCGGTTCTTTTCATTAAATAACATCTAATGGG
26	PSOP12 KO 5' R	TAGATCTATGGCATTCATAAGGTGC
27	PSOP12 KO 3' F	AGACCATGGCTGACATAACATTAGCAATCATATGTCC
28	PSOP12 KO 3' R/GFP WR R	AGACCTAGGTCATTCTTGACATATAATCATGTGACG
29	PSOP12 GFP F	AGACCGGTATGATTTATCAATTAATTTAAACGATCG
30	PSOP12 GFP R	AGAGTCGACAAATGTAATATCATAGTACTTATTATTTGCG
31	SUB2 GFP F	AGACCGGTGGAAGAGGTGTTAGATATATTAACAAGGAC
32	SUB2 GFP R	AGAGTCGACATCATCAAGTTGATTCATTGCTTTATTAA
33	OBCIp KO 5' F	TGACCGCGGATTAGTAAATATATTGTTATATCCACGTGG
34	OBCIp KO 5' R	GTACTAGTCCAAGAAAAGGTACTTATAAAAATATATACG
35	OBCIp KO 3' F	AGACCATGGTATGAATTGAGATCCAATGCCAACAAGG
36	OBCIp KO 3' R	AGACCTAGGTTATTTATTATGTTGATAGTAAAGTGTTTG
37	OBCIp GFP F	AGACCGGTCAAATGTATGCTATATTCATTGACAGG
38	OBCIp GFP R	AGAGTCGACTAATTTCTCCTTAAAAACTTCCAGC
39	Myc F	TCGACGGAGAACAAAAACTAATATCAGAAGAAGACCTAAGCC
40	Myc R	CCGGGGCTTAGGTCTTCTTCTGATATTAGTTTTTGTTCTCCG
41	Polylinker F	GGCCGACCGGTTGGCGCGCCTTGATCATGCTAGCTTTCGTCGACATTGC
42	Polylinker R	GGCCGCAATGTCGACGAAAGCTAGCATGATCAAGGCGCGCCAACCGGTC
43	GFP 5' R	GACAACTCCAGTGAAAAGTTCTTCTCC
44	GFP 3' F	GATATTGAGCAGAGGATATGCGC
45	PSOP12 GFP 5' F UP	GTAAAAGAAATTAAAAGAAACCATACAACAAC
46	PSOP12 GFP 3' R DOWN	TGATGCCTATCTAATGTTACCTGTAGTGAG
47	SUB2 GFP 5' F UP	CTTTACAGGTACTTCTATGGCTGCTC
48	SUB2 GFP 3' R DOWN	CCAAAAAGGTGTATAAAACTGAAAGTG
49	OBCIp GFP UP F	TTTATGTCATATTGTATATACAACATATATTAAAAACG
51	OBCIp GFP DOWN R	GAAAGTTATGATTGAATCTATATCAGATGGTG
51	GFP integration 5' R internal	GACAACTCCAGTGAAAAGTTCTTCTCC
52	GFP integration 3' F internal	GATATTGAGCAGAGGATATGCGC
53	KO integration 5' R internal	CAAAATGCTTAAGACAGATCTTCGG
54	KO integration 3' F internal	GAACATATTTATTAAATCTAGAATTCATATCG
55	GEST KO 5' F UP	TTGTTTTTATTCGAGTAGCTGTATGAAG
56	GEST KO 3' R DOWN	CATATCATACTTAATTTGTTCATTTTAGG
57	PSOP12 KO 5' F UP	CCTTTTTTCTTCTTCATTACTACATTATGG
58	PSOP12 KO 3' R UP	AATGTGATTACAACTATTCTTGACTCTCC
59	OBCIp KO 5' F UP	TATCTTTTAGCATGTAATAGAATGTATGGAC
60	OBCIp KO 3' R DOWN	CTGTTATTTTGATATTTTTCCATAGTGC

Results

Generation of stable *pfg377*KO parasites

The previously published characterization of the function of protein Pfg377 was performed producing and analysing *pfg377*KO parasites in which the gene was disrupted through single recombination of the *pfg377*KO plasmid into the *pfg377* gene locus via a *pfg377* homology region. As reversion of the single integration event was reported in that publication to reconstitute the WT *pfg377* coding sequence, albeit with very low efficiency, new *pfg377*KO parasite lines were produced in which the gene was stably disrupted by the gene replacement construct pHH-TK-Pfg377, irreversibly inserting a selection cassette in the *pfg377* coding sequence.

The plasmid pHH-TK-Pfg377, enabling negative and positive selection of the transfected parasites, was used to disrupt the *pfg377* locus through a double cross-over (DXO) recombination promoted by two distinct *pfg377* homology regions flanking a selection cassette where expression of the *h-dhfr* gene, conferring resistance to WR99210, was directed by parasite gene regulatory regions. *P. falciparum* 3D7 parasites were transfected. Transfectants were first positively selected for WR99210 resistance, and surviving parasites were negatively selected by incubation with Ganciclovir.

Parasite lines from two independent transfections (GT5 and GT8) were obtained and characterized.

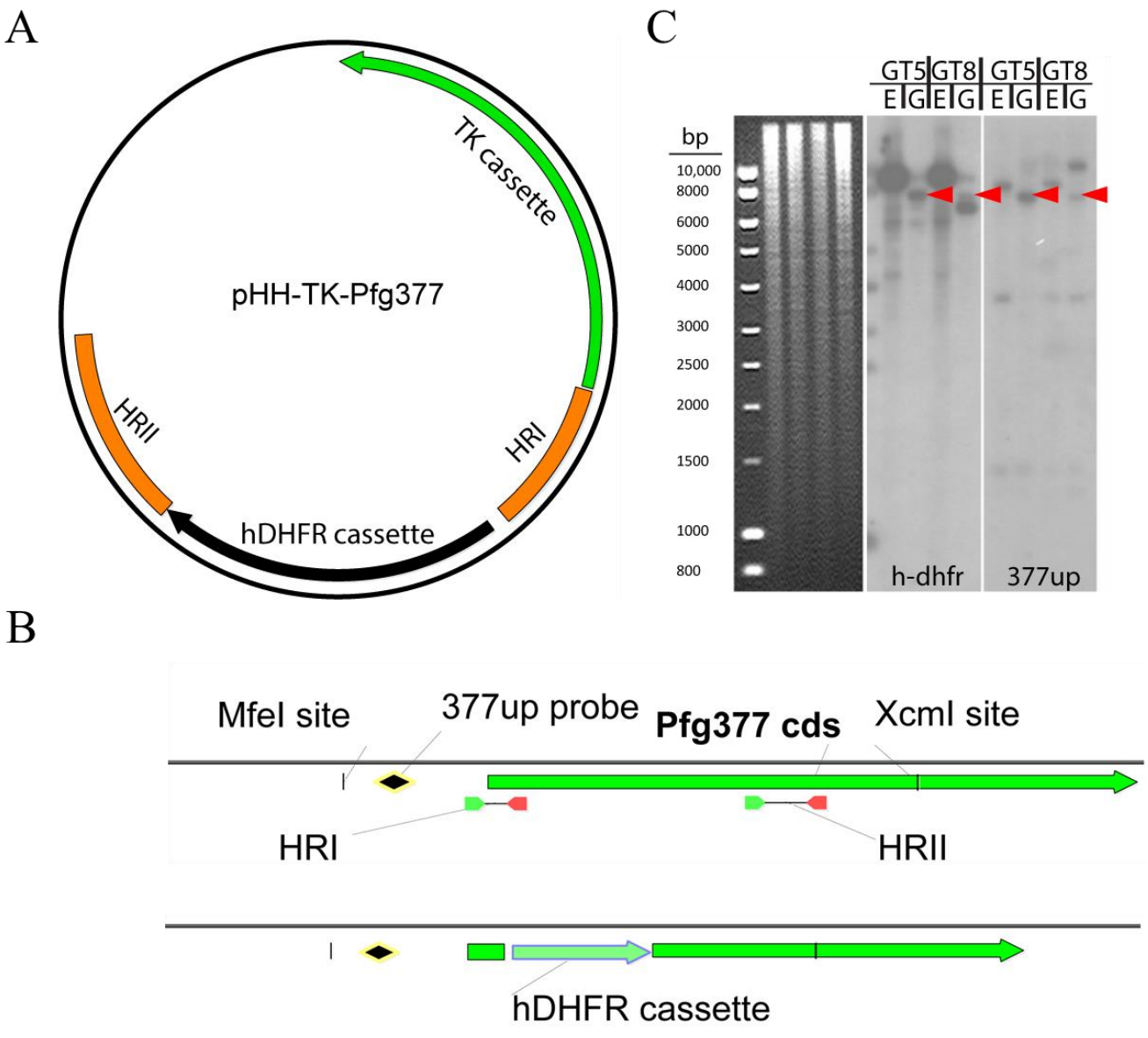
Southern blot analysis of the transfected parasite lines

The independent parasite lines obtained as described above were analysed by Southern blot using a probe for the genomic *pfg377* locus and one for the selection marker *h-dhfr*. The *pfg377*-specific probe was amplified by PCR using primers 5 and 6 and the one for the *h-dhfr* cassette was amplified using adequate primers described in [17]. Genomic DNA was obtained in both transfections by parasites after the positive selection, expected to mainly contain the construct as episomal multicopy plasmid (Samples E in Figure 1) and after the negative selection with Ganciclovir (Samples G in Figure 1). Genomic DNAs were digested with MfeI and XcmI, and blotted after electrophoresis to be hybridized with the above probes. The Southern blot analysis was designed so that the patterns of the resulting hybridization bands with the two probes were diagnostic of the four genomic structures possibly resulting from 1) absence of plasmid integration; 2) plasmid integration through the 5' homology region; 3) plasmid integration through the 3' homology region and; 4) DXO recombination (see Table 1 below). This analysis showed presence of hybridization bands diagnostic of the DXO recombination in both GT5 and GT8 parasites (figure 1).

Table 1. Expected MfeI-XcmI hybridization bands (in bps):

Status of *pfg377* locus	***h-dhfr* probe**	***pfg377* probe**
wt	no signal	8261
5' integration	9406	9406
3' integration	6709	10289
DXO	7378	7378

Figure 1. Southern analysis of *pfg377*KO transfectants. **A**: Map of the pHH-TK-Pfg377 disruption plasmid. **B**: *pfg377* genomic locus before and after DXO integration. (1) WT locus. (2) Recombinant locus. **C**: Ethidium bromide stained gel and hybridization bands with the *pfg377* and the *h-dhfr* probes indicated on genomic DNAs from GT5 and GT8 parasites. Samples E: DNA from WR resistant parasites, before selection with ganciclovir; samples G: DNA from parasites after selection with ganciclovir. Red arrows indicate bands from the disrupted locus after DXO integration of the *h-dhfr* cassette. HR: homology Region. TK: thymidine kinase. hDHFR: human dihydrofolate reductase. cds: coding sequence.

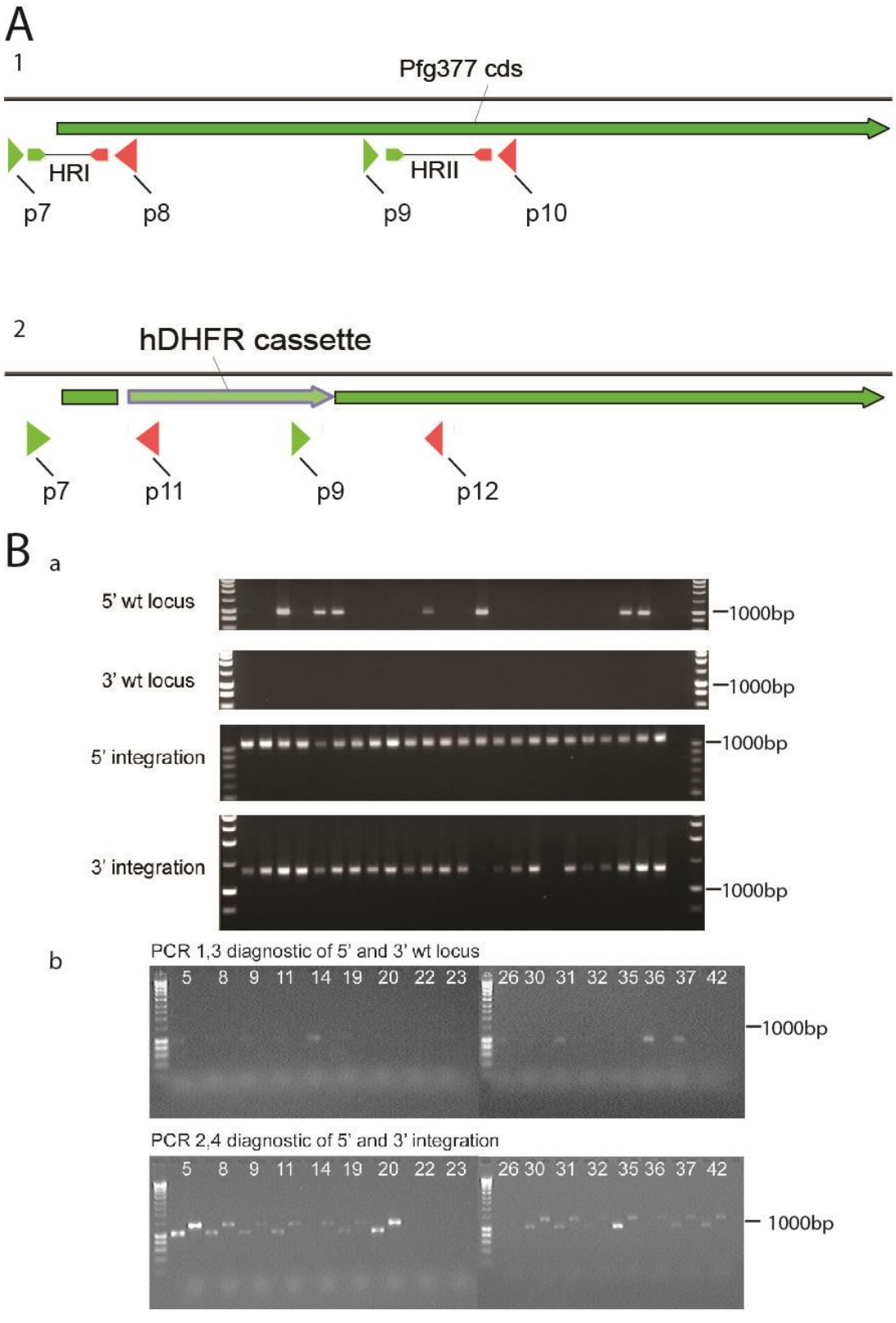


The independent GT5 and GT8 parasite lines were cloned by limiting dilution and 25 and 18 parasite clones, respectively, were analysed by PCR with primers diagnostic of the integration of the selection cassette through the 5' or the 3' homology region (table 2). In several clones PCR amplified products diagnostic of plasmid integration through both the 5' and the 3' homology regions and could not amplify fragments diagnostic for the WT locus, indicating successful occurrence of the expected gene replacement in the *pfg377* target site. As a result from this analysis, parasite clones 5-2, 8-20 and 8-42 from the GT5 and GT8 independent transfections were selected for further study.

Table 2. Expected lengths of diagnostic PCR products for the analysis of the disruption of the *pfg377* locus (in bp).

Detected *pfg377* locus	**Reaction**	**Primer pair**	**Expected length**
5'WT	PCR1	7-8	1008 bp
5'integration	PCR2	7-11	1060 bp
3'WT	PCR3	9-10	1308 bp
3'integration	PCR4	10-12	1378 bp

Figure 2. PCR analysis on the parasite clones from the GT5 and GT8 lines. **A**: Structure of (1) WT locus and fragment amplified in diagnostic PCR 1 (primers 7-8) and 3 (primers 9-10), (2) Double Crossover Recombinant locus and fragments amplified by diagnostic PCR 2 (primers 7-11) and 4 (primers 10-12). **B**. Electrophoresis of fragments from diagnostic PCRs on parasite clones from lines GT-5 and GT-8. (a) GT-5 clones 1 to 25. (b) GT-8 clones.



Characterization of the *pfg377*KO parasites

Analysis of the asexual growth of parasite cultures of clones *pfg377*KO 5-2 and 8-20 showed no obvious difference with WT parasites in growth rate (data not shown). Long term culture propagation, needed for the generation of the transgenic parasites, was reported to negatively affect gametocyte conversion rates [33], an analysis was conducted to detect any loss in gametocyte production in parasites 5-2 and 8-20. Giemsa-stained smears of gametocyte cultures from these clones showed no differences in gametocyte production and in their morphology compared to WT, confirming what was observed in the characterization of the previously published *pfg377*KO strains [13].

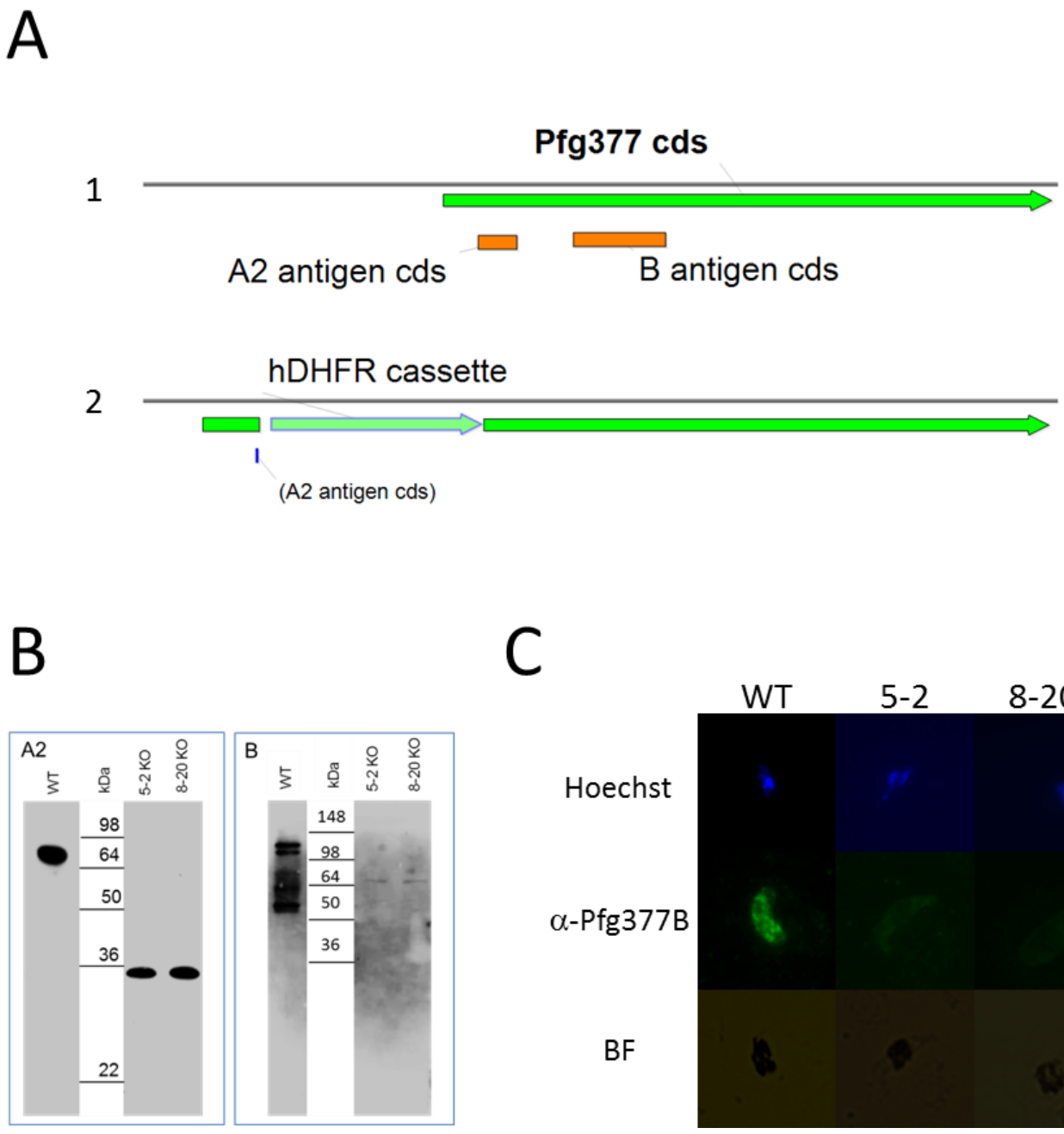
Pfg377 is not expressed in *pfg377*KO clones

Western blot (WB) and immunofluorescence analysis (IFA) were performed to confirm the expected lack of the Pfg377 protein in the clones with the disrupted locus. Antibodies against the A2 and B regions of the Pfg377 protein detect different fragments of the protein, presumably derived from protein processing of the full length protein of 377 kDa, in WB analysis of WT parasites. WB analysis conducted on stage V gametocytes of clones 5-2 and 8-20 showed that none of the antibodies could detect a WT protein pattern in the *pfg377*KO clones. No bands were detected in pfg377 KO gametocytes by the anti-B antibodies. In contrast, with the anti-A2 antibody, a signal was still detectable as a band of 33kDa, corresponding to a shorter Pfg377 peptide. This result can be explained by the production of a Pfg377 fragment derived from a residual 5' portion of the disrupted coding sequence, which overlapped with the portion of *pfg377* coding for the peptide used in the production of the A2 antibody. This would predict a residual protein of 22.7 kDa, which is in reasonable agreements with the detected fragment of around 33kDa (figure 3A and B).

Immunofluorescence analysis was performed with α-B and α-A2 antibodies on mature (stage V) gametocytes fixed on acetone. WT parasites consistently showed the characteristic granular pattern associated to Pfg377, while no specific signal was detectable on stage V gametocytes of the *pfg377*KO parasites (figure 3C).

Together, these results showed that the Pfg377 protein was undetectable in mature gametocytes of the 5-2 and 8-20 clones, confirming that disruption of the *pfg377* locus was successful.

Figure 3. Pfg377 is not expressed in the *pfg377*KO parasites. **A**: Diagram of *pfg377* locus. (1) WT locus. (2) Recombinant locus after DXO recombination of pHH-TK-Pfg377. Coding sequences (CDS) of the antigens used for the production of A2 and B antibodies are shown in orange. **B**: WB on samples from mature gametocytes of WT or *pfg377*KO parasites (left) Rat α-Pfg377A2 antibodies, (right) rabbit α-Pfg377B antibodies. **C**: IFA shows Pfg377 signal in WT gametocytes, and no signal in *pfg377*KO gametocytes. Rabbit α-Pfg377B antibodies were used.



Phenotypic analysis of the *pfg377*KO parasites from clones 5-2 and 8-20

Electron microscopy observations on gametocytes from the previously published *pfg377KO* parasites showed a striking reduction in the number of OBs compared to WT gametocytes [13]. To determine whether the newly generated *pfg377KO* clones showed the same phenotype, mature gametocytes from the independent clones 5-2 and 8-20 were examined in transmission electron microcopy and compared to WT gametocytes. Results showed that in the case of WT gametocytes, typical OBs could be observed in the periphery of the cell, under the IMC, while in sections from *pfg377*KO gametocytes the number of OBs was dramatically reduced. In the examination of the residual OBs still detectable in the KO gametocytes, it was observed that OB sections from mutant gametocytes presented a smaller and more circular shape than in WT parasites. This apparent difference in shape was more carefully evaluated in a morphometric analysis by measuring the eccentricity of OBs from WT and mutant parasites. Eccentricity is a measure of how much a conic section deviates from being circular, with a circular section having 0 eccentricity and ellipses ranging from 0 to 1 in eccentricity. In this analysis it was confirmed that OBs from *pfg377*KO gametocytes were significantly less eccentric than those from WT parasites.

Figure 4. Representative transmission electron microscopy images of WT and *pfg377*KO gametocytes. Arrows indicate OBs.

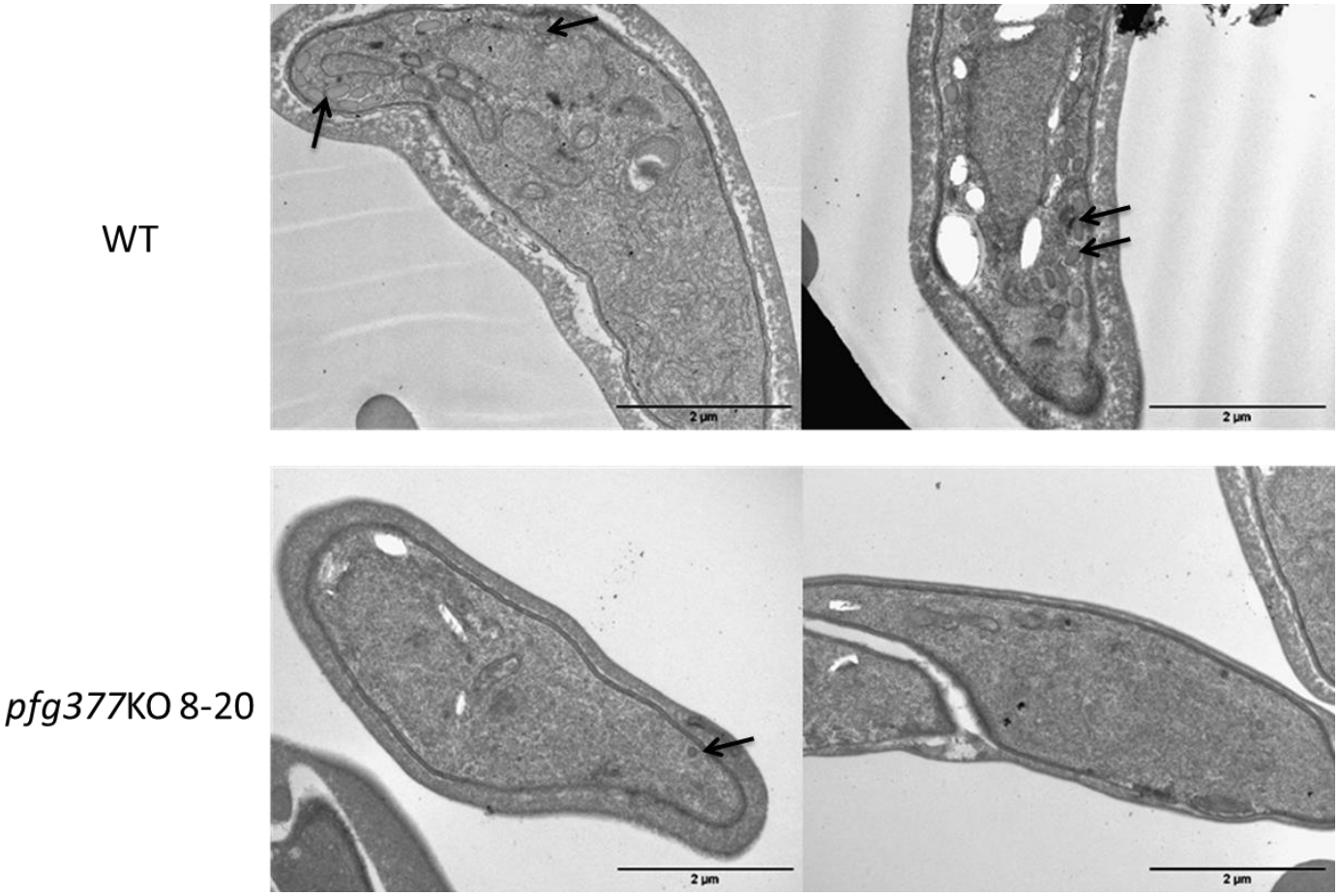


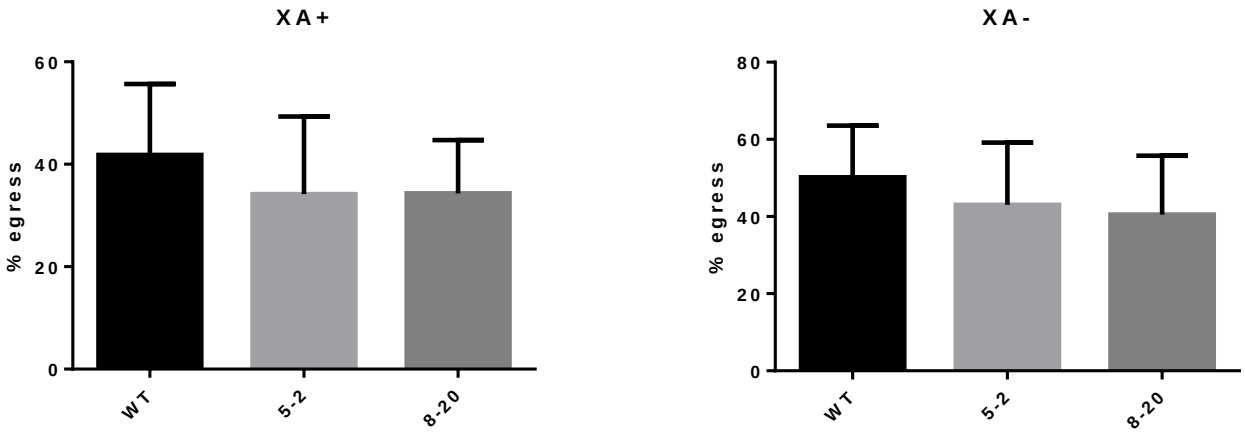
Table 3. Morphometric analysis of OBs in WT and *pfg377*KO clones. SD: standard deviation.

parasite	**n. OBs**	**major axis (nm)**		**minor axis (nm)**		**eccentricity**	
		mean	SD	mean	SD	mean	SD
WT	117	202,1	46,1	103,3	16,9	0,83	0,09
GT5-2	40	107,0	26,2	85,8	17,8	0,47	0,28
GT5-24	41	121,7	35,3	92,9	14,8	0,54	0,23
GT5 mean	81	114,5	31,8	89,4	16,6	0,50	0,26
WT	161	203,2	45,2	104,2	24,1	0,83	0,10
GT8-20	61	116,3	30,6	91,9	26,5	0,54	0,24
GT8-42	67	123,5	29,5	91,3	21,8	0,56	0,26
GT8 mean	128	120,1	30,2	91,6	24,1	0,55	0,25
WT mean	278	202,8	45,6	103,7	21,4	0,80	0,10
KO mean	209	118,0	30,9	90,8	21,5	0,50	0,25

The efficiency of the *pfg377*KO parasite clones in egress from the erythrocyte at gametogenesis was measured using the newly established protocol described in Part II. Egress efficiency was measured at a fixed time of 15 minutes from gametogenesis induction. No significant differences between WT and *pfg377*KO parasites could be detected in our experiments. This confirms the results obtained with the previously published *pfg377*KO gametocytes, and further supports the hypothesis that Pfg377 is not necessary for an efficient egress in *P. falciparum* (figure 5).

Figure 5. Egress efficiency of WT and *pfg377*KO parasites.

Efficiency of gamete egress was measured after 15 minutes from activation, and it is shown as % of WGA negative spherical gametes over total spherical gametes. Left: Gametogenesis in presence of XA. Right: Gametogenesis in absence of XA.



Identification of new OB resident proteins through comparative label-free quantitative proteomic analysis of OB+ and OB- gametocytes.

The dramatic depletion of OBs observed in the gametocytes of the *pfg377*KO parasites was exploited to investigate the protein composition of OBs and to identify novel proteins residing in these organelles. The approach undertaken was to perform a comparative label-free proteomic analysis of OB+ and OB- gametocytes, based on the assumption that proteins residing in OBs in WT parasites will be underrepresented in the OB-depleted KO parasites.

WT, *pfg377*KO 5-2 and *pfg377*KO 8-20 mature gametocytes were cultured and purified in parallel, using a single stock of blood and human serum through the experiment. Samples of $2x10^7$ mature gametocytes from each strain were prepared through magnetic-activated cell sorting (MACS) purification and stored for mass spectrometry analysis. Several quality control steps were performed before submitting the three samples to proteomic analysis. Giemsa-stained smears from the cultures prior to samples purification were examined to confirm that all gametocytes were at stage V of maturation and that the gametocyte sex ratio was similar among the different cultures, showing the typical high proportion of female to male gametocytes (data not shown). No asexual parasites were observed in these preparations, and amount of cellular debris (resulting from the overgrowth of the cultures to trigger gametocytogenesis) was found in similar amounts in all cultures. After gametocyte purification, the number of unifected RBC (uRBC) was less than 20% of the total cells in all the purified samples. It was also determined that the percentage of spontaneously activated gametes varied from 5 to 15% in the three samples.

Preparation of the three parallel gametocyte samples from WT and *pfg377*KO gametocytes was repeated to obtain a total of three independent biological replicates, producing a total of nine different samples.

Label free quantitative mass spectrometry identification of the proteins contained in each sample was performed as described in Materials and Methods, resulting in nine datasets. Only proteins detected in all samples (n=1964) were included in the MaxQuant quantitative determination to calculate the relative abundance of each protein in each sample. This was done by normalizing values, for which the standard score (Z-score) was calculated.

The abundance of a protein in each one of the two *pfg377*KO samples relative to its abundance in the WT sample was calculated for the 1964 selected protein as it was assumed that proteins significantly underrepresented in the OB-defective gametocytes may be considered as candidates for being OB resident proteins.

In order to conduct an overall analysis of the nine proteomic profiles from the three biological replicates, the Z-values generated in the mass spectrometry analysis were used to calculate the geometrical distance between samples by cluster analysis. This grouped samples according to their overall similarity to each other.

The cluster analysis provided two relevant pieces of information. The first was that in two out of three biological replicates the proteomic profiles of the two independent *pfg377*KO gametocyte samples were more similar with each other than with the WT gametocyte sample. The second was that the three biological replicates were classified together, indicating that overall difference in the proteomic profiles between the three experiments was higher than that between proteomic profiles of the WT and the *pfg377*KO gametocyte samples (figure 6).

The high variability among biological replicates therefore suggested to apply a stringent statistical threshold in the selection of proteins which appeared underrepresented in *pfg377*KO gametocytes, which were assumed to represent candidates for being OB resident proteins.

The most stringent confidence level included proteins for which underrepresentation (KO/WT ratios) was statistically significant in all six *pfg377*KO to WT gametocytes comparisons. The second confidence level included proteins statistically underrepresented in five out of six comparisons, which ensured that they were underrepresented in all three biological replicates in at least one of the *pfg377*KO clones. Only two proteins appeared in the most stringent category. One was Pfg377, as expected, and the second was the Putative Secreted Ookinete Protein 12 (PfPSOP12). Seven proteins were included by the second most stringent criteria, which resulted in a total of nine OB candidate proteins, including the already described Pfg377 (table 4). An additional criterion was applied to select candidate gene products for the subsequent functional analysis, based on the fact that OBs appear in stage III gametocytes, from day 4 of sexual differentiation. For this reason we selected proteins for which previous data indicated that they are upregulated in mature gametocytes. Data from a previously published comparative proteomic analysis between trophozoite, early gametocytes and mature gametocytes [3] were inspected, revealing that three of the above proteins were overrepresented in mature gametocytes, apart from Pfg377. This motivated the choice to concentrate on proteins PF3D7_0513700 (PfPSOP12), PF3D7_1247800 (Dipeptidyl peptidase 2, PfDPAP2) and Pf3D7_1136900 (Subtilisin-like protease 2, PfSUB2) for further studies.

As an additional control to evaluate the presence of proteins attributed to the asexual cycle of the parasites in the analyzed samples, proteins overrepresented in asexual stages [3] were taken into account. The presence of these proteins in KO samples relative to the presence in WT samples (KO/WT ratio) results in a value of 1.02, indicating that no major contamination of asexual proteins

is present in the samples. In a similar manner, a list of proteins specific of female and male stage V gametocytes [30] was used to address the possibility of a sex-ratio change between the different samples. Average ratio for these proteins in this case was 1.2 for both sets of proteins, indicating that no major differences in sex-ratio were present in the selected samples.

Figure 6. Cluster analysis of Z scores from proteomic profiles of samples from WT (A) and *pfg377*KO (B, C) gametocytes. Numbers indicate the three independent experimental replicates.

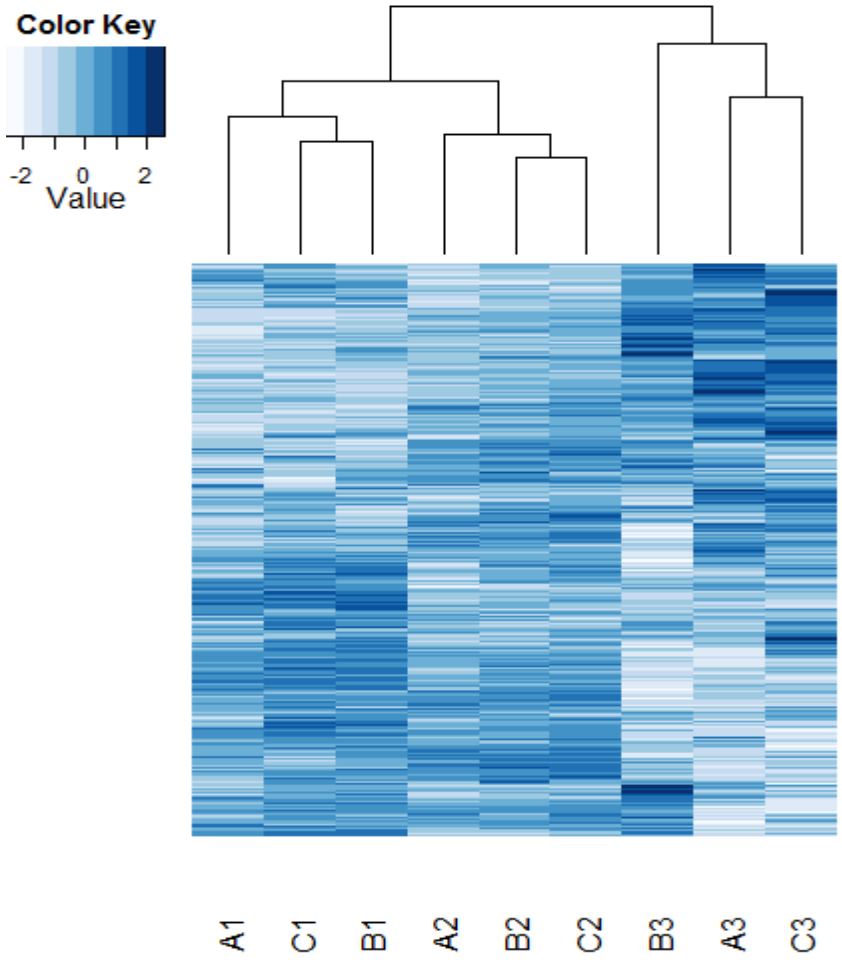


Table 4. Candidate gene products selected for being OB resident proteins (OBCs) from the whole cell proteomic comparison of OB- and OB+ gametocytes. Proteins found to be upregulated in late gametocytes in [3] are marked in green. Gene ID and Product description are taken from PlasmoDB [34].

Gene ID	Product Description	CONFIDENCE CLASS
PF3D7_1250100	Osmiophilic body protein (Pfg377)	I
PF3D7_0513700	Secreted ookinete protein, putative (PSOP12)	I
PF3D7_1247800	Dipeptidyl peptidase 2, putative (DPAP2)	II
PF3D7_1136900	Subtilisin-like protease 2 (SUB2)	II
PF3D7_1456800	V-type H-translocating pyrophosphatase, putative	II
PF3D7_1116800	Heat shock protein 101 (HSP101)	II
PF3D7_0501600	Rhoptry-associated protein 2 (RAP2)	II
PF3D7_0922200	S-adenosylmethionine synthetase (SAMS)	II
PF3D7_1471100	Exported protein 2 (EXP2)	II

Identification of Pfg377 co-immunoprecipitated proteins

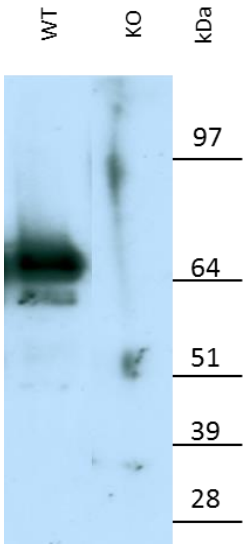
In another approach to identify OB resident proteins, a co-immunoprecipitation (co-IP) experiment was conducted using Pfg377 as bait. The absence of Pfg377 in KO gametocyte samples provided an optimal negative control in this experiment, allowing to perform parallel co-IP experiments with antibodies specific for the B fragment of Pfg377 on extracts from WT and *pfg377*KO gametocytes. This approach can reveal the potential false positive hits due to non-specific binding of proteins to the anti-bait antibody, as these proteins will also be present in the final eluate from *pfg377*KO gametocites.

Samples of $3x10^7$ WT or *pfg377*KO gametocytes were cultured, purified and processed in parallel, using rabbit polyclonal antibodies against Pfg377B fragment as bait. Western Blot analysis was performed on the final co-IP eluates from WT and *pfg377*KO samples using rat polyclonal antibodies against Pfg377A2 fragment. The absence of a Pfg377 signal in the KO samples, and its presence in WT samples, confirmed that the anti-bait antibody was able to bind to its antigen in the co-IP experiment, resulting in the presence of Pfg377 in the final eluate (figure 7).

The whole final co-IP eluates were analyzed by mass spectrometry as described in Material and Methods. This resulted in two data sets of detected proteins and their relative LFQ values. The

WT/KO LFQ ratio was obtained and an arbitrary threshold of a 100 fold overrepresentation in WT samples was set to select candidates for being proteins directly or indirectly interacting with Pfg377. Three proteins were overrepresented in WT samples over the threshold: Pfg377, PSOP12, also identified in the above whole cell proteomic analysis, and protein PF3D7_1214800. The presence of Pfg377 confirmed that the anti-bait antibody was able to bind to the Pfg377 epitopes in the experiment. After this experiment, PF3D7_1214800 was added to the list of potential OB localized proteins as OBCIp (OB candidate Ip protein).

Figure 7. Western Blot analysis on co-IP eluates of WT and *pfg377*KO gametocyte samples. Co-IP was performed using a rabbit α-Pfg377B antibody, and Western blot analysis was performed with rat α-Pfg377A2 antibodies (1:2000 dilution).



Characterization of the *Plasmodium falciparum* homologue of the *P. berghei* protein PbGEST.

The *P. berghei* protein PbGEST had been previously shown to localize to the OBs in *P. berghei* gametocytes [11]. We explored the localization of the *P. falciparum* homologue PfGEST, which has been previously detected in gametocytes [3, 31], as a possible additional candidate for being an OB resident protein. A mouse polyclonal antibody against the C terminal region of PfGEST was produced. The selected coding sequence was cloned by PCR into the GST-tagging plasmid pGEX-6P-1, and the resulting plasmid was transformed into *Escherichia coli*. The chimeric peptide GST-PfGEST^{Cterm} was then expressed and purified, and used to immunize a mouse to produce α-PfGEST antibodies (See materials and methods).

To investigate if PfGEST localizes to OBs, immunoelectron microscopy was performed using α-PfGEST antibodies on WT mature gametocytes. The results show localization of the antibodies to the OBs of gametocytes, indicating PfGEST resides in these organelles (figure 8A).

IFA analysis using the α-PfGEST antibodies was performed on mature WT gametocytes. Results showed a fluorescent granular pattern resembling that observed in IFA with antibodies against the OBs protein Pfg377 on mature WT gametocytes. Double IFA with α-Pfg377 and α-PfGEST antibodies was performed, and results showed colocalization of the fluorescence pattern associated to the two antibodies. This supports the results obtained in immunoelectron microscopy, indicating that PfGEST resides in the same compartment as Pfg377, the OBs (figure 8B superior panel).

Male gametocytes do not show reaction to α-Pfg377 antibodies, making it possible to distinguish between female and male gametocytes. To explore if PfGEST is expressed in male gametocytes, IFA was performed on mature gametocytes with α-Pfg377 and α-PfGEST antibodies. Gametocytes negative for Pfg377-associated fluorescence were identified as male, and evaluated for the presence of PfGEST-associated fluorescence. All examined male gametocytes showed a granular pattern of PfGEST-associated fluorescence, suggesting that PfGEST is expressed in male gametocytes and directed to a yet non-described cellular compartment in these cells (figure 8B central panel).

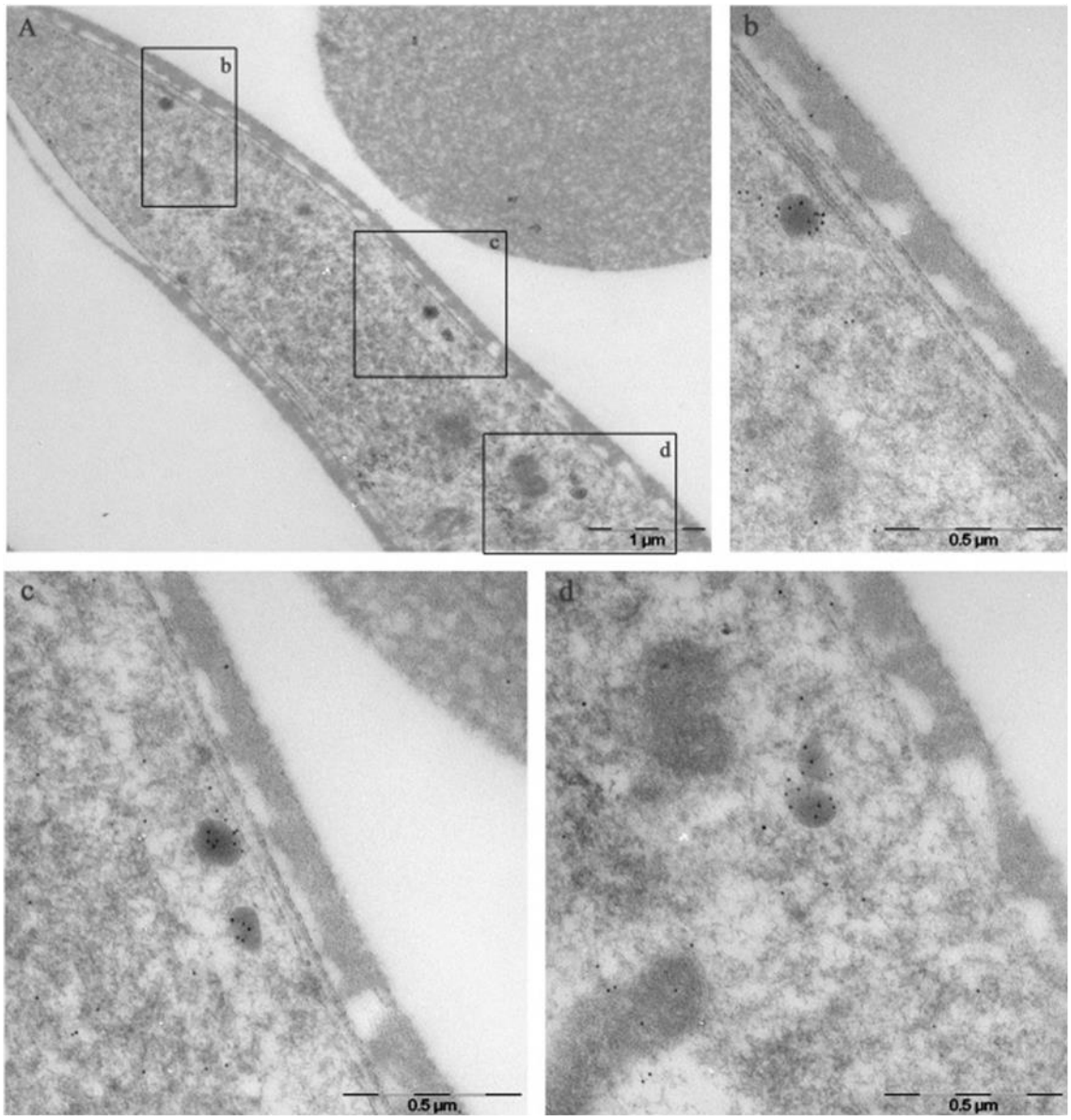
When IFA was performed on mature OB-depleted *pfg377*KO gametocytes, while fluorescence associated with Pfg377 was not observed (as was expected), α-PfGEST marking maintained a pattern similar to that of WT parasites. This could be explained by the targeting of PfGEST to the residual OBs found in these gametocytes (figure 8B inferior panel).

WB analysis using α-PfGEST antibodies shows a band in samples from sexual stages that is absent in samples from asexual parasites, in agreement with the expected expression of the target protein. The molecular weight corresponding to the anti-PfGEST positive band is lower than 28kDa, expected from the protein predicted aminoacidic sequence, possibly due to processing of the protein. Additionally, the signal is stronger in samples from mature gametocytes, indicating expression of the protein preferentially in late gametocytes, in agreement with the generation of OBs in theses stages. The PfGEST-associated band is also present in samples from *pfg377*KO gametocytes, in agreement with the results obtained in IFAs. The secretion of OBs content during gamete egress poses the question of whether OBs-associated proteins are released to the medium during this process. This was evaluated in the WB analysis in samples from WT and *pfg377*KO gametes. In both cases, the PfGEST-associated signal was present, indicating that PfGEST is present in gametes after egress (figure 8C).

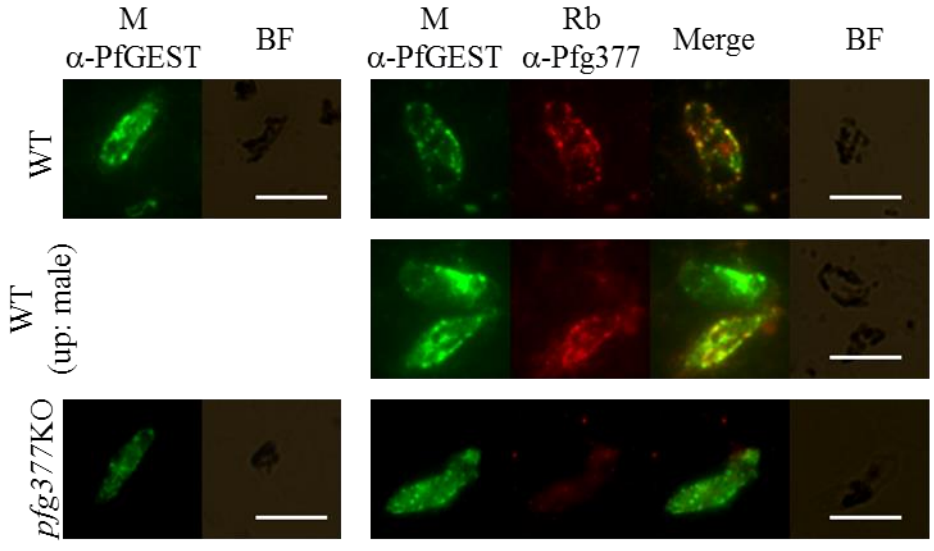
The above experiments indicate that PfGEST localizes to the OBs of *P. falciparum*.

Figure 8. PfGEST characterization. **A**: Representative immunoelectron micrographs using mouse α–PfGEST antibodies (1:100 dilution) on WT gametocytes. **B**: IFA assay using mouse α–PfGEST (1:100 dilution) and rabbit α–Pfg377B (1:800 dilution) antibodies. Acetone fixation was performed on mature gametocytes and gametes from WT and *pfg377*KO cultures. BF: bright field. **C**: Western blot assay using mouse α–PfGEST antibodies (1:1000 dilution). Samples from WT and from *pfg377*KO parasites were used. Rabbit α–Pf27 antibodies (1:3000 dilution) were used as loading control. uRBC: uninfected RBC, Asex: asexual parasites, gII: stage II gametocytes, gV: stage V gametocytes, G: gametes. $5x10^{10}$ parasites (or RBCs) loaded per lane.

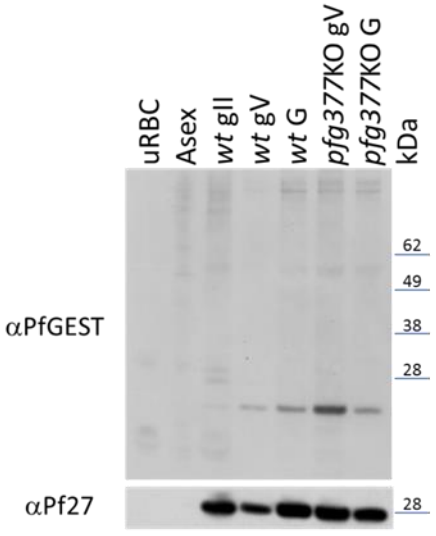
A


A
b
c
d
1 µm
b
0.5 µm
c
0.5 µm
d
0.5 µm

B


M
α-PfGEST
BF
M
α-PfGEST
Rb
α-Pfg377
Merge
BF
WT
WT
(up: male)
pfg377KO

C

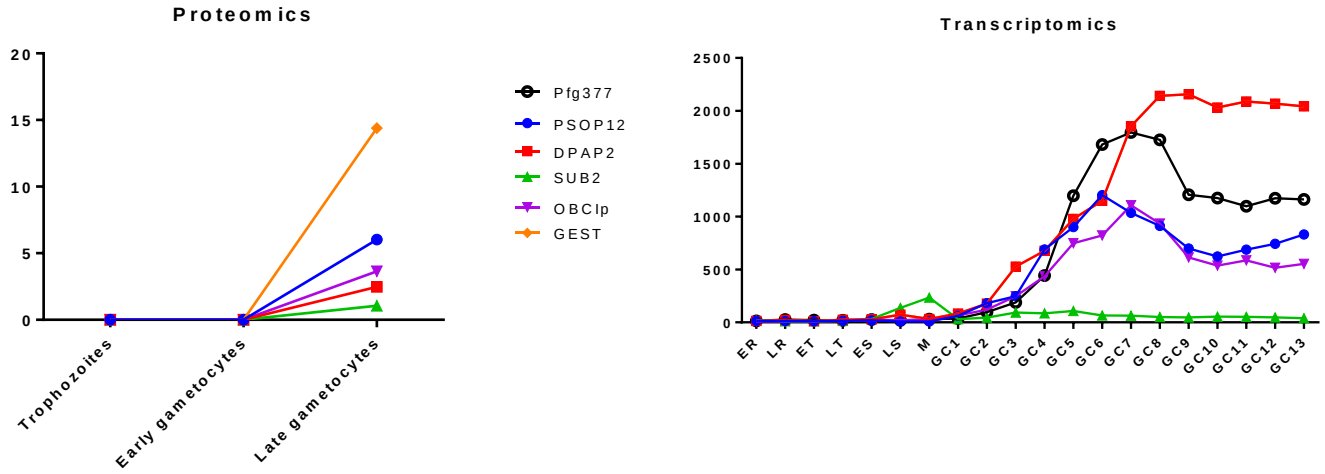

uRBC
Asex
wt gII
wt gV
wt G
pfg377KO gV
pfg377KO G
kDa
αPfGEST
αPf27
62
49
38
28
28

Functional characterization of Osmiophilic Body Candidate proteins (OBCs)

The results of the above work led to the identification of 4 OBC from the whole cell comparative proteomic analysis and the Pfg377 co-IP approaches. PfGEST, whose localization in OBs in *P. falciparum* has been described above, was also included in the group of proteins selected for further study. The selection process therefore yielded a final list of 5 candidate proteins.

Transcriptional and expression profiles found in previous publications [3, 35] were analyzed for the selected candidates. Proteomic data indicate prevailing presence of these proteins in mature gametocytes, compared to early gametocytes or asexual forms [3]. Transcription of all five candidates is upregulated in gametocytes stages, with a peak in mid to late gametocytogenesis, strongly resembling Pfg377 transcription profile [31] (figure 9A). Their predicted aminoacidic sequence was also analyzed in search of protein domains typically associated to membranous organelles-localized proteins. All five proteins include a signal peptide and a transmembrane domain in their predicted sequence, which strongly suggest these proteins are targeted to an alternative cellular location other than the cytoplasm.

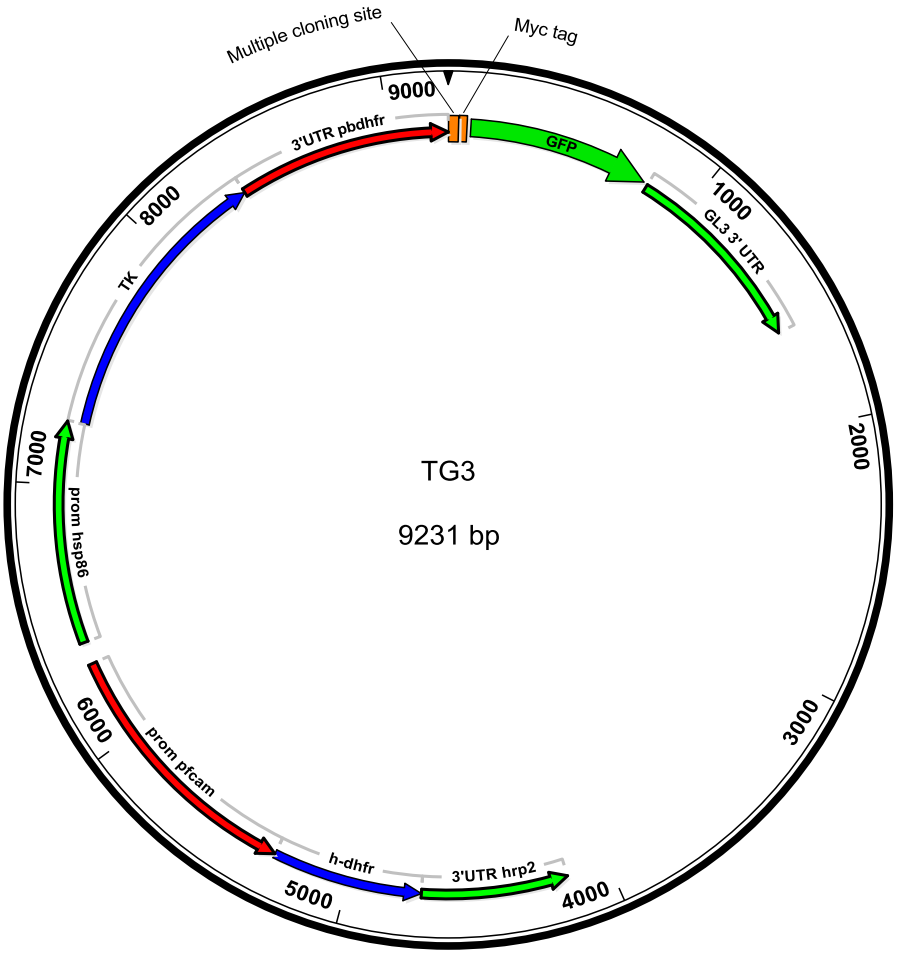
Figure 9. Proteomic and transcriptomic analysis of OBCs and Pfg377. Data from Silvestrini et al. 2010 (left) and Young et al. 2005 (right). Values in expression profiles correspond to emPAI (Exponentially modified protein abundance index) values. (Pfg377 value in proteomics is 445, detected only in late gametocytes; excluded for clarity). Values in transcription profiles correspond to MOID (Match-only Integral Distribution algorithm) values. ER: early rings, LR: late rings, ET: early trophozoites, LT: late trophozoites, ES: early schizonts, LS: late schizonts, M: merozoites, GC: gametocytes from day 1 to day 13.



Generation of transgenic OBC-GFP parasites

To further study OBCs, several transgenic lines were produced to generate parasites harboring a GFP-tagged chimeric version of the protein coded by the targeted gene. TK-GFP-ROM3 (TG3), a new GFP-tagging vector, was developed. It contains a multiple cloning site followed by a Myc immunotag, the GFP coding sequence and finally the 3' UTR region of PfROM3, a gene highly expressed in late gametocytes (G. Siciliano unpublished results). Importantly, the Myc tag was surrounded by Gly and Pro residues, which contribute to flexibility of the region, through the free mobility of Gly bonds and the helix-breaking effect of Pro residues. As a positive selection cassette, TG3 includes the hDHFR cassette, giving resistance to WR99210. TG3 also includes the TK (thymidine kinase) cassette, a negative selection cassette for Plasmodium giving sensitivity to ganciclovir. It has been observed that parasites with episomal copies of the cassette die faster than that with the integrated plasmid under ganciclovir selection (G. Camarda unpublished results), making it possible to select parasites with the integrated plasmid over parasites with the episomal plasmid (figure 10).

Figure 10. TG3 plasmid. GFP, hDHFR cassette and TK cassette are depicted. To generate the corresponding GFP-tagging vector, the 3' coding sequence of the target gene is cloned into the multiple cloning site.



Polylinker site of TG3 was then used to clone the 3' regions of PSOP12, SUB2 and OBCIp in frame with Myc tag and GFP coding sequence, producing plasmids pTG3-PSOP12, pTG3-SUB2 and pTG3-OBCIp. Each of these plasmids is able to recombine with the target gene through homologous recombination between the cloned gene fragment and the genomic site, introducing the coding sequence for Myc and GFP at the 3' end of the endogenous loci, followed by the 3' UTR region of GL3. This recombination event is promoted by drug selection using WR99210.

Regarding the rest of OBCs, localization of PfGEST had been determined through the production of mouse multiclonal antibodies as described above, while a clone from a DPAP2-GFP tagged line was kindly provided by M. Klemba, Virginia Tech, Virginia, USA (unpublished work). This clone was named DPAP2-GFP1.

Plasmids were transfected into 3D7 WT parasites and transfected parasites were selected under presence of WR99210. After resistant parasites were obtained, they were cultured for seven days without drug selection, and then WR99210 was reintroduced, together with the negative selector TK for five days, which was introduced to eliminate parasites containing episomal plasmids still present in the culture. Two additional 7 days cycles of WR99210 presence and absence were conducted to minimize the presence of episomal plasmid in the resistant strains.

Diagnostic PCRs were conducted on bulk cultures to investigate the correct integration of the plasmids into the parasite genome. PCR products indicative of the integrated form of each of the three plasmids were generated using genomic DNAs of the correspondent transfected parasites as templates. Products indicative of the WT form of each locus were also detected after PCR in each of the genomic DNAs, indicating a mixed population of parasites with recombinant and WT target loci (data not shown).

In an attempt to maximize the recombinant population in the transfected cultures, clones from the different bulk cultures were obtained by limiting dilution. Two clones from each strain were obtained, and diagnostic PCR analyses were conducted on genomic DNA from these clones. Products of the expected length indicating the presence of the recombinant sites in each of the genomic DNAs from the selected clones were detected after PCR. The product indicative of the presence of the WT site was also detected in the case of DNA from pTG3-PSOP12 and one of the clones of pTG3-SUB2-transfected parasites, indicating a mixed population of recombinant and WT parasites in these clones.

Finally, clone 1 from each of the three strains was selected for further characterization, naming the clones PSOP12-GPF1, SUB2-GFP1 and OBCIp-GFP1 respectively (figure 11 and 12 and table 5). The homogeneity of these clones will be examined in the future through Southern blot analysis.

Figure 11. Analysis of GFP-tagged mutants. Diagram of recombination events showing plasmid and original and recombinant locus. Primers used for diagnostic PCR are marked as: 5' F UP (5' F upstream), 3' R DOWN (3' R downstream), 5' R IN (5' R internal), 3' F IN (3' F internal). GFP: GFP coding sequence. HR: Homologous region. AUG: target gene's start codon. STOP: target gene's stop codon.

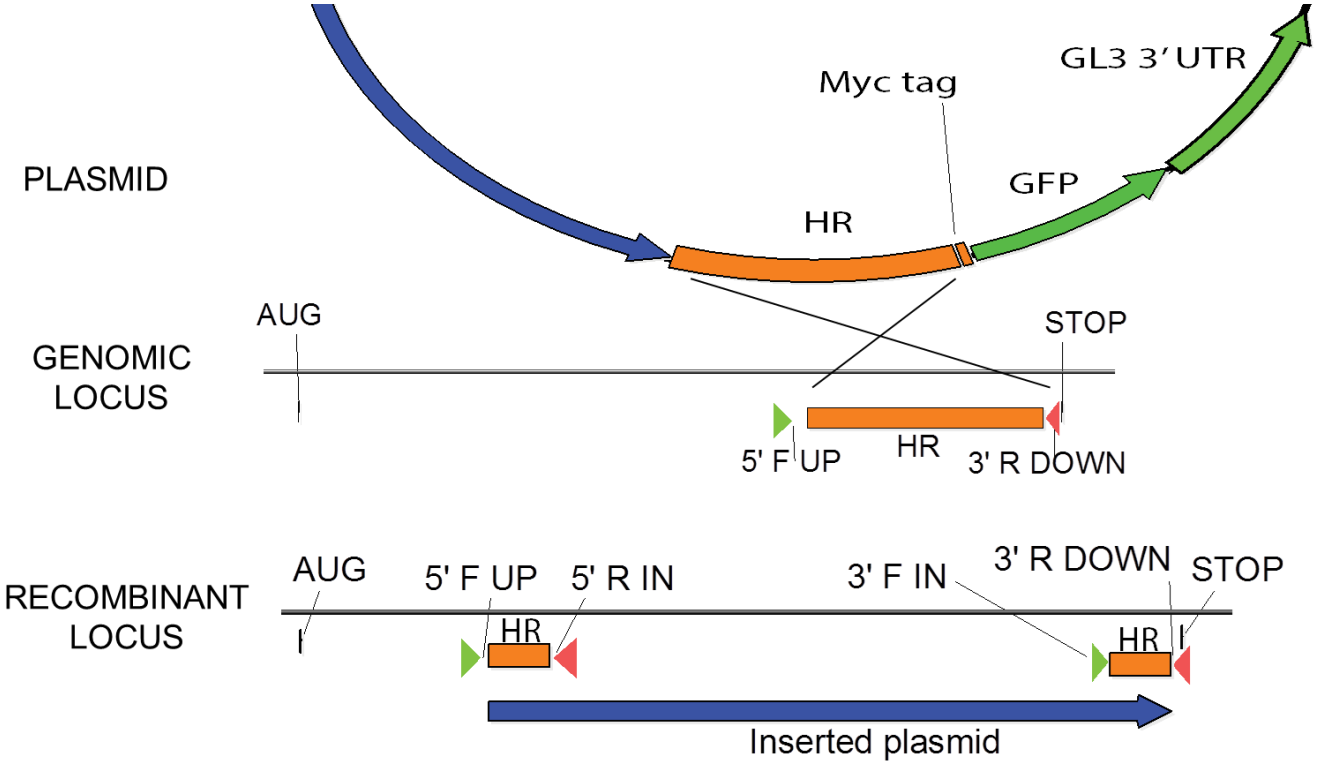
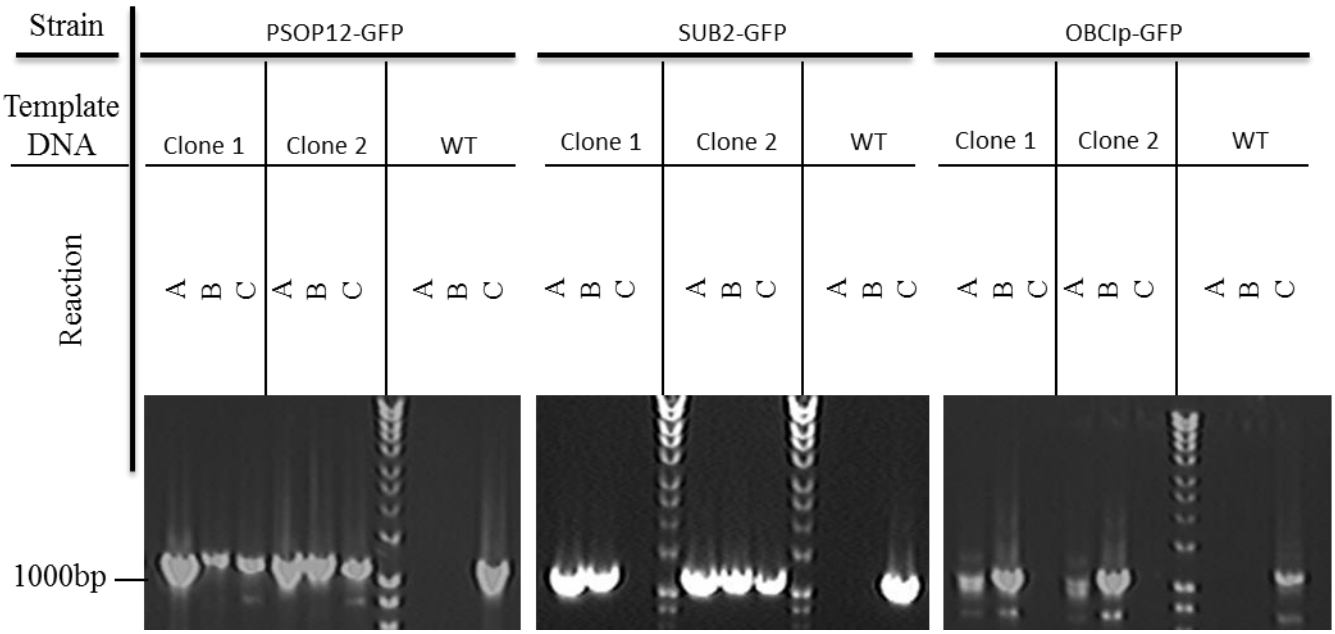


Table 5. Primers and expected lengths in diagnostic PCRs for GFP-tagging vectors.

Target gene	Reaction	Primer pair	Expected lenght (bp)	Product indicative of:
pfPSOP12	A	45-51	1179	5' recombinant locus
	B	52-46	1192	3' recombinant locus
	C	45-46	1160	WT locus
pfSUB2	A	47-51	1237	5' recombinant locus
	B	52-48	1249	3' recombinant locus
	C	47-48	1207	WT locus
pfOBCIp	A	49-51	1075	5' recombinant locus
	B	52-50	1024	3' recombinant locus
	C	49-50	1120	WT locus

Figure 12. Diagnostic PCR of parasites transfected with GFP-tagging plasmids.

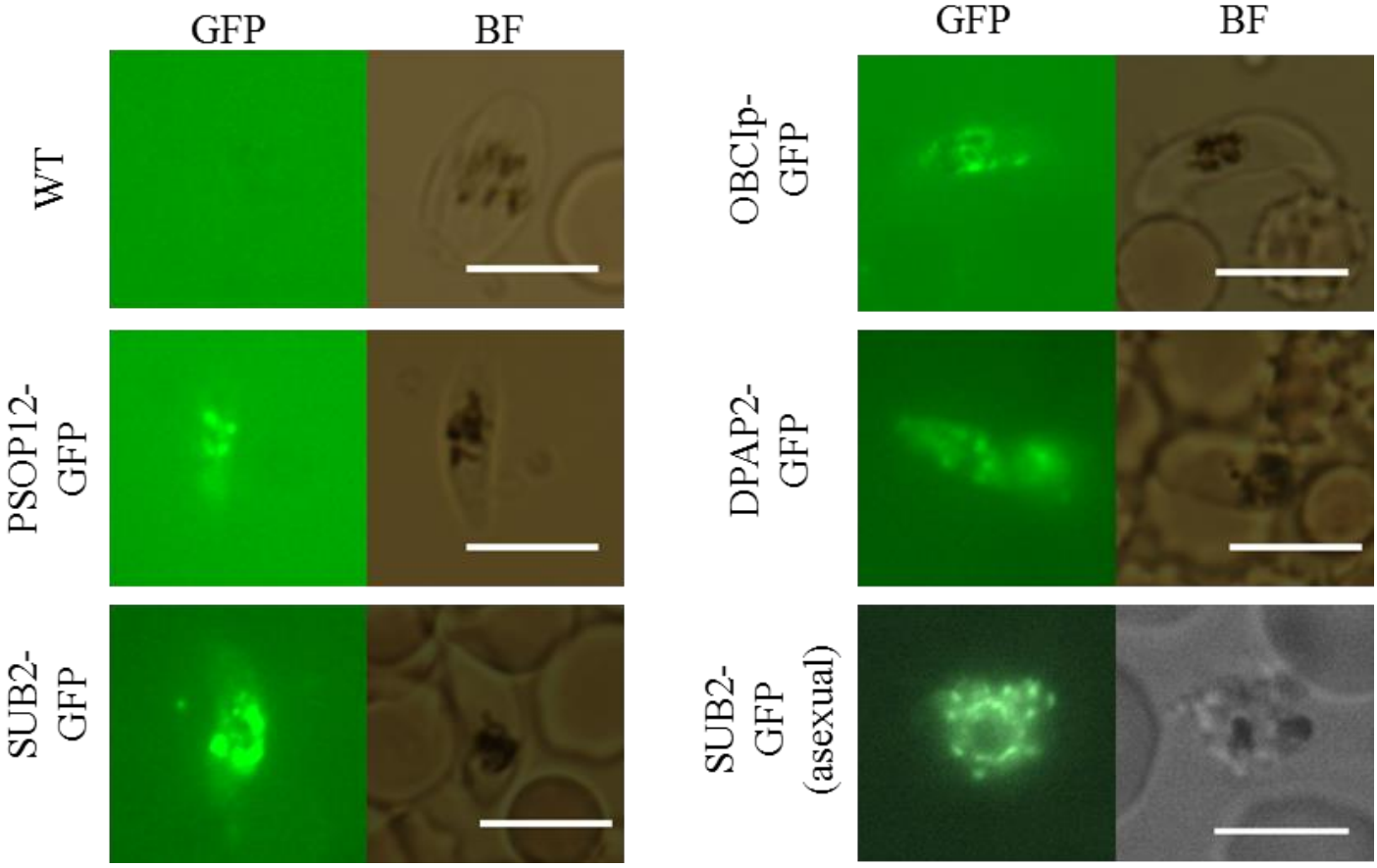


Characterization of OBC-GFP parasites

Mature gametocytes from PSOP12-GFP1, SUB2-GFP1, OBCIp-GFP1 and DPAP2-GFP1 clones were analyzed for GFP fluorescence. Live parasites were attached to poly-lysine coated Petri dishes and observed for GFP-emitted fluorescence. Mature SUB2-GFP1 and OBCIp-GFP1 gametocytes showed a granular, peripheral fluorescence pattern in live mature gametocytes, which was less obvious in the case of DPAP2-GFP. In the case of PSOP12-GFP parasites, no clear signal could be observed in live parasites. Additionally, fluorescence was detected in live asexual SUB2-GFP schizonts, in agreement with the established presence of PfSUB2 in merozoite micronemes [36] (figure 13).

These results show that parasites from at least three of the four OBC-GFP parasites produce a functional GFP-tagged version of their respective OBC protein, and that the expression pattern and timing of such protein is in agreement with its presence in the OBs.

Figure 13. Live OBC-GFP parasites. Mature gametocytes (and schizonts in the case of SUB2-GFP) were attached to polylisin-coated Petri dishes and observed for GFP-emitted fluorescence. WT: Wild Type parasites. BF: Bright Field. Scale bar: 5μm.



To explore the possible localization of the tagged proteins to the OBs, IFA analyses were performed on acetone-fixed parasites of the OBC-GFP lines. For this, commercially available α-GFP antibodies from two different origins were used. The GFP-associated fluorescence pattern observed in live parasites was reproduced in mature gametocytes incubated with α-GFP antibodies in the cases of SUB2-GFP1, OBCIp-GFP1 and DPAP2-GFP1 (figures 14 and 15A, left panels).

Pfg377 was then used as a marker for OBs. α-Pfg377 antibodies were used in conjunction with α-GFP antibodies in IFAs, which showed colocalization of the fluorescences associated to these two antibodies in gametocytes of the SUB2, OBCIp and DPAP2 tagged strains, indicating colocalization of the GFP-tagged protein and Pfg377 (figures 14 and 15A, right panels). Mature SUB2-GFP1 schizonts were also acetone-fixed and probed with antibodies against GFP and the micronemal protein AMA-1. The resulting fluorescence patterns from the two proteins were coincident, confirming the tagged protein is targeted to the micronemes, as described for the native form of PfSUB2. SUB2-GFP parasites did not show any obvious growth alteration, which could arise from steric or conformational problems caused by the GFP tag in the essential PfSUB2 protein [36]. These results indicate that PfSUB2, PfOBCIp and PfDPAP2 localize to the OBs in *P. falciparum* gametocytes.

In the case of PSOP12-GFP1 gametocytes, the previous PCR analysis indicated a mixed population of recombinant and WT parasites. In IFAs using antibodies against GFP on samples of acetone-fixed PSOP12-GFP1 gametocytes, the vast majority of parasites were positive for fluorescence, indicating that PfPSPOP12-GFP was expressed in these parasites. This indicates that the vast majority of parasites from clone PSOP12-GFP1 are recombinant, making it possible to analyze the localization of recombinant GFP in such parasites.

When PSOP12-GFP1 gametocytes were analyzed through IFA using α-GFP antibodies, they showed a diffuse GFP-associated pattern in the apices of gametocytes (figures 14 and 15, left panel). This diffuse apical signal found in single GFP marking was observed again in double IFA using α-Pfg377 antibodies in conjunction with α-GFP antibodies, and it did not colocalize to the granular pattern associated to α-Pfg377A2 antibodies (figure 14, right panel). There was an apparent partial coincidence between the α-GFP and α-Pfg377B signals in these parasites as well (figure 15, right panel). This was not the case when α-Pfg377A2 antibodies were used, which prompted the question of whether different parts of Pfg377 could reside in more or less diverse ensembles of OBs. To explore this, WT mature gametocytes were acetone-fixed and incubated with antibodies against both Pfg377 fragments. The resulting fluorescence patterns associated to each of the antibodies were very similar, indicating no obvious difference in localization between the two Pfg377 fragments (figure

16). The observed Pfg377B-associated signal coincident with PSOP12-GFP associated signal in PSOP12-GFP gametocytes is apparently different from the usual signal observed with α-PFG377B antibodies, which could be observed only in these transfected parasites. PfPSOP12 is predicted to interact with Pfg377B based on our co-IP data, an interaction that could be affected by a change in the properties of the tagged protein. The alteration of this interaction could provoke a different distribution of Pfg377B in mature gametocytes of PSOP12-GFP parasites, explaining the results in IFA of PfPSOP12-GFP gametocytes.

Figure 14. IFA of OBC-GFP parasites using Rabbit α-GFP antibodies. Mature gametocytes from the tagged strains were fixed on acetone and incubated with the indicated antibodies. Fluorescein and rhodamine were used as the secondary antibodies for α-GFP and α-Pfg377 antibodies respectively. Rabbit α-GFP antibodies (1:100 dilution) and Rat α-Pfg377A2 antibodies (1:400 dilution) were used. Left panel: single α-GFP IFA. Right panel: Double α-GFP/α-Pfg377 IFA. WT: Wild Type parasites. BF: Bright field. Rb: Rabbit antibodies. Rt: Rat antibodies. Scale bar: 5μm.

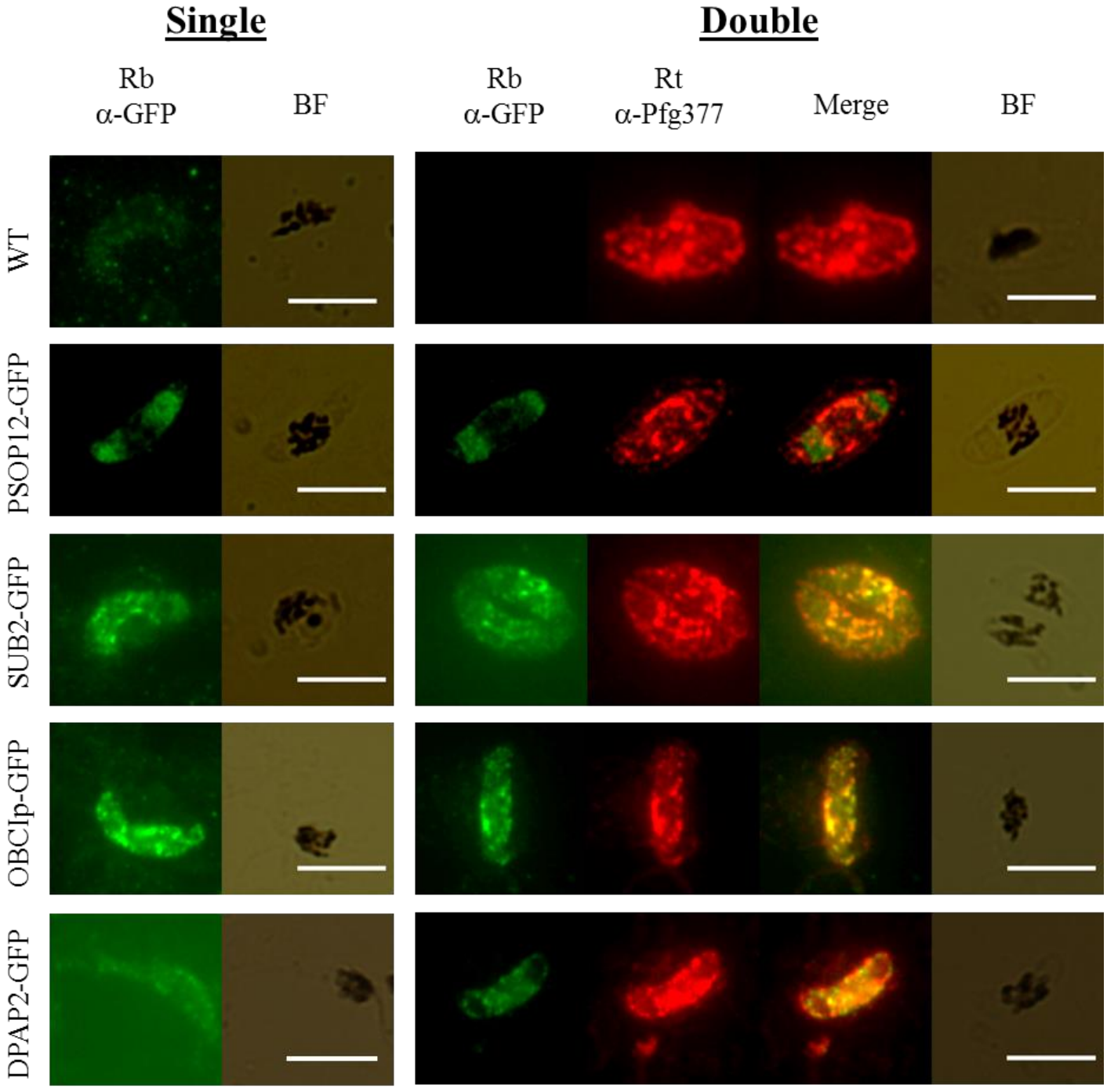
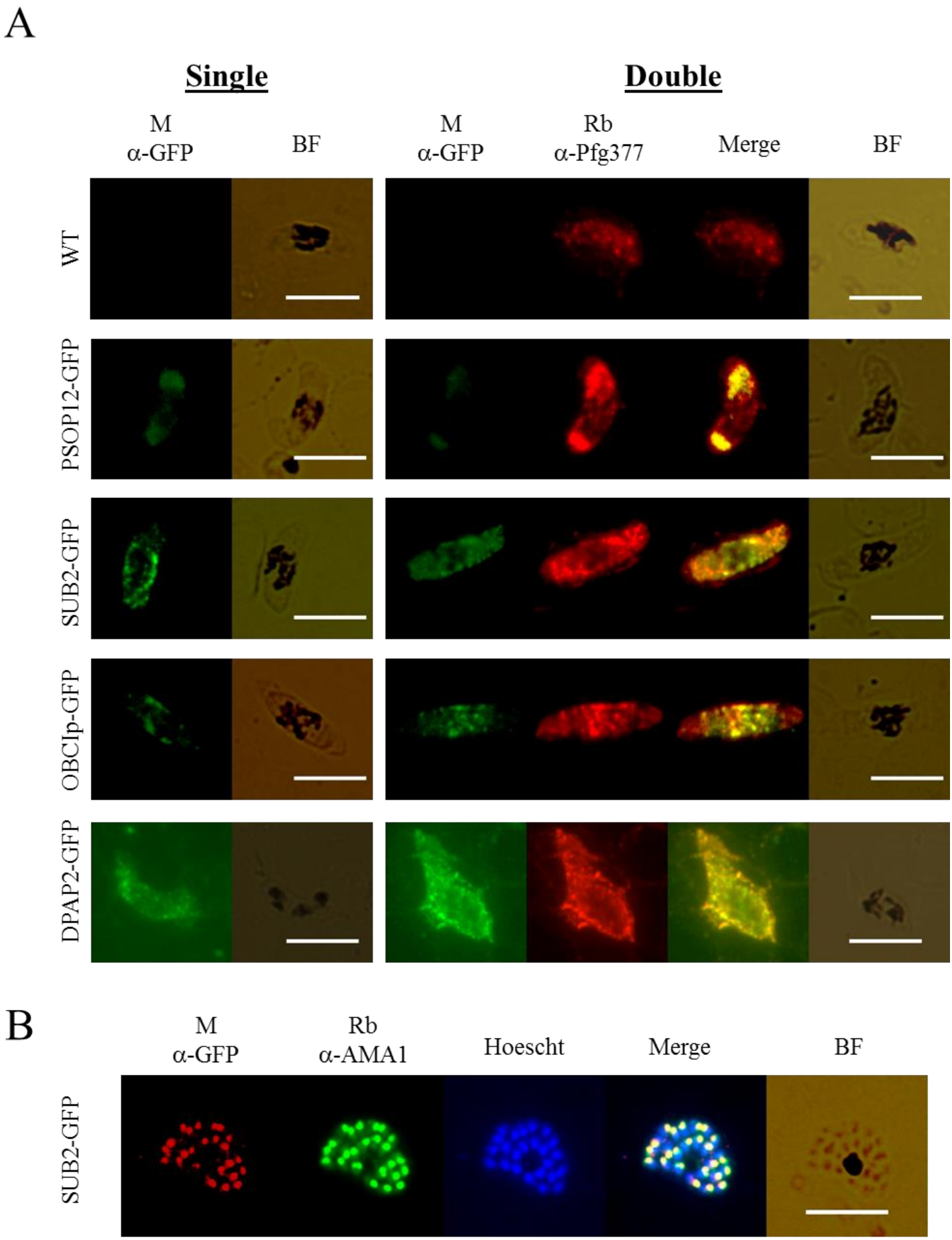


Figure 15. IFA of OBC-GFP parasites using Mouse α-GFP antibodies. Mature gametocytes (and schizonts in the case of SUB2-GFP) from the tagged strains were fixed on acetone and incubated with the indicated antibodies. Fluorescein and rhodamine were used as the secondary antibodies for GFP and Pfg377 respectively. Mouse α-GFP antibodies (1:100 dilution) and Rabbit α-Pfg377B antibodies (1:800 dilution) were used. **A**: mature OBC-GFP gametocytes. Left panel: single α-GFP IFA. Right panel: Double α-GFP/α-Pfg377 IFA. **B**: mature SUB2-GFP schizont IFA. BF: Bright field. M: Mouse antibodies. Rb: Rabbit antibodies. Scale bar: 5μm.


A
Single
Double
M
α-GFP
BF
M
α-GFP
Rb
α-Pfg377
Merge
BF
WT
PSOP12-GFP
SUB2-GFP
OBCIp-GFP
DPAP2-GFP
B
M
α-GFP
Rb
α-AMA1
Hoescht
Merge
BF
SUB2-GFP

OBs are much more abundant in female gametocytes than in male gametocytes, and α-Pfg377 antibodies are able to detect Pfg377 in IFA only in female parasites. To investigate the presence of OBC proteins in male gametocytes, OBC-GFP gametocyte samples were acetone-fixed and incubated with antibodies against Pfg377 and GFP. Samples were then examined for gametocytes negative to Pfg377-associated fluorescence, considered to be male gametocytes. Subsequently, the presence or absence of GFP-associated fluorescence in male gametocytes was determined. For SUB2-GFP and OBCIp-GFP parasites, all male gametocytes were negative for GFP-associated fluorescence (figure 17). PSOP12-GFP male gametocytes showed the bipolar GFP-associated fluorescence pattern described in female gametocytes, while for DPAP2-GFP not enough parasites have been examined so far to evaluate the presence or absence of the tagged protein in male gametocytes (data not shown). These results support a possible sex-specific expression of SUB2 and OBCIp in gametocytes.

Figure 16. IFA of WT gametocytes using antibodies against different fragments of Pfg377. Acetone-fixed gametocytes were incubated with Rabbit α-Pfg377B antibodies (1:800 dilution) and Rat α-Pfg377A2 antibodies (1:400 dilution). Rb: Rabbit antibodies. Rt: Rat antibodies. WT: Wild Type. BF: Bright Field. Scale bar: 5μm.

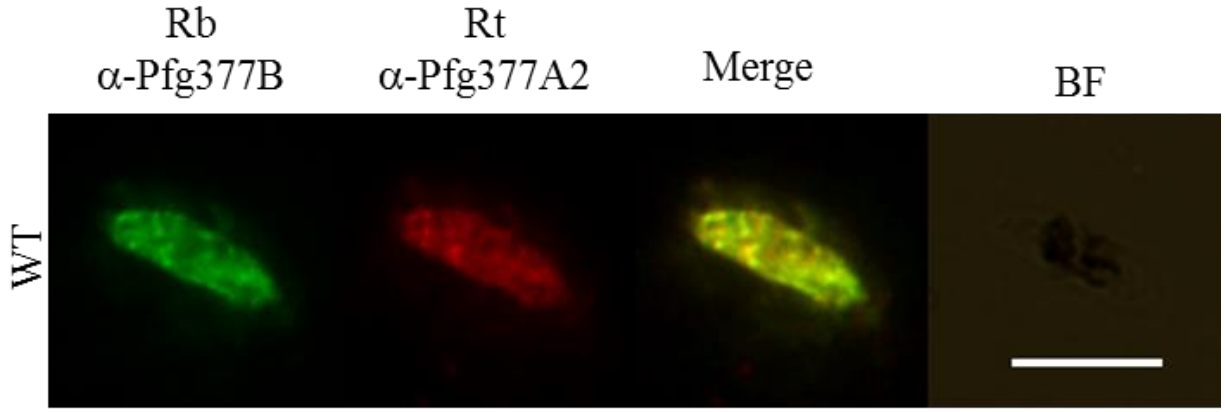
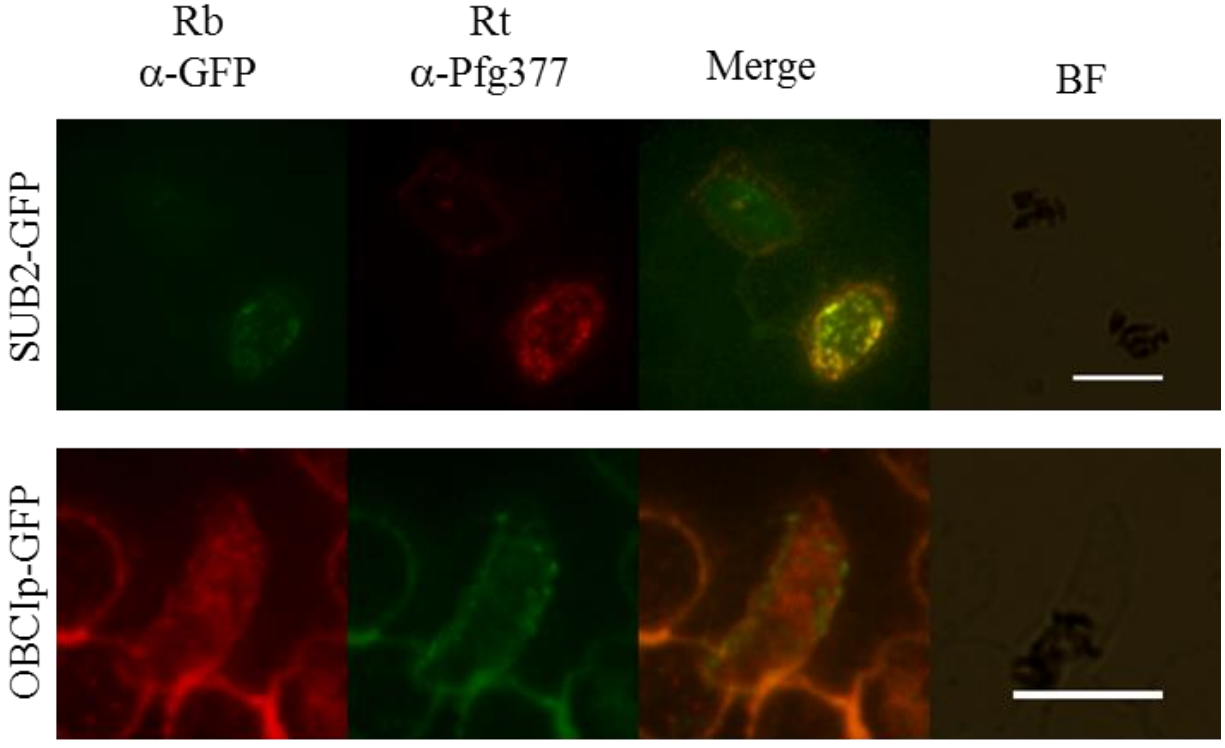


Figure 17. Male gametocytes of the SUB2-GFP and OBCIp-GFP strains do not show presence of GFP in IFAs. For SUB2-GFP, a male (up-left) and a female (down-right) gametocyte are shown. Gametocytes from the indicated strains were acetone-fixed and incubated with rabbit α-GFP antibodies (1:100 dilution) and α-Pfg377 antibodies (1:400 dilution). At least 100 gametocytes were examined for each strain. Rb: Rabbit antibodies. Rt: Rat antibodies. BF: Bright field. Scale bar: 5μm.



Taken together, our results show that the GFP-tagged SUB2, OBCIp and DPAP2 localize to the OBs in mature gametocytes of the correspondent OBC-GFP parasites. This validates both the OB-/OB+ and the co-IP approaches used to select OBCs.

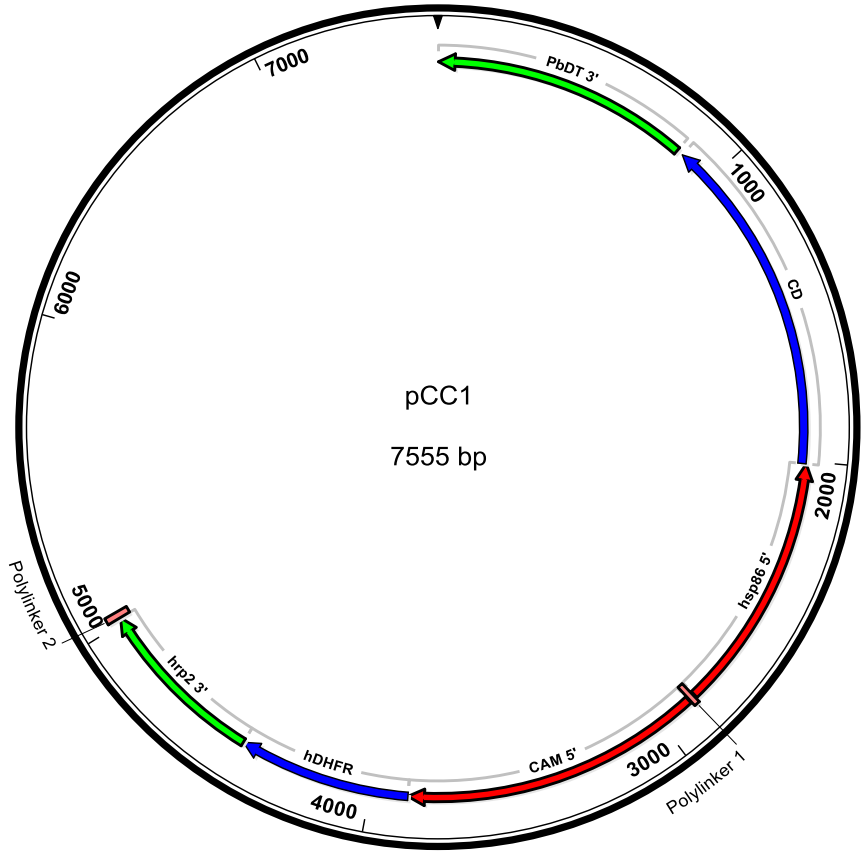
In the case of PSOP12, no clear fluorescence signal was observed in gametocytes harboring a GFP-tagged version of the gene coding this protein, making it not possible to determine its location within the parasite.

Generation of KO strains of OBCs

OBC genes were then selected to be disrupted through double crossover recombination, using plasmid pCC1 [17]. Plasmids pCC1-GESTKO, pCC1-PSOP12KO and pCC1-OBCIpKO were constructed and transfected into parasites, to generate the GEST-KO, PSOP12-KO and OBCIp-KO strains, respectively. DPAP2-KO1, a clone from a DPAP2-KO strain, was kindly provided by Kim Williamson [37]. In the case of SUB2, disruption of the gene has been unsuccessful previously [38], which supports the notion that the protein is essential for the asexual cycle, and therefore a plasmid to generate SUB2-KO parasites was not produced.

pCC1 plasmid contain a hDHFR (human dihydrofolate reductase) positive selection cassette, that confers resistance to WR99210. hDHFR cassette is flanked by two polylinker sites to clone two fragments of the target gene, which will act as the homology regions (HR) needed in recombination. Double crossover recombination is ensured to take place by the presence in the backbone of the plasmid of the CD (cytosine deaminase) cassette. CD allows the conversion of 5-fluorocytosine to the toxic 5-fluorouracil, constituting a negative selector of cells containing this expression cassette. Only cells that have incorporated the hDHFR cassette and lost the CD cassette through double crossover recombination will survive a combined treatment with WR99210 and 5-fluorocytosine. This selection system has been proven useful although with limited efficiency in the context of the very few available genetic selection systems for *P. falciparum*[39].

Figure 17. pCCI plasmid used to generate OBC-KO plasmids. Homology Region I and II of the targeted gene are cloned in polylinker 1 and 2 sites respectively to generate the correspondent OBC-KO plasmid.



Parasites were transfected, and cultures resistant to WR99210 were obtained, and then grown without drug selection to eliminate episomal plasmids. After reintroducing WR99210, 5-fluorocytosine was added and parasites resistant to both drugs obtained. Genomic DNA from these bulk cultures was isolated from asexual parasites and probed to investigate the correct disruption of the targeted genes.

PCR products indicative of the expected recombination events were produced from DNA of all three of the OBC-KO parasites. The product associated to the WT locus was also detected in all three cases, indicating the presence of mixed populations of recombinant and WT parasites in the cultures (data not shown).

These bulk OBC-KO cultures were then cloned by limiting dilution. Several clones were obtained from each strain, and genomic DNA was purified from these clones. Diagnostic PCRs using these DNAs as template showed the products of the expected length indicative of double crossover integration in PSOP12-KO and OBCIp-KO parasites. No PCR product corresponding to the WT locus was obtained when the specific primers were used in DNA from OBCIp-KO parasites, while PSOP12-KO showed a very faint signal corresponding to the presence of the WT locus. These results indicate successful disruption of PSOP12 and OBCIp (figures 18 and 19 and table 6). This will be confirmed in the future through Southern analysis of the DNA from OBC-KO clones.

These clones were named PSOP12-KO1 and OBCIp-KO1 and were selected for further study along with DPAP2-KO1.

Figure 18. Schematic representation of the double crossover recombination events between pOBC-KO and the targeted OBC, resulting in disruption of the targeted OBC. Primers used to determine the presence of the genomic locus and the recombinant locus are indicated. CD: Cytosine Deaminase. HR: Homology Region. hDHFR: human Dihydrofolate Reductase. AUG: Start codon. UP: Upstream. DOWN: Downstream.

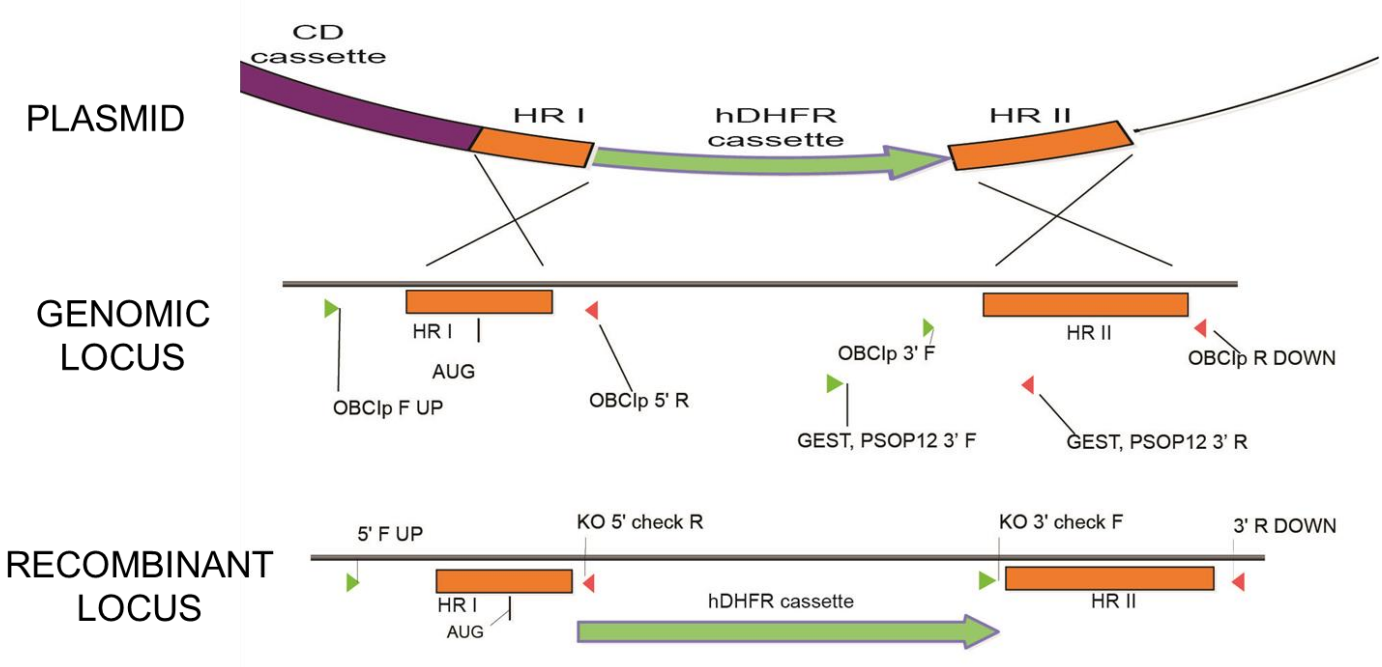
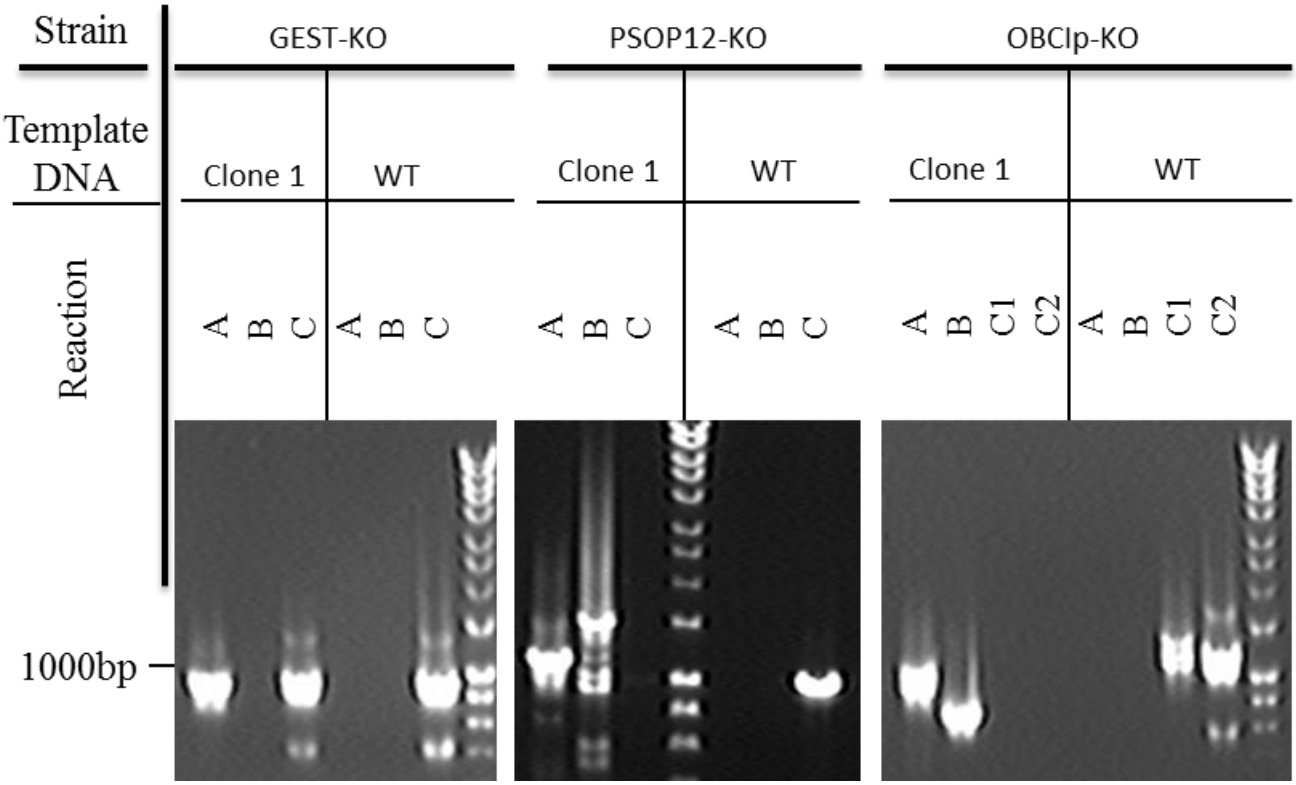


Table 6. Primers and expected lengths in diagnostic PCRs for OBC-KO vectors.

Target gene	Reaction	Primer pair	Expected lenght (bp)	Product indicative of:
pfGEST	A	55-53	985	Recombination through HRI
	B	54-56	1221	Recombination through HRII
	C	23-24	972	Presence of WT or single recombinant locus
pfPSOP12	A	57-53	1134	Recombination through HRI
	B	54-58	1213	Recombination through HRII
	C	29-30	1004	Presence of WT or single recombinant locus
pfOBCIp	A	59-53	1134	Recombination through HRI
	B	54-60	790	Recombination through HRII
	C1	59-38	1401	Presence of WT locus (5' end)
	C2	37-60	1289	Presence of WT locus (3' end)

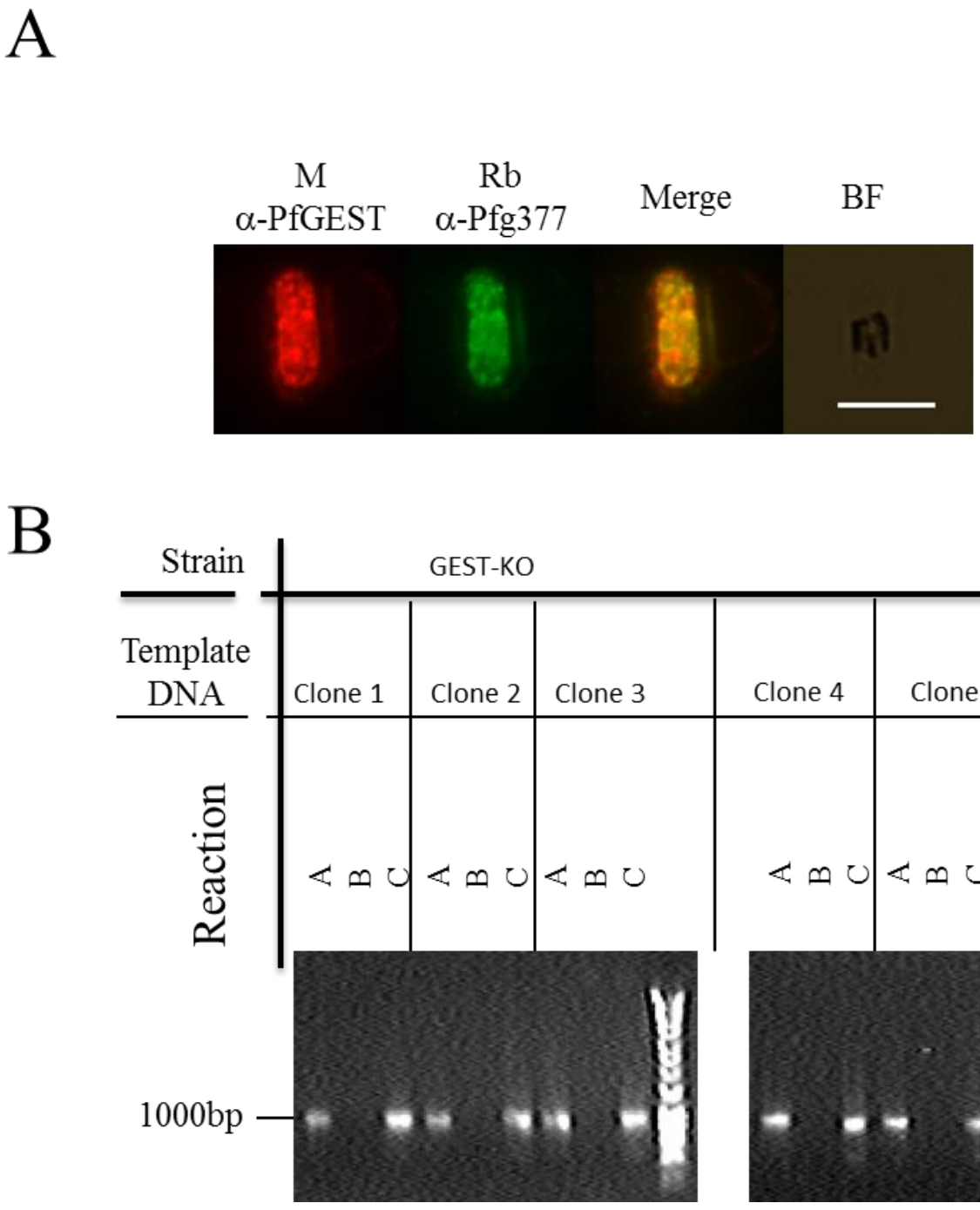
Figure 19. Diagnostic PCR of parasites transfected with KO plasmids.



DNA from the GEST-KO clone showed the product indicative of an integration of the plasmid in the 5' region of the target gene through homologous recombination of HRI, but it showed no product associated to recombination through HRII in the 3' region of the gene. In principle, this should result in parasites resistant to WR99210 but sensitive to 5-fluorocytosine, but these parasites are resistant to both drugs. An aberrant recombination event may have occurred eliminating the CD cassette from the integrated plasmid, without recombination through HRII, explaining the resistance of the parasites to 5' fluorocytosine. Correct integration through HRI implies the lack of 5' untranslated region (5'UTR) in the resulting *pfGEST* site. This could cause a defect in GEST expression, which was evaluated through IFA analysis of GEST-KO gametocytes with α-PfGEST antibodies. PfGEST-associated fluorescence in GEST-KO gametocytes showed no difference compared to that of WT parasites. This indicated that the selected clone of GEST-KO parasites was not disrupted in its expression of the GEST protein (figure 20A). 5' UTR of the coding gene has been shown to be non-essential for the correct expression of other proteins in Plasmodium [40], and our results suggest this is the case for PfGEST.

Other GEST-KO clones from the same transfection were analyzed through diagnostic PCR performed on genomic DNA obtained from such clones. Only the PCR product associated to the 5' recombination event was observed, while the PCR product associated to the WT locus was produced in samples from all the selected clones (figure20B). In another attempt to achieve *pfGEST* disruption, three additional transfections were performed with pGEST-KO, obtaining parasites resistant to WR99210 in all of them. When 5-fluorocytosine was introduced in the cultures of the additional transfections, no resistant parasites emerged after the expected time.

Figure 20. GEST-KO gametocytes recombinant in the 5' region of *pfGEST* show normal PfGEST-associated fluorescence in IFAs. **A**: IFA of mature GEST-KO gametocytes from clone 1. Mouse α-PfGEST antibodies 1:100 dilution, rabbit polyclonal α-Pfg377 antibodies 1:800 dilution. M: Mouse. Rb: Rabbit. BF: Bright field. Scale bar: 5μM. **B**: Additional clones of GEST-KO parasites show integration of the plasmid only through the 5' integration site. Primers used and expected lengths for diagnostic PCRs are described in table 4.



Characterization of the egress phenotype of OBC-KO gametocytes.

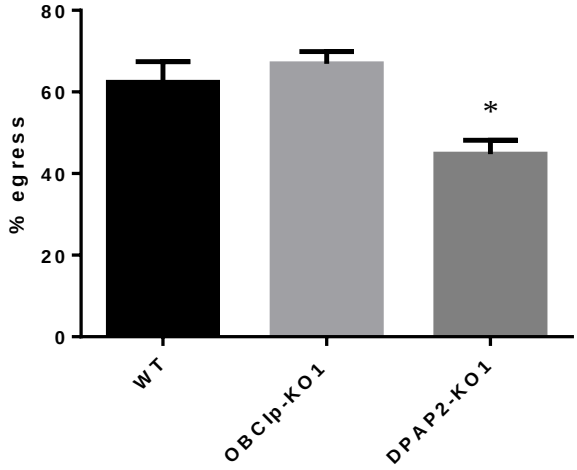
Although our results indicate that *pfg377*KO parasites can egress efficiently from the host RBC, OBs in *P. falciparum* could play an important role in egress, as suggested by their discharge during gametogenesis and the importance in this process of at least two proteins present in the OBs of *P. berghei* [10, 11].

We evaluated the egress efficiency of parasites of OBC-KO clones that produced enough mature gametocytes: OBCIp-KO1 and DPAP2-KO1. PSOP12-KO1 did not produce gametocytes in repeated attempts of induction of sexual development (data not shown).

Egress efficiency was measured based on the presence or absence of the RBC surrounding the rounded-up gamete, marked by the staining of RBC membrane with WGA-conjugated fluorophores [12], as described in part II.

No difference in egress efficiency was found in OBCIp-KO parasites compared to WT parasites, while DPAP2-KO gametocytes showed a reduced egress efficiency (from 60% to 45%), which was found to be statistically significant (figure 21).

Figure 21. Egress efficiency of OBC-KO gametocytes. Egress is given as % of WGA negative spherical gametes over total spherical gametes. WT and OBC-KO mature gametocytes were activated in presence of XA and their egress efficiency evaluated in 4 replicate experiments. Error bar: SD. n=200. ∗: Significant egress deficiency compared to WT gametes.



Discussion and future prospects

Early mosquito phases of Plasmodium parasites are still poorly understood. Few proteins have been identified to play a role in gametogenesis, and the molecular mechanisms that result in rounding up of the falciform mature gametocyte and egress of the resulting gamete from the host RBC have not been clarified.

Pfg377 was shown to be important in the biogenesis of OBs, and was proposed to be important for gamete egress based on previous reports [13]. The newly generated *pfg*377KO mutants confirm the importance of Pfg377 in the generation of OBs, which appear in much reduced numbers in these parasites. The reduction in size and eccentricity in the residual OBs of *pfg377*KO gametocytes suggests that incipient OBs are still synthetized in these parasites, but the lack of Pfg377 affects its developments into fully functional organelles. It is possible that the smaller dimensions of these OBs result in less organelles being observed in electron microscopy sections, while the total number of organelles remain the same. However, recent reports of a *pbg377*KO *P. berghei* mutant showing a reduction in size but not in number of OBs using similar electron microscopy techniques [41] supports the notion of a real reduction in the number of OBs in *pfg377*KO gametocytes.

The lack of a defect in gamete egress efficiency observed in the newly produced OB-depleted parasites confirms what was described in our recent work [12] and is also supported by a minimal, if any, egress defect reported in *P. berghei* gametes defective for the homologue Pbg377 [41].

As the article describing the egress defect of the *pfg377*KO gametocytes also showed that these parasites failed to efficiently infect mosquitoes, such a mosquito infectivity phenotype needs to be re-evaluated as well.

Identification of new proteins of the OBs in *P. falciparum* gametocytes

To describe proteins present in OBs we have taken advantage of the OBs depletion observed in *pfg377*KO gametocytes. Initially, efforts were done to isolate the OBs from WT parasites, but enrichment of OBs could not be achieved despite repeated attempts (data not shown). Comparative whole cell proteomic analysis was therefore chosen to continue the experiments.

The almost complete depletion of a specific organelle in a mutant cell is not a common occurrence, as usually all organelles are essential for the correct functioning of the cell, and mutants lacking a specific organelle cannot be stably produced. The only other case of organelle depletion in Plasmodium to date is the generation of apicoplast-depleted parasites through chemical treatment, which were dependent on chemical rescue for survival [42]. The presence of OBs only in the sexual

forms of Plasmodium and the generation of *P. falciparum* mutants through culture of the asexual forms of the parasite alone result in the possibility of generating stable *pfg377*KO parasites, which produce gametocytes depleted of OBs.

Pfg377 resides in OBs alone, and therefore it is not expected to have a regulatory role in the expression of other proteins of the OBs. Therefore, the expression of such proteins is not predicted to be altered by the lack of Pfg377 in the mutant parasites. However, we hypothesized that in OBs-depleted *pfg377*KO gametocytes, proteins directed to the tiny and scarce residual OBs could not be efficiently incorporated to them. Such proteins, probably transported to OBs through Golgi vesicles in a similar manner to proteins of other secretory organelles in Plasmodium [43], would accumulate in the cytoplasm, making it possible that they are more exposed to degradation, resulting in a lower amount of these proteins in the OB-depleted mature gametocytes compared to WT parasites. This degradation would not be efficient, resulting in the affected proteins still present in the OB-depleted gametocytes. Therefore, it was very important for the success of the comparative study to obtain quantitative data from multiple independent experiments. In this way, it would be possible to detect relatively small variations in the amount of specific proteins between WT and mutant parasites. Different proteins would be more or less susceptible to this degradation, depending on their solubility and folding properties. In our experiments, all selected proteins found to be underrepresented in *pfg377*KO gametocytes possess a transmembrane domain. It is possible that proteins targeted specifically to the membrane of OBs are more subject to degradation than other possible proteins directed to the lumen of OBs, which would have remained undetected in our comparative proteomic approach.

Results from the co-IP experiments using Pfg377 as bait suggest that this protein is able to bind to several other proteins of the gametocyte, including at least one protein from OBs. This opens the question of whether Pfg377 interacts with other components of OBs and facilitate their targeting to these organelles or their role in subsequent parasite stages. Co-IP experiments resulted in identifying an additional candidate (OBCIp) to the list of, and also identified one of the proteins selected in the above comparative proteomics study (PfPSOP12).

Combining previous knowledge from the murine model, whole cell comparative proteomics and co-IP, we were able to select five OBC, with finally four of them localizing to the OBs as shown in IFA experiments. PfGEST was also validated as an OB protein through immunoelectron microscopy, and similar experiments will be conducted in the rest of selected proteins as well.

PSOP12 was the only protein identified as potential OB protein in both whole cell comparative proteomics and Pfg377 co-IP experiments. However, it was the only selected candidate not identified as a component of these organelles.

Because we have not been able to detect expression of PSOP12-GFP to a specific location in gametocytes in our experiments, we cannot exclude that the endogenous protein is directed to the OBs. On the other hand, there are several explanations for an absence of PfPSOP12-GFP in the OBs. Recent reports show the homologue PbPSOP12 protein localizes to the surface of gametes [43]. Assuming that PfPSOP12 is not targeted to the OBs, it is possible that Pfg377 interacts with it (as suggested by our co-IP results), once gametogenesis takes place, retaining it in the surface of gametes. The spontaneously rounded-up *pfg377*KO gametes in the samples used for the proteomic analysis could have retained a smaller amount of PfPSOP12 than the ones in WT samples, explaining the lower levels of PfPSOP12 detected in the mutant parasites. Another possible explanation for the lack of a clear flurescence pattern in PSOP12-GFP gametocytes could be that the tagged protein is unable to fold correctly, resulting in deficient fluorescence activity, antibody recognition and/or targeting to its target organelle. The physiological amounts of PSOP12-GFP protein produced in the cell could also be under the detection limit for live GFP-associated fluorescence, although this is more improbable in the case of antibody-associated fluorescence in IFAs.

PSOP12 has been previously studied as a predicted ookinete secreted protein. *P. berghei* gametocytes lacking the homologue PbPSOP12 showed no significant viability defect in their mosquito stages, but recent reports confirm PbPSOP12 location to the surface of early mosquito stages and show that antibodies against this protein have a transmission-blocking effect *in vivo*. It will be interesting to evaluate if PfPSOP12 is also found in the surface of gametes, and its potential as an antigen for transmission-blocking antibodies.

Characteristics of the newly identified OB proteins

Of the identified OB proteins, PfGEST and PfOBCIp have an unknown mode of action, and the lack of predicted functional domains in these proteins makes it difficult to predict their *in vivo* function.

While no PfOBCIp homologue has been studied to date, PbGEST, the homologue of PfGEST in *P. berghei*, has been shown to be important for gamete egress and sporozoite traversal. PfGEST may play a similar role in *P. falciparum*, but it is not known how the protein intervenes in these processes.

The two other proteins newly localized to the OBs, PfSUB2 and PfDPAP2, are both known proteases.

PfSUB2 is an essential serine protease in the asexual parasite cycle, where it is expressed in late schizonts, localizing to the merozoite micronemes. It plays a critical role in merozoite invasion after relocalizing to the merozoite surface through microneme secretion. Here, it causes shedding of the merozoite adhesins MSP1 and AMA1 [45], in a process essential for productive erythrocyte invasion. In reference to this activity, PfSUB2 is also known as a sheddase. In this shedding process PfSUB2 translocates from the anterior to the posterior end of the merozoite in an actin-dependent movement as the merozoite enters into the host erythrocyte [46].

Ookinetes are capable of invading cells from the mosquito midgut while traversing to its basal side. The *P. berghei* homologue protein PbSUB2 has been reported to be present in ookinetes, where it was observed to be secreted to the cytoplasm of cells invaded by ookinetes [47]. It is interesting to hypothesize that PfSUB2 may be present in the surface of mosquito invasive forms, after being released from the OBs to the surface of the cell during gametogenesis. The possible role of PfSUB2 in this phase remains to be understood.

DPAP2 is an exopeptidase expressed only in the sexual stages of Plasmodium parasites [37]. Homologues PfDPAP1 and 3 described previously in *P. falciparum* have been shown to be essential for parasite survival, with roles in haemoglobin degradation and merozoite egress from the RBC, respectively [48, 49].

DPAP2-defective *P. falciparum* and *P. berghei* parasites present a normal gametocytogenesis, and in the case of *P. berghei* mutants, they are able to complete the whole life cycle (no evaluation of the mosquito infectivity in the described *pfDPAP2*KO parasites was performed in this report). However, a significative upregulation of *pbDPAP1* transcription was observed in *pbDPAP2*KO parasites, suggesting that a compensation mechanism was taking place [37]. Although PfDPAP1 is expressed in both sexual and asexual stages, its location has only been studied in the latter, where it is localized to the food vacuole. It will be important to study the location of DPAP1 in *P. falciparum* gametocytes

and its possible overexpression in DPAP2-defective parasites. The very mild gamete egress defect we observe in DPAP2KO parasites will have to be evaluated taking these compensation mechanisms into account.

In contrast, cellular distribution of the PfDPAP3 homologue, expressed only in asexual stages, has been described as punctuated in merozoites, possibly due to a targeting to secretory vesicles, with similarity to our observation of PfDPAP2 in OBs, the secretory organelles of gametocytes. PfDPAP3 has a role in PfSUB1 maturation in merozoites, suggesting a possible role for sex-specific DPAP2 in the maturation of PfSUB2 or other yet unidentified proteins of OBs [49].

Future directions

Characterization of the newly identified OB proteins will continue with analysis of the state of these proteins in gametes of OBC-GFP parasites. The observation that PfGEST remains in gamete samples in similar amounts to gametocyte samples asks whether this is the case for the rest of identified OB proteins. IFA and WB experiments will be conducted to evaluate the fate of the GFP tagged proteins in gametes. WB experiments on OBC-GFP gametocyte samples will be useful to validate the observations in IFA experiments too, by detecting OBC-GFP peptides of the expected lengths.

Generation of at least a second clone of each OBC-KO strain will be important to validate the results obtained in egress efficiency experiments and in future mosquito infectivity experiments.

Authorship statement

I performed the experiments described, with exception of the following: *pfg377*KO parasites were produced by Pietro Alano and cloned by Pietro Alano and Anna Rosa Sannella. Electron microscopy experiments were performed by Lucia Bertuccini. Mass spectrometry was performed by Edwin Lasonder and Vikram Sharma. Mouse immunization and serum extraction were performed by Leonardo Picci.

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