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SIGNALLING IN MALARIA PARASITES
THE MALSIG CONSORTIUM#

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Summary:

Depending on their developmental stage in the life cycle, malaria parasites develop within or outside host cells, and in extremely diverse contexts such as the vertebrate liver and blood circulation, or the insect midgut and hemocoel. Cellular and molecular mechanisms enabling the parasite to sense and respond to the intra- and the extra-cellular environments are therefore key elements for the proliferation and transmission of *Plasmodium*, and therefore are, from a public health perspective, strategic targets in the fight against this deadly disease. The MALSIG consortium, which was initiated in February 2009, was designed with the primary objective to integrate research ongoing in Europe and India on *i)* the properties of *Plasmodium* signalling molecules, and *ii)* developmental processes occurring at various points of the parasite life cycle. On one hand, functional studies of individual genes and their products in *Plasmodium falciparum* (and in the technically more manageable rodent model *Plasmodium berghei)* are providing information on parasite protein kinases and phosphatases, and of the molecules governing cyclic nucleotide metabolism and calcium signalling. On the other hand, cellular and molecular studies are elucidating key steps of parasite development such as merozoite invasion and egress in blood and liver parasite stages, control of DNA replication in asexual and sexual development, membrane dynamics and trafficking, production of gametocytes in the vertebrate host and further parasite development in the mosquito. This article, which synthetically reviews such signalling molecules

Résumé : SIGNALISATION CELLULAIRE CHEZ *PLASMODIUM* - LE CONSORTIUM MALSIG

Les parasites responsables du paludisme sont confrontés à des environnements extrêmement divers au cours du cycle parasitaire ; ils peuvent se trouver dans la circulation sanguine chez l'hôte vertébré et hémocoele chez le moustique vecteur, ainsi qu'à l'intérieur de divers types cellulaires (hépatocytes et érythrocytes de l'hôte vertébré, intestin et glandes salivaires de l'insecte). Les mécanismes moléculaires et cellulaires permettant au parasite de reconnaître et de répondre à ces environnements intra ou extracellulaires jouent un rôle crucial dans sa survie et son programme développemental, tant pour la prolifération que pour la transmission au vecteur, et représentent donc des cibles potentielles pour l'intervention chimiothérapeutique. L'objectif essentiel du consortium MALSIG, initié en février 2009, est d'intégrer la recherche en cours en Inde et en Europe sur i) *les propriétés des molécules impliquées dans la signalisation chez* Plasmodium, *et* ii) *les processus développementaux caractéristiques des étapes-clés du cycle parasitaire. D'une part, des études fonctionnelles chez* Plasmodium falciparum *et chez le modèle murin* Plasmodium berghei *révèlent des informations sur les protéine-kinases et phosphatases, ainsi que sur les molécules régissant les voies du calcium et des nucléotides cycliques. D'autre part, des approches cellulaires et moléculaires visent à élucider les étapes-clés du développement parasitaire, telles que l'invasion et la sortie des cellules-hôtes aux stades hépatique et sanguin, le contrôle de la réplication du génome parasitaire aux stades*

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and cellular processes, aims to provide a glimpse of the global frame in which the activities of the MALSIG consortium will develop over the next three years.

KEY WORDS : malaria, *Plasmodium*, *Toxoplasma*, signalling.

MOTS CLÉS : *malaria*, Plasmodium, Toxoplasma, *signalisation*

asexué et sexué, le trafic intracellulaire et la dynamique des membranes, la production des gamétocytes chez l'hôte vertébré et la poursuite du développement sexué chez le moustique. Cette revue présente de façon synthétique les molécules de signalisation et les processus cellulaires cités ci-dessus, et vise à définir le cadre général dans lequel les activités du consortium MALSIG vont se développer au cours des trois prochaines années.

INTRODUCTION

Malaria parasites still impose a huge Public Health burden in the developing world, with 40 % of the world population at risk, and an estimated 350-500 million clinical cases annually. This translates into 1-3 million deaths each year, mostly amongst young children in sub-Saharan Africa. In view of the growing concern caused by the emergence and spread of drug resistance in the apicomplexan parasite *Plasmodium falciparum*, the species responsible for the vast majority of lethal malaria cases, the development of novel control agents is an urgent task (Olliaro, 2005). The identification of novel targets for intervention is now facilitated by the availability of genomic databases for several *Plasmodium* species (Bahl *et al.*, 2003) (including *P. falciparum* and the widely used rodent malaria parasite *P. berghei*) and for *Toxoplasma gondii*, an apicomplexan parasite that is recognised as a valuable model for specific aspects of *Plasmodium* biology (Baum *et al.*, 2008).

Malaria pathogenesis is caused by the asexual multiplication of parasites in red blood cells (RBCs). After invasion of the RBC, the infecting merozoite develops into a so-called ring form, which grows to form a trophozoite, in which DNA synthesis is initiated around 30 hours post invasion. Several rounds of genome replication and nuclear mitoses lead to the formation of multinucleated schizonts, which eventually (48 hours post invasion in *P. falciparum*, 24 hours in *P. berghei* and *P. knowlesi*) rupture and release 16-32 new merozoites. Some merozoites, after invasion of the host RBC, arrest their cell cycle and differentiate into male or female gametocytes. These sexual cells do not contribute to pathology, but are required for transmission to the mosquito vector (reviewed in Sinden *et al.*, 1996). Upon ingestion by the insect, they complete their development to gametes; for male gametocytes, this involves three rounds of division leading to the release of eight flagellated gametes, a process known as exflagellation. Fertilisation and subsequent meiosis lead to the development of a motile ookinete, which crosses the midgut epithelium and establishes infection as an oocyst at the basal lamina of the epithelium. Sporogony, the asexual generation of several thousand sporozoites, occurs within the oocyst. Upon release from the oocyst, sporozoites invade the insect's salivary glands, where they are primed to infect a new human host during a subsequent blood meal taken by the mosquito. Injected sporozoites invade hepatocytes and undergo exo-erythrocytic schizogony, whereby several thousand RBC-infective merozoites are produced.

The transition between successive developmental stages of this complex life cycle, as well as the completion of the developmental programme of each of the individual stages, is most likely regulated by extensive signalling pathways. Surprisingly little is currently known about the organisation of such pathways and their role in parasite development, despite recent advances *i)* in the characterisation of signalling pathway components (*e.g.* protein kinases, nucleotide cyclases/phosphodiesterases, calcium signalling, chaperones, etc), and *ii)* in the description of the biology of developmental stages. In this review, we will first (Section 1) provide an overview of current knowledge pertaining to *Plasmodium* signalling molecules, and second (Section 2), review selected biological processes from the perspective of signalling pathways.

1 - *PLASMODIUM* SIGNALLING PATHWAYS COMPONENTS

Although several signal transduction pathway components had been identified in *Plasmodium* prior to the genomics era, the relatively recent availability of genomic databases, in particular PlasmoDB (Aurrecoechea *et al.*, 2009), has allowed exhaustive searches for such molecules. Over recent years, reverse genetics techniques were developed to assess the biological function of specific genes. Many approaches, particularly for regulatable expression (Herm-Gotz *et al.*, 2007; Meissner *et al.*, 2007), were pioneered in *T. gondii* (Fig. 1) and applied subsequently to *Plasmodium* (Armstrong & Goldberg, 2007), while others were designed directly for malaria parasites, notably those involving stage-specific recombination (Carvalho et *al.*, 2004; Combe *et al.*, 2009). Thus, the conjunction of enhanced bio-informatics capabilities with reverse genetics tools has allowed considerable advances in investigating signalling pathway components in Apicomplexa, as discussed in the following sections.

Protein kinases (PKs)

Two published studies of the *P. falciparum* kinome (Anamika *et al.*, 2005; Ward *et al.*, 2004) yielded a pic-

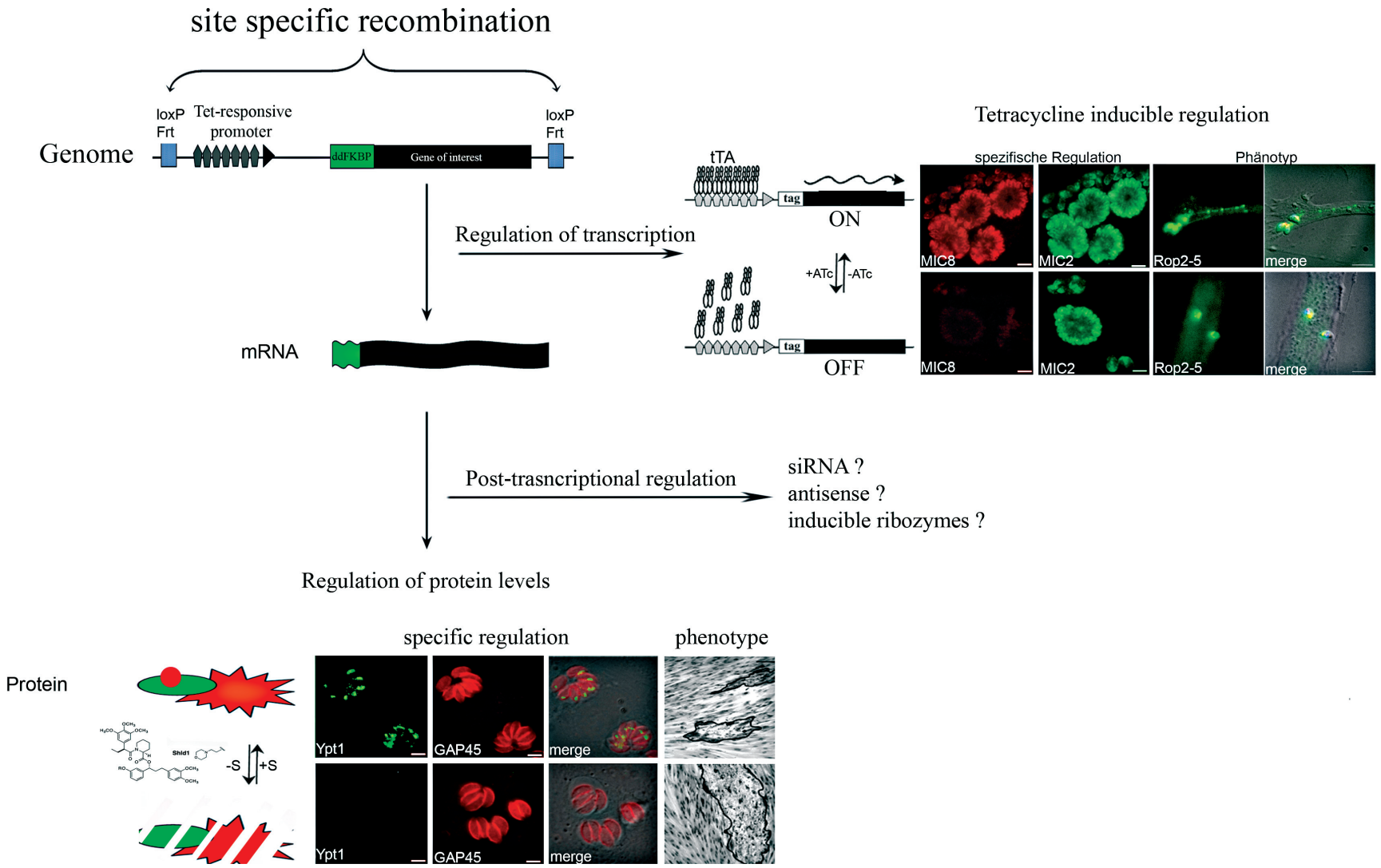

Fig. 1. – Overview of ectopic regulation systems established in *Toxoplasma gondii*.
Site specific recombinase (Flp or Cre) allows the generation of KO mutants by the selective removal of a gene of interest (GOI) that is flanked by the respective recognition sequences (Frt or Cre). The tetracycline inducible system enables a reversible regulation of a GOI and consequently the establishment conditional KOs. The depicted example demonstrates specific regulation of the essential micronemal protein 8 (MIC8). Reduction of MIC8 expression results in parasites that are unable to invade the host cell due to a defect in rhoptry secretion. Other proteins like MIC2 are not affected in their expression. An alternative for the generation of conditional mutants is the employment of the ddFKBP system that allows direct regulation of protein levels. In absence of the inducer Shield-1 a fusion protein between ddFKBP and the protein of interest is immediately degraded. In presence of Shield-1 the whole protein is stabilised. The example demonstrates the essential role of the small G-protein Ypt1. Conditional dominant-negative expression results in a significant reduction in parasite growth. Courtesy of Markus Meissner.

ture of profound divergences between *Plasmodium* and mammalian PKs, at the levels of *i)* the composition of the kinome itself, *ii)* the organisation of signalling pathways, and *iii)* the properties of individual enzyme orthologues (reviewed in Doerig & Meijer, 2007).

• Divergences in kinome and in the organisation of signalling pathways.

Most established eukaryotic PK groups have members in *Plasmodium*, with the notable absence of tyrosine protein kinases (TyrK) and a group of PKs involved in mitogen-activated protein kinase (MAPK) pathways (STEs). Conversely, many "orphan" PKs do not cluster with any of the PK families established from the yeast and mammalian kinomes, *e.g.* the FIKK kinases, members of which are found only in apicomplexan parasites (Schneider & Mercereau-Puijalon, 2005; Ward *et al.*, 2004). Finally, several plasmodial PKs do cluster within established families, but branch off near the base of the branch, precluding the assignment of any precise orthology with specific mammalian enzymes - these are often called "semi-orphan" kinases. Malaria parasites possess several genes encoding calcium-dependent protein kinases (CDPKs), a PK family characterised by the presence of an EF-hand calcium-binding domains on the same polypeptide chain as the kinase domain, which is also found in ciliates and plants, but not in mammalian cells (Bradley *et al.*, 2005). Two atypical MAP kinases are present in *P. falciparum* and the absence of typical MAPKKs (STEs) in the malarial kinome indicates that their mode of activation differs from that of MAPKs of other eukaryotes. For example, malaria parasites do not possess classical 3-component (MEKK-MEK-MAPK) pathways of the ERK1/2, p38 or SAPK/JNK families (Anamika *et al.*, 2005; Dorin *et al.*, 2005; Ward *et al.*, 2004).

• Divergences in the structural properties of individual PKs.

Many "orthologous" or "semi-orphan" plasmodial PKs display atypical characteristics, such as large insertions/extensions, or variant regulatory sites. Furthermore, several plasmodial PKs are "composite" enzymes, displaying primary structure features from distinct PK groups in a single molecule (Bracchi-Ricard *et al.*, 2000; Doerig, 2004; Doerig & Meijer, 2007; Dorin *et al.*, 2005). The two crystal structures of plasmodial PKs that have been published so far (Holton *et al.*, 2003; Merckx *et al.*, 2008a) indicate that primary structure differences between mammalian and *Plasmodium* kinases translates into spatial divergences in the catalytic cleft that can be exploited for selective inhibition, explaining observed differential susceptibility to chemical inhibitors.

• Functional analysis of Plasmodium protein kinases has been initiated only recently.

The inability to knock-out a given locus, together with the ability to modify the allele in a way that does not cause loss-of-function of the gene product, can be interpreted as strongly indicative of an essential role of that locus during asexual blood-stages. This approach led to the identification of *P. falciparum* PKs that are essential for erythrocytic schizogony, such as PfCK2 (Holland *et al.*, 2009) and Pfmap-2 (Dorin-Semblat *et al.*, 2007). The orphan kinase PfPK7 was shown to be dispensable for asexual multiplication, but asexual growth rate and sporogony were affected, with both phenotypes being rescued by knocking-in an extra copy of the gene in *pfpk7*$^{-}$ parasites (Dorin-Semblat *et al.*, 2008). Another phenotype in asexual parasites that is caused by inactivation of a *P. falciparum* PK is the inability of *pfeiK1*$^{-}$ clones to respond to amino-acid starvation by phopshorylating of the eIF2a translation factor (Fennell *et al.*, 2009). Genetic manipulations are significantly more straightforward in the rodent malaria *P. berghei* than in *P. falciparum*, because *i)* gene replacement by double cross-over recombination occurs at a much higher rate in the former than in the latter, and *ii)* the selection of transformed parasites occurs in the mouse and is much faster than the *in vitro* cultivation of *P. falciparum*. Using classical gene knock-out approaches, Pbcrk-1, a CDK-related kinase, was shown to be essential for schizogony in *P. berghei* (Rangarajan *et al.*, 2006), and several PKs were demonstrated to have essential roles in *P. berghei* sexual development. The first was a member of the CDPK family (see above), CDPK4: male gametocytes lacking this kinase are unable to undergo gametogenesis ("exflagellation") in the mosquito vector (Billker *et al.*, 2004). Similarly, a MAPK "semi-orphan", Pbmap-2, is also required for completion of male gametogenesis (Khan *et al.*, 2005; Rangarajan *et al.*, 2005; Tewari *et al.*, 2005). Interestingly, the orthologue in *P. falciparum*, Pfmap-2, is required for asexual growth (Dorin-Semblat *et al.*, 2007). The initial steps of sporogony following fertilisation in *P. berghei* require Pbnek-4, a NIMA-related kinase with a role in meiosis (Reininger *et al.*, 2005), and a second enzyme of this family was recently shown to also be essential for meiosis in both *P. berghei* and *P. falciparum* (Reininger *et al.*, 2009). PbCDPK3 is required for traversal of the mosquito midgut epithelium (Ishino *et al.*, 2006; Siden-Kiamos *et al.*, 2006).

In summary: *i) Plasmodium* PKs are divergent from their human counterparts, and it is difficult to reconstruct signalling pathways from sequence databases, *ii)* a collection of parasite clones lacking specific kinases has been constituted and is growing (in both *P. berghei* and *P. falciparum*), allowing the study of phenotypes mostly in sexual development/sporogony, *iii)* some PKs have been demonstrated to be essential for the asexual cycle, and *iv)* several recombinant PKs are available for biochemical studies and drug discovery activities.

CYCLIC NUCLEOTIDE SIGNALLING

The *P. falciparum* genome encodes two guanylyl cyclases (Carucci *et al.*, 2000; Linder *et al.*, 1999) and two adenylyl cyclases (Muhia *et al.*, 2001; Muhia *et al.*, 2003). The guanylyl cyclases have a paired catalytic domain reminiscent of mammalian G protein-dependent adenylyl cyclases, but also have a unique, potentially bifunctional structure incorporating an N-terminal P-type ATPase-like domain. One of these, PfGCα is refractory to gene deletion and may therefore be essential for asexual blood stage development. Deletion of PfGCβ, however, has no effect on either asexual or sexual blood stage development, even though cGMP synthesis is halved in mutant gametocytes. There are four cyclic nucleotide phosphodiesterases in malaria parasites (Wentzinger *et al.*, 2008). Both PfPDEα and PfPDEδ are dispensable for asexual growth (Taylor *et al.*, 2008; Wentzinger *et al.*, 2008). Disruption of PfPDEδ (transcription of which is highly upregulated in gametocytes) leads to abnormally high intracellular levels of cGMP and severely reduces the ability of the parasite to undergo gametogenesis. This suggests that a premature increase in cGMP levels is detrimental to sexual development (Taylor *et al.*, 2008).

The cGMP-dependent protein kinase of *P. falciparum* (PfPKG) is likely to be a major effector of cGMP (Deng & Baker, 2002). Highly specific inhibitors of apicomplexan cGMP-dependent protein kinase (PKG) used in conjunction with parasites expressing an inhibitor-insensitive PKG show that PKG plays an essential role in the initiation of *Plasmodium* gametogenesis (McRobert *et al.*, 2008). These PKG inhibitors have been used to indicate a role for PKG in motility and invasion in related coccidian parasites (Donald *et al.*, 2002).

Unlike the guanylyl cyclases, the malarial adenylyl cyclases are structurally quite distinct, but both have a key catalytic domain substitution that suggests they may be regulated by bicarbonate ions (Muhia *et al.*, 2003). The first (PfACα) has a unique bifunctional structure that incorporates an N-terminal potassium channel-like domain, suggesting that cAMP synthesis is coupled to changes in membrane potential (Weber *et al.*, 2004). This gene is not essential for asexual blood stage development, but phenotype analysis has not been carried out on gametocytes or mosquito stages. The *P. berghei* orthologue has been disrupted indicating a role for the enzyme in sporozoite apical regulated exocytosis and hepatocyte infection (Ono *et al.*, 2008). The second (PfACβ) is predicted to be soluble, is expressed at high levels in asexual blood stage parasites, and is refractory to deletion.

The parasite also possesses a single cAMP-dependent protein kinase (PKAc) catalytic subunit (Syin *et al.*, 2001), as well as a single PKA regulatory subunit that, when overexpressed, inhibits both parasite growth and anion transport across the erythrocyte plasma membrane (Merckx *et al.*, 2008b). This argues that PKA regulates both anion transport and parasite growth and that reduced growth may be due to the failure in anion transport. The role of erythrocyte versus parasite PKA in regulating anion transport across the RBC membrane has been recently discussed (Merckx *et al.*, 2009).

CALCIUM SIGNALLING

Calcium (Ca^{2+}) is a universal secondary messenger in eukaryotic cells. Binding to effector proteins changes their properties and thereby allows them to regulate a variety of cellular events. The importance of Ca^{2+} acquisition and homeostasis in the life cycle of the malaria parasite has long been recognized (Moreno & Docampo, 2003). Since RBCs do not possess intracellular organelles to store Ca^{2+}, these cells are dependent on a pump for Ca^{2+} extrusion. The Ca^{2+} content in RBCs increases after infection by the parasite and there is an enhancement of RBC-membrane permeability after infection. Ca^{2+} homeostasis within the parasite is thought to rely on an unusual acidic and a more conventional ER-like compartment (Moreno & Docampo, 2003). The anti-malarial drug artemisinin was recently shown to target the parasite's SERCA Ca^{2+} ATPase (PfATP6) (Eckstein-Ludwig *et al.*, 2003) and parasites possess another atypical P type calcium ATPase (PfATP4). The release of Ca^{2+} from intracellular stores is sensitive to phospholipase C (PLC) inhibitors. It was proposed that the circadian rhythm of the parasite is controlled by melatonin in a Ca^{2+}-dependent manner (Beraldo *et al.*, 2005; Hotta *et al.*, 2000). Alteration of Ca^{2+} levels influences sporozoite survival and egress from hepatocytes (Sturm *et al.*, 2006). The critical importance of calcium signalling for motility and microneme secretion is well documented for other apicomplexan zoite stages and signalling through Ca^{2+} is thought to be important during erythrocyte invasion by the merozoites, as well as intra erythrocytic parasite development. However, the exact function Ca^{2+} plays in these processes remains to be determined. The best defined function of Ca^{2+} as a second messenger come from studies utilizing transgenic parasites expressing a Ca^{2+} reporter in *P. berghei* gametocytes in which cytosolic Ca^{2+} mobilization occurs within seconds once cells are exposed to conditions encountered in the mosquito midgut and is critical for the initiation of gametogenesis (Billker *et al.*, 2004).

These and other examples highlight the importance of calcium homoeostasis for parasite development, emphasizing the need to study Ca^{2+} mediated signalling in the parasite. The *Plasmodium* genomes code for several proteins with Ca^{2+} binding motifs *e.g.* EF hands (Aravind *et al.*, 2003). Among these are a family of six calcium-dependent protein kinases (CDPKs), which are major mediators of Ca^{2+} signalling in plants (Ward

et al., 2004). PfCDPK1 was originally suggested to be exported to the parasitophorous vacuole in a myristoylation/palmitoylation-dependent manner (Moskes *et al.*, 2004) but more recent studies suggest that it is associated with the parasite plasma membrane and has a role in merozoite egress and invasion (Green *et al.*, 2008; Kato *et al.*, 2008). Studies on CDPK3 (Ishino *et al.*, 2006) (Siden-Kiamos *et al.*, 2006) and CDPK4 (Billker *et al.*, 2004) in *P. berghei* showed that these enzymes are important for development of sexual stages. While CDPK4 controls differentiation and cell cycle progression in male gametocytes, probably in response to xanthurenic acid-induced calcium mobilisation (with the MAPK Pfmap-2 as a possible downstream effector of CDPK4 (Tewari *et al.*, 2005)), CDPK3 is required for gliding motility and mosquito midgut invasion. *Plasmodium* also has homologues of other eukaryotic Ca^{2+} binding proteins like calmodulin, troponin-C and centrin. Although calmodulin inhibitors were shown to block invasion (Matsumoto *et al.*, 1987), the identity of calmodulin targets has remained largely elusive. Ca^{2+}/calmodulin was shown to regulate a *P. falciparum* protein kinase B-like enzyme, PfPKB (Kumar *et al.*, 2004), and Ca^{2+} levels needed for this interaction were modulated by phospholipase C (PLC). These studies resulted in identification of a novel parasite-specific signalling pathway (Vaid & Sharma, 2006). A unique phosphatidylinositol 4-phosphate 5-kinase (PIP5K) found in apicomplexan parasites has an N-terminal neuronal calcium sensor (NCS)-like domain that comprises EF hands. The *P. falciparum* PIP5K domain expressed in *E. coli* was shown to be activated by ADP-ribosylation factor (ARF1), but not phosphatidic acid (Leber *et al.*, 2009). The unexpected linkage of the two domains could indicate that phosphatidylinositol 4,5-bisphosphate production in malaria parasites is regulated by changes in intracellular Ca^{2+} levels.

The importance of Ca^{2+} throughout the *Plasmodium* life cycle is now well recognized, yet the knowledge of most specific Ca^{2+}-signalling pathways has remained limited. Extracellular triggers of Ca^{2+} mobilization and their molecular mediators are largely unidentified. The roles of individual calcium stores in signalling events are unknown. Only for some of the Ca^{2+}-regulated protein kinases have specific functions been suggested. For few of these has it so far been possible to identify downstream molecular pathways, or targets for phosphorylation, although CDPK1 phosphorylates two components of the myosin A-dependent molecular motor, Myosin Tail domain Interacting Protein (MTIP) and Gliding Associated Protein (GAP) 45 (Green *et al.*, 2008) and PKB phosphorylates GAP45 (Vaid *et al.*, 2008). Clearly, Ca^{2+}-dependent pathways need to be dissected to provide insights of molecular mechanisms underlying parasite development.

2 - BIOLOGICAL PROCESSES FROM A SIGNALLING PERSPECTIVE

Asexual blood stage development

The asexual blood stage of the malaria parasite life cycle is responsible for the pathogenesis and is therefore a major target of research into drug discovery and vaccine design. The asexual blood stage of the malaria parasite life cycle is responsible for the disease and is therefore a major target of research into drug discovery and vaccine design. The following sections review key events in the asexual erythrocytic cycle from a signalling perspective.

• Gliding motility and cell invasion

Apicomplexan parasites use a conserved actin-myosin motor to drive both host cell invasion and gliding motility across a substrate (Soldati-Favre, 2008). The myosin is anchored to the inner membrane complex by a group of proteins that includes Myosin Tail domain Interacting Protein (MTIP) and the Gliding Associated Proteins (GAP) –45 and –50 (see Fig. 2 for an illustration of GAP45/MTIP colocalisation). GAP45 is known to be phosphorylated by at least two different kinases, CDPK1 and PKB (Green *et al.*, 2008) (Vaid *et al.*, 2008). MTIP is also phosphorylated by CDPK1 (Kato *et al.*, 2008) (Green *et al.*, 2008) and recently it has been shown in *T. gondii* that dephosphorylation of GAP45 is a prerequisite for its association with MTIP (Gilk *et al.*, 2009). Thus, phosphorylation/dephosphorylation appears to have a role in controlling motor function. Nonetheless, it is unclear how malaria parasites regulate the motor at the different stages of the life cycle and whether or not motility can be differentiated from invasion.

The invasion of host cells by apicomplexan parasites is a complex process. Studies in *T. gondii* suggest that tachyzoite invasion of host cells comprises at least seven steps (Carruthers & Boothroyd, 2007). Malarial merozoites are restricted to invasion of anucleated erythrocytes, but the process is equally complex. Following contact between parasite and host cell there is a complex cascade of events that results in the parasite entering and modifying the red cell ready for intracellular growth, development and replication. Cell binding is followed by parasite reorientation so that its apical end contacts the host cell surface, and invasion ensues, with the following steps: secretion of microneme content onto the parasite surface; formation of a tight junction between parasite and host cell and invagination of the red cell plasma membrane; secretion of the contents of the rhoptry organelles; engagement of the parasite's actomyosin motor and movement of the parasite into the developing parasitophorous vacuole; finally, closure of the vacuole once the parasite has entered the cell.

At the molecular level this process involves many protein-protein interactions and modifications that regulate the process through signalling and switches that control function. For example, interactions between receptor and ligand, and protein phosphorylation and proteolytic cleavage are key processes in the invasion cascade. From the formation of the merozoite, with its marked cellular polarity, to the colonization of the new cell, the process involves a multitude of proteins in distinct organelles and extensive post-translational modifications and signalling pathways. One such group of organelles are the micronemes, which contain proteins like EBA175, AMA1 and the protease SUB2, all of which have been shown to play critical roles in erythrocyte invasion. Following discharge from the micronemes, EBA175 binds to sialic acid residues on glycophorin A (Sim *et al.*, 1994). AMA1 is another protein that plays a critical role in invasion of erythrocytes by merozoites (Triglia *et al*., 2000); the protein has a C-terminal cytoplasmic tail, with certain residues conserved across species, a structure that is suggestive of a mechanism of signal transduction through AMA1 by phosphorylation (Treeck *et al.*, 2009). Whether AMA1 is involved in the apical reorientation following initial interaction (Mitchell *et al*., 2004), or at a later step of invasion (Treeck *et al.*, 2009) is still unclear. SUB2 then translocates across the merozoite surface causing the shedding of AMA1, the MSP1 complex (Harris *et al.*, 2005) and M-TRAP (Green *et al.*, 2006) from the surface (Harris *et al.*, 2005). The signalling events that lead to the regulated release of microneme proteins to the merozoite surface to allow receptor engagement and the subsequent steps of invasion have not yet been defined. In the case of *T. gondii*, an increase in intracellular Ca^{+2} has been shown to lead to secretion of microneme proteins such as MIC2 to the tachyzoite surface (Lovett *et al.*, 2002) (Wetzel *et al*., 2004) and it seems likely that Ca^{2+} may play a similar role in *Plasmodium* merozoites. Erythrocyte invasion by merozoites involves several steps of proteolytic modification of merozoite surface and vacuolar proteins (O'Donnell & Blackman, 2005) (Yeoh et al, 2007). At least two of these are mediated by subtilisin-like serine proteases: SUB1 and SUB2. SUB1 is released into the parasitophorous vacuole (PV) and is responsible for the so-called 'primary processing' of a number of accessible proteins, including merozoite surface proteins MSP1, MSP3, MSP6 and MSP7, as well as PV proteins of the SERA family (Koussis *et al.*, 2009). SUB2 is a membrane-associated protease released on to the merozoite surface. These two processing steps are temporally and spatially regulated, and the proteases have distinct substrate preferences.

• DNA replication in asexual blood stage parasites

Successful invasion and initial development of a single parasite within the erythrocyte leads to the multiple rounds of DNA replication inside erythrocytes (amplification from 1N DNA content up to 16-32 N). Further, DNA replication in *Plasmodium* does not follow typical duplication and segregation, but it is asynchronous in nature making it both fascinating and difficult to study. In eukaryotes, the key player for DNA replication initiation is a six-protein complex called origin recognition complex (ORC). Till now genes for three members of the ORC family of proteins (namely PfORC1, 2 and 5) have been cloned from *P. falciparum* and their role in erythrocytic replication has been established (Gupta *et al.*, 2006; Mehra *et al.*, 2005). Besides ORCs, several other replicating proteins have also been identified and characterized in *P. falciparum*, namely minichromosomal maintenance proteins (MCM2-7), DNA polymerase enzymes, PCNA1 and PCNA2, PfRPA (Ridley *et al*., 1991; Patterson *et al.*, 2002; Gupta *et al.*, 2006; Nunthawarasilp *et al.*, 2007; Voss *et al.*, 2002) although their precise role has not been elucidated in parasite biology.

Studies using radioactive nucleotides incorporation in parasite culture have shown a window (~22-36 hours post-invasion) where replication takes place during asexual blood stages (Inselburg & Banyal, 1984; Arnot & Gull, 1998). Recently, it has been shown that replication in *Plasmodium* occurs in replicative factories, and that the timing of formation and progression of replication foci matches with the radiolabelled nucleotide uptake studies. Disruption of these foci by known replication inhibitors like hydroxyurea leads to the stall of parasite growth and loss of replication foci formation (Gupta *et al.*, 2008).

Cyclin-dependent kinases (CDKs) (reviewed in Morgan, 1977) are thought to be important for the regulation of DNA replication and cell cycle progression during asexual blood stage development. Expression profiles of CDK related kinases and cyclins have revealed that some kinases and cyclins are expressed during the replicative phase of the parasite life cycle (Merckx *et al.*, 2003). They are mainly, but not limited to, PfPK5, PfCrk1, PfCrk5, Pfmap1, PfGSK3, PfCyc2, and PfCyc4 (Doerig *et al.*, 2002). Some of them may be involved in regulating DNA replication, but their cellular targets and molecular mechanism are still not known.

Several other kinases that have recently been shown to be essential for asexual multiplication include the cyclic nucleotide-dependent kinases PfPKA, PfPKG, MAPKs, CDPKs, NIMA-related kinases (reviewed herein) and casein kinases PfCK1 (Barik *et al.*, 1997) and PfCK2 (Holland *et al.*, 2009). Elucidating the role of these kinases in parasite development, DNA replication, as well as cell cycle progression will not only be interesting to understand the parasite biology, but it will also help us to identify new targets for therapy.

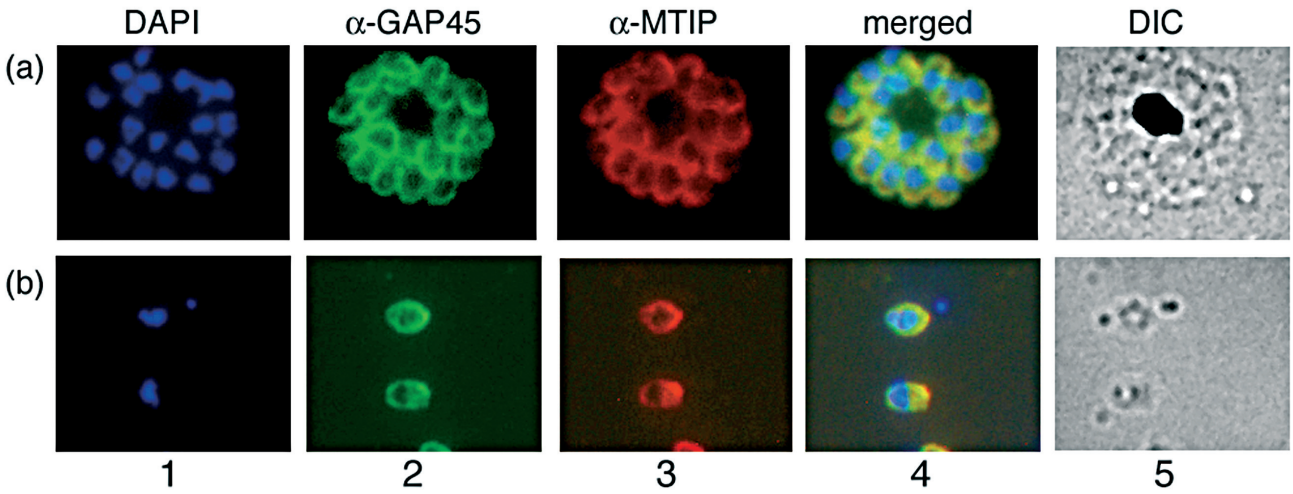

Fig. 2. – GAP45 and MTIP are located at the periphery of developing and free merozoites.
Immunofluorescence assays performed using both mouse PfGAP45 antiserum and rabbit anti-PfMTIP on (a) a mature schizont and (b) free merozoites. Panel 1 shows nuclei stained by DAPI, panels 2 and 3 show patterns of fluorescence corresponding to PfGAP45 and PfMTIP respectively. The merged images are shown in panel 4 and the Differential Interference Contrast (DIC) image is shown in panel 5. Courtesy of Antony Holder.

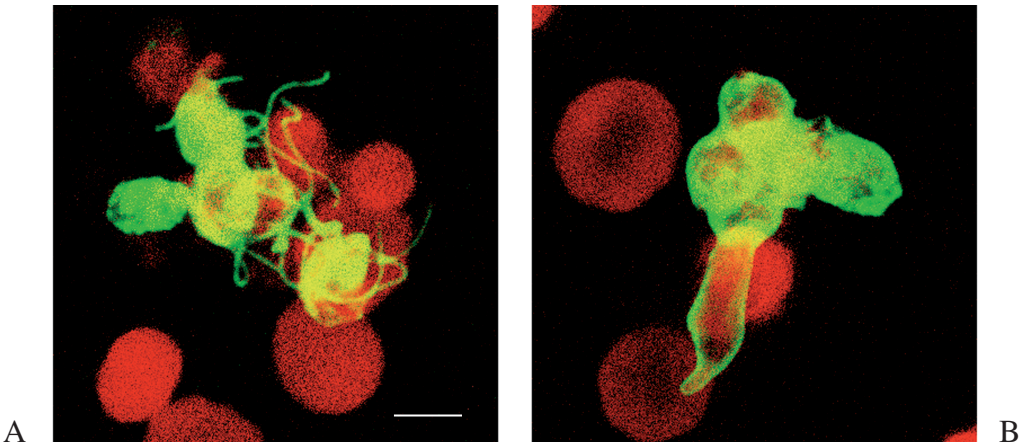

Fig. 3. - Sexual stage parasites of *P. falciparum*.
A. Immunofluorescence image depicting a cluster of five exflagellating activated microgametocytes, which are in the process of forming microgametes (shown in green by labelling with antibodies against alpha-tubulin II in combination with Alexa Fluor488).
B. Immunofluorescence staining of a cluster of five zygotes, one of which is transforming into an ookinete (shown in green by labelling with antibodies against the surface protein Pfs25 in combination with Alexa Fluor488). Erythrocytes are highlighted via Evans Blue-labelling (shown in red). Bar, 5 µm. Courtesy of Gabi Pradel.

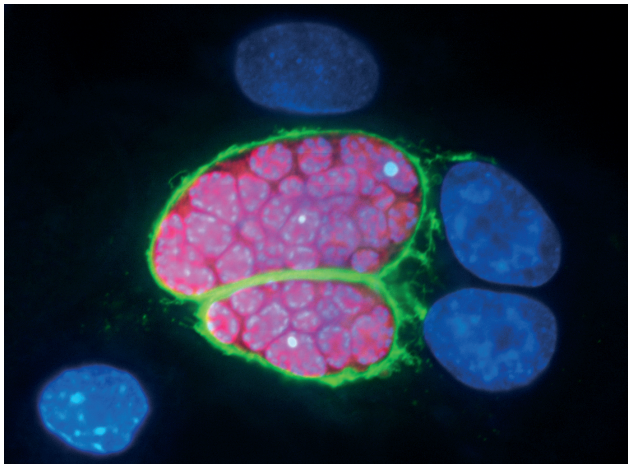

Fig. 4. – *P. berghei* hepatocytic schizont.
HepG2 cells were infected with *P. berghei* expressing the Red Fluorescent Protein under a strong constitutive promoter. RFP fluorescence of parasites was monitored by live imaging. Immediately after live imaging, cells were fixed and stained with an antiserum directed against the parasitophorous vacuole membrane protein Exp1 (green). DNA was visualised with DAPI (blue). Parasites were still clearly visible by the remaining RFP fluorescence (red). Note the large DAPI-stained host cell nuclei. Courtesy of Volker Heussler.

SEXUAL DEVELOPMENT

Sexual differentiation and sporogony are highly complex processes encompassing commitment to gametocytogenesis (including sex determination), production of mature gametocytes within the red blood cell (a process requiring ten days in *P. falciparum* and only one day in *P. berghei*), gametogenesis and fertilisation in the mosquito gut, transformation into a motile ookinete (Fig. 3), traversal of the insect's midgut wall, and production of an oocyst which will finally develops to yield thousands of sporozoites. Sexual development and sporogony are absolutely required for parasite transmission, and are thus crucial to the spread of disease. Therefore, decreasing parasite transmission using drugs (*e.g.* artemisinin derivatives, which are gametocytocidal), or vaccines, has the potential to have a large impact on disease control.

Results from functional work in *P. berghei*, and to a lesser extent in *P. falciparum*, have clarified some aspects of this process, particularly with respect to the role of signalling molecules. The protein kinases CDPK4 and Pbmap-2 have been associated with the production of male gametes (Billker *et al.*, 2004; Khan *et al.*, 2005; Rangarajan *et al.*, 2005), Pbnek-4 and Pbnek-2 with ookinete maturation (Reininger *et al.*, 2005; Reininger *et al.*, 2009), and PbCDPK3 and guanylate cyclise beta with ookinete physiology (Ishino *et al.*, 2006; Siden-Kiamos *et al.*, 2006; Hirai *et al.*, 2006). The role of signalling molecules in several other phases of sexual differentiation is however completely unknown, despite the fact that some of these processes, such as the establishment of the gametocyte parasitophorous vacuole, or gamete emergence are intrinsically complex cellular rearrangements, which proceed in response to specific cellular and environmental signals.

In a parallel line of investigation, several parasite molecules specifically expressed in gametocyte/gamete development have been identified and characterised. In most cases these are abundant proteins, specifically produced at various stages of the sexual cycle. Often they do not have significant homology to other eukaryotic proteins, preventing predictions regarding their function, or homology is restricted to specific motifs (*e.g.* the multi-adhesive protein family CCp/LAP, conserved between *P. falciparum* and *P. berghei* – reviewed (Pradel, 2007). Evidence is accumulating that some of these molecules are subject to protein modification directly or indirectly mediated by signalling cascades. Examples are phosphorylation of the abundant early gametocyte protein Pfg27 (Kumar, 1997), or proteolysis of the fertilisation protein Pfs230 (Brooks & Williamson, 2000). Significant progress in understanding sexual development is expected to come from the merging of functional studies on signalling and effector molecules with the biochemical and functional characterisation of several sexual stage-specific molecules.

INTRA-HEPATOCYTE DEVELOPMENT

When *Plasmodium* sporozoites enter hepatocytes, signalling events occur in both the parasite and the host cell during the process. Host cell invasion by *Toxoplasma* tachyzoites has been shown to be critically dependent on actin polymerization in the parasite, without requiring actin polymerization in the host cell. Based on this study and others performed on *Plasmodium* zoites, it is still currently assumed that Apicomplexa in general use their actin-myosin motor to enter host cells that remain essentially passive, a view that is consistent with the rapidity of the zoite internalization process (several seconds or tens of seconds). The current dogma that actin polymerization in the host cell is dispensable for apicomplexan zoite entry has been re-evaluated and new evidence contradicts this view. Live video and confocal microscopy indicate that actin polymerization is in fact important for entry of *P. berghei* sporozoites and *T. gondii* tachyzoites (Gonzalez *et al.*, 2009). Apicomplexan invasion of host cells thus appears to require not only the parasite motor, but also *de novo* polymerization of host actin at the entry site, for anchoring the junction on which the parasite pulls in order to penetrate the host cell.

Recent progress has been made in our understanding of how, after differentiation of sporozoites into merozoites inside hepatocytes (see Fig. 4), merozoites leave the host cell and reach the blood. The Heussler laboratory has recently shown that upon disruption of the PVM, merozoites mix freely with the host cell cytoplasm and host cell death is induced (Sturm *et al.*, 2006). This parasite-induced host cell death clearly differs from apoptosis in that caspases are not involved and the host cell scramblase is not activated. On the other hand, activation of non-caspase cysteine proteases induces damage of mitochondria and Ca^{2+} release. Before this release induces scramblase activity, intracellular merozoites accumulate Ca^{2+} and thus inhibit exposure of phosphatidyl serine (PS) residues to the outer leaflet of the cell membrane. As long as merozoites are viable and maintain a low Ca^{2+} concentration in the host cell cytoplasm, infected cells will not be recognized by macrophages expressing PS receptors. It has previously been speculated that putative parasite cysteine proteases of the SERA family are critically involved in the process of PVM destruction in infected erythrocytes (Miller *et al.*, 2002) and in infected hepatocytes (Sturm & Heussler, 2007), but experimental evidence is needed to confirm this attractive hypothesis.

In vitro, infected hepatocytes detach upon cell death and float freely in the culture supernatant and merozoite-filled vesicles, called merosomes, bud off the cell. Intravital imaging of mouse livers infected with GFP-expressing *P. berghei* clearly supports this *in vitro* observation

(Sturm *et al.*, 2006). At the end of the liver stage, *Plasmodium* merosomes can be detected in liver sinusoids. They can stay in contact with the infected cell for several hours, increasing considerably in size by transport of parasite material from the host cell to the merosome. These findings confirm that merozoites are not immediately liberated from the host cell, but are first transported in merosomes to the blood stream. A prerequisite for the parasite differentiation and rapid growth during the liver phase is an extensive intracellular remodelling. Signalling *via* Rab proteins and the formation and transport of vesicles are thought to play key roles in these processes and might represent potent targets for intervention in the parasite's life cycle (Quevillon *et al.*, 2003). Identification of other key signalling events during the liver phase and/or proteases initiating parasite liberation at the end of the liver stage would also be important for understanding hepatocyte infection and subsequent potential applications, such as development of new anti-malarial strategies at a very early and non-pathogenic infection phase in hepatocytes.

Membrane dynamics and trafficking

Inside erythrocytes and hepatocytes the *Plasmodium* parasite resides within the parasitophorous vacuole and is separated from the host cell cytosol by a membrane called the PVM. Export of substances (like parasite-derived proteins such as EMP1) out of the parasite to the host cell surface, and import of molecules from the host cell cytosol (like haemoglobin) or from the blood flow (like nutrients or drugs), thus requires traffic across up to three membrane barriers: the parasite's plasma membrane, the PVM and the erythrocyte's plasma membrane. How the parasite regulates import and export across these membrane barriers is a fascinating problem of cell biology.

Both classical and non-classical secretory pathways operate in *P. falciparum*-infected erythrocytes. Peptide motifs have now been identified within a large number of secreted proteins that direct their targeting to the red blood cell cytosol, the apicoplast and the food vacuole (Maier *et al.*, 2009). In addition, motifs that direct proteins to rhoptries have recently been described (Richard *et al*., 2009), but in contrast the motifs necessary for targeting to micronemes have yet to be defined. Moreover, the type of vesicle in which these proteins are transported to their destinations, and the signalling pathways that regulate the process, are unknown.

Plasmodium parasites clearly posses a classical secretory pathway: Rab GTPases are well-established regulators of vesicular traffic in eukaryotes, and a family of *Plasmodium* Rabs has been described (Quevillon *et al.*, 2003; Langsley *et al.*, 2008). When not associated with a vesicle, Rabs are bound to a carrier protein called rabGDI, a homologue of which has also been described in *P. falciparum* (Attal & Langsley, 1996). In mammalian cells rabGDI can interact with a specialized membrane-associated recycling Hsp90/Hsp70 chaperone system on the vesicle membrane, and this results in the coordinated triggering of calcium-dependent events (Sakisaka *et al.*, 2002). The endoplasmic reticulum (ER) is a major store of calcium and calcium fluxes are known to be involved in regulating parasite invasion of host cells, most likely through the activation of Ca^{2+}-dependent proteins, such as ATPases and kinases (Nagamune & Sibley, 2006). Ca^{2+} and phosphatidylinositol signalling regulate Rab-mediated vesicular traffic in eukaryotic cells; the identification of a *P. falciparum* PI3-phosphate binding protein located in the food vacuole (McIntosh *et al.*, 2007) may be linked to trafficking regulation.

Normally, the direction of Rab-mediated vesicular traffic is given by the association of a vesicle with a Rab-kinesin motor and hence to the microtubule network (reviewed in (Caviston & Holzbaur, 2006). However, to date a role in vesicular transport for microtubules has not been described in *Plasmodium* (Chattopadhyay *et al.*, 2000). In contrast, a role for an actin-myosin motor called the "Glideosome" has recently been demonstrated in driving Rab11A-mediated transport of GAP45 in both *Plasmodium* and *Toxoplasma* (Agop-Nersesian *et al*., 2009). The Glideosome motor has a well-established role in invasion of *Plasmodium* and *Toxoplasma* parasites (Soldati-Favre, 2008). Thus, phosphorylation/dephosphorylation of motor components like, but not exclusively, GAP45 (Gilk *et al.*, 2009) and MTIP (Kato *et al*., 2008) could regulate not only invasion (Green *et al.*, 2008), but also Rab11A-mediated vesicular transport. Understanding how signal transduction pathways regulate vesicular traffic is a central theme of modern cell biology and although certain key players have been identified in *Plasmodium*, we still do not understand how the parasite regulates vesicular traffic and membrane dynamics. Clearly, certain proteins crucial to invasion have to be transported to the rhoptries and micronemes and then released (Agop-Nersesian *et al*., 2009), but precisely how this is regulated remains to be determined.

CONCLUDING REMARKS

It is apparent from this brief review that there is a clear and definite lack of understanding of the way complex processes in *Plasmodium* developmental biology are controlled by signalling events, and that the molecular tools (especially with respect to signalling molecules) and the biological assays/systems required to efficiently address this question are now becoming available. This augurs well for a deepening in our understanding of how signalling pathways coordinate to regu-

late the developmental changes that govern the *Plasmodium* life cycle.

The MALSIG consortium comprises expertise in all aspects of signalling components and parasite biology discussed above. The purpose behind this collaborative venture is that the "melting pot" thus constituted will foster progress in this field, which is fascinating in terms of fundamental biology and highly relevant to the search for novel targets for intervention against a deadly pathogen.

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