Executive Summary:

Malaria: an acute public health problem of the developing world.

Malaria is a major public health problem in the developing world and is endemic in many regions of India. Novel control agents are urgently required in view of the emergence and spread of resistance of malaria parasites against even the latest generation of antimalarial drugs. The development of novel strategies for malaria control requires a better understanding of the biology of malaria parasites.

Malaria parasites (genus Plasmodium) develop within and outside host cells, and in diverse contexts such as the human liver and blood circulation, or a mosquito gut or salivary glands. Cellular and molecular mechanisms enabling the parasite to sense and respond to diverse environments are crucial for proliferation and transmission of Plasmodium, and therefore represent strategic targets in the fight against this deadly disease.

Our strategy: integrate science, synergize and focus on defined targets. The primary objective of MALSIG was to integrate research ongoing in Europe and India on the properties of Plasmodium signalling molecules and on the developmental processes occurring along the parasite life cycle. The project merged two lines of investigation that had thus far been conducted largely independently from each other:

(i) The characterisation of molecular components of signalling pathways in malaria parasites;
(ii) The study of specific biological and developmental processes during the life cycle of malaria parasites, namely:
- Erythrocyte infection (invasion, parasite proliferation, egress)
- Sexual development (gametocytogenesis, gametogenesis, transmission to the mosquito vector)
- Hepatocyte infection (invasion, parasite proliferation, egress)
- Membrane dynamics (trafficking, transporters).

Integration of these two fields of malaria research ensured complementarity and synergy between the partner Institutions, which fostered significant advances in our understanding of the molecular regulation of the parasite's life cycle. Seminal discoveries were made within the MALSIG consortium, notably with respect to the importance of host cell signalling pathways in liver and red blood cells, and to the identification (through reverse genetics) of signalling, trafficking, motility and transporter molecules that are essential for specific stages of the life cycle (and are therefore genetically validated as potential targets for intervention). The research implemented by the MALSIG consortium led to 177 articles, many of which were published in high impact journals. It is significant that the systematic functional analysis of malaria parasite kinases in both rodent and human parasites came from the MALSIG consortium, and the proteome-wide identification of kinase target proteins in P. falciparum were entirely generated within this network. Altogether, the output of the MALSIG consortium has brought a highly significant contribution to knowledge in the field of basic biology of malaria parasites; this provides a strong basis for further developments in translation research, which are now ongoing at an accelerated pace in many institutions (notably with respect to drug discovery based on protein kinase inhibition).

Linking EU and Indian malaria scientists for a global impact.

The MALSIG consortium brought together around 150 malaria scientists in 20 Institutions from India and five European countries. This effort has strengthened existing links and created new ones, and helped foster and strengthen a culture of scientific cooperation between EU and India in this globally crucial public health issue, and, through identification and characterization of potential therapeutic targets for intervention, paved the way for novel intervention strategies and populated the antimalarial drug development pipeline.

Project Context and Objectives:

Context and objectives

Malaria parasites still impose a huge burden in the developing world, with 40% of the world's 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 Apicomplexa parasite Plasmodium falciparum, the species responsible for the vast majority of lethal malaria cases, the development of novel control agents is an urgent task [1]. Malaria is endemic in many regions of India with around 70 million cases reported each year, approximately half of which are attributed to P. falciparum and the other half to P. vivax [2]. The malaria transmission and incidence rates in parts of the country such as Orissa are comparable to those reported from hyperendemic regions of Africa with P. falciparum accounting for greater than 80% of malaria cases in these regions [3, 4]. The epidemiology of malaria in these regions is very similar to that reported from Africa, with children bearing the brunt of the disease and adults developing clinical immunity [4]. Although the number of deaths due to malaria in India is low compared to that reported from sub-Saharan Africa largely due to widespread availability of chloroquine and the high proportion of P. vivax (versus the more lethal P. falciparum infections), the spread of chloroquine resistance and the unavailability of next generation anti-malarials could rapidly change this scenario [2]. The identification of novel targets for intervention is now facilitated by the availability of genomic databases for several Plasmodium species [5] (including P. falciparum and the widely used rodent malaria parasite P. berghei) and for Toxoplasma gondii, an Apicomplexa parasite that is recognised as a valuable model for specific aspects of Plasmodium biology.

Malaria pathogenesis is caused by the asexual multiplication of parasites in erythrocytes. After invasion of the red blood cell (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) 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 [6]). Upon ingestion by the insect, they complete their development to gametes; for male gametocytes, this implies 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 as an oocyst at the basal lamina of the epithelium. Sporogony, the asexual generation of several thousand sporozoites, occurs within 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 first establish an infection inside a hepatocyte, which results in the generation of several thousand merozoites (exo-erythrocytic schizogony) able to infect erythrocytes.

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, must imply 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) and (ii) in the description of the biology of developmental stages (see section 1.2 for the current state-of-the-art regarding these two topics, and section 2.3.1 for the distribution of expertise on these topics between consortium members). The general objective of this project was to remedy this blatant lack of information on a very fundamental aspect of Plasmodium biology. The concept behind the project was to merge two broad lines of investigation that have until then been conducted largely independently, one of which focused on the characterisation of signalling pathways components, and the other on the biology of specific developmental stages of the Plasmodium life cycle. The consortium was constituted so as to include prominent researchers involved in either line of investigation, as well as colleagues working on Toxoplasma, a model that will be useful in addressing some of the workpackages listed below.

The Specific Aims were to understand the signalling events involved in three life cycle stages, and they defined the first three Workpackages of this project:

1. Erythrocyte invasion and the asexual blood stage
2. Sexual development and sporogony
3. Hepatocyte infection by the sporozoite

In addition, a fourth Workpackage focused on the signalling-dependent regulation of membrane biology at various stages:

4. Transporters, membranes and trafficking

A secondary Objective of the project was to strengthen existing, and foster novel, collaborations between malaria researchers in the EU and in India (see below, "Impact").

A further Workpackage (WP5) covered the managerial aspects of the project.

1. Olliaro, P., Drug resistance hampers our capacity to roll back malaria. Clin Infect Dis, 2005. 41 Suppl 4: p. S247-57.
2. Sharma, V.P., Battling the malaria iceberg with chloroquine in India. Malar J, 2007. 6(1): p. 105.
3. Sharma, S.K., et al., Epidemiology of malaria transmission and development of natural immunity in a malaria-endemic village, San Dulakudar, in Orissa state, India. Am J Trop Med Hyg, 2004. 71(4): p. 457-65.
4. Sharma, S.K., et al., Epidemiology of malaria transmission in forest and plain ecotype villages in Sundargarh District, Orissa, India. Trans R Soc Trop Med Hyg, 2006. 100(10): p. 917-25.
5. Bahl, A., et al., PlasmoDB: the Plasmodium genome resource. An integrated database providing tools for accessing, analyzing and mapping expression and sequence data (both finished and unfinished). Nucleic Acids Res, 2002. 30(1): p. 87-90.

6. Sinden, R.E., et al., Regulation of infectivity of Plasmodium to the mosquito vector. Adv Parasitol, 1996. 38: p. 53-117.

Project Results:
1. WP 1: Signalling in erythrocyte invasion and the asexual blood stage

WP1 Coordinators: T. Holder (partner 3a) and C. Chitnis (partner 14a).

Objectives
An understanding of the importance of signalling and effector molecules in parasite invasion of red blood cells and their continued intracellular development and replication within the infected cell.

The signalling molecules to be investigated will include calcium, cAMP/cGMP and protein kinases, including calcium dependent kinases.

The effector molecules include DNA replication initiation proteins, proteins of the micronemes such as AMA1, EBA175 and SUB2, and proteins of the actomyosin motor such as MTIP, GAP45, GAP50 and myosin.

The biological processes are all integral to parasite multiplication within the bloodstream, and include parasite entry into red blood cells (focusing on the role of the apical organelles such as the micronemes, the importance of proteases in protein processing and activation, and the structure and function of the motor that drives red blood cell invasion) and parasite replication inside the host cell (focusing on the control of DNA replication).

Accomplishments at a glance

Signalling events in the release and activation of micronemal proteins during invasion of erythrocytes

Methods have been developed to isolate viable invasive merozoites, enabling analysis of cellular and biochemical processes partaking in invasion. This advance has allowed us to establish the importance of potassium concentration in the control of microneme release and in triggering changes in intracellular calcium. cAMP and phospholipase C have also been incriminated as important second messengers. Considerable progress has been made in the area of merozoite egress from the infected red blood cell. Two kinases, CDPK5 and PKG, as well as the protease SUB1, have been implicated in this process. The FKBP destabilisation domain (DD) is being used in a variety of systems as a way of controlling the level of specific kinases, and should it prove successful will provide a useful way of regulating kinase activity. Several proteins including, kinases such as CDPK1 and their substrates such as GAP45 are acylated by myristoylation and palmitoylation, a process that targets the proteins to a cell membrane. (Ref: 179).

The regulation of invasion and parasite replication by signalling cascades: the proteins, their structure and their function
Antibodies and epitope or GFP tags have been used to localise various protein kinases within the cell, since location may provide a clue to function. A range of kinases have been expressed in active recombinant form, which facilitates the generation of specific antibodies and in vitro investigation of enzymatic properties, as well as the development of inhibitors. Highly specific and potent inhibitors of certain kinases are being developed, which will allow the role of the target kinase within the cell to be dissected in detail. This approach is complemented by an extensive programme of systematic gene knockouts and tagging coupled with analysis of the resultant phenotype, which enable specific

kinases to be implicated in certain biological processes. The importance of kinases in the initiation and completion of DNA replication during the asexual blood stage is thus being established. Structural studies on a number of kinases are ongoing and are complemented by using homology modelling-based approaches. (Ref: 97)

The actomyosin motor: The complex is comprised of several proteins including myosin (MyoA), myosin tail domain interacting protein (MTIP) and glideosome associated proteins (GAP) 45 and 50, and is anchored on the inner membrane complex (IMC), which underlies the plasmalemma. A ternary complex of MyoA, MTIP and GAP45 is formed that then associates with GAP50. Characterization of molecular mechanisms governing the actomyosin motor led to identify Interaction and dynamics of the P. falciparum MTIP-MyoA complex, a key component of the invasion motor in the malaria parasite, the key determinants of binding at the MTIP/MyoA interface, and the first structural study on the complex in solution using protein NMR. (Ref: 64).

In another study the subcellular location, phosphorylation and assembly into the motor complex of GAP45 during P. falciparum schizont development was characterised. We show that full length GAP45 labelled internally with GFP is assembled into the motor complex and transported to the developing IMC in early schizogony, where it accumulates during intracellular development until merozoite release. We show that GAP45 is phosphorylated by calcium dependent protein kinase 1 (CDPK1), and identify the modified serine residues. Replacing these serine residues with alanine or aspartate has no apparent effect on GAP45 assembly into the motor protein complex or its subcellular location in the parasite. The early assembly of the motor complex suggests that it has functions in addition to its role in erythrocyte invasion. (Ref: 156).

Genome-wide functional kinomics and phosphoproteomics: The consortium produced kinome-wide reverse genetics studies in both P. berghei and P. falciparum. To identify pathways essential for parasite transmission between their mammalian host and mosquito vector, we undertook a systematic functional analysis of ePKs in the genetically tractable rodent parasite P. berghei. Modelling domain signatures of conventional ePKs identified 66 putative Plasmodium ePKs. Kinomes are highly conserved between Plasmodium species. Using reverse genetics, we show that 23 ePKs are redundant for asexual erythrocytic parasite development in mice. Phenotyping mutants at four life cycle stages in Anopheles stephensi mosquitoes revealed functional clusters of kinases required for sexual development and sporogony. Roles for a putative SR protein kinase (SRPK) in microgamete formation, a conserved regulator of clathrin uncoating (GAK) in ookinete formation, and a likely regulator of energy metabolism (SNF1/KIN) in sporozoite development were identified (see WP2 for additional details). (Ref: 76).

With P. falcaliparum, we combined global phospho-proteomic analysis with kinome-wide reverse genetics to assess the importance of protein phosphorylation in asexual proliferation. We identified 1177 phosphorylation sites on 650 parasite proteins that are involved in a wide range of general cellular activities such as DNA synthesis, transcription and metabolism as well as key parasite processes such as invasion and cyto-adherence. Several parasite protein kinases are themselves phosphorylated on putative regulatory residues, including tyrosines in the activation loop of PfGSK3 and PfCLK3; we show that phosphorylation of PfCLK3 Y526 is essential for full kinase activity. A

kinome-wide reverse genetics strategy identified 36 parasite kinases as likely essential for erythrocytic schizogony. These studies not only reveal processes that are regulated by protein phosphorylation, but also define potential anti-malarial drug targets within the parasite kinome (Ref: 97).

Specific studies on individual kinases that are essential for asexual proliferation. A number of essential kinas have been the subject of specific investigations. We will not discuss each of these in detail (see the list of publications for an exhaustive overview); these include:

The cyclic GMP-dependent protein kinase PfPKG: A specific PKG inhibitor (compound 1, a trisubstituted pyrrole) prevented the progression of P. falciparum schizonts through to ring stages in erythrocyte invasion assays. Addition of compound 1 to ring-stage parasites allowed normal development up to 30 h post-invasion, and segmented schizonts were able to form. However, synchronized schizonts treated with compound 1 for greater than or =6 h became large and dysmorphic and were unable to rupture or liberate merozoites. To conclusively demonstrate that the effect of compound 1 on schizogony was due to its selective action on PfPKG, we utilized genetically manipulated P. falciparum parasites expressing a compound 1-insensitive PfPKG. The mutant parasites were able to complete schizogony in the presence of compound 1, but not in the presence of the broad-spectrum protein kinase inhibitor staurosporine. This shows that PfPKG is the primary target of compound 1 during schizogony and provides direct evidence of a role for PfPKG in this process. Discovery of essential roles for the P. falciparum PKG in both asexual and sexual development (see below, WP2) demonstrates that cGMP signalling is a key regulator of both of these crucial life cycle phases and defines this molecule as an exciting potential drug target for both therapeutic and transmission blocking action against malaria. (Ref: 65).

Calcium-dependent protein kinase 5 (CDPK5): The coordinated processes of parasite egress from and invasion into erythrocytes are rapid and tightly regulated. We have found that the plant-like calcium-dependent protein kinase PfCDPK5, which is expressed in invasive merozoite forms of P. falciparum was critical for egress. Parasites deficient in PfCDPK5 arrested as mature schizonts with intact membranes, despite normal maturation of egress proteases and invasion ligands. Merozoites physically released from stalled schizonts were capable of invading new erythrocytes, separating the pathways of egress and invasion. The arrest was downstream of cyclic guanosine monophosphate-dependent protein kinase (PfPKG) function and independent of protease processing. Thus, PfCDPK5 plays an essential role during the blood stage of malaria replication. (Ref: 67).

Protein kinase CK2: Protein kinase CK2 (casein kinase 2) is a eukaryotic serine/threonine protein kinase with multiple substrates and roles in diverse cellular processes, including differentiation, proliferation, and translation. The mammalian holoenzyme consists of two catalytic alpha or alpha' subunits and two regulatory beta subunits. We report the identification and characterization of a P. falciparum CK2alpha orthologue, PfCK2alpha, and two PfCK2beta orthologues, PfCK2beta1 and PfCK2beta2. Recombinant PfCK2alpha possesses protein kinase activity, exhibits similar substrate and cosubstrate preferences to those of CK2alpha subunits from other organisms, and interacts with both of the PfCK2beta subunits in vitro. Gene disruption experiments show that the presence of PfCK2alpha is crucial to asexual blood stage parasites and

thereby validate the enzyme as a possible drug target. PfCK2alpha is amenable to inhibitor screening, and we report differential susceptibility between the human and P. falciparum CK2alpha enzymes to a small molecule inhibitor. Taken together, our data identify PfCK2alpha as a potential target for antimalarial chemotherapeutic intervention. (Ref: 5).

We subsequently showed that the genes encoding both regulatory PfCK2 subunits (PfCK2ß1 and PfCK2ß2) can't be disrupted. Using immunofluorescence and electron microscopy, we examined the intra-erythrocytic stages of transgenic parasite lines expressing hemagglutinin (HA)-tagged catalytic and regulatory subunits (HA-CK2a, HA-PfCK2ß1 or HA-PfCK2ß2), and localized all three subunits to both cytoplasmic and nuclear compartments of the parasite. The same transgenic parasite lines were used to purify PfCK2ß1- and PfCK2ß2-containing complexes, which were analyzed by mass spectrometry. The recovered proteins were unevenly distributed between various pathways, with a large proportion of components of the chromatin assembly pathway being present in both PfCK2ß1 and PfCK2ß2 precipitates, implicating PfCK2 in chromatin dynamics. We also found that chromatin-related substrates, such as nucleosome assembly proteins (Naps), histones, and two members of the Alba family are phosphorylated by PfCK2a in vitro. Our reverse-genetics data show that each of the two regulatory PfCK2 subunits is required for completion of the asexual erythrocytic cycle. Our interactome study points to an implication of PfCK2 in many cellular pathways, with chromatin dynamics being identified as a major process regulated by PfCK2. (Ref: 144).

Aurora kinases: Aurora kinases compose a family of conserved Ser/Thr protein kinases playing essential roles in eukaryotic cell division. To date, Aurora homologues remain uncharacterized in the protozoan phylum Apicomplexa. In malaria parasites, the characterization of Aurora kinases may help understand the cell cycle control during erythrocytic schizogony where asynchronous nuclear divisions occur. We revisited the kinome of P. falciparum and identified three Aurora-related kinases, Pfark-1, -2, -3. Among these, Pfark-1 is highly conserved in malaria parasites and also appears to be conserved across Apicomplexa. By tagging the endogenous Pfark-1 gene with the green fluorescent protein (GFP) in live parasites, we show that the Pfark-1-GFP protein forms paired dots associated with only a subset of nuclei within individual schizonts. Immunofluorescence analysis using an anti-a-tubulin antibody strongly suggests a recruitment of Pfark-1 at duplicated spindle pole bodies at the entry of the M phase of the cell cycle. Unsuccessful attempts at disrupting the Pfark-1 gene with a knockout construct further indicate that Pfark-1 is required for parasite growth in red blood cells. This study provided new insights into the cell cycle control of malaria parasites and reports the importance of Aurora kinases as potential targets for new antimalarials. (Ref: 49).

Tyrosine kinase-like kinase 3 (PfTKL3): We performed a thorough characterisation of PfTKL3 (PF13_0258), an enzyme that belongs to the tyrosine kinase-like kinase (TKL) group. We demonstrate by reverse genetics that PfTKL3 is essential for asexual parasite proliferation in human erythrocytes. PfTKL3 is expressed in both asexual and gametocytes stages, and in the latter the protein co-localises with cytoskeleton microtubules. Recombinant PfTKL3 displays in vitro autophosphorylation activity and is able to phosphorylate exogenous substrates, and both activities are dramatically dependent on the presence of an N-terminal

"sterile alpha-motif" domain. This identified PfTKL3 as a validated drug target amenable to high-throughput screening. (Ref: 53)

NIMA-related kinase Pfnek-1: The P. falciparum kinome includes a family of four protein kinases (Pfnek-1 to -4) related to the NIMA (never-in-mitosis) family, members of which play important roles in mitosis and meiosis in eukaryotic cells. Only one of these, Pfnek-1, which we previously characterized at the biochemical level, is expressed in asexual parasites. The other three (Pfnek-2, -3 and -4) are expressed predominantly in gametocytes, and a role for nek-2 and nek-4 in meiosis has been documented. Here, we show by reverse genetics that Pfnek-1 is required for completion of the asexual cycle in red blood cells and that its expression in gametocytes in detectable by immunofluorescence in male (but not in female) gametocytes, in contrast with Pfnek-2 and Pfnek-4. This indicates that the function of Pfnek-1 is non-redundant with those of the other members of the Pfnek family and identifies Pfnek-1 as a potential target for antimalarial chemotherapy. A medium-throughput screen of a small-molecule library provides proof of concept that recombinant Pfnek-1 can be used as a target in drug discovery. (Ref: 98).

Development of a scalable new transfection technology for the generation of mutant parasite lines: In malaria parasites, the systematic experimental validation of drug and vaccine targets by reverse genetics is constrained by the inefficiency of homologous recombination and by the difficulty of manipulating adenine and thymine (A+T)-rich DNA of most Plasmodium species in Escherichia coli. We overcame these roadblocks by creating a high-integrity library of P. berghei genomic DNA (greater than 77% A+T content) in a bacteriophage N15-based vector that can be modified efficiently using the lambda Red method of recombineering. We built a pipeline for generating P. berghei genetic modification vectors at genome scale in serial liquid cultures on 96-well plates. Vectors have long homology arms, which increase recombination frequency up to tenfold over conventional designs. The feasibility of efficient genetic modification at scale will stimulate collaborative, genome-wide knockout and tagging programs for P. berghei. (Ref: 116).

A host erythrocyte signalling pathway required for parasite survival: We demonstrated that a protein kinase-mediated signalling pathway involving host RBC PAK1 and MEK1, which do not have orthologues in the Plasmodium kinome, is selectively stimulated in P. falciparum-infected (versus uninfected) RBCs, as determined by the use of phospho-specific antibodies directed against the activated forms of these enzymes. Pharmacological interference with host MEK and PAK function using highly specific allosteric inhibitors in their known cellular IC50 ranges results in parasite death. Furthermore, MEK inhibitors have parasiticidal effects in vitro on hepatocyte and erythrocyte stages of the rodent malaria parasite P. berghei, indicating conservation of this subversive strategy in malaria parasites. These findings have profound implications for the development of novel strategies for antimalarial chemotherapy. (Ref: 99).

WP 2: Signalling in sexual development and sporogony

WP2 Coordinators: P. Alano (partner 2) and S. Dhar (partner 15).

Workpackage activities were organised to study signalling in sexual development and sporogony on three levels of investigation: i) signalling molecules, ii) effectors molecules, and iii) stage-specific processes.

Accomplishments at a glance:

i) Signalling molecules

Work in the MALSIG consortium functionally analysed a large number of kinases and signalling molecules. The above described production of the global gene disruption analysis of kinase encoding genes in P. berghei and P. falciparum provided the unique opportunity to assess phenotypic consequences of gene disruption in sexual development for such an unprecedented number of kinase-encoding genes, yielding important information on their role in sexual development.

Reverse genetics of the P. berghei kinome: information gained on kinases essential for sexual development. The work conducted on the rodent malaria parasite, in particular, specifically focussed on evaluating effect of gene disruption in sexual development. Results were that the systematic functional analysis of Plasmodium protein kinases identified essential regulators of mosquito transmission. Although eukaryotic protein kinases (ePKs) contribute to many cellular processes, only three Plasmodium falciparum ePKs have thus far been identified as essential for parasite asexual blood stage development. To identify pathways essential for parasite transmission between their mammalian host and mosquito vector, we undertook a systematic functional analysis of ePKs in the genetically tractable rodent parasite Plasmodium berghei. Modeling domain signatures of conventional ePKs identified 66 putative Plasmodium ePKs. Kinomes are highly conserved between Plasmodium species. Using reverse genetics, we show that 23 ePKs are redundant for asexual erythrocytic parasite development in mice. Phenotyping mutants at four life cycle stages in Anopheles stephensi mosquitoes revealed functional clusters of kinases required for sexual development and sporogony. Roles for a putative SR protein kinase (SRPK) in microgamete formation, a conserved regulator of clathrin uncoating (GAK) in ookinete formation, and a likely regulator of energy metabolism (SNF1/KIN) in sporozoite development were identified. (Ref: 76)

Besides the above general systematic analysis, some KO lines were investigated in particular detail:

CDPK1. One of such studies revealed that the Plasmodium Calcium-Dependent Protein Kinase 1 (CDPK1) controls zygote development and transmission by translationally activating repressed mRNAs. Calcium-dependent protein kinases (CDPKs) play key regulatory roles in the life cycle of the malaria parasite, but in many cases their precise molecular functions are unknown. Using the rodent malaria parasite Plasmodium berghei, we show that CDPK1, which is known to be essential in the asexual blood stage of the parasite, is expressed in all life stages and is indispensable during the sexual mosquito life-cycle stages. Knockdown of CDPK1 in sexual stages resulted in developmentally arrested parasites and prevented mosquito transmission, and these effects were independent of the previously proposed function for CDPK1 in regulating parasite motility. In-depth translational and transcriptional profiling of arrested parasites revealed that CDPK1 translationally activates mRNA species in the developing zygote that in macrogametes remain repressed via their 3' and 5'UTRs. These findings indicate that CDPK1 is a multifunctional protein that translationally regulates mRNAs to ensure timely and stage-specific protein expression. (Ref: 170)

PKRP. In another study it was revealed that The kinase-related protein (PKRP) from Plasmodium berghei mediates infection in the midgut and salivary glands of the mosquito: The putative kinase-related protein (PKRP) that is predicted to be an atypical protein kinase, which is conserved across many species of Plasmodium. The pkrp gene encodes a RNA of about 5300 nucleotides that is expressed as a 90kDa protein in sporozoites. Targeted disruption of the pkrp gene in Plasmodium berghei, a rodent model of malaria, compromises the ability of parasites to infect different tissues within the mosquito host. Early infection of mosquito midgut is reduced by 58-71%, midgut oocyst production is reduced by 50-90% and those sporozoites that are produced are defective in their ability to invade mosquito salivary glands. Midgut sporozoites are not morphologically different from wild-type parasites by electron microscopy. Some sporozoites that emerged from oocysts were attached to the salivary glands but most were found circulating in the mosquito hemocoel. These findings indicate that a signalling pathway involving PbPKRP regulates the level of Plasmodium infection in the mosquito midgut and salivary glands. (Ref: 84)

Pfnek2. One study revealed an essential role for the Plasmodium Nek-2 Nima-related protein kinase in the sexual development of malaria parasites We previously showed that a Plasmodium gametocyte-specific NIMA-related protein kinase, nek-4, is required for completion of meiosis in the ookinete, the motile form that develops from the zygote in the mosquito vector. Here, we show that another NIMA-related kinase, Pfnek-2, is also predominantly expressed in gametocytes, and that Pfnek-2 is an active enzyme displaying an in vitro substrate preference distinct from that of Pfnek-4. A functional nek-2 gene is required for transmission of both Plasmodium falciparum and the rodent malaria parasite Plasmodium berghei to the mosquito vector, which is explained by the observation that disruption of the nek-2 gene in P. berghei causes dysregulation of DNA replication during meiosis and blocks ookinete development. This has implications (i) in our understanding of sexual development of malaria parasites and (ii) in the context of control strategies aimed at interfering with malaria transmission. (Ref: 3)

Pfnek-4 and the commitment to sexual differentiation. The Plasmodium falciparum, Nima-related kinase Pfnek-4 appear to mark asexual parasites committed to sexual differentiation. The expression and function of the Nima-related kinase Pfnek-4 during the early sexual development of the human malaria parasite Plasmodium falciparum were investigated, using three types of transgenic Plasmodium falciparum 3D7 lines: (i) episomally expressing a Pfnek-4-GFP fusion protein under the control of its cognate pfnek-4 promoter; (ii) episomally expressing negative or positive selectable markers, yeast cytosine deaminase-uridyl phosphoribosyl transferase, or human dihydrofolate reductase, under the control of the pfnek-4 promoter; and (iii) lacking a functional pfnek-4 gene. Parasite transfectants were analysed by fluorescence microscopy and flow cytometry. In vitro growth rate and gametocyte formation were determined by Giemsa-stained blood smears. The Pfnek-4-GFP protein was found to be expressed in stage II to V gametocytes and, unexpectedly, in a subset of asexual-stage parasites undergoing schizogony. Culture conditions stimulating gametocyte formation resulted in significant increase of this schizont subpopulation. Moreover, sorted asexual parasites expressing the Pfnek-4-GFP protein displayed elevated gametocyte formation when returned to in vitro culture in presence of fresh red blood cells, when compared to GFP- parasites from the same initial population. Negative selection of asexual parasites expressing pfnek-4 showed a marginal reduction in

growth rate, whereas positive selection caused a marked reduction in parasitaemia, but was not sufficient to completely abolish proliferation. Pfnek-4- clones are not affected in their asexual growth and produced normal numbers of stage V gametocytes.

The results indicate that Pfnek-4 is not strictly gametocyte-specific, and is expressed in a small subset of asexual parasites displaying high rate conversion to sexual development. Pfnek-4 is not required for erythrocytic schizogony and gametocytogenesis. This is the first study to report the use of a molecular marker for the sorting of sexually-committed schizont stage P. falciparum parasites, which opens the way to molecular characterization of this pre-differentiated subpopulation. (Ref: 142).

PKG role in ookinete. A cyclic GMP signalling module that regulates gliding motility in a malaria parasite

The ookinete is a motile stage in the malaria life cycle, which forms in the mosquito blood meal from the zygote. Ookinetes use an acto-myosin motor to glide towards and penetrate the midgut wall to establish infection in the vector. The regulation of gliding motility is poorly understood. Through genetic interaction studies we here describe a signalling module that identifies guanosine 3', 5'-cyclic monophosphate (cGMP) as an important second messenger regulating ookinete differentiation and motility. In ookinetes lacking the cyclic nucleotide degrading phosphodiesterase delta (PDEdelta), unregulated signalling through cGMP results in rounding up of the normally banana-shaped cells. This phenotype is suppressed in a double mutant additionally lacking guanylyl cyclase beta (GCbeta), showing that in ookinetes GCbeta is an important source for cGMP, and that PDEdelta is the relevant cGMP degrading enzyme. Inhibition of the cGMP-dependent protein kinase, PKG, blocks gliding, whereas enhanced signalling through cGMP restores normal gliding speed in a mutant lacking calcium dependent protein kinase 3, suggesting at least a partial overlap between calcium and cGMP dependent pathways. These data demonstrate an important function for signalling through cGMP, and most likely PKG, in dynamically regulating ookinete gliding during the transmission of malaria to the mosquito. In addition to protein kinases, other clases of molecules closely related to signalling pathways were found to have functional role in specific sexual stage processes. (Ref: 22).

PLC. In one example, multiple roles for P. berghei phosphoinositide-specific phospholipase C in regulating gametocyte activation and differentiation were identified. Critical events in the life cycle of malaria parasites are controlled by calcium-dependent signalling cascades, yet the molecular mechanisms of calcium release remain poorly understood. The synchronized development of Plasmodium berghei gametocytes relies on rapid calcium release from internal stores within 10 s of gametocytes being exposed to mosquito-derived xanthurenic acid (XA). Here we addressed the function of phosphoinositide-specific phospholipase C (PI-PLC) for regulating gametocyte activation. XA triggered the hydrolysis of PIP(2) and the production of the secondary messenger IP(3) in gametocytes. Both processes were selectively blocked by a PI-PLC inhibitor, which also reduced the early Ca(2+) signal. However, microgametocyte differentiation into microgametes was blocked even when the inhibitor was added up to 5 min after activation, suggesting a requirement for PI-PLC beyond the early mobilization of calcium. In contrast, inhibitors of calcium release through ryanodine receptor channels were active only during the first minute of gametocyte

activation. Biochemical determination of PI-PLC activity was confirmed using transgenic parasites expressing a fluorescent PIP(2) /IP(3) probe that translocates from the parasite plasmalemma to the cytosol upon cell activation. Our study revealed a complex interdependency of Ca(2+) and PI-PLC activity, with PI-PLC being essential throughout gamete formation, possibly explaining the irreversibility of this process. (Ref: 118).

ii) Effector molecules

An objective of the Workpackage was to identify the role of effector molecules linking the signalling network to specific processes occurring in sexual differentiation and sporogony.

Alveolins: In one of such study it was found that he Alveolin IMC1h Is Required for Normal Ookinete and Sporozoite Motility Behaviour and Host Colonisation in Plasmodium berghei. Alveolins, or inner membrane complex (IMC) proteins, are components of the subpellicular network that forms a structural part of the pellicle of malaria parasites. In Plasmodium berghei, deletions of three alveolins, IMC1a, b, and h, each resulted in reduced mechanical strength and gliding velocity of ookinetes or sporozoites. Using time lapse imaging, we show here that deletion of IMC1h (PBANKA_143660) also has an impact on the directionality and motility behaviour of both ookinetes and sporozoites. Despite their marked motility defects, sporozoites lacking IMC1h were able to invade mosquito salivary glands, allowing us to investigate the role of IMC1h in colonisation of the mammalian host. We show that IMC1h is essential for sporozoites to progress through the dermis in vivo but does not play a significant role in hepatoma cell transmigration and invasion in vitro. Colocalisation of IMC1h with the residual IMC in liver stages was detected up to 30 hours after infection and parasites lacking IMC1h showed developmental defects in vitro and a delayed onset of blood stage infection in vivo. Together, these results suggest that IMC1h is involved in maintaining the cellular architecture which supports normal motility behaviour, access of the sporozoites to the blood stream, and further colonisation of the mammalian host. (Ref: 169).

Proteases. Work on the role of proteases in various aspects of gametocytogenesis and gametogenesis revealed that malaria proteases mediate inside-out egress of gametocytes from red blood cells following parasite transmission to the mosquito. Gametocyte egress is a crucial step for the parasite to prepare for fertilization, but the molecular mechanisms of egress are not well understood. Via electron microscopy, we show that Plasmodium falciparum gametocytes exit the erythrocyte by an inside-out type of egress. The parasitophorous vacuole membrane (PVM) ruptures at multiple sites within less than a minute following activation, a process that requires a temperature drop and parasite contact with xanthurenic acid. PVM rupture can also be triggered by the ionophore nigericin and is sensitive to the cysteine protease inhibitor E-64d. Following PVM rupture the subpellicular membrane begins to disintegrate. This membrane is specific to malaria gametocytes, and disintegration is impaired by the aspartic protease inhibitor EPNP and the cysteine/serine protease inhibitor TLCK. Approximately 15 min post activation, the erythrocyte membrane ruptures at a single breaking point, which can be inhibited by inhibitors TLCK and TPCK. In all cases inhibitor treatment results in interrupted gametogenesis. (Ref: 127).

PfCCP adhesive proteins: Other effector molecules were identified for their ability to form multimolecular complexes in gametocyte development.

This work revealed that sexual stage adhesion proteins form multi-protein complexes in the malaria parasite Plasmodium falciparum. Among these are six secreted proteins with multiple adhesion domains, termed P. falciparum LCCL domain-containing protein (PfCCp) proteins, which are expressed in the parasitophorous vacuole of the differentiating gametocytes and which are later associated with macrogametes. Although the majority of the PfCCp proteins are implicated in parasite development in the mosquito vector, their functions remain unknown. In the present study we investigated the molecular interactions between the PfCCp proteins during gametocyte development and emergence. Using five different gene-disruptant parasite lines, we show that the lack of one PfCCp protein leads to the loss of other PfCCp family members. Co-immunoprecipitation assays on gametocyte lysates revealed formation of complexes involving all PfCCp proteins, and affinity chromatography co-elution binding assays with recombinant PfCCp domains further indicated direct binding between distinct adhesion domains. PfCCp-coated latex beads bind to newly formed macrogametes but not to gametocytes or older macrogametes 6 or 24 h post-activation. In view of these data, we propose that the PfCCp proteins form multi-protein complexes that are exposed during gametogenesis, thereby mediating cell contacts of macrogametes. (Ref: 33).

iii) Stage-specific processes

Stage specific processes potentially governed by signalling molecules were studied in depth, and some were in fact identified for the first time.

Nanotubes, a novel features in malaria parasite biology. One example is the ability of malaria parasites to form filamentous cell-to-cell connections during reproduction in the mosquito midgut. Novel filamentous cell-cell contacts have been identified in different types of eukaryotic cells and termed nanotubes due to their morphological appearance. Nanotubes represent small dynamic membranous extensions that consist of F-actin and are considered an ancient feature evolved by eukaryotic cells to establish contact for communication. We here describe similar tubular structures in the malaria pathogen Plasmodium falciparum, which emerge from the surfaces of the forming gametes upon gametocyte activation in the mosquito midgut. The filaments can exhibit a length of greater than 100 µm and contain the F-actin isoform actin 2. They actively form within a few minutes after gametocyte activation and persist until the zygote transforms into the ookinete. The filaments originate from the parasite plasma membrane, are close ended and express adhesion proteins on their surfaces that are typically found in gametes, like Pfs230, Pfs48/45 or Pfs25, but not the zygote surface protein Pfs28. We show that these tubular structures represent long-distance cell-to-cell connections between sexual stage parasites and demonstrate that they meet the characteristics of nanotubes. We propose that malaria parasites utilize these adhesive "nanotubes" in order to facilitate intercellular contact between gametes during reproduction in the mosquito midgut. (Ref: 102).

Gametocyte-infected rbc mechanical properties: In this area of investigation, distinct changes in cell mechanical properties were identified for the first time in gametocyte development. In particular it was identified a switch in infected erythrocyte deformability at the maturation and blood circulation of Plasmodium falciparum transmission stages. We show here that mature GIEs are more deformable than immature stages using ektacytometry and microsphiltration methods, and that a

switch in cellular deformability in the transition from immature to mature gametocytes is accompanied by the deassociation of parasite-derived STEVOR proteins from the infected erythrocyte membrane. We hypothesize that mechanical retention contributes to sequestration of immature GIEs and that regained deformability of mature gametocytes is associated with their release in the bloodstream and ability to circulate. These processes are proposed to play a key role in P falciparum gametocyte development in the host and to represent novel and unconventional targets for interfering with parasite transmission. (Ref: 152).

Egress and osmiophilic bodies. Another line of work aimed to investigate the biogenesis and mobilisation of the egress-related osmiophilic bodies in the gametocytes of P. falciparum. Gametocytes, the blood stages responsible for P. falciparum transmission, contain electron dense organelles, traditionally named osmiophilic bodies, that are believed to be involved in gamete egress from the host cell. In order to provide novel tools in the cellular and molecular studies of osmiophilic body biology, a P. falciparum transgenic line in which these organelles are specifically marked by a reporter protein was produced and characterized. A P. falciparum transgenic line expressing an 80-residue N-terminal fragment of the osmiophilic body protein Pfg377 fused to the reporter protein DsRed, under the control of pfg377 upstream and downstream regulatory regions, was produced. The transgenic fusion protein is expressed at the appropriate time and stage of sexual differentiation and is trafficked to osmiophilic bodies as the endogenous Pfg377 protein. These results indicate that a relatively small N-terminal portion of Pfg377 is sufficient to target the DsRed reporter to the gametocyte osmiophilic bodies.This is the first identification of a P. falciparum amino acid sequence able to mediate trafficking to such organelles. To fluorescently tag such poorly characterized organelles opens novel avenues in cellular and imaging studies on their biogenesis and on their role in gamete egress. (Ref: 153).

Interactions with the mosquito vector. Process involving the crosstalk between sexual stages and the insect host were also investigated and identified. One example is the characterisation of parasite ookinete destruction within the mosquito midgut lumen, which explains Anopheles albimanus refractoriness to P. falciparum (3D7A) oocyst infection. A. albimanus is generally refractory to oocyst infection with allopatric isolates of the human malaria parasite P. falciparum. However, the reasons for the refractoriness of A. albimanus to infection with such isolates of P. falciparum are unknown. In the current study, we investigated the infectivity of the P. falciparum clone 3D7A to laboratory-reared A. albimanus and another natural vector of human malaria, A. stephensi. P. falciparum gametocytes grown in vitro were simultaneously fed to both mosquito species and the progress of malaria infection compared. In 22 independent paired experimental feeds, no mature oocysts were observed on the midguts of A. albimanus 10days after blood feeding. In contrast, high levels of oocyst infection were found on the midguts of simultaneously fed A. stephensi. Direct immunofluorescence microscopy and light microscopical examination of Giemsa-stained histological sections were used to identify when the P. falciparum clone 3D7A failed to establish mature oocyst infections in A. albimanus. Similar densities of macrogametes/zygotes, and immature retort-form and mature ookinetes were found within the blood meals of both mosquito species. However, in A. albimanus ookinetes were seldom associated with the peritrophic matrix, and were neither observed in the ectoperitrophic

space nor the midgut epithelium. In contrast, ookinetes were frequently observed in these midgut compartments in A. stephensi. Additionally, young oocysts were observed on the midguts of A. stephensi, but not A. albimanus 2 days after bloodfeeding. Vital staining of the immature retort-form and mature ookinetes found within the luminal bloodmeal, demonstrated that a significantly greater proportion of these malaria parasite stages were non-viable in A. albimanus compared with A. stephensi. Overall, our observations indicate that ookinetes of the P. falciparum clone 3D7A are destroyed within the bloodmeal of A. albimanus and that the midgut lumen, rather than the midgut epithelium, is the site of mosquito refractoriness in this particular malaria parasite-mosquito vector combination. (Ref: 175)

WP 3: Liver stage development

WP3 Coordinator: V. Heussler (partner 4).

WP3 concentrated on the molecular basis of parasite-host cell interaction during the liver phase of the parasite. The main achievements have been the generation of various transgenic parasite strains to elucidate the function of parasite proteins or their localization. Participation of Partner 7 (BPRC) into WP3 during the project allowed the MALSIG consortium to investigate the function of kinases during the dormant hypnozoite stage of P. cynomolgi. This added a new and very important facet to the analysis of liver stage parasites.

Accomplishments at a glance:

Functional studies of AMA1 and RON proteins during invasion by the sporozoite. During invasion, apicomplexan parasites form an intimate circumferential contact with the host cell, the tight junction (TJ), through which they actively glide. The TJ, which links the parasite motor to the host cell cytoskeleton, is thought to be composed of interacting apical membrane antigen 1 (AMA1) and rhoptry neck (RON) proteins. We showed that, in P. berghei, while both AMA1 and RON4 are important for merozoite invasion of erythrocytes, only RON4 is required for sporozoite invasion of hepatocytes, indicating that RON4 acts independently of AMA1 in the sporozoite. Further, in the Toxoplasma gondii tachyzoite, AMA1 is dispensable for normal RON4 ring and functional TJ assembly but enhances tachyzoite apposition to the cell and internalization frequency. We propose that while the RON proteins act at the TJ, AMA1 mainly functions on the zoite surface to permit correct attachment to the cell, which may facilitate invasion depending on the zoite-cell combination. (Ref: 104).

Role of PbPKG in liver stages. Parasites blocked at an early developmental stage inside hepatocytes elicit a protective host immune response, making them attractive targets in the effort to develop a pre-erythrocytic stage vaccine. We generated parasites blocked at a late developmental stage inside hepatocytes by conditionally disrupting the P. berghei cGMP-dependent protein kinase in sporozoites. Mutant sporozoites are able to invade hepatocytes and undergo intracellular development. However, they remain blocked as late liver stages that do not release merosomes into the medium. These late arrested liver stages induce protection in immunized animals. This suggests that, similar to the well studied early liver stages, late stage liver stages too can confer protection from sporozoite challenge. (Ref: 82).

FLP/FRT-mediated conditional mutagenesis in pre-erythrocytic stages of P. berghei. We developed a highly efficient procedure for conditional mutagenesis in Plasmodium. The procedure uses the site-specific recombination FLP-FRT system of yeast and targets the pre-erythrocytic stages of the rodent Plasmodium parasite P. berghei, including the sporozoite stage and the subsequent liver stage. The technique consists of replacing the gene under study by an FRTed copy (i.e., flanked by FRT sites) in the erythrocytic stages of a parasite clone that expresses the flip (FLP) recombinase stage-specifically--called the 'deleter' clone. We present the available deleter clones, which express FLP at different times of the parasite life cycle, as well as the schemes and tools for constructing new deleter parasites. We also outline and discuss the various strategies for exchanging a wild-type gene with an FRTed copy and for generating conditional gene knockout or knockdown parasite clones. Finally, we detail the protocol for obtaining sporozoites that lack a protein of interest and for monitoring sporozoite-specific DNA excision and depletion of the target protein. The protocol should allow the functional analysis of any essential protein in the sporozoite, liver stage or hepatic merozoite stages of rodent Plasmodium parasites. (Ref: 125).

Lipoic acid scavenging. Lipoic acid is an essential cofactor for enzymes that participate in key metabolic pathways in most organisms. While in mammalian cells lipoylated proteins reside exclusively in the mitochondria, apicomplexan parasites of the genus Plasmodium harbour two independent lipoylation pathways in the mitochondrion and the apicoplast, a second organelle of endosymbiotic origin. Protein lipoylation in the apicoplast relies on de novo lipoic acid synthesis while lipoylation of proteins in the mitochondrion depends on scavenging of lipoic acid from the host cell. Here, we analyse the impact of lipoic acid scavenging on the development of P. berghei liver stage parasites. Treatment of P. berghei-infected HepG2 cells with the lipoic acid analogue 8-bromo-octanoic acid (8-BOA) abolished lipoylation of mitochondrial enzyme complexes in the parasite while lipoylation of apicoplast proteins was not affected. Parasite growth as well as the ability of the parasites to successfully complete liver stage development by merosome formation were severely impaired but not completely blocked by 8-BOA. Liver stage parasites were most sensitive to 8-BOA treatment during schizogony, the phase of development when the parasite grows and undergoes extensive nuclear division to form a multinucleated syncytium. Live cell imaging as well as immunofluorescence analysis and electronmicroscopy studies revealed a close association of both host cell and parasite mitochondria with the parasitophorous vacuole membrane suggesting that host cell mitochondria might be involved in lipoic acid uptake by the parasite from the host cell. (Ref: 166).

Role of proteases in liver stages. Plasmodium parasites must control cysteine protease activity that is critical for hepatocyte invasion by sporozoites, liver stage development, host cell survival and merozoite liberation. We showed that exoerythrocytic P. berghei parasites express a potent cysteine protease inhibitor (PbICP, P. berghei inhibitor of cysteine proteases) that has an important function in sporozoite invasion and is capable of blocking hepatocyte cell death. Pre-incubation with specific anti-PbICP antiserum significantly decreased the ability of sporozoites to infect hepatocytes and expression of PbICP in mammalian cells protects them against peroxide- and camptothecin-induced cell death. PbICP is secreted by sporozoites prior to and after hepatocyte invasion, localizes to the parasitophorous vacuole as well as to the

parasite cytoplasm in the schizont stage and is released into the host cell cytoplasm at the end of the liver stage. Like its homolog falstatin/PfICP in P. falciparum, PbICP consists of a classical N-terminal signal peptide, a long N-terminal extension region and a chagasin-like C-terminal domain. In exoerythrocytic parasites, PbICP is posttranslationally processed, leading to liberation of the C-terminal chagasin-like domain. Biochemical analysis has revealed that both full-length PbICP and the truncated C-terminal domain are very potent inhibitors of cathepsin L-like host and parasite cysteine proteases. These results suggest that the inhibitor plays an important role in sporozoite invasion of host cells and in parasite survival during liver stage development by inhibiting host cell proteases involved in programmed cell death. (Ref: 70).

Merosome biogenesis. The transition to the blood stage was previously shown to involve the packaging of exoerythrocytic merozoites into membrane-surrounded vesicles, called merosomes, which are delivered directly into liver sinusoids. However, it was unclear whether the membrane of these merosomes was derived from the parasite membrane, the parasitophorous vacuole membrane or the host cell membrane. This knowledge is required to determine how phagocytes will be directed against merosomes. Here, we fluorescently label the candidate membranes and use live cell imaging to show that the merosome membrane derives from the host cell membrane. We also demonstrate that proteins in the host cell membrane are lost during merozoite liberation from the parasitophorous vacuole. Immediately after the breakdown of the parasitophorous vacuole membrane, the host cell mitochondria begin to degenerate and protein biosynthesis arrests. The intact host cell plasma membrane surrounding merosomes allows Plasmodium to mask itself from the host immune system and bypass the numerous Kupffer cells on its way into the bloodstream. This represents an effective strategy for evading host defenses before establishing a blood stage infection. (Ref: 111).

In vitro culturing system for P. cynomolgi liver stages. Amongst the Plasmodium species in humans, only P. vivax and P. ovale produce latent hepatic stages called hypnozoites, which are responsible for malaria episodes long after a mosquito bite. Relapses contribute to increased morbidity, and complicate malaria elimination programs. A single drug effective against hypnozoites, primaquine, is available, but its deployment is curtailed by its haemolytic potential in glucose-6-phosphate dehydrogenase deficient persons. Novel compounds are thus urgently needed to replace primaquine. Discovery of compounds active against hypnozoites is restricted to the in vivo P. cynomolgi-rhesus monkey model. Slow growing hepatic parasites reminiscent of hypnozoites had been noted in cultured P. vivax-infected hepatoma cells, but similar forms are also observed in vitro by other species including P. falciparum that do not produce hypnozoites. P. falciparum or P. cynomolgi sporozoites were used to infect human or Macaca fascicularis primary hepatocytes, respectively. The susceptibility of the slow and normally growing hepatic forms obtained in vitro to three antimalarial drugs, one active against hepatic forms including hypnozoites and two only against the growing forms, was measured. The non-dividing slow growing P. cynomolgi hepatic forms, observed in vitro in primary hepatocytes from the natural host M. fascicularis, can be distinguished from similar forms seen in P. falciparum-infected human primary hepatocytes by the differential action of selected anti-malarial drugs. Whereas atovaquone and pyrimethamine are active on all the dividing hepatic forms observed, the P. cynomolgi slow growing forms are highly resistant to treatment by

these drugs, but remain susceptible to primaquine. Resistance of the non-dividing P. cynomolgi forms to atovaquone and pyrimethamine, which do not prevent relapses, strongly suggests that these slow growing forms are hypnozoites. This represents a first step towards the development of a practical medium-throughput in vitro screening assay for novel hypnozoiticidal drugs. (Ref: 122).

1. WP 4: Trafficking and membrane dynamics

WP4 Coordinators: G. Langsley (partner 1b) and P. Sharma (partner 16).

Accomplishments at a glance:

Signalling molecules:
PKA: One of the prototype mammalian kinases is PKA and various roles have been defined for PKA in malaria pathogenesis. The recently described phospho-proteomes of P. falciparum introduced a great volume of phospho-peptide data for both basic research and identification of new anti-malaria therapeutic targets. We discuss the importance of phosphorylations detected in vivo at different sites in the parasite R and C subunits of PKA and highlight the inhibitor sites in the parasite R subunit. The N-terminus of the parasite R subunit is predicted to be very flexible and we propose that phosphorylation at multiple sites in this region likely represent docking sites for interactions with other proteins, such as 14-3-3. The most significant observation when the P. falciparum C subunit is compared to mammalian C isoforms is lack of phosphorylation at a key site tail implying that parasite kinase activity is not regulated so tightly as mammalian PKA. Phosphorylation at sites in the activation loop could be mediating a number of processes from regulating parasite kinase activity, to mediating docking of other proteins. The important differences between Plasmodium and mammalian PKA isoforms that indicate the parasite kinase is a valid anti-malaria therapeutic target. (Ref: 147).

PKG: Evidence suggests that PKG regulates merozoite egress from P. falciparum blood stage schizonts and P. berghei liver stage schizonts. Regulatory roles have also been found for PKG in the changes that take place in preparation for gamete egress, as well as in P. berghei ookinete development and motility. The intracellular targets of PfPKG, which as a consequence are key players in the progression of the parasite life cycle, remain unknown. PKG probably has numerous downstream substrates and since it is acting at multiple stages of the parasite life cycle, it is likely to have some functionally distinct, stage-specific substrates. As advances are made in the field of phosphoproteomics and specific analyses of PKG-dependent phosphoproteomes are assembled we will be able to build a clearer picture of the consequences, at the protein level, that arise from inhibition of PKG, and how these relate to the phenotypic observations that have arisen from genetic and chemical knockdown of PKG. (Ref: 168).

PI3-K: Polyphosphorylated phosphoinositides (PIPs) are potent second messengers, which trigger a wide variety of signalling and trafficking events in most eukaryotic cells. However, the role and metabolism of PIPs in malaria parasite Plasmodium have remained largely unexplored. Our present studies suggest that PfPI3K, a novel phosphatidylinositol-3-kinase (PI3K) in Plasmodium falciparum, is exported to the host erythrocyte by the parasite in an active form. PfPI3K is a versatile enzyme as it can generate various 3'-phosphorylated PIPs. In the

parasite, PfPI3K was localized in vesicular compartments near the membrane and in its food vacuole. PI3K inhibitors wortmannin and LY294002 were effective against PfPI3K and were used to study PfPI3K function. We found that PfPI3K is involved in endocytosis from the host and trafficking of hemoglobin in the parasite. The inhibition of PfPI3K resulted in entrapment of hemoglobin in vesicles in the parasite cytoplasm, which prevented its transport to the food vacuole, the site of hemoglobin catabolism. As a result, hemoglobin digestion, which is a source of amino acids necessary for parasite growth, was attenuated and caused the inhibition of parasite growth. (Ref: 94).

Kinase Substrates: In this study, we present a detailed analysis of the phosphoproteome of P. falciparum schizonts. Phosphorylated peptides were purified with TiO2 beads and analysed with liquid chromatography-tandem mass spectrometry, resulting in the identification of 919 phosphoproteins and mapping of 2541 unique phosphorylation sites, including 871 novel sites. Prominent roles for cAMP-dependent Protein Kinase A (PKA)- and phosphatidylinositol-signalling were identified following analysis by functional enrichment, phosphoprotein interaction network clustering and phospho-motif identification tools. Furthermore, we observed that most key enzymes in the inositol pathway are phosphorylated, which strongly suggests additional levels of regulation and crosstalk with other protein kinases (such as PKA) that co-regulate different biological processes. A distinct pattern of phosphorylation of proteins involved in merozoite egress and red blood cell invasion was noted. The analyses also revealed that cAMP-PKA signalling is implicated in a wide variety of processes including motility. We verified this finding experimentally using an in vitro kinase assay, and identified three novel PKA substrates associated with the glideosome motor complex: Myosin A, Glideosome Associated Protein 45 (GAP45) and calcium dependent protein kinase 1 (CDPK1). Therefore, in addition to an established role for CDPK1 in the motor complex, this study reveals the co-involvement of PKA, further implicating it as an important regulator of host cell invasion. (Ref: 183).

ii) Effector molecules

Rabs. Rabs are important regulators of vesicular traffic due to their capacity to recruit specific effectors. In order to identify P. falciparum Rab (PfRab) effectors, we first built a Ypt-interactome by exploiting genetic and physical binding data available at the Saccharomyces genome database (SGD). We then constructed a PfRab-interactome using putative parasite Rab-effectors identified by homology to Ypt-effectors. We demonstrate its potential by wet-bench testing three predictions; that casein kinase-1 (PfCK1) is a specific Rab5B interacting protein and that the catalytic subunit of cAMP-dependent protein kinase A (PfPKA-C) is a PfRab5A and PfRab7 effector. The establishment of a shared set of physical Ypt/PfRab-effector proteins sheds light on a core set Plasmodium Rab-interactants shared with yeast. The PfRab-interactome should benefit vesicular trafficking studies in malaria parasites. The recruitment of PfCK1 to PfRab5B+ and PfPKA-C to PfRab5A+ and PfRab7+ vesicles, respectively, suggests that PfRab-recruited kinases potentially play a role in early and late endosome function in malaria parasites. (Ref: 149).

Apicomplexa contain membranous sacs (alveoli) beneath the plasma membrane, termed the Inner Membrane Complex (IMC). During parasite replication the IMC is formed de novo within the mother cell in a process described as internal budding. We hypothesized that an alveolate specific

factor is involved in the specific transport of vesicles from the Golgi to the IMC and identified the small GTPase Rab11B as an alveolate specific Rab-GTPase that localises to the growing end of the IMC during replication of Toxoplasma gondii. Conditional interference with Rab11B function leads to a profound defect in IMC biogenesis, indicating that Rab11B is required for the transport of Golgi derived vesicles to the nascent IMC of the daughter cell. Curiously, a block in IMC biogenesis did not affect formation of sub-pellicular microtubules, indicating that IMC biogenesis and formation of sub-pellicular microtubules is not mechanistically linked. We propose a model where Rab11B specifically transports vesicles derived from the Golgi to the immature IMC of the growing daughter parasites. (Ref: 7).

Heat Shock proteins: Using a pharmacological inhibitor of Hsp90 in cultured malarial parasite, we have previously implicated P. falciparum Hsp90 (PfHsp90) as a drug target against malaria. During this project, we have biochemically characterized PfHsp90 in terms of its ATPase activity and interaction with its inhibitor geldanamycin (GA) and evaluated its potential as a drug target in a preclinical mouse model of malaria. Our studies with full-length PfHsp90 showed it to have the highest ATPase activity of all known Hsp90s; its ATPase activity was 6 times higher than that of human Hsp90. Also, GA brought about more robust inhibition of PfHsp90 ATPase activity as compared with human Hsp90. Mass spectrometric analysis of PfHsp90 expressed in P. falciparum identified a site of acetylation that overlapped with Aha1 and p23 binding domain, suggesting its role in modulating Hsp90 multichaperone complex assembly. Indeed, treatment of P. falciparum cultures with a histone deacetylase inhibitor resulted in a partial dissociation of PfHsp90 complex. Furthermore, we found a well-known, semisynthetic Hsp90 inhibitor, namely 17-(allylamino)-17-demethoxygeldanamycin, to be effective in attenuating parasite growth and prolonging survival in a mouse model of malaria. In all, our biochemical characterization, drug interaction, and animal studies supported Hsp90 as a drug target and its inhibitor as a potential drug against protozoan diseases. (Ref: 95).

iii) Transporters

Glucose transporters. Glucose is the primary source of energy and a key substrate for most cells. Inhibition of cellular glucose uptake (the first step in its utilization) has, therefore, received attention as a potential therapeutic strategy to treat various unrelated diseases including malaria and cancers. For malaria, blood forms of parasites rely almost entirely on glycolysis for energy production and, without energy stores, they are dependent on the constant uptake of glucose. P. falciparum is the most dangerous human malarial parasite and its hexose transporter has been identified as being the major glucose transporter. Progress was achieved regarding the validation and development of the P. falciparum hexose transporter as a drug target, highlighting the importance of robust target validation through both chemical and genetic methods. A P. falciparum hexose transporter (PfHT) has previously been shown to be a facilitative glucose and fructose transporter. Its expression in Xenopus laevis oocytes and the use of a glucose analogue inhibitor permitted chemical validation of PfHT as a novel drug target. Following recent re-annotations of the P. falciparum genome, other putative sugar transporters have been identified. To investigate further if PfHT is the key supplier of hexose to P. falciparum and to extend studies to different stages of Plasmodium spp., we functionally analysed the hexose transporters of both the human parasite P. falciparum and the rodent parasite P. berghei using gene targeting strategies. We

demonstrated an essential function of pfht for the erythrocytic parasite growth, as it was not possible to knockout pfht unless the gene was complemented by an episomal construct. Also, we showed that parasites are rescued from the toxic effect of a glucose analogue inhibitor when pfht is overexpressed in these transfectants. We found that the rodent malaria parasite orthologue, P. berghei hexose transporter (PbHT) gene, was similarly refractory to knockout attempts. However, using a single crossover transfection strategy, we generated transgenic P. berghei parasites expressing a PbHT-GFP fusion protein suggesting that locus is amenable for gene targeting. Analysis of pbht-gfp transgenic parasites showed that PbHT is constitutively expressed through all the stages in the mosquito host in addition to asexual stages. These results provide genetic support for prioritizing PfHT as a target for novel antimalarials that can inhibit glucose uptake and kill parasites, as well as unveiling the expression of this hexose transporter in mosquito stages of the parasite, where it is also likely to be critical for survival. (Ref: 120).

Transgenic P. berghei parasites expressing GFP-tagged PbH showed that Pbht-gfp transgenic parasites constitutively express the fusion protein through all stages in the mosquito host in addition to asexual stages. Additionally, PbHT is expressed through the liver stages of infection. Analysis of pbht-gfp transgenic parasites shows PbHT expression at both 24 h and 48 h post-invasion of human hepatoma cells. Initial pharmacolical studies using a D-glucose-derived specific inhibitor of plasmodial hexose transporters, compound 3361, demonstrated the importance of D-glucose uptake during liver and transmission stages of P. berghei. Initially, we confirmed the expression of PbHT during liver stage development, using a green fluorescent protein (GFP) tagging strategy. Compound 3361 inhibited liver-stage parasite development, with a 50% inhibitory concentration (IC50) of 11 µM. This process was insensitive to the external D-glucose concentration. In addition, compound 3361 inhibited ookinete development and microgametogenesis, with IC50s in the region of 250 µM (the latter in a D-glucose-sensitive manner). Consistent with our findings for the effect of compound 3361 on vector parasite stages, 1 mM compound 3361 demonstrated transmission-blocking activity. These data indicate that novel chemotherapeutic interventions that target PfHT may be active against liver and, to a lesser extent, transmission stages, in addition to blood stages. (Ref: 121).

Anion transporters: Malaria symptoms occur during P. falciparum development into red blood cells. During this process, the parasites make substantial modifications to the host cell in order to facilitate nutrient uptake and aid in parasite metabolism. One significant alteration that is required for parasite development is the establishment of an anion channel, as part of the establishment of New Permeation Pathways (NPPs) in the red blood cell plasma membrane, and we have shown previously that one channel can be activated in uninfected cells by exogenous protein kinase A. Here, we present evidence that in P. falciparum-infected red blood cells, a cAMP pathway modulates anion conductance of the erythrocyte membrane. In patch-clamp experiments on infected erythrocytes, addition of recombinant PfPKA-R to the pipette in vitro, or overexpression of PfPKA-R in transgenic parasites lead to down-regulation of anion conductance. Moreover, this overexpressing PfPKA-R strain has a growth defect that can be restored by increasing the levels of intracellular cAMP. Our data demonstrate that the anion channel is indeed regulated by a cAMP-dependent pathway in P. falciparum-infected

red blood cells. The discovery of a parasite regulatory pathway responsible for modulating anion channel activity in the membranes of P. falciparum-infected red blood cells represents an important insight into how parasites modify host cell permeation pathways. These findings may also provide an avenue for the development of new intervention strategies targeting this important anion channel and its regulation. (Ref: 6).

Potential Impact:

1. Scientific Impact: understanding of Plasmodium developmental biology, as a platform for subsequent translational research.

Existing knowledge about how Plasmodium development is controlled by signalling events was extremely fragmentary prior to implementation of the MALSIG project. Merging investigations on signalling pathways on the one hand, and on specific aspects of Plasmodium cell biology on the other hand, clearly had a high impact on such knowledge. A large body of joint publications originated from the collaborations established in the consortium, with a significant impact on this field of research worldwide (see the scientific report and lists of Deliverables/publications for details).

The research programme of this Consortium addressed the fundamental biology of malaria parasites, a prerequisite for the rational development of subsequent translational research. The long-term goal of furthering our understanding of signalling events, which clearly progressed significantly through the output of the MALSIG consortium, is indeed to discover novel targets for antimalarial drug and vaccine development. The MALSIG project output provides a very strong basis in fundamental biology that can now be (and indeed is) used for subsequent drug discovery activities.

2. Startegic Impact: structuring national and transnational malaria research, EU and India

The Call document stated that "Specific International Cooperation Actions (SICA) will be implemented in the areas identified through bi-regional dialogues with third countries/regions on the basis of mutual interest and mutual benefit". There is no doubt that all partners, EU-based and Indian alike, benefitted from being part of a Consortium that offered such a wide range of specific expertises (provided in combination by both EU and Indian laboratories). To adequately address the theme of signalling in major developmental stages of the malaria parasite required establishing a large scientific consortium, which represented an ideal tool for generating and sharing of knowledge in an India-EU transnational setting. The significant presence of several Indian partners (one quarter of the total) in all horizontal and transversal activities of MALSIG ensured that the consortium structure was suited for optimally achieving the bi-regional mutual interests and benefits mentioned above.

Within India : In addition to building links between Indian and European researchers, the MALSIG project also presented an opportunity for five leading Indian groups working on malaria to build collaborative links in a formal and funded way. Two out of the three annual MALSIG network meetings were held in Indian and were open to non MALSIG members of the malaria community. The five Indian laboratories have complementary strengths and interests. This project encouraged exchange of ideas, methods and human resources between these groups that give rise to synergistic collaborative efforts. It is expected that the links thus established will outlive the project and will strengthen the malaria research effort in India.

Within the EU: The present project represented a novel, distinct contribution to the integration of malaria research at the EU level. Being a SICA, its scope was obviously different from that of the

BioMalPar / EviMalaR NoEs, as the focus was on attaining specific research objectives, rather than on promoting interactions between EU institutions. It was also complementary to the ANTIMAL project, as the proposed activities are situated in the most upstream section of the drug discovery process. Thus, despite its relatively large size, the present project has proven to be complementary to, rather than redundant or competing with, co-existing EU initiatives in malaria research.

Globally, establishment of a large consortium of leading European laboratories combined with a sizeable proportion of the leading Indian laboratories active in molecular/cellular aspects of malaria research, contributed to increasing European competitivity with respect to the United States, not only in the particular field relevant to this proposal, but also in the ability to establish partnerships between European and Indian research institutions.

An important aspect of the Consortium is that its members were both established senior malaria researchers, and promising junior group leaders, who were in the process of developing their research teams. Belonging to the MALSIG Consortium, and therefore being supported financially in an environment of high scientific quality, has been instrumental in facilitating/consolidating the establishment of several "young" groups within the malaria research community at large, which is of considerable importance at the European level. Indeed MALSIG was a major source of funding for several such emerging groups.

EU-India: A major objective of SICA projects is to promote links between scientists in the EU and colleagues abroad working on topics that are important for the targeted country. The present project obviously meets all aspects of this criterion. Although some of the Indian and EU partners did cooperate prior to the start of the MALSI project, the number and scope of such trans-national collaborations clearly increased during the implementation phase.

Long-term perspectives
It is important to mention that the national and trans-national links that have been established through this project are sustainable, and will be maintained beyond the duration of the project itself. Clearly, the topics under investigation will remain relevant areas of research for years to come.

3. Dissemination activities and exploitation of results

Results from the fundamental research undertaken within the MALSIG project were published in peer-reviewed journals, many of which implement an Open Access policy. Due to the high level of complementarity between the different partners, a majority of the publications arising from this work involve at least two MALSIG partner laboratories (see List of Publications).

The activities of the Consortium have been presented via a Website. The website will remain accessible, with links to the publications arising from the project.

The MALSIG consortium has a preliminary agreement with Wiley Publishers to produce a book on signalling in malaria parasites, with chapters on their respective specialised field of research contributed by Full and Affiliated members from the consortium. This book will contribute to a

wide dissemination of the scientific results, and general scientific context and issues, of the research topics addressed by the MALSIG consortium.

List of Websites:

http://www.malsig.lille.inserm.fr/