Functional characterization of four CDK-like kinases and one Calmodulin-dependent kinase of the human malaria parasite *Plasmodium falciparum*

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Würzburg, Februar 2010

Shruti Agarwal

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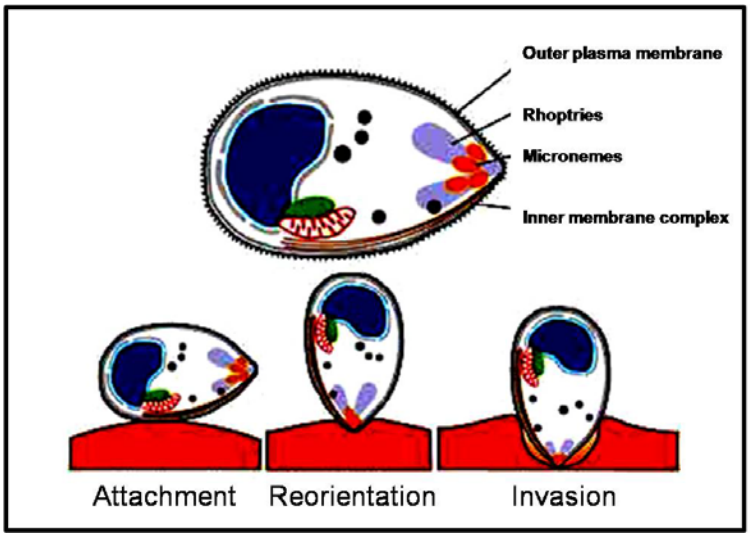
1. Introduction

1.1 The tropical disease, Malaria

Approximately 300 million people worldwide are affected by the tropical disease malaria and about 1.0 million people die from it every year (World malaria report, 2008). The disease is prevalent mostly in the sub-Saharan region, Southeast Asia, India, South and Central America (Hyde, 2005) with greatest mortality levels in sub-Saharan Africa, where children under 5 years of age account for 90% of all deaths. The tropical climate including the temperature and rainfall provide breeding opportunities to the *Anopheles* mosquito within which the parasite develops into its sexual stages (Greenwood et al., 2008).

Malaria is caused by eukaryotic, unicellular, protozoan parasite of the genus *Plasmodium* belonging to the Apicomplexa phylum and is transmitted by the female *Anopheles* mosquito. Apicomplexa is a broad category of protists containing an apical complex that helps the parasite to penetrate the host cell as exemplified by the invasion of the *P. falciparum* into host erythrocytes during the blood stage infection. The apical complex consists of anteriorly located structures called micronemes and two rhoptries which secrete enzymes to assist in the entry to the host cell (Figure 1.1).



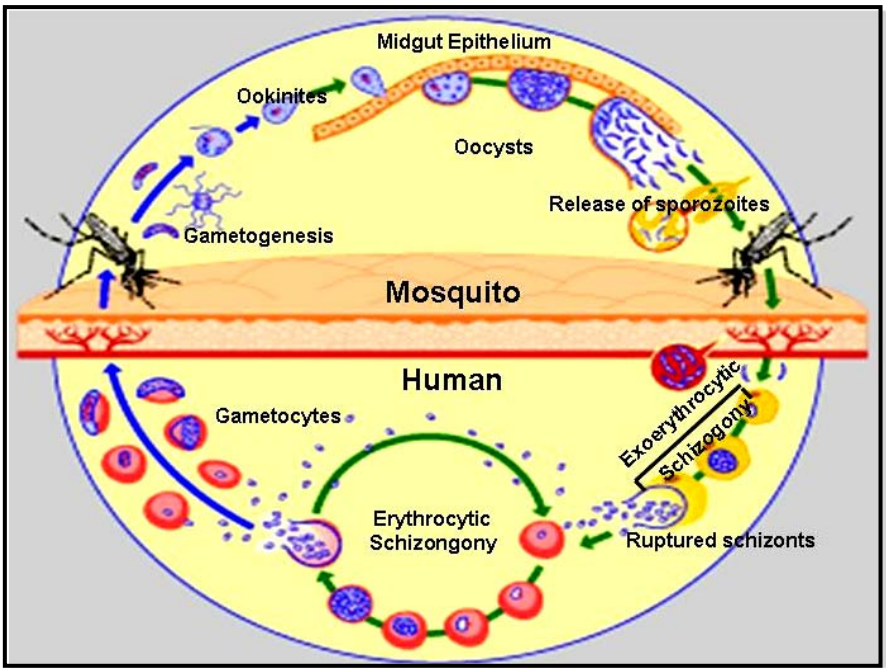
(Adapted from: www.wehi.edu)

Figure 1.1: A *P. falciparum* merozoite and its mode of entry into the red blood cell. The process involves attachment, reorientation towards the apical end and invasion.

In order to gain entry into the host cell, the merozoite first attaches itself to the erythrocyte membrane, reorients the micropore and invades the cell. This ability of the parasite to attach and invade erythrocytes during its asexual life cycle accounts for the high mortality (Greenwood et al., 2008).

The five species of *Plasmodium* that infect humans include *P. falciparum, P. knowlesi* (Ong et al., 2009)*, P. vivax, P. malariae,* and *P. ovale*, with *P. falciparum* being the deadliest. The parasite dwells in the human host and the mosquito vector. Over 430 species of anopheline mosquito exist, out of which only 40 transmit malaria (www.cdc.gov).

The life cycle of the parasite switches between the human and the female anopheline mosquito (Figure 1.2). Following a mosquito bite, the sporozoites are injected into the human host and migrate through the blood vessels to the liver. Here, they invade hepatocytes, and following an asexual replication, they give rise to the hepatic schizonts (exoerythrocytic schizogony).



(Source: http://www.geocities.com/aaadeel/malaria.html)

Figure 1.2: Schematic of life cycle of the malaria parasite, *Plasmodium falciparum*

The liver schizonts then rupture in about seven days to release thousands of merozoites into the blood. Each merozoite invades an erythrocyte and upon mitotic division result in the erythrocytic schizont (erythrocytic schizogony) that contains about 16-32 merozoites which can further reinfect

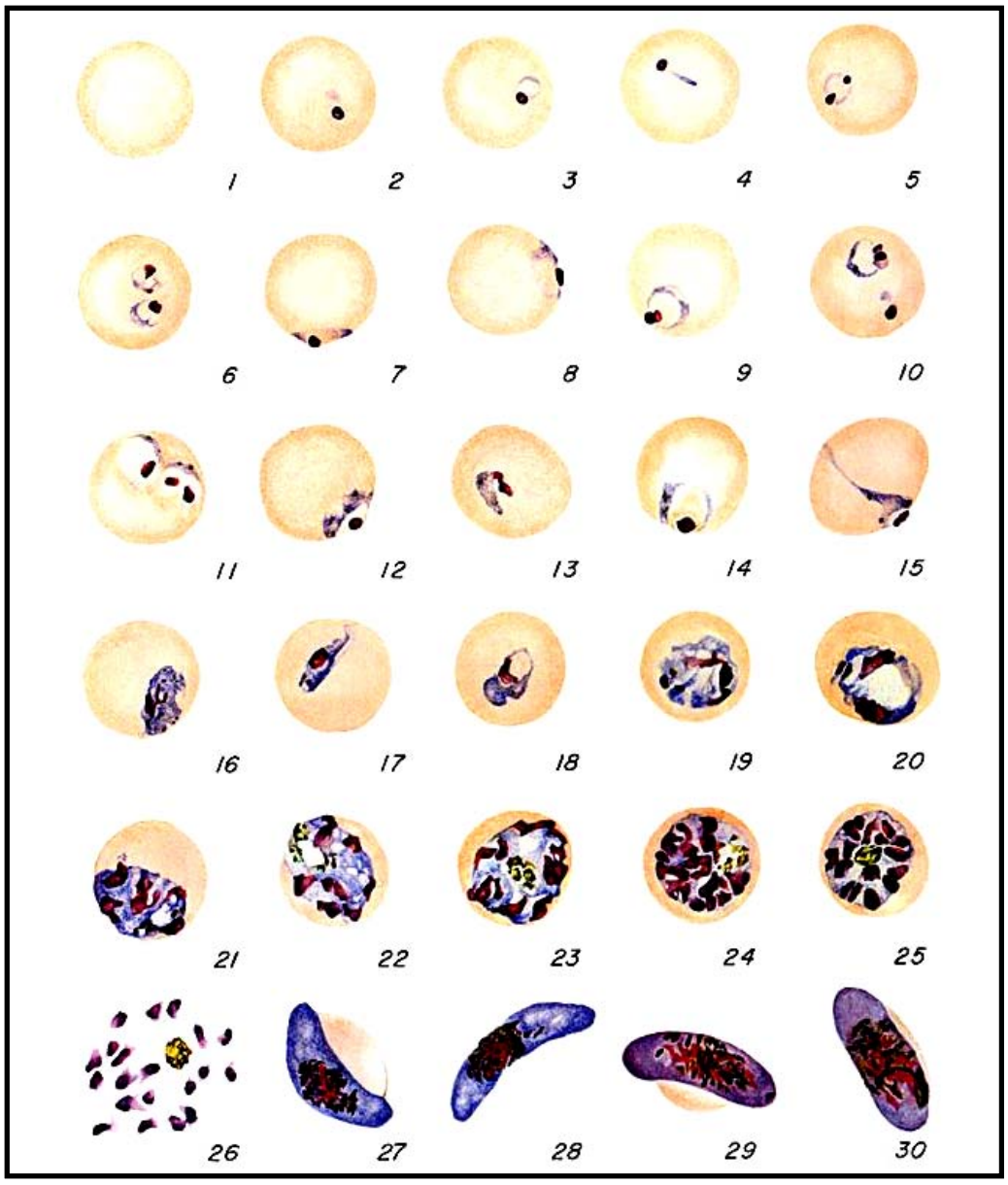
further erythrocytes. This results in a cyclic blood-stage infection that occurs every 48h. Some unknown factors stimulate few precursor merozoites to develop into male and female gametocytes (Hill, 2006). *P. falciparum* blood stages can be distinguished under the light microscope in thin blood smears followed by staining with giemsa solution (Figure 1.3).

When these gametocytes are ingested by the female mosquito during a blood meal, they give rise to the sexual gametes. Gametogenesis, the emergence of gametes from erythrocytes, is triggered by diverse factors within the mosquito midgut, including shifts in temperature, pH, and CO_2 tension, as well as the mosquito molecule, xanthurenic acid (Billker et al., 1998; Arai et al., 2001). Subsequently, complexes of multiple gametes, gametocytes and erythrocytes are formed, termed fertilization centres, which promote fertilization. In this process, the male gametocyte undergoes three rounds of DNA replication resulting in development of eight flagellated male gametes, referred as microgametes, a process commonly referred to as exflagellation. Each microgamete then fertilizes a female macrogamete to form a zygote. The resulting zygote develops into a motile ookinete that traverse the midgut epithelium in upto 24 h after the blood meal. This diploid ookinete undergoes meiosis and gives rise to an oocyst once it reaches the basal side of the midgut. The resulting oocyst undergoes several rounds of mitotic division releasing thousands of haploid sporozoites in the mosquito haemocoel (Whitten et al., 2006). These sporozoites then transit to the salivary gland of the mosquito where they are released into the blood stream of the human host, thus perpetuating the disease.

1.2 Malaria control strategies

In most endemic countries, drug therapy and disease control measures are undermined by the spread of drug resistance (Figure 1.4), HIV, extreme poverty and poor health services (Martin-Blondel et al., 2009). The other factors that contribute to increased malaria incidence include migration of refugees, climatic and environmental changes. Not only have there been high rates of resistance against the widely used anti-malarial drug chloroquine and the antifolate combination pyrimethamine-sulfadoxine but also, the alternative

therapies are too expensive to be afforded by the inhabitants of widely affected countries (Weisner et al., 2003).



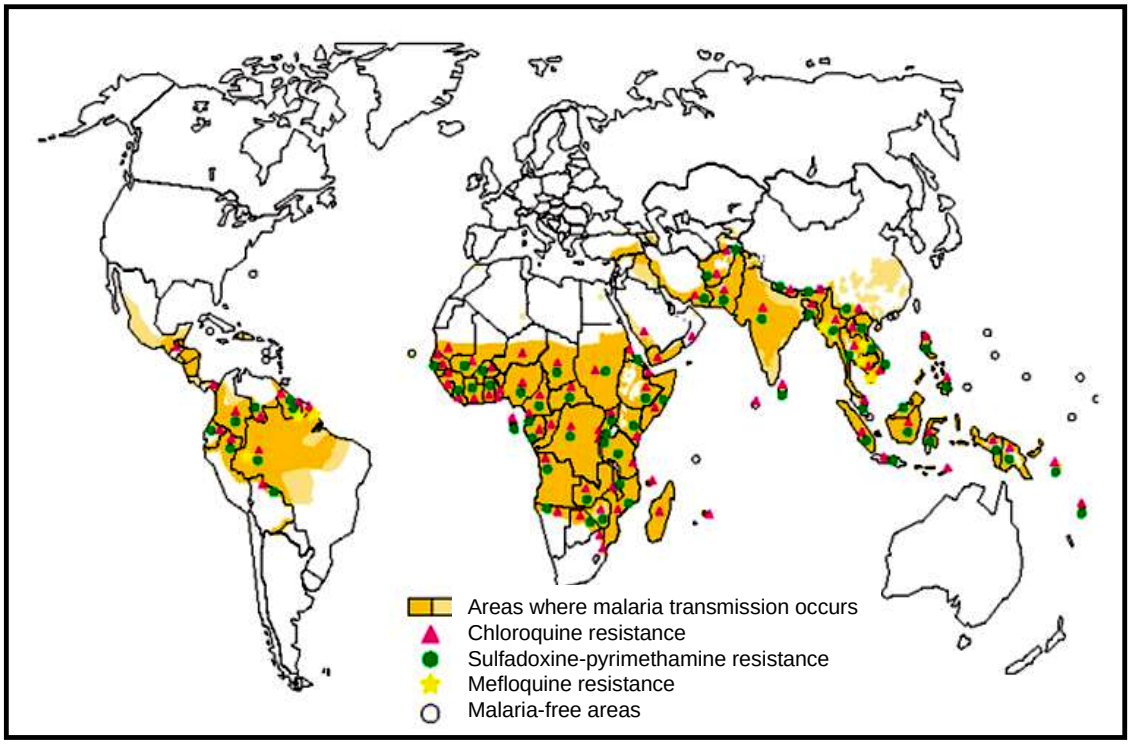
(Source: www.dpd.cdc.gov)

Figure 1.3: Giemsa stained thin blood smears show life cycle stages of the human malaria parasite, *P. falciparum*. 1: normal blood cell; 2-9: young and old rings; 10-18: young and old trophozoites; 19-26: schizonts; 27-28: mature female gametocytes; 29-30: mature male gametocytes.

Therefore, there is an urgent need for the development of new effective and affordable vaccines and anti-malarial drugs.

1.2.1 Vaccine research

The complex life cycle of the malaria parasite, which alternates between the human host and an Anopheline mosquito vector, provides a multitude of potential targets (Zhang et al., 2001; Hill et al., 2006; White et al., 2008). Vaccine development research is categorized into different stages of the parasite's life cycle. These include broadly the pre-erythrocytic stages, the blood stage and the sexual stages that are prime targets of transmission blocking vaccines (Richards and Beeson, 2009).



(Adapted from: World Malaria Report, WHO 2005)

Figure 1.4: Rapid spread of drug resistance to *P. falciparum* in sentinel sites.

Two main sub-categories of pre-erythrocytic vaccines are the anti-sporozoite vaccine and the anti-liver stage vaccines. Anti-sporozoite vaccine candidate, RTS,S is a protein particle vaccine (comprising of polypeptides RTS and S) in a complex adjuvant and is under phase II clinical trials (Stoute et al., 1997; Snounou et al., 2005). This vaccination comprises of the particles that constitute Hepatitis B S antigen (HBsAg) and a CSP (circumsporozoite protein) polypeptide. The C-terminal of the polypeptide is fused with the N-terminal of the S antigen (Gordon et al., 1995). The anti-liver

stage vaccine however, is based on the evidence implicating T-cell immunity in the protection against liver-stage parasites (Hill, 2006).

Another phase in the parasite life cycle is the blood stage which is least understood in terms of immunity. It is the stage which presents clinical symptoms and pathology like cerebral malaria and anaemia (Miller et al., 1994). Unlike most other vaccines where the whole antigen complex of the organism is in attenuated or killed form, this is not possible in case of malaria. It is due to the requirement of human erythrocytes for culturing the organism and is considered to be unsafe due to high prevalence of the disease and about 40% population under the risk of developing the disease (reviewed in Good, 2001). Therefore, a subunit vaccine comprising of only a part of the organism, like a recombinant protein, is used. Examples of blood stage vaccine candidates which are under clinical trials or under development include a multicomplex of merozoite surface protein 1 (MSP1), MSP2 and ring infected surface antigen (RESA). In addition, individual recombinant proteins of MSP1 and MSP2 and apical membrane antigen 1 (AMA1) recombinant protein are also considered (Sercarz et al., 1993; Herrera et al., 1992; reviewed in Good, 2001).

Further, parasite sexual stages exposed in the mosquito midgut are the focus of transmission blocking vaccines (TBV). These rely on human antibodies that are taken up by the mosquito during a blood meal on an infected individual. These would then inhibit the parasite stages within the mosquito midgut in addition to the human complement factors that are involved in the process (Carter et al., 2000; Carter et al., 2001; Stowers and Carter 2001; Kaslow 1997; Kaslow, 2002). The known TBV candidates till date are expressed in gametocytes and gametes, e.g. *Pfs*48/45 and *Pfs*230, where a natural infection would boost the immunity against these proteins. Some proteins like *Pfs*25 and *Pfs*28 are expressed in mosquito-specific stages such as gametes, zygotes, ookinetes and are restricted to the mosquito vector. Other prime candidates for TBV include proteins possessing important roles in development of the parasite in the mosquito vector such as CTRP (circumsporozoite protein [CSP] and thrombospondin-related adhesive protein [TRAP]-related protein). These proteins are differentially expressed in the sporozoites derived from oocysts and mosquito salivary glands. They also have a role in entry of the parasite into

the hepatocytes (Robson et al., 1995). Another family comprising of six secreted *Pf*CCp (Limulus coagulation factor C domain-containing proteins; Pradel et al., 2004) proteins has multiple adhesion domains (reviewed in Pradel, 2007) and has been shown to represent potential candidates for TBVs. *Pf*CCP proteins are expressed in the parasitophorous vacuole during gametocyte maturation and assemble to multiprotein complexes during gametogenesis (Simon et al., 2009).

Apart from vaccines, the complex life cycle of *P. falciparum* offers various targets for drug intervention.

1.2.2 Malaria chemotherapy

Several anti-malarial compounds available till date have targeted the various life cycle stages of the parasite. As vaccines, drugs also target the symptomatic asexual erythrocytic cycle, the liver stages and the sexual stages of the parasite.

Drugs targeting the erythrocytic stages are required for the treatment of the disease while drugs that target asymptomatic liver stages are for prophylactic therapy to those who intend to travel to endemic areas (reviewed in Doerig, 2009). Many of the available anti-malarial compounds are schizonticidal, i.e. they target the hepatocytic as well as erythrocytic schizonts while some others reduce gametocyte transmission (Table 1.1). Examples include the classical drug Quinine, which is an alkaloid and very effective against *P. falciparum*. Chloroquine, further was the drug of choice until the emerging drug resistant parasite strains reduced its efficacy about five decades ago (Sidhu et al., 2002). It prevents the biocrystallization of hemozoin thus poisoning the parasite. It has been mainly known to act against trophozoites and early schizonts but also against immature gametocytes of *P. falciparum*.

Another class of schizonticidal drugs is Pyrimethamine which acts by inhibiting dihydrofolate reductase thus blocking purine and pyrimidine biosynthesis. It is especially advised in *P. falciparum* chloroquine-resistant strains in combination with Sulphadoxine (White 2004; Mugittu et al., 2005). It inhibits DNA synthesis along with cell division and replication. Likewise, Mefloquine acts against schizonts, although other life cycle stages are also known targets of antiplasmodial compounds.

Intervention with the parasite sexual stages offers targets that could reduce or block transmission of the disease by either acting at the level of gametocytogenesis or within the mosquito host during sporogony (reviewed in Pradel 2007 and Doerig, 2009). Hence, such compounds are referred as transmission blocking drugs (TBD). These include Primaquine which acts against liver stages and sexual blood stages of *Plasmodium*. (Schlitzer, 2003) and Artemisinin that apart from acting on trophozoites, regress the disease by blocking development of early gametocytes (Dutta et al., 1989; Kumar and Zheng 1990).

All anti-malarial drugs act by either altering or inhibiting various metabolic processes in various cell organelles or in the cytoplasm of the host cell (Greenwood et al., 2008). Recent reports suggest drug resistance to even the latest generation of anti-plasmodial compounds (Dondorp et al., 2009) while the other reports contradict, due to undefined dosing regimes which vary according to pharmacokinetic properties of a specific population (Hodel et al., 2009).

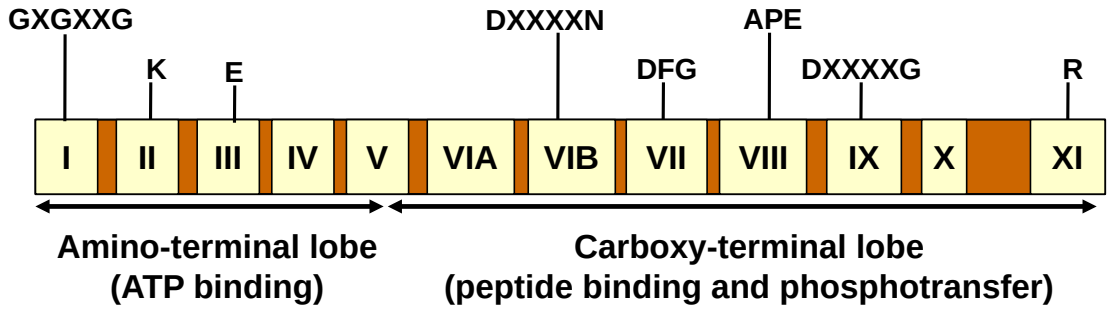
Table 1.1: Widely used anti-malarial compounds and their targets in the life cycle stages of the malaria parasite.

Drug	**Target life cycle stage of the parasite**
Chloroquine	Trophozoites, early schizonts, immature gametocytes
Pyrimethamine	Schizonts
Sulphadoxine	Schizonts
Proguanil	Hypnozoites, weakly on schizonts
Mefloquine	Schizonts
Primaquine	Hypnozoites, sexual blood stages
Artemesinin	Trophozoites
Artemether	Gametocytes
Artesunate	Gametocytes
Dihydroartemesinin	Schizonts, weakly on gametocytes

In order to prevent the increasing drug resistance of the parasite it is important to design anti-malarials that have important properties. These include combination of two different compounds targeting different metabolic reactions so that the chance of developing rapid mutation is decreased. Secondly, the drug should be cleared fast so that surviving parasites are not exposed to sub-therapeutic levels of the medication. Third, the potential antimalarial should also be able to block transmission of the parasite (White 2004; Doerig et al., 2005). Finally, cross-resistance should also be approached which might result due to similar chemical structure of small molecules as exemplified by longer half-life of Mefloquine than Chloroquine in the combination therapy. All above approaches would be a consideration for the present global malaria eradication strategy (Feachem et al., 2008; Greenwood et al., 2008).

1.3 Evaluation of kinases as drug targets

In the search for new drugs in malaria therapy, research focuses on novel metabolic pathways of the parasite. Here, various developmental processes involve tightly regulated signal pathways. These in turn include a number of proteins, which are controlled by phosphorylation and dephosphorylation reactions by different protein kinases and phosphatases (Anamika et al., 2005). Importance of eukaryotic protein kinases (ePKs) in cell proliferation and differentiation is reflected by the number of these enzymes present in eukaryotes. Approximately 2% of the genes in the human genome encode protein kinases (Kostich et al., 2002; Manning et al., 2002). Database mining of eukaryotic genomes led to the discovery of proteins that have or might have a kinase activity. This is based on the presence of a conserved kinase domain in a protein sequence. The kinase domain is further classified into eleven sub-domains, each of which carries a conserved motif required for the kinase activity (described by Hanks, 2003; Figure 1.5). Domains I to V comprise the N-terminal lobe essential for ATP binding and stretch of domains from V to XI is referred as C-terminal which has a functional role in peptide binding and phosphotransfer. Lysine in sub domain II and aspartate in sub domain VII function in support and anchoring of ATP. An aspartate in VI B is likely to be involved in phosphotransfer reaction (Hanks, 2003).



(Modified from: Hanks, 2003)

Figure 1.5: Schematic showing a typical ePK catalytic domain. The eleven sub-domains with respective conserved motifs on top are represented in roman numbers. Based on the crystal structure, the kinase domain is divided into two lobes separated by a hinge region.

The distant relation between humans and *P. falciparum* and extensive gene duplication on the human side resulted in divergence between the two genomes (Ward et al., 2004). Divergence from the mammalian kinases is the outcome due to several features of *Plasmodium* kinase family. Firstly, there are no TyrK (Tyrosine kinase) and members of the STE (ePKs identified in sterile yeast mutant) family. Secondly, presence of “atypical” or “orphan” protein kinases with characteristics of more than one family places them in a distinct category than mammalian cells. Thirdly, the presence of CDPKs (calcium-dependent protein kinases) with a fused Calmodulin-like domain is unique to *Plasmodium* apart from plants and alveolates but not present in metazoans (Zhang et al., 2001). Lastly, *Plasmodium* lacks clearly clustered orthologues of mammalian ePKs (Hanks and Quinn, 1991; Doerig et al., 2008).

Further divergence occurs due to one or more modifications in the kinase sequence. These changes include either long insertions or extensions, which might result in structural changes (reviewed in Kappes et al., 1999). The Apicomplexan structure has some variations as compared with enzymes of higher eukaryotes, e.g. protein kinase-G (PKG) of higher eukaryotes possesses two cGMP-responsive regulatory domains whereas apicomplexan parasites such as *P. falciparum* (Deng et al., 2002) and *T. gondii* (Donald et al., 2002) have three such sites. The extra site is predicted to provide more stringent allosteric regulation of the kinase activity when compared to PKG of higher eukaryotes (Salowe et al., 2002). Additional divergence in the parasite includes the example of the activation sight in MAPKs (Mitogen activated protein kinases) which is displayed by a conserved motif Thr-X-Tyr (Garrington et al.,

1999). In *Pf*map-2 of *Plasmodium*, this motif is replaced by Thr-Ser-His along with tiny insertions in the activation site (Dorin et al., 1999). In *Pf*map-1 however, the motif of the typical MAPKs is conserved. This highlights the structural changes within one protein family.

Although most parasite kinases are classified into specific families, there are examples of “hybrid kinases”. *Pf*PK6 for example shares features of both CDKs and MAPKs (Bracchi-Ricard et al., 2000). While few structural changes on one hand still categorize the enzyme in the same sub-family while the ones diverged to greater extent might place the kinase in a completely different family. This is exemplified by *Pf*nek-1 which has a high sequence homology with NIMA (Never in mitosis/Aspergillus) related parasite kinases but on the other hand has an activation site that is similar to MAPKK (MAPK kinase). The ability of *Pf*nek-1 and *Pf*nek-3 (NIMA-related protein) to phosphorylate *Pf*map-2 in vitro (Dorin et al., 2001; Lye et al., 2006) and the absence of a MAPKK encoding gene homologue in *P. falciparum* raises a question whether the two members of the NIMA family have a link to the lost MAPKK in *Plasmodium*.

Other members of the NIMA family like *Pf*nek-2 do not show such a correlation with *Pf*map-2 (Reininger et al., 2009) thus showing that divergence in terms of substrate specificity is exhibited even by members of the same family. Protein kinase *Pf*PK7, although shares a high homology with MAPKK but has no potential phosphorylation residue where the activation site of MAPKK normally lies (Dorin-Semblat et al., 2007; reviewed in Doerig 2004). Thus, proteins encoded by the parasite genome are sufficiently divergent at the level of sequence, structure and function with their counterparts in vertebrate hosts. These differences raise a possibility of the specific kinase inhibition (Ward et al., 2004).

Furthermore, selective protein kinase inhibitors have been successfully employed to treat various cancers and inflammatory diseases which enhances the prospects of kinases as targets for malaria therapy (Agaimy and Schneider, 2009; Cohen et al., 2009; Eurich et al., 2009).

1.4 The *Plasmodium falciparum* kinome

Via genome annotation, 99 hypothetical kinases have so far been identified (Ward et al., 2004; Anamika et al., 2005). The collectivity of these kinases is termed as the *P. falciparum* kinome and can be classified into seven major groups (Ward et al., 2004). Generally, parasite protein kinases display a high identity at the amino acid sequence level to their homologues in other organisms. Thus, based on the primary sequence alone, most kinases can be classified into one of the seven groups. These comprise of the Casein kinase group, which includes *Pf*CK1 (Barik et al., 1997), the AGC group that comprises *Pf*PKA, *Pf*PKB and *Pf*PKG (Syin et al., 2001; Deng and Baker, 2002; Kumar et al., 2004), although *Pf*PKB was later described to have a role in Ca^{2+}/calmodulin mediated signalling pathway (Vaid et al., 2008), Ca^{2+}/CaMKs (calcium-calmodulin-dependent kinase) include *Pf*CDPK1 to *Pf*CDPK6. A CaMK activity in *P. gallinaceum* has been described to be crucial for ookinete development in the mosquito vector (Silva-Neto et al., 2002). The fourth group includes the CMGC kinases which comprise members from four different families and thus forms the largest group in the parasite kinome. It includes five cyclin-dependent kinases (CDK) (Doerig et al., 2002); two mitogen-activated protein kinases (MAPK), *Pf*map-1 and *Pf*map-2 (Lin et al., 1996; Graeser et al., 1997; Doerig CM et al., 1996; Dorin et al., 1999; Dorin-Semblat et al., 2007), three glycogen-synthase kinases (GSK3) (Kappes et al., 1995; Droucheau et al., 2004) and four cyclin-dependent kinase-like kinases (CLK) , a group where only one has been previously described (Li et al., 2001; Doerig et al., 2008). The fifth group in the parasite kinome describes Tyrosine-like kinases (TLK group) which comprise five plasmodial enzymes. The NIMA group further constitutes four *Pf*Nek kinases (Dorin et al., 2001; O'Connell et al., 2003; Reininger et al., 2009). The ePK family has two more members which belong to the STE and TyrK group. No malarial kinase has been thus far clustered under these groups (Ward et al., 2004).

Several studies in *P. falciparum* as well as in the rodent malaria model *P. berghei* have unveiled functions of multiple kinases, predominantly in the asexual blood and the sexual transmission stages of the parasite (e.g. Billker et al., 2004; Rangarajan et al., 2005; Reininger et al., 2005, 2009; Tewari et al., 2005; Ishino et al., 2006; Siden-Kiamos et al., 2006; Dorin-Semblat et al., 2007,

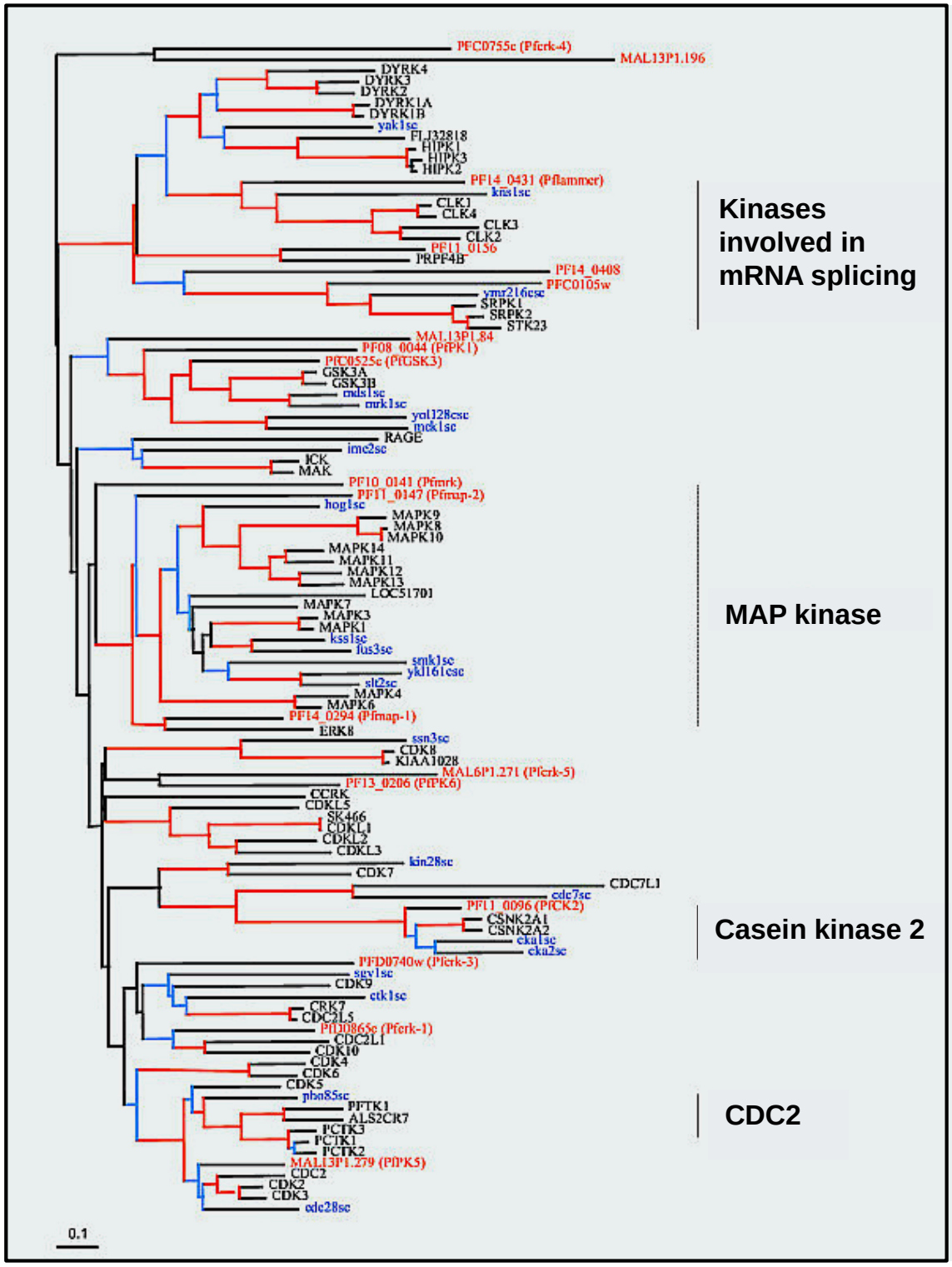
2008; McRobert et al., 2008). Although, both the *Plasmodium* species originated from the same lineage, considerable differences have been documented in their biochemical characteristics. This is well exemplified by the cysteine protease of the two species, the Bergheipain and the Falcipain which differ in terms of pH, substrate specificity and sensitivity to the inhibitors (Chan et al., 2005; Ramjee et al., 2006). The divergence between the two plasmodial species clearly underlines the necessity to investigate additional regulatory enzymes of *P. falciparum*. Therefore, characterization of additional kinases forms a prelude to widen the search for small molecule inhibitors.

A phylogenetic tree of the CMGC group representing the kinomes of human, *P. falciparum* and yeast was compiled by aligning the conserved motifs (Figure 1.6, Ward et al., 2004).

1.5 The CMGC group

The CMGC (cyclin-dependent, mitogen-activated-, glycogen-synthase and cyclin-dependent kinase-like kinases) group, one of the major groups of the parasite kinome, includes some of the most important kinases like the above mentioned MAPKs and CDPKs. About eighteen malarial kinases exist within this group, making it central to the *Plasmodium* kinome. CMGC kinases are known to regulate many cell proliferation and developmental processes in eukaryotic systems. Thus, their abundance in the *P. falciparum* kinome may reflect their similar role in the life cycle of the malaria parasite (Ward et al., 2004). Whereas plasmodial enzymes of the CDK, MAPK and GSK3 families have been investigated extensively, very little information is available regarding the fourth family in the CMGC group, the *Pf*CLK kinases.

Various kinases clustering within the families of CMGC group have been extensively characterized to different life cycle stages of the parasite. These studies provide the basis of further search on antiplasmodial targets.



(Adapted from: Ward et al., 2004)

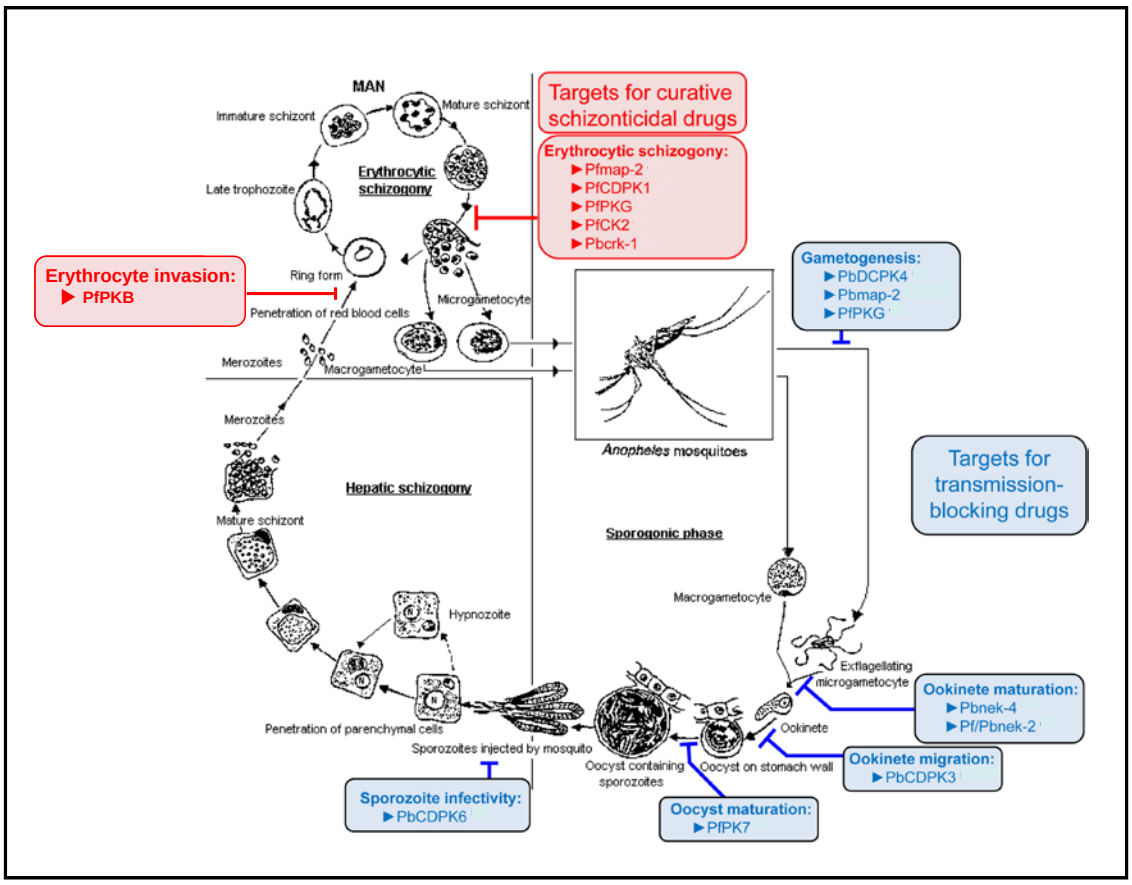
Figure 1.6: Phylogenetic tree representing *P. falciparum,* yeast and human kinases of the CMGC group represented in red, blue and black colour respectively.

As introduced before, targets for malaria therapy include the liver and blood stages within the human host, which would either help to prevent an infection or combat symptoms of the disease. The parasite sexual stages, on the other hand, are formed during reproduction in the mosquito vector and

represent promising targets for transmission blocking strategies. These strategies arrest the development of parasite in the mosquito so as to reduce or eliminate the transmision of the disease. Several parasite kinases, including the ones from the CMGC group have been assigned to play essential roles in both asexual development in the human host and during transmission stages in the mosquito vector (Figure 1.7).

Several studies have recently affirmed reverse genetics as a tool for the target analysis. Though, kinase inhibition could also be studied by the use of pharmacological inhibitors, but their specificity still remains a critical consideration (Davies et al., 2000; Bain et al., 2003; Bain et al., 2007). Indeed a combination of reverse genetics and pharmacological approach is expected to give rational information. This is well exemplified by *Pf*PKG where transgenic parasites together with the c-GMP dependent protein kinase (PKG) inhibitors were employed to demonstrate the role of PKG in induction of gametogenesis (McRobert et al., 2008). Further, both MAP kinases of *P. falciparum* were characterized using reverse genetics. Contrary to the role of *Pb*map-2 in male gametogenesis, *Pf*map-2 was assessed to be essential for erythrocytic schizogony as its deletion was lethal to the parasites, indicating the divergence between the two species which is thought to date back about 68 million years ago (Polley et al., 2005). *Pf*map-1, on the other hand is dispensable for the asexual development, although no clear phenotype could be observed in the mutant parasites (Dorin-Semblat et al., 2007). As both are present throughout the asexual stages of the parasite, there might exist a compensatory mechanism. A large set of the plasmodial kinases have been recently assigned essential for the erythrocytic schizogony, amongst them are *Pb*crk-1, *Pf*map-2, *Pf*CK2. The casein kinase, *Pf*CK2 is a serine/threonine kinase with multiple substrate specificity. Recently, it has been shown to be amenable to inhibitor screening thus exemplifying an essential kinase that is validated as a drug target (Holland et al., 2009).

Thus far, investigation on the family comprising of CDK-like kinases (CLK) has been scarce. Bioinformatic analysis categorized these kinases to be involved in mRNA splicing, a major phenomenon in gene regulation (Ward et al., 2004).



(Modified from Doerig et al., 2009)

Figure 1.7: Characterization of *P. falciparum* and *P. berghei* kinases by reverse genetics or by inhibitor studies. Previously published articles have revealed the life cycle stage at which the above mentioned kinases act thereby, representing potential targets for schizonticidal or transmission blocking strategies.

It therefore becomes crucial to investigate this family of regulatory enzymes to widen the knowledge on spliceosomal machinery in the parasite that is hitherto unknown.

1.6 CDK-like kinases in the malaria pathogen, *P. falciparum*

*Pf*CLK kinases constitute a family of the largest group in the *P. falciparum* kinome, the CMGC group. Members of the CLK group are implicated in cell proliferation and development processes and their abundance in the *P. falciparum* kinome may reflect a similar role in the life cycle of the malaria parasite (Ward et al., 2004).

In recent years, important advances have been made regarding the characterization of a number of malaria kinases. In other eukaryotes, CLKs are major regulators of mRNA splicing by phosphorylation of Serine/Arginine-rich (SR) proteins, which function in the RNA processing pathway, its nuclear

export, maintaining the stability and quality of mRNA and protein synthesis (reviewed in Huang and Steitz, 2005; Sanford et al., 2005; Godin and Varani, 2007). Thus, *Pf*CLK kinases participate in the control of gene expression, and may be particularly important in malaria parasites, in view of the importance of post-transcriptional regulation of gene expression in these protozoa (Deitsch et al., 2007).

Four plasmodium kinases cluster within the CLK family which were assigned here as *Pf*CLK-1/Lammer (PF14_0431), *Pf*CLK-2 (PF14_0408), *Pf*CLK-3 (PF11_0156), and *Pf*CLK-4 (PFC0105w). Protein sequences of *Pf*CLK kinases derived from the genome database of *P. falciparum* (PlasmoDB) were submitted into a database search to derive architectural information about the domains present. All four kinases were assigned to the serine/threonine kinase category with a C-terminal kinase domain for *Pf*CLK-1, *Pf*CLK-2, *Pf*CLK-3 and an N-terminal kinase domain for *Pf*CLK-4 (Figure 1.8).

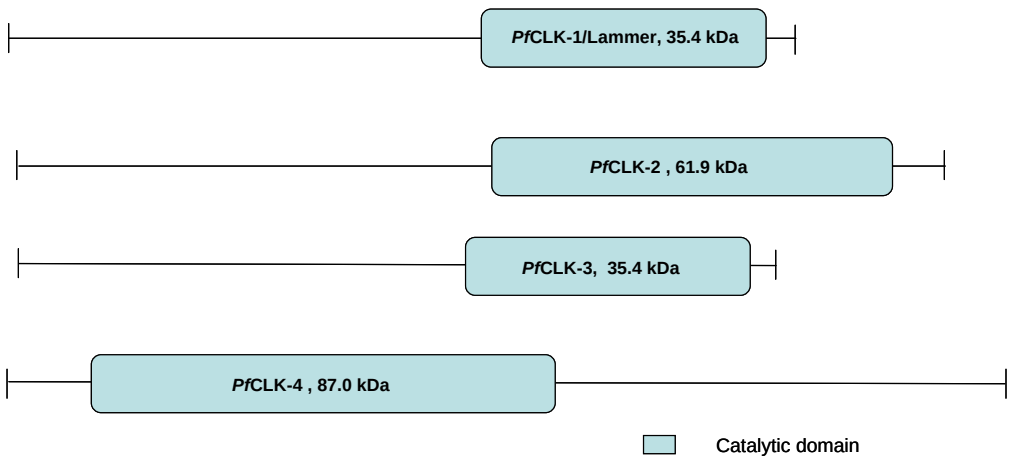


Figure 1.8: Schematic representing the position and molecular mass of kinase domains of four CLK kinases.

Amongst them, only *Pf*CLK-1/Lammer was previously studied as the plasmodial homologue of the mammalian Lammer kinase (Li et al., 2001) based on its preserved sequence motif in sub-domain X. Members of the Lammer protein kinase family are present throughout the eukaryotes and are characterized by a highly identical amino acid sequence and a similar structure (Nikolakaki et al., 2001).

Lammer kinases in other eukaryotes bind to and phosphorylate SR-rich mRNA splicing factors (Colwill et al., 1996; Nayler et al., 1997). They exist

throughout eukaryotes including yeast, mammals and plants. Table 1.2 summarizes the known Lammer kinases with their assigned function and substrate specificity wherever identified.

Table 1.2: Summary of Lammer kinases thus far known in eukaryotes other than *P. falciparum*.

Lammer kinase in eukaryotes	**Function/ knock-out phenotype**	**Mode of action**
Schizosaccharomyces pombe, Lkh1(Lammer kinase homologue 1) (Kang et al., 2007).	negative regulation of filamentous growth and flocculation.	Phosphorylation of RNA binding protein, csx1 in response to oxidative stress. Csx1 shows low identity to plasmodial RNA binding proteins.
Saccharomyces cerevisiae, Kns1 (Padmanabha et al., 1991; Lee et al., 1996).	no detectable phenotype, probably critical for asexual stages.	thus far not known.
Arabidopsis thaliana, AFC1 (*Arabidopsis* FUS3-complementing gene 1) kinase (Bender and Fink, 1994).	activates STE12 protein and its dependent processes.	activation of yeast STE12 (sterile mutant of yeast).
Mammalian Clk/Sty kinase having alternatively spliced transcripts (Colwill et al., 1996; Prasad et al., 1999).	shows co-localization with SR proteins in nuclear speckles. Kinase over expression leads to a redistribution in nucleus and cytoplasm.	SR family members ASF/SF2 (Alternative splicing factor).
Drosophila melanogatser, Doa kinase (Darkner Of Apricot) (Yun et al., 2000; Kpebe and Rabinow, 2008).	regulates alternative splicing and thus sex determination.	affects splicing of dsx (doublesex) pre-mRNA and phosphorylates SR and SR-like proteins.
Nicotiana tabacum, PK12 (Sessa et al., 1996; Savaldi-Goldstein et al., 2000).	is regulated by plant hormone, ethylene.	phosphorylates plant splicing factor, SRp34/SR1.

Sequence based analogy revealed presence of only one Lammer kinase in *P. falciparum* and its sub-cellular localization, life cycle expression, activity assays have not been conducted till date. Similarly, for the other three *Pf*CLK kinases, no data regarding their functional characterization has been previously reported.

CDKs are serine/threonine kinases which have been assigned a role in control of cell cycle progression, transcriptional regulation and neuronal function. Several kinases in the eukaryotic genome exist which apart from having crucial roles in the cell cycle regulation, share primary sequence similarities with CDK kinases. Such kinases might be regarded as the CDK-like kinases. For e.g. the sequence based search in the human and the mouse genomes reflected the presence of several CDK and CLK kinases. CDK

kinases further require a regulatory sub-unit for their catalytic activity (Malumbres and Barbacid, 2005). Thus, CLK kinases might share part of CDK-like features. Considering the importance of CDK kinases in the plethora of cellular mechanisms and in the absence of knowledge on malarial CLK kinases, characterization of CLK kinases appears to be essential.

1.7 The cluster of Calmodulin-dependent protein kinases in *P. falciparum*

The calcium modulated protein (CaM) or Calmodulin binds to intracellular calcium and is expressed by all eukaryotic cells. CaM is an acidic molecule that contains four EF-hand motifs. Each of these motifs binds to a calcium ion (Ca^{2+}). The calcium/calmodulin protein complex can bind to and activate several target proteins such as Calmodulin-dependent kinases (CaMKs), thus, regulating the cellular machinery and calcium-dependent signalling cascades in mammals (Stevens 1983; Chin and Means, 2000; Ishino et al., 2006).

Calcium-dependent protein kinases (CDPKs) however, have been described in plants, algae, ciliates and malarial parasites but not in mammals. They themselves possess calmodulin-like domains which carry EF-hand motifs and are activated by calcium when calmodulin and phospholipids are absent (Harper and Harmon, 2005). Calcium plays diverse roles in different stages of the parasite development and proliferation. It has been described crucial for maintenance of cell morphology, as changes in intracellular calcium levels leads to shape changes (Berridge et al., 2000). Calcium alone and in complex with Calmodulin has been reported crucial for parasite mediated cell invasion (Bonhomme et al., 1999; Griffith 2004). For example the calcium/calmodulin complex activates *Pf*PKB (protein kinase B), activating a signal cascade that aids the parasite in erythrocyte invasion (Vaid et al., 2006).

The phylogenetic tree depicting the kinome of *P. falciparum* signifies the importance of calcium signalling in the parasite by identification of thirteen kinases categorized into the calcium/calmodulin branch (Ward et al., 2004).
Five of the CDPKs (CDPK1-5) have been previously described while a sixth one, *Pf*CDPK6 was recently reported (Zhao et al., 1994; Farber et al., 1997; Li et al., 2000; Billker et al., 2004; Ward et al., 2004; Coppi et al., 2007). *Pb*CDPK3 is produced in the ookinete stage and has a critical role in parasite

transmission to the mosquito vector and is required for migration of the ookinete through midgut epithelial cells (Ishino et al., 2006; Siden-Kiamos et al., 2006). Cell invasion by calcium signalling has been described not only in *Plasmodium* but also in the related parasites like *Toxoplasma gondii* (Carruthers and Sibley, 1999; Mota et al., 2002). These studies are suggestive of important roles of CaMK and CDPKs in maintaining cellular morphology and in invasion processes.

Apart from a role in invasion, CDPKs have also been implicated in gametogenesis and fertilization. For example, the *P. berghei*, *Pb*CDPK4 triggers cell cycle progression in microgametocytes (Billker et al., 2004), while *Pf*CDPK1 and *Pf*CDPK2 are crucial for eryhtrocytic schizogony (Farber et al., 1997; Kato et al., 2008). Further, CDPK6 has been recently described to be involved in activation of sporozoites for invasion (Coppi et al., 2007).

While all the above mentioned plasmodial CDPKs comprise four EF-hand motifs, there exist a CDPK-related kinase which carries only one such motif. Another enzyme, *Pf*PK2 represents a previously characterized CaMK which has no EF-hand motif (Zhao et al., 1992). An additional family, closely related with CDPKs comprise of six kinases, out of which only one has an EF-hand and the calcium/calmodulin dependent kinase of *P. gallinaceum* which blocks morphological differentiation of zygotes to ookinetes could be associated with one of them (Silva-Neto et al., 2002; Ward et al., 2004). Thus, calcium mediated cell signalling is central to signal cascades which in turn control various developmental pathways.

Considering that most of the calcium/calmodulin kinases have been implicated in sexual developmental processes of the parasite, it would be promising to characterize additional CaM kinases to evaluate their potential as transmission blocking candidates.

The PKRP (putative kinase related protein) kinase belongs to the group of CamK (Calmodulin-dependent kinases) and is expected to be involved in signalling events of the parasite. Further, the PKRP protein is conserved across many *Plasmodium* species. The *pkrp* gene of *P. berghei* encodes a transcript of 5300bp and a PKRP protein of 90kDa in sporozoites. Gene disruptant parasites have reduced ability to infect the mosquito midgut and a reduced oocyst production. Although, oocyst derived sporozoites are morphologically

similar to the wild type, they lack the capacity to invade mosquito salivary glands (Purcell et al., 2010). Therefore, evaluation of its *P. falciparum* orthologue, *Pf*PKRP might reveal signalling events crucial in transmission of the disease. Further, *Pb*PKRP and *Pf*PKRP share approximately 75% identity across their catalytic domain (Purcell et al., 2010).

The hypothetical protein *Pf*PKRP is a 295 kDa serine/threonine protein kinase comprising two exons. It possesses an N-terminal catalytic domain present in the second exon and a long C-terminal extension (Figure 1.9). Although these extensions are unique to the respective PKRP kinase, they possess some residues that are conserved across the serine/threonine protein kinases. *Pf*PKRP shares 26% identity with the human testis-specific serine kinase (hTSSK) in its catalytic domain (Hao et al., 2004; Purcell et al., 2010). No other significant domain was identified by in silico studies.

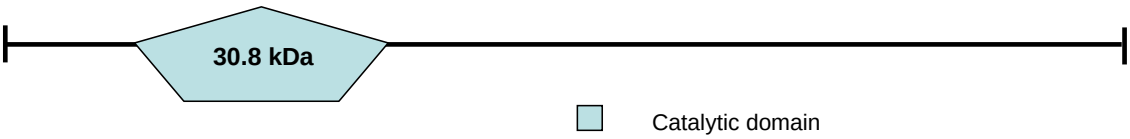


Figure 1.9: Schematic representing position and molecular mass of the *Pf*PKRP N-terminal catalytic domain.

In its 30.8 kDa catalytic domain, *Pf*PKRP comprises the ATP binding site, with GGGXG instead of GXGXXG and a PE motif required for its structural stability categorizing it as a putative kinase.

1.8 Aim of the present study

The sequencing of the *P. falciparum* genome in 2002 has provided the opportunity to identify proteins that might represent targets for novel approaches in malaria immune regimes or chemotherapy. As kinases represent important regulatory enzymes, elucidation of their roles in *Plasmodium* would unwind their complex cross-talks and enhance the scope of the yet limited small molecule based pharmacological intervention in the malaria treatment.

The phylogenetic tree of *P. falciparum* kinome categorizes the four *Pf*CDK-like kinases, *Pf*CLK kinases to the branch of the CMGC (cyclin-dependent, mitogen-activated-, glycogen-synthase and cyclin-dependent

kinase-like kinases) group which is involved in the mRNA splicing process. Several CLK kinases in other organisms have been attributed to the phosphorylation of serine/arginine-rich splicing factors which govern the mRNA splicing regulation in concert with other proteins. However, the proteins which are part of this multiplex have not yet been described. Thus, unwinding these complex interactions is required to gain deeper insight into the malarial spliceosomal complex.

Thus, the aim of the present study was to functionally characterize four *Pf*CLK kinases in the life cycle of the parasite with a special focus on *Pf*CLK-1/Lammer and *Pf*CLK-2. Issues related with the analysis of respective *Pf*CLK kinase stage-specific expression, localization of kinases in the parasite life cycle stages, identification of kinase interaction partners and substrates phosphorylated by these kinases were aimed to be addressed in this thesis.

The second aim of my PhD thesis was to characterize a calmodulin-dependent putative kinase related protein, *Pf*PKRP. It is presumed to play an essential role in the sexual development of the parasite, thus representing a valid target for transmission blocking drugs. Since kinases function in co-ordination with other proteins and in view of existing examples of *P. falciparum* kinases crucial to sexual development of the parasite, we aimed to investigate the *Pf*PKRP kinase in detail. By generation of antibodies directed against the catalytic domain of *Pf*PKRP and transcript analysis, the localization and stage-specific expression of the kinase at both transcription and protein level were to be investigated. Further, gene disruption of the kinase was planned in order to analyze the resulting phenotype in the life cycle of the parasite.

Investigation of stage-specific expression of these five novel kinases at both transcript and protein level would set the platform for their further characterization. Deeper insight into the kinase function would be realized by generation of gene disruptant parasites. All these studies, put together can be threaded to generate the respective signal cascade that these enzymes catalyze thus verifying their potential as prospective anti-malarial drug targets.

2. Materials and Methods

2.1 Materials

2.1.1 Computer programmes for in silico analysis

Prediction of phosphorylation site	http://kinasephos.mbc.nctu.edu.tw
Prediction of nuclear localization signals	cubic.bioc.columbia.edu/predictNLS
Multiple sequence alignment tool	www.ebi.ac.uk/clustalw
Formatting multiple sequence alignments	espript.ibcp.fr
Deduction of catalytic domains	smart.embl-heidelberg.de.
Mascot programme	www.matrixscience.com

2.1.2 Instruments, chemicals and disposables

Instrument	**Company**
AccuJet® pro	Brand, Wertheim
Amicon® Ultra-4, Ultra-15 filter units	Millipore, Schwalbach
Binocular FOMI B 50	Zeiss, Oberkochen
Incubator Model 100-800	Memmert, Schwabach
Bunsen burner	Schütt, Göttingen
Chromatography column PolyPrep®	Bio-Rad, München
Cell culture flask 25-cm^2, 75-cm^2	Becton Dickinson, Falcon, Heidelberg
Centrifuge Megafuge 1.OR	Heraeus, Hanau
Diamond knife Diatom Ultra 45°	Provac, Austria-Winkel
Electron microscope	Zeiss, Oberkochen
Electrophoresis chamber MIDI 1, MAXI	Roth, Karlsruhe
Electrophoresis chamber Mini-Protean 3	Bio-Rad, München
Developing mashine CURIX 60	Agfa, Köln
Fluorescence microscope Axiolab HBO 50/AC	Zeiss, Oberkochen
Fluorescence microscope Axiphot	Zeiss, Oberkochen
French® Press FA078	Heinemann, Schwäbisch Gmünd
Gel documenter Gel Doc 2000	Bio-Rad, München
Gel drying apparatus 14 x 14 cm	Roth, Karlsruhe

Gel Dryer	Bio-Rad, München
Heat Block Bio TBD-100, TBD-120	Lab-4you, Berlin
Incubator HERAcell	Heraeus, Hanau
Incubation chamber	Genheimer, Höchberg
Incubator, kinase assay	Eppendorf thermomixer, Hamburg
Light microscope Leica DMLS	Leica, Solms
Light microscope Leitz Laborlux 11	Leitz, Wetzlar
Microscope camera AxioCam	Zeiss, Oberkochen
Microscope camera MP 5000	INTAS, Göttingen
Mini-Rocker MR1	Lab-4you, Berlin
PCR thermocycler primus 25 advanced	Peq-Lab, Erlangen
pH-Meter inoLab	WTW, Weilheim
Power source PowerPac HC High Current Power supply	Bio-Rad, München
Shaker SM 30 control	Edmund Bühler GmbH, Tübingen
Sequencer ABI Prism®3100	Applied Biosystems, Darmstadt
Southern blot transfer	VacuGene XL, Pharmacia Biotech, Freiburg
Sterile bench HERAsafe	Heraeus, Hanau
Sonication device Sonoplus HD70	Bandelin, Berlin
Ultracentrifuge Beckmann J2-HC	Beckmann, München
Ultrospec 3100 pro UV/visible spectrophotometer	Amersham Bioscience, München
UV Stratalinker® 1800	Stratagene, Amsterdam, Netherlands
Vortexer Power mix Model L46	Labinco, Breda, Netherlands
Water bath Hecht 3185 WTE	Karl Hecht KG, Sondheim
Water bath Typ WB20	PD Industriegesellschaft, Dresden
Western blot apparatus Mini-Trans-Blot	Bio-Rad, München
X-Ray film Konica A3 Medical film	Konica, Hobenbrunn

Chemicals

AppliChem, Darmstadt
ATCC, Manassas, USA
Dianova, Hamburg
GE Healthcare/Amersham Bioscience, München
Invitrogen/Gibco/Molecular Probes, Karlsruhe
Merck/Novagen, Darmstadt
Pharmacia/Pfizer, Wien
Roth, Karlsruhe
Santa Cruz Biotechnology, Heidelberg
Sigma/Fluka, Taufkirchen
WAK Chemie, Darmstadt

Disposable material

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

Miscellaneous

- Blood and Serum of A+ blood group for cell culture was purchased from Bayerisches Rotes Kreuz (BRK), Würzburg.

- Six weeks old NMRI-female mice for immunization were obtained from Charles River laboratories, Sulzfeld.

- For gassing the cell culture flasks, 50 l of gas cylinder containing a mixture of 5% O_2, 5% CO_2, in 90% N_2 was purchased from Tyczka Industriegase, Würzburg.

- The cell culture medium RPMI 1640 + 25 mM Hepes + L-Glutamine + Sodium bicarbonate was purchased from Invitrogen/Gibco, Karlsruhe.

- The pCAM-BSD based vector was kindly provided by Prof. Christian Doerig.

- ^{32}P γ-ATP was purchased from Hartmann Analytic GmbH, Braunschweig.

2.1.3 Purification kits

BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Darmstadt
DIG High Prime DNA Labeling and Detection Starter Kit II	Roche, Mannheim
Amersham ECL direct nucleic acid labeling and detection system	GE Healthcare, Braunschweig
Epoxy Embedding Medium Kit	Fluka, Taufkirchen
QIAamp Blood Mini Kit	Qiagen, Hilden
QiAprep Spin Miniprep Kit	Qiagen, Hilden
QIAquick PCR purification Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
SuperScriptTM First Strand Synthesis System for RT-PCR	Invitrogen, Karlsruhe

2.1.4 Enzymes and DNA/Protein Ladders

DNA Ladders, MBI/Fermentas, St. Leon-Rot

- Gene Ruler^{TM}-1 kb Ladder (bp)
- Gene Ruler^{TM}-100 bp Ladder (bp)

Protein Ladder, MBI/Fermentas,

- Page-Ruler^{TM}-pre stained-Protein standard (~ kDa)

2.1.5 Medium and buffers for cell culture

Hypoxanthine Stock Solution (1000x)

0.5 g Hypoxanthine were added and mixed to 5 ml 1M NaOH. Further, NaOH was added dropwise till the solution became clear and the final volume of 10 ml was obtained by adding dH_2O.

Gentamycin stock solution

50 mg of Gentamycin powder was dissolved in 1 ml of dH_2O to obtain 0.1 M (1000 x) stock solution.

Pyrimethamine Stock Solution (1000x) for 10 ml end volume

15 mg Pyrimethamine (GIBCO, Karlsruhe) were added to 500 µl DMSO. The solution was dissolved by keeping it in a 37ºC waterbath and by vortexing it in between. From this Pyrimethaine/DMSO solution, 13 µl were added to 10 ml RPMI incomplete medium and stored at 4°C.

Blasticidin Stock Solution

5 mg of Blasticidin were dissolved in 1 ml of dH_2O and frozen in aliquots of 280 µl. A final concentration of 2.5 µg/ml was obtained after its addition to 550 ml of RPMI medium.

A+ human serum for P. falciparum cultivation

Commercially available serum was steril filtered, aliquoted in 50-ml falcon tubes and heat inactivated at 55°C for 50 min. The falcon tubes were then stored at -20°C till further use.

A+ erythrocytes (50% Haematocrit) for P. falciparum cultivation

Purchased erythrocyte concentrate was aliquoted in a volume of 30 ml and centrifuged at 2500xg for 10 min. Supernatant was discarded and the pellet was resuspended in 50 ml RPMI-Incomplete medium and washed two more times. Erythrocyte pellet was resuspended with incomplete RPMI in a ratio 1:1 and stored at 4°C for a maximum of four weeks. For transfection experiments fresh blood was used (maximum one week old).

RPMI 1640 Complete A+ Medium

To commercially available RPMI 1640 medium containing L-Glutamine and 25 mM HEPES (GIBCO, Karlsruhe), 50 ml sterile heat inactivated serum A+, 550 µl Hypoxanthine (1000x) and 550 µl Gentamycin (10 mg/ml, GIBCO, Karlsruhe) were added. The RPMI complete A+ medium was stored at 4ºC.

RPMI 1640 Complete Pyrimethamine Medium

To RPMI 1640 Complete A+ Medium, 550 µl Pyrimethamine (1000x) was added and the solution was stored at 4ºC.

RPMI 1640 Complete Blasticidin Medium

To RPMI 1640 Complete A+ Medium, 275 µl Blasticidin (5mg/ml) was added and the solution was stored at 4ºC.

RPMI Incomplete, 1000 ml

In a sterile beaker, 5.94 g of Hepes were mixed with commercially available 10 g RPMI 1640 powder (GIBCO), 1 ml Gentamycin (10 mg/ml) was added to this above mixture and finally made up to the volume of 1000 ml using cell culture grade water. The ingredients were dissolved by magnetic-stirring for 30 min.

Cytomix Buffer used for Electroporation (Wu et al., 1995)

In a sterile beaker, 8.95 g KCl, 0.017 g CaCl, 0.76 g EGTA, 1.02 g $MgCl_2$, 0.871 g K_2HPO_4, 0.68 g KH_2PO_4, 7.08 g Hepes were dissolved in dH_2O in a final volume of 1000 ml with dH_2O. pH was adjusted to 7.6 and finally the buffer was sterile-filtered.

Accudenz

For 6%, 11% and 16% of accudenz solution, 0.9 g, 1.65 g and 2.4 g of accudenz powder were dissolved in 15 ml incomplete medium respectively.

10% Saponin

5 g Saponin was dissolved in 1 x PBS to make an end volume of 50 ml.

5% Sorbitol

2.5 g Sorbitol was dissolved in dH_2O to a final volume of 50 ml.

1 mM Xanthurenic acid

0.05 g Xanthurenic acid was dissolved 1 ml 0.5 M NH_4OH and a final volume of 245 ml was prepared with dH_2O.

Glycerolyte 57

300 mg KCl, 517 mg Na_2PO_4 Monohydrate, monobasic, 1242 mg Na_2PO_4 anhydrous, dibasic were dissolved in 1000 ml dH_2O.

All solutions used for cell culture were sterile filtered either using syringe filter (0.22µm) or via bottle-top filter (Millipore).

2.1.6 Solutions for nucleic acid isolation and Southern blot

DEPC- H_2O

0.1% DEPC-H_2O was prepared by adding 1 ml of DEPC (Roth, Karlsruhe) in 1000 ml dH_20, mixed overnight and autoclaved.

1 M Tris-HCl

12 g Tris (Roth, Karlsruhe) was dissolved in 100 ml distilled H_2O and pH 7.0 was adjusted with HCl.

3M NaAc (Sodium Acetate)

13.6 g Sodium Acetate was dissolved in 100 ml H_2O and the pH was adjusted to 4.6 with HCl.

Solutions for maxi-prep.

Solution 1: 50 ml 1 M Glucose was added to 20 ml 0.5 M EDTA pH 8.0 and 10 ml 1 M Tris-HCl pH 8.0 to make a final volume of 1000 ml with dH_2O.

Solution 2: 50 ml 1 M NaOH was added to 12.5 ml 20% SDS to make a final volume of 250 ml dH_2O.

Solution 3: 600 ml 5 M Calcium acetate was added to115 ml Acetic acid to make a final volume of 1000 ml with dH_2O.

1x TE (Tris/EDTA)

10 mM Tris/HCl pH 8.0 was added to 1 mM EDTA pH 8.0.

4M $NaClO_4$, pH 4.6

280.92 g $NaClO_4$ (Roth, Karlsruhe) was dissolved in 300 ml dH_2O. The pH was adjusted to 4.6 using HCl and the volume was finally made up to 500 ml with dH_2O.

8M LiCl

169.56 g LiCl (Roth) were dissolved in dH_2O and the final volume adjusted to 500 ml.

4M GuSCN + 4% Triton X-100

To 236.32 g of Guanidine Thiocyanate (GuSCN, Roth, Karlsruhe), 4% Triton X–100 detergent (Roth, Karlsruhe) was added.

TSE for genomic DNA isolation

20 mM Tris, pH 8.0 was added to 100 mM NaCl and 50 mM EDTA, pH 8.0.

Malstat Reagent

2 ml 10% Triton X-100, 2 g L-Lactate, 0.66 g Tris and 66 mg APAD were mixed to a final volume of 200 ml with dH_2O.

Maleic acid buffer

0.1M Maleic acid powder and 0.15 M NaCl were added to dH_2O, the pH was adjusted to 7.5 using HCl and the final volume 1000 ml of the solution was prepared.

Detection solution

0.1 M NaCl was mixed with 0.1 M Tris/HCl pH 9.5 and the final volume of 500 ml was prepared using dH_2O.

2.1.7 Reagents and solution for protein purification, SDS-PAGE and Western blot

RIPA- protein lysis buffer

30 mM Tris pH 8.0, 150 mM NaCl, 20 mM $MgCl_2$**,** 1x complete protease inhibitor cocktail, 10 mM NaF, 10 mM β-glycerophosphate were dissolved in the required volume of dH_2O.

Lysis buffer (protein purification)

5 M NaCl, 20% IGEPAL**,** 10 mM NaF, 10 mM β-glycerophosphate and 1 mM EDTA pH 8.0 were dissolved in the required volume of dH_2O.

Lysis buffer (Inclusion bodies)

50 mM Tris/HCl pH 8.0, 0.25% Sucrose and 1 mM EDTA pH 8.0 were dissolved in the required volume of dH_2O.

Detergent buffer (Inclusion bodies)

20 mM Tris/HCl pH 7.5, 2 mM EDTA pH 8.0, 200 mM NaCl, 1% Deoxycholic acid and 1% Nonidet-P 40 were dissolved in dH_2O to make a final volume of 1000 ml.

Elution buffer, GST-tagged protein purification

50 mM Tris/HCl, 10 mM reduced Glutathione were dissolved in the required volume of dH_2O.

Wash buffer (Inclusion bodies)

0.5% Triton X-100, 1 mM EDTA pH 8.0 were dissolved in dH_2O to make a final volume of 1000 ml.

Wash buffer 3 (protein purification)

50 mM Tris/HCl pH 8.0, 350 mM NaCl, 1 mM beta-Mercaptoethanol, 10% Glycerin were dissolved in dH_2O to make a final volume of 1000 ml.

Wash buffer 5 (protein purification)

50 mM Tris/HCl pH 8.0, 350 mM NaCl, 1 mM beta-Mercaptoethanol, 10% Glycerin and 60 mM Imidazol were dissolved in dH_2O to make a final volume of 1000 ml.

PBS 10 x Solution

80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g NaH_2PO_4 were dissolved in dH_2O. The pH was adjusted to 7.4 with HCl and a final volume of 1000 ml was prepared.

2x Sample buffer

2.5 ml 500 mM Tris/HCl pH 6.8, 2 ml glycerin, 4 ml 10%SDS and 0.5 ml 0.1% bromophenol blue were added to dH_2O and a final volume of 10 ml was prepared.

10 x PAGE running buffer

29 g Tris, 144 g Glycerin and 10 g SDS were dissolved in dH_2O to obtain a final volume of 1000 ml.

Transfer Buffer

3.03 g Tris, 14.4 g Glycerin and 200 ml Methanol were added to dH_2O to obtain a final volume of 1000 ml.

10 x TBS

12.1 g Tris and 87.3 g NaCl were dissolved in dH_2O. The pH 7.5 was adjusted with HCl to obtain a final volume of 1000 ml.

TBS-Milk

3g of skimmed-milk powder was dissolved in 100 ml of 1 x TBS to obtain a 3% TBS-Milk solution.

Blocking solution

0.5 g BSA (fraction V Albumin) and 2.5 g milk powder were added to 50 ml 1 xTBS.

Equilibriation buffer

12.1 g Tris, 5.8 g NaCl and 10.2 g $MgCl_2$ were mixed in dH_2O to obtain a final volume of 1000 ml.

Stop buffer

1.2 g Tris and 0.4 g EDTA were dissolved in dH_2O. The pH 8.0 was adjusted and a final volume of 1000 ml was prepared.

5% SDS-resolving gel (2 mini-gels)

0.6 ml 30% Acrylamide, 1 ml 1.5 M Tris/HCl pH 8.8, 12 µl 10% SDS were added to 2.4 ml dH_2O. 12 µl 10% APS and 4 µl TEMED were added to this solution and the gel was poured immediately.

12% SDS-resolving gel (2 mini-gels)

4.0 ml 30% Acrylamide, 2.5 ml 1.5 M Tris/HCl pH 8.8 and 100 µl, 10% SDS were added to 3.3 ml dH_2O. 100 µl 10% APS and 4 µl TEMED were added to proceed as above.

15% SDS-resolving gel (2 mini-gels)

7.5 ml 30% Acrylamide, 5.6 ml 1.5 M Tris/HCl pH 8.8 and 150 µl 10% SDS were added to 1.75 ml dH_2O. 120 µl 10% APS and 15 µl TEMED were added to proceed as above.

5% Stacking gel (2 mini-gels)

0.6 ml 30% Acrylamide, 1 ml 0.5 M Tris/HCl pH 6.8 and 12 µl 10% SDS were added to 2.4 ml dH_2O. 12 µl 10% APS and 4 µl TEMED were added to proceed as above.

Buffer A and B for nuclear extraction

Buffer A: 10 mM HEPES, pH 7.9, 10 mM KCl 0.1 mM EDTA, 1 mM DTT, 10% IGEPAL and 1x complete protease inhibitor cocktail (Roche) were added to the required volume of dH_2O.

Buffer B: 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% Glycerol, 1 mM DTT and 1x complete protease inhibitor cocktail were added to the required volume of dH_2O.

Kinase buffer

20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM NaF, 10 mM β-glycerophosphate, 10 µM ATP and 0.1 MBq [γ-32P] ATP were added to the required volume of dH_2O. A volume of 6 µl was used per reaction.

Permeablization solution, immunofluorescence

0.5% BSA and 0.01% Saponin were dissolved in 1 x PBS pH 7.4 to obtain the required volume.

2.1.8 Medium and agar plates for bacterial cultivation

Lysogeny Broth (LB)-medium

10g Tryptone, 5 g yeast extract, 5 g NaCl, dH_2O was added to obtain a final

volume of 1000 ml.

LB-Agar

10g Tryptone, 5 g yeast extract, 5 g NaCl, 15 g Agar, dH_2O was added to obtain a final volume of 1000 ml.

Super Optimal broth with Catabolite repression (SOC)-medium

20g Tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 0.25 M KCl, 5 ml $MgCl_2$, 20 ml 1 M glucose and dH_2O was added to obtain a final volume of 1000 ml.

Ampicillin stock (1000 x)

100 mg Ampicillin was dissolved in 1ml dH_2O to obtain 100 mg/ml Ampicillin stock.

2.1.9 Cell lines and bacteria

Cell lines of P. falciparum

Plasmodium falciparum WT NF54 strain: is a gametocyte-producing strain. It is chloroquine sensitive and isolated in 1982 from The Netherlands (Ponnudurai et al., 1981).

Plasmodium falciparum NF54 clone 3D7: is derived by limiting dilution (Rosario, V. 1981).

Plasmodium falciparum 3D7 derived clone F12: is a gametocyte-less strain obtained by limiting dilution after cultivation of 3D7 isolate for 20 months. It was kindly provided by Dr. Pietro Alano, Rome (Walliker et al., 1987).

Cell lines of Escherichia coli (E.coli)

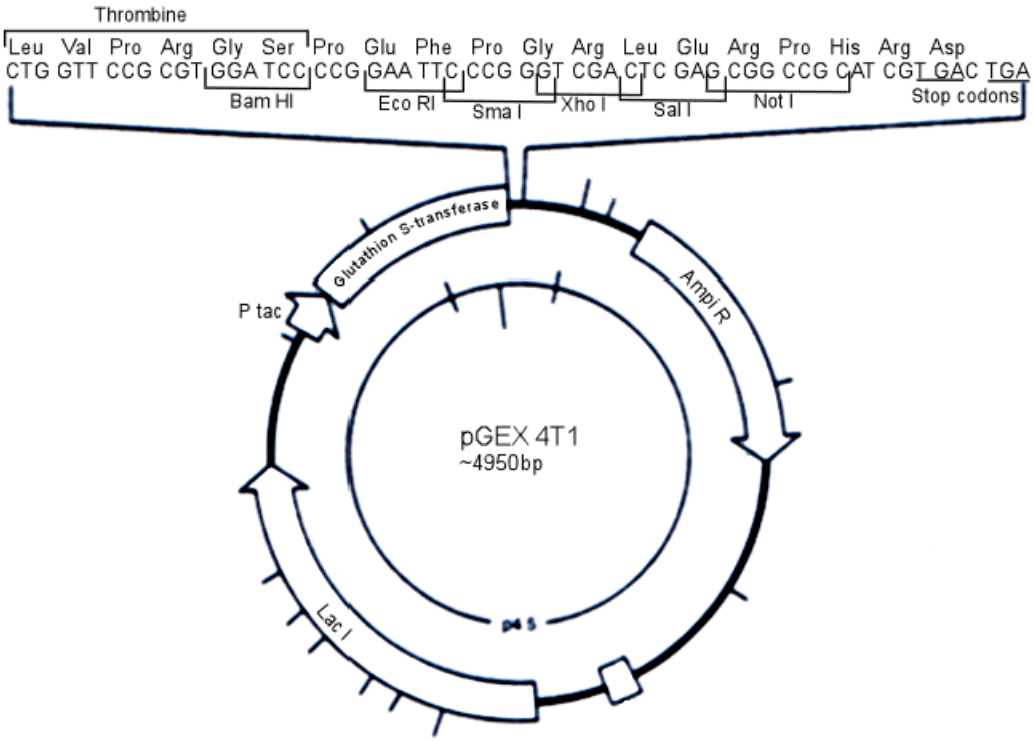
E. coli-protein expression cell line: BL21-CodonPlus®-(DE3)-RIL, Stratagene.

E. coli-transformation cells : *OneShot*®-*Top10*-*Competent*-*Cells*, Invitrogen

2.1.10 Plasmids

pGEX-4T1

It is a high-copy protein expression vector with Ampicillin resistance and Glutathione-S-transferase (GST) tag obtained from Amersham Biosciences.



pCAM-BSD vector

In order to achieve gene-disruption, pCAM-BSD vector was used for a single cross-over homologous recombination (Sidhu et al., 2005). The vector carries a Blasticidin and ampicillin resistance cassettes. The plasmid was kindly provided by Prof. Christian Doerig, Lausanne.

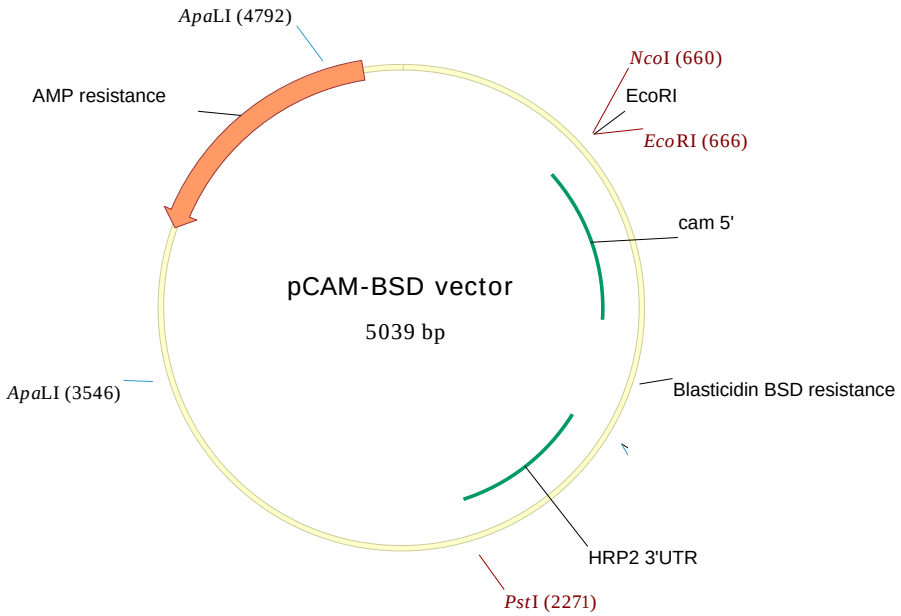


Figure 2.1.1 Vector pCAM-BSD with the Blasticidin selective cassette.

2.1.11 Antibodies

Primary antibodies

Antibody	Dilution	Source
Anti-*Pf*CLK-1, mouse polyclonal	1:50 WB, IFA, IEM	AG Pradel
Rabbit anti-*Pf*CLK-1, peptide upstream to the catalytic domain : NRTKTSDTEDKKER,	1:50 WB	Biogenes, Berlin
Mouse anti-*Pf*CLK-2, peptide upstream to the catalytic domain : CISYNEKENKYNDQD,	1:50 WB, IFA, IEM	Biogenes, Berlin
Rabbit anti-*Pf*PKRP, peptide upstream to the catalytic domain : YEYKFIRDKNDDTL,	1:100 WB, IFA	Biogenes, Berlin
Anti-*Pf*CCp1rp1, mouse polyclonal	1:200 WB	Scholz et. al 2008
Anti-*Pf*39, mouse polyclonal	1:100 WB	Scholz et. al 2008
Anti-GST, mouse polyclonal	1:100 WB	Simon et. al 2009

Anti-*Pf*s25, rabbit polyclonal	1:1000 IFA	ATCC
Anti-α-tubulinII, rabbit polyclonal	1:1000 IFA	ATCC
Anti-*Pf*MSP-1, rabbit polyclonal	1:500 IFA	ATCC
Neutral mouse serum	1:100 IFA	Scholz et. al 2008
Neutral rabbit serum	1:100 IFA	Biogenes, Berlin
Neutral goat serum	1:100 IFA	Sigma
Anti-*Pf*AMA-1, rabbit polyclonal	1:1000 IFA	ATCC
Anti-Myc-Tag 71D10, rabbit monoclonal	1: 800 WB	Cell Signaling Technology

Secondary antibodies

Antibody	**Dilution**	**Source**
Goat-anti-mouse IgG Alexa-488	1:1000 IFA	Molecular Probes
Goat-anti-mouse IgG Alexa-594	1:1000 IFA	Molecular Probes
Goat-anti-rabbit IgG Alexa-488	1:1000 IFA	Molecular Probes
Goat-anti-rabbit IgG Alexa-594	1:1000 IFA	Molecular Probes
Goat-anti-mouse IgG alkaline Phophatase	1:5000 WB	Sigma
Goat-anti-rabbit IgG alkaline phophatase	1:5000 WB	Sigma
Anti-mouse12-nm-colloidal gold antibody	1:10 IEM	Dianova

2.1.12 Primers for Reverse Transcriptase PCR for *Pf*CLK kinases

Gene locus	**Primer sequence 5'–3'**
*Pf*CLK-1-1S	GATACGCATTTTGGACAA
*Pf*CLK-1-1AS	TTCCCATCTTGAGATGGA
*Pf*CLK-1-2S	GAACCATTAGGTCCATCA
*Pf*CLK-1-2AS	TCCATCAGTAACCCTTCT
*Pf*CLK-2-1S	AGATGCTTGGAAACAACC

*Pf*CLK-2-1AS	GCCAAAGTTAGGTGATGA
*Pf*CLK-2-2S	TTGTTAGAACTCATGGTC
*Pf*CLK-2- 2AS	TAATTGACCCAAAGTTCC
*Pf*CLK-3-1S	GATTCTAGCAACGACGAA
*Pf*CLK-3-1AS	CGAATCGCTACTAGTAGA
*Pf*CLK-3-2S	ATGATGGAATATAAG GGC
*Pf*CLK-3-2AS	GCTATTTCCCTTCAACCA
*Pf*CLK-4-1S	GGAAGTGATGAATACTGC
*Pf*CLK-4-1AS	TTCACATTTGGCTGATTC
*Pf*CLK-4-2S	ATGATGGATCATGACACA
*Pf*CLK-4-2AS	CTCCTCGTAATCATCATC
*Pf*PKRP-1S	ATGATGGATCATGACACA
*Pf*PKRP-1AS	CTCCTCGTAATCATCATC
*Pf*39-1S	CTT GAA CAC CAT GAT GTA
*Pf*39-1AS	TCC ACT TTC ATG AGC AGG
*Pf*AMA-1S	GGA TTA TGG GTC GAT GGA
*Pf*AMA-1AS	GAT CAT ACT AGC GTT CTT
*Pf*CCp1-S	GAA GAT GGA GAT GGG AAA
*Pf*CCp1-AS	GCT GTT CAA ATT CCC ATC
*Pfs*25-1S	CCA TGT GGA GAT TTT TCC
*Pfs*25-1AS	TAC ATT GGG AAC TTT GCC

2.1.13 Recombinant protein primers

Gene locus	**Primer sequence 5'-3'**
*Pf*CLK-1rp 1S	TAGGTCGACGATAGAGAACAGAAGCGT
*Pf*CLK-1rp 1AS	TAGCGGCCGCTTAATTATCGGACGACGTTCT
*Pf*CLK-1rp 2S	TAGGTCGACTCGTCCATTTTAGTTGGAAG
*Pf*CLK-1rp 2AS	TAGCGGCCGCTTACGGTCGAAGTGTTGGATC
*Pf*CLK-2rp 1S	TAGGTCGACTCAAGAAGAGACACCTAACG
*Pf*CLK-2rp 1AS	TAGCGGCCGCTTAAGCACTTGATAATTCATCATC
*Pf*CLK-2rp 2S	TAGGTCGACTCACACAAGTATTCCTACCA
*Pf*CLK-2rp 2AS	TAGCGGCCGCTTATGGCTGAAGCCATGGGTG

*Pf*CLK-1^{mut}1S	CTACGCTGTAATGGTTGTTCGAAAC
*Pf*CLK-1^{mut}1AS	GTTTCGAACAACCATTACAGCGTAG

2.1.14 Primers for gene disruption using the pCAM-BSD vector

Gene	Primer sequence 5'-3'
pCAM-CLK1KOS	GGATCCTTTGGTAGAGTTTTATTATGTCAA
pCAM-CLK1KOAS	GCGGCCGCTTAAGCTCGATATTGTCTAGT
pCAM-CLK2KOS	GGATCCTTGTTAGAACTCATGGTC
pCAM-CLK2KOAS	GCGGCCGCTTACACATTCGATGTCTTTTC
pCAM-CLK3KOS	GGATCCCCTGTAGCTGTAAAAGTT
pCAM-CLK3KOAS	GCGGCCGCTTATGCTCTATAAAATCTACT
pCAM-CLK4KOS	GGATCCTGTACGAGCAGTAAAGAA
pCAM-CLK4KOAS	GCGGCCGCTTAGTCGTTCTTTTCGGAATC
pCAM-PKRPKOS	GGATCCGGTGGGAATGGTTTATCA
pCAM-PKRPKOAS	GCGGCCGCTTACAAACATTTTTTTATAATCTTC

2.1.15 Primers for gene tagging using the pCAM-BSD vector

Gene	Primer sequence 5'-3'
pCAM-CLK1-tag-S	CTGCAGCGCTCAGCTAAAATTGAA
pCAM-CLK1KI-AS	GGCAGATCTATAGTACTCATAATTTTCTTCAAGGA ACTTGTGC
pCAM-CLK2-tag-S	CTGCAGCAAACTCGATCTTATAGA
pCAM-CLK2-tag-AS	GGATCCTAAACCGTCTTTATACAA
pCAM-CLK3-tag-S	CTGCAGGGAAGTGCAAGTGATATATCA
pCAM-CLK3-tag-AS	GGATCCTTCATTTTGAGATTTTGA
pCAM-CLK4-tag-S	CTGCAGGGACAAGAACATGATGCT
pCAM-CLK4-tag-AS	GGATCCAGTATATGCACAAGAGTT
pCAM-PKRP-tag-S	CTGCAGCCAGTTGATTCTTTAAATCATTC
pCAM-PKRP-tag-AS	GGATCCTTTTATTTTTTTAAAAAAATAAGGAC

2.1.16 Primers for genotype characterization

Gene	Primer sequence 5'-3'
pCAM-BSD-CLK1KO	Primer 1: TCGTCCATTTTAGTTGGAAG Primer 2: CGGTCGAAGTGTTGGATC Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK1-Myc	Primer 1: AGAGTTTTATTATGTCAA Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK2KO	Primer 1: TCACACAAGTATTCCTACCA Primer 2: TTATGGCTGAAGCCATGGGTG Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK2-Myc	Primer 1: TTGTTAGAACTCATGGTC Primer 2:GAGAAATGTTATTTCCACT Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK3KO	Primer 1: AGTAAGGGAAATGCAGATACA Primer 2: GCCCTTATATTCCATCAT Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK3-HA	Primer 1: CTTGTAGCTGTAAAAGTT Primer 2: TGCACATACAACTATGCA Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-CLK4KO	Primer 1: GAATCAGCCAAATGTGAA Primer 2: GCCCTTATATTCCATCAT Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG

pCAM-BSD-CLK4-HA	Primer 1: ATGATGGATCATGACACA Primer 2: AAATGTACCCGTTAGGTT Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-PKRPKO	Primer 1: TCTCTGTCGTATAAGTTGGCT Primer 2: CCAATCATGATTAAGAGCTTC Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG
pCAM-BSD-PKRP-GFP	Primer 1: CTGCAGCCTGTTCATGTGCCACCT Primer 2: GTTTATATTGAACCTTCT Primer 3: TATTCCTAATCATGTAAATCTTAAA Primer 4: CAATTAACCCTCACTAAAG

2.1.17 Primers for amplification of probes for Southern blot analysis

Gene locus	**Primer sequence 5'-3'**
*Pf*CLK-1S	TACTACGATACGCGAAAT
*Pf*CLK-1AS	GCTTCGGGATCTACTTCT
*Pf*CLK-2S	CTGCAGCAAACTCGATCTTATAGA
*Pf*CLK-2AS	GGATCCTAAACCGTCTTTATACAA
*Pf*PKRP-1S	CTGCAGCCAGTTGATTCTTTAAATCATTC
*Pf*PKRP-1AS	GGATCCTTTTATTTTTTTAAAAAAATAAGGAC

2.1.18 PlasmoDB gene IDs

The following gene identifiers are assigned to sequences derived from PlasmoDB.

Gene ID	**Gene Name**
PF14_0431	*Pf*CLK-1
PF14_0408	*Pf*CLK-2
PF11_0156	*Pf*CLK-3
PFC0105w	*Pf*CLK-4
PFC0485w	*Pf*PKRP

2.2. Methods

2.2.1 Cell biology methods

In order to perform the characterization of kinases included in the study, *P. falciparum* was used as the model organism. They were cultivated and harvested at their respective optimum conditions for further experiments.

2.2.1.1 Cultivation and storage of *Plasmodium falciparum*

Thawing of *P. falciparum* blood cultures

P. falciparum blood cultures were initiated by transferring the contents of the cryovial into a 15-ml falcon tube and dropwise adding 200 µl of 12 % NaCl solution. The solution was incubated for 2 min and then 10ml 1.6% NaCl solution was added dropwise while gently shaking the culture in the falcon tube. The resuspension was centrifuged at 2500xg for 5 min and the supernatant was discarded. 10 ml of 0.2% Dextrose/0.9% NaCl solution was added dropwise and centrifuged again at 2500xg for 5 min. The pellet was resuspended in 5ml RPMI medium with 5% haematocrit.

Culturing of *P. falciparum*

To maintain continuous cultures, parasites were cultivated in a small 25-cm^2 cell culture flask in which the medium (2.1.5) was replenished everyday followed by gassing. Once a parasitemia of 2% was reached, the culture was passaged. The process involved dilution of parasites in RPMI complete medium to reach about 1% end parasitemia and adding human A+ blood to a final haematocrit of 5%. Gassing (mixture of 5% CO_2 and 5% O_2 in N_2) of flasks was finally done. To purify various blood stages, the remaining culture from passaging was cultivated in 75-cm^2 cell culture flasks. The parasitemia was determined from the Giemsa stained blood smears. For this about 100 µl of the parasite culture was transfered into a 1.5-ml eppendorf tube and centrifuged at 3400xg for 1 min. The supernatant was discarded and the pellet was

resuspended in an equal volume of the medium. A smear of the resuspended pellet was prepared on a glass slide (Figure 2.1).

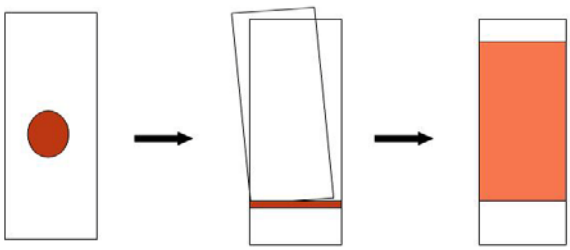


Figure 2.1: Schematic depicting preparation of the culture smear for determination of parasitemia.

The air-dried smear was rinsed in methanol for fixation and dried again. It was then incubated in Giemsa solution which was diluted 1:25 in dH_2O. This was followed by rinsing the slide with dH_2O and air dried. The blood smear thus prepared was then viewed with a light microscope under 1000x magnification using the immersion oil to determine the cell density and to calculate the parasitemia.

Freezing parasite cultures

Once a parasitemia of about 3-4 % was attained (preferably a high percentage of ring stages), the culture was centrifuged at 2000xg for 5 min and the supernatant was discarded. The pellet was resuspended in 5 volumes of glycerolyte solution and stored at -80°C or in liquid nitrogen until further required.

Synchronization and purification of asexual parasite blood stages

Upon having about 2% early ring stages, the parasite cultures were synchronized. The culture was transferred into a 50-ml falcon tube and centrifuged at 2,200xg for 5 min. The supernatant was discarded and the pellet was resuspended in five volumes of 5% prewarmed sorbitol solution and incubated at 37ºC for 10 min. The suspension was centrifuged at 2,200xg for 10 min and the supernatant was discarded. The resulting pellet was

resuspended in 5 ml and 20 ml medium according to its further transfer into a 25-cm^2 or 75-cm^2 cell culture flask respectively. Above procedure was repeated once at an interval of 4 h. Purification of the desired asexual parasite stages i.e. trophozoites and schizonts were achieved by centrifuging the parasite culture at 4,000xg for 10 min. The resulting pellet was resuspended in 10 ml PBS. For erythrocyte lysis, 50 µl of 10% Saponin w/v in PBS was added to the above falcon tube. The sample was centrifuged at 4,000xg for 10 min resulting in a black pellet that was resuspended in 50-100 µl PBS.

Purification of Gametocytes

A stage V gametocyte culture was transferred into a 50-ml falcon tube, centrifuged at 4,000xg for 10 min and resuspended in 10 ml incomplete medium (section 2.1.5). A gradient of 11%-16% Accudenz was created by carefully pouring 15 ml of 11% Accudenz over an equal volume of 16% Accudenz in a 50-ml falcon tube (Figure 2.2). The blood suspension was layed on the above gradient and the falcon tube was centrifuged for 10 min at 2,000xg (in a fixed angle rotor). The interface between 5–30 ml was collected, centrifuged at 2,000xg for 10 min, and the gametocyte pellet was obtained for further experiments.

Figure 2.2: Purification of gametocytes and zygotes via the Accudenz gradient method.

Exflagellation assay and purification of zygotes

Suitability of the gametocyte culture for purification of zygotes was verified by an exflagellation assay. This involved the transfer of about 500 µl of a gametocyte culture into a 1.5-ml eppendorf tube which was centrifuged at 6,000xg for 1 min. The supernatant was discarded and the pellet was resuspended in 300 µl of human A+ serum followed by incubation for 12 min at

room temperature (RT). Following this, approximately 50 µl of the culture in serum was transfered onto a glass slide and visualized under 400x magnification of a light microscope for the presence of exflagellation centres. Presence of these centres verifies a mature gametocyte culture suitable for purification of zygotes. Zygotes can be purified by the same method as described for gametocytes above. However, for zygotes, an Accudenz gradient of 6% and 11% is used instead of 11% and 16%. The cultures were grown until gametocytes were mature and further verified by exflagellation assay described above. These were transferred into 50-ml falcon tubes and centrifuged at 4,000xg for 10 min. The supernatant was aspirated and the pellet was resuspended in approximately two volumes of human serum. It was then incubated at room temperature for 20 h, and determined for exflagellation as mentioned above. The culture was centrifuged at 4,000xg for 10 min and resuspended in 10 ml incomplete medium (section 2.1.5). 15 ml of 6% Accudenz were then poured carefully over the same volume of 11% Accudenz already dissolved in incomplete RPMI and centrifuged again at 2,200xg for 10 min. Samples were processed further as described for gametocytes.

2.2.1.2 Transfection

Once the cloning was accomplished, the vector was quantified to ensure the required concentration for the following transfection experiment. In the current study our aim was to transfect the infected erythrocytes in the *Plasmodium* culture with the knock-out construct so that it is integrated into the parasite genome via single-cross over homologous recombination.

Synchronization of ring stage continuous culture was performed (section 2.2.1.1) and parasites were diluted into 25-ml cell culture flasks. Once 4-6% parasitemia with about 70% ring stages was obtained, transfection was performed using 4 ml culture per transfection. The medium was replenished 3-4 h before transfection. The culture was centrifuged at 2000xg for 4min and the pellet was resuspended in 4 ml of cytomix buffer (section 2.1.5). The culture was centrifuged as above and the pellet was resuspended in 300 µl of cytomix buffer containing 60 µg of pCAM-BSD-kinase cloned plasmid. The parasite suspension containing plasmid was transferred into a cooled Gene Pulser

cuvette (0.2 cm electrode gap) and cells were electroporated using the following conditions (Fidock et al., 1997): Voltage; 0.31 KV, Capacity; 975 uFD, Time constant; 10–13 s**.** The electroporated infected erythrocytes were then transferred to a 25-cm^2 cell culture flask containing 3 ml RPMI complete medium and 5% haematocrit. The remaining sample was rinsed from the cuvette by washing it two times with 1 ml complete medium which were also transferred to the flask mentioned above. Transfectants were replenished with fresh medium 4-6 h post transfection. 24 h later, smears were prepared followed by adding 2.5 µg/ml Blasticidin. This introduces a drug pressure wherein only the parasites containig the plasmid with Blasiticidin resistance cassette would survive. As a control, parasites were electroporated only with cytomix buffer and cultivated for the same length of time. The parasites disappeared in 2-3 d after transfection and about 100 µl of fresh human blood was added per week until parasites reappeared in about three weeks. They were splitted when 2% parasitaemia was achieved.

When parasites started growing rapidly even in the presence of the drug, it was considered to be a sign of integration of the knock–out construct into the *Plasmodium* genome. Diagnostic PCR of the disrupted locus was done to verify the successful gene disruption (2.2.2.2).

2.2.1.3 Clonal dilution and Malstat assay

Once the integrant population is confirmed by diagnostic PCR, it is important to isolate single clones of the integrant population to separate them from the mixed parasite population. For doing this, a thin blood smear was prepared (section 2.2.1.1) and parasites and erythrocytes per field were counted for at least 8-10 fields.

Average parasitemia was calculated as follows:

$$\frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes}} * 100$$

Number of cells/ml was then counted using a Neubauer chamber as:

$$\frac{\text{Number of erythrocytes}}{\text{Number of chamber squares counted}} * 10000 * \text{dilution factor}$$

Total number of parasites / ml (p/ml) can be calculated as:

$$\frac{\text{Average parasitemia}}{100} * \frac{(\text{p/ml})}{\text{total number of cells}}$$

In a series of dilution (usually 3 times 1:50), about 12 parasites were finally added to a 96-well micro-titre plate with 5% haematocrit and placed in the incubator within an air-tight chamber. The chamber was gassed for 3 min. The medium was replenished every third day and cultivated for about three weeks. Every third time, the medium was replenished, 1% and blood was added to each well. After three weeks, a Malstat assay was carried out by measuring the parasite enzyme lactate dehydrogenase. Here, 20 µl of infected erythrocytes from the 96-well plate was aspirated after resuspension into a new plate. To each well 100 µl of Malstat reagent (section 2.1.5) was added. Thereafter, 20 µl of NBT and Diaphorase mixed in a ratio of 1:1 were added to each well. The 96-well plate containing the infected erythrocytes, Malstat reagent and NBT-Diaphorase mixture was incubated on a rocker for 30-50 min. A change in the colour of the malstat reagent was observed in the wells containing live parasites. Optical density of the culture in the 96-well plate was measured at wavelength 630 nm. A 10 µl sample was withdrawn from the original 96-well culture plate to be verified by Giemsa staining. Positive cultures were transferred from the 96-well plate to 25 cm^2 cell culture flask and genomic DNA was isolated once sufficient parasitemia was reached. Diagnostic PCR then verified the clones with integrated locus and loss of the wild type.

2.2.1.4 Parasite culture and membrane feeds

Asexual parasites and mature gametocytes of the *P. falciparum* NF54 isolate or of the gametocyte-less F12 strain were cultivated in vitro as described (Ifediba and Vanderberg, 1981). Exflagellation was tested by incubating mature gametocyte cultures in 100 µM xanthurenic acid for 15 min

at RT. To obtain mosquito-specific parasite stages, i.e. ookinetes and sporozoites, these mature gametocyte cultures of wild-type NF54 were membrane fed to mosquitoes (Bishop and Gilchrist, 1946). Ookinetes were obtained by preparing midgut smears 24 h post-feeding and sporozoites were isolated from salivary glands 18-22 d after feeding.

2.2.1.5 Indirect immunofluorescence assay

Parasite preparations for immunofluorescence microscopy included mixed asexual stages, mature gametocytes of the *P. falciparum* NF54 isolate as well as mosquito midgut smears and isolated sporozoites. Preparations were air dried on slides and fixed for 10 min in -80°C methanol. For membrane permeabilization and blocking of non-specific binding, fixed cells were incubated in 0.01% saponin, 0.5% BSA and 1% neutral goat serum in PBS for 30 min each time. Preparations were then incubated for 2 h at 37°C with anti-*Pf*CLK-1/Lammer and *Pf*CLK-2 kinase immune sera 1:50 diluted in PBS solution containing 0.01% saponin and 0.5% BSA. Binding of primary antibody was visualized using fluorescence-conjugated goat anti-mouse or goat anti-rabbit (Alexa Fluor 488 or Alexa Fluor 594) antibodies diluted 1:1000 in the same solution as the primary antibody. For double-labelling experiments, specimens were consecutively incubated with the respective first antibody followed by Alexa Fluor 488-conjugated secondary antibody, before incubation with the respective second antibody followed by Alexa Fluor 594-conjugated secondary antibody. Nuclei were highlighted by incubating the specimens with Hoechst nuclear stain (Molecular probes) prepared in a dilution of 1:5000 in dH_2O for 1 min. For counterstaining of red blood cells, 0.05% Evans Blue solution diluted in 1x PBS was added on each well for 1 min. Labelled specimens were examined with a Zeiss Axiolab microscope in combination with an Axiocam camera. Digital images were processed using Adobe Photoshop CS software.

2.2.1.6 Immunoelectron microscopy

Asexual parasites of *P. falciparum* NF54 were fixed in 4% paraformaldehyde in PBS overnight at 4°C. After a PBS wash they were

treated with 50 mM NH_4Cl in PBS for 15 min at RT to block aldehyde groups. Specimens were dehydrated under rotating condition in increasing concentrations of ethanol starting from two times 15 min at 30% and 4°C to further dehydration in 50%, 70%, and 90% ethanol each two times for 30 min. 100% ethanol was dehydrated two times 60 min each time at -25°C. 70% and 90% ethanol were additionally mixed with 0.2% uranyl acetate. Thereafter, the samples were transferred to glass tubes to be incubated for 1 h in a 1:1 mixture of 100% ethanol and LR White resin at 4°C. Specimens were subsequently embedded in LR White at 40°C for 3-4 d. Ultrathin sections of the respective specimens were then subjected to post-embedding labelling. After a 5 min wash in PBS, samples were blocked in 1% BSA and 0.1% Tween20 in PBS for 10 min prior to immunolabelling with the primary antibodies, anti-*Pf*CLK-1/Lammer and anti-*Pf*CLK-2 respectively in a dilution of 1:50 with a subsequent incubation for 1 h in a humid chamber. After washes with 0.1% BSA and 0.1% Tween20 in PBS, samples were incubated for 1 h with the 12 nm gold-conjugated goat anti-mouse secondary antibody. Ultrathin sections were subsequently post-fixed with 1% glutaraldehyde in PBS, washed three times 5 min each in PBS and dH_2O to be finally post-stained with 2% uranylacetate. Photographs were taken with a Zeiss EM10 transmission electron microscope and scanned images were processed using Adobe Photoshop.

2.2.2 Molecular biology methods

2.2.2.1 Genomic DNA isolation

20 ml of parasite culture were transferred into a falcon tube and centrifuged at 2,200xg for 10 min. The resulting pellet was resuspended in 5 ml PBS, and lysed for 5 min by adding 75 µl 10% saponin. The lysed pellet was centrifuged at 2,200xg for 10 min and the supernatant was discarded. The resulting parasite pellet was resuspended in PBS, transferred to an eppendorf tube and washed twice with 1 ml PBS. The washed pellet was then taken in 500 µl TSE, 100 µl 10% SDS, and 50 µl 6 M $NaClO_4$ (section 2.1.6) and rocked overnight at room temperature. To this one volume of phenol was added, rocked for 5 min, and centrifuged for 5 min at 16,000xg. The colourless

aqueous phase (top) was transferred into a clean tube and half a volume of phenol followed by half a volume of chloroform was added. The sample was rocked for 5 min followed by centrifugation for 5 min at 16,000xg. The colourless aqueous phase (top) was transferred again to a clean tube. One volume of chloroform was added to it, rocked for 5 min, and centrifuged for 5 min at 16,000xg. The top layer was transferred to a clean tube, 2 volumes of pure ethanol were added and rocked gently for 5 min. This was then centrifuged for 5 min at 16,000xg. The pellet was finally washed with ice-cold 70% ethanol, air-dried and gently resuspended in 20 µl water by heating it at 55-60ºC. Once the genomic DNA was isolated, it was employed as a template in PCR-amplification.

2.2.2.2 Polymerase chain reaction

Gene-specific sequences from PlasmoDB (www.plasmodb.org) were chosen to design primers for each kinase. Master Mix for the PCR reaction was prepared as follows:

dH_2O	38.0 µl
Pfx Buffer (10x stock)	5.0 µl
$MgCl_2$ (50 mM)	1.5 µl
DNA template (1 µg/µl)	2.0 µl
dNTPs (10 mM stock)	1.0 µl
Pfx DNA polymerase	0.5 µl
Primers: Sense (100 pmol/µl)	1.0 µl
Antisense (100 pmol/µl)	1.0 µl
	50.0 µl

Following programme was employed for diagnostic reverse transcriptase PCR:

95°C	Denaturation	4 min	
95°C	Denaturation	30 sec	30 cycles
50°C	Annealing	30 sec	
62°C	Elongation	1min/700bp	
72°C	Final Elongation	3 min	
4°C	Store	Forever	

The amplification was verified on 1.4% agarose gel

2.2.2.3 Spin purification and digestion

Once amplification was confirmed, the PCR product was spin purified (Qiagen PCR purification kit, Hilden) as per the manufacturer's instructions. DNA elution was done by adding 50 µl autoclaved water to the column followed by a brief incubation for 2 min. at RT and centrifugation at 13, 000 rpm for 1 min. The spin-purified insert was digested to further prepare it for ligation with the vector.

Digestion of the insert was done as follows:

DNA (from above purification)	43.0 µl
10x Buffer	5.0µl
restriction enzyme 1	1.0 µl
restriction enzyme 2	1.0 µl
	50.0 µl

Above-mentioned reaction mixture was incubated for 3-4 h at 37°C. The digested PCR product was spin-purified once again. The insert was thus optimized for cloning, while the vector required was obtained in high concentration by performing large-scale (maxi) preparation.

2.2.2.4 Plasmid DNA Maxipreparation (for 500-1000ml overnight culture)

The vector used for maxipreparation was obtained from available glycerol stocks in the laboratory. 20 µl from this were added to 1000 ml of LB medium along with Ampicillin (100 µg/ml). The culture was grown overnight at 37ºC and centrifuged at 5,000xg for 10 min. The pellet was resuspended in 10 ml of Solution 1 (section 2.1.6). 20 ml of freshly prepared Solution 2 were added (see section 2.1.6) and mixed by inverting the tube 5 times followed by incubation for 5 min at room temperature. To this, 15 ml of Solution 3 (see section 2.1.6) was added, gently mixed by inverting the tube 4-5 times and incubated for 5 min. 30 ml of sodium perchlorate (see section 2.1.6) was added and mixed by inverting the tube 5 times which resulted in DNA precipitation. It was then centrifuged for 10 min at 10,000xg. The supernatant was filtered through folded filters and transferred into a new tube. To the filtered

supernatant 50 ml of isopropanol were added and centrifuged at 10,000xg for 30 min at 4°C. The resulting pellet was dissolved in 2 ml 0,1x TE. To this 8 ml of 5 M LiCl (section 2.1.6) were added and mixed. This was again centrifuged at 10,000xg for 30 min to precipitate the DNA. The resulting supernatant was transferred to a new tube and 10 ml of Bind Mix (pH 6) was added to it. Bind mix was prepared by resuspending the appropriate amount of Silica in 10 ml 4 M GuSCN and 4% Triton X-100 (section 2.1.6). 100 mg Silica was added to 100 ml of the overnight culture for high copy plasmids and 20 mg Silica for 100 ml of the overnight culture for low copy plasmids. Supernatant/Bind mix was incubated for 3 min at room temperature and mixed. The Silica was then pelleted at 2000xg for 3 min. The resulting pellet was resuspended in 5 ml 4M GuSCN and 4% Triton-X and centrifuged again for 3 min at 2,000xg. Silica pellet was then resuspended in 5 ml 50% EtOH followed by centrifugation for 3 min at 2,000xg. This step was repeated again. The resulting Silica pellet was resuspended in 2 ml 1xTE and incubated for 3 min at 65°C. This was further centrifuged for 5 min at 8,000xg. The supernatant was transferred to a clean tube. DNA can be further concentrated by NaAc/EtOH-precipitation. To the above supernatant, 2 volumes of 100% ice-cold EtOH and 1/50 volume of 5 M NaAc was added. It was then incubated on ice for 20 min. This was followed by centrifugation for 30 min at 8,000xg. The resulting pellet was washed with 80% EtOH and then air-dried. The pellet was finally resuspended in 200 µl H_2O.

Once the vector was obtained by following the above-mentioned protocol, it was digested with Sac II/Not I restriction enzymes so as to derive the vector required for desired cloning.

2.2.2.5 Digestion and gel purification of the vector

Vector (approx. 4 µg)	4 µl
dH_2O	38 µl
10x Buffer	5 µl
restriction enzyme 1	1 µl
restriction enzyme 2	1 µl
CIP	1 µl
	50 µl

Above reaction mix was incubated for 3-4 h at 37°C.

Once digested, the vector was loaded onto 1.4% agarose gel and gel-purified (QIAquick, PCR purification kit, Hilden) by following the enclosed instruction manual.

2.2.2.6 Ligation

The amount of insert required for ligation was calculated using the following formula:

$$\text{Amount of insert (ng)} = \text{5x amount of vector (ng)} \times \frac{\text{Length of the insert (bp)}}{\text{Length of the vector (bp)}}$$

Following the above formula, the ligation reaction was prepared as follows:

	Ligation (µl)
PCR product	x
Vector	4
dH_2O	(13-x)
10X Ligation buffer	2
T4 Ligase	1
	20

Above mentioned ligation reaction was incubated either at RT for 4-6 h or overnight at 16ºC. Following ligation, the vector with cloned insert was transformed into Top10 competent *E. coli* cells (section 2.2.2.12).

2.2.2.7 Control Digestion

dH_2O	7 µl
10 x Buffer	2 µl
Miniprep-DNA	8 µl
restriction enzyme 1	0.5 µl
restriction enzyme 2	0.5 µl
	20 µl

The above-mentioned digestion reaction was incubated for about 4-6 h at 37°C and run on 1.4% agarose gel along with the DNA ladder to confirm the ligation. Once the insert size and the vector were confirmed from control digestion, the purified DNA from mini-preparation was quantified for sequencing.

2.2.2.8 Generation of *Pf*CLK-1/Lammer kinase-dead mutant

The PCR fragment (SalI/NotI digested) used for generation of recombinant *Pf*CLK-1/Lammer was digested and replaced with a mutated fragment (Figure 5). A catalytically inactive mutant was constructed by performing site directed mutagenesis of Lys^{581} to Met using overlap extension PCR (Ho et al., 1989). This included a first round of amplification with forward primer, rp 2S (1, Figure 2.3) of the *Pf*CLK-1/Lammer recombinant protein and reverse primer for *Pf*CLK-1^{mut}, rp 1AS ^{mut}(2, figure 2.3). The second round of PCR amplification was performed with rp $1S^{mut}$ as the forward primer and rp 2AS as the reverse primer (3 and 4, figure 2.3). These two independently amplified PCR products were employed as templates with primers 1 and 4 in a third PCR amplification. The right size PCR product was gel purified, digested with SalI/NotI enzymes and ligated to digested pGEX-4T1 vector. The sequence was confirmed and kinase dead mutant was purified (section 2.2.2.9) for subsequent kinase assays.

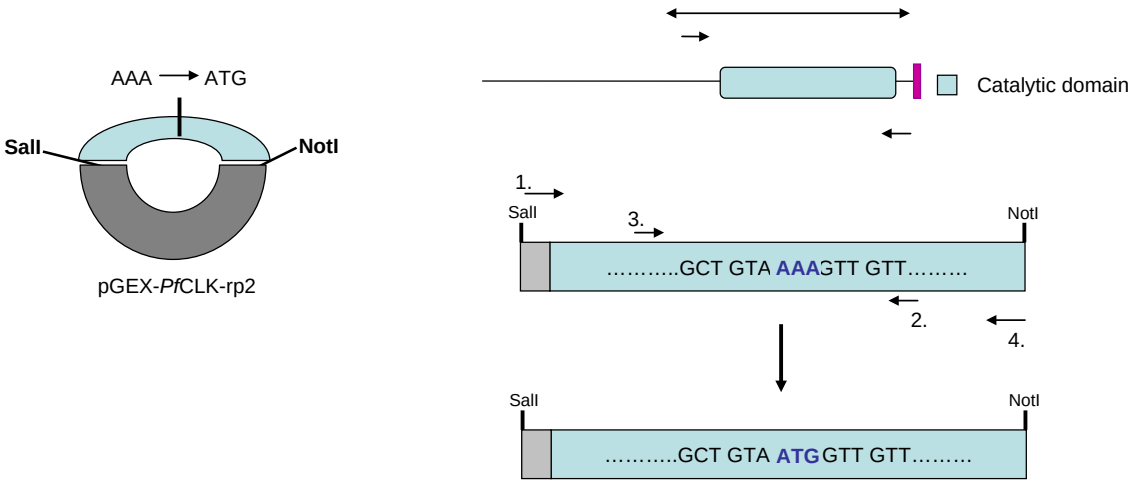


Figure 2.3: Strategy for generation of a kinase dead mutant.

2.2.2.9 Sequencing

Each clone was PCR-amplified using a pair of gene-specific and a pair of vector primers (section 2.1.13-16). The sequencing results thus obtained ensure the successful ligation of the insert having the right orientation within the vector.

The reaction mix consists of the following:

5 X Premix	2 µl
5X Sequencing Buffer	2 µl
DNA Template (from miniprep)	400 ng
Primer (50 pm each primer)	0.5 µl

The final reaction volume was made up to 10 µl with sequencing water. Following PCR programme was used for sequencing:

95°C	Denaturation	4 min	
95°C	Denaturation	30 sec	30 cycles
50°C	Annealing	30 sec	
62°C	Elongation	4 min	
72°C	Final Elongation	3 min	
4°C	Store	Forever	

Precipitation of sequencing product was done with ethanol as follows:

PCR product	10 µl
Sequencing water	90 µl
3M NaAc, pH 4.6	10 µl
100% Ethanol	250 µl

The reaction mixture was centrifuged at 13,000xg for 15 min. The supernatant was discarded and 700 µl of 70% ethanol was added and again centrifuged at 13,000xg for 15 min. The supernatant was discarded after centrifugation and the pellet was dried completely and then resolved in 25 µl of Hi-Di formamide. Once the correct kinase-vector sequence was confirmed with

sequencing results, 20 µl of the overnight subculture was used for maxi preparation (section 2.2.2.4).

2.2.2.10 RNA isolation and cDNA preparation

RNA from each blood stage of the parasite, *P. falciparum* was isolated using the Trizol method. The concentration of parasites was determined by preparing 1:50 dilution in PBS and counted by adding a drop on a clean Neubauer chamber. The cells were then aliquoted in $1x10^7$ portions in 1.5ml eppendorf tubes, centrifuged at 13,000xg for 1 min and the supernatant was discarded. The pellet was resuspended in 1ml of Trizol reagent by repetitive pipetting and incubated for 5 min at room temperature on a rocker. To this, 200 µl of chloroform were added. Tubes were shaken vigorously for 15 s and incubated at RT for 10 min on a rocker and then centrifuged at 12,000xg for 15 min at 4ºC. The colourless aqueous phase (top) was transferred to a clean tube, 500 µl of isopropanol were added, mixed and incubated at room temperature for 10 min on a rocker. The tubes were further centrifuged at 12,000xg for 15 min at 4ºC. The pellet was washed once with 1 ml of 75% ethanol, vortexed and centrifuged at 7,500xg for 5 min at 4ºC. The RNA pellet was briefly air-dried and resuspended in 50 µl DEPC-water (Diethylpyrocarbonate-water) by heating it for 10 min at 60ºC. The concentration was then determined spectrophotometrically ($A_{260/280}$ > 1.8) and RNA was further processed to cDNA.

For removal of all genomic DNA, 2.5 µl DNaseI stock solution was added to 5 µg of RNA and 10 µl of buffer RDD. The volume was made up to 100 µl with RNase free water and incubated at room temperature for 40 min.

Before cDNA was synthesized, RNA was treated to remove any contaminating salt or protein. 150 µl of phenol, 150 µl of chloroform and 250 µl of DEPC-water were added to the purified RNA. The reaction tube was incubated on the rocker for 5 min and centrifuged for 5 min at 13,000xg. The aqueous phase was then transferred to a new tube and 300 µl of chloroform were added. This was rocked again for 5 min followed by centrifugation for 5 min at 13,000xg. The aqueous phase was transferred to a new eppendorf tube, RNA was precipitated by adding 30 µl of 3M NaOAc and 1 ml of EtOH. It was then incubated on ice for 50 min and centrifuged for 20 min at 12,000xg at

4ºC. The pellet was washed once with 1 ml of ice-cold, RNase free 75% ethanol, vortexed and finally centrifuged at 7,500xg for 5 min at 4ºC. The RNA pellet was briefly air-dried and resuspended in 50 µl DEPC-water. For cDNA synthesis, SuperScript First-Strand Synthesis system for RT-PCR was used and the components were mixed in a PCR tube as follows:

	Sample	**0-RT control**
DEPC H_2O	(10.5-X) µl	(10.5-X) µl
RNA (5 µg)	X µl	X µl
Random hexamers	0.5 µl	0.5 µl
10 mM dNTPs	1 µl	1 µl
	12 µl	

Samples were then incubated at 65ºC for 5 min followed by incubation on ice for 1 min. Following reaction mixture was prepared:

10x RT buffer	2 µl
50 mM $MgCl_2$	2 µl
0.1 M DTT	2 µl
RNAse OUT inhibitor	1 µl
	7 µl / reaction

7 µl of the reaction mixture was added to each 12 µl of RNA/primer mixture, gently mixed and centrifuged at 6,000xg for 1min followed by incubation at 25ºC for 2 min. To the above reaction mixture, 1 µl (50U) of Super Script II reverse transcriptase was added except for the tube which was kept as negative control lacking reverse transcriptase (0-RT control). This reaction mix was incubated for 10 min at 25ºC. Thereafter, reaction mixtures were incubated for 50 min at 42ºC and finally terminated by incubating the tubes at 70ºC for 15 min followed by chilling on ice. The reaction mixtures were centrifuged at 6,000xg for 1 min followed by addition of 1 µl RNase H to cleave any RNA impurities and then incubated for 20 min at 37ºC.

cDNA prepared above from various blood stages of the parasite was employed to determine the stage-specific transcript expression for the four CLK kinases under study by sequence-specific amplification via diagnostic Reverse-Trancriptase PCR. For this, two sets of primers were designed from different regions of each kinase (section 2.1.12). Primers were designed such that the length of each PCR product was 200-250 bp.

For determining the sequence-specific amplification, the following master mix for all reactions was prepared:

H_2O	38.0 µl
Pfx Buffer (10x stock)	5.0 µl
$MgCl_2$ (50 mM)	1.5 µl
DNA template (5 µg)	2.0 µl
dNTPs (10 mM stock)	1.0 µl
Pfx DNA polymerase	0.5 µl
Primers: Sense (100 pmol/ µl)	1.0 µl
Antisense (100 pmol/ µl)	1.0 µl
	50.0 µl

Once the above reaction mixture was set up, the tubes were subjected to PCR using a thermocycler and amplified products were verified on 1.4% agarose gel.

Apart from the diagnostic reverse transcriptase PCR, Real Time reverse transcriptase PCR was also performed for quantitation of the transcript using SYBR-Green. It is based on the principle that SYBR-Green intercalates in the double strand of the DNA and when the amount of its expression is above a certain threshold level, it emits a signal. The reaction mix for each of the Real Time RT–PCR primer pair was prepared as mentioned below. The primers were used at a concentration of 1pm/µl. The following reaction mixtures of 1x were prepared:

Primer alone:

Water	6.0 µl
Primer Mix	5.0 µl
cDNA(water)	1.0 µl
Master Mix	12.0 µl

For each malarial blood stage

Water	6.24 µl
Primer Mix	5.0 µl
cDNA (2µg)	1.0 µl
Master Mix	12..2 µl

Primer efficiency (the concentration of cDNA at which primers themselves give the lowest signal) was previously verified using diluted cDNA samples (Pradel et al., 2004). Once the reaction mix was prepared 20 µl of it were added in the respective wells. To this 5 µl of gene-specific primers were added. The plate was covered with optical film, centrifuged at 2,200xg for 5 min and carefully placed in the Real Time thermocycler

The programme used for Real Time RT-PCR was as follows:

95°C	Polymerase activation	10 min		
95°C	Denaturation	30 sec	⎫	
50°C	Hybridization	30 sec	⎬	40 cycles
62°C	Elongation	1 min	⎭	
15°C	Store	48 h		

The results obtained were then analyzed for transcript abundance for each the four *Pf*CLK kinase in blood stages and zygotes of the malaria parasite.

2.2.2.11 Cultivation and storage of bacteria

Bacterial cells were grown in LB medium with selective antibiotics overnight in a shaker incubator at 37°C with 180-220 revolutions per minute. For long term storage bacterial cells were resuspended in 20% sterile glycerol in LB and stored at -80°C.

2.2.2.12 Transformation of competent bacterial cells

The Top10 cells were thawed on ice and 20 µl of these cells were transfered into 1.5 ml eppendorf tubes. 2 µl from each of the ligation products

were added into the respective eppendorf tubes with Top10 cells. This mixture was incubated for 30 min on ice. Heat shock was given at 42°C for 30 s, followed by placing on ice for 2 min. 200 µl of SOC medium were added and incubated at 37°C for 1 h in a shaker incubator. The transformation product was then plated completely onto LB/Ampicillin plates and incubated overnight in the 37°C incubator. Clones were picked and subcultured the following day. Subcultures comprised of 3 ml LB media in small tubes together with an appropriate antibiotic (Ampicillin, 100 µg/ml). Each clone was grown overnight in the 37°C incubator shaker followed by small-scale plasmid DNA purification using the Qiagen Miniprep DNA Purification System.

2.2.3.13 Plasmid construction for single cross-over homologous recombination

The Knock-Out (KO) strategy

A reverse genetic approach was performed to analyse kinase functions. The five constructs for gene disruptions were named as pCAM-BSD-CLK1KO, pCAM-BSD-CLK2KO, pCAM-BSD-CLK3KO, pCAM-BSD-CLK4KO and pCAM-BSD-PKRP-KO. They were designed such that homologous integration led to disruption of the kinase domain (introducing a stop codon at the end of the insert) such that the disrupted locus either lacks the kinase activity or is insufficient, thus termed as the Knock-out (KO) strategy (Figure 2.4). Primers introduced BamHI/NotI restriction sites in the PCR fragments which were ligated to the BamHI/NotI-cut pCAM-BSD vector. All fragments were amplified using *P. falciparum* NF54 genomic DNA as a template using gene-specific primers (section 2.1.14).

Gene	Fragment length
pCAM-BSD-CLK1KO	543 bp
pCAM-BSD-CLK2KO	512 bp
pCAM-BSD-CLK3KO	664 bp
pCAM-BSD-CLK4KO	524 bp
pCAM-BSD-PKRP-KO	478 bp

The gene-tagging strategy

Recombinogenecity of the gene locus was verified by generation of C'-terminal tagged kinases by inserting last 600-800 bp of the gene (without stop codon) in the pCAM-BSD vector containing double Myc tag (HA or a GFP-tag) and 3'UTR from *P. berghei* DHFR-ts into the multiple cloning site (Figure 2.1.15) thus encoding a full-length tagged kinase and therefore being termed as a gene-tagging strategy. Asexual parasites of the 3D7 clone of *P. falciparum* were cultivated (section 2.2.1.1) and employed in transfection experiments (section 2.2.1.2). Primers introduced PstI/BglII restriction sites in the case of pCAM-BSD-CLK1-Myc and PstI/BamHI-disgested in the PCR fragments of the other four kinases which were ligated to the PstI/BamHI-cut pCAM-BSD vector. All fragments were amplified using *P. falciparum* 3D7 isolate genomic DNA (section 2.2.2.1) template using gene-specific primers (section 2.16).

Gene	Fragment length
pCAM-BSD-CLK1Myc	873 bp
pCAM-BSD-CLK2Myc	621 bp
pCAM-BSD-CLK3Myc	906 bp
pCAM-BSD-CLK4Myc	648 bp
pCAM-BSD-PKRP-GFP	566 bp

2.2.2.14 Genotype characterization

After 60-90 days of drug pressure, the respective transfected cultures were investigated for plasmid-integration by a diagnostic PCR assay. Primers from the kinase locus and from the flanking regions of pCAM-BSD-KO vector were employed for investigation of wild-type, 5'- and 3'-integrations and for the presence of episomes (Figure 2.4).

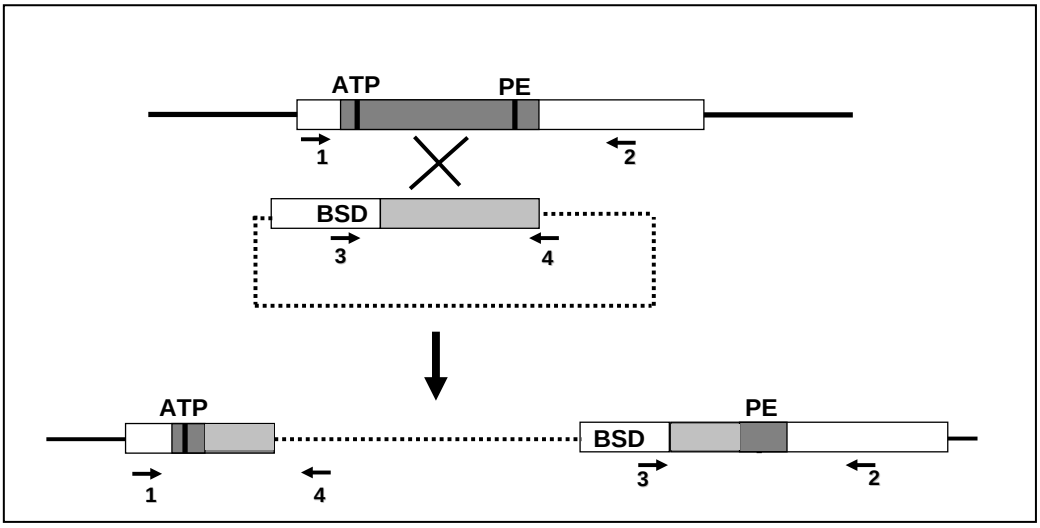


Figure 2.4: Schematic of Knock-out (KO)-strategy by single cross-over homologous recombination using the pCAM-BSD vector

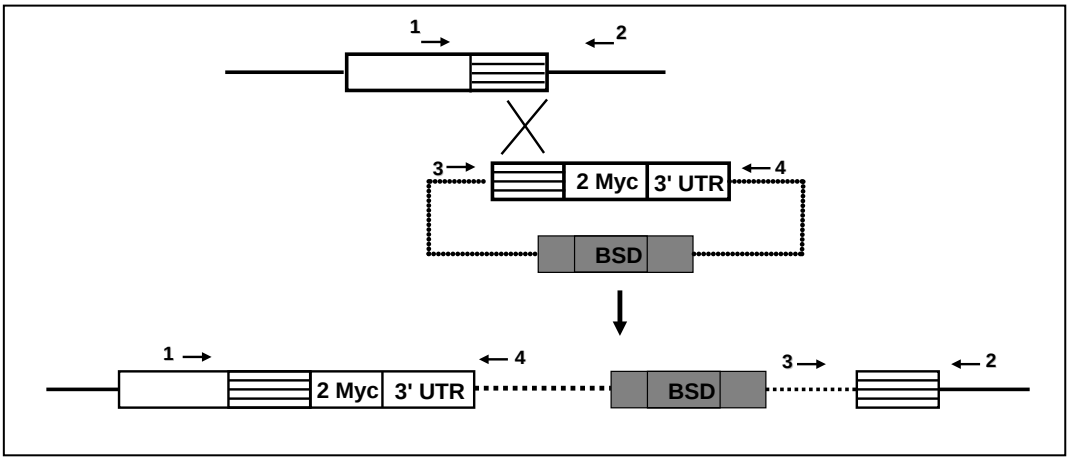


Figure 2.5: Verification of the locus recombinogenecity by C'-terminal tagging

Primers 1+2	amplification of Wild type (wt) gene locus
Primers 3+4	amplification of episome
Primers 1+4	diagnostic of 5'-integration
Primers 3+2	diagnostic of 3'-integration
BSD	Blasticidin drug resistance cassette
2 Myc	Double Myc-tag
3'UTR	3'-Untranslated region from *P.berghei*-DHFR-ts gene
ATP	Adenosine triphosphate binding motif, ATP binding
PE	Proline-glutamate motif , structural stability

Similarly, recombinogenicity of the gene locus was verified by a diagnostic PCR on the gDNA isolated from the respective culture transfected with pCAM-BSD-tag construct. The sequence-specific primers were chosen from the flanking sequence of both the gene locus at the 3'-end of the kinase.

to test the presence of the wild-type, 5'-integration, 3'-integration and episomal integration (Figure 2.5).

2.2.2.15 Southern blot analysis

Successful gene disruption of *Pf*PKRP and gene-tagging of *Pf*CLK-1/Lammer and *Pf*CLK-2 were verified by Southern blot analysis. Genomic DNA was isolated from asexual 3D7 wild-type and the disruptant parasites after saponin lysis using the DNA purification kit (Epicentre Biotechnologies) as per manufacturer's instructions. Restriction digest assays diagnostic of wild-type versus disruptant loci were guided by analysis of the genome nucleotide sequence of the extended gene loci (section 2.2.2.5, section 4.4). 2.5 µg of genomic DNA was digested with EcoRI for analysing the CLK-1 and with EcoRV and BglII for the CLK-2 locus. Restriction digested products were separated by 0.7% agarose gel electrophoresis and transferred to a Hybond N+ membrane. A hybridization probe of approximately 500 bp was used for detection of integration in CLK genes.

The probe for CLK-1 was amplified by PCR from 5' to the integration. For *Pf*CLK-2, the insert from the pCAM-BSD vector was used as the probe (section 2.1.17). The spin-purified hybridization probe was labelled with digoxygenin (DIG) and Southern blot hybridization was performed according to the manufacturer's instructions using the DIG High Prime DNA Labelling and Detection Starter Kit II. For *Pf*PKRP, 3µg of genomic DNA isolated from knock-out mutants G4, G8 and WT were digested with ScaI for one set and with BseRI and BamHI for a second set. The cloned insert of about 480 bp was used as the probe. Southern hybridization with enhanced chemiluminescence was performed according the instructions from the manufacturer of Amersham ECL direct nucleic acid labelling and detection system.

2.2.3 Protein biochemistry methods

2.2.3.1 Expression of recombinant protein

Recombinant proteins were expressed to generate polyclonal mouse antibodies for characterization of kinase expression and localization. The

design of recombinant protein constructs were from N-terminal region outside the catalytic domain as well as from the C-terminal kinase domain for two of the CLK kinases, *Pf*CLK-1 and *Pf*CLK-2 (Figure 6). Recombinant proteins were expressed as fusion proteins with a GST-tag using the pGEX 4T1 vector (Amersham Bioscience, München). Cloning was mediated by addition of restriction sites to the ends of PCR-amplified gene fragments corresponding to the respective domains (section 2.2.2.3). Recombinant proteins were expressed in BL21 (DE3) RIL cells by transformation and cultivation of bacterial cells (section 2.2.2.11 and 2.2.2.12).

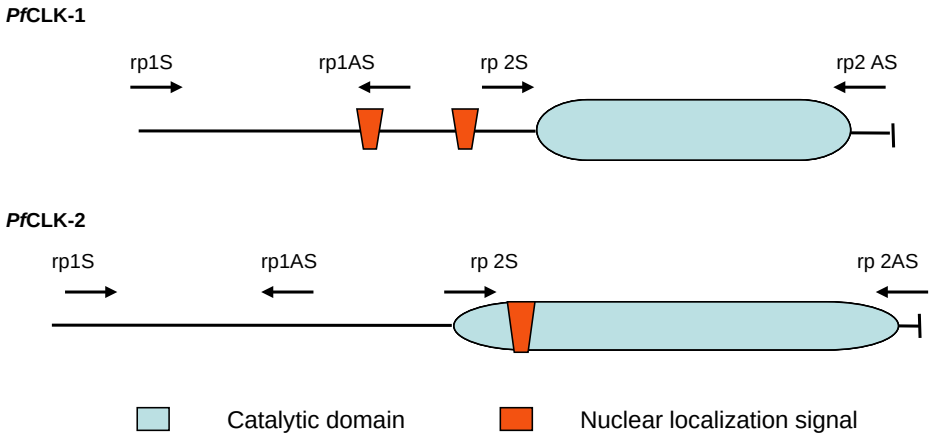


Figure 2.6: Schematic representing the location of primers chosen for generation of recombinant *Pf*CLK-1 and *Pf*CLK-2. rp: recombinant protein; S: sense primer; AS: antisense primer.

Mini-protein expression

Five clones from the transformation (section 2.2.2.12) were cultivated in 3 ml overnight LB medium and diluted 1:5 with LB medium containing Ampicillin (100 mg/ml). The diluted cultures were grown further for 2 h until the bacteria reached an absorbance at wavelength 600 (A_{600}) equal to 0.6. The presence of the lac operon within the plasmid, pGEX-4T1, enabled the induction of protein expression by an artificial substrate, IPTG (Isopropyl-β-D-thio galactoside) at a concentration of 0.75 mM. The cultures were induced for about 2.5 h and uninduced cultures were also cultivated under same conditions as a negative control. Induction was performed at 30°C and 30 µl of samples were obtained from each of the induced and the uninduced cultures. Samples

were diluted 1:1 with 2x sample buffer and 25 mM DTT and boiled at 95°C for 10 min. Samples were either subjected to SDS-PAGE (section 2.2.3.4) or stored at -20°C until further required.

Maxi-protein expression

Once the induction of selective recombinant protein was observed by mini-expression protein was produced in large scale. 100 ml of overnight culture was diluted 1:10 after overnight shaking at 37°C. Induction was performed with 0.75 mM IPTG as mentioned above and after about 5 h of induction, cultures were centrifuged at 5000xg at 4°C to obtain bacterial pellets which were either stored at -20°C until use or lysed to purify the recombinant protein.

2.2.3.2 Purification of recombinant protein

Purification of soluble recombinant protein

Bacterial pellets obtained above were resuspended in 50 mM Tris, pH 8.0, and 10% glycerol. Resulting suspension was incubated with lysis buffer (350 mM NaCl, 0.2% IGEPAL (20% stock), 10 mM sodium fluoride, 10 mM β-glycerophosphate) for 1 h at 4°C on a shaker. Cell disruption was further performed using a French® Press by applying 1200 psi pressure in three subsequent cycles followed by DNA disruption by pulse sonicating the sample for 2 min at 50% intensity and 50 cycles. The resulting sample was centrifuged at 30,000xg at 4°C. The supernatant was collected and filtered with a 0.22 µm syringe filter. Meanwhile, Glutathione-Sepharose-*4FastFlow* beads were washed three times with 1x PBS and 500 µl were added to the above supernatant followed by overnight incubation at 4°C under gentle rotation. The mixture was loaded onto a PolyPrep® column and allowed to pass through the matrix where protein selectively bound to GST-sepharose was retained. The column was washed four times with PBS, two times with wash buffer 3, one time with wash buffer 5, two times further with PBS. Finally, the protein was eluted in three fractions of 1 ml each of GST elution buffer. Fractions were stored at -20°C and also verified by SDS-PAGE (section 2.2.3.4).

Purification of Inclusion bodies

Bacterial pellets after IPTG induction were resuspended in lysis buffer (section 2.1.7). 200 mg lysozyme were added to the lysis buffer and incubated on ice for 30 min. The sample was sonicated as described before and the lysate was resuspended in 200 ml detergent buffer followed by centrifugation at 5000xg for 10 min at 4°C. The pellet was resuspended in 250 ml washing buffer, centrifuged at 5000xg for 10 min and thoroughly washed with 250 ml of 70% ethanol. The pellet was resuspended in a small volume of freshly prepared PBS and sonicated to enable the protein to pass through a 23 G needle used for later immunization of mice. Protein concentration was estimated by comparison with the protein ladder to calculate the appropriate amount for immunization.

Immunization and generation of mouse antiserum

Specific immune sera were generated by the immunization of 6 week-old female NMRI mice with 100 µg recombinant protein emulsified in Freund's incomplete adjuvant followed by a boost after 4 weeks. Mice were anesthesized by intraperitoneal injection of a mixture of ketamine and xylazine according to the manufacturer´s protocol (Sigma-Aldrich), and immune sera were collected 10 d after the second immunization via heart puncture. Immunization of mice and collection of immune sera was performed in collaboration with Dr. Gabriele Pradel, Ludmilla Sologub and Nina Simon. Sera from non-immunized mice served as a control for antibody reagent studies. As a second source of antibody for *Pf*CLK-1/LAMMER, antipeptide sera directed against the peptide sequence NRTKTSDTEDKKER of the protein were produced by immunization of two rabbits. For *Pf*CLK-2, antisera from two mice were generated against the peptide sequence CISYNEKENKYNDQD. Both peptides were directed against the catalytic domain of the kinases.

2.2.3.3 Preparation of parasite and nuclear extracts

Parasite cultures with 6-8% parasitemia were lysed in 0.15% Saponin. The pellet was resuspended in 200 µl lysis buffer (Nunes et al., 2007, section

2.1.7) and sonicated with 50% amplitude and 50 cycles. Centrifugation was performed at 13,000xg, 4°C for 10 min. Supernatant was then separated and protein concentration was measured by Bradford assay.

For preparation of nuclear extracts, parasite pellets were resuspended in lysis buffer and washed with PBS. Pellets were resuspended in Buffer A (section 2.1.7) and incubated at room temperature for 10 min followed by centrifugation at 13,000xg for 10 min at 4°C. The resultant pellet was resuspended in Buffer B (section 2.1.7). The resuspension was vigorously shaken for 2 h at 4°C and centrifuged at 13,000xg at 4°C for 5 min. The supernatant was taken as the nuclear fraction. Nuclear fraction was verified by Hoechst staining.

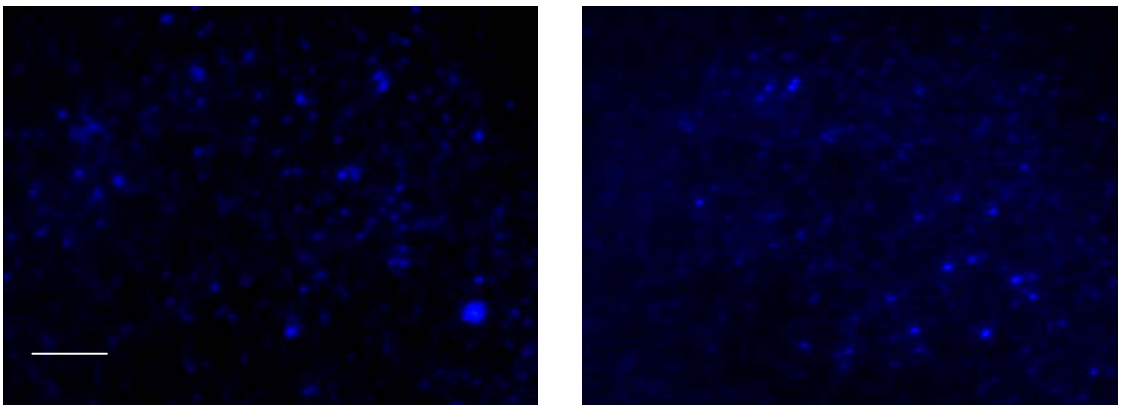


Figure 2.7: Nuclear fraction of the parasites was extracted and stained with Hoechst nuclear stain. Bar, 5 µm.

2.2.3.4 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a biochemical technique used for separation of proteins on the basis of their electrophorectic mobility. Varying percentage of resolving gels (6%-15%) were prepared according to the size of the desired protein to be identified.A stacking gel of about 5% served as a starting zone for the protein separation in the resolving gel. The samples were loaded onto the pockets prepared and allowed to resolve with an initial voltage of 85V until they migrated out of the stacking gel and about 125V until the desired separation was reached in the resolving gel. The gel was then either used in a western

blot (section 2.2.3.5) or after three times washing with distilled water, stained for about 1 h with *GelCode®-Blue-Stain* on a rocker. Once the desired staining was achieved, the gel was washed with water for about 2 h or until the desired stain was remaining. Protein concentrations were estimated according to the 5 µl protein ladder considering each band corresponded to 1µg of protein. For long term gel preservation, the gel was incubated with a solution of 10% Glycerol and 20% Ethanol for about 30 min and then dried between two clear cellophane sheets in the gel drying frame for one day at RT.

2.2.3.5 Western blot analysis

Parasite pellets after saponin lysis or Percoll gradient-enriched gametocytes were resuspended in PBS and SDS-PAGE loading buffer (section 2.1.7). Parasite proteins were separated by SDS-PAGE and was transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, München) using the Mini-Trans-Blot-apparatus from Bio-Rad according to the manufacturer's instructions using transfer buffer (section 2.1.7). The sponge pads and whatman papers were pre-soaked in the transfer buffer for 1 min. The gel was placed on the layer of the sponge pad and whatman paper, followed by a nitrocellulose membrane over them. The membrane was further layered with the whatman paper and sponge pad and the surface was smoothened by a pipette to remove the trapped air. The protein transfer was performed either at 15 V o/n or at 25 V for 2 h. After the transfer, membrane was washed briefly in 1x TBS followed by blocking for non-specific binding by incubation in Tris-buffered saline (TBS) containing 5% skim milk and 1% BSA fraction V. The membrane was then incubated with primary antibodies such as with anti-*Pf*CLK-1/Lammer, anti-*Pf*CLK-2 and anti-*Pf*PKRP diluted 1:50 respectively in 1x TBS (section 2.1.7, 2.1.11) containing 3% skim milk (3%TBSM) for 2 h at RT. Subsequent washes were performed one time with 3% TBSM, two times with TBSM and 0.1% Tween and one time with TBSM before incubating the membrane with alkaline phosphatase-conjugated secondary antibody (section 2.1.11) for 1 h at RT. To remove unbound antibody, further washes were performed with TBS and TBS/0.1% Tween followed by adding the equilibration buffer. Nitrocellulose membrane was developed in a solution of

nitroblue tetrazolium chloride (NBT) and 5-brom-4-chlor-3-indoxylphosphate (BCIP) for 5-30 min and the reaction was stopped by addition of stop buffer.

2.2.3.6 Co-immunoprecipitation assay

Parasite pellets obtained from wild-type *Pf*CLK kinases after saponin lysis were resuspended in 200 µl lysis buffer and sonicated with 50% amplitude and 50 cycles followed by centrifugation at 13,000xg and 4°C for 10 min. Pre-purification of lysate was done by consecutive incubation with 5% v/v pre-immune mouse sera and 20 µl of protein G-beads for 30 min each at 4°C. After centrifugation, the supernatant was incubated for 1 h at 4°C with 16 µl of mouse antisera raised against the respective *Pf*CLK kinase. A volume of 20 µl protein G-beads was added and incubated for another hour. The beads were centrifuged, washed with PBS, and mixed with an equal volume of loading buffer and loaded onto a 12% SDS gel. Gels were subsequently stained with colloidal Coomassie. Selected protein bands were then subjected to mass spectrometry. Parasite extracts obtained from Myc-tagged PfCLK kinases were pre-purified by consecutive incubation with 5% v/v pre-immune rabbit sera and 20 µl of protein G-beads for 30 min each at 4°C. After centrifugation, the supernatant was incubated for 1 h at 4°C with 2 µl anti-Myc-tag rabbit monoclonal antisera. A volume of 20 µl protein G-beads was added and incubated for another hour. The beads were centrifuged, washed with PBS, and employed in the kinase activity assay (section 2.2.3.8).

2.2.3.7 Pull down assay

For the Pull-down assay, recombinant *Pf*CLK-1 was expressed as a GST-fusion protein in *E. coli* BL21 (DE3) RIL with 0.75 mM Isopropyl Thiogalactoside (IPTG) (section 2.2.3.1). Bacteria were grown at 30°C for 5 h and centrifuged to obtain a bacterial pellet and resuspended in 1x PBS. Protein extraction was performed by digestion with 1 mg/ml lysozyme w/v in PBS for 1 h on ice. Sonication was performed (section 2.2.3.2) and bacterial lysate was cleared by centrifugation at 13,000xg, 10 min at 4°C. The lysate was incubated with washed 300 µl glutathione beads for 2 h followed by four washes with 1x cold PBS. The bacterial lysate was incubated with 150 µg parasite extract

(section 2.2.3.3) from asexual stages of the parasite for 1 h at 4°C with rotation, centrifuged and washed three times with lysis buffer (section 2.1.7) including protease and phosphatase inhibitors. A last wash was performed with the kinase buffer before proceeding with the kinase assay.

2.2.3.8 Kinase activity assay

A kinase reaction of 30 µl was carried out in a standard kinase buffer (section 2.1.7) and prepared as follows:

Kinase buffer	: 6 µl
Recombinant kinase	: 2 µg
Exogenous substrates	: 5 µg
(Histone H1, MBP, α/ β –caseins)	
dH_2O	: x µl
	25 µl

60 µM ATP non- radiolabelled was prepared by adding 75 µl dH2O + 5 µl 1 mM ATP. Further, a mixture of 4.75 µl 60 µM ATP and 0.25 µl 0.1 MBq [γ 32P] ATP was prepared per reaction. This 5 µl mixture of radiolabelled and non-radiolabelled ATP was added to the above 25 µl reaction, scaling the final volume of 30 µl kinase reaction. Reactions were incubated at 37°C for 1 h under constant agitation and terminated by addition of 8 µl of 2 x sample buffer (section 2.1.7) for 5 min at 100°C. Samples were separated on 12% SDS-PAGE (section 2.1.7 and 2.2.4.4) and the gel was dried followed by exposing it to an X-ray film. The film was incubated at -20°C for 2-20 h and developed to detect the phosphorylation signal.

2.2.3.9 Mass spectrometry

Co-immunoprecipitation of native *Pf*CLK-1/Lammer and *Pf*CLK-2 was performed from 3D7 parasite extracts using the respective mouse antisera. Proteins were resolved via SDS-PAGE. Similarly treated uninfected erythrocytes were taken as negative control. The selected protein bands were subjected to in-gel trypsin digestion before mass spectrometry analysis as

described previously (Hellmann et al., 1995). The peptide mixtures from the tryptic digests were desalted and concentrated using ZipTips™ columns made from the reverse chromatography resins Poros and Oligo R3. The bound peptides were washed with a solution of 0.5% formic acid and eluted from the column in 1 µl of 33% (v/v) acetonitrile/0.1% trifluoroacetic acid solution saturated with α-cyano-4-hydroxycinnamic directly onto a MALDI target plate and air dried before analysis in the mass spectrometer. Measurement was performed on an Ultraflex-TOF TOF tandem mass spectrometer. Peptide mass fingerprint spectra were acquired in the reflectron positive mode with a pulsed extraction using approximately 100 laser shots. The spectra were acquired after an external calibration using reference peptides. After internal calibration using trypsin autolysis peaks as internal standards (842.5100, 2211.1046 Da) the monoisotopic masses were assigned and processed using Biotools ™ and FlexAnalysis ™ software. Subsequently they were submitted to the Mascot programme for searches against the non-redundant NCBI database. The parameters used in the Mascot peptide mass fingerprint searches were as follows: Taxonomy, *P. falciparum* and *Homo sapiens*; search all molecular masses and all isoelectric points; allow up to one missed proteolytic cleavage site and a peptide mass tolerance of 100 ppm. Methionine oxidation was considered as an optional modification and cysteine carbamidomethylation as a fixed modification in all the searches. Matches to human proteins were considered unambiguous when the probability score was significant using the Mascot score with a p value < 0.05.

3. Results

3.1 Functional characterization of *Pf*CLK kinases

The largest group of the *P. falciparum* kinome, the CMGC group has been widely investigated except for the family constituting the four CDK-like kinases (CLK) proposed to be involved in mRNA splicing. These kinases are here referred as *Pf*CLK-1/Lammer, *Pf*CLK-2, *Pf*CLK-3 and *Pf*CLK-4. *Pf*CLK-1/Lammer belongs to the Lammer kinase family which is conserved in eukaryotes. Similarity of the latter three kinases to the existing protein families has not been yet described.

3.1.1 In silico analysis of *Pf*CLK kinases

Annotation via PlasmoDB revealed that the four *Pf*CLK kinases represent putative Serine-Threonine kinases, with a possible additional function of Tyrosine phosphorylation. Gene sequences of *PfCLK-1/Lammer*, *PfCLK-2* and *PfCLK-4* are intron-less whereas, *PfCLK-3* is an intron-rich sequence, encoding large polypeptides.

The predicted catalytic domains of the four *Pf*CLK kinases are located at the C-terminus for *Pf*CLK-1 to *Pf*CLK-3 and N-terminal in *Pf*CLK-4 (Figure 3.1). In silico analysis further predicted two nuclear localization signals for *Pf*CLK-1 and one signal for *Pf*CLK-2, located upstream of the C-terminal catalytic domains (Figure. 3.1), indicating a possible localization within nuclear speckles. No nuclear signals could be detected for *Pf*CLK-3 or *Pf*CLK-4 although the sub-cellular localization is suggestive of promyelocytic leukemia bodies (PML bodies) and nucleoplasm for the two *Pf*CLK kinases respectively (section 2.1.1).

In collaboration with Prof. Thomas Dandekar and Dr. Armin Robubi, the catalytic domain sequences of the four *Pf*CLK kinases under study were aligned with the homologous kinase Sky1p of *Saccharomyces cerevisae* (Nolen et al., 2001). Sky1p is a well studied non-essential Serine-Arginine protein kinase that has been reported to be involved in mRNA splicing and mRNA transport in the yeast (Siebel et al., 1999). Sequence alignment confirmed the presence of all conserved kinase domains in *Pf*CLK kinases (Figure 3.2). The

positively charged Lysine at position 30 is likely to form a bridge with the negatively charged Glutamate at position 45. The sequence DLKPEN with the conserved Aspartate 126 is present and is considered to be the catalytic base. The loop at positions 169-193 signifies the activation segment, starting with Glutamate 169 and ending with sequence APE in case of *Pf*CLK-1/Lammer, *Pf*CLK-3, *Pf*CLK-4 and SPE in case of *Pf*CLK-2 and Sky1p. Furthermore, the ATP-binding domain GXGXXG is present at positions 8-13 for *Pf*CLK-1 and GXGXXS for *Pf*CLK-3, *Pf*CLK-4 and Sky1p, but is missing in *Pf*CLK-2.

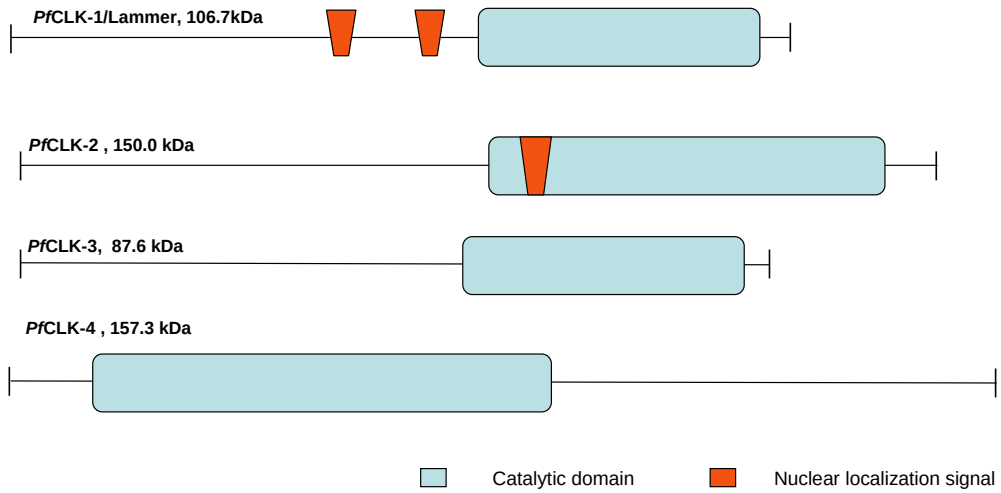
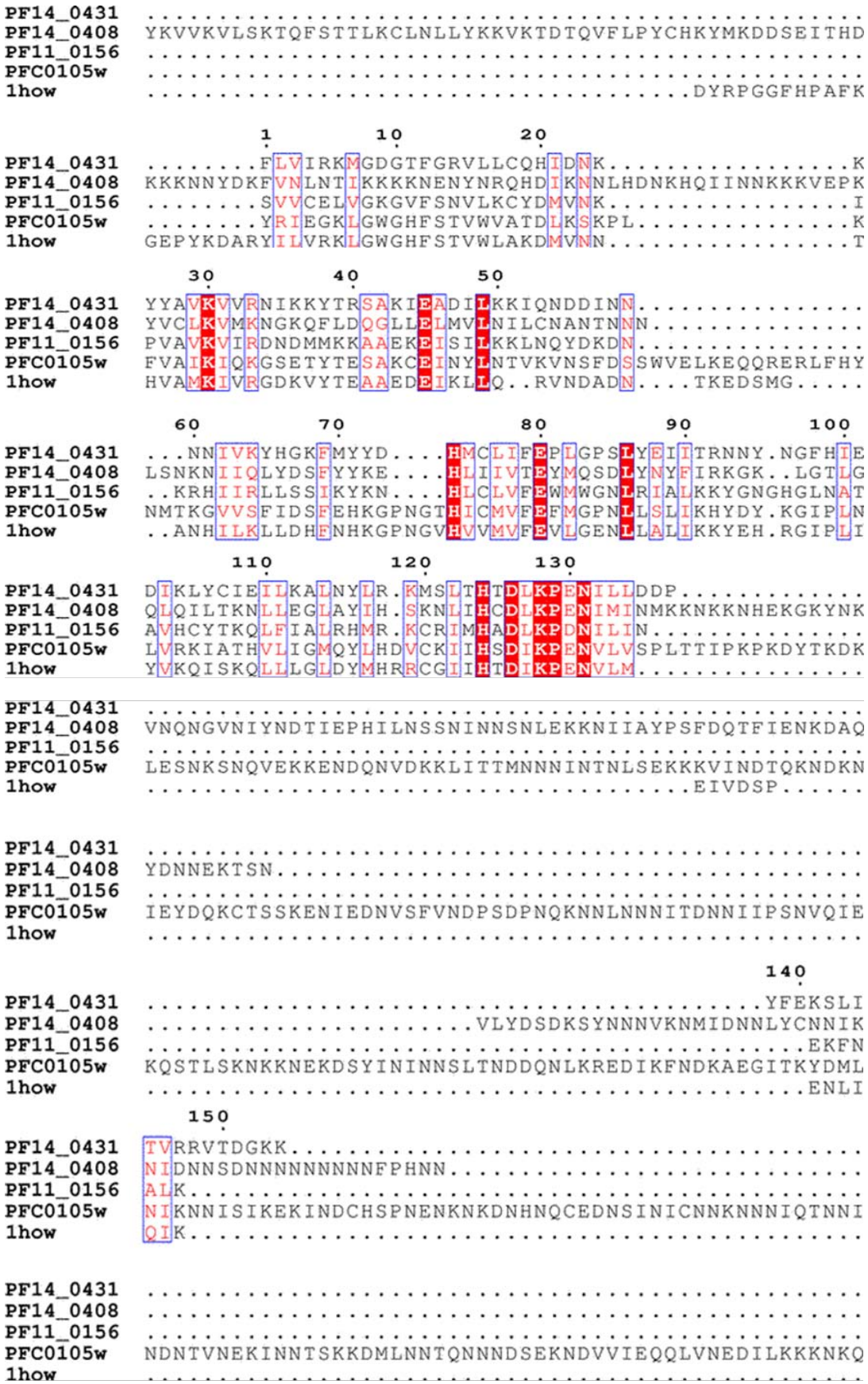


Figure 3.1: Domain architecture of *Pf*CLKs under study. *Pf*CLK-1/Lammer and *Pf*CLK-2 possess putative nuclear localization signals upstream to the catalytic domain.

Sequence alignment with Sky1p further revealed matches between substrate binding residues of the kinases with the substrate binding site of Sky1p. These residues include Arginine 187, Tyrosine 189, Arginine 190 and Glutamate 215 (Figure 3.2). Interestingly, the yeast Sky1p kinase has a specific substrate, Npl3p, which represents a shuttle-protein between the yeast nucleus and the cytoplasm (Siebel et al., 1999; Lukasiewicz et al., 2007). We therefore investigated possible *P. falciparum* homologues of the yeast substrate in silico and identified various putative splicing factors that are homologous to the Sky1p substrate, Npl3p and to the mammalian CLK kinase substrate, ASF/SF2 (Alternative splicing factor). These include the hypothetical proteins PFE0865c, PF10_0217, and PF10_0047 which were common to the BLAST searches of the yeast kinase substrate, Npl3p and to the mammalian kinase substrate, ASF/SF2 sequences against the *P. falciparum* genome. Interestingly, all three protein hits are putative splicing factors or the RNA binding proteins. Additional

proteins having high homology in *P. falciparum* genome like PF11_0205, MAL13P1.120, and PF11_0205 were also identified.



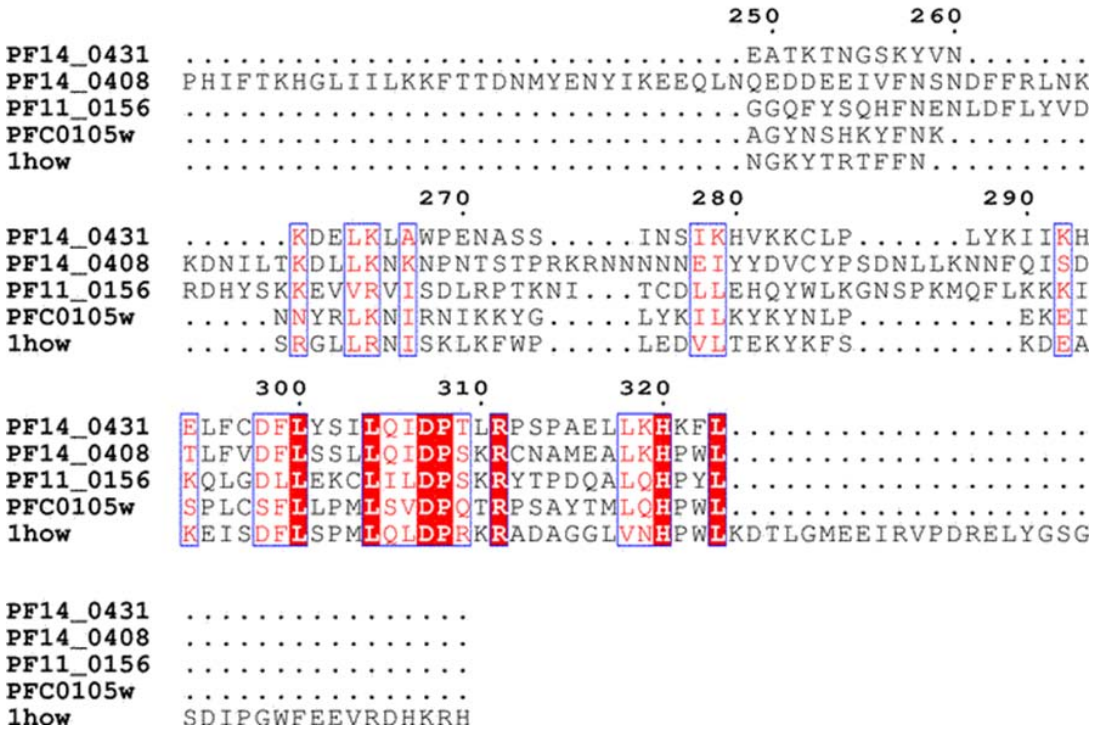


Figure 3.2: Alignment of kinase domains of the four hypothetical CLK kinases with Sky1p, the only member of SRPK in *Saccharomyces cerevisae* (PDB ID; 1how). The amino acid residues shown in red indicate highly conserved sequences essential for the kinase activity.

3.1.2 Stage-specific transcriptional analysis of *Pf*CLK kinases

To analyze the stage-specific transcript expression of the kinases under study, various blood stages of the parasite were purified from parasite cultures throughout the study (Figure 3.3).

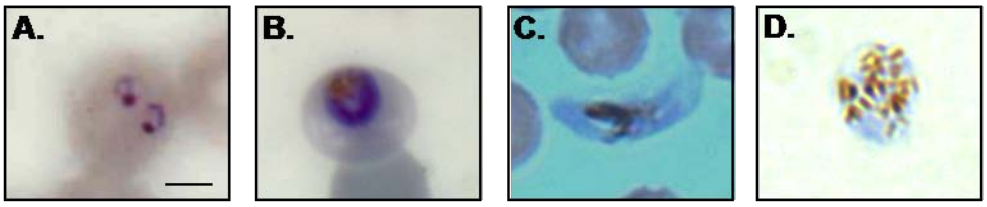


Figure 3.3: Giemsa staining of *P. falciparum* blood stages and zygote
Parasite cultures were synchronized using Albumax II (section 2.2.1.1), smears were prepared and stained in Giemsa solution. **A.** early ring and **B.** schizont stages were visible in the smears obtained from a continous culture flask. **C.** Stage V gametocytes were purified after cultivation of parasite cultures for about three weeks. **D.** Zygotes were obtained by incubation of stage V gametocytes with human serum for 20 h at RT. Bar, 5µm.

Once the blood stages were purified by Accudenz density gradient, total RNA was isolated using the Trizol (Invitrogen) method and cDNA was prepared to investigate the stage-specific expression of these kinases (section 2.2.2.10).

Stage-specific transcript expression of the four *Pf*CLK kinases at various stages was deduced using the sequence-specific PCR amplification of cDNA. This was obtained from the stage-specific RNA using reverse transcriptase. The profile of transcript expression shows that the four kinases were expressed at varied levels during the four different stages analysed. *PfCLK-1/Lammer*, *PfCLK-2* and *PfCLK-3* show transcript abundance in trophozoites while *PfCLK-4* transcript is expressed at low levels (Figure 3.4). Transcript expression of *PfCLK-1/Lammer* and *PfCLK-4* is more predominant in gametocytes than that of *PfCLK-2* and *PfCLK-3*. None of the four kinases under study showed any transcript expression in zygotes. Moreover, no bands were detected in samples lacking the reverse transcriptase (0-RT). Known stage-specific genes were taken as positive controls for the cDNA of respective blood stage. Positive control used for trophozoites was *Pf39*, which encodes a protein localized to endoplasmic reticulum (Templeton et al., 1997), while for schizonts it was *AMA-1* (Apical Membrane Antigen-1, Urquiza et al., 2000).

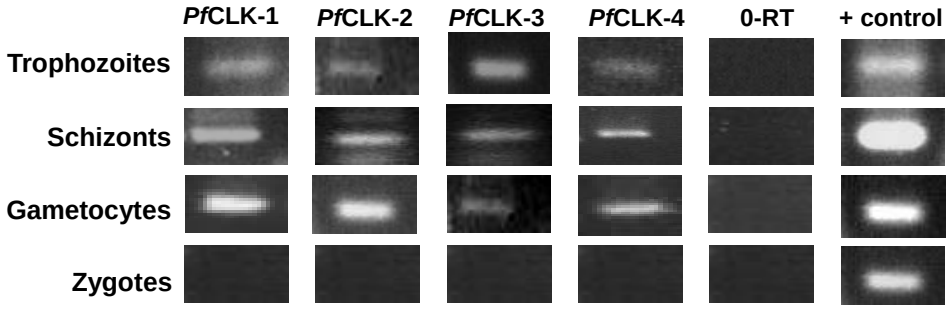


Figure 3.4: Transcript expression of selected *Pf*CLK hypothetical kinases in blood stages of *P. falciparum*. 0-RT refers to the negative control where no reverse transcriptase was added. Positive controls used were, for trophozoites, *Pf39*; for scizonts, *AMA-1*; for gametocytes, *PfCCp1*; for zygotes, *Pfs25*.

Positive control employed for gametocytes was *PfCCp1*. It encodes for a multidomain adhesion protein that localizes with the parasite membrane in the parasitophorous vacuole (Pradel et al., 2004; Pradel et al., 2006) while for zygotes, *Pfs25*, which encodes a surface marker that identifies unfertilized macrogametes, zygotes and ookinetes (Kaslow et al., 1988; Kaslow et al., 1994) was employed as the positive control.

In addition to diagnostic RT-PCR, more sensitive Real Time RT-PCR was also employed (Figure 3.5) in order to compare the expression levels of the four kinases in various blood stages with the known stage-specific proteins.

All the four kinases under study were analysed for the pattern of their expression in various blood stages, both sexual and asexual. Those genes which were known to be expressed in the particular stages were taken as positive controls. This ensured a valid comparison between the levels of expression of the respective unknown kinase under study with the gene that is already known to be expressed in that particular blood stage. All the expression levels that were detected by SYBR Green between threshold crossing cycles 15–25 were considered to be highly expressed whereas those primer signals that were detected above 25 were considered to have a low level expression. The results were analyzed and expressed in the form of a graph (Figure 3.5). As indicated in the graph, the lower the threshold cycle number at which signal is detected, the higher is the transcript expression. Thus, all the four kinases are compared with each other in terms of their transcription expression at trophozoites, schizonts, gametocytes and zygote stages of the parasite.

*Pf*CLK-1, *Pf*CLK-2 and *Pf*CLK-3 are predominantly expressed in trophozoites whereas the expression is low in *Pf*CLK-4. However, contrary to results obtained from real time RT-PCR, diagnostic RT-PCR shows absence of expression of *Pf*CLK-3 and a low level expression of *Pf*CLK-4 in trophozoites. In schizonts and gametocytes all four kinases show high transcript expression.

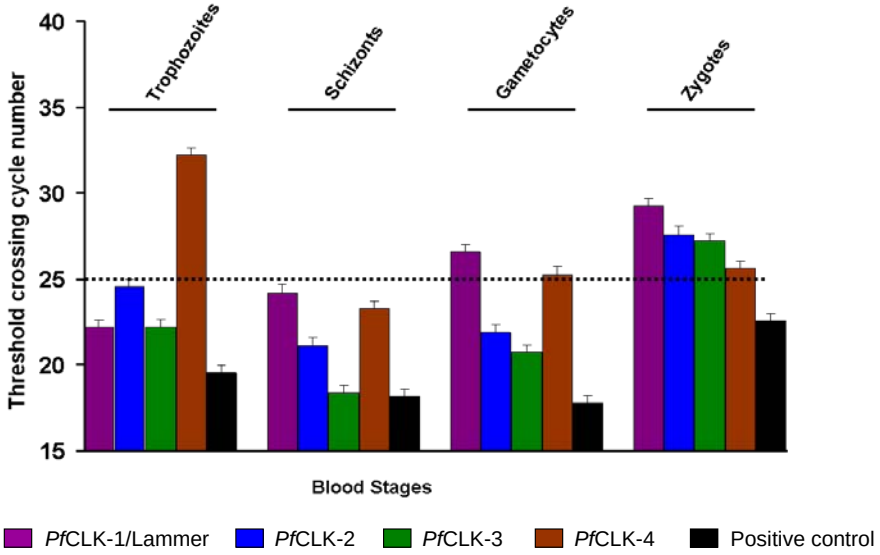


Figure 3.5 Stage-specific expression of selected *Pf*CLK kinases obtained by Real-Time RT-PCR. The threshold crossing cycle number represents the number of cycles above which expression was detectable. The dotted line at threshold cycle 25 indicates that the expression levels detected below this limit represent high expression. The positive control refers to the proteins known to be expressed in each blood stage analysed.

Transcript of *Pf*CLK-4 is highly expressed in schizonts and gametocytes, while has low level expression in zygotes. Positive controls were employed as mentioned for diagnostic RT-PCR. All the positive controls showed expected transcript abundance. Real-time RT-PCR data indicated that the four *Pf*CLK genes are highly expressed in the blood stage parasites. Transcript expression was also found in gametocytes, but decreases in the zygote stage (Figure. 3.5).

3.1.3 Protein expression analysis of *Pf*CLK-1/Lammer and *Pf*CLK-2 in blood stages of the malaria parasite

Expression profile of the two CLK kinases, *Pf*CLK-1/Lammer and *Pf*CLK-2 at the protein level was investigated. For the detection of *Pf*CLK-1/Lammer, a 35.8 kDa portion of the catalytic domain was chosen to be expressed as a recombinant kinase in *E.coli* expression system. The portion of the catalytic domain was fused to an N-terminal 26 kDa GST tag. This made the expected size of the recombinant *Pf*CLK-1/Lammer protein fragment as 61.8 kDa. Overnight bacterial cultures were induced with IPTG (section.2.2.3.1) to express the protein fragment (Figure 3.6). Additional attempts to express the recombinant protein from the region N-terminal to the catalytic domain were not successful. Therefore, further studies were carried out with the recombinant protein encompassing the catalytic domain. A polyclonal mouse antibody against this recombinantly expressed portion of the catalytic domain was generated. In accord with the transcription data, Western blot analysis using antibodies against *Pf*CLK-1/Lammer revealed protein expression in lysates of asexual blood stages (Asex, using the gametocyte-less strain F12) and of gametocytes (GC, using the gametocyte-producing strain NF54) (Figure. 3.8A). The detected protein bands did not migrate at the calculated molecular weight of 107 kDa, but at a band of approximately 60 kDa.

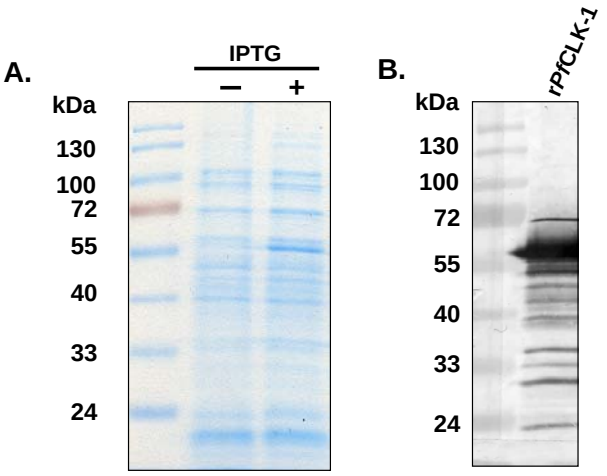


Figure 3.6: Generation of GST-tagged recombinant PfCLK-1/Lammer. **A.** A fragment from the catalytic domain was fused with GST at N-terminal and recombinantly expressed in bacteria. Mice were immunized to generate anti-*Pf*CLK-1/Lammer antibody. **B.** Western blot on the recombinant *Pf*CLK-1 (r*Pf*CLK-1) was performed to verify the detection of the 61. 8 kDa protein by the antiserum generated against it.

As a second source, antisera were generated in rabbit against a peptide sequence upstream of the catalytic domain of *Pf*CLK-1/Lammer. A similar 60 kDa protein band was detectable in Western blot on lysates of asexual and gametocyte stages of the parasite (Figure 3.7). An additional faint protein band with an approximate molecular weight of approximately 100 kDa was observed, which might represent the 107 kDa full-length kinase protein. No protein bands were detected in the lysates of uninfected erythrocytes (EC) or in neutral rabbit serum (NRS) used as negative controls.

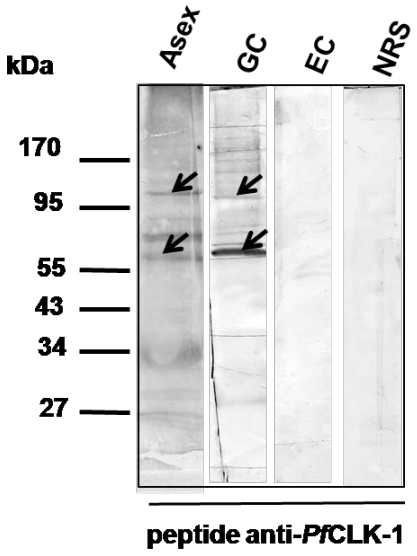


Figure 3.7: Protein expression of *Pf*CLK-1/Lammer. Western blot analysis was performed on the parasite lysates using rabbit antisera generated against a peptide sequence upstream of the *Pf*CLK-1/Lammer catalytic domain. Protein bands at sizes 60 kDa and approximately at 100 kDa were detected. For protein detection in asexual parasites, lysates of the gametocyte-less strain F12 (ASEX) and for detection of gametocytes, lysates of the gametocyte-producing strain NF54 (GC) were used. Lysates of uninfected erythrocytes (EC) was used as negative control.

To experimentally verify the presence of the kinase into the parasite nucleus, the nuclear pellet from mixed asexual parasite stages was collected. Via Western blot analysis, the above 60 kDa protein band was also detected in the nuclear pellet (NP, Figure 3.8A). No protein bands were detected in lysates of uninfected erythrocytes (EC) or in the neutral mouse serum (NMS), used as negative controls (Figure 3.8A). The Western blot data is indicative of the possible processing of the *Pf*CLK-1/Lammer during its expression and its shuttle to the nucleus such that only the C-terminal part comprising the catalytic domain is directed to the nucleus for further activity.

Protein expression was further investigated by immunofluorescence assay on *P. falciparum* strain, NF54. Immunolabelling revealed a punctuated expression of *Pf*CLK-1/Lammer in a subset of asexual parasites, particularly in trophozoites, where kinase expression was mainly observed in association with the parasite nucleus (Figure 3.8B). *Pf*CLK-1/Lammer was also found in gametocytes in association with the gametocyte nucleus (Figure 3.8C).

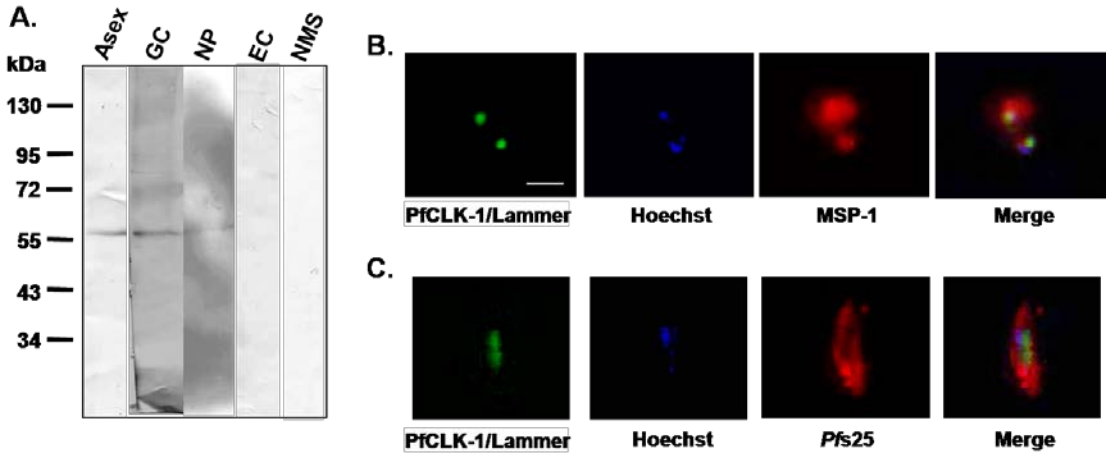
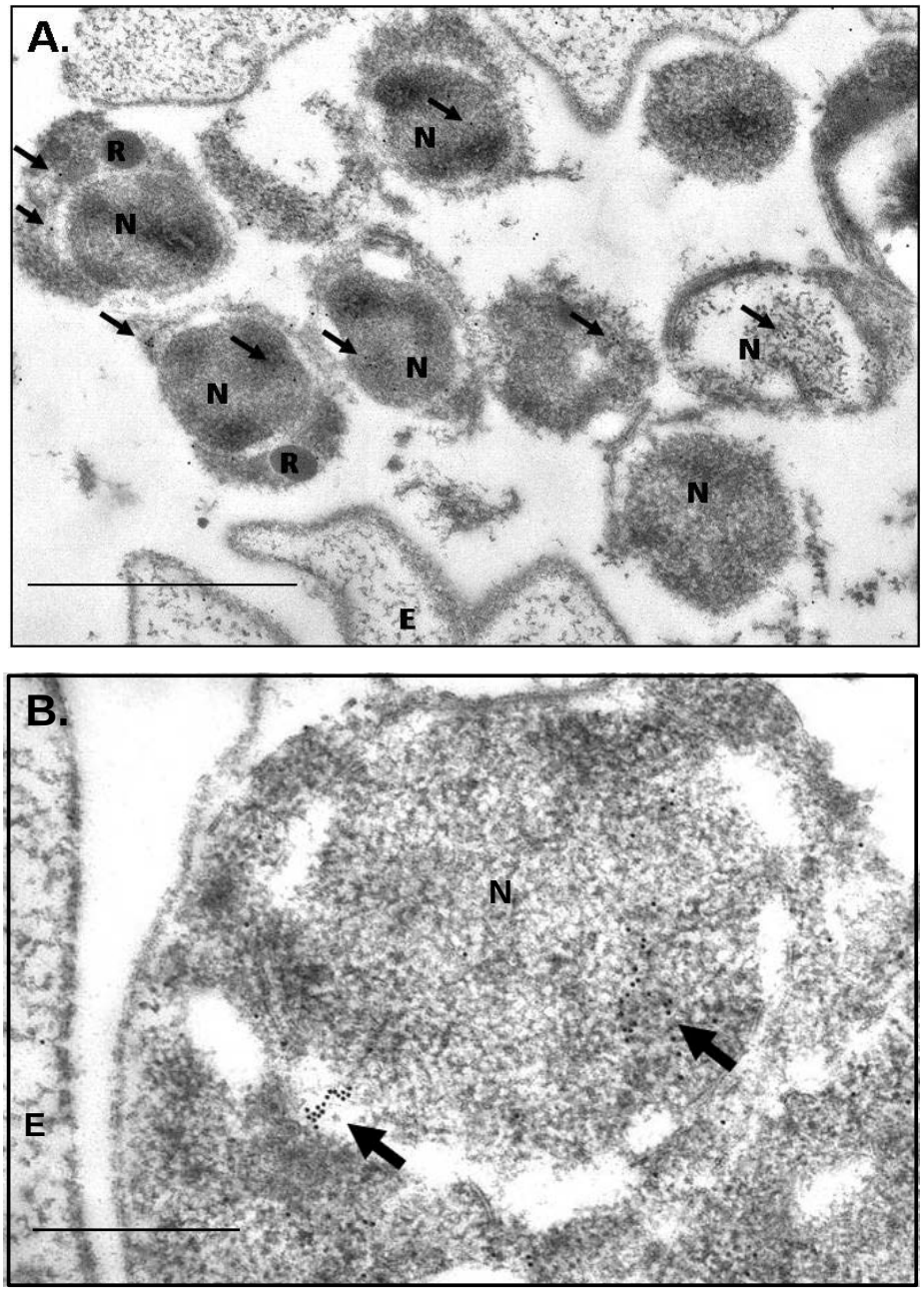


Figure 3.8: Protein level expression of *Pf*CLK-1 in blood stages of the parasite.
A. Western blot on lysates of asexual (Asex., gametocyte-less strain, F12), gametocyte (GC., gametocyte-producing strain, NF54) stages of the parasite show a protein migrating at approximately 60 kDa. Parasite nuclear pellet (NP) was isolated and the 60 kDa, *Pf*CLK-1 could be detected. Uninfected erythrocytes (EC) and neutral mouse serum (NMS) were used as the negative controls. **B.** Indirect immunofluorescence assay was performed using mouse polyclonal antibodies generated from the catalytic domain of *Pf*CLK-1. Punctuated protein expression was detected in asexual parasites. **C.** Protein expression in association with the nucleus was also detectable in gametocytes. Asexual parasites were detected with antibodies against the merozoite surface protein MSP-1, and gametocytes were labelled with antibodies against the sexual stage protein *Pfs*25. Nuclei were highlighted by Hoechst staining. Bar, 5 µm.

Asexual stage parasites were highlighted by labelling of the merozoite surface protein MSP-1 (Herrera et al., 1993) and gametocytes by labelling of *Pf*s25 (Carter et al., 1989). The protein *Pf*s25 is abundantly expressed on the surface of macrogametes and zygotes, but is also present in mature gametocytes within vesicular structures (Scholz et al., 2008).

Protein expression was subsequently investigated by immunoelectron microscopy, via post-embedding labelling using gold-conjugated secondary antibodies for detection. Gold-labelling was mostly found in association with the nuclei, but to a lower extent also in the cytoplasm of malaria parasites.



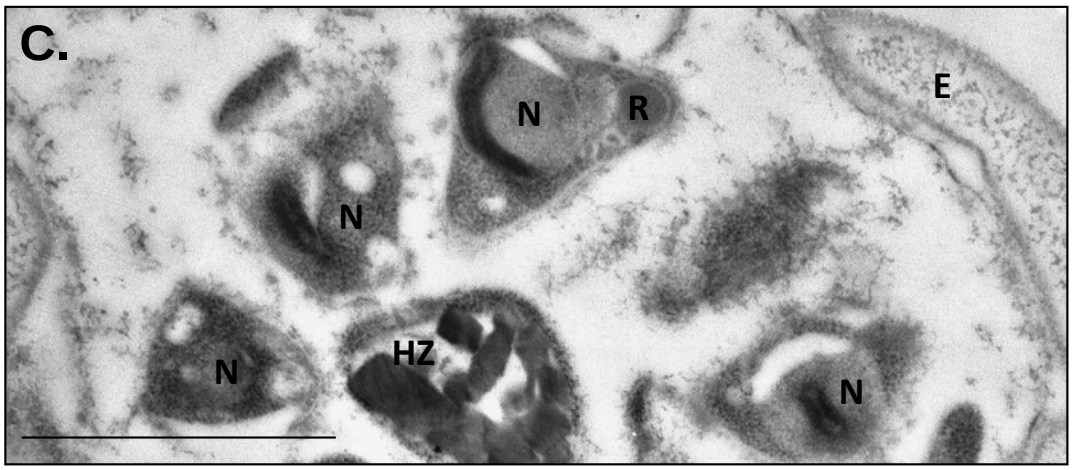


Figure 3.9: Post-embedding immunoelectron microscopy labelling with secondary immunogold antibody revealed protein expression in the nucleus as well as the cytoplasm of an asexual blood stage parasite. **A, B** *Pf*CLK-1 labelling was particularly detected in a passage between nucleus (N) and cytoplasm. **C.** Only secondary antibody was used as a negative control where no unspecific labelling was detected in nucleus, haemozoin (HZ), rhoptries (R) or unifected erythrocytes. Arrows indicate gold particles. Bar: - A, C: 1 µm; B: 0.5 µm.

At one instance, intense gold-labelling was detected at a passage between nucleus and cytoplasm, possibly a nuclear pore (Fig. 3.9A, B), indicating that the kinase might be involved in phosphorylating the splicing factors and to its possible shuttle between the nucleus and the cytoplasm during this process. No unspecific labelling for *Pf*CLK-1/Lammer was found in the rhoptries of merozoites, or in the erythrocyte cytoplasm (Figure 3.9C).

For the detection of stage-specific protein expression of *Pf*CLK-2, attempts to generate a recombinant protein were made using primers from regions within and outside to the catalytic domain (Figure 2.6, section 2.2.3.1). Although, the recombinant protein from the N-terminus region could be purified (Figure 3.10A, B), the antiserum generated against *Pf*CLK-2 could not detect the kinase by a Western blot analysis or by an immunofluorescence assay. However, the antiserum thus generated recognised the recombinant *Pf*CLK-2 protein (Figure 3.10C). Attempts to generate the recombinant protein from the catalytic domain were not successful.

Therefore, in a third attempt, a mouse polyclonal antibody against a peptide sequence upstream of the catalytic domain was generated (section 2.1.11). Western blot data on parasite lysates indicated presence of the kinase in asexual parasites (Asex) as well as in gametocytes (GC) (Figure 3.11A). The

detected protein bands migrated at the expected molecular weight of 150 kDa. Occasionally, additional bands with approximate molecular weights of 70, 50 and 40 kDa were detectable, indicating protein processing. Full-length protein band was also detected in the nuclear fraction (NP). No protein bands were detected in lysates of uninfected erythrocytes (EC) or in neutral mouse serum (NMS) (Figure 3.11A).

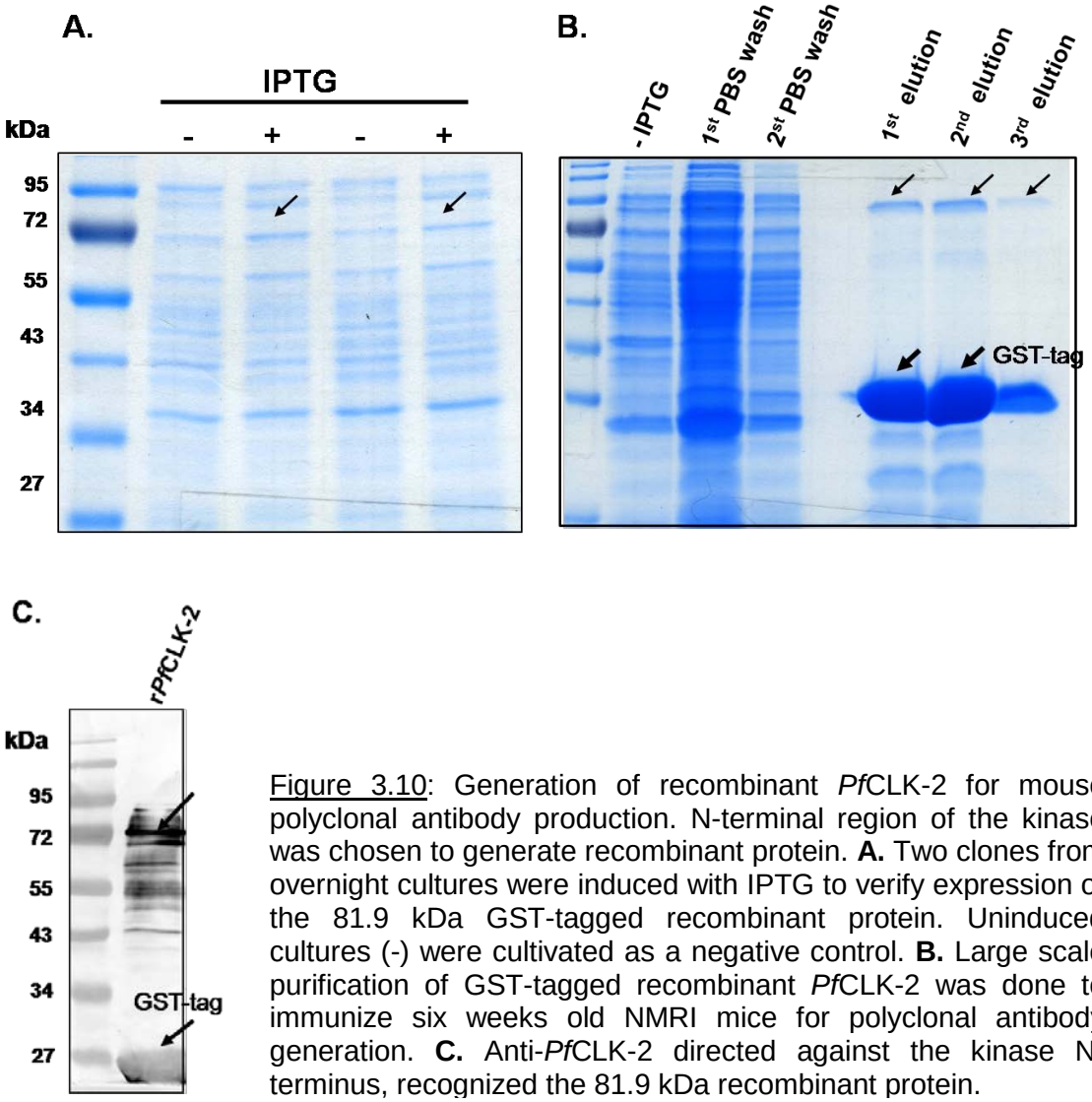


Figure 3.10: Generation of recombinant *Pf*CLK-2 for mouse polyclonal antibody production. N-terminal region of the kinase was chosen to generate recombinant protein. **A.** Two clones from overnight cultures were induced with IPTG to verify expression of the 81.9 kDa GST-tagged recombinant protein. Uninduced cultures (-) were cultivated as a negative control. **B.** Large scale purification of GST-tagged recombinant *Pf*CLK-2 was done to immunize six weeks old NMRI mice for polyclonal antibody generation. **C.** Anti-*Pf*CLK-2 directed against the kinase N-terminus, recognized the 81.9 kDa recombinant protein.

In immunofluorescence assays, the anti-*Pf*CLK-2 antibody labelled the nuclei as well as the cytoplasm of asexual blood stages (Figure 3.11B) and gametocytes (Figure 3.11C). Asexual stage parasites were highlighted by MSP-1 labelling, and gametocytes by labelling of alpha-tubulin II (Rawlings et al., 1992).

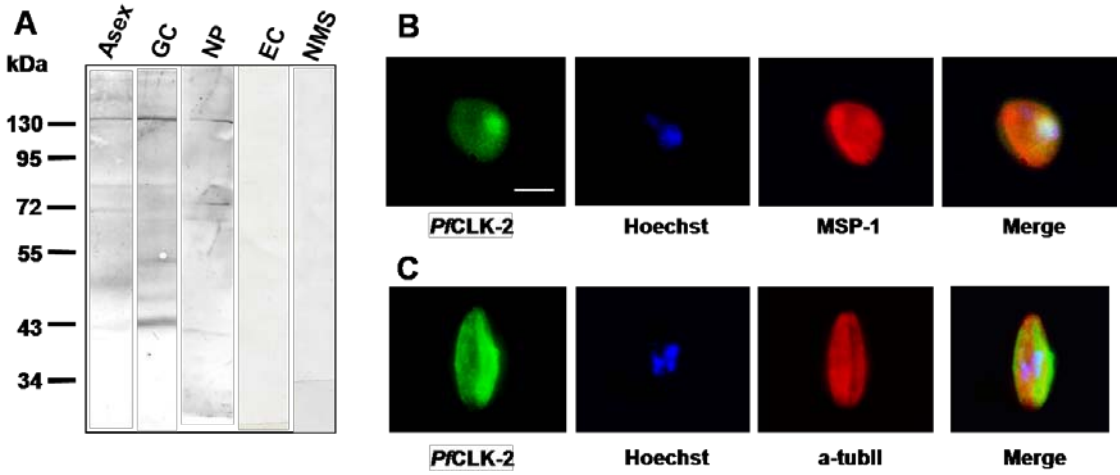


Figure 3.11: Blood stage expression of *Pf*CLK-2 and its co-localization with the parasite nucleus. **A.** Western-blot analysis on parasite lysates, using mouse peptide antibody against a region upstream of the *Pf*CLK-2 catalytic domain, revealed a full size protein band at 150 kDa, as well as lower molecular weight protein bands at 70, 50 and 40 kDa for asexual (Asex., gametocyte-less strain, F12) and gametocyte (GC., gametocyte-producing strain, NF54) stages. Protein bands also migrated at these sizes in a nuclear pellet fraction (NP). Lysates of uninfected erythrocytes (EC) and neutral mouse serum (NMS) were used as negative controls. **B.** Indirect immunofluorescence assay showed protein expression in the cytoplasm and in association with the nucleus of asexual parasites. **D.** A similar protein expression pattern was also detected in gametocytes. Asexual parasites were labelled with antibodies against MSP-1, and gametocytes were labelled with antibodies against alpha-tubulin II. Nuclei were highlighted by Hoechst staining. Bar, 5 µm.

The intracellular localization of *Pf*CLK-2 was confirmed by immunoelectron microscopy, where gold-labelling was detected in the nuclei and the cytoplasm of blood stage parasites. No labelling for *Pf*CLK-2 was found in the food vacuole, the merozoite rhoptries or in erythrocytes (Figure 3.12A, B).

To investigate expression of both kinases in transmission stages of mosquito, mature gametocyte cultures were membrane fed to *Anopheles stephensi* (section 2.2.1.4). Mosquitoes were dissected at different intervals to obtain mosquito midgut stages of the parasite.

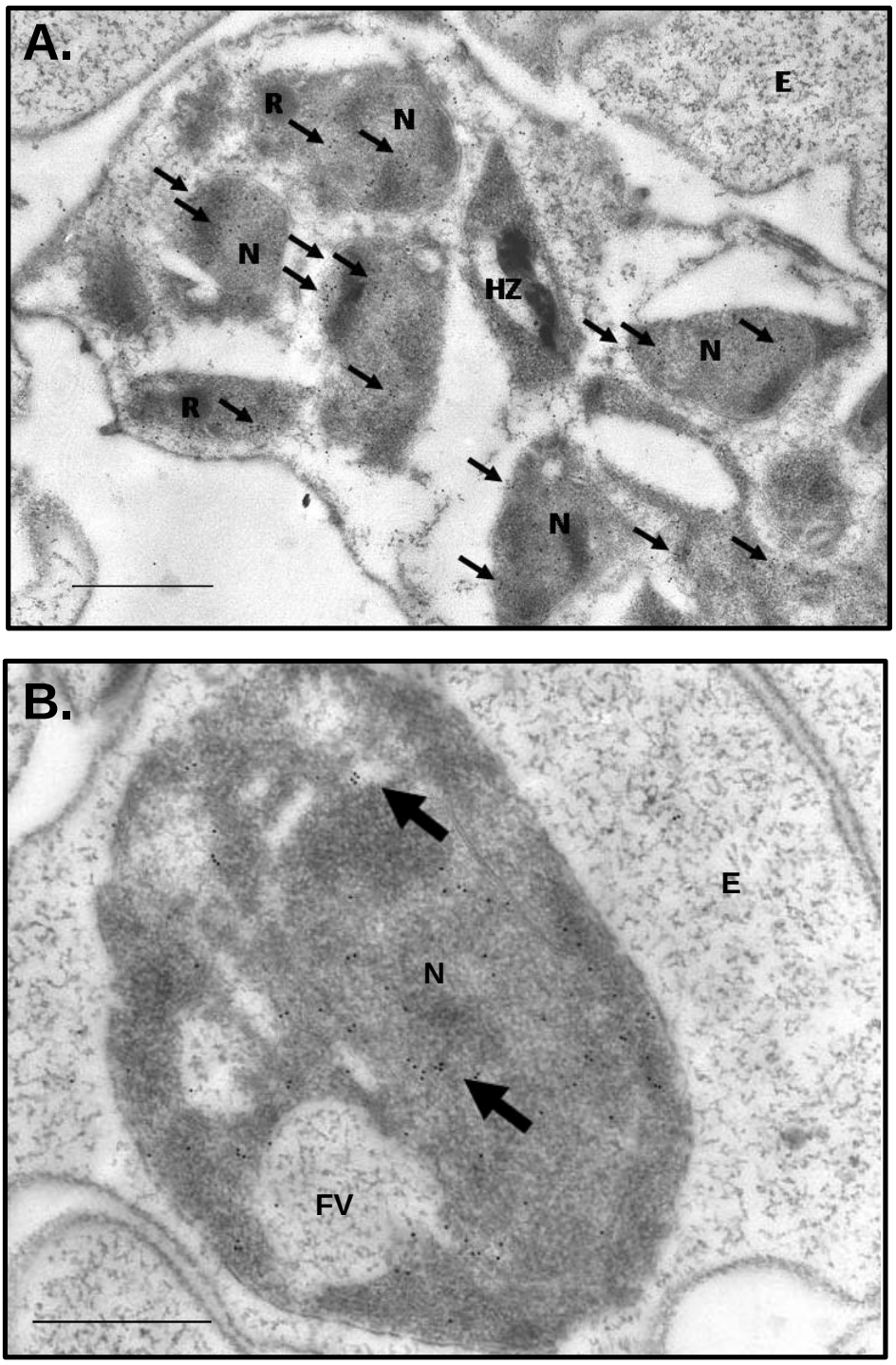
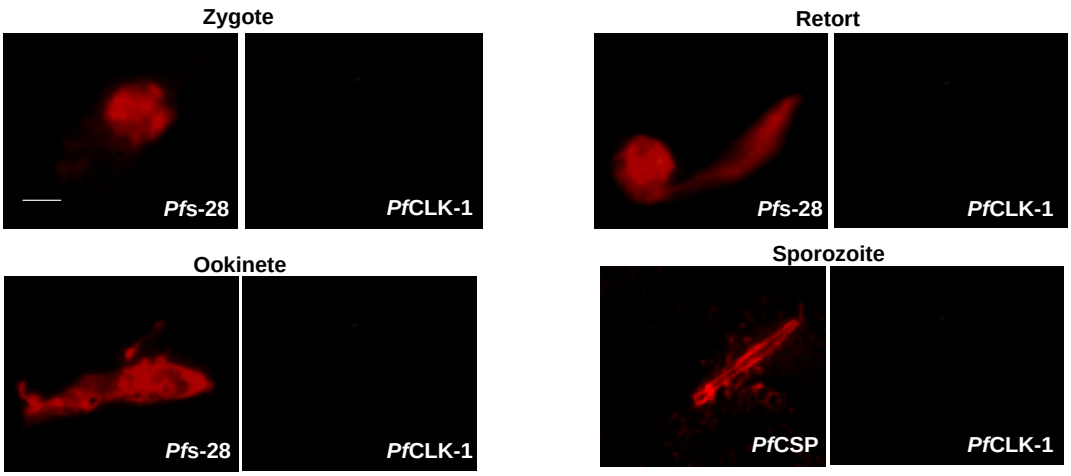
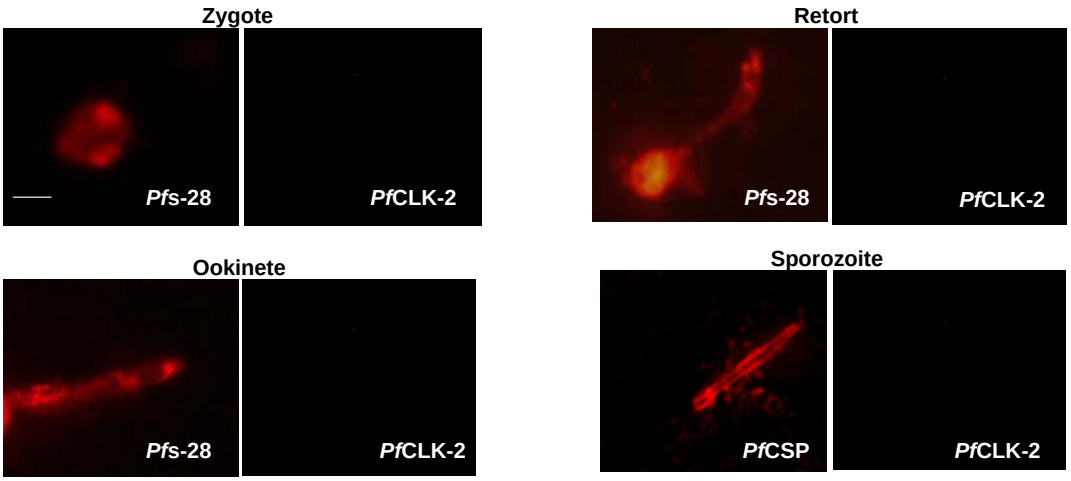


Figure 3.12: Post-embedding immunoelectron microscopy labelling with secondary immunogold antibody revealed protein expression of *Pf*CLK-2 in the nucleus as well as the cytoplasm of an asexual blood stage parasite. **A.** No labelling was detected in uninfected erythrocytes (E), hemozoin (HZ), rhoptries (R), bar: - 1 µm or in **B.** food vacuole (FV). Bar, 0.5 µm.

A. Expression of *Pf*CLK-1 in transmission stages of *P. falciparum*



B. Expression of *Pf*CLK-2 in transmission stages of *P. falciparum*



C. Antibody controls

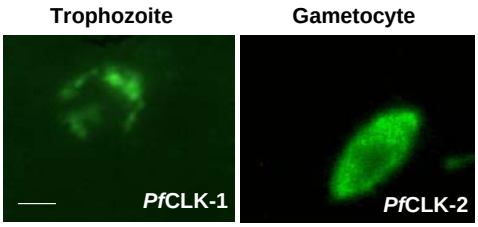


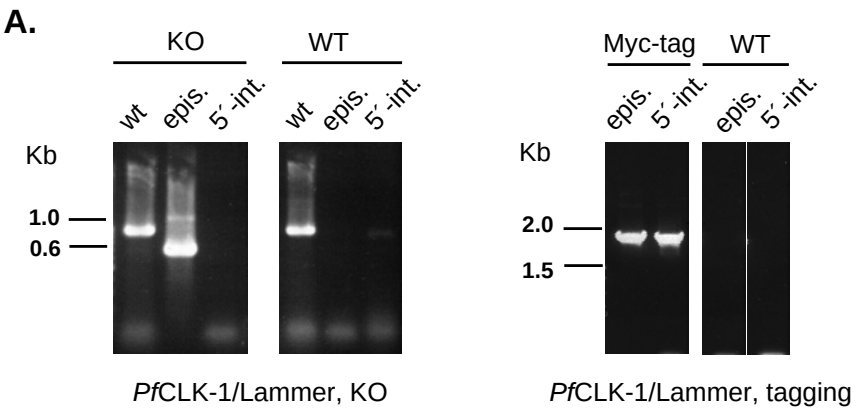
Figure 3.13: Expression of *Pf*CLK-1/Lammer and *Pf*CLK-2 during the transmission stages in the mosquito was investigated via indirect immunofluorescence assay. **A.** No labelling of *Pf*CLK-1/Lammer (in green) was detected in zygotes, retorts, ookinetes and sporozoites suggesting, that its expression is limited to blood stages of the parasite. **B.** Similarly, no expression of *Pf*CLK-2 was detected in the parasite transmission stages. A double-labelling.of midgut stages was performed with stage-specific protein, *Pf*s28 (in red) and sporozoites were labelled with the *Pf*CSP protein (in red). **C.** Expression of both *Pf*CLK-1/Lammer and *Pf*CLK-2 was detected (in green) in the blood stages co-ingested with the mature gametocyte culture by the mosquito during the membrane feeding procedure. Bar, 5 µm.

Midgut smears prepared 24 h post-feeding were tested for zygotes, retort and ookinete stages. For sporozoites, mosquito salivary glands were dissected about 22 days post feeding. Immunofluorescence assay revealed the absence of expression of both *Pf*CLK-1/Lammer (Figure 3.13A) and *Pf*CLK-2 (Figure 3.13B) in the zygote, retort/ookinete and sporozoite stages. However, the blood stage parasites co-ingested by the mosquito during membrane feeding showed labelling with the respective kinase antisera (Figure 3.13C). Mosquito midgut stages were kindly provided by Dr. Matthias Scheuermayer.

3.1.4 Reverse genetics studies on *Pf*CLK kinases

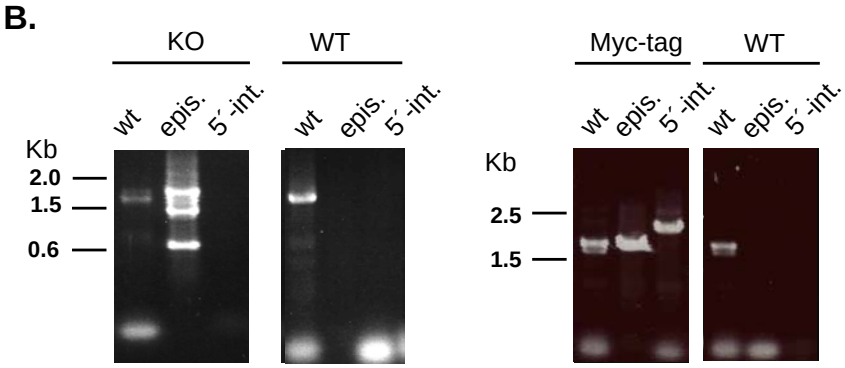
In order to investigate the function of *Pf*CLK kinases in the parasite life cycle, gene-disruptions were generated (Figure 2.4) using a recently described reverse genetics approach (Dorin-Semblat et al., 2007). It aimed at analysing if the kinases play an essential role in the parasite replication. Firstly, knock-out (KO) of the respective *Pf*CLK kinases was attempted to generate by a single cross-over homologous recombination technique, using the pCAM-BSD vector (Sidhu et al., 2005). The KO vector contained an insert corresponding to a portion of the kinase catalytic domain as well as a cassette conferring resistance to blasticidin. Integration of such a vector into the respective *Pf*CLK gene would result in a gene-disruptant (pseudo-diploid) gene locus, in which the conserved ATP-binding region and the proline-glutamate (PE) motif of the catalytic domain are separated (Figure 2.4) rendering expression of a truncated non-functional enzyme.

The ring stage parasites of *P.faciparum* strain 3D7 were electroporated with pCAM-BSD-based *Pf*CLK-KO vectors. After onset of drug pressure, the number of parasites was initially reduced, but a sub-population of blasticidin-resistant parasites emerged after three weeks. However, even after prolonged culture of over 20 weeks, only the parasites containing non-integrated episomes were detected. No integration of the respective *Pf*CLK-KO vector could be detected for any of the four kinase genes by diagnostic PCR (Figure. 3.14). Noteworthy, isochronously the locus of *Pf*PKRP kinase was disrupted by the same strategy (S. Agarwal, C. Doerig, R. Schillig, G. Pradel, unpublished observations), indicating correct conduction of the method



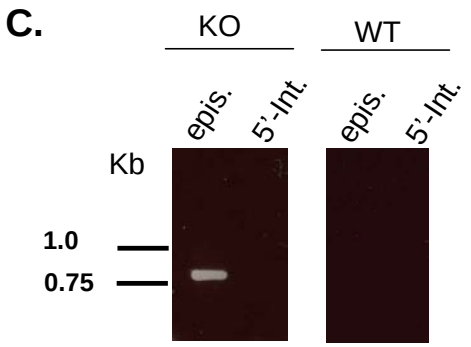
*Pf*CLK-1/Lammer, KO

*Pf*CLK-1/Lammer, tagging

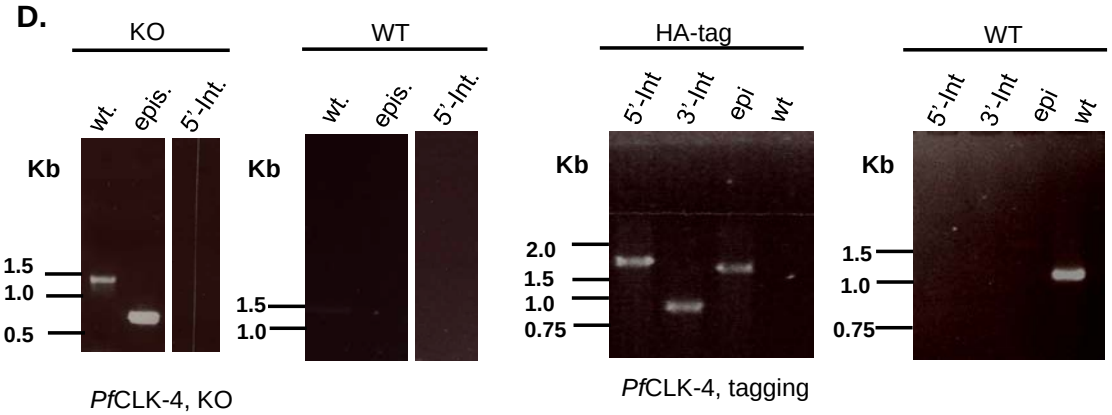


*Pf*CLK-2, KO

*Pf*CLK-2, tagging



*Pf*CLK-3, KO



*Pf*CLK-4, KO

*Pf*CLK-4, tagging

Figure 3.14: Molecular analysis of four *Pf*CLK kinases by reverse genetics approach. Diagnostic PCR from genomic DNA (gDNA) isolated from parasites transfected with the pCAM-BSD-KO vector showed amplification only in the DNA bands correlating to the wild-type (WT) and of the episome. No amplification was observed at the expected sizes of the 5'- or 3'- integrations for any of the four CLKs under investigation. Recombinogenicity of the *Pf*CLK genes was assessed by integration of the 3'-tagged kinase into the respective gene locus. **A.** *Pf*CLK-1-KO showed amplification band of the wild-type (WT) gene locus (977 bp) and episome (672 bp). Diagnostic PCR, revealing bands for episome (1.93 kb) and 5'-integration (1.92 kb) in case of *Pf*CLK-1Myc confirmed successful integration. **B.** Similarly, only bands for the WT gene locus (1.631 kb) and episome (652 bp), but no band for 5'-integration were detected, when gDNA from parasites transfected with the *Pf*CLK-2-KO-vector was PCR amplified. Recombination of the *Pf*CLK-2-Myc vector was identified by bands for episome (1.67 kb) and 5'-integration (2.34 kb) together with bands for the WT gene locus (1.63 kb) from gDNA of transfected mixed cultures. **C.** Only the episomal band (794 bp) was observed for *Pf*CLK-3-KO with no expected WT (1.48 kb). No clear amplification could be obtained from gDNA isolated from *Pf*CLK-3-HA tagged transfected cultures. **D.** gDNA from *Pf*CLK-4-KO on the other hand gave both the WT band (1.44 kb) and the episomal band (672 bp) but no amplification at the expected integration sizes. *Pf*CLK-4-HA however successfully integrated with detectable 5'-integration (2.6 kb), 3'-integration (899 bp) and episome (2.36 kb) and no wild type (1046 bp). Untransfected wild-type 3D7 parasites were used as positive control for each of the WT gene locus. PCR amplifications were regularly tested until 24 weeks post transfection. WT parasites of strain 3D7 were used as a control for the presence of WT gene locus.

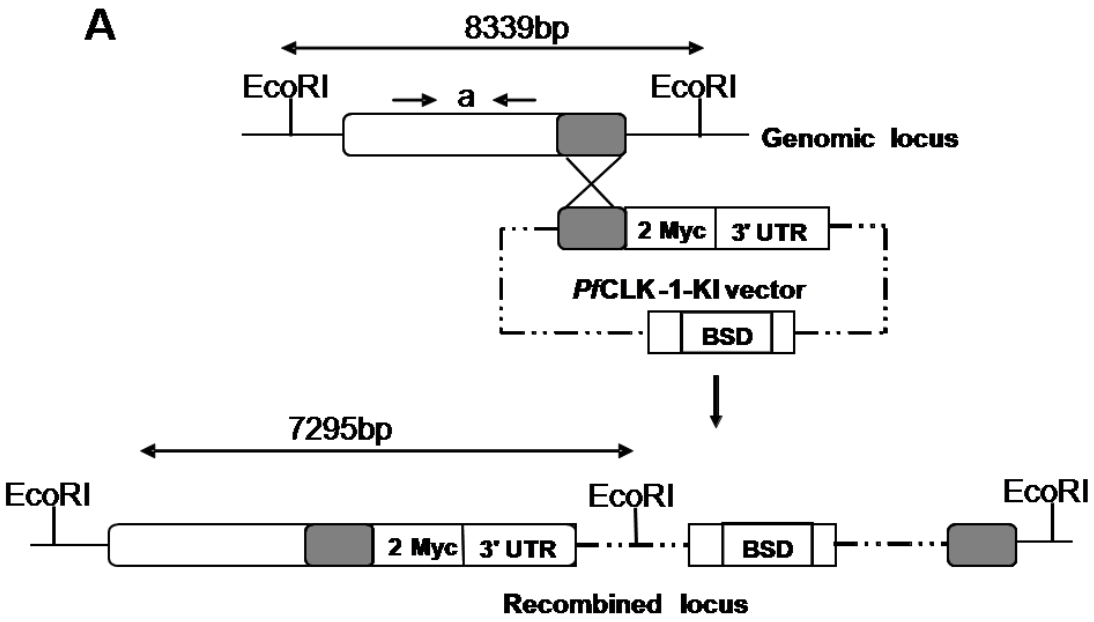
To verify that the genomic loci for the four *Pf*CLK kinases were accessible for recombination, a second pCAM-BSD-based vector was generated. These vectors contained an insert homologous to the 3´-end of the respective kinase gene, which was fused to the sequence either of a Myc/HA/GFP-epitope, followed by the 3´-untranslated region from the *P. berghei dhfr-ts* gene (Figure 2.5). Single cross-over homologous integration of the vector would result in a complete kinase gene followed by a 3´-located tag-sequence. The reverse genetics approach would be expected to result in the expression of a functional, tagged kinase and prove that the technique is feasible.

The ring stage parasite cultures were electroporated with *Pf*CLK-tagged vectors and treated with blasticidin as described above. A subpopulation of drug-resistant parasites was observed after approximately three weeks. gDNA was isolated and investigated for vector integration by a diagnostic PCR (Figure 3.14) using gene-specific primers and the primers from the flanking vector sequences (section 2.1.15). gDNA was isolated from parasite cultures 60 d post-transfection and PCR amplification readily demonstrated the integration of tagged kinases into the respective gene locus. *Pf*CLK-1 was

tagged with both Myc- as well as HA-epitope. *Pf*CLK-2 was successfully Myc-tagged while *Pf*CLK-4 integrated with a fused HA-tag. PCR amplification of *Pf*CLK-4-HA was performed in collaboration with Selina Kern.

The successful integration of tagged kinase was subsequently confirmed by Southern blot analysis for *Pf*CLK-1/Lammer in mixed Myc-tagged parasite cultures using the two HA clones A11 and G12 which were isolated by clonal dilution method (section 2.2.1.3). Recombination in the gene locus was tested by restriction digest pattern of recombined locus with respect to the wild-type locus in 3D7 parasites (Figure 3.15A). *Pf*CLK-1/Lammer was digested with EcoRI restriction enzyme present on either side of the kinase sequence and the relative band sizes of the digested WT (8339 bp) vs recombined locus (7295 bp) were calculated. The difference of the digestion pattern was detected with a probe ‘a’, located 5’ to the insert cloned in the tagging vector (Figure 3.15A). WT band was observed neither in the mixed population of Myc-tagged parasites nor in the isolated HA-tagged clones, A11 and G12 (Figure 3.15B).

Western blot of Myc-tagged *Pf*CLK-1/Lammer demonstrated an immunoreactive band at approximately 60 kDa while no full-size band for *Pf*CLK-1/Lammer at 107 kDa could be detected. No protein band was detected in the non-integrated WT lysate used as a control (Figure 3.15C).



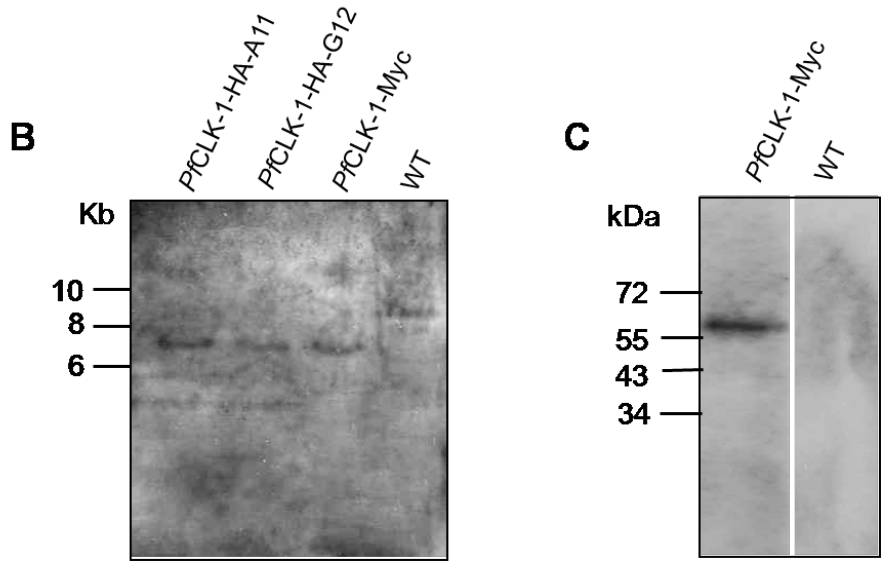


Figure 3.15: *Pf*CLK-1/Lammer genomic locus is accessible for recombination.
A. Schematic of *Pf*CLK-BSD-KI vector containing an insert homologous to the 3´-end of the respective kinase gene, fused to the sequence of double Myc or double HA-epitope, followed by the 3´-untranslated region from the *P. berghei dhfr-ts* gene together with a blasticidin resistance cassette. Schematic shows EcoRI digestion sites with the size of expected fragments in WT and recombined locus. Oligonucleotide probe 'a' was chosen from the 5'-end of the gene. **B.** Southern blot showing successful recombination of *Pf*CLK-1-tagging vector with the genomic locus. HA and Myc-tagged recombined fragment (7295 bp) could be separated by agarose gel electrophoresis and detected by labelled probe 'a'. WT gDNA showed an expected band size of 8339 bp as expected for the WT locus. **C.** Lysate of Myc-tagged *Pf*CLK-1/Lammer parasites was used to detect the tagged parasites using a rabbit anti-Myc antibody via a Western blot. A protein band was detected at approximately 60 kDa and no band was detected in lysate of WT 3D7 parasites used as a negative control.

Recombination for the *Pf*CLK-2 gene locus was investigated by comparing restriction digest pattern of EcoRV and BglII using the gDNA isolated from integrated and non-integrated parasites. Detection was based on hybridization of a labelled probe (a) derived from the PCR amplification of the insert from the *Pf*CLK-2-tagging construct (Figure 3.16 A). Selection of probe within the recombination locus enabled the detection of both the 5'-integration and the 3'-integration.

As confirmed via Southern blot analysis, upon recombination the integrated parasite population yielded a digested fragment of 6955 bp as 5'-integration and a 9060 bp fragment as 3'-integration (shown by arrows) An episomal band at approximately 6000 bp was additionally detected. The non-integrated 3D7 parasites retained a 9968 bp band corresponding to the wild type gene locus (Figure 3.16 B).

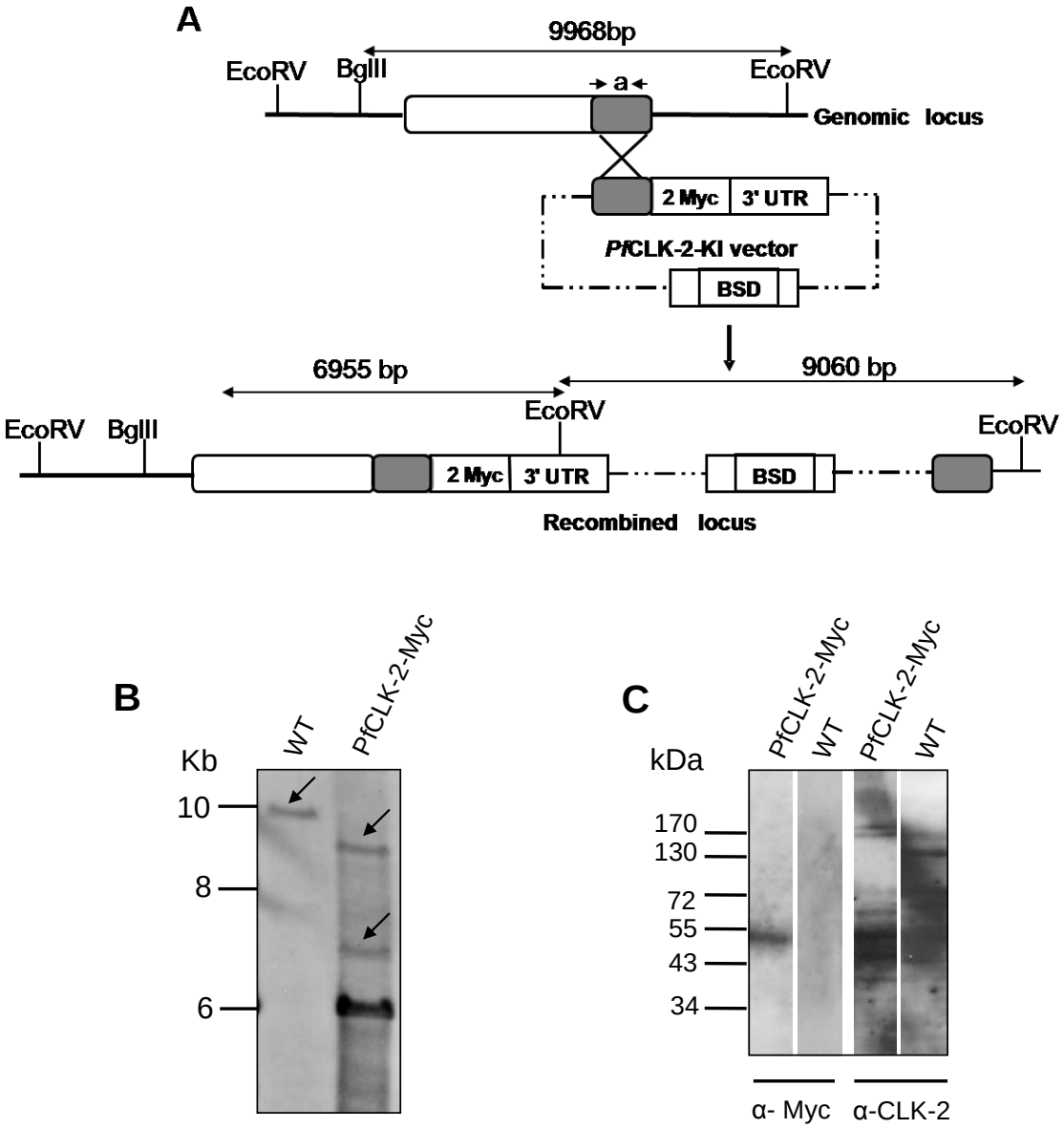


Figure 3.16: *Pf*CLK-2 locus is amenable for homologous recombination.
A. A schematic showing digestion pattern in the gene locus of the recombined and non-integrated 3D7 paraites. Oligonucleotide probe 'a' was PCR amplified and labelled for the detection. **B.** Southern blot analysis showed that EcoRV and BglII digest the respective gene loci resulting in a specific digestion pattern yielding fragments of 6955 bp and 9060 bp (arrows) in integrated population of mixed parasites.An additional band at approximately 6000 bp was detected for episome. A fragment of 9968 bp was detected in WT 3D7 parasites. **C.** Western blot detected the 50 kDa protein band in Myc-tagged *Pf*CLK-2 parasite lysate. No band was detected in the WT control. Stripped blot was detected with mouse polyclonal peptide anti-*Pf*CLK-2 antibody and a 50 kDa band was detected in Myc tagged lysate whereas additional 150 kDa and 70 kDa bands migrated at the full-length and processed protein respectively.

The presence of Myc-tagged protein was demonstrated by Western blot analysis on lysate of Myc-tagged mixed *Pf*CLK-2 culture. Detection of

Myc-tagged *Pf*CLK-2 by Western blotting revealed a protein band of 50 kDa. Neither the full size protein band of 150 kDa, nor protein bands with approximate molecular weights of 70 and 40 kDa were detected (Figure 3.12A, 3.16C). The blot was therefore stripped and protein bands at 150 kDa, 70 kDa and 50 kDa were detected using the mouse anti-*Pf*CLK-2 antibody. Labelling with this antibody revealed that no full size *Pf*CLK-2 protein and no 70 kDa protein are present in the Myc-tagged cultures, as described in lysates of WT control (Figure 3.16C). A 40 kDa protein was detected in the lysates of both Myc-tagged mixed and WT cultures. This could be a result of either a processed N'-terminal kinase or a result of unspecific cross-reactivity. Myc-tagged *Pf*CLK-2 therefore appears to result in a predominant 50 kDa processed protein.

The combined reverse genetics data suggest that the gene loci for *Pf*CLK-1/Lammer and *Pf*CLK-2 are amenable to homologous recombination. The inability to disrupt the respective genes is thus likely to be due to an essential role of the two kinases in the asexual replication cycle of the parasite.

3.1.5 Kinase activity assays on *Pf*CLK-1/Lammer and *Pf*CLK-2

In order to test the capacity of recombinant *Pf*CLK-1/Lammer to phosphorylate the exogenous substrates, kinase assays were performed as described (Reininger et al., 2009). Recombinant GST-tagged *Pf*CLK-1/Lammer was expressed from the catalytic domain of the kinase and purified to be employed in the kinase activity assay in which a mixture of Histone HI, myelin basic protein (MBP), and α/β caseins was used as substrates (section 2.2.3.8). Further, in order to analyse, if an intrinsic substrate from the parasite extract activates the kinase, recombinant *Pf*CLK-1/Lammer was coated on the Glutathione-sepharose beads and incubated with heat inactivated parasite extract of strain 3D7 before proceeding with the kinase assay. Autoradiography was performed to visualize the phosphorylation signal (Figure. 3.17).

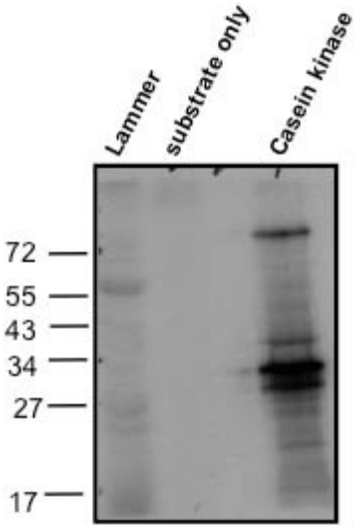
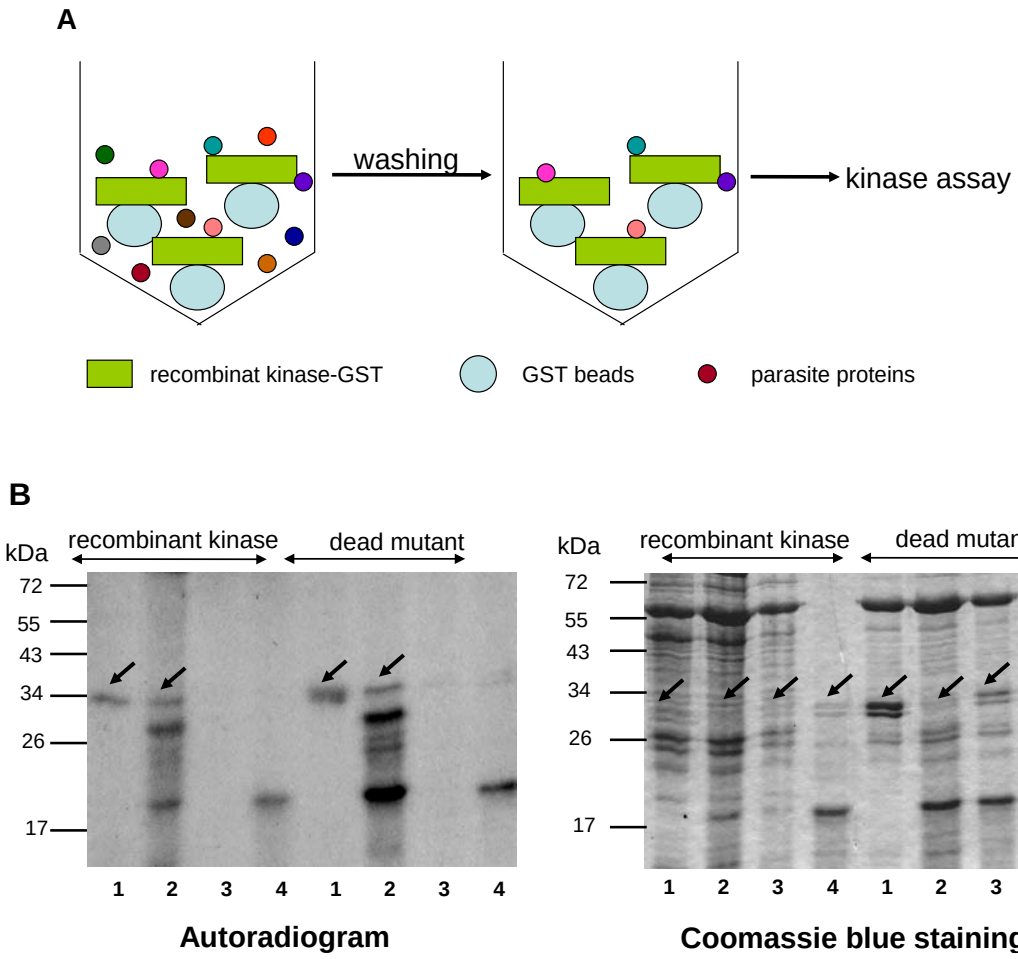


Figure 3.17: Inactivated *P. falciparum* strain 3D7 extract is associated with a faint autophosphorylation of *Pf*CLK-1/Lammer.
Heat inactivated 3D7 parasite lysate was incubated with recombinant *Pf*CLK-1/Lammer coated on Glutathione-beads. Addition of exogenous substrates in a kinase buffer was subsequently performed to visualize the phosphorylation pattern. Only exogenous substrates on uncoated beads incubated with parasite extract served as a negative control. Casein kinase incubated with exogenous substrates was taken as a positive control for the kinase assay.

A faint phosphorylation was observed at an approximately 60 kDa, the size of recombinant *Pf*CLK-1/Lammer protein hinting its possible autophosphorylation, but no significant phosphorylation of the exogenously added substrates was observed. The exogenous substrate coated beads served as a negative control. Casein kinase 2 (CK2), a serine/threonine *P. falciparum* kinase (Holland et al., 2009) was tested as a positive control for the kinase assay. Intense signals at approximately 28 kDa and 30 kDa demonstrates strong phophorylation of α/β caseins by CK 2 (Figure 3.17).

Subsequent verification of specific phophorylation in presence of radiolabelled ATP by recombinant *Pf*CLK-1/Lammer was investigated by construction of a kinase dead mutant where a conserved lysine at position 30 (Figure 2.3) was replaced by a methionine (section 2.2.2.8). This replacement renders an inactive kinase that is expected to loose its capacity to phosphorylate substrates. Both *Pf*CLK-1/Lammer and its kinase dead mutant were recombinantly expressed in *E.coli* (section 2.2.3.1). A pull down assay was performed where both recombinant proteins coated on Glutathione-sepharose beads were incubated with active saponin lysed parasite extract (Figure 3.18).



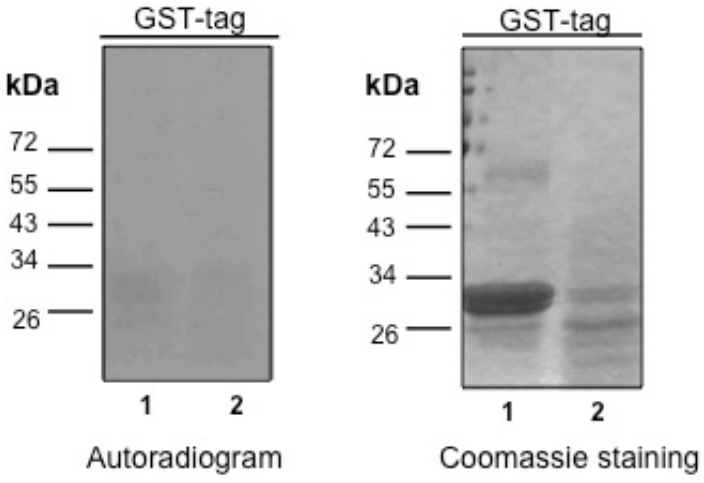
Lane 1. *Pf*CLK-1/Lammer + Histone HI + parasite extract
Lane 2. *Pf*CLK-1/Lammer + Histone HI+ MBP/α/β caseins + parasite extract
Lane 3. *Pf*CLK-1/Lammer + Histone HI+ MBP, w/o parasite extract
Lane 4. Parasite extract + Histone HI+ MBP, w/o *Pf*CLK-1/Lammer

Figure 3.18: Kinase assay on recombinantly expressed *Pf*CLK-1/Lammer and its dead-mutant reveal an in vivo interaction. **A.** Schematic of a pull-down assay. Catalytic domain derived recombinant *Pf*CLK-1 and its dead-mutant were coated onto the Glutathione-sepharose beads and incubated with the parasite extract to proceed with the kinase assay. **B.** The kinase activity assay was performed with radiolabelled ATP in the presence or absence of active parasite extract. The autoradiography of SDS-gel separated proteins revealed phosphorylation of the substrates histone, MBP and α/β casein (~33k Da, 18k Da, 25 kDa), when parasite extract and *Pf*CLK-1/Lammer were present, but not, when one of the two components was absent. Background phosphorylation of MBP in samples lacking recombinant kinase was observed. A similar phosphorylation pattern was observed, in the kinase dead version indicating that the kinase is associated in a complex with phosphorylation activity. Coomassie blue staining of radiolabelled gel was used as a loading control.

This experiment ensured the availability of potential interaction partners from the parasite extract that *Pf*CLK-1/Lammer might need to interact with for its activation to further phosphorylate the substrates directly or in association with

other binding partners. However, due to replacement of the activating lysine residue in the kinase-dead mutant, the final phosphorylation is not expected.

Phosphorylation of Histone or of MBP and α/β caseins was detectable in the autoradiography, when active parasite extract was present. Significant phosphorylation of physiological substrates was observed only in combination of beads coated with recombinant proteins (section 2.2.3.2) and active parasite extract (lanes 1 and 2, autoradiograph; Figure 3.18B). Further, no phosphorylation was detected, when beads coated with the recombinant *Pf*CLK-1/Lammer were used in absence of the parasite extract, or when the assay was performed with uncoated beads in presence of the parasite extract. Interestingly, similar phosphorylation activity was observed, when a kinase dead version of recombinant *Pf*CLK-1/Lammer, in which the conserved Lysine 30 was replaced by Methionine, was used (Figure 3.18B) signifying that *Pf*CLK-1/Lammer acts in a protein complex rather than directly phosphorylating its substrates. In addition, no phosphorylation was observed when GST-tag alone was incubated with the parasite extract (Figure 3.19).



Lane 1: GST-tag + parasite extract + Histone HI
Lane 2: GST-tag + parasite extract + MBP/α/β caseins

Figure 3.19: GST-tag alone does not phosphorylate physiological substrates when incubated with proteins from the parasite extract.

Such studies on recombinant *Pf*CLK-2 could not be pursued as the expression of this kinase from the catalytic domain was not successful. Therefore, the phosphorylation activities of two CLK kinases were tested on

native immunoprecipitated Myc-tagged kinases. The two *Pf*CLKs were immunoprecipitated from the respective Myc-tagged cultures using Myc-antibody (section 2.2.3.6). Wild type 3D7 was immunoprecipitated with Myc antibody as a negative control. After several washes of the immunocomplex, a kinase assay was performed with the exogenous substrates (Figure 3.20) and the proteins were subjected to SDS-gel electrophoresis. Signals of phosphorylation were further detected by autoradiography. Both endogenous kinases significantly phosphorylated the exogenous substrates showing that there might other proteins from the parasite extract that assist the substrate phosphorylation by the two kinases. Recombinantly expressed protein kinase 6 (rPK6; Bracchi-Richard et al., 2000) that predominantly phosphorylates MBP was used as a positive control for the kinase assay.

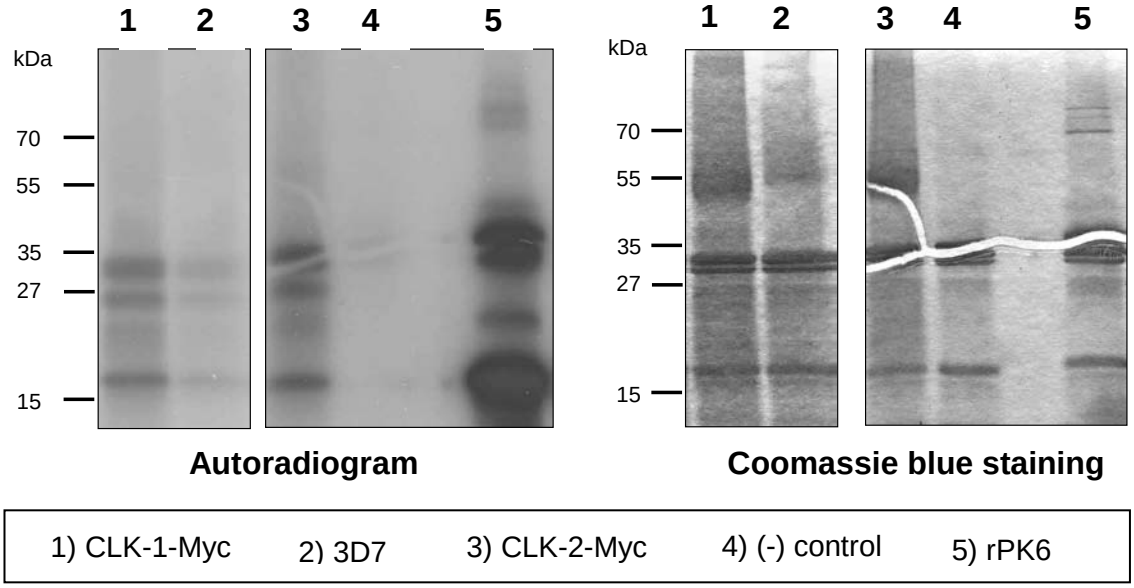


Figure 3.20: Immunoprecipitated native *Pf*CLK-1/Lammer and *Pf*CLK-2 show kinase activity. Myc-tagged *Pf*CLK-1/Lammer and *Pf*CLK-2 were immunoprecipitated from respective parasite cultures with Myc-antibody. A mixture of exogenous substrates histone HI (~33k Da), Myelin basic protein (MBP, ~18k Da) and α/β caseins (~25 kDa), was added to the assay along with radiolabelled ATP and signals were detected via autoradiography. All the substrates were phosphorylated. Untagged wild type parasites of 3D7 strain were also precipitated by anti-Myc antibody and did not show any phosphorylation. Recombinant protein kinase 6 (rPK6), a kinase that predominantly phosphorylates MBP was taken as a positive control for the kinase assay. Coomassie blue staining of SDS-gel served as a loading control.

In silico studies localize both *Pf*CLK-1/Lammer and *Pf*CLK-2 in the nuclear speckles, the site for storage of splicing factors. Both possess putative nuclear localization signals and the antibodies generated against the catalytic domain detect the two proteins in association with the parasite nucleus.

Therefore, it was worth testing if both kinases are a part of the same protein complex. Parasite lysate from Myc-tagged *Pf*CLK-1/Lammer was used to immunoprecipitate *Pf*CLK-1/Lammer using Myc-antibody. Precipitated proteins were separated by SDS-PAGE and the presence of *Pf*CLK-2 was investigated using anti-*Pf*CLK-2 antibody. No full-size *Pf*CLK-2 was detected, indicating that the two kinases do not interact (Figure 3.21). A possible interaction of the processed kinase could not be investigated, because on the SDS-gel such protein bands were covered by prominent protein bands of the precipitating antibody running at an approximate molecular weight of 55 kDa and 23 kDa.

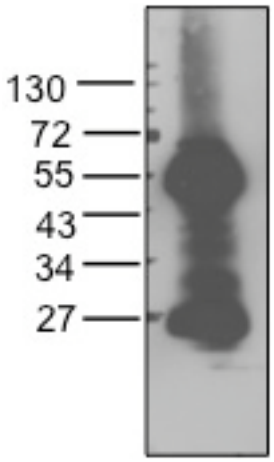


Figure 3.21: *Pf*CLK-1/Lammer and *Pf*CLK-2 do not interact. Lysate of Myc-tagged *Pf*CLK-1/Lammer was used to precipitate PfCLK-1 with anti-Myc antibody and investigated for the possible interaction with *Pf*CLK-2 using anti-*Pf*CLK-2 antibody. No full-length (150 kDa) protein was detected. Processed kinase fragments were covered by thick antibody bands and therefore could not be detected.

3.1.6 Investigation of potential binding partners of *Pf*CLK-1/Lammer and *Pf*CLK-2

As results from kinase assays were suggestive of the kinase activity in presence of proteins from the parasite extract, co-immunoprecipitation was performed on wild type cultures of strain 3D7 using mouse antisera directed against the two kinases. The proteins were separated on a SDS-gel and stained with colloidal coomassie blue stain. Selected bands were then analysed by mass spectrometry (section 2.2.3.9) in collaboration with Dr. Jude Przyborski and Dr. Stefan Baumeister, Marburg (Figure 3.22). Uninfected erythrocytes were also used in immunoprecipitation assays in the similar way as a negative control.

The above pattern of separated protein bands was obtained from colloidal coomassie and the highlighted bands (Figure 3.22) were analysed by mass spectrometry.

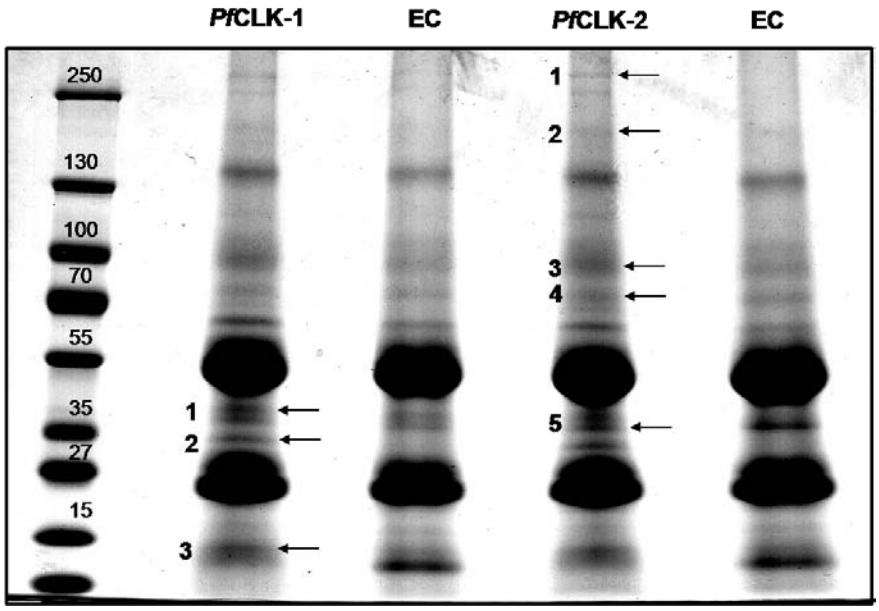


Figure 3.22: *Pf*CLK-1/Lammer and *Pf*CLK-2 interact with in vivo parasite proteins. Native kinases were immunoprecipitated using respective polyclonal mouse antibody directed against *Pf*CLK-1 and *Pf*CLK-2. The resulting proteins were separated on a SDS-gel and stained by coomassie blue stain. Several protein bands were stained and the ones unique to kinases were extracted for mass spectrometric analysis. Protein bands used for analysis are numbered and arrows indicate the exact band. Uninfected erythrocyte (EC) lysates immunoprecipitated with anti-*Pf*CLK-1/Lammer or with anti-*Pf*CLK-2 were used as a negative control.

These were mainly the proteins that were unique to either *Pf*CLK-1/Lammer or to the *Pf*CLK-2 but not to their respective erythrocyte controls. The protein bands were identified according to their PlasmoDB gene ID. Further analysis in terms of investigating these possible interaction partners was executed by two approaches. The first one included a sequence similarity search using blast analysis. The second approach determined the neighbouring proteins on the same chromosome since proteins with similar functions are known to cluster together.

Band 1 in *Pf*CLK-1/Lammer was identified as a hypothetical *P. falciparum* protein in the database search with a Protein knowledgebase ID Q8ILX6_PLAF7. It corresponds to the open reading frame (ORF) sequence of PF14_0117 in *P. falciparum*. Blast analysis identified the protein sequence as a putative s1/p1 nuclease. It is also significantly homologous to 3'-nucleotidases/nucleases in *P.yoelii*. Further, nuclear transport factor, PF14_0122 lies in a close proximity of this interaction partner. Band 2 and band 3 correspond to Glyceraldehyde-3-phosphate dehydrogenase and membrane

antigen pf12 of *P. falciparum*, which might arise due to high abundance of these housekeeping genes in the parasite extract.

Table 3.1: Identification of potential interaction partners of: **A.** *Pf*CLK-1/Lammer and **B.** *Pf*CLK-2 by Mass spectrometry.

A.

*Pf*CLK-1	Possible role	Gene cluster
Band 1 (PF14_0117)	S1/p1 nuclease	Nuclear transport factor-2
Band 2 (PF14_0598)	Glyceraldehyde-3-phosphate	-
Band 3 (PFF0615c)	Membrane antigen pf12	-

B.

*Pf*CLK-2	Possible role	Gene cluster
Band 1 (PFL1445w)	protein-protein interaction	heat shock protein
Band 2 (PF13_0187)	similarity with helicases	histone H3
Band 3 (PFI1190w)	hypothetical	RNA binding protein and Palatin-like phospholipases
Band 4 (PFL1220w)	hypothetical	Ubiquitin-activating enzyme
Band 5 (PF11_0084)	hypothetical	Nucleic acid binding protein

In case of *Pf*CLK-2, Band 1 had a hit with Q8I5C0_PLAF7 which corresponds to the gene ID PFL1445w of *P. falciparum* protein. A blast with its protein sequence resulted in a similarity with PF14_0175 which is a Leucine rich repeats (LRR) containing protein. Such proteins are known to be important

in protein-protein interactions. Its chromosomal location is further near another gene PFL1465c which encodes a heat shock protein (hslV). These are threonine proteases and might play a role in the cleavage of the kinase. Band 2 identified a protein Q8IDY9_PLAF7 which matched the gene ID PF13_0187. Blast searches revealed its similarity with DNA replication helicase, DNA-2 and Histone H3 (PF13_0185) present in the near vicinity. Band 3 identified with Q812R5_PLAF7 which has a corresponding ORF, PFI1190w. This gene lies in a close proximity of the gene PFI1175c which encodes a RNA binding protein, gene PFI1180w that encodes a Palatin-like phospholipase and gene PFI1195c that encodes Thiamine pyrophosphokinase. Band 4 recognized with Q8I5G4_PLAF7 which is similar to PFL1220w. It is syntenic with a gene that encodes ubiquitin activating enzyme, E1. It has been shown that E1 could be phosphorylated and localized into the nucleus of a cell with an ATP hydrolysis reaction (Stephen et al., 1996). Band 5 was identified as Q8IIT0_PLAF7 which is similar to PF11_0084. A close neighbour of this gene PF11_0083 encodes for a nucleic acid binding protein. In *P. yoelii*, it is an RNA recognition motif. The potential binding partners of *Pf*CLK-1/Lammer and *Pf*CLK-2 identified by mass spectrometry and the proteins encoded by genes in the same cluster are summarized in Table 3.1.

Taken together this mass spectrometry data strongly suggests that the kinases are in close association with other cytoplasmic or nuclear proteins which assist them in phosphorylation of specific substrates thereby controlling the gene expression.

3.2 Functional characterization of *Pf*PKRP kinase

The hypothetical kinase *Pf*PKRP (putative kinase related protein, PFC0485w) belongs to the group of CamK (Calmodulin-dependent kinases) and is expected to be involved in signaling events of the parasite. Domain architecture of this 296 kDa protein revealed an N-terminal kinase domain (Figure 3.23A) with no signal peptide or transmembrane domains that could be depicted by in silico analysis.

3.2.1 Transcript and protein expression analysis of *Pf*PKRP kinase

Transcript abundance of *Pf*PKRP in asexual and gametocyte stages of the parasite was investigated by PCR amplification of cDNA obtained from respective stages using sequence-specific primers. A strong amplification band at 246 bp was detectable for *Pf*PKRP in gametocytes purified from NF54 stain of the parasite while a lower transcript level was observed from the asexual cDNA obtained from the gametocyte less starin, F12 (Figure 3.23B). *PfCCp1* and *AMA-1* were used as positive controls and showed abundant transcript expression. Samples without addition of reverse transcriptase served as negative control to ensure no contamination from the genomic DNA. Reverse transcriptase studies were performed in collaboration with Walentina Diez.

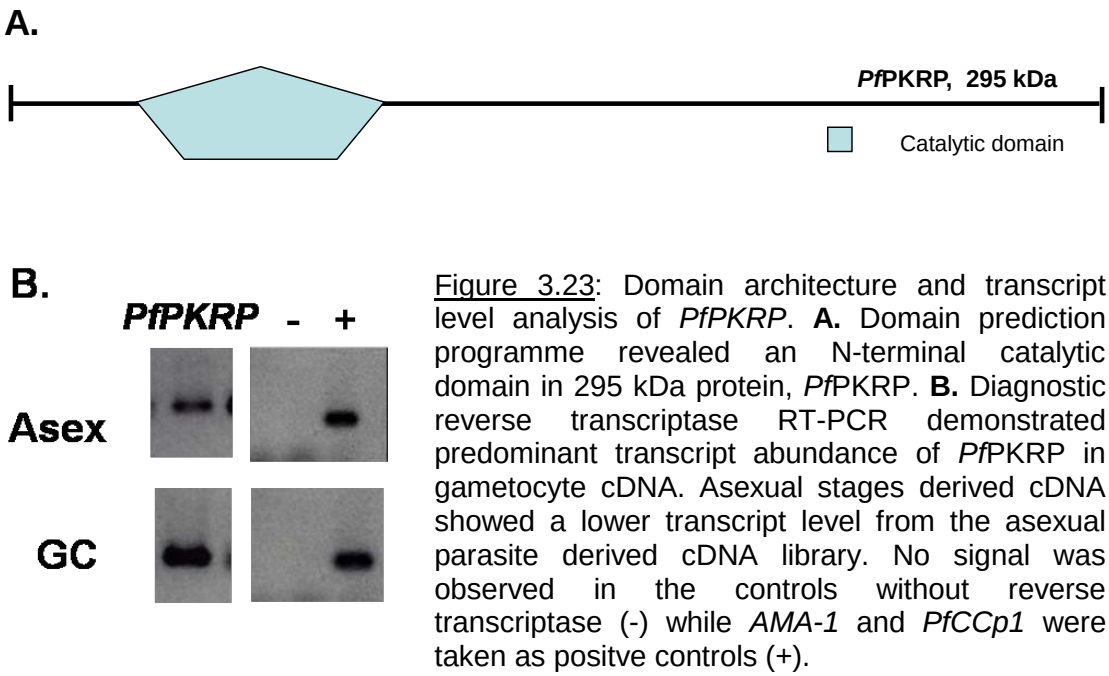


Figure 3.23: Domain architecture and transcript level analysis of *PfPKRP*. **A.** Domain prediction programme revealed an N-terminal catalytic domain in 295 kDa protein, *Pf*PKRP. **B.** Diagnostic reverse transcriptase RT-PCR demonstrated predominant transcript abundance of *Pf*PKRP in gametocyte cDNA. Asexual stages derived cDNA showed a lower transcript level from the asexual parasite derived cDNA library. No signal was observed in the controls without reverse transcriptase (-) while *AMA-1* and *PfCCp1* were taken as positve controls (+).

Further, to investigate the stage-specific protein expression of *Pf*PKRP, rabbit polyclonal antibody was obtained against a peptide sequence a few amino acids upstream of the *Pf*PKRP catalytic domain (section 2.1.11). Western blot analysis was performed on purified blood stage lysates and revealed the kinase expression in asexual parasites (Asex, in gametocyte-less strain, F12) and in gametocytes (GC, in gametocyte-producing strain, NF54, Figure 3.24A). The detected protein band migrated at the calculated molecular weight of 295 kDa (arrow). Additional protein bands were observed at approximately 170 kDa and 75 kDa (arrows) indicating that the CamK kinase might be processed during the expression. Uninfected erythrocyte lysate (EC)

and neutral rabbit serum (NRS) did not show any protein band in response to the peptide antibody. Indirect immunofluorescence assays on mixed asexual cultures of gametocyte-less strain, F12 localized the kinase expression mainly in schizonts. No immunofluorescence signal could be detected in the trophozoite blood stage of the parasite. Immunofluorescence assay showed predominant expression in stage II-V gametocytes mainly in the cytoplasm in a punctuated manner (Figure. 3.24B). Protein expression studies on *Pf*PKRP were performed in collaboration with Rebecca Schillig and Selina Kern.

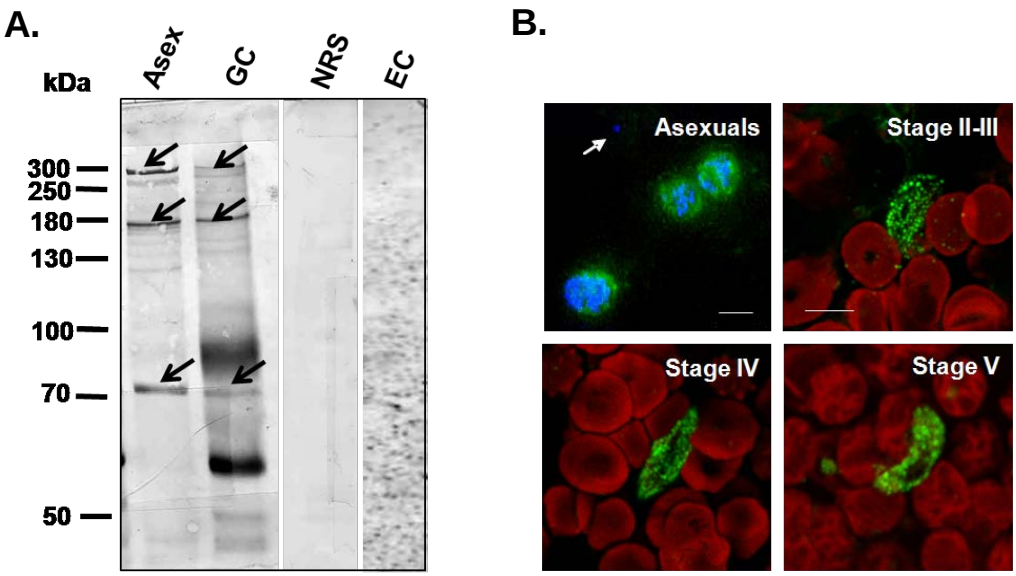


Figure 3.24: Protein expression profile of *Pf*PKRP kinase. **A.** Western-blot analysis using rabbit polyclonal peptide antibody against *Pf*PKRP showed expression of the kinase in asexual parasites, Asex using F12, gametocyte-deficient *P. falciparum* strain and gametocytes, GC derived from NF54, gametocyte-producing *P. falciparum* strain. In addition to the expected molecular weight of 295 kDa, the protein migrated at approximately 170 kDa and 75 kDa (indicated by arrows). Lysate of uninfected erythrocytes (EC) and neutral rabbit serum (NRS) were used as negative controls. **B.** Indirect immunofluorescence assay revealed intracellular protein expression in schizonts, (kinase shown in green, nuclei highlighted by Hoechst in blue), but not in trophozoites (arrow). The kinase was further expressed in the cytoplasm of gametocytes during differentiation in a punctuated pattern (kinase shown in green, erythrocytes counterstained with Evans Blue in red). Bar, 5 µm.

Expression of *Pf*PKRP was also investigated in the mosquito midgut derived sexual stages like zygote, retort/ookinete and sporozoites from the mosquito salivary gland. No expression could be localized in zygotes or retort/ookinete while a faint signal was detectable in sporozoites (Figure 3.25). Sporozoites were labelled with *Pf*CSP antisera as a positive control. Since both

*Pf*PKRP and *Pf*CSP antisera were generated in rabbit, a double-labelling could not be shown. Immunofluorescence assay was performed in collaboration with Dr. Matthias Scheuermayer.

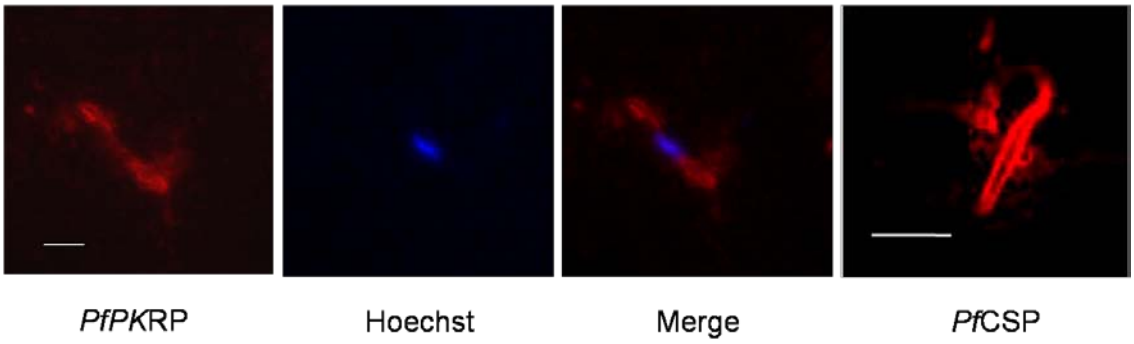


Figure 3.25: Salivary gland sporozoites express low level *Pf*PKRP. Indirect immunoflurescence assay using immune sera directed against the catalytic domain of *Pf*PKRP revealed no expression of *Pf*PKRP (red) kinase in zygotes, retorts/ookinetes while a low level expression was observed in sporozoites derived from the salivary glands of the mosquito. Sporozoites were labelled with the *Pf*CSP protein as a positive control. Parasite nucleus was stained with Hoechst (blue), Bar 5 µm.

3.2.2 Generation of *Pf*PKRP gene-disruptant parasites

*Pf*PKRP orthologue, *Pb*PKRP has recently been knocked out in the *P. berghei* rodent malaria model (Purcell et al., 2010). Further, disruption of *Pf*PKRP was attempted.

*Pf*PKRP gene locus was targeted by single cross-over homologous recombination to investigate the phenotype of the kinase in the parasite life cycle. By a similar reverse genetics approach as described (section 3.1.4), we aimed to investigate if *Pf*PKRP is essential for the survival of asexual parasite blood stages. pCAM-BSD based vector was used to clone a 500 bp fragment of *Pf*PKRP catalytic domain in collaboration with Rebecca Schillig. The cultures of ring-stage parasites were transfected as described (section 2.2.1.2). Unlike *Pf*CLK kinases, it was possible to disrupt the *Pf*PKRP locus within the catalytic domain separating the ATP binding motif from the motif for the structural stability (section 3.1.4). Diagnostic PCRs were performed on genomic DNA derived from parasite cultures four months post transfection to test the integration of pCAM-*Pf*PKRP-KO into the wild type gene locus using sequence-specific primers (section 2.1.15).

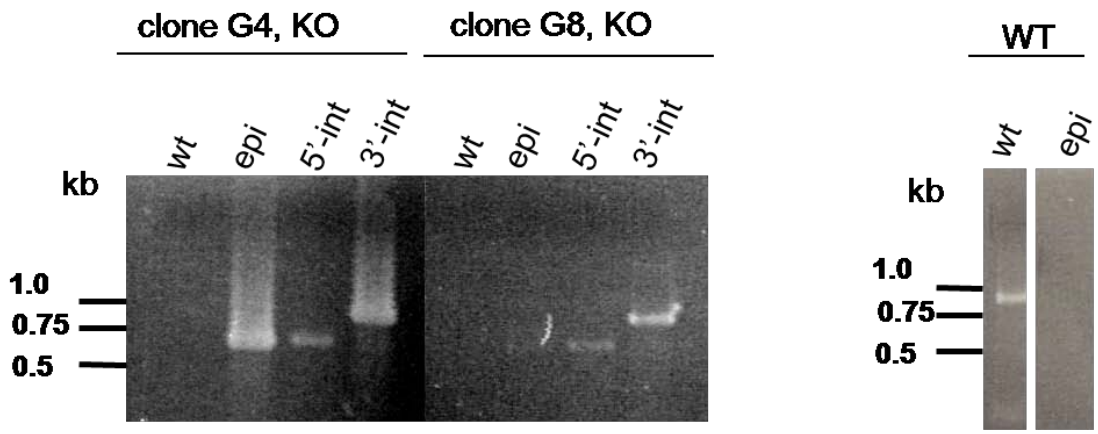
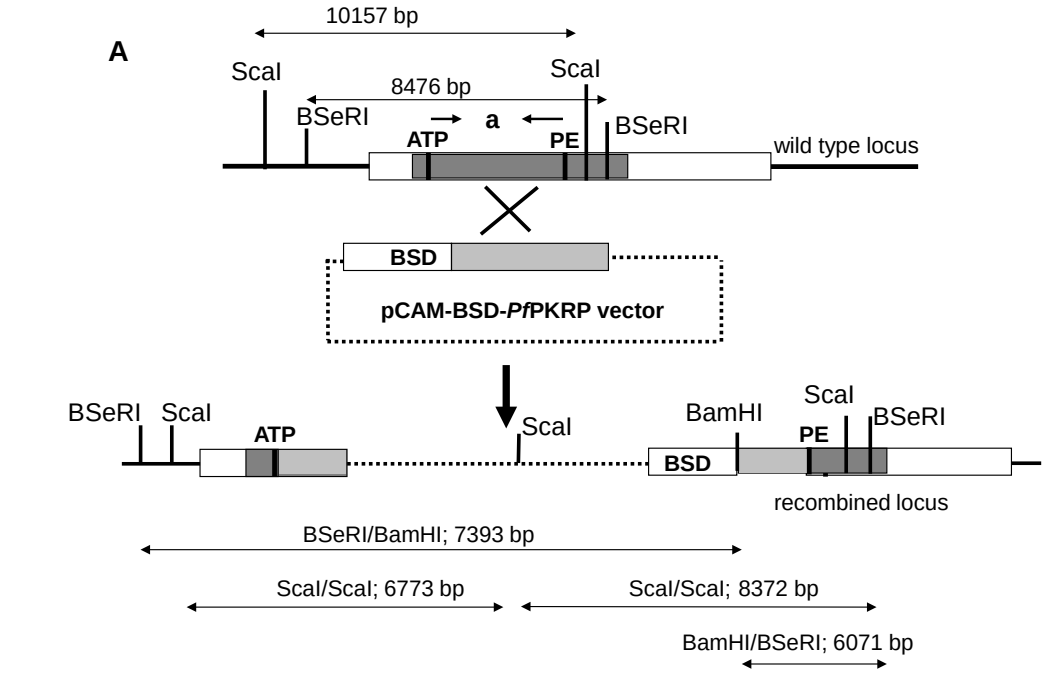


Figure 3.26**:** A diagnostic PCR showing successful disruption of *Pf*PKRP gene locus. Mixed population of transfected parasites was diluted and cultivated to isolate several single clones. Two clones G4 and G8 comprised only the integrant population without wild-type. Diagnostic PCR from genomic DNA (gDNA) isolated from G4 and G8 KO clones of *Pf*PKRP showed only amplification bands correlating to the episome (615 bp), for 5´-integration (634 bp) and 3'-integration (811 bp) of the vector at 120 d post-transfection.

In order to isolate single integrant parasite clones from the mixed population dilution cloning followed by a Malstat assay were performed (section 2.2.1.3). Several clones were isolated out of which two clones G4 and G8 were tested positive by a diagnostic PCR for wild-type lacking pure integrant population (Figure 3.26). Successful gene integration was tested using sequence specific primers (section 2.1.14) leading to amplification bands of episome (615 bp), 5'-integration (634 bp) and 3'-integration (811 bp). No WT band was detected after isolation of single parasite clones G4 and G8 from the mixed parasite population.

Via Southern blot analysis gene recombination of clone G4 was further confirmed. Digestion pattern of the recombined locus was assessed using restriction enzyme ScaI and a combination of enzymes BSeRI/BamHI. The oligonucleotide probe was derived by amplifying the insert 'a' (Figure 3.27A) from *Pf*PKRP-KO vector by PCR amplification (section 2.2.2.2). The probe was labelled and its hybridization with the gene locus was detected by autoradiography (Figure 3.27B).



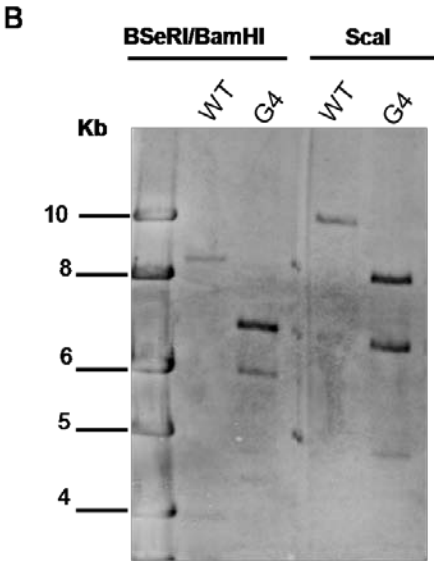


Figure 3.27: *Pf*PKRP is important in the development of sexual stages of the parasite. **A.** Schematic showing the generation of *Pf*PKRP-KO with expected fragment sizes upon restriction digestion with ScaI and BSeRI/BamHI. Oligonucleotide probe 'a' was chosen from the region cloned into the pCAM-BSD vector. **B**. Southern blot was performed to confirm the recombined locus. With ScaI digestion, band sizes of 10157 bp (WT), 6773 bp (5'-integration), 8372 bp (3'-integration), 4987 bp (episome) were obtained. Digestion with BSeRI/BamHI resulted in 8476 bp (WT), 7393 bp (5'-integration), 6071 bp (3'-integration). gDNA isolated from untransfected strain 3D7 was taken as a negative control and thus showed only wild type specific band size.

Successful gene disruption of *Pf*PKRP signifies that it is dispensable for the asexual parasite cycle and that it could be essential for transmission stages in the mosquito as its *P. berghei* orthologue, *Pb*PKRP. Therefore, the expression of the mutant in various sexual stages of the parasites needs to be further investigated.

As mentioned above for *Pf*CLK kinases (section 3.1.4), *Pf*PKRP was also tested for C'-terminal tagging via generation of knock-in constructs. *Pf*PKRP was fused to HA/GFP/Myc-epitopes and out of these GFP was further analysed for recombination. Diagnostic PCR using gene-specific primers

(section 2.1.16) showed successful integration with episome and 3'-integration bands migrating at 2.28 kb and 2.42 kb respectively (Figure 3.28). Tagging of kinases in addition to verifying recombinognecity of the gene locus, provide a tool to further characterize the kinase expression in the parasite life cycle by Western blot, immunofluorescence and co-immunoprecipitation assays.

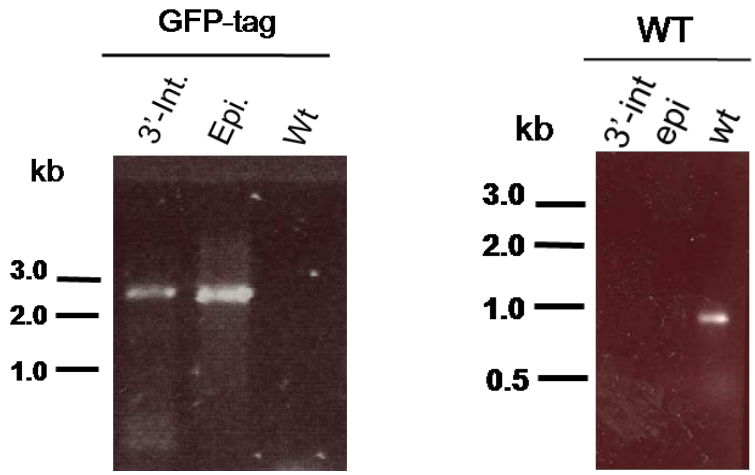


Figure 3.28: *Pf*PKRP was successfully fused to GFP epitope.
A 600 bp fragment was fused to GFP-epitope to generate a full-length tagged kinase by a tagging strategy. Successful gene integration was demonstrated by a diagnostic PCR on the gDNA isolated from the tagged *Pf*PKRP parasite culture. An episomal band (2.28 kb) and 3'-integration band (2.42 kb) were observed after cultivation of the transfected parasites for about 24 weeks. WT band was not detectable in the mixed parasite population. In gDNA derived from the WT parasites of 3D7 isolate, only WT band at 822 bp was detectable with no 3'-integration or the episomal band.

3.2.3 Ultrastructure of the *Pf*PKRP gene-disruptant parasites

Gene disruptant clone of *Pf*PKRP, G4 was analysed for its development during the blood stages. Stage III-V gametocytes were developed with the normal morphology (Figure 3.29) although the formation of exflagellation centres was reduced as compared with the WT. Quantification of this observed reduction process is under progress.

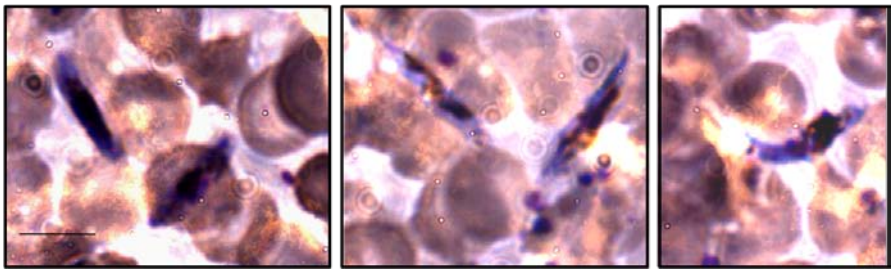
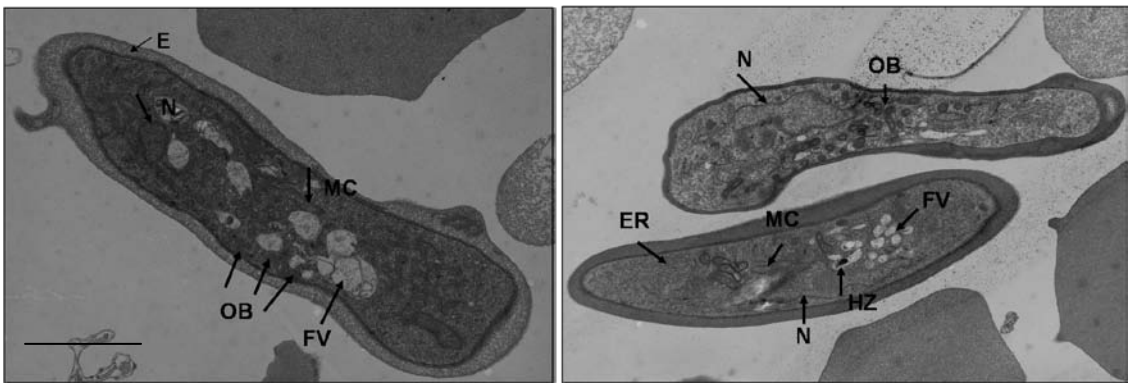


Figure 3.29: Giemsa staining showing stage III-V gametocytes formed in gene disruptant *Pf*PKRP parasites. Bar 5 µm.

Via immunoelectron microscopy, possible changes in the ultrastructure of the G4 mutant parasites were investigated. Stage III-V gametocytes were embedded in epon medium and thin sections were cut to be analysed further. Embedding was performed in collaboration with Dr. Matthias Scheuermayer and Andrea Kuehn. Gametocyte morphology was compared between the mutant (Figure 3.30A) and the WT parasites (Figure 3.30B). Both erythrocyte (E) and the parasite membranes appeared normal, nucleus (N), osmiophilic bodies (OB), food vacuole (FV), mitochondria (MC), endoplasmic reticulum (ER) and hemozoin (HZ) could also be observed.

A. Ultrastructure of *Pf*PKRP mutant gametocyte



B. Ultrastructure of a wild type gametocyte

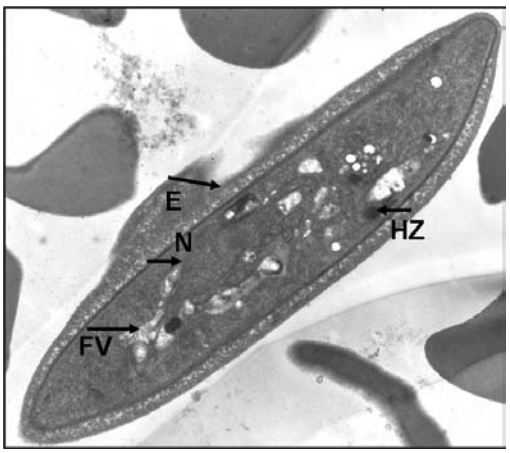


Figure 3.30: Morphology of *Pf*PKRP disruptant parasites remains unchanged. **A.** Gametocytes were purified from the G4 mutant parasites and embedded in epon medium. Thin sections were cut and analysed by immunoelectron microscopy to visualize any possible morphological changes introduced due to disruption of *Pf*PKRP. **B.** No discernable changes could be seen between the mutant gametocytes when compared with the WT gametocytes as nucleus (N), endoplasmic reticulum (ER), Mitochondria (MC), hemozoin (HZ), osmiophilic bodies (OB), food vacuole (FV) were clearly visible with a normal architecture. Bar 0.5 µm.

4. Discussion

4.1 Functional analysis of *Pf*CLK kinases involved in mRNA splicing

Out of 5300 genes, unraveled by genome sequencing of *Plasmodium falciparum*, only about one-tenth encode for enzymes (Gardner et al., 2002). Yet, up to date knowledge on *P. falciparum* kinases limits to a handful of kinases being characterized and targeted for the chemotherapy. Malaria is a devastating disease taking on an average one life every 10-30 s (http://www.who.int). Predominantly affected areas include region of sub-Saharan Africa where malaria tropica is a leading cause of death in young children. Other affected areas include the Amazon region and South-east Asia (Hyde, 2005). Several factors such as population movements, poor health services, malnutrition and associated opportunistic infections contribute to elevation of malaria burden in endemic countries (World malaria report, 2008). To counteract, further advancements in search of effective therapeutics is clearly needed (Greenwood et al., 2008). Extensive divergence of parasitic protein kinases from their vertebrate counterpart however enables their selective inhibition (Doerig, 2004).

Genome-wide analysis of the parasite led to identification of about 99 hypothetical kinases based on in silico studies (Anamika et al., 2005). Construction of a phylogenetic tree categorized these kinases into seven major groups, the collectivity being termed as the parasite “kinome”. The largest of these groups comprises of the CMGC kinases which includes Cyclin dependent (CDK), Mitogen-activated (MAPK), Glycogen-synthase (GSK) and CDK-like kinases (CLK). In other eukaryotes, CDKs that play an important role in cell cycle progression, have been extensively investigated and are validated drug targets. However, cell cycle control and regulation in *P. falciparum* is still poorly understood. Therefore, characterization of malarial CLKs is important in order to understand the mechanisms involved leading to identification of additional drug targets. In the present study, four plasmodial serine/threonine CLK kinases namely *Pf*CLK-1/Lammer (PF14_0431), *Pf*CLK-2 (PF14_0408), *Pf*CLK-3 (PF11_0156) and *Pf*CLK-4 (PFC0105w) were chosen for characterization in the parasite life cycle stages.

4.2 In silico analysis of *Pf*CLK kinases

Using various bioinformatics parameters, *Pf*CLK kinases were previously assigned to the branch of CMGC kinases which is predicted to be involved in mRNA splicing (Ward et al., 2004). *Pf*CLK-1/Lammer has a motif "EHLAMMESII" that resembles the "EHLAMMERILG" signature motif conserved in the lammer kinases of other eukaryotes. Since this motif is responsible in substrate recognition, the exchange of 'S' with 'R' in LAMMER motif of *Pf*CLK-1 might alter its substrate specificity (Li et al., 2001). Thus far, four murine Lammer kinases (Ben-David et al., 1991), three in human (Johnson and Smith, 1991; Hanes et al., 1994), three in *Arabidopsis thaliana* (Bender and Fink, 1994), one in *Drosophila melanogaster* (Yun et al., 1994), one in *Saccharomyces cerevisiae* (Padmanabha et al., 1991), one in *Schizosaccharomyces pombe* (database accession number Q10156) and one in *Nicotiana tabacum* (Sessa et al., 1996) are known. Cataltyic domains of Lammer kinase share 56-60% similarity and 37-41% identity amongst other members of the family. Further, *Pf*Lammer has highest homology to the Lammer kinase of *A. thaliana*, AFC3 kinase, with almost 41% identity (Bender and Fink., 1994). AFC3 is a close homologue of AFC1 which has been documented to activate STE12 (Sterile 12) dependent pathway in yeast. STE12 is a transcription factor that is required for expression of mating-specific genes in *S. cerevisiae* and is phosphorylated by FUS3, a yeast kinase (Elion et al., 1993; Peter et al., 1993).

*Pf*CLK-1/Lammer comprises of two putative nuclear localization signals upstream of the kinase catalytic domain (Li et al., 2001). *Pf*CLK-2 on the other hand is not well characterized and possesses only one putative nuclear localization signal at the N-terminal of the kinase catalytic domain. The here described in silico analysis depicted the sub-nuclear localization of these two kinases in speckles. Speckles are subnuclear structures enriched in pre-messenger RNA splicing factors (Lamond and Spector, 2003). *Pf*CLK-3 is an orthologue of human PRP4, a kinase that is associated with mRNA splicing and histone deacetylation. *S. pombe* pre-mRNA splicing kinase, Prp4p is enriched in SC35-containing nuclear speckles (Dellaire et al., 2002). *Pf*CLK-4 on the other hand clusters with Serine/Arginine protein kinases (SRPK) (Ward et al., 2004). Sequence based analysis revealed that although, both *Pf*CLK-3 and

*Pf*CLK-4 lack the nuclear localization signals, their in silico localization studies place them to promyelocytic leukemia (PML) bodies and to the cell nucleoplasm respectively. PML bodies localized in a mammalian nucleus are typically 0.3 µm-1.0 µm in diameter and have been implicated a role in transcription regulation (reviewed in Spector, 2001). Nucleoplasm on the other hand, is a dense organic viscous liquid that fills the interior of the nucleus with nucleotides and enzymes.

Reversible phosphorylation of Serine/Arginine-rich (SR) proteins is mediated by SRPK and CLK kinases. SRPK1 phosphorylation of splicing factors is restricted by a specific docking interaction with the substrates whereas CLK activity is less constrained (Bullock et al., 2009) but both are implicated in phosphorylation of splicing factors.

Catalytic domains of all four kinases align well with the catalytic domain of SR kinase in yeast (Sky1p), the only member of SRPK family in *S. cerevisiae* (Siebel et al., 1999; Nolen et al., 2001). As per the homology prediction, all the four kinases appeared to be closely related to the yeast kinase. The alignment reflects the presence of all the important conserved domains that are essential for the kinase activity. The conserved sequence DLKPEN for example around position 130 is conserved with D (Aspartate) 126, which is considered to be the catalytic base. The loop at the end (position 170-193), signifies the activation segment that has a regulatory function for the kinase activity. The fact that the ATP-binding domain GXGXXG is present only in *Pf*CLK-1 but is replaced by GXGXXS in case of *Pf*CLK-3, *Pf*CLK-4 and Sky1p raises an interesting question if this domain is really important for the activity or whether it could be compensated by the presence of other domains. Further, the domain is completely absent in *Pf*CLK-2 although the other kinase domains are present. Several putative kinases have been previously reported to lack all three Gs of this triad like the three *P. falciparum* proteins which include, PFA0380w, PFI1415w, PFL0080c (Ward et al., 2004). Absence of one or more conserved motifs might enhance the possibility that these kinases interact in association with other proteins to fulfill the ATP binding function which is normally believed to be performed by the GXGXXG motif.

Sky1p has remarkable substrate specificity for Npl3p, a protein that shuttles between the nucleus and the cytoplasm (Lee et al., 1996). Cytoplasmic

Sky1p phosphorylates Npl3p at a single serine residue located in the C-terminal RS (Arginine/serine) domain which defines its distribution between the two cellular compartments. Absence of Sky1p leads to accumulation of Npl3p in the cell cytoplasm with increased affinity to poly-adenylated RNA (Gilbert et al., 2001). Phosphorylation status of SR proteins thus to some extent determines their association and dissociation with mRNA and hence possibly govern the splicing events in the nucleus and export of mature mRNA into the cell cytoplasm. As mentioned above, not only catalytic domains of the four *Pf*CLK kinases have homology with Sky1p but they also belong to the branch of the *P. falciparum* kinome that is involved in mRNA splicing. Interestingly, Sky1p also shares the substrate binding residues with the kinases under the study, therefore, sequences homologous to Npl3p were searched against the *P. falciparum* genome. In addition, plasmodial sequences homologous to the human CLK substrate, alternative splicing factor (ASF/SF2) were also investigated. It has been shown previously that ASF/SF2-associated protein p32 interacts directly with the human CDK-like kinase, CDC2L5, which has a possible role in regulation of transcription and mRNA splicing (Even et al., 2006). Via genome annotation, three putative splicing factors of *P. falciparum*, i.e. PFE0865c, PF10_0217 and PF10_0047, which show similarities with Npl3p, as well as with mammalian ASF/SF2, were identified. Protein expression for all three splicing factors to determine whether they are the in vivo substrates for the CLK kinases is under progress.

4.3 Expression profile of *Pf*CLK kinases in parasite blood stages

Characterization of kinase expression during the parasite life cycle was invstigated at both transcription and protein level. The transcript expression of the four kinases was investigated in trophozoite, schizont, gametocyte and zygote stages of *P. falciparum* which can be easily purified from in vitro parasite cultures. The transcript profile suggests that the kinases under study are predominantly transcribed at mRNA level in schizonts and gametocytes. Noteworthy, *PfCLK-1/Lammer* has been previously described to be a sexual stage-specific kinase (Li et al., 2001) observed via Northern blotting but in the current study its transcript expression has been observed in asexual stages as well as gametocytes. However, apart from the variable expression levels of

selected kinases observed in asexual stages and gametocytes, there seems to be no detectable expression of the kinases under study in zygotes. Therefore, it is possible that these kinases are expressed in the intraerythrocytic parasite stages including gametocytes and that the expression ceases once the gametes are formed.

In parallel to diagnostic RT-PCR, more sensitive Real-Time RT-PCR was also conducted in order to verify the transcript abundance. *PfCLK-1/Lammer* and *PfCLK-2* show considerable expression in trophozoites. All four kinases appear to be predominantly expressed in schizonts and gametocytes. High transcript expression levels are referred to those where threshold crossing cycles lie in the range of 15–25, while those lying between threshold crossing cycles 25-30, were considered to be weakly expressed. Thus, the sooner the expression is detected in terms of minimum threshold crossing cycle, the higher is the stage specific transcript abundance. Positive control, *Pf39* used in case of trophozoites is transcribed in both sexual and asexual stages of the malaria parasite. *Pf*39 is localized to an intracellular membranous compartment suggestive of endoplasmic reticulum (Templeton et al., 1997). The expression levels in schizonts were compared with the *AMA-1* (Apical Membrane Antigen-1), used as the positive control. AMA-1 is particularly implicated in erythrocyte binding and predicted to have role in merozoite invasion in new erythrocytes (Urquiza et al., 2000). The positive control used for verifying gametocyte transcript expression is *PfCCp1*. The gene product is known to be a surface-associated multidomain adhesion protein in mature gametocytes (Pradel et al., 2004, Pradel et al., 2006). Apart from the asexuals and gametocytes, zygote stage was also analysed. In case of zygotes, except for *PfCLK-4* where low level transcript was detected, none of the other genes analyzed had significant expression. The gene product of *Pfs*25 used as a positive control in zygote is a sexual stage specific antigen expressed mostly on surface of zygotes and female gametes (Kaslow et al., 1988; Kaslow et al., 1994). The positive controls showed abundant transcript expression in the respective stages.

For determination of kinase expression at protein level, mouse antisera directed against the catalytic domains of *Pf*CLK-1/Lammer and *Pf*CLK-2 respectively, was used. Catalytic domains are crucial to the activity of the

kinase and comprise eleven highly conserved sub-domains (Ward et al., 2004), and therefore these regions were chosen for the antibody production. Further, N-terminal regions of both *Pf*CLK-1 and *Pf*CLK-2 were tested for the production of recombinant protein but the respective protein could not be expressed. This could be due to the lack of essential sequences from the kinase catalytic domain. Another reason could be due to the difficulty in expressing AT-rich eukaryotic gene in a prokaryotic system. It has been observed that only about 50% of constructs express the recombinant protein. Generation of full-length protein with natural start and stop codon was tested by incorporating the complete kinase sequence but this attempt was also unsuccessful, possibly due to large-size inserts. Therefore, in view of established importance of the conserved sub-domains in kinase activity, it might be predicted that presence of all domains is essential for the intracellular functioning of the kinase.

Western blot analysis on asexual and gametocyte lysates obtained from NF54 strain of *P. falciparum*, detected a processed protein band at approximately 60 kDa for *Pf*CLK-1 instead of a 107 kDa full-length protein. This might be due to the fact that long N-terminal extension in *Pf*CLK-1 is unstable and prone to cleavage by in vivo proteases and only the catalytic domain containing C-terminal kinase is remaining which is detected by anti-*Pf*CLK-1 generated against this region. In case of *Pf*CLK-2, the antisera directed against the catalytic domain detected a 150 kDa, full-length protein band on the lysates of asexual and gametocyte stages. Additionally, 70 kDa, 50 kDa and 40 kDa protein bands, were also detectable. Western blot analysis on the purified nuclear pellets also detected the processed 60 kDa protein band of *Pf*CLK-1 and the full-length 150 kDa protein band of *Pf*CLK-2. Purification of nuclear pellet was confirmed with both Giemsa and Hoechst staining procedures. Presence of kinases in the nuclear pellet is in concordance with the predicted association of the CLK kinases with nuclear speckles where pre-mRNA splicing factors that include snRNPs (small nuclear-ribonucleoprotein particles) and SR proteins are localized. Previous studies document that mammalian Clk/sty kinases involved in phosphorylation of factors of splicing machinery also reside in the nuclear speckles. Thus, these kinases together with splicing factors form a part of transcription regulators and splicing machinery (Ngo et al., 2005, Lamond and Spector, 2003).

Localization of the two CLK kinases, *Pf*CLK-1/Lammer and *Pf*CLK-2 was further verified by immunoelectron microscopy. Both kinases were detected within the nucleus as well as the cytoplasm. At one instance, *Pf*CLK-1 was even detected at the passage between the nucleus and the cytoplasm. It is possible that the C-terminal part of the kinase comprising the catalytic domain migrates between the two cellular compartments and assists in the phosphorylation of the target splicing factor(s). Later, it might dissociate to orchestrate the events that lead to splicing, maturation of mRNA and its final transport into the cytoplasm to proceed with the translation process. A similar event has been documented before, where cytoplasmic Sky1p reversibly phosphorylates the SR protein Npl3p and leads to its shuttle between the nucleus and the cytoplasm (Gilbert et al., 2001). Localization of the *Pf*CLK-1/Lammer and *Pf*CLK-2 kinase was further investigated by an immunofluorescence assay on asexual and gametocyte stages of the parasite. Here too, the kinases co-localized with both the parasite nucleus and the cytoplasm. Protein expression was further identified in all blood stages and the expression appears to decrease during the parasite transmission into the mosquito and completely ceases during the transmission stages such as zygotes, retorts/ookinetes and sporozoites. This is in contrast to several previously characterized kinases which were reported to have important roles in sexual stages of the parasite. For instance, *Pf*nek-2 is important in ookinete maturation, *Pb*CDPK3 aids in ookinete migration, while *Pf*PK7 has been reported to be essential in oocysts maturation (Ishino et al., 2006; Siden-Kiamos et al., 2006; Dorin-Semblat et al., 2008; Reininger et al., 2009). However, absence of *Pf*CLK-1/Lammer and *Pf*CLK-2 in the transmission stages further illustrates the important role of these kinases predominantly in the propagation of parasite asexual stages.

4.4 *Pf*CLK kinases are essential for intraerythrocytic multiplication of the asexual parasites

Investigation of kinase phenotype was attempted by generation of gene-disruptant parasites by single cross-over homologous recombination using a recently described strategy (Dorin-Semblat et al., 2007). The disruption in the catalytic domain separates the GXGXXG ATP binding domain and the

PE motif responsible for the structural stability of the enzyme (Dorin-Semblat et al., 2007). Several essential kinases have so far been reported in both *P. berghei* and *P. falciparum* like *Pb*crk-1, *Pf*map-2, and *Pf*CK2 all of which are indispensable for erythrocytic schizogony (Rangarajan et al., 2006; Dorin-Semblat et al., 2007; Holland et al., 2009). On the other hand, several kinases have been successfully disrupted by the same reverse genetics approach. This is exemplified by *Pf*map-1 which has a normal phenotype in all parasite stages (Dorin-Semblat et al., 2007), by *Pf*elk1, also having no developmental block in the parasite life cycle stages (Fenell et al., 2009). *Pf*nek-4 blocks ookinete development (Reininger et al., 2009) and *Pf*PK7 in addition to displaying slow growth rates, blocks oocysts development (Dorin-Semblat et al., 2008; all above examples are additionally reviewed in Doerig et al., 2009).

Gene disruption approaches not only investigate the essentiality of the kinase for replication of the asexual parasite stages but also provide a tool to determine the application of specific kinase inhibition. *Pf*CK2α for example has been shown to be selectively inhibited by kinase inhibitors, ML-7 and Rottlerin. The IC_{50} of these compounds were compared between the CK2α subunit of *P. falciparum* (*Pf*) and *Homo sapiens* (*Hs*). A much higher concentration of the compounds was required to inhibit the enzyme in *Hs* as compared with *Pf*, thus indicating the possibility of a differential inhibition. This is despite the fact that CK2 has a high identity (65%) between the two genomes (Holland et al., 2009).

For none of the four CLK kinases gene disruption could be successfully pursued. This lead to the conclusion that either the kinase locus is non-recombinogenic or the kinase is essential for the survival of asexual parasites (Rangarajan et al., 2006). Therefore, in a second approach, a Myc/HA/GFP-epitope was fused to the 3'-end of the respective kinase resulted in gene tagging, without loss-of-function. Integrants were readily detected via diagnostic PCR and were further verified by Southern blot. The success with this experiment ensured that the kinase locus is recombinogenic, and it is due to the essentiality of these kinases involved in the parasite replication cycle that resulted in the inability to observe the integrant population.

Fusion of a tag at 3' end of the kinase sequences after a single cross-over homologous recombination would produce a tagged functional enzyme which even after a recombination process does not lead to a "loss of

function" as opposed to the knock-out strategy where the kinase domain is separated. The tagging of kinases allowed in proving the involvement of the kinases in the asexual replication cycle on one hand and on the other provided a tool for further characterization of the kinases using for e.g. anti-Myc antibody. Myc-tagged *Pf*CLK-1 and *Pf*CLK-2 were verified by Western blot analysis using the antisera against the Myc-tag. On lysates of Myc-tagged *Pf*CLK-1/Lammer, the same 60 kDa band was detectable as on the lysates obtained from WT 3D7 parasites. On Myc-tagged *Pf*CLK-2 lysates, only the 50 kDa protein band was detectable instead of the 150 kDa band obtained when parasite lysates of endogenous kinase were used. This suggests that possibly the 70 kDa, 50 kDa and 40 kDa are either the processed or degraded products of the kinase and only 50 kDa retains the Myc-tag. This was further confirmed when the same blot was stripped and proteins bands of sizes 150 kDa, 70 kDa and 50 kDa were detectable with anti-*Pf*CLK-2. The absence of 40 kDa band could be due to a cross-reaction. PCR amplification demonstrated successful tagging for *Pf*CLK-4 with HA-epitope and no clear integration bands could be detected for *Pf*CLK-3 tagging. Further characterization of this tagged kinase is under progress.

Together, these results concluded that all four CLK kinases are important to parasite asexual survival, which is also in accord to their mRNA level expression and protein level expression detected throughout asexual blood stages.

4.5 *Pf*CLK-1/Lammer and *Pf*CLK-2 associate with a kinase activity

Repeated trials on recombinant *Pf*CLK-1 from the catalytic domain region failed to demonstrate any kinase activity in terms of autophosphorylation and phosphorylation of physiological substrates provided in vitro. This could have been due to some missing residues in the recombinant protein. A faint phosphorylation signal was observed when the recombinant *Pf*CLK-1 was incubated with heat inactivated parasite lysate that served as the substrate for the reaction. This experiment ensured the availability of possibly required endogenous proteins as substrates. Heat treatment of the parasite lysate inactivated the kinases that might interfere in the assay. Such heat inactivation

of kinases in activity assays has been previously described (Solyakov et al., 2004).

Substrate phosphorylation by recombinant *Pf*CLK-1/Lammer was further investigated in presence of the active parasite extract. Here, phosphorylation of all physiological substrates was observed. A similar phosphorylation activity was observed when a kinase dead mutant of the protein, in which a conserved Lys-581 was replaced by a methionine residue. This Lysine residue in subdomain II is considered essential for the phosphotransfer reaction and a substitution renders the kinase inactive (Carrera et al., 1993). Negligible background phosphorylation was observed when only the parasite extract or when only the uncoated Glutathione beads were used in the kinase assay. Therefore, the phosphorylation was observed only in combination of parasite extract with *Pf*CLK-1/Lammer coated beads. The phosphorylation activity of the kinase dead mutant as well as the necessity of additional proteins from the parasite extract for phosphorylation suggest that *Pf*CLK-1/Lammer might not itself carry out phosphorylation, but that it is associated in a complex with other parasite proteins possessing phosphorylation activity.

Endogenous Myc-tagged *Pf*CLK-1/Lammer and *Pf*CLK-2, immunoprecipitated using anti-Myc antibody revealed phoshorylation activity, when exogenous substrates i.e, histone H1, MBP and α/β caseins were added to the immunoprecipitated kinase in the presence of radiolabelled ATP. This result further showed the requirement of in vivo binding partners for kinase activity.

Further, mass spectrometric analysis was performed in order to extricate the possible interaction partners. Several protein bands were analysed against the *Plasmodium* genome. None of these binding partners identified any protein with known function in mRNA splicing. However, a number of proteins with presumed roles in nucleic acid processing and signal transduction were identified. A binding partner of *Pf*CLK-1/Lammer was identified as PF14_0117 which revealed similarity with nucleases and nucleotidases from parasites such as *Plasmodium* sp. and *Leishmania major*. A neighboring gene in *P. falciparum* as well as in its orthologue, *P. vivax* was identified as a nuclear transport factor-2 which binds to RanGDP and is required for an efficient transport of nuclear localization signal (NLS) containing proteins (Moore and Blobel, 1994; Zhao et

al., 2006). A second neighboring gene encodes for p1/s1 nucleases which cleave RNA and single-stranded DNA. A number of potential binding partners for *Pf*CLK-2 on the other hand, were identified as plasmodial proteins, although none of them had a known function. One of the identified partner was the *P falciparum* protein encoded by PFL1445w. This protein has a sequence homology to proteins comprising leucine rich repeats (LRR) that are involved in protein-protein interactions such as RNAse inhibitor, ribonucleoprotein binding U2A protein. In addition, this binding partner of *Pf*CLK-2 also shares homology to a heat shock protein, hslV (heat shock loci V) that belongs to a class of threonine proteases. Previous studies have reported that kinases such PKN are cleaved by a protease, caspase-3 (Takahashi et al., 1998). A second binding partner, PF13_0187 cluster with the histone H3 encoding gene and a blast analysis revealed *P. yeolli* DNA replication helicase 2 has a high homology with this binding partner. Three other binding partners encoded by genes in *Plasmodium* were identified. Each of them was associated with proteins like RNA binding protein, palatin-like phospholipase, nucleic acid binding proteins, phosphatases etc.

Thus, mass spectrometry data revealed several hypothetical proteins from *Plasmodium* genome that could be correlated with proteins like nucleases, phospholipases, histone, RNA binding proteins and nuclear transport factor. Most of these proteins were in synteny or in shared synteny with the proteins identified as kinase binding partners. Synteny describes the physical co-localization of genetic loci on the same chromosome within an individual or species. It is a shared synteny when the preserved co-localization of genes is on chromosomes of related species, in this case, *Plasmodium* species such as *P. vivax* or *P. yoelli*.

The activity of kinases in presence of parasite derived proteins and linkage of identified binding partners with proteins related to one or the other process in nucleus or transport and assistance in mRNA export strengthens the idea that function of both *Pf*CLK-1/Lammer and *Pf*CLK-2 is interwoven in a multiprotein complex rather than direct phosphorylation of their in vivo substrates.

4.6 *Pf*CLK kinases possess a role in transcriptional regulation of *P. falciparum*

As genes of similar function are thought to cluster together, the findings from mass spectrometry further strongly corroborate that the two CLK kinases phosphorylate splicing factors that are integral part *P. falciparum* spliceosomal machinery. SR proteins were traditionally thought to be crucial to mRNA splicing, but more recently they have been identified to play a central role in assisting mRNA from its transcription in the nucleus, its nuclear export, maintaining its stability followed by translational modifications in the cytoplasm to its final degradation of the protein in the cytoplasm. Thus, these are one of the major mRNA binding proteins in addition to messenger ribonucleoprotein particles (mRNPs). SR proteins contain a couple of RNA recognition motifs (RRM) at their terminals which are rich in arginine-serine repeats (RS domains). At these serine residues, the SR proteins are phosphorylated by a number of kinases (Bourgeois et al., 2004). Some SR proteins remain in the speckles in the interphase nucleus and some shuttle between the nucleus and the cytoplasm where they become phosphorylated by the residing kinases (reviewed in Huang and Steitz, 2005). Upon phosphorylation they are exported to the sites of pre-mRNA synthesis where they in addition to participating in splicing process are channeled into the transcriptional regulation in association with naïve mRNPs that transport the mRNA into the cell cytoplasm (reviewed in Bouregeois et al., 2004; Dimaano and Ullmann, 2004).

Interestingly, the activity of SR proteins is controlled by interplay of phosphorylating CLK kinases and dephosphorylating phosphatases, and only dephosphorylated splicing factors appear to accompany mRNA from the nucleus to the cytoplasm, while rephosphorylation facilitates their release from the mRNPs as shown for the shuttle protein Npl3p (Gilbert and Gunthrie, 2004). A very recent study documented that human serine/threonine CLK kinase, CDKL5 encoded by a human X-linked gene controls the morphology of nuclear speckles. It phosphorylates in vitro a nuclear protein MeCP2 (Mari et al., 2005; Lin et al., 2005; Kameshita et al., 2008). Abnormality in CDKL5 kinase results in large and irregular shaped speckles. Mutations in either or both MeCP2 and CDKL5 result in neurodevelopmental disorders and Rett syndrome (Ricciardi et al., 2009). Both MePC2 and CDKL5 have been implicated in binding with DNA

methyltransferase 1, an enzyme that methylates CpG dinucleotides after DNA replication (Kameshita et al., 2008). Furthermore, MeCP2 is a nuclear protein while CDKL5 shuttles between the nucleus and the cytoplasm through a CRM1-mediated nuclear export mechanism (Rusconi et al., 2008). Although, CDKL5 has an influence on RNA splicing, its nuclear distribution is not affected by inhibition of cellular transcription or by association with RNA (Ricciardi et al., 2009). These results are well correlated with serine/threonine *Pf*CLK-1 and *Pf*CLK-2 kinases both of which are compartmentalized in the nucleus as well as in the cytoplasm and are expected to have a role in mRNA splicing as depicted by bioinformatics tools and by blast analysis of Npl3p homologous sequences in the *Plasmodium* genome.

A hypothesis (Figure 4.1) based on the mass spectrometric data suggests that *Pf*CLK-1/Lammer and *Pf*CLK-2 interact in a protein complex leading to the phosphorylation of splicing factors which aid in pre-mRNA splicing. Once the mature mRNA moiety is formed, phosphatases dephosphorylate the splicing components to aid their nuclear export. However, the splicing factors remain attached to the nascent mRNA to stabilize it until the translation has occurred. It is possible that the cytoplasmic *Pf*CLK-1/Lammer and *Pf*CLK-2 rephosphorylate a set of cytosolic splicing factors that leads to their release from the mRNA followed by their nuclear re-import.

The control of transcript processing is an important process in *P. falciparum* due to the role of post-transcriptional regulation of expression of the intron-rich parasite genes. At least 60% of the *Plasmodium* genome is transcriptionally active during intraerythrocytic development (Arvind et al., 2003). Alterations in the kinases phosphorylating these splicing factors could cause metabolic defects. One study has reported that abrupt alternative splicing of the calcium/calmodulin-dependent kinase IIδ transcript results in cardiomyocytes deficient in ASF/SF2 leading to a defect in hypercontraction (Xu et al., 2005).

Splicing repression during heat shock by a SR protein, SRp38 contributes to the cell survival under stress conditions (Shin et al., 2004). Previous studies also provide evidence of some of the SR proteins like ASF/SF2 and Npl3p to be associated with 80S ribosomes and polysomes

thereby reflecting their roles not only in mRNA splicing but also during translation process (Sanford et al., 2004; Windgassen et al., 2004).

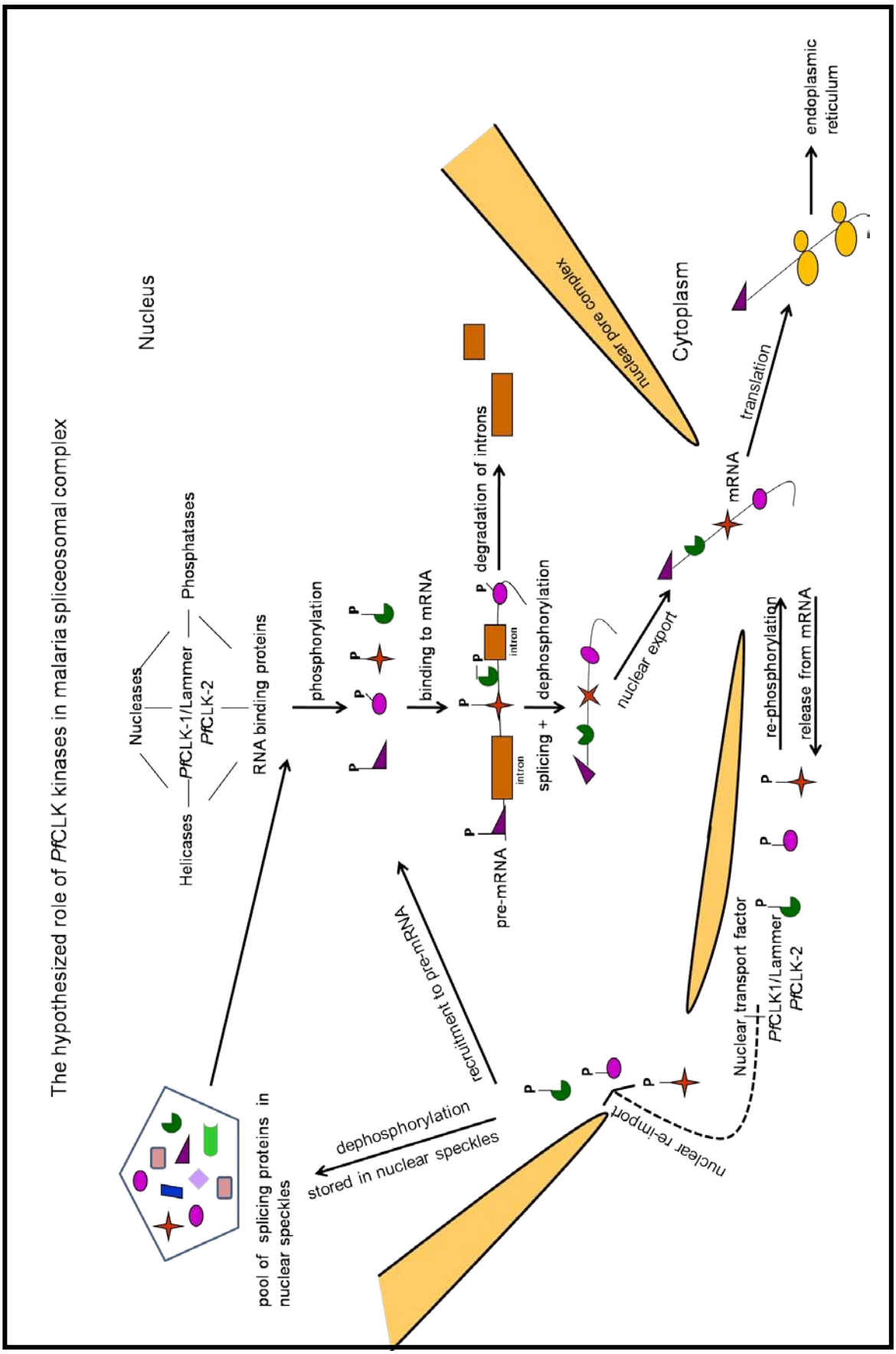


Figure 4.1: A hypothesis based on the role of *Pf*CLK kinases in malaria spliceosomal machinery. In the present study, kinase assays revealed that *Pf*CLK-1/Lammer and *Pf*CLK-2 assemble in a protein complex. Further, mass spectrometry analysis detected potential binding partners of the two *Pf*CLK kinases. Since genes of the similar function cluster together, products of syntenic genes were analysed in silico. Association of *Pf*CLK kinases was therefore correlated with helicases, nucleases, phosphatases and RNA binding protein. Additionally, the expression profile of *Pf*CLK-1/Lammer and *Pf*CLK-2 at the protein level localized both kinases in the parasite nucleus and cytoplasm. The role of SR-rich splicing proteins in mRNA splicing and export has been previously described. Also it has been reviewed that phosphorylated splicing factors are recruited at the splice sites on the pre-mRNA. While being still associated with the mRNA moiety they are dephosphorylated by in vivo phosphatases and aid in the mRNA nuclear export. Furthermore, previous reports suggest that re-phosphorylation of the mRNA bound splicing factors by yet unknown kinases results in their release from the mRNA to aid the translation process. It is possible that the two cytoplasmic *Pf*CLK kinases possess a role during the phosphorylation process. The re-phosphorylated splicing factors are somehow re-imported to the nucleus. These factors could then either be directly recruited to splice sites again or dephosphorylated to be stored or processed in the nuclear compartment called the nuclear speckles. By in silico studies, the two *Pf*CLK kinases have been found to localize within the nuclear speckles. Via immunoelectron microscopy, *Pf*CLK-1/Lammer was also detected at the passage between the nucleus and cytolasm. Moreover, chromosomal location of a gene encoding the nuclear transport factor was found close to a potential binding partner as detected by mass spectrometry. Our in silico analysis as well as protein expression data together with the previous studies hint towards the shuttle of the two *Pf*CLK kinases between the parasite nucleus and cytoplasm thereby regulating the phosphorylation status of the splicing factors during their entry and exit from the parasite nucleus.

Thus in the present study, it has been shown that *Pf*CLK-1/Lammer and *Pf*CLK-2 localize in the nucleus and the cytoplasm of asexual parasites and gametocytes All four CLK kinases are essential for intraerythrocytic multiplication of the asexual parasites. Successful tagging of the kinases provided a tool to further characterize the kinases by Western blot, co-immunoprecipitation studies and kinase assays. A similar approach has revealed the sub-cellular localization of NIMA-related kinase with microtubular structures in gametocytes (Reininger et al., 2009) and CDPK4 with the plasma membrane of gametocytes (Ranjan et al., 2009).

Kinase activity assays on immunoprecipitated *Pf*CLK-1/Lammer and *Pf*CLK-2 raised the possibility that these proteins associate in a protein complex for accomplishing their purpose of existence in the parasite.

All the above cited examples of eukaryotic CLK kinases correlate well with their role in regulation of phosphorylation of SR proteins and snRNPs which in turn is crucial to the splicing machinery to control gene expression. However, up to date, the modes of action of mRNA splicing and the

composition of mRNPs remain largely unknown. Experimental evidence indicating mode of function of plasmodial spliceosomal machinery is limited although recently some studies identifying alternative splicing in *P. falciparum* genome are available (Iriko et al., 2009). Furthermore, the *Plasmodium* genome resource provides yeast-2-hybrid (Y2H) interactions of each protein by in silico analysis but most of these proteins have unknown function and are purely hypothetical. None of the binding partners that were identified by mass spectrometry belong to this Y2H interactions documented. It is worth mentioning here that another study determined the protein-protein interactions in *P. falciparum* by high throughput Y2H assay that clustered discrete protein domain in sub-networks which can implicate proteins relevant to a process. Based on such networks, *Pf*CLK-3, the protein closely related with human Prp4 was shown to share domains with PFI1715w which is a *Plasmodium* exported protein with an unknown function whereas, *Pf*CLK-4 shares domains with another kinase PF14_0294 (LaCount et al., 2005). PF14_0294 is the well characterized mitogen-activated protein kinase1 (*Pf*map-1) which has roles in cell proliferation and signal transduction (Doerig et al., 1996).

The current study provides first detailed characterization of at least two CLK kinases of *P. falciparum* which are nucleus-localized, show association with phosphorylation activity and play an essential role for blood stage replication. All above results corroborate with the hypothetical role of *Pf*CLKs in mRNA splicing. Thus, these findings form the platform for future studies on unveiling the malaria spliceosome, which is so far unexplored. Understanding the underlying mechanisms might lead to the discovery of unknown targets for chemotherapy. Several kinases have been characterized essential to the parasite erythrocytic schizogony which could symbolize the targets for curative schizonticidal drugs.

Protein expression analysis of *Pf*CLK-3 and *Pf*CLK-4, and identification of splicing factors that might represent the substrates of the four *Pf*CLK kinases is currently under progress.

4.7 Calcium-mediated signaling in malaria parasites

Ca^{2+}/Calmodulin-dependent protein kinases are serine/threonine protein kinases possessing N-terminal kinase domain. Principally, calmodulin binds to

calcium so that it may be utilized by eukaryotes in a number of ways. Calcium influences a great number of biological processes like cell motility, muscle contraction, cytoplasmic streaming, chromosome movement, glycogen metabolism (Cheung et al., 1980). For calcium to exert its effects it must be bound to receptors. Calmodulin is the primary receptor of calcium and is present in all eukaryotic cells where it functions as a mediator of calcium mediated pathways and act as a regulator of calcium-dependent enzymes (Cheung et al., 1980). The calcium-calmodulin binding results in a 10-100 thousand fold increase in the affinity of the receptor protein for target molecules such as hormones (Wang et al., 1985). These chemical substances represent first messengers which bind to intracellularly located secondary messengers like cyclic-AMP. cyclic-AMP in a signal cascade that activates protein kinases which further phosphorylate their substrates for control of gene expression.

Both calcium dependent protein kinases (CDPK) and calmodulin-dependent kinases (CaMK) have been investigated in *P. falciparum* underlining the importance of calcium signaling in the parasite. Around thirteen such *P. falciparum* eukaryotic protein kinases have been identified in the parasite kinome. Calmodulin comprises of four “EF-hand motifs” each of which binds to a Ca^{2+} ion. Upon activation by calcium, calmodulin further acts on kinases thereby activating them. Six plasmodial CDPKs have been previously described (Doerig et al., 2009). Furthermore, it has been documented that CDPKs have a calmodulin-like domain and are activated by calcium ions during unavailability of calmodulin. In addition, the CaMK family includes calcium-dependent protein kinases (CDPKs), as well as a distinct group that does not contain EF-hand motifs (important for calcium-binding) (Ward et al., 2004)..

Till date, the Ca^{2+} /CaM kinases, that have been shown to be essential for erythrocytic schizogony include few examples like *Pf*CDPK1 (Kato et al., 2008) and *Pf*PKB kinase that plays a role in the erythrocyte invasion (Vaid et al., 2007). Other existing examples of *Pb*CDPKs show a blockage in transmission stages in the mutant parasites. The fact that humans lack CDPKs, their selective inhibition in *Plasmodium* is achievable which makes the CDPK family, a candidate for the transmission blocking strategy. CDPK3 of rodent malaria parasite, *P. berghei* is produced in ookinetes and is involved in ookinete penetration through basal lamina of the mosquito midgut. In disrupted

parasites, the ability of the ookinetes to infect mosquito midgut is significantly reduced. In wild type parasites extracellular calcium provided together with a functional CDPK3 aid the invasion process. Thus, CDPK3 is shown to be involved in a calcium dependent signalling pathway (Ishino et al., 2006).

Calcium-dependent cell signaling drives shape changes in many cells (Berridge et al., 2000). Such changes in *Plasmodium* have also been documented where invasion of erythrocytes and intraerythrocytic development of merozites was affected by calcium triggers (Deighton et al., 1992; Garcia et al., 1999; Hotta et al., 2000). Calcium-derived shape changes have been documented via two mechanisms. The first one is by the direct interaction of calcium with cytoskeleton polymerization proteins while the second one is the complex of calcium-calmodulin that activates target proteins (Acuto et al., 2000). One of the target proteins is the CaMK family that includes CaMK I, II and IV which further activates the signalling eventleading to an elevation of intracellular calcium (Means et al., 2000; Soderling et al., 2000). Upon activation, CaM kinases play essential roles in maintaining cell morphology and cytoskeleton assembly by phosphorylation of associated proteins (le Gouvello et al., 1998; Sanchez et al., 2000). Several constituents of calcium signalling pathway including CDPKs, Ca^{2+}-ATPases, noncytosolic EF-hand Ca^{2+} binding proteins have been investigated without any conclusive roles in the parasite infection or development (Garcia et al., 1999). Further CaMK play roles in the cell growth and elongation (Mean et al., 2000; Sanchez et al., 2000; Oberstart et al., 1997; Yamauchi et al., 1998).

4.8 *P. falciparum* calmodulin-dependent protein kinase, *Pf*PKRP is predominantly expressed in gametocytes

The hypothetical kinase *Pf*PKRP (Calmodulin-dependent protein kinase related protein) belongs to the group of CaMK and is expected to be involved in signaling events of the parasite. Its orthologue *Pb*PKRP, has recently been knocked-out in the *P. berghei* rodent malaria model (Purcell et al., 2010). Both *Pf*PKRP and *Pb*PKRP lack EF-hand motifs, both enzymes possess only a single glycine in their glycine triad (Purcell et al., 2010). The PKRP orthologues were closely aligned using bioinformatics tools with a human enzyme, TSSK1 which is specifically expressed in human testes having a role in fusion of the

sperm and the oocyte (Hao et al., 2004). Interestingly *Pb*PKRP has also been assigned a role in cell invasion (Purcell et al., 2010). Parasites like *Toxoplasma gondii* and *Cryptosporidium parvum* additionally require CDPK-like proteins during cell invasion (Bonhomme et al., 1999; Nagamune and Sibley, 2006). Further, CDPK6 is involved in the signal cascade that leads to the activation of sporozoites for invasion (Coppi et al., 2007).

Phenotype characterization of the *Pb*PKRP knock-out mutants revealed normal sexual stage as well as ookinete and oocyst development. Sporozoites that develop within the oocysts show morphological similarities with the wild type, however, sporozoite transition to the salivary glands is inhibited. This phenotype is in striking coincidence with the knock-out phenotypes of select *Pf*CCp proteins, multi-adhesion domain proteins that are predominantly expressed in gametocytes (Pradel *et al.*, 2004; Scholz *et al.*, 2008; reviewed in Pradel, 2007).

Transcription level analysis of *PfPKRP* was performed by a diagnostic RT-PCR using asexual and gametocyte derived cDNA libraries. Transcript abundance in gametocytes as compared with cDNA derived from asexual stages of the parasite suggested that the gene is predominantly transcribed in gametocyte stage of the parasite. For determination of kinase expression at protein level, antiserum against the catalytic domain of *Pf*PKRP using a peptide sequence was generated. As mentioned for CLK kinases, kinase domain plays an important role in kinase activity and stability and therefore this region was chosen for kinase characterization. The domain architecture of the kinase reveals only an N-terminal kinase domain with no EF-hand and no signal peptide indicating that the kinase is cytoplasmic. Immunofluorescence localized faint kinase expression in schizonts but copious expression in the cytoplasm of stage II-V gametocytes in a punctuated pattern. No expression was detected in gametes. This signifies that the kinase has a predominant role in gametocyte differentiation and the expression decreases after gametocyte maturation. Unlike *Pf*CLK kinases, no expression was observed in association with the parasite nucleus as observed by Hoechst staining, furthermore confirming its cytoplasmic localization. Western blot analysis detected a protein band migrating at the full-length weight of 295 kDa. Additional protein bands at

approximately 170 kDa and 75 kDa were detected, indicating that the *Pf*PKRP kinase might be processed during expression as observed in *Pf*CLK kinases.

4.9 *Pf*PKRP as a potential candidate for transmission blocking drugs

Previously described reverse genetic approaches were applied for functional analysis of *Pf*PKRP (Dorin-Semblat et al., 2007). Diagnostic PCRs determined successful disruption of the gene locus separating the essential ATP binding GXGXXG motif and the PE motif required for structural stability of the kinase. As asexual parasites used for transfection are haploid and the genes under consideration exist in a single copy, it is possible to obtain a null mutant by only one round of drug selection (reviewed in Doerig et al., 2009). Since the population of parasites obtained after integration is a mixture of parasites having a recombined gene locus and a wild type locus, clonal isolation becomes necessary for characterization of the knock-out phenotype. Clonal dilution strategy was used to isolate single clones namely, G4 and G8 mutants representing only the parasites possessing recombined locus. Both mutants were verified for recombined locus via Southern blot, however only G4 appeared to have an integrant population free of the wild type gene locus. Emergence of spontaneous wild type in G8 clone could be due to the reversion of parasites from *Pf*PKRP^{-} to the wild type parasites. The characterization of disrupted gene locus could not be verified by Western blot analysis or by immunofluorescence assay due to the lack of an appropriate anti-*Pf*PKRP antibody. Generation of *Pf*PKRP antiserum from C-terminal region is currently in progress.

It is a challenging task to ascertain the phenotype of the genes essential to the asexual survival of parasites as gene-disruptant parasites do not survive. Genes like *Pf*PKRP however, are categorized under the genes that are dispensable for the parasite replication cycle and could have an important role in the infection of mosquito vector, thus representing a target for a transmission blocking drug. *Pb*CDPK4 was the first reported knock-out where parasite clones had a normal asexual growth pattern and gametogenesis but could not lead to an infection in mosquito due to a blockage in the exflagellation of male gametocytes (Billker et al., 2004). In case of G4 mutant of *Pf*PKRP, a typical asexual growth pattern is observed leading to the formation of gametocytes

from stage II-V. However, even after repeated observations for exflagellation centers, rarely a two-three centers could be located in about 10 fields investigated. Wild type gametocytes cultivated for same length of time on the other hand show 2-4 exflagellation centers per microscopic field observed. Induction of exflagellation was also attempted by addition of mosquito derived molecule, Xanthurenic acid (XA) in addition to the human serum. XA is an inducer of differentiation of gametocytes into male and female gametes but even in presence of this chemical, no significant change in the exflagellation event was observed. Mosquitoes were fed with wild type, stage V gametocytes to localize the *Pf*PKRP expression in mosquito derived parasite stages. No expression was detectable in zygotes, retorts/ookinetes while a faint labeling was detected in salivary gland derived sporozoites. This could imply that the kinase might be expressed at very low levels in the sporozoites which are injected by the mosquito into the human host during a blood meal.

Expression remains low during asexual blood stages but becomes higher during the gametocyte differentiation. Lack of this CaM-dependent protein kinase in mature gametocytes might affect the signal cascade leading to gametocyte activation thereby causing abnormalities in the development of a microgametocyte into a microgamete. This could also explain extremely less or complete lack of exflagellation in the *Pf*PKRP mutant parasites. Via, immunoelectron microscopy it has been demonstrated that ookinetes are not able to access the midgut epithelial cells in parasites lacking CDPK3. In contrast, when the morphology of G4 mutant was analysed by immunoelectron microscopy, no architectural deformation could be observed despite the fact that calcium alterations mediate shape changes (Berridge et al., 2000). All parasite organelles like nucleus, osmiophilic bodies, food vacuole, mitochondria, endoplasmic reticulum and erythrocyte membrane were present and were comparable to the wild type parasites.

Thus, the *Pf*PKRP mutant parasite line shows a normal architecture, normal growth of asexual blood stage parasites, but reduced or a blocked exflagellation. We are currently investigating if this is associated with a reduced number of male or female gametocytes using sex-specific proteins as controls. Gametocytes are said to be developmentally arrested while they are circulating in the bloodstream of vertebrates, but are readily activated in the mosquito

midgut after a blood meal (Billker et al., 2004). Similarly, the microgametocyte undergoes three rounds of endomitotic division before the motile male gametes are formed. Although the key players of this phenomenon in *P. falciparum* are not till date known but previous inhibitor studies allude the involvement of several kinases and secondary messengers such as calcium, cyclic nucleotides in sexual stage development of the parasite (Kawamoto et al., 1990; Martin et al., 1994).

Essential functions of the protein kinases in parasite proliferation and development have rendered them as representatives of anti plasmodial drug targets or as targets of transmission blocking drugs. Amongst the important targets are, *Pf*PK5, PKG, *Pf*PK7 (Holton et al., 2003; Doerig, 2004; Ward et al., 2004; Doerig et al., 2005; Doerig and Meijer, 2007; Merckx et al., 2008; Klein M et al., 2009).

In view of the established significance of calcium in signal cascades and available examples of kinases involved in transmission stages, CaM-dependent protein kinases form an important class of enzymes that could be targeted for antiplasmodial compounds. Recently in *P. gallinaceum*, it has been shown that calcium/calmodulin dependent protein kinases have a role in blocking morphological differentiation from zygotes to ookinetes. This study was based on using calmodulin antagonists, W-7 and calmidazoilum and specific CaMK inhibitor, KN-93 (Silva-Neto et al., 2002).

Several studies have also reported that CaM antagonists block gametogenesis in *P. falciparum* and *P. berghei* (Kawamoto et al., 1990; Kawamoto et al., 1993) placing further evidences that CaMK do have important roles in parasite growth, cell invasion and differentiation. Investigation of *Pf*PKRP so far highlights that it is dispensable for asexual parasite development, but the mutant G4 shows significant reduction in exflagellation process with a normal morphology as compared with the wild type parasites.

Further detailed study on the protein expression of *Pf*PKRP is ongoing to gain a deeper insight into the role of *Pf*PKRP in development of sexual stages in the parasite life cycle. Trivial anti-malarial compounds like Primaquine and chloroquine have been shown to be effective in clearing and reducing gametocytemia respectively. While primaquine clears sub-microscopic levels of gametocytes, chloroquine on the other hand, is effective on immature

gametocytes (Shekalaghe et al., 2007; Smalley, 1977). Both drugs additionally exhibit as CaM antagonists at low concentration, inhibiting the activity of CaMKs in vitro (van Weert et al., 2000). Thus, CaMKs represent prospective enzymes for transmission blocking strategies particularly when the propensity of the parasites to develop drug resistance, clearly elevates the need for research on novel drug targets.

5. Future perspectives

Available data shows that the four members of CLK kinases are transcribed throughout the blood stages of the parasite and the expression ceases after fertilization. Reverse genetics approach exhibited that the kinases are essential for replication of the parasite. In accord to the above results, protein level investigations of *Pf*CLK-1/Lammer and *Pf*CLK-2 reflected the presence of the predominant kinase expression in the parasite nucleus. In other eukaryotes, CLK kinases have been reported for their interaction with Serine/Arginine-rich splicing factors. In view of this available information and due to the fact that *Pf*CLK kinases belong to the mRNA splicing branch of the parasite kinome, it becomes crucial to further investigate the signal cascade where these kinases are involved. The expression and sub-cellular localization studies, in this thesis shed light on the characterization and localization of kinases in the parasite. Kinase activity assays on recombinant *Pf*CLK-1/Lammer gave the first hint of the kinase been involved in a protein complex. As an attempt to investigate the interaction partners, mass-spectrometry was performed on the co-immunoprecipitated proteins. The database search reflected that most of the binding partners identified by this strategy have one or the other function corresponding to nuclear transport, RNA binding, dephosphorylation processes. Though these gave a generalized view of the possible kinase interactions, specific binding partner(s) is yet to be investigated.

Similar studies on protein expression profile via immunofluorescence assay, immunoelectron microscopy and Western blot analysis would be conducted for *Pf*CLK-3 and *Pf*CLK-4. Specific binding partners of all four *Pf*CLK kinases via co-immunoprecipitation and mass spectrometry will be investigated. Previous studies have shown that Sky1p, a yeast serine/threonine kinase has high homology to the kinase domains of the four *Pf*CLK kinases. It has a specific substrate, Npl3p which is a nuclear shuttle protein. Kinase assays using GST-tagged Npl3p as a substrate would be performed to test the activity of the four *Pf*CLK kinases. The GST-tagged Npl3p has been kindly provided by Dr. Gourisankar Ghosh, University of California.

Mammalian CLK kinases utilize ASF/SF2 as their substrate. Thus we extracted the plasmodial homologues of Np3p and ASF, represented by three plasmodial proteins, PFE0865c, PF10_0217, and PF11_0205 which are putative splicing factors. Interaction studies are now being carried out by two approaches. One approach would investigate the direct interaction between the kinase and putative splicing factors via Yeast-2-hybrid analysis. The second approach relies on the recombinant expression of three proteins to be further exploited for co-immunoprecipitation assay and a kinase assay. This would reveal if they are the prospective substrates of *Pf*CLK kinases. Knowledge on all these aspects would further aid in understanding the hitherto unexplored malarial spliceosomal complex.

In the second part of my PhD thesis, I showed successful gene disruption of *Pf*PKRP, indicating a potential role of the enzyme in sexual stage development of the parasite. Expression at both transcript and protein level suggested its predominant expression in gametocytes. Morphological analysis of *Pf*PKRP mutant did not display any architectural deformity as compared with the wild type, although preliminary results, show reduced exflagellation. Therefore, quantification of microgametes will be performed by immunofluorescence assay on activated stage V gametocyte cultures. To ensure the phenotype of the *Pf*PKRP mutant, its expression through the transmission stages of the parasite in mosquito vector would be studied in more detail by membrane feeding of mosquitoes with the mutant parasite line. In addition, its possible sex-specific expression would be investigated by quantifying the gender ratio in comparison with the wild type parasites. Further determination of CaMK involvement in gametocyte differentiation would be evaluated by adding specific calmodulin inhibitors at early stages of gametocyte development. Exflagellation assays on mature gametocyte cultures using these inhibitors are currently under progress to test the effect of CaMKs on gametogenesis.

Thus, in presence of the current data, a detailed analysis would provide a better understanding in unveiling the kinase mediated signalling cascades. This would ameliorate our knowledge on complex signalling mechanisms that are being utilized by the parasite in hijacking the host cell machinery.

6. Summary

Malaria still persists as one of the deadliest infectious disease in addition to AIDS and tuberculosis. It is a leading cause of high mortality and morbidity rates in the developing world despite of groundbreaking research on global eradication of the disease initiated by WHO, about half a century ago. Lack of a commercially available vaccine and rapid spread of drug resistance have hampered the attempts of extinguishing malaria, which still leads to an annual death toll of about one million people. Resistance to anti-malarial compounds thus renders search for new target proteins imperative.

The kinome of the human malaria parasite *Plasmodium falciparum* comprises representatives of most eukaryotic protein kinase groups, including kinases which regulate proliferation and differentiation processes. Several reports till date have suggested involvement of parasite kinases in the human host and as well as in the mosquito vector. Kinases essential for life cycle stages of the parasite represent promising targets for anti-malarial compounds thus, provoking characterization of additional malarial kinases.

Despite extensive research on most plasmodial enzymes, very little information is available regarding the four identified members of the cyclin-dependent kinase like kinase (CLK) family. Thus, the present thesis dealt with the functional characterization of four members of the *Pf*CLK kinase family of the parasite denoted as *Pf*CLK-1/Lammer, *Pf*CLK-2, *Pf*CLK-3 and *Pf*CLK-4 with a special focus on the first two kinases. Additionally, one Ca^{2+}/Calmodulin dependent putative kinase-related protein, *Pf*PKRP, presumed to be involved in sexual stage development of the parasite, was investigated for its expression in the life cycle of the parasite.

In other eukaryotes, CLK kinases regulate mRNA splicing through phosphorylation of Serine/Arginine-rich proteins. Transcription analysis revealed abundance of *PfCLK* kinase genes throughout the asexual blood stages and in gametocytes. By reverse genetics approach it was demonstrated that all four kinases are essential for completion of the asexual replication cycle of *P. falciparum*. *Pf*CLK-1/Lammer possesses two nuclear localization signals and *Pf*CLK-2 possesses one of these signals upstream of the C-terminal catalytic domains. Protein level expression and sub-cellular localization of the

two kinases was determined by generation of antiserum directed against the kinase domains of the respective kinase. Indirect immunofluorescence, Western blot and electron microscopy data confirm that the kinases are primarily localized in the parasite nucleus, and in vitro assays show that both enzymes are associated with phosphorylation activity. Finally, mass spectrometric analysis of co-immunoprecipitated proteins shows interactions of the two *Pf*CLK kinases with proteins, which have putative nuclease, phosphatase or helicase functions.

*Pf*PKRP on the other hand is predominantly expressed during gametocyte differentiation as identified from transcriptional analysis. Antiserum directed against the catalytic domain of *Pf*PKRP detected the protein expression profile in both asexual and gametocyte parasite lysates. Via immunofluorescence assay, the kinase was localized in the parasite cytoplasm in a punctuated manner, mostly in the gametocyte stages. Reverse genetics resulted in the generation of *Pf*PKRP gene-disruptant parasites, thus demonstrating that unlike CLK kinases, *Pf*PKRP is dispensable for asexual parasite survival and hence might have crucial role in sexual development of the parasite.

On one hand, characterization of *Pf*CLK kinases exemplified the kinases involved in parasite replication cycle. Successful gene-disruption and protein expression of *Pf*PKRP kinase on the other hand, demonstrated a role of the kinase in sexual stage development of the parasite. Both kinase families therefore, represent potential candidates for anti-plasmodial compounds.

7. Zusammenfassung

Malaria stellt neben AIDS und Tuberkulose weiterhin eine der bedeutendsten Infektionskrankheiten dar. Trotz intensiver, auf die Auslöschung der Krankheit abzielender Forschung, welche vor etwa 50 Jahren durch die Weltgesundheitsorganisation initiiert wurde, bleibt Malaria einer der Hauptgründe für hohe Mortalität und Morbidität in Entwicklungsländern. Das Fehlen eines Impfstoffes und die schnelle Ausbreitung von Resistenzen erschweren die Versuche, Malaria zu eliminieren, welche jährlich weiterhin eine Todesrate von einer Millionen Menschen aufweist. Aufgrund der Zunahme an Resistenzen ist die Suche nach neuen Angriffspunkten für Antimalariamedikamente zwingend erforderlich.

Das Kinom des humanpathogen Parasiten *Plasmodium falciparum* besteht aus Vertretern der meisten eukaryotischen Proteinkinasegruppen, einschließlich einiger Kinasen, welche Proliferations- und Differenzierungsprozesse regulieren. Verschiedenen Berichten zufolge ist eine Rolle von Parasitenkinasen sowohl im menschlichen Wirt als auch in der die Krankenheit übertragende Mücke denkbar. Kinasen, welche für verschiedene Parasitenstadien essentiell sind, stellen viel versprechende Angriffspunkte für Malariamedikamente dar. Dies bestätigt die Bedeutung der Erforschung von weiteren, bisher uncharakterisierten Kinasen.

Trotz extensiver Forschungsarbeit an den meisten Enzymen des Parasiten ist bisher sehr wenig über die vier identifizierten Mitglieder der Proteinfamilie Zyklin-abhängige-Kinase-ähnlicher Kinasen (cyclin-dependent kinase like kinases, CLK) bekannt. Aufgrund dessen war die Charakterisierung der vier Mitglieder der *Pf*CLK-Kinasefamilie, *Pf*CLK-1/*Pf*LAMMER, *Pf*CLK-2, *Pf*CLK-3 und *Pf*CLK-4 Bestandteil dieser Arbeit. Der Forschungsschwerpunkt lag hierbei auf den beiden erstgenannten Kinasen. Zusätzlich wurde die stadienspezifische Expression von *Pf*PKRP, einer Kinase, welche vermutlich in der Entwicklung der Sexualstadien des Parasiten beteiligt ist, untersucht.

In anderen Eukaryoten regulieren die CLK kinases das Spleißen von mRNA durch die Phosphorylierung von Serin-/Arginin-reichen Proteinen. Untersuchungen hinsichtlich der Expression der CLK kinase zeigten eine Transkriptabundanz in allen asexuellen Blutstadien sowie in Gametozyten. Mit Hilfe der *Reverse-Genetics*-Technik, wurde festgestellt, dass alle vier Kinasen essentiell sind für die asexuelle

Replikation von *P. falciparum*. *Pf*CLK-1/Lammer besitzt zwei Kernlokalisationssequenzen, während *Pf*CLK-2 ein solches Signal stromaufwärts der C-terminalen katalytischen Domäne aufweist. Die Expression auf Proteinebene sowie die subzelluläre Lokalisation der beiden Kinasen wurde durch die Herstellung von Antiseren gegen die jeweilige Kinasedomainen hergestellt. Indirekte Immunfluoreszenzstudien, Westernblots und elektronenmikroskopische Daten bestätigten die Lokalisation vornehmlich in Zellkern des Parasiten. *In-vitro*-Studien demonstrierten, das beide Enzyme mit Phosphorylierungsaktivität assoziierte sind. Die massenspektrometrische Analyse von ko-immunopräzipitierten Proteinen zeigten Interaktionen der beiden *Pf*CLK-Kinasen mit Proteinen, welche vermutlich Nuklease-, Phosphatase- oder Helikase-Funktion besitzen.

Im Gegensatz zu den CLK-Kinasen wird *Pf*PKRP wird hauptsächlich während der Differenzierung der Gametozyten exprimiert wie Transkriptanalysen zeigten. Antiseren gegen die katalytische Domäne von *Pf*PKRP detektierten jedoch Proteinexpression sowohl in Lysaten asexueller Parasiten als auch in Gametozytenlysaten. In Immunfluoreszenzstudien wurde ein punktiertes Expressionsmuster im Zytoplasma beobachtet, wobei die Expression vornehmlich in Gametozyten stattfand. Die Tatsache, dass die Herstellung einer *Pf*PKRP-Knock-out-Mutante möglich war, zeigt, dass *Pf*PKRP für das Überleben asexueller Parasiten entbehrlich ist, weshalb eine wichtige Rolle in der sexuellen Entwicklung der Parasiten möglich ist.

Zum Einen dient die Charakterisierung der *Pf*CLK-Kinasen als Beispiel für Kinasen, welche eine wichtige Rolle im Replikationszyklus der Parasiten spielen. Das erfolgreiche Ausschalten von *PfPKRP* sowie Untersuchungen zur Expression der *Pf*PKRP-Kinase lassen zum Anderen eine Rolle in den Sexual- oder Transmissionstadien vermuten. Aufgrund dessen stellen beide Kinasefamilien viel versprechende Kandidaten für die Herstellung von malariamedikamenten dar.

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Abbreviations

AFC	*Arabidopsis* FUS3-complementing gene
ALP	Alkaline phosphatase
AMA	Apical membrane antigen
APAD	3-Acetylpyridine adenine dinucleotide
APS	Ammonium peroxide sulphate
Approx.	Approximately
Asex	Asexual
ASF	Alternative splicing factor
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoxylphosphate
bp	Base pair
BRK	Bayerisches Rotes Kreuz
BSA	Bovine serum albumin
BSD	Blasticidin
Ca^{2+}	Calcium ion
PKRP	Putative kinase related protein
CaMK	Calmodulin-dependent protein kinase
CDC	Centers for disease control and prevention
CDK	Cyclic dependent kinase
CDPK	Calcium dependent protein kinase
cDNA	Complementary DNA
c-GMP	Cyclic-guanosine monophosphate
CK	Casein kinase
CLK	CDK-like kinase
CMGC	CDK-MAPK-GSK-CLK
cm	Centimeter
CO_2	Carbon dioxide
Co-IP	Co-immunoprecipitation
conc.	Concentration
CSP	Circumsporozoite protein
CTRP	CSP and TRAP-related proteins
d	days
°C	Degree Celsius
DHFR-ts	Dihydrofolate reductase-temperature sensitive
dH_2O	Distilled water
DIG	Digoxygenine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Doa	Darkner of apricot
Dsx	Doublesex

dsDNA	Doublestranded DNA
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EC	Uninfected erythrocytes
EDTA	Ethylene diamide tetracetic acid
e.g.	Example
ePK	Eukaryotic protein kinase
ER	Endoplasmic reticulum
et al.	et altera
EtBr	Ethidium bromide
Etc.	Et cetera
Et-OH	Ethanol
FV	Food vacuole
g	Gram
xg	Gravitational force, g = 9.81 m/s^2
GC	Gametocyte
gDNA	Genomic DNA
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
GST	Glutathione-S-transferase
h	Hour
HA	Hemagglutinin
HIV	Human Immunodeficiency virus
Hs	*Homo sapiens*
hslV	Heat shock loci V
HZ	Hemozoin
IB	Inclusion bodies
IC5O	Inhibitory concentration
IEM	Immunoelectron microscopy
IFA	Immunofluorescence assay
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB	Lysogeny broth/Luria broth/Luria-Bertani
LCCL	*Limulus* coagulation factor C
Lkh	Lammer kinase homologue
LRR	Leucine rich repeat
kDa	Kilodaltons
KO	Knock-out
µ	Micro
M	Molar
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MBP	Myelin basic protein

MC	Mitochondria
min	Minutes
mg	Milli-gram
ml	Milli-litre
mM	Milli-molar
mRNA	Messenger-RNA
mRNPs	Messenger-ribonucleoproteins
MSP	Merozoite surface protein
Mwt.	Molecular weight
NBT	Nitroblue tetrazoliumchloride
NEB	New England Biolabs
ng	Nanograms
NGS	Neutral goat serum
NIMA	Never in mitosis/*Aspergillus*
NMS	Neutral mouse serum
NP	Nuclear pellet
OB	Osmiophilic bodies
O/N	Overnight
%	Percentage
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PE	Proline-Glutamate motif
*Pf*CCP	*Pf* LCCL proteins
PML	Promyelocytic leukemia
pmol	Picomoles
‘R’	Arginine
RESA	Ring infected surface antigen
RNA	Ribonucleic acid
rp	Recombinant protein
rPK	Recombinant protein kinase
RPMI	Roswell-Park-Memorial-Institute-Medium
RT	Room temperature
RT	Reverse transcriptase
‘S’	Serine
SDS	Sodium dodecyl sulphate-
s	Second
SR proteins	Serine/arginine-rich proteins
SRPK	Serine/Arginine protein kinase
STE	Sterile yeast mutant
TBD	Transmission blocking drug
TBS	Tris buffered saline

TBSM	Milk powder in TBS
TBV	Transmission blocking vaccine
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TKL	Tyrosine Kinase Like-kinases
T. gondii	*Toxoplasma gondii*
TRAP	Thrombospondin-related adhesion protein
TyrK	Tyrosine kinase
U	Unit
UTR	Untranslated region
v/v	Volume/volume
w/v	Weight/volume
WB	Western blot
WHO	World health organization
WT	Wild type
XA	Xanthurenic acid
Y-2-H	Yeast-2-hybrid

Publications and participation in conferences

Publications

Kumar, R., Goswami, R., **Agarwal, S.**, Israni, R., Singh, S.K., Rani, R. (2007). Association and interaction of the TNF-alpha gene with other pro-and anti-inflammatory cytokine genes and HLA genes in patients with type1 diabetes from North India. *Tissue Antigens*, 69(6) 557-567.

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Conferences

Rupp, I., **Agarwal, S**., Doerig, C., Pradel, G. (2006). Characterization of various sexual stage-specific proteases and kinases as potential targets in the malaria pathogen *Plasmodium falciparum*. 58th Meeting of the German Society of Hygiene and Microbiology, Würzburg, *Int J Med Microbiol* 296 (Suppl. 3), Abstract EKP02. (Poster).

Rupp, I., **Agarwal, S**., Doerig, C., Pradel, G. (2006). 3rd Malaria Meeting of the Paul-Ehrlich-Society, Missionsärztliche Klinik, Würzburg. (Poster).

Agarwal, S., Doerig, C., Bosse, R., Pradel, G. (2008). Blood stage kinases in the human malaria parasite, *Plasmodium falciparum.* Eukaryontische Krankheitserreger, DGHM-Fachgruppe, Würzburg. (Oral presentation).

Agarwal, S., Doerig, C., Pradel, G. (2008). Characterization of blood stage kinases of *Plasmodium falciparum*. 19th Molecular Parasitology Meeting, Woods Hole, U.S.A. (Poster).

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microbiology (DGHM), Göttingen, Germany. *Int J Med Microbiol* 299 (Suppl 1) Abstract EKV 11 (Oral presentation).

Agarwal, S., Kern, S., Doerig, C., Przyborski, J.M., Baumeister, S., Dandekar, T., Pradel, G. (2010). Functional characterization of four CDK-like kinases in the blood stages of the human malaria parasite *Plasmodium falciparum.* 24th Annual meeting of German society of parasitology, Düsseldorf. (Poster).

Kinases sequences

1. *Pf*CLK-1/Lammer (PF14_0431)

RT-PCR Primers

Recombinant protein primers

Peptide for rabbit anti-*Pf*CLK-1/Lammer

pCAM-BSD-KO primers

pCAM-BSD-tagging primers

Catalytic Domain 552 – 874

Location of nuclear localization signals

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1     ATG ACT GAA TTG AAT ATT ATT GAT AAT AAT GAA AAA CAT AGT AAA   45
1      M   T   E   L   N   I   I   D   N   N   E   K   H   S   K    15

46    TAT AAA TTA TAT AAA TAT TGT AGA AAT TAT ATA AAT GAT TTT AAA   90
16     Y   K   L   Y   K   Y   C   R   N   Y   I   N   D   F   K    30

91    AAT AAA TAT ATG ATG GGT TAT TCA TCG AAT TTG TAT AAA AAT AGT   13
31     N   K   Y   M   M   G   Y   S   S   N   L   Y   K   N   S    45

136   CCC AAC AAA TAT TAC TAC GAT ACG CGA AAT AAT AGT AAT AAT AAT   180
46     P   N   K   Y   Y   Y   D   T   R   N   N   S   N   N   N    60

181   AAT AAT GGT TAT TAT TAC CAT AAT TAT AAT AAT GGT TAT CAA ATA   225
61     N   N   G   Y   Y   Y   H   N   Y   N   N   G   Y   Q   I    75

226   CGA AAT AAA AGA AAT CGT AGT TTT GAG ACT GTA AGT AAT TCA AAA   270
76     R   N   K   R   N   R   S   F   E   T   V   S   N   S   K    90

271   AAC AAA CAT AAA ATT TCA AAA AAA TAT AAA TAT AAT AAT GAA CCC   315
91     N   K   H   K   I   S   K   K   Y   K   Y   N   N   E   P    105

316   CAT GAA AGT TTT AGA GAC TAT GAT TAC AAA AGT AAA CCT AGT TAT   360
106    H   E   S   F   R   D   Y   D   Y   K   S   K   P   S   Y    120

361   TCT TCT GTA AAA AAA ATT TAT GAT AGA GAA CAG AAG CGT TAT AAC   405
121    S   S   V   K   K   I   Y   D   R   E   Q   K   R   Y   N    135

406   AAG ATG TTT GTG GAA TCA AGG TAT AAT GAA AAT CAT AAG AAT AAA   450
136    K   M   F   V   E   S   R   Y   N   E   N   H   K   N   K    150

451   TAT ATA AAT AAT TAC AAT CTA TCA AGG AAA AGA AAT TAT TAT TCT   495
151    Y   I   N   N   Y   N   L   S   R   K   R   N   Y   Y   S    165

496   TAT AAA AAA AAA ATA TAT ACT GAT AGA AGA AAT TTT GAT ACG CAT   540
166    Y   K   K   K   I   Y   T   D   R   R   N   F   D   T   H    180

541   TTT GGA CAA GGT AAT ATA ATA TAT AAT AAT GAA TAT TAC TTA ATA   585
181    F   G   Q   G   N   I   I   Y   N   N   E   Y   Y   L   I    195

586   AAT GAT GGT TCA AGT TTA AAT AAA AAA AGG TTG CAT TAT GCT AAG   630
196    N   D   G   S   S   L   N   K   K   R   L   H   Y   A   K    210

631   GGT AGT TTT AGA AGT AGA TCC CGA AGC AAA AAT TAT AAT ACC TAT   675
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211    G   S   F   R   S   R   S   R   S   K   N   Y   N   T   Y    225

676   AAT TTG GAA AAG TCA AAG AAA ATG AAA TAT AGT AGT AGA CAT AAC   720
226    N   L   E   K   S   K   K   M   K   Y   S   S   R   H   N    240

721   ATT TAT AGA TAT CAT AAT GGA AAT AAA AAT AAA TCC ATC TCA AGA   765
241    I   Y   R   Y   H   N   G   N   K   N   K   S   I   S   R    255

766   TGG GAA GAT AAT TAT ACA TAC AAA GAT ATA TAT CAA CCC TCA AGT   810
256    W   E   D   N   Y   T   Y   K   D   I   Y   Q   P   S   S    270

811   TCC TTG CAT AAA TAC AAA AGG AGG AGT AAT TTT ACG AGG GAT GAC   855
271    S   L   H   K   Y   K   R   R   S   N   F   T   R   D   D    285

856   AAT TCT ATA AAA TCG AAG ATG TAT TAT GAA AAT AAA AAG ACG GAT   900
286    N   S   I   K   S   K   M   Y   Y   E   N   K   K   T   D    300

901   CAC CTT TAT AGT TAT GAG GAC AAA TAT TAC AAC AAG TAT GAT GAC   945
301    H   L   Y   S   Y   E   D   K   Y   Y   N   K   Y   D   D    315

946   AAG TAT GAT GAC AAG TAT GAT GAC AAG TAT GAT GAC AAG TAT GAT   990
316    K   Y   D   D   K   Y   D   D   K   Y   D   D   K   Y   D    330

991   GAC AAA TAT GAT GAC AAA TAT GAT GAC AAA TAT GAT GAC AAA TAT  1035
331    D   K   Y   D   D   K   Y   D   D   K   Y   D   D   K   Y    345

1036  GAT GAT AAA TAT GAT GAC AAT TAT GAT GAC AAG TAT GAT TAT GGA  1080
346    D   D   K   Y   D   D   N   Y   D   D   K   Y   D   Y   G    360

1081  GAA GAA GAA CAT AGA CAA GAA TAT TAT AAG AAG AAA AAA ATA TCT  1125
361    E   E   E   H   R   Q   E   Y   Y   K   K   K   K   I   S    375

1126  TTT AAT AAT AGT ACT AAT AAT AAG AGT AGT ATA AAT TAT GAT GAT  1170
376    F   N   N   S   T   N   N   K   S   S   I   N   Y   D   D    390

1171  GTT AAA AGA GAA TTA AAG AAA AGG AAA AAA AAA AAA TAT TCA AAT  1215
391    V   K   R   E   L   K   K   R   K   K   K   K   Y   S   N    405

1216  GAA TCC AAA GTT TAT GAT TCA TTA AAA AGA GAT GAA ACT AAT CAT  1260
406    E   S   K   V   Y   D   S   L   K   R   D   E   T   N   H    420

1261  CAT ACT AAT AAT AAT TCT TAT TTA AAT GAG ATA AAT AAA AAA AAT  1305
421    H   T   N   N   N   S   Y   L   N   E   I   N   K   K   N    435

1306  TCG AAT TTA TCA AAT AAT TAT GTA GCA GTA AGA AAT AAA AAA AGA  1350
436    S   N   L   S   N   N   Y   V   A   V   R   N   K   K   R    450

1351  GAT AAA GAA TAT ATC AGT GAT AGT AAT AAA AGT GGG TTT TCA AAT  1395
451    D   K   E   Y   I   S   D   S   N   K   S   G   F   S   N    465

1396  AAA GGT AGC TAT TAT ATG CAA AAG AAA AAA TTG CAT GTA GAC AAG  1440
466    K   G   S   Y   Y   M   Q   K   K   K   L   H   V   D   K    480

1441  TAT GAT AAT GAT TCA CAT AAT AGA ACT ATT TCT AGA ACG TCG TCC  1485
481    Y   D   N   D   S   H   N   R   T   I   S   R   T   S   S    495

1486  GAT AAT TAT TCT AGA AAA AAA TAT ACA CAT AAA AGA AAT AGG ACG  1530
496    D   N   Y   S   R   K   K   Y   T   H   K   R   N   R   T    510

1531  AAA ACA TCT GAT ACA GAA GAT AAA AAA GAA AGG AAA AAG AAG AAA  1575
511    K   T   S   D   T   E   D   K   K   E   R   K   K   K   K    525

1576  AAA AAA AAG GAA AAT AAT GAA TCA GAT GAT GAA ATT GTC CAT TTT  1620
526    K   K   K   E   N   N   E   S   D   D   E   I   V   H   F    540

1621  AGT TGG AAG AAA GGT ATG CTA TTA AAT AAT GCC TTT TTA GTT ATA  1665
541    S   W   K   K   G   M   L   L   N   N   A   F   L   V   I    555

1666  AGA AAA ATG GGA GAT GGG ACA TTT GGT AGA GTT TTA TTA TGT CAA  1710
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556    R   K   M   G   D   G   T   F   G   R   V   L   L   C   Q    570

1711  CAT ATA GAT AAT AAA AAA TAC TAC GCT GTA AAA GTT GTT CGA AAC   1755
571    H   I   D   N   K   K   Y   Y   A   V   K   V   V   R   N    585

1756  ATA AAG AAA TAT ACA CGC TCA GCT AAA ATT GAA GCA GAT ATT TTA   1800
586    I   K   K   Y   T   R   S   A   K   I   E   A   D   I   L    600

1801  AAA AAA ATA CAA AAT GAT GAT ATT AAT AAT AAT AAT ATT GTT AAG   1845
601    K   K   I   Q   N   D   D   I   N   N   N   N   I   V   K    615

1846  TAT CAT GGG AAA TTT ATG TAT TAT GAC CAT ATG TGT TTA ATA TTT   1890
616    Y   H   G   K   F   M   Y   Y   D   H   M   C   L   I   F    630

1891  GAA CCA TTA GGT CCA TCA TTA TAT GAA ATA ATT ACA AGG AAT AAT   1935
631    E   P   L   G   P   S   L   Y   E   I   I   T   R   N   N    645

1936  TAT AAC GGA TTC CAT ATA GAA GAT ATT AAA TTA TAT TGT ATA GAA   1980
646    Y   N   G   F   H   I   E   D   I   K   L   Y   C   I   E    660

1981  ATA TTA AAA GCT TTA AAT TAT TTA CGT AAA ATG TCT TTA ACG CAT   2025
661    I   L   K   A   L   N   Y   L   R   K   M   S   L   T   H    675

2026  ACA GAT TTA AAA CCT GAA AAT ATT TTA TTA GAT GAT CCA TAT TTT   2070
676    T   D   L   K   P   E   N   I   L   L   D   D   P   Y   F    690

2071  GAA AAA TCA TTA ATA ACT GTT AGA AGG GTT ACT GAT GGA AAA AAA   2115
691    E   K   S   L   I   T   V   R   R   V   T   D   G   K   K    705

2116  ATA CAA ATT TAT AGA ACC AAG TCT ACT GGT ATA AAA TTA ATT GAT   2160
706    I   Q   I   Y   R   T   K   S   T   G   I   K   L   I   D    720

2161  TTT GGT TGC GCC ACA TTT AAA AGT GAT TAT CAT GGT TCT ATT ATT   2205
721    F   G   C   A   T   F   K   S   D   Y   H   G   S   I   I    735

2206  AAC ACT AGA CAA TAT CGA GCT CCA GAA GTT ATA TTA AAT TTG GGT   2250
736    N   T   R   Q   Y   R   A   P   E   V   I   L   N   L   G    750

2251  TGG GAT GTA TCT AGT GAT ATG TGG AGT TTT GGT TGT GTT TTG GCT   2295
751    W   D   V   S   S   D   M   W   S   F   G   C   V   L   A    765

2296  GAA TTA TAT ACA GGT TCT TTA TTA TTT AGA ACC CAT GAA CAT ATG   2340
766    E   L   Y   T   G   S   L   L   F   R   T   H   E   H   M    780

2341  GAA CAT CTG GCT ATG ATG GAA AGT ATT ATT CAA CCT ATA CCA AAA   2385
781    E   H   L   A   M   M   E   S   I   I   Q   P   I   P   K    795

2386  AAT ATG TTA TAT GAA GCA ACC AAA ACA AAT GGA TCC AAA TAT GTT   2430
796    N   M   L   Y   E   A   T   K   T   N   G   S   K   Y   V    810

2431  AAT AAG GAT GAG TTG AAA TTA GCT TGG CCA GAA AAT GCA TCC AGT   2475
811    N   K   D   E   L   K   L   A   W   P   E   N   A   S   S    825

2476  ATT AAT TCT ATT AAA CAT GTT AAA AAA TGT TTA CCC TTG TAT AAA   2520
826    I   N   S   I   K   H   V   K   K   C   L   P   L   Y   K    840

2521  ATA ATT AAA CAT GAA CTA TTT TGT GAT TTC TTA TAT TCC ATA TTA   2565
841    I   I   K   H   E   L   F   C   D   F   L   Y   S   I   L    855

2566  CAA ATA GAT CCA ACA CTT CGA CCG TCA CCC GCC GAA TTA TTA AAG   2610
856    Q   I   D   P   T   L   R   P   S   P   A   E   L   L   K    870

2611  CAC AAG TTC CTT GAA GAA AAT TAT GAG TAC TAT TAA    2646
871    H   K   F   L   E   E   N   Y   E   Y   Y   *
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2. *Pf*CLK-2 (PF14_0408)

RT-PCR Primers

pCAM-BSD-KO primers

pCAM-BSD-tagging primers

Peptide for rabbit anti-*Pf*CLK-1/Lammer

Catalytic Domain 696 – 1259

Location of nuclear localization signals

Same primer for KO antisense and RTPCR 2 Sense

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1     ATG AGT AAG ACA GAA TAT GAT ATT CTT TCA CTT GTA AAT GCA GTT   45
1      M   S   K   T   E   Y   D   I   L   S   L   V   N   A   V    15

46    GAA GAA TTT AGC AAA TAT TGT TCA GAA TAT AAG AAG AGA CAC CTA   90
16     E   E   F   S   K   Y   C   S   E   Y   K   K   R   H   L    30

91    ACG AAT TAT ATA CAA TCT TGT TCT GTT AAA AAC CAA ATT AAT AAC   135
31     T   N   Y   I   Q   S   C   S   V   K   N   Q   I   N   N    45

136   AAT GAA GAG GTA ATA TTT AAA ACA ACC TTA CAA GAT TTT ATG AAC   180
46     N   E   E   V   I   F   K   T   T   L   Q   D   F   M   N    60

181   AAG GTT AAT ATG TTA GGT AAT AAT ATT TTG CAA ATT GAA AAA ATA   225
61     K   V   N   M   L   G   N   N   I   L   Q   I   E   K   I    75

226   AAT AAA TGT CAG ATA CCT GAG AAT ATA TTA TAT GAA AAA AAT GGA   270
76     N   K   C   Q   I   P   E   N   I   L   Y   E   K   N   G    90

271   AAT AAA ATG ATA TAT GAT CCA AAA CAT TAC AAT GAA GAC AAA TTA   315
91     N   K   M   I   Y   D   P   K   H   Y   N   E   D   K   L   105

316   AAT AAT GAA AAT ATA TTG GAA GAT AAA AAT AAA AGA AAT ATT TGC   360
106    N   N   E   N   I   L   E   D   K   N   K   R   N   I   C   120

361   GAA CAC ATA AAA TCC AAA ATA AAT GAC AAA AAC TTT GTA CAA AAT   405
121    E   H   I   K   S   K   I   N   D   K   N   F   V   Q   N   135

406   AAT GAT TAT GGA AAA AAT ATA AAA ATA ATT AAT AAT ATA AAT GAA   450
136    N   D   Y   G   K   N   I   K   I   I   N   N   I   N   E   150

451   CAA AAT TTA TTA ATG AAA GAT AAA AAT GAT TAT ACC AAT ATT TCT   495
151    Q   N   L   L   M   K   D   K   N   D   Y   T   N   I   S   165

496   GAA AAT TTA TAT ATG AGG AAA ATA TCC ACT GAA TAT ACT TCA TCA   540
166    E   N   L   Y   M   R   K   I   S   T   E   Y   T   S   S   180

541   GAC AGA AGC AGT GAA GAT AAT GTA TTA TTG CAT AAT ATA AAT AAT   585
181    D   R   S   S   E   D   N   V   L   L   H   N   I   N   N   195

586   GTG CTT GTG AAA TAT GTA AGT GGA TTA GAT TAT ACA CTG AAT ATT   630
196    V   L   V   K   Y   V   S   G   L   D   Y   T   L   N   I   210

631   AGA AGA ATA TCA AGA AAA GAT TTA ATC GAA GAA ATT AAA CAA TTT   675
211    R   R   I   S   R   K   D   L   I   E   E   I   K   Q   F   225

676   TAT ATT ATA CAT AAT AAT AAT ATT CAA TTA AAT GAT TAT TCT ACA   720
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226    Y   I   I   H   N   N   N   I   Q   L   N   D   Y   S   T    240

721   TAT CTA AGT GAT GAA GTA ATA AAT ATT CTG AAA CAA AAA AAA GAT   765
241    Y   L   S   D   E   V   I   N   I   L   K   Q   K   K   D    255

766   AAT ATA AAA TGT TTA GAT AAA AAT AAT CTC TTT ATT ATA GAA AAA   810
256    N   I   K   C   L   D   K   N   N   L   F   I   I   E   K    270

811   AAT GTT GAT TTT TAT AAA AAC AAT TTT TTG CCT TTT TTT TAT ATT   855
271    N   V   D   F   Y   K   N   N   F   L   P   F   F   Y   I    285

856   CCT TGT AAT TAT TTA TTT ATA AAA AAT TAT TGT AAT GAT AAT GAA   900
286    P   C   N   Y   L   F   I   K   N   Y   C   N   D   N   E    300

901   AGA TGC TTG GAA ACA ACC TAT GAT ACA AAT GAT GAA AAA AAT TCT   945
301    R   C   L   E   T   T   Y   D   T   N   D   E   K   N   S    315

946   ACA ACA GAA AAT TCT TAT ATA TTA TCA TCT CCT AAT CAT ATG TAT   990
316    T   T   E   N   S   Y   I   L   S   S   P   N   H   M   Y    330

991   AAT ACA AAT ACG AAT AGT TAT AAT TCA CAT TCA AAT AGT ATA ATA  1035
331    N   T   N   T   N   S   Y   N   S   H   S   N   S   I   I    345

1036  GCT AGA AAT ACT TCT TCA AAT AAA TGT AAC TCA CCT ATT TTT GGA  1080
346    A   R   N   T   S   S   N   K   C   N   S   P   I   F   G    360

1081  ATT AAT GAA CGA AAC TCA TCA CCT AAC TTT GGC ATA TTC AAT GAA  1125
361    I   N   E   R   N   S   S   P   N   F   G   I   F   N   E    375

1126  AGT ATC AAA AAC CAT AAG GAT CAA TAT AAT ATG GAT CCT AAT TAT  1170
376    S   I   K   N   H   K   D   Q   Y   N   M   D   P   N   Y    390

1171  TTA ATC ATG AGA TAT AAT TTA AAC GAA GAA GTA CAT ATT AAA GAT  1215
391    L   I   M   R   Y   N   L   N   E   E   V   H   I   K   D    405

1216  GAT AGA GTT ATT AAT TCT CCA AAC AAT ATA AAA TCT ATG AAT GAT  1260
406    D   R   V   I   N   S   P   N   N   I   K   S   M   N   D    420

1261  AAT AGA TAT AAT TCA CCT AAT TTT AAT ATA GAA CAT ATA CAT ATT  1305
421    N   R   Y   N   S   P   N   F   N   I   E   H   I   H   I    435

1306  AAT AAT AAT GAT ACA CAA ATA AAC AAT TAT GAT ATT TAT GAT ATA  1350
436    N   N   N   D   T   Q   I   N   N   Y   D   I   Y   D   I    450

1351  AAT AAT ATG GAT GGA AAG AAA AAT AAC CTT AAT AGC AAT ACT AGC  1395
451    N   N   M   D   G   K   K   N   N   L   N   S   N   T   S    465

1396  GAT TTT TAT CAA TCC ATA AGC GAT TTT TAT AAA AAT AAT GAA AAG  1440
466    D   F   Y   Q   S   I   S   D   F   Y   K   N   N   E   K    480

1441  AAT CTT ATT TGC CAA GAA CAA AAT AAC AAT AAT ACA TAT ATC CAA  1485
481    N   L   I   C   Q   E   Q   N   N   N   N   T   Y   I   Q    495

1486  AAA TGC GCC TTT TTC TTA AAT AGT AAT GAA ACT AAT GTA CTA ACA  1530
496    K   C   A   F   F   L   N   S   N   E   T   N   V   L   T    510

1531  AAT AAT AAA AAT CAT CAC AAG AGT GGA AAA AAT CTA ATT AGC TTA  1575
511    N   N   K   N   H   H   K   S   G   K   N   L   I   S   L    525

1576  TGC TTT GAT GAT GAA TTA TCA AGT GCT ATA ACT ATA AAC AAC GAT  1620
526    C   F   D   D   E   L   S   S   A   I   T   I   N   N   D    540

1621  TCC TCT TTA AAT AAA GCA ATA TAC AAT GAA TTC AAT AAC TCT GAA  1665
541    S   S   L   N   K   A   I   Y   N   E   F   N   N   S   E    555

1666  AAA GAC ATT GTC GAA GAT ATA TGT AAC ACA ACA AAT TAT AAA GAT  1710
556    K   D   I   V   E   D   I   C   N   T   T   N   Y   K   D    570

1711  ATA AAT ATG GAA CAT ATG AAC ACA TAT CAA AAT GAT GAA AGA AAA  1755
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571    I   N   M   E   H   M   N   T   Y   Q   N   D   E   R   K    585

1756  TGG ATG GAT GAA TAT TGC AAT GGA GAT TAT AAT TTA TTT AGA TCA   1800
586    W   M   D   E   Y   C   N   G   D   Y   N   L   F   R   S    600

1801  AAG CAA TTA AAA AAG AAA AAA GAA CTA CTG TTA TCC TGC ATA TCT   1845
601    K   Q   L   K   K   K   K   E   L   L   L   S   C   I   S    615

1846  TAT AAT GAA AAG GAA AAT AAA TAT AAC GAT CAA GAT AAT ATG ATA   1890
616    Y   N   E   K   E   N   K   Y   N   D   Q   D   N   M   I    630

1891  AAT GTT GAA ATA AAT AAT GAT ACA AAA GTA AAA AAC ATT TTA TAT   1935
631    N   V   E   I   N   N   D   T   K   V   K   N   I   L   Y    645

1936  GAC ACA GGC AAT ATT TCC TTT GAT GAA AAT ATT GGA ATT AAT AAT   1980
646    D   T   G   N   I   S   F   D   E   N   I   G   I   N   N    660

1981  CAT TAT AAT ACT ATA AAT TTA AAG GTT ATA TAT GAA GCT AAC AAA   2025
661    H   Y   N   T   I   N   L   K   V   I   Y   E   A   N   K    675

2026  AGT GAA TAT GGA AAT AAT GAA CGT TTG AAT TTT ATT AAA GGA CAA   2070
676    S   E   Y   G   N   N   E   R   L   N   F   I   K   G   Q    690

2071  ATT ATT TTG AAT AAA TAT AAA GTA GTC AAA GTT TTA TCA AAA ACA   2115
691    I   I   L   N   K   Y   K   V   V   K   V   L   S   K   T    705

2116  CAA TTT AGT ACT ACA CTT AAA TGT CTC AAT TTA TTA TAT AAA AAA   2160
706    Q   F   S   T   T   L   K   C   L   N   L   L   Y   K   K    720

2161  GTA AAA ACT GAT ACA CAA GTA TTC CTA CCA TAT TGT CAT AAA TAT   2205
721    V   K   T   D   T   Q   V   F   L   P   Y   C   H   K   Y    735

2206  ATG AAA GAC GAT TCA GAA ATA ACA CAT GAT AAA AAA AAA AAC AAT   2250
736    M   K   D   D   S   E   I   T   H   D   K   K   K   N   N    750

2251  TAT GAT AAA TTT GTA AAT TTA AAT ACT ATA AAA AAA AAA AAA AAT   2295
751    Y   D   K   F   V   N   L   N   T   I   K   K   K   K   N    765

2296  GAG AAT TAT AAT AGA CAA CAT GAT ATT AAA AAT AAT TTA CAT GAT   2340
766    E   N   Y   N   R   Q   H   D   I   K   N   N   L   H   D    780

2341  AAT AAA CAC CAA ATA ATA AAT AAT AAA AAA AAG GTA GAA CCT AAA   2385
781    N   K   H   Q   I   I   N   N   K   K   K   V   E   P   K    795

2386  TAT GTT TGT TTA AAA GTA ATG AAA AAT GGA AAA CAA TTT TTA GAT   2430
796    Y   V   C   L   K   V   M   K   N   G   K   Q   F   L   D    810

2431  CAG GGA TTG TTA GAA CTC ATG GTC TTA AAT ATA CTA TGT AAT GCA   2475
811    Q   G   L   L   E   L   M   V   L   N   I   L   C   N   A    825

2476  AAT ACA AAT AAT AAT TTA TCT AAT AAA AAC ATT ATA CAA TTA TAT   2520
826    N   T   N   N   N   L   S   N   K   N   I   I   Q   L   Y    840

2521  GAT TCC TTT TAT TAT AAA GAA CAT TTA ATT ATA GTA ACA GAA TAC   2565
841    D   S   F   Y   Y   K   E   H   L   I   I   V   T   E   Y    855

2566  ATG CAA AGT GAT TTA TAT AAT TAT TTC ATT AGG AAA GGA AAA TTA   2610
856    M   Q   S   D   L   Y   N   Y   F   I   R   K   G   K   L    870

2611  GGA ACT TTG GGT CAA TTA CAA ATA TTG ACT AAA AAC TTA TTA GAA   2655
871    G   T   L   G   Q   L   Q   I   L   T   K   N   L   L   E    885

2656  GGA TTA GCA TAT ATA CAT TCG AAG AAT TTA ATA CAT TGT GAT TTA   2700
886    G   L   A   Y   I   H   S   K   N   L   I   H   C   D   L    900

2701  AAG CCA GAA AAT ATT ATG ATA AAT ATG AAA AAA AAT AAG AAG AAT   2745
901    K   P   E   N   I   M   I   N   M   K   K   N   K   K   N    915

2746  CAT GAA AAG GGT AAA TAT AAT AAA GTT AAT CAA AAT GGA GTT AAT   2790
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916    H   E   K   G   K   Y   N   K   V   N   Q   N   G   V   N    930

2791  ATA TAT AAC GAT ACA ATA GAA CCA CAT ATT CTG AAT TCT TCA AAT   2835
931    I   Y   N   D   T   I   E   P   H   I   L   N   S   S   N    945

2836  ATA AAT AAC TCA AAT TTA GAA AAA AAG AAC ATA ATT GCG TAT CCA   2880
946    I   N   N   S   N   L   E   K   K   N   I   I   A   Y   P    960

2881  TCA TTT GAT CAA ACC TTT ATT GAA AAT AAA GAT GCA CAA TAT GAT   2925
961    S   F   D   Q   T   F   I   E   N   K   D   A   Q   Y   D    975

2926  AAT AAT GAA AAG ACA TCG AAT GTG TTA TAT GAT TCT GAT AAA TCA   2970
976    N   N   E   K   T   S   N   V   L   Y   D   S   D   K   S    990

2971  TAT AAC AAT AAT GTA AAA AAT ATG ATA GAT AAT AAT TTA TAT TGT   3015
991    Y   N   N   N   V   K   N   M   I   D   N   N   L   Y   C   1005

3016  AAT AAT ATA AAA AAT ATT GAT AAT AAT AGT GAT AAT AAT AAT AAT   3060
1006   N   N   I   K   N   I   D   N   N   S   D   N   N   N   N   1020

3061  AAT AAT AAT AAT AAT TTT CCA CAT AAT AAT ATA AAT ATA TAT AAT   3105
1021   N   N   N   N   N   F   P   H   N   N   I   N   I   Y   N   1035

3106  ACT AAA CAG TTT GAC AAG ATC AAA ATA ATC GAT TTT AAT AGT TGT   3150
1036   T   K   Q   F   D   K   I   K   I   I   D   F   N   S   C   1050

3151  ATA TAT GAA AGC GAT AAA TTA GAA ATG TAT ATA CAA ACT CGA TCT   3195
1051   I   Y   E   S   D   K   L   E   M   Y   I   Q   T   R   S   1065

3196  TAT AGA TCA CCT GAA GTT TTA TTA CAA CAA AAC TAT GAT AGG AAA   3240
1066   Y   R   S   P   E   V   L   L   Q   Q   N   Y   D   R   K   1080

3241  ATT GAC ATA TGG AGT TTA GGA TGT ATA TTA TTT GAA TTT TTA ACA   3285
1081   I   D   I   W   S   L   G   C   I   L   F   E   F   L   T   1095

3286  AAA AAA ATA TTA TTT GAT TAT CAA AAT ATT TAT CGT TTT ATA TAT   3330
1096   K   K   I   L   F   D   Y   Q   N   I   Y   R   F   I   Y   1110

3331  TCA ATT GTT TCT TAT ATT GGA CCT TTC CCA TTT TAT ATG ATA AAT   3375
1111   S   I   V   S   Y   I   G   P   F   P   F   Y   M   I   N   1125

3376  AAC TGT AGG ATA CCA CAT ATC TTT ACA AAA CAC GGA TTA ATA ATA   3420
1126   N   C   R   I   P   H   I   F   T   K   H   G   L   I   I   1140

3421  TTA AAA AAG TTT ACG ACA GAT AAT ATG TAC GAG AAT TAT ATA AAA   3465
1141   L   K   K   F   T   T   D   N   M   Y   E   N   Y   I   K   1155

3466  GAA GAA CAA TTA AAT CAA GAA GAT GAT GAA GAA ATT GTT TTT AAT   3510
1156   E   E   Q   L   N   Q   E   D   D   E   E   I   V   F   N   1170

3511  TCT AAT GAT TTC TTT CGT TTA AAT AAA AAA GAT AAT ATC CTT ACA   3555
1171   S   N   D   F   F   R   L   N   K   K   D   N   I   L   T   1185

3556  AAA GAT TTA TTA AAA AAT AAA AAT CCA AAT ACA TCT ACT CCA AGA   3600
1186   K   D   L   L   K   N   K   N   P   N   T   S   T   P   R   1200

3601  AAA CGT AAC AAT AAC AAT AAT GAG ATT TAT TAT GAT GTA TGT TAT   3645
1201   K   R   N   N   N   N   N   E   I   Y   Y   D   V   C   Y   1215

3646  CCA AGT GAT AAC TTA TTA AAA AAT AAT TTT CAA ATA AGT GAC ACA   3690
1216   P   S   D   N   L   L   K   N   N   F   Q   I   S   D   T   1230

3691  TTG TTT GTT GAT TTT CTT TCA TCC TTG TTA CAA ATA GAT CCC TCT   3735
1231   L   F   V   D   F   L   S   S   L   L   Q   I   D   P   S   1245

3736  AAA AGG TGT AAT GCT ATG GAA GCC TTA AAG CAC CCA TGG CTT CAG   3780
1246   K   R   C   N   A   M   E   A   L   K   H   P   W   L   Q
1260
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3781  CCA AAT TTG TAT AAA GAC GGT TTA TAA   3807
1261   P   N   L   Y   K   D   G   L   *
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3. *Pf*CLK-3 (PF11_0156)

RT-PCR Primers

pCAM-BSD- KO Primers

pCAM-BSD-tagging primers

Catalytic Domain 417 - 739

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1     ATG TCC AAA GAT AAG AGA AAC TCG TTT GCA TCC AAT TCT TTT GAT   45
1      M   S   K   D   K   R   N   S   F   A   S   N   S   F   D    15

46    TCT AGC AAC GAC GAA AAA AAA TCT AAG AAT GGA AAT AAA ATT TAT   90
16     S   S   N   D   E   K   K   S   K   N   G   N   K   I   Y    30

91    AAA TCA AAA CAT GAA GAG AAT AGT CCT GAT GGT GAT TCA TAT AAA   135
31     K   S   K   H   E   E   N   S   P   D   G   D   S   Y   K    45

136   ATA AAT AAT AAC GAA AAA GAG AAA AGT AAA GAA AAA TTA AAA AAA   180
46     I   N   N   N   E   K   E   K   S   K   E   K   L   K   K    60

181   GAT CAA AAG AAA AAA TCT AAA GAA ATT TAT AAT TCA TTT AAT TCT   225
61     D   Q   K   K   K   S   K   E   I   Y   N   S   F   N   S    75

226   CCT AAT TCT ACT AGT AGC GAT TCG GAT GGA AAT GGA TTA CAT CTA   270
76     P   N   S   T   S   S   D   S   D   G   N   G   L   H   L    90

271   AAT TTT TCC AAC GCA TCA AGT AAT ATT TTT AAT ATA TAT GCT ATA   315
91     N   F   S   N   A   S   S   N   I   F   N   I   Y   A   I   105

316   TGT AAA TAT CCG TAT TAT ATA TGT AGC ATG TAT ATC TTT TTT AAA   360
106    C   K   Y   P   Y   Y   I   C   S   M   Y   I   F   F   K   120

361   AAA AAG ATA ATA AAT ATA TAT ATA TAT ATA TAT ATA TAT TTG GTT   405
121    K   K   I   I   N   I   Y   I   Y   I   Y   I   Y   L   V   135

406   TAT TAT GTA GGT TCG AGT AGT GAA AAC GGA TTT AAG ATA CTA CGA   450
136    Y   Y   V   G   S   S   S   E   N   G   F   K   I   L   R   150

451   ACA CAA GAA AAT GAG GAT AAA CTT CTA GAA GAA AGA AGA AGA AAA   495
151    T   Q   E   N   E   D   K   L   L   E   E   R   R   R   K   165

496   AGA GAA GCA TTA AAA GAA AAA TTA AAA AAC ATG GTT AAG GAA AAT   540
166    R   E   A   L   K   E   K   L   K   N   M   V   K   E   N   180

541   GAA CAA AAT AAT GAT GCG AAT GAA ATA CTA CAG AAT GAT CAG ATA   585
181    E   Q   N   N   D   A   N   E   I   L   Q   N   D   Q   I   195

586   AAT AAA GAT TAT AAC AAT GAA ACT TTT TTG TTA AGT GAA AAT AAA   630
196    N   K   D   Y   N   N   E   T   F   L   L   S   E   N   K   210

631   AAT GAT AAT GAT ATA ATA ACA AAT GAA ATA CCA TCT AAT CCA TCA   675
211    N   D   N   D   I   I   T   N   E   I   P   S   N   P   S   225

676   TAT ATC GAC CAA AAT GAT GCG GCC TGC ATT TTC GCA CCC AAC AAT   720
226    Y   I   D   Q   N   D   A   A   C   I   F   A   P   N   N   240
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721   GAT GTT ATT GAA GAT ACG TGC TCA TCA CTC TCA TCA GAT CAT GAA   765
241    D   V   I   E   D   T   C   S   S   L   S   S   D   H   E   255

766   ATT ATA GAA GAA AAA CAA AAT AAA GAA AAA CCA GAA GCA GTA AAA   810
256    I   I   E   E   K   Q   N   K   E   K   P   E   A   V   K   270

811   GAG TGT AGT GAT TTG TAT AAT GAT TTA AAA AAA AAA ATT GAT GAA   855
271    E   C   S   D   L   Y   N   D   L   K   K   K   I   D   E   285

856   GAA AAG GCC AAA ATT AGG TCA TTT ATA ATC AAA CAG AAA GAA TTA   900
286    E   K   A   K   I   R   S   F   I   I   K   Q   K   E   L   300

901   CAT GAA AGA TTA AAA ATG AAT GTG GAT GAT AGT TTA TAT GTG AAT   945
301    H   E   R   L   K   M   N   V   D   D   S   L   Y   V   N   315

946   AAA AGT AAG GGA AAT GCA GAT ACA CAT AAT AAT TTA ACT AAT AAG   990
316    K   S   K   G   N   A   D   T   H   N   N   L   T   N   K   330

991   AAG AGT CCT CTT GAA AAT GAA GAA GAT GAA ATG CAA GAA GAA TAC  1035
331    K   S   P   L   E   N   E   E   D   E   M   Q   E   E   Y   345

1036  GAT GAG GAT AAT GAT GAT TTT GAT ATG TTT TCC TGT GTA CAA GCA  1080
346    D   E   D   N   D   D   F   D   M   F   S   C   V   Q   A   360

1081  AAT AAA AAA AGA AAA GTT GAA AAA GTA CAT ATA ACT GAT TAT TAC  1125
361    N   K   K   R   K   V   E   K   V   H   I   T   D   Y   Y   375

1126  ACA ACA GGA AAT AAT GCA AAT TTG TCA GAT AAT TGG AAT GAC TCA  1170
376    T   T   G   N   N   A   N   L   S   D   N   W   N   D   S   390

1171  GAG GGA TAT TAC AAG GTT AAT AAA ATA TAT ATG TAT TTT ATA AAT  1215
391    E   G   Y   Y   K   V   N   K   I   Y   M   Y   F   I   N   405

1216  GCT ATG GTT GGC GAG GTT ATT GAT AAA AGA TAC AGT GTT GTG TGT  1260
406    A   M   V   G   E   V   I   D   K   R   Y   S   V   V   C   420

1261  GAA CTG GTT GGG AAA GGT GTT TTT TCA AAT GTA TTA AAG TGT TAT  1305
421    E   L   V   G   K   G   V   F   S   N   V   L   K   C   Y   435

1306  GAT ATG GTA AAT AAA ATT CCT GTA GCT GTA AAA GTT ATT AGA GAT  1350
436    D   M   V   N   K   I   P   V   A   V   K   V   I   R   D   450

1351  AAT GAT ATG ATG AAA AAG GCT GCA GAA AAA GAA ATA TCT ATT TTG  1395
451    N   D   M   M   K   K   A   A   E   K   E   I   S   I   L   465

1396  AAG AAG TTA AAT CAA TAT GAT AAG GAC AAT AAA AGG CAC ATC ATT  1440
466    K   K   L   N   Q   Y   D   K   D   N   K   R   H   I   I   480

1441  CGT TTA TTA AGT AGT ATA AAA TAT AAA AAT CAT TTA TGT TTA GTA  1485
481    R   L   L   S   S   I   K   Y   K   N   H   L   C   L   V   495

1486  TTT GAG TGG ATG TGG GGT AAC TTA AGA ATA GCA CTG AAA AAG TAT  1530
496    F   E   W   M   W   G   N   L   R   I   A   L   K   K   Y   510

1531  GGA AAT GGA CAT GGA CTA AAC GCA ACA GCC GTT CAT TGT TAC ACA  1575
511    G   N   G   H   G   L   N   A   T   A   V   H   C   Y   T   525

1576  AAA CAA TTA TTT ATA GCC CTA AGA CAT ATG AGA AAA TGT CGA ATA  1620
526    K   Q   L   F   I   A   L   R   H   M   R   K   C   R   I   540

1621  ATG CAT GCT GAT CTA AAA CCG GAT AAT ATT CTT ATT AAT GAA AAA  1665
541    M   H   A   D   L   K   P   D   N   I   L   I   N   E   K   555

1666  TTT AAC GCC TTA AAA GTT TGC GAT TTA GGA AGT GCA AGT GAT ATA  1710
556    F   N   A   L   K   V   C   D   L   G   S   A   S   D   I   570

1711  TCA GAA AAT GAA ATT ACG TCA TAT TTA GTT AGT AGA TTT TAT AGA  1755
571    S   E   N   E   I   T   S   Y   L   V   S   R   F   Y   R   585
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1756  GCA CCT GAA ATT ATT TTG GGT TTT CGA TAC GAC GCT CAG ATT GAT   1800
586    A   P   E   I   I   L   G   F   R   Y   D   A   Q   I   D   600

1801  GTA TGG TCA GCT GCT GCA ACT GTT TTT GAA TTA GCA ACG GGT AAA   1845
601    V   W   S   A   A   A   T   V   F   E   L   A   T   G   K   615

1846  ATC TTG TTT CCG GGT AAA TCA AAT AAT CAT ATG ATA AAA CTG ATG   1890
616    I   L   F   P   G   K   S   N   N   H   M   I   K   L   M   630

1891  ATG GAA TAT AAG GGC AAA TTT TCA CAT AAA ATG ATA AAA GGT GGG   1935
631    M   E   Y   K   G   K   F   S   H   K   M   I   K   G   G   645

1936  CAA TTT TAT TCT CAA CAT TTT AAT GAA AAT TTA GAT TTT CTT TAT   1980
646    Q   F   Y   S   Q   H   F   N   E   N   L   D   F   L   Y   660

1981  GTG GAT AGA GAT CAT TAT TCC AAA AAA GAA GTT GTT AGA GTT ATA   2025
661    V   D   R   D   H   Y   S   K   K   E   V   V   R   V   I   675

2026  TCT GAT TTG AGA CCT ACG AAA AAT ATA ACA TGT GAT TTA TTG GAG   2070
676    S   D   L   R   P   T   K   N   I   T   C   D   L   L   E   690

2071  CAT CAA TAT TGG TTG AAG GGA AAT AGC CCT AAA ATG CAA TTT TTG   2115
691    H   Q   Y   W   L   K   G   N   S   P   K   M   Q   F   L   705

2116  AAA AAA AAA ATA AAA CAA CTA GGA GAT TTA TTA GAG AAA TGT TTA   2160
706    K   K   K   I   K   Q   L   G   D   L   L   E   K   C   L   720

2161  ATT CTA GAT CCA TCT AAA CGA TAT ACT CCA GAT CAA GCT TTA CAA   2205
721    I   L   D   P   S   K   R   Y   T   P   D   Q   A   L   Q   735

2206  CAT CCT TAT TTA AGA GAA TCT ATT CAT TTT TCA AAA TCT CAA AAT   2250
736    H   P   Y   L   R   E   S   I   H   F   S   K   S   Q   N   750

2251  GAA TAA   2256
751    E   *
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4. *Pf*CLK-4 (PFC0105w)

RT-PCR Primers

pCAM-BSD- KO Primers

pCAM-BSD-tagging primers

Catalytic Domain 58 - 850

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1     ATG AGT TTT AGT AAT ACA TGC TCA CTA TCC AAT AAC AGC AAC AGT   45
1      M   S   F   S   N   T   C   S   L   S   N   N   S   N   S   15

46    TCT AGT AGT AGT GAA GAT GCT ACT TCT GGT AAA TTA CAA TAC ACC   90
16     S   S   S   S   E   D   A   T   S   G   K   L   Q   Y   T   30

91    GAA AGT GAT GAT GAA GGA AGT GAT GAA TAC TGC GAA GGA GGG TAT   135
31     E   S   D   D   E   G   S   D   E   Y   C   E   G   G   Y   45

136   CAC CCA GTC AAA ATT AAT GAA ATA TAT AAT GAT AGA TAT AGA ATT   180
46     H   P   V   K   I   N   E   I   Y   N   D   R   Y   R   I   60

181   GAA GGA AAA TTA GGT TGG GGA CAT TTT TCA ACC GTT TGG GTT GCT   225
61     E   G   K   L   G   W   G   H   F   S   T   V   W   V   A   75
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226   ACT GAT TTA AAA AGT AAA CCC TTA AAA TTT GTT GCT ATA AAA ATT   270
76     T   D   L   K   S   K   P   L   K   F   V   A   I   K   I    90

271   CAA AAA GGA TCA GAA ACT TAT ACT GAA TCA GCC AAA TGT GAA ATT   315
91     Q   K   G   S   E   T   Y   T   E   S   A   K   C   E   I   105

316   AAT TAT TTA AAT ACA GTC AAA GTA AAT TCT TTT GAT TCT TCA TGG   360
106    N   Y   L   N   T   V   K   V   N   S   F   D   S   S   W   120

361   GTT GAA TTA AAA GAA CAA CAA AGA GAA AGA TTA TTT CAT TAT AAT   405
121    V   E   L   K   E   Q   Q   R   E   R   L   F   H   Y   N   135

406   ATG ACT AAA GGA GTT GTC TCT TTT ATT GAT AGT TTT GAA CAT AAA   450
136    M   T   K   G   V   V   S   F   I   D   S   F   E   H   K   150

451   GGT CCA AAT GGT ACT CAT ATT TGT ATG GTC TTT GAA TTT ATG GGT   495
151    G   P   N   G   T   H   I   C   M   V   F   E   F   M   G   165

496   CCT AAT TTA TTA TCC CTA ATA AAA CAT TAT GAT TAT AAA GGA ATT   540
166    P   N   L   L   S   L   I   K   H   Y   D   Y   K   G   I   180

541   CCA TTA AAT TTG GTC AGA AAA ATT GCT ACA CAT GTG TTA ATA GGA   585
181    P   L   N   L   V   R   K   I   A   T   H   V   L   I   G   195

586   ATG CAA TAT TTA CAT GAT GTC TGT AAA ATT ATA CAT AGT GAT ATC   630
196    M   Q   Y   L   H   D   V   C   K   I   I   H   S   D   I   210

631   AAA CCA GAA AAT GTT TTG GTC TCA CCA TTG ACT ACT ATT CCA AAA   675
211    K   P   E   N   V   L   V   S   P   L   T   T   I   P   K   225

676   CCA AAG GAT TAT ACC AAA GAT AAA TTA GAA TCA AAT AAA TCT AAC   720
226    P   K   D   Y   T   K   D   K   L   E   S   N   K   S   N   240

721   CAA GTT GAA AAA AAA GAA AAT GAC CAA AAT GTA GAT AAG AAA TTA   765
241    Q   V   E   K   K   E   N   D   Q   N   V   D   K   K   L   255

766   ATT ACT ACA ATG AAT AAT AAC ATA AAT ACA AAT CTA AGT GAA AAA   810
256    I   T   T   M   N   N   N   I   N   T   N   L   S   E   K   270

811   AAA AAA GTT ATT AAT GAT ACA CAA AAA AAT GAT AAA AAT ATA GAA   855
271    K   K   V   I   N   D   T   Q   K   N   D   K   N   I   E   285

856   TAT GAT CAA AAA TGT ACG AGC AGT AAA GAA AAT ATT GAA GAT AAT   900
286    Y   D   Q   K   C   T   S   S   K   E   N   I   E   D   N   300

901   GTA TCC TTT GTA AAT GAT CCA AGT GAT CCT AAT CAA AAG AAT AAT   945
301    V   S   F   V   N   D   P   S   D   P   N   Q   K   N   N   315

946   CTA AAT AAT AAT ATA ACG GAT AAT AAT ATC ATA CCC AGT AAT GTA   990
316    L   N   N   N   I   T   D   N   N   I   I   P   S   N   V   330

991   CAA ATA GAA AAA CAA TCT ACA TTA AGT AAA AAT AAA AAA AAT GAA  1035
331    Q   I   E   K   Q   S   T   L   S   K   N   K   K   N   E   345

1036  AAA GAT TCA TAT ATA AAT ATA AAC AAT TCT CTT ACA AAT GAT GAT  1080
346    K   D   S   Y   I   N   I   N   N   S   L   T   N   D   D   360

1081  CAA AAT TTA AAA AGA GAA GAT ATC AAA TTT AAT GAT AAA GCG GAA  1125
361    Q   N   L   K   R   E   D   I   K   F   N   D   K   A   E   375

1126  GGG ATT ACC AAA TAT GAT ATG TTA AAT ATT AAA AAT AAT ATA TCT  1170
376    G   I   T   K   Y   D   M   L   N   I   K   N   N   I   S   390

1171  ATT AAA GAA AAA ATA AAT GAT TGT CAT TCA CCC AAT GAA AAT AAA  1215
391    I   K   E   K   I   N   D   C   H   S   P   N   E   N   K   405

1216  AAT AAA GAT AAT CAT AAT CAA TGT GAA GAC AAT TCG ATC AAC ATA  1260
406    N   K   D   N   H   N   Q   C   E   D   N   S   I   N   I   420
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1261  TGT AAT AAC AAA AAT AAT AAT ATT CAA ACA AAT AAT ATT AAT GAT   1305
421    C   N   N   K   N   N   N   I   Q   T   N   N   I   N   D    435

1306  AAC ACT GTT AAC GAA AAA ATT AAT AAT ACA TCA AAG AAG GAT ATG   1350
436    N   T   V   N   E   K   I   N   N   T   S   K   K   D   M    450

1351  TTA AAT AAT ACA CAA AAT AAT AAT GAT TCC GAA AAG AAC GAC GTT   1395
451    L   N   N   T   Q   N   N   N   D   S   E   K   N   D   V    465

1396  GTT ATT GAA CAA CAA TTG GTA AAT GAA GAT ATT TTA AAA AAA AAA   1440
466    V   I   E   Q   Q   L   V   N   E   D   I   L   K   K   K    480

1441  AAC AAA CAA ACA AAA AAA AAA AAA AAT ATA AAT GAA CCT CCA TAT   1485
481    N   K   Q   T   K   K   K   K   N   I   N   E   P   P   Y    495

1486  GTT AAA CAT AAA CTA AGA CCA TCA AAT TCG GAT CCT TCT TTG CTC   1530
496    V   K   H   K   L   R   P   S   N   S   D   P   S   L   L    510

1531  ACA TCT TAT TCT AAT ATA CAT GCA CTT CAA GAA ACC TTG ACA AGG   1575
511    T   S   Y   S   N   I   H   A   L   Q   E   T   L   T   R    525

1576  AAA CCA TAT CAT TAT AAT ACC TAT TTT TTA AAC AAC CCC GAA AAA   1620
526    K   P   Y   H   Y   N   T   Y   F   L   N   N   P   E   K    540

1621  TAT AGA GAT AAT AAA ATG AAT CCA TAC TTA CAC AGA TTG CCA AAT   1665
541    Y   R   D   N   K   M   N   P   Y   L   H   R   L   P   N    555

1666  GAT TGC TTG AAA AAA ATC GAT CAA GAT GAT AGT GAT GAA ACG GAA   1710
556    D   C   L   K   K   I   D   Q   D   D   S   D   E   T   E    570

1711  GAG GAG GAT GAT CTT TCA GAT GTA GAC CAA AAT AAG GAA CAA AAT   1755
571    E   E   D   D   L   S   D   V   D   Q   N   K   E   Q   N    585

1756  AAG AAC CAA TTA GAG GTC AAC TTG CCA AAT AAT AAA TAT CCA AAT   1800
586    K   N   Q   L   E   V   N   L   P   N   N   K   Y   P   N    600

1801  TCC AAT GAT GTG TAT AAA TTT TTT GAA AAA GAT ATT AAT AAA TTT   1845
601    S   N   D   V   Y   K   F   F   E   K   D   I   N   K   F    615

1846  CCC ATA TAC TGC GAC ATG TTT AAT CAT CTT ATA CAT CCA GAA GCC   1890
616    P   I   Y   C   D   M   F   N   H   L   I   H   P   E   A    630

1891  TTA CGA TTA CAT GAA TTA TAT ATG AAA AAT AAA AAA AAC ATC GAT   1935
631    L   R   L   H   E   L   Y   M   K   N   K   K   N   I   D    645

1936  TCT AAC AAT ACA ATG AAT GAT TTA GGT AAT AAT CAA AAT AGT CAT   1980
646    S   N   N   T   M   N   D   L   G   N   N   Q   N   S   H    660

1981  AAA GTA GTA TAT ATA AAT ACT GAA GAT GGA GAA TAT TGT ATT AGG   2025
661    K   V   V   Y   I   N   T   E   D   G   E   Y   C   I   R    675

2026  CCA TAC GAT CCG TCT GTT TAT TAT CAT GAA AAA TCA TGT TAT AAA   2070
676    P   Y   D   P   S   V   Y   Y   H   E   K   S   C   Y   K    690

2071  ATA TGT GAC CTA GGA AAT AGT TTG TGG ATA GAT GAA TCA AGA TAT   2115
691    I   C   D   L   G   N   S   L   W   I   D   E   S   R   Y    705

2116  GCC GAA ATT CAA ACT AGA CAA TAT CGA GCC CCT GAA GTT ATT TTA   2160
706    A   E   I   Q   T   R   Q   Y   R   A   P   E   V   I   L    720

2161  AAA AGT GGG TTC AAT GAA ACA GCA GAT ATA TGG TCC TTT GCA TGC   2205
721    K   S   G   F   N   E   T   A   D   I   W   S   F   A   C    735

2206  ATG GTA TTC GAA TTA GTA ACA GGA GAC TTT TTA TTT AAT CCA CAA   2250
736    M   V   F   E   L   V   T   G   D   F   L   F   N   P   Q    750

2251  AAA GGT GAT AGA TAT GAT AAA AAT GAA GAA CAT TTA AGT TTT ATA   2295
751    K   G   D   R   Y   D   K   N   E   E   H   L   S   F   I    765
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2296  ATT GAA GTG TTA GGA AAT ATA CCA AAG CAT ATG ATT GAT GCA GGG   2340
766    I   E   V   L   G   N   I   P   K   H   M   I   D   A   G   780

2341  TAT AAT TCC CAT AAA TAT TTT AAC AAA AAT AAT TAT CGA CTT AAA   2385
781    Y   N   S   H   K   Y   F   N   K   N   N   Y   R   L   K   795

2386  AAT ATA AGA AAT ATT AAA AAA TAT GGT TTA TAT AAA ATA TTA AAA   2430
796    N   I   R   N   I   K   K   Y   G   L   Y   K   I   L   K   810

2431  TAT AAA TAT AAT CTT CCT GAA AAG GAA ATT AGC CCC TTA TGT AGT   2475
811    Y   K   Y   N   L   P   E   K   E   I   S   P   L   C   S   825

2476  TTC TTA TTA CCC ATG TTA TCT GTG GAT CCA CAA ACG CGC CCC TCA   2520
826    F   L   L   P   M   L   S   V   D   P   Q   T   R   P   S   840

2521  GCA TAT ACC ATG CTT CAA CAC CCA TGG CTT AAT ATG GTA TCA TTA   2565
841    A   Y   T   M   L   Q   H   P   W   L   N   M   V   S   L   855

2566  GAA GAA GGG GAT GAC ATG TAT ATT AAT GAT GAA TCA TAT TCT ATT   2610
856    E   E   G   D   D   M   Y   I   N   D   E   S   Y   S   I   870

2611  AAT AAT GAT AGA AAC ATG AAA AAT AAT AGT AAT AGT AAT AAT TTC   2655
871    N   N   D   R   N   M   K   N   N   S   N   S   N   N   F   885

2656  ATC TAC GAC GGT CAT AAT AGT AGT AAA AAT AAA AAT TCT TCA AAT   2700
886    I   Y   D   G   H   N   S   S   K   N   K   N   S   S   N   900

2701  AAA AAA AAA ATT GAT GTA AAC TAC AAA ATT GGT AAT AAT GGA AAT   2745
901    K   K   K   I   D   V   N   Y   K   I   G   N   N   G   N   915

2746  AAT GCT TAT AAC GAT AAC TAT TAT AAT AAA AAT TAT AAA AAT AAT   2790
916    N   A   Y   N   D   N   Y   Y   N   K   N   Y   K   N   N   930

2791  AAA AAT AAT AAA AAT TTT AAT GAT GAT GTT GTA GAA CCA TCA CCA   2835
931    K   N   N   K   N   F   N   D   D   V   V   E   P   S   P   945

2836  GAT CAA TAT ATG CAT GCA AAT TAT AAT AAT GAT ATT GTG CAT GCA   2880
946    D   Q   Y   M   H   A   N   Y   N   N   D   I   V   H   A   960

2881  GTT TTG TAT GAA AAG CCA TAT AAT TCA AAT AAT GTC ATT TCA TAC   2925
961    V   L   Y   E   K   P   Y   N   S   N   N   V   I   S   Y   975

2926  ACT AAT AAC AAA GGA CAT AAA AAT AAT TTT GAT ATT AAT TAT TTA   2970
976    T   N   N   K   G   H   K   N   N   F   D   I   N   Y   L   990

2971  CAA CAT AGG AAT GAT AAT AAT TCG AAC AAA CAA AAT ATT TCA TTA   3015
991    Q   H   R   N   D   N   N   S   N   K   Q   N   I   S   L   1005

3016  ACT ACA AAC GAT TAT ACA TTT AAT TCG GAT TAT ATT GCT AAT ATG   3060
1006   T   T   N   D   Y   T   F   N   S   D   Y   I   A   N   M   1020

3061  ATG GAT CAT GAC ACA TAT AGA AAA CAA ATA ATA AAA AAT ATT CCT   3105
1021   M   D   H   D   T   Y   R   K   Q   I   I   K   N   I   P   1035

3106  GCA CAT CAA ATT TCA AAA CTA AAA GAT GGT AAA AAT TTT AAG GCA   3150
1036   A   H   Q   I   S   K   L   K   D   G   K   N   F   K   A   1050

3151  TAT AAT GAA TCT ATT CAA TAT GAA ATG CAT GAT TTT CAA CAA TAC   3195
1051   Y   N   E   S   I   Q   Y   E   M   H   D   F   Q   Q   Y   1065

3196  AAT GAA CAT GAT TTT GAA TAC AAA TTT AAT AAA AGA TTT GAA CAT   3240
1066   N   E   H   D   F   E   Y   K   F   N   K   R   F   E   H   1080

3241  GCA CAT CAT ATA AAA GAA ATG AAA CAT AAC GAT GAT GAT TAC GAG   3285
1081   A   H   H   I   K   E   M   K   H   N   D   D   D   Y   E   1095

3286  GAG GAA GAT GAA GAT GAA GAT GAC GAT GAT GAA GAT TAT GAA AGT   3330
1096   E   E   D   E   D   E   D   D   D   D   E   D   Y   E   S   1110
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3331  GAT GTT GAT TAT GAT GAT GAT GAT GAA TAT GAT GAA GGA CAA GAA  3375
1111   D   V   D   Y   D   D   D   D   E   Y   D   E   G   Q   E  1125

3376  CAT GAT GCT GAT CAA GAT GAA AAA AAC AAC GAT AAC GAA AAA CAA  3420
1126   H   D   A   D   Q   D   E   K   N   N   D   N   E   K   Q  1140

3421  CAA GAA CAA CAA AAT TAC GGT GAA AAA TAT AAT TAT GAA CAT TAT  3465
1141   Q   E   Q   Q   N   Y   G   E   K   Y   N   Y   E   H   Y  1155

3466  GAA AAT AAT ATG GGT TAT AAT AAA AAC ATT CAA CAA TTG TCA TAT  3510
1156   E   N   N   M   G   Y   N   K   N   I   Q   Q   L   S   Y  1170

3511  ACA AAT AAT AAT GAT GAT GAA AAT AAT TTT TGT GAG ACA CAA AAT  3555
1171   T   N   N   N   D   D   E   N   N   F   C   E   T   Q   N  1185

3556  ATA TAT ATA TTA CAA AAC AAA AGA GAT ATA AAT TTT AAA GAA TGT  3600
1186   I   Y   I   L   Q   N   K   R   D   I   N   F   K   E   C  1200

3601  ACA CCA CGA AAT AAT ATC AAC AAA GAA ATA AAA AGT GAT AAA TAT  3645
1201   T   P   R   N   N   I   N   K   E   I   K   S   D   K   Y  1215

3646  CAA TCC AGT AAA GTT ATA AAT CAA AAA GAT AAT TAT TGG AAT TAC  3690
1216   Q   S   S   K   V   I   N   Q   K   D   N   Y   W   N   Y  1230

3691  AAA ATC AAA GAA AAC ACA AAA TTA AGA GAA CAT GCA AAA AAA CAA  3735
1231   K   I   K   E   N   T   K   L   R   E   H   A   K   K   Q  1245

3736  CAT TAT AGC AAC AAC AAT AAT ATC AAT AAA AAT GAT AAT ACT AAT  3780
1246   H   Y   S   N   N   N   N   I   N   K   N   D   N   T   N  1260

3781  ATA ATG AAC CAA ATA GAT ACC AAA GAT CAA ATA TCC AAA AAT TTA  3825
1261   I   M   N   Q   I   D   T   K   D   Q   I   S   K   N   L  1275

3826  CAT GAT TTA TCA ACA AAT AAC AAT ATG GAC CAA AAA CAC GGT GCA  3870
1276   H   D   L   S   T   N   N   N   M   D   Q   K   H   G   A  1290

3871  TTA CAA AAA ATG CAT ATG AAC GAA AAA ACA AAC CAA GAC AAA CCA  3915
1291   L   Q   K   M   H   M   N   E   K   T   N   Q   D   K   P  1305

3916  TTA AAT GAC GAA GAA ATT TTA ATC GAA AAT AGA GAT GAC CAG AAT  3960
1306   L   N   D   E   E   I   L   I   E   N   R   D   D   Q   N  1320

3961  GTT AAT AAA ATC AAT TGC AAA GTT ATT AAC AAA AAA AAC TCT TGT  4005
1321   V   N   K   I   N   C   K   V   I   N   K   K   N   S   C  1335

4006  GCA TAT ACT TAA   4017
1336   A   Y   T   *
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5. *Pf*PKRP (PFC0485w)

RT-PCR Primers

Peptide for rabbit anti-*Pf*PKRP

pCAM-BSD-KO primers

pCAM-BSD-tagging primers

Catalytic Domain 161-441

Overlapping primer for tagging sense and RT-PCR sense

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1     ATG CAA TTA AAA AAC AAT TTT TAC CTT TCA GAA AAA AAA GAC AAC   45
1     Met Gln Leu Lys Asn Asn Phe Tyr Leu Ser Glu Lys Lys Asp Asn   15

46    GAA AAA TTG GTT AAT AAG AAT ACA CAT TTA GAT AAT TCA ATA ATA   90
16    Glu Lys Leu Val Asn Lys Asn Thr His Leu Asp Asn Ser Ile Ile   30

91    AAA AAA ATC TAT ACT TTT TAT AAA AAT AAA GAT GAG AAA GTG GTA   135
31    Lys Lys Ile Tyr Thr Phe Tyr Lys Asn Lys Asp Glu Lys Val Val   45

136   GAT AAT TTT GAT TGG ACG TTA GAA ATT CTG AAT AAC CTT TAT AAT   180
46    Asp Asn Phe Asp Trp Thr Leu Glu Ile Leu Asn Asn Leu Tyr Asn   60

181   GTC GAA TTA CCA ATA TTA TAT AAA ATG TTT GAT ATA AAT AGT ATT   225
61    Val Glu Leu Pro Ile Leu Tyr Lys Met Phe Asp Ile Asn Ser Ile   75

226   TGT GTA TCT TTA AAG TTG AAA TTG AAA ATA AAA AAT AAT AAT GAG   270
76    Cys Val Ser Leu Lys Leu Lys Leu Lys Ile Lys Asn Asn Asn Glu   90

271   GCA TCA AAT AAA AAT TAT TTT TTA TAT TTC GAT ATA AAT CCT GGA   315
91    Ala Ser Asn Lys Asn Tyr Phe Leu Tyr Phe Asp Ile Asn Pro Gly   105

316   GGA ATC ATG TCC AAA AAT CAA GAA AAA ATT TAT TAT GAG TAT AAA   360
106   Gly Ile Met Ser Lys Asn Gln Glu Lys Ile Tyr Tyr Glu Tyr Lys   120

361   TTT ATT AGG GAT AAA AAT GAC GAC ACT TTG GAA AAG ATG CCA AGG   405
121   Phe Ile Arg Asp Lys Asn Asp Asp Thr Leu Glu Lys Met Pro Arg   135

406   GAA CAA AAA ATG AAA TAT TTT TCT TCT CTG TCG TAT AAG TTG GCT   450
136   Glu Gln Lys Met Lys Tyr Phe Ser Ser Leu Ser Tyr Lys Leu Ala   150

451   GGG AAA GTC ATA GAG TAT AAA GAA TCA AAG TAT AAA TTG ATA AAC   495
151   Gly Lys Val Ile Glu Tyr Lys Glu Ser Lys Tyr Lys Leu Ile Asn   165

496   GTG TTA CAG TCA GCT ATT TAC GGG AGT GTG TAT TTA TCA GAA GTT   540
166   Val Leu Gln Ser Ala Ile Tyr Gly Ser Val Tyr Leu Ser Glu Val   180

541   GTT GAA GGT GGT GGG AAT GGT TTA TCA AAG AGA TAT AAG GCT ATA   585
181   Val Glu Gly Gly Gly Asn Gly Leu Ser Lys Arg Tyr Lys Ala Ile   195

586   AAA ATA TTG TCT AAG CAT CTG ATT GAG ATG GCT AAA GAT AAA GTA   630
196   Lys Ile Leu Ser Lys His Leu Ile Glu Met Ala Lys Asp Lys Val   210

631   CAA GAG GAC CCA TTA TCT GAA TAT TAT TAT AGA GAT AGT ATG AGT   675
211   Gln Glu Asp Pro Leu Ser Glu Tyr Tyr Tyr Arg Asp Ser Met Ser   225

676   GGT CAT AGT AAT ATA TTA AGT TGT GAT AAC ATA TTT GAT GAC AAT   720
226   Gly His Ser Asn Ile Leu Ser Cys Asp Asn Ile Phe Asp Asp Asn   240
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721   TTA TAT ATA TAT ATG GTA ATG CCT TTT GCT GTA CAT GGG GAT TTA   765
241   Leu Tyr Ile Tyr Met Val Met Pro Phe Ala Val His Gly Asp Leu   255

766   TTT GAG GTT ATG AAA AAT AGG AAT AAG TGT TTT AAT GAA GAA GAA   810
256   Phe Glu Val Met Lys Asn Arg Asn Lys Cys Phe Asn Glu Glu Glu   270

811   GCA AGG TAT TTA TTT CAT CAG ATA TTA TTA GCG ATA AAA TTT TTA   855
271   Ala Arg Tyr Leu Phe His Gln Ile Leu Leu Ala Ile Lys Phe Leu   285

856   CAT TCG AAA AAA ATG GCT TTA CGC GAT ATA TCT TTA GAA AAT ATC   900
286   His Ser Lys Lys Met Ala Leu Arg Asp Ile Ser Leu Glu Asn Ile   300

901   CTT TTA TTT GAG AAT GAG AAA AAT GGA TTA ATA TAT CCA GTT TTA   945
301   Leu Leu Phe Glu Asn Glu Lys Asn Gly Leu Ile Tyr Pro Val Leu   315

946   AAT GAT CCT GGA CAA GCA ATA TAT TTT AAT GTG AAT AAA AGA AAT   990
316   Asn Asp Pro Gly Gln Ala Ile Tyr Phe Asn Val Asn Lys Arg Asn   330

991   AAT GTA ATA TTA GAA GAT TAT AAA AAA ATG TTT GGT AAA ATA TTT   1035
331   Asn Val Ile Leu Glu Asp Tyr Lys Lys Met Phe Gly Lys Ile Phe   345

1036  AGA CCT CCT GAA ATA TAT ATG AAA TGT AAA TAT GAT CCA ACA AAG   1080
346   Arg Pro Pro Glu Ile Tyr Met Lys Cys Lys Tyr Asp Pro Thr Lys   360

1081  GTA GAT ATA TTT TGT GTT GGA TAT ATT CTT TAT TTT TGT TTA ACA   1125
361   Val Asp Ile Phe Cys Val Gly Tyr Ile Leu Tyr Phe Cys Leu Thr   375

1126  AAA CAT GAA CTT TTT AAA TGT TCT TTA GAC AAA GAT ATT TAT TGG   1170
376   Lys His Glu Leu Phe Lys Cys Ser Leu Asp Lys Asp Ile Tyr Trp   390

1171  AAA ATG TTT AAA AAT AAA AAT TAT AAA CAA TTA TTA AAA GAA AAA   1215
391   Lys Met Phe Lys Asn Lys Asn Tyr Lys Gln Leu Leu Lys Glu Lys   405

1216  AAA GGA TTA CAT TTA TCA AAA CAA GTT ATT GAT TTA ATT TTT AAT   1260
406   Lys Gly Leu His Leu Ser Lys Gln Val Ile Asp Leu Ile Phe Asn   420

1261  TGT TTA CAT CCA AAT TTC AAT ATA AGG TAT AAT ATA AAT GAA GCT   1305
421   Cys Leu His Pro Asn Phe Asn Ile Arg Tyr Asn Ile Asn Glu Ala   435

1306  CTT AAT CAT GAT TGG TTC AAA GGA AAT ATA TTT CCT ATA CAC AAT   1350
436   Leu Asn His Asp Trp Phe Lys Gly Asn Ile Phe Pro Ile His Asn   450

1351  TTT AAT TTA TAT ATA AAC AAA GAT AAT AAT AAC AAA AAT AAC ACA   1395
451   Phe Asn Leu Tyr Ile Asn Lys Asp Asn Asn Asn Lys Asn Asn Thr   465

1396  TCT AAT AAT AAA ACG AAT AAC AAT AAT ATG TGT AAT AAT AAT GAT   1440
466   Ser Asn Asn Lys Thr Asn Asn Asn Asn Met Cys Asn Asn Asn Asp   480

1441  GAA GAC AAA GGA AAA AAT TTA TGT TCT TTC AAA CTT TCT CTT CAA   1485
481   Glu Asp Lys Gly Lys Asn Leu Cys Ser Phe Lys Leu Ser Leu Gln   495

1486  ATA GAA AAA TAT GCG AAA AAA AAT AAT ATC CCT ATA TAT CAT GAT   1530
496   Ile Glu Lys Tyr Ala Lys Lys Asn Asn Ile Pro Ile Tyr His Asp   510

1531  TCC ATT ATA CAA TTT TAT ATT TAT GAG CAT GTA TTT GTC TCT TCC   1575
511   Ser Ile Ile Gln Phe Tyr Ile Tyr Glu His Val Phe Val Ser Ser   525

1576  TCT GAT AAT TCA TTA AAT CAA AAG AAT CGA TTA CCA AAT AAA ATA   1620
526   Ser Asp Asn Ser Leu Asn Gln Lys Asn Arg Leu Pro Asn Lys Ile   540

1621  TAT CCT TTT CAT AAT AAA ATA GAT ATA GTA TAT AAA GAA AAT GAT   1665
541   Tyr Pro Phe His Asn Lys Ile Asp Ile Val Tyr Lys Glu Asn Asp   555

1666  GGA GAT GAT TAT AAA AAG AAT ACT TTG TTA TAT AAA AAT ATG CAT   1710
556   Gly Asp Asp Tyr Lys Lys Asn Thr Leu Leu Tyr Lys Asn Met His   570

1711  GTG CCA CTG AAT TTA ACA CGT ATT AAT GGT AAC AAC AGT GGA CAG   1755
571   Val Pro Leu Asn Leu Thr Arg Ile Asn Gly Asn Asn Ser Gly Gln   585
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1756  CCA CCT TGT AAT GTT CCC AAG TTT GAA GAG CCG AAA AAA AAA TTT   1800
586   Pro Pro Cys Asn Val Pro Lys Phe Glu Glu Pro Lys Lys Lys Phe   600

1801  AAT AAT AAT AAT AAT AAT AAT AAT AAA AAT AAA AGT AAT AAC AAT   1845
601   Asn Asn Asn Asn Asn Asn Asn Asn Lys Asn Lys Ser Asn Asn Asn   615

1846  AAC AAT AAT AAT AAC AAT AAT AAT AAC AAT AAT AAT AAT AAT AAC   1890
616   Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn   630

1891  AAT TTG GAT AAC ATA AAA AAT AAA AAT GCT GTT TTC ATT GAA AAG   1935
631   Asn Leu Asp Asn Ile Lys Asn Lys Asn Ala Val Phe Ile Glu Lys   645

1936  GTA AAA CAA AAT AAT GTT CAG GAT AAT ATA TCT ATG AAA CCA CAT   1980
646   Val Lys Gln Asn Asn Val Gln Asp Asn Ile Ser Met Lys Pro His   660

1981  AAT GGA AAT GTT GCC AAA AGG GAA TGT ATA CAT ATG AAT AAA GGA   2025
661   Asn Gly Asn Val Ala Lys Arg Glu Cys Ile His Met Asn Lys Gly   675

2026  CGT TTT GTT CGA GGC GGT GCT TAC ACA AAA GTG TGT GAG AGA AAT   2070
676   Arg Phe Val Arg Gly Gly Ala Tyr Thr Lys Val Cys Glu Arg Asn   690

2071  ACC GAA CGT GTT AAA AGG AAT AAT TAT TGT GGT AAA ATG TAC TTA   2115
691   Thr Glu Arg Val Lys Arg Asn Asn Tyr Cys Gly Lys Met Tyr Leu   705

2116  GAT GAG GGA GAT ATT TAC AAC CGT TTA TGT AAT GAT AAT AAA AAA   2160
706   Asp Glu Gly Asp Ile Tyr Asn Arg Leu Cys Asn Asp Asn Lys Lys   720

2161  GGA GAA TGT GAT CCT AGG CAT ATA GAA GAT GAT AAA AAA AAT AAA   2205
721   Gly Glu Cys Asp Pro Arg His Ile Glu Asp Asp Lys Lys Asn Lys   735

2206  GTT CAT GAT AAA GTG ATT AAT AAT ATG TGT TAT GGA TTG ATG AAC   2250
736   Val His Asp Lys Val Ile Asn Asn Met Cys Tyr Gly Leu Met Asn   750

2251  TCT AAT GAT GAT CTT AAC GAA ACG TTC AAA ACG AAT ATT CTC AAC   2295
751   Ser Asn Asp Asp Leu Asn Glu Thr Phe Lys Thr Asn Ile Leu Asn   765

2296  AGT TAT CAA AAA GGT ATT TAC AAG TTG ATA GAA CTA AGA ACA AAT   2340
766   Ser Tyr Gln Lys Gly Ile Tyr Lys Leu Ile Glu Leu Arg Thr Asn   780

2341  GAA AAG AAA ATA AAA AAT TTG GAT ATA ACA TCT GAT GTG AAT ACA   2385
781   Glu Lys Lys Ile Lys Asn Leu Asp Ile Thr Ser Asp Val Asn Thr   795

2386  AAG GAT ACA TCT CTG AAT GTT TTG TTA ACT AAA GAA ATG GTA AAT   2430
796   Lys Asp Thr Ser Leu Asn Val Leu Leu Thr Lys Glu Met Val Asn   810

2431  AAG AAA AAG GAA GGT CCT CCT TTT TTC AAT AAT AAA GGA GAG ATG   2475
811   Lys Lys Lys Glu Gly Pro Pro Phe Phe Asn Asn Lys Gly Glu Met   825

2476  TGC TTG GAA AAT CAT GAA GAT ATG GTA GAC ATT TTT GGA GAA GGT   2520
826   Cys Leu Glu Asn His Glu Asp Met Val Asp Ile Phe Gly Glu Gly   840

2521  AAA ATG AAA GGA ATT AAA AAT GTG GTG GAT ACA TAT GAC AAG CAC   2565
841   Lys Met Lys Gly Ile Lys Asn Val Val Asp Thr Tyr Asp Lys His   855

2566  AAT AAT GAT AAT AAT AAT AAT AAT GAT AGT AGT AGT AGT AAT AAA   2610
856   Asn Asn Asp Asn Asn Asn Asn Asn Asp Ser Ser Ser Ser Asn Lys   870

2611  TGT TGT AAT AAT TGT TGT AGT AGT AGT TAT GAT AAG GGG AAA GAA   2655
871   Cys Cys Asn Asn Cys Cys Ser Ser Ser Tyr Asp Lys Gly Lys Glu   885

2656  AAA AAG AAA CAA ACG ACA GAG ATC AAT ATT TTG ATA AAC AGT ATG   2700
886   Lys Lys Lys Gln Thr Thr Glu Ile Asn Ile Leu Ile Asn Ser Met   900

2701  TGT GAC ATA AAT AAC TAC ACA CAT ACA AAT ACA CAT TCT GTA ATT   2745
901   Cys Asp Ile Asn Asn Tyr Thr His Thr Asn Thr His Ser Val Ile   915

2746  ATA AAA GAA GAT TAT GAT AAA ATA CAA CAA AAT AAG ATA TCT TCA   2790
916   Ile Lys Glu Asp Tyr Asp Lys Ile Gln Gln Asn Lys Ile Ser Ser   930
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2791  AAA AAT GAT ACC TTC AAT GAA TAT TCA TCA TTT GTA TTT AGT TTA   2835
931   Lys Asn Asp Thr Phe Asn Glu Tyr Ser Ser Phe Val Phe Ser Leu   945

2836  AAT ATG AAC ACG CAA ATA TTG AAA AAC AAA TTA CTA GAG ATG AAA   2880
946   Asn Met Asn Thr Gln Ile Leu Lys Asn Lys Leu Leu Glu Met Lys   960

2881  AAA AAA AAT GAT TTA GAT ATG TAC GGG TGT AAC GAA ATA TTG AAG   2925
961   Lys Lys Asn Asp Leu Asp Met Tyr Gly Cys Asn Glu Ile Leu Lys   975

2926  GGT GAA AAT GAG ATT GGT ATG GAC CCT TTA ATG AAA ATT GAT CAG   2970
976   Gly Glu Asn Glu Ile Gly Met Asp Pro Leu Met Lys Ile Asp Gln   990

2971  ACG AAT AAA ATT GTT AGT AAG GTG GAC GGT AGT AAT TTT AAT AAG   3015
991   Thr Asn Lys Ile Val Ser Lys Val Asp Gly Ser Asn Phe Asn Lys   1005

3016  GTA GAT GGT ATT AAT TTT AAT AAG ACA GAT GGT AGT AAT TTT AAT   3060
1006  Val Asp Gly Ile Asn Phe Asn Lys Thr Asp Gly Ser Asn Phe Asn   1020

3061  AAG ATA GAT GGT ATT AAT TTT AAT AAG ACA GAT GGT ATT AAT TTT   3105
1021  Lys Ile Asp Gly Ile Asn Phe Asn Lys Thr Asp Gly Ile Asn Phe   1035

3106  AAT AAG ACA GAT GGT ATT AAT TTT AAT AAG ACA GAT GGT ATT AAT   3150
1036  Asn Lys Thr Asp Gly Ile Asn Phe Asn Lys Thr Asp Gly Ile Asn   1050

3151  TTT AAT AAG GTA GAT GAT AAT ATT TTC AAT AAA ATA AAA GAT GAG   3195
1051  Phe Asn Lys Val Asp Asp Asn Ile Phe Asn Lys Ile Lys Asp Glu   1065

3196  GTA GAA AAA TAT GTA GAT CCT TTG CCG TCA CAT ATA AGA ACA GAT   3240
1066  Val Glu Lys Tyr Val Asp Pro Leu Pro Ser His Ile Arg Thr Asp   1080

3241  GAC ATG AGA AAA AAA AAG TCG GAA TTA TTA TTA TCC AAA GAC GGT   3285
1081  Asp Met Arg Lys Lys Lys Ser Glu Leu Leu Leu Ser Lys Asp Gly   1095

3286  TCT ATA ATT ATA TCG AAT TTG GAT ACT TCA CAT TTT GAA ATA AAT   3330
1096  Ser Ile Ile Ile Ser Asn Leu Asp Thr Ser His Phe Glu Ile Asn   1110

3331  CTG TCT CGG AGC GAG ATA CAG AAT GAA ATG TGT AAG GAA AAT AGT   3375
1111  Leu Ser Arg Ser Glu Ile Gln Asn Glu Met Cys Lys Glu Asn Ser   1125

3376  TTT GTT AAA TGT CAA CTG GAA AAT AAA CTA ATT TTA GAA TTA GAA   3420
1126  Phe Val Lys Cys Gln Leu Glu Asn Lys Leu Ile Leu Glu Leu Glu   1140

3421  AAA GAA ATA AAA GAC GAA GAA AAA AAT CTG CAA AAT GAA CTA GAG   3465
1141  Lys Glu Ile Lys Asp Glu Glu Lys Asn Leu Gln Asn Glu Leu Glu   1155

3466  AGA AGT AAC TGG TCT ATA GAT ATA GAA GAT CTT GAT AAA GAT TTA   3510
1156  Arg Ser Asn Trp Ser Ile Asp Ile Glu Asp Leu Asp Lys Asp Leu   1170

3511  ATA ATT AAT AAA GAA AGT AGA GAT ATT AAA TAT AAA CAT TGG ATA   3555
1171  Ile Ile Asn Lys Glu Ser Arg Asp Ile Lys Tyr Lys His Trp Ile   1185

3556  GAT ATA AAT AAA GAT AAC TAT ATG ATG ATA TAT CAA GAT AAC AAA   3600
1186  Asp Ile Asn Lys Asp Asn Tyr Met Met Ile Tyr Gln Asp Asn Lys   1200

3601  TGT GGG AGA CGA AAA AAA ATG ATA TCA CAA AAT AAA CTA CTT ATA   3645
1201  Cys Gly Arg Arg Lys Lys Met Ile Ser Gln Asn Lys Leu Leu Ile   1215

3646  AAA AAG AAA AGG ATA AAA ATG AGA AAT CAT GAG AAA AAA AGA AAA   3690
1216  Lys Lys Lys Arg Ile Lys Met Arg Asn His Glu Lys Lys Arg Lys   1230

3691  ATT CGT TTT TTT TTT AAA TTA TAT AAA AGA AAT GAT ACA CAT AAA   3735
1231  Ile Arg Phe Phe Phe Lys Leu Tyr Lys Arg Asn Asp Thr His Lys   1245

3736  AAA TTA AGG CCC ATA CGG TTT GTA CGA CAT GTA GAT GTG AAG TTG   3780
1246  Lys Leu Arg Pro Ile Arg Phe Val Arg His Val Asp Val Lys Leu   1260

3781  GAT AAT CTG AAT GAT AAG ACT GTC ATG TTA AAA AAT GAA ATA AGG   3825
1261  Asp Asn Leu Asn Asp Lys Thr Val Met Leu Lys Asn Glu Ile Arg   1275
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3826  GAT GTG AAA GGA GAG GAT AAA GGA GAG GAT GTA TAT TTT GAT TTT   3870
1276  Asp Val Lys Gly Glu Asp Lys Gly Glu Asp Val Tyr Phe Asp Phe   1290

3871  TTA AAT AAA GAT AAT AAT ATG GGA AAT ATG GAA AAT AAG AAA AAT   3915
1291  Leu Asn Lys Asp Asn Asn Met Gly Asn Met Glu Asn Lys Lys Asn   1305

3916  GTA AAA AAT GTG AAA AAT GTA AAA AAT GTG AAT AAT GTG AAA GAT   3960
1306  Val Lys Asn Val Lys Asn Val Lys Asn Val Asn Asn Val Lys Asp   1320

3961  GTC AAA AAT GTG AAT AAT GTC AAA AAT GTG AAT AAT GTG AAT AAT   4005
1321  Val Lys Asn Val Asn Asn Val Lys Asn Val Asn Asn Val Asn Asn   1335

4006  GTG AAT AAT GTG AAA GAT GTG AAA AAT ATG GAA CAT ATC GAT AAA   4050
1336  Val Asn Asn Val Lys Asp Val Lys Asn Met Glu His Ile Asp Lys   1350

4051  TAT AAT AAA AAA GAG GTG ATG ATA AAA AAA AAA GGA GAA TCG AAT   4095
1351  Tyr Asn Lys Lys Glu Val Met Ile Lys Lys Lys Gly Glu Ser Asn   1365

4096  AAT GTA CCG CAC AAA GAG AAA CAC AAT AAT AAA AAG AAT TAT TGT   4140
1366  Asn Val Pro His Lys Glu Lys His Asn Asn Lys Lys Asn Tyr Cys   1380

4141  AAT TAT GAT TTG GGA ATG CAT TCA TTA CAA AAT AGA CAT ACT ATT   4185
1381  Asn Tyr Asp Leu Gly Met His Ser Leu Gln Asn Arg His Thr Ile   1395

4186  ACA TCA GAA GTA TCA TCC AAA TTT TTA TGT AAA AAC ATG AAA AAT   4230
1396  Thr Ser Glu Val Ser Ser Lys Phe Leu Cys Lys Asn Met Lys Asn   1410

4231  TAT TTT GAT AAG TCT AAT AAT TCA ATT GAA ATA CAC AAA ATA AGC   4275
1411  Tyr Phe Asp Lys Ser Asn Asn Ser Ile Glu Ile His Lys Ile Ser   1425

4276  GCT TCT AAT ATT TTT AGA CAT ACG ATG TGT GTG GCA AGC AAT ATA   4320
1426  Ala Ser Asn Ile Phe Arg His Thr Met Cys Val Ala Ser Asn Ile   1440

4321  AAA GGT GAA AAT AAA AAT AAT GGA AAT AAT ATT AAT TAT AAA GGA   4365
1441  Lys Gly Glu Asn Lys Asn Asn Gly Asn Asn Ile Asn Tyr Lys Gly   1455

4366  CCA GCT ACC AAA GCG TTA GTT AAT AAA TTG TTT ATA TCA AAA AAG   4410
1456  Pro Ala Thr Lys Ala Leu Val Asn Lys Leu Phe Ile Ser Lys Lys   1470

4411  GAG AGT AAG AGA GCT ATT ACA TCA TCC AAA AAA AGG GAT GAT GAT   4455
1471  Glu Ser Lys Arg Ala Ile Thr Ser Ser Lys Lys Arg Asp Asp Asp   1485

4456  AAT ATA AAT GTG ATA AAA AAA ATT AAT ACA CCA TCC CAA AAA GTA   4500
1486  Asn Ile Asn Val Ile Lys Lys Ile Asn Thr Pro Ser Gln Lys Val   1500

4501  AGT GAA AAA AGA AAC AAC AAC AAT AAT AAT AAT AAT GTA CTA GGA   4545
1501  Ser Glu Lys Arg Asn Asn Asn Asn Asn Asn Asn Asn Val Leu Gly   1515

4546  GAC AAG AAC AAA AAT AAA AAT AAC GAT GAA CTA TTC ACA AAG GAG   4590
1516  Asp Lys Asn Lys Asn Lys Asn Asn Asp Glu Leu Phe Thr Lys Glu   1530

4591  ATT AAG AAA AGT ACT ATT TCT AAA CAA AAA AAA GGG AAG AAT GAA   4635
1531  Ile Lys Lys Ser Thr Ile Ser Lys Gln Lys Lys Gly Lys Asn Glu   1545

4636  GGA AAC ACA AAA ACG CAT AAA GAT AAT ATT AAC ATA TTG AAT GAA   4680
1546  Gly Asn Thr Lys Thr His Lys Asp Asn Ile Asn Ile Leu Asn Glu   1560

4681  GAC GTA GAC CAT TTC AAA CAA CCA AGT TTA CGT TTG GAA GTT ACC   4725
1561  Asp Val Asp His Phe Lys Gln Pro Ser Leu Arg Leu Glu Val Thr   1575

4726  AAA AAA AAT AAT AAA AAT AAT AAA AAT AAT AAA AAT AAT AAA AAA   4770
1576  Lys Lys Asn Asn Lys Asn Asn Lys Asn Asn Lys Asn Asn Lys Lys   1590

4771  TTT AAT GAT AAT TAT AAT AAT AAT CAT AAT AAT AAT AAT AGT AAT   4815
1591  Phe Asn Asp Asn Tyr Asn Asn Asn His Asn Asn Asn Asn Ser Asn   1605

4816  GAT TTC GAA GAA TAT AAA GAG GAA CAT ATT GCC ACT AAT GAA ATT   4860
1606  Asp Phe Glu Glu Tyr Lys Glu Glu His Ile Ala Thr Asn Glu Ile   1620
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4861  GTT CGA GAA TCA GAA AGT GAT TTG TAT GTT AGT TGT GAT GAG GGG   4905
1621  Val Arg Glu Ser Glu Ser Asp Leu Tyr Val Ser Cys Asp Glu Gly   1635

4906  TGT TAT AAA AAT GGA GAT ATA TAC AAT ATG GAA ATT ATA AAC AAT   4950
1636  Cys Tyr Lys Asn Gly Asp Ile Tyr Asn Met Glu Ile Ile Asn Asn   1650

4951  GTG GAT AAT ATA CAC AAG ATA GAT AAA AAG GGT AAT GAT ATA GAT   4995
1651  Val Asp Asn Ile His Lys Ile Asp Lys Lys Gly Asn Asp Ile Asp   1665

4996  TAT AAG GAT AAA TCA TTG GAA AAT AAT AAA AAA AAA CAG ATG AAA   5040
1666  Tyr Lys Asp Lys Ser Leu Glu Asn Asn Lys Lys Lys Gln Met Lys   1680

5041  GGT CTC ATC AAA ATA TTA CCC TCG TCA TAT ACA GAA AAG GAA AAA   5085
1681  Gly Leu Ile Lys Ile Leu Pro Ser Ser Tyr Thr Glu Lys Glu Lys   1695

5086  GAA AAA GAA AAG GAA AAA GAA AGA AAA AAA AAA AAA AAT ATA TAT   5130
1696  Glu Lys Glu Lys Glu Lys Glu Arg Lys Lys Lys Lys Asn Ile Tyr   1710

5131  ATA CCA TTA ACA ATA GCA AGT AGG AAG ACA GTA GCA TAT CCA ATC   5175
1711  Ile Pro Leu Thr Ile Ala Ser Arg Lys Thr Val Ala Tyr Pro Ile   1725

5176  AAC GAT ACG ACA AAG GAT GTC AAA AAT AAG TTA CAT ACT TTA AAA   5220
1726  Asn Asp Thr Thr Lys Asp Val Lys Asn Lys Leu His Thr Leu Lys   1740

5221  AGA AAT ACA TGT GTA ACA TAT TGT AAT GTG GAT AAT ATA CAG AAT   5265
1741  Arg Asn Thr Cys Val Thr Tyr Cys Asn Val Asp Asn Ile Gln Asn   1755

5266  AAA AAA AAA AAA GGA GAC GAT AAA AAA AAC ATC AAG AGG GAT CAA   5310
1756  Lys Lys Lys Lys Gly Asp Asp Lys Lys Asn Ile Lys Arg Asp Gln   1770

5311  CAT GTA GGG CTA GAA AAG TTT CTT GAT GAA ATG TCG GCA ATG TTT   5355
1771  His Val Gly Leu Glu Lys Phe Leu Asp Glu Met Ser Ala Met Phe   1785

5356  GAA AAA AAA AAA AAA ATT AAA AAG GAT GAT ATA AAT AAA AAG GAA   5400
1786  Glu Lys Lys Lys Lys Ile Lys Lys Asp Asp Ile Asn Lys Lys Glu   1800

5401  GAT ATA AAT AAA AAG GAT GAC ATA AAT AAA AAG GAT AAC ATA AAT   5445
1801  Asp Ile Asn Lys Lys Asp Asp Ile Asn Lys Lys Asp Asn Ile Asn   1815

5446  AAA AAG AAT GAT ATA AAT AAA AAG GAC GAT ATA AAT AAA AAG GAC   5490
1816  Lys Lys Asn Asp Ile Asn Lys Lys Asp Asp Ile Asn Lys Lys Asp   1830

5491  GAT ATA AAT AAA AAG GGT GAC ATA AAT AAA AAG GAC GAT ATA AAT   5535
1831  Asp Ile Asn Lys Lys Gly Asp Ile Asn Lys Lys Asp Asp Ile Asn   1845

5536  AAA AAA AAT AAT TAC AAT AAT AAT AGT AAT AAT AAC AAT GTT GTG   5580
1846  Lys Lys Asn Asn Tyr Asn Asn Asn Ser Asn Asn Asn Asn Val Val   1860

5581  AAA AAA TTC TCA AAG ACA CAT CAA AAT GAA GAA AAG ATT AAG GGA   5625
1861  Lys Lys Phe Ser Lys Thr His Gln Asn Glu Glu Lys Ile Lys Gly   1875

5626  AAT ATT ACT GTT ATA AGA AAC AAG TTA AAG GAC AAA GGT AAA AAG   5670
1876  Asn Ile Thr Val Ile Arg Asn Lys Leu Lys Asp Lys Gly Lys Lys   1890

5671  GAA AAT ATC GGA TTA AAA AAA AAA AAA ATA GAA AGG AAA AAC ACA   5715
1891  Glu Asn Ile Gly Leu Lys Lys Lys Lys Ile Glu Arg Lys Asn Thr   1905

5716  ACA ACA ATT GCT ACA AAG AAA CAT GAT AAT ATT ATA GAT ATA AAG   5760
1906  Thr Thr Ile Ala Thr Lys Lys His Asp Asn Ile Ile Asp Ile Lys   1920

5761  AAA AAG AAT GAA AAA GAA AAT AAA ATG ATA AAG AAT TCC AAA TTT   5805
1921  Lys Lys Asn Glu Lys Glu Asn Lys Met Ile Lys Asn Ser Lys Phe   1935

5806  CAA TGT TTA AAA AAC AAG GGA ACG CAA ATA GAA AAT AAA AAG AAT   5850
1936  Gln Cys Leu Lys Asn Lys Gly Thr Gln Ile Glu Asn Lys Lys Asn   1950

5851  ATG ATT ATT TCC GGA GGG AAA AAA ACT AAT CAA TTA GTA AGG ATG   5895
1951  Met Ile Ile Ser Gly Gly Lys Lys Thr Asn Gln Leu Val Arg Met   1965
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5896  AAA AAT AGC AAG CAT CAA AAT AAA GTG GAA TAT AAT TTA AAT AAT   5940
1966  Lys Asn Ser Lys His Gln Asn Lys Val Glu Tyr Asn Leu Asn Asn   1980

5941  ATT TCA AAA GAA TTA GAA AAA AAG AAA ATA TAC ATG CGT TAT TTT   5985
1981  Ile Ser Lys Glu Leu Glu Lys Lys Lys Ile Tyr Met Arg Tyr Phe   1995

5986  AAA AAG GGT ATA AAG GAT AAG ATA GAA AAT ATG GGC AAT CTA AAA   6030
1996  Lys Lys Gly Ile Lys Asp Lys Ile Glu Asn Met Gly Asn Leu Lys   2010

6031  GTT TCT AGA GAT ATG AAA AAA AAG AAA AAG AGC GAT CTT ATA AAA   6075
2011  Val Ser Arg Asp Met Lys Lys Lys Lys Lys Ser Asp Leu Ile Lys   2025

6076  AAT AAG GAG GGA GGA TTA TTG TAT GAA TGG TAT AAT AAG TAT ATA   6120
2026  Asn Lys Glu Gly Gly Leu Leu Tyr Glu Trp Tyr Asn Lys Tyr Ile   2040

6121  TAT AAT GGG GAC GGA AAA GAA AAG TTA AAA ATG GAT GAT TCG AAA   6165
2041  Tyr Asn Gly Asp Gly Lys Glu Lys Leu Lys Met Asp Asp Ser Lys   2055

6166  AAT TAC AAG GAT AAT ATA TGT GAT AAA AAT AAA AAC AAT ATG TGT   6210
2056  Asn Tyr Lys Asp Asn Ile Cys Asp Lys Asn Lys Asn Asn Met Cys   2070

6211  GAT AAA AGT AAA AAC AAT ATG TGT GAT AAA AAT AAA AAC AAT ATG   6255
2071  Asp Lys Ser Lys Asn Asn Met Cys Asp Lys Asn Lys Asn Asn Met   2085

6256  TGT GAT GAT AAT AAA AAC AAT ATG TGT GAT AAA AGT AAA AAC AAT   6300
2086  Cys Asp Asp Asn Lys Asn Asn Met Cys Asp Lys Ser Lys Asn Asn   2100

6301  ATG TGT GAT GAT AAT AAA AAG GAA ATC GAA ATA GTA AAT ATA ATT   6345
2101  Met Cys Asp Asp Asn Lys Lys Glu Ile Glu Ile Val Asn Ile Ile   2115

6346  CAT ACA AGT AAT GCT AGA CAA AAT TAT AAT AAA ACT AAC GAA AAG   6390
2116  His Thr Ser Asn Ala Arg Gln Asn Tyr Asn Lys Thr Asn Glu Lys   2130

6391  ATA AAA AAT ATA TCA AGG GAA TAT ATG ACA GAA TCA TAT GGA GAT   6435
2131  Ile Lys Asn Ile Ser Arg Glu Tyr Met Thr Glu Ser Tyr Gly Asp   2145

6436  ATA TCT ACT CCT TTG AAA AAA AAT GTA AAT GTA AAT GTA AAT AAA   6480
2146  Ile Ser Thr Pro Leu Lys Lys Asn Val Asn Val Asn Val Asn Lys   2160

6481  GAT ATA CAT ATA ATA AAT GAT AAA CAC GAA AGG ATA TGT ACC AAA   6525
2161  Asp Ile His Ile Ile Asn Asp Lys His Glu Arg Ile Cys Thr Lys   2175

6526  AAT AAC AAC CCA AAT CTA CAT ATT TGT CAG CCC ACT AAT GAT CAG   6570
2176  Asn Asn Asn Pro Asn Leu His Ile Cys Gln Pro Thr Asn Asp Gln   2190

6571  GAT GGG GAT AAA AAA AAA AAT ATA GTT AAA TTT AAA AGT GTA GAG   6615
2191  Asp Gly Asp Lys Lys Lys Asn Ile Val Lys Phe Lys Ser Val Glu   2205

6616  AAT AGA ACT CCT CAT ACA TAT TTA CTA TTT AAA AAT GAT GAG AAC   6660
2206  Asn Arg Thr Pro His Thr Tyr Leu Leu Phe Lys Asn Asp Glu Asn   2220

6661  AAA GCA TAT TTA GAA ATG GTA AAG AGT GTT AAT TAT ATG AAT AAA   6705
2221  Lys Ala Tyr Leu Glu Met Val Lys Ser Val Asn Tyr Met Asn Lys   2235

6706  AAG AAA GGA GCA CAA CAT AAT ATT ACA AAC AAG ATA GAT GTT GAG   6750
2236  Lys Lys Gly Ala Gln His Asn Ile Thr Asn Lys Ile Asp Val Glu   2250

6751  AAT AGC TCT CCT GTT CAT GTG CCA CCT CAT AAT ATA GGA TAC AAA   6795
2251  Asn Ser Ser Pro Val His Val Pro Pro His Asn Ile Gly Tyr Lys   2265

6796  AAA GTA GAT TAT AAT GAA GGA GAA AAT ATA AAA GTA TCA TCA CAA   6840
2266  Lys Val Asp Tyr Asn Glu Gly Glu Asn Ile Lys Val Ser Ser Gln   2280

6841  AAA CAA ATA AAT AAT AAT AAT AAT AAT AAT AAT AAT AAT TAT AAT   6885
2281  Lys Gln Ile Asn Asn Asn Asn Asn Asn Asn Asn Asn Asn Tyr Asn   2295

6886  AAT AAT AAT AAT AAT AAT TAT AAT AAA AAT AAT TAT AAT AAA AAT   6930
2296  Asn Asn Asn Asn Asn Asn Tyr Asn Lys Asn Asn Tyr Asn Lys Asn   2310
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6931  AAT TAT AAT AAT AAT TAT TAT GAT TAT CCT AGC TTA TAT ATT AAC   6975
2311  Asn Tyr Asn Asn Asn Tyr Tyr Asp Tyr Pro Ser Leu Tyr Ile Asn   2325

6976  AAT CCA GTT GAT TCT TTA AAT CAT TCT AAA GGG GTT CAG AAA AAA   7020
2326  Asn Pro Val Asp Ser Leu Asn His Ser Lys Gly Val Gln Lys Lys   2340

7021  AAT GAT GAT ACG GAT AAG AAC AAT ATA ACG TCA GCT TGT GAA AAT   7065
2341  Asn Asp Asp Thr Asp Lys Asn Asn Ile Thr Ser Ala Cys Glu Asn   2355

7066  GAG GAA AAT AAA AAG TCC TCA TGT TTA ATA AAT AAT AAA ACA TTA   7110
2356  Glu Glu Asn Lys Lys Ser Ser Cys Leu Ile Asn Asn Lys Thr Leu   2370

7111  TAT TTT ATA TAT CCA CCA AAA AAC AGG TCG TCA TGT TAT GGA AAG   7155
2371  Tyr Phe Ile Tyr Pro Pro Lys Asn Arg Ser Ser Cys Tyr Gly Lys   2385

7156  ACG TAT GAT TAT ATT AAT CAT ATA AAA TGT GAG GGG GGA AAT AAA   7200
2386  Thr Tyr Asp Tyr Ile Asn His Ile Lys Cys Glu Gly Gly Asn Lys   2400

7201  AAT AAA AGT AAA AAT AAT GAT ATT AAA AGG TAT ATT AAT TTT ACA   7245
2401  Asn Lys Ser Lys Asn Asn Asp Ile Lys Arg Tyr Ile Asn Phe Thr   2415

7246  CAT TTA ACA AAA GAA CAG GAT ATT ACG AAC AAG TGT GTT GAG AAA   7290
2416  His Leu Thr Lys Glu Gln Asp Ile Thr Asn Lys Cys Val Glu Lys   2430

7291  AAT AGA ATG GAT GAT ATA TAT AGA AAT GAT GAC AGG AAA TAT ATG   7335
2431  Asn Arg Met Asp Asp Ile Tyr Arg Asn Asp Asp Arg Lys Tyr Met   2445

7336  AAG TCT TTA CAT TTT TAT AAT AAT TAT TTA AAT ACT ACA CGT GGT   7380
2446  Lys Ser Leu His Phe Tyr Asn Asn Tyr Leu Asn Thr Thr Arg Gly   2460

7381  ACA TAT GTT GCA CAT CCC TAT TAT TAT AAT AAC ATG AGA AGT AAT   7425
2461  Thr Tyr Val Ala His Pro Tyr Tyr Tyr Asn Asn Met Arg Ser Asn   2475

7426  AAT AAA ACA CAG TGT TCT GAA CAA AAA AAT AAA GGA ATA GCA GTC   7470
2476  Asn Lys Thr Gln Cys Ser Glu Gln Lys Asn Lys Gly Ile Ala Val   2490

7471  GGA AGG GAA GAT AAT AAA AAG AAT ACT AAT TCT ATA AAC TTC ATA   7515
2491  Gly Arg Glu Asp Asn Lys Lys Asn Thr Asn Ser Ile Asn Phe Ile   2505

7516  CTA GAT CCT TAT TTT TTT AAA AAA ATA AAA TAA   7548
2506  Leu Asp Pro Tyr Phe Phe Lys Lys Ile Lys End
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