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1 Introduction

1.1 Malaria:

Malaria is the most devastating parasitic infection Although it has been eradicated from several occidental countries, malaria is still present worldwide with the majority of cases in the developing world [1]. According to the World Health Organization's (WHO) report, malaria was endemic in 109 countries in 2008 and in 2006 there were approximately 247 million of cases and 3.3 billion people at risk. Each year, there are about 1 million of deaths and most of them are children under five years old leaving in Sub-Saharan Africa. Furthermore malaria cases lead to hundreds millions of days of illness annually in Africa. In light of the above the economists conclude that the disease has a huge impact on economy, being a definitive cause of poverty in endemic regions [1]. For health and socioeconomic reasons there is an urgent need for an anti-malaria treatment, which becomes more and more vital because of the spread of resistances that parasites as well as mosquitoes have developed against various drugs (like chloroquine) or insecticides (like DTT) which now makes these solutions inefficient in many countries [2]

1.2 Parasite life cycle

The Parasite responsible of the disease is *Plasmodium* which besides *Toxoplasma* and *Theileria* is part of the Apicomplexa phylum. There are five species of *Plasmodium* able to infect human: *P.malariae. P.vivax, P.ovale, P.falciparum* and *P.knowlesi*. Among them *P.falciparum* is causing the most severe and lethal form of malaria [3, 4].

P.falciparum is transmitted by the *Anopheles* mosquito. Infection of the human host starts by the bite of a female *Anopheles*. During the blood meal, motile sporozoites present in the salivary glands are directly transferred in the human blood stream. Sporozoites then circulate in the blood before they reach the liver where they invade hepatocytes.Each sporozoite present in a liver cell divides mitotically into merozoites (which correspond to the first asexual stage). After 10 to 12 days, infected hepatocytes burst, releasing several thousand merozoites into the blood stream where they invade erythrocytes. In the red blood cells merozoites differentiate into immature trophozoites stage called rings. A few hours later rings will complete their growth and reach the trophozoites mature stage. Late trophozoites subsequently enter in several cycles of nuclear divisions without cytokinesis and reach the so called schizont stage because of this schizogonic multiplication. In a single schizont there can be up to 32 daughters' merozoites which will be finally released directly into the blood stream when the rupture of the schizont appears.

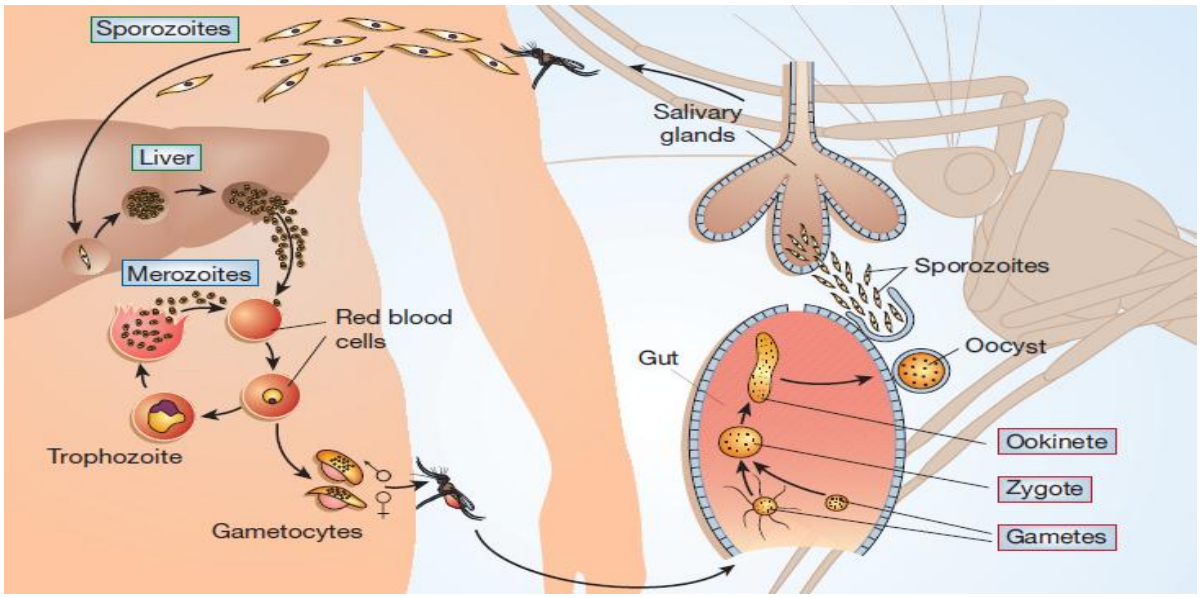


Figure 1 Plasmodium life cycle [5]

This intra-erythrocytic cycle of asexual replication is responsible for the disease. The alternation of fever and chill observed on patient reflects directly steps of invasion and growth in red blood cells [1-3](http://www.icp.ucl.ac.be/~opperd/parasites/malaria4.htm). For reasons that are still not completely defined, some parasites differentiate into sexual stages generating male and female gametocytes called micro and macro gametocytes, respectively. These gametocytes stay in erythrocytes waiting for their uptake by another mosquito during a new blood meal. Gametogenesis begins in the stomach of the mosquito and is followed by the fusion of the gametes to form a zygote. The zygote maturation leads to a motile ookinete which can penetrate the gut epithelium cells and develops into an oocyst. The oocyst then enters in many cycles of asexual replication resulting in the generation of several sporozoites that, after having reached salivary glands, can be transmittes to another human host during a next blod meal.

http://www.dpd.cdc.gov/DPDx/HTML/malaria.htm

http://www.tulane.edu/~wiser/malaria/mal_lc.PDF

1.3 Plasmodium falciparum kinases:

As mentioned before, the spread of drug resistance in *Plasmodium* and mosquito makes discovery of new compounds an urgent task. To achieve this goal, various approaches are used like the development of new vaccines [6], chemical modification of drugs already existing and search for innovative molecules [7] as well as genetically modified mosquitoes [8]. However, in this work, I will focus only on a recently developed approach based on proteins kinases studies. Protein phosphorylation and dephosphorylation, controlled by kinases and phosphatases, is one of the major mechanisms of regulation of cellular processes. Indeed, changes in the phosphorylation state of a protein can induce changes in their function either by increasing or decreasing their activity, by modifying its cellular localisation or by changing its interaction with other proteins or by triggering its own destruction [9]. Moreover deregulation of this cellular process proved to be involved in various diseases like cancer,

neurodegenerative diseases, diabetes and inflammation, which is why development of specific kinases inhibitors has been and is still extensively studied to target all the aforementioned human diseases [10, 11].

Based on the success story of different kinases inhibitors to treat cancers it has become interesting to study these molecules in other diseases like *Plasmodium falciparum*. Thanks to bioinformatics, the kinome of *P.falciparum* has been sequenced and it has revealed some homologies between human and *P.falciparum* proteins but also some divergences which can be explained by the phylogenetic distance between malaria parasite and human [12].This separation between the host and the parasite makes the use of inhibitors against *Plasmodium* kinases even more interesting since targeting the parasites proteins seems feasible without disturbing human proteins [4, 12].

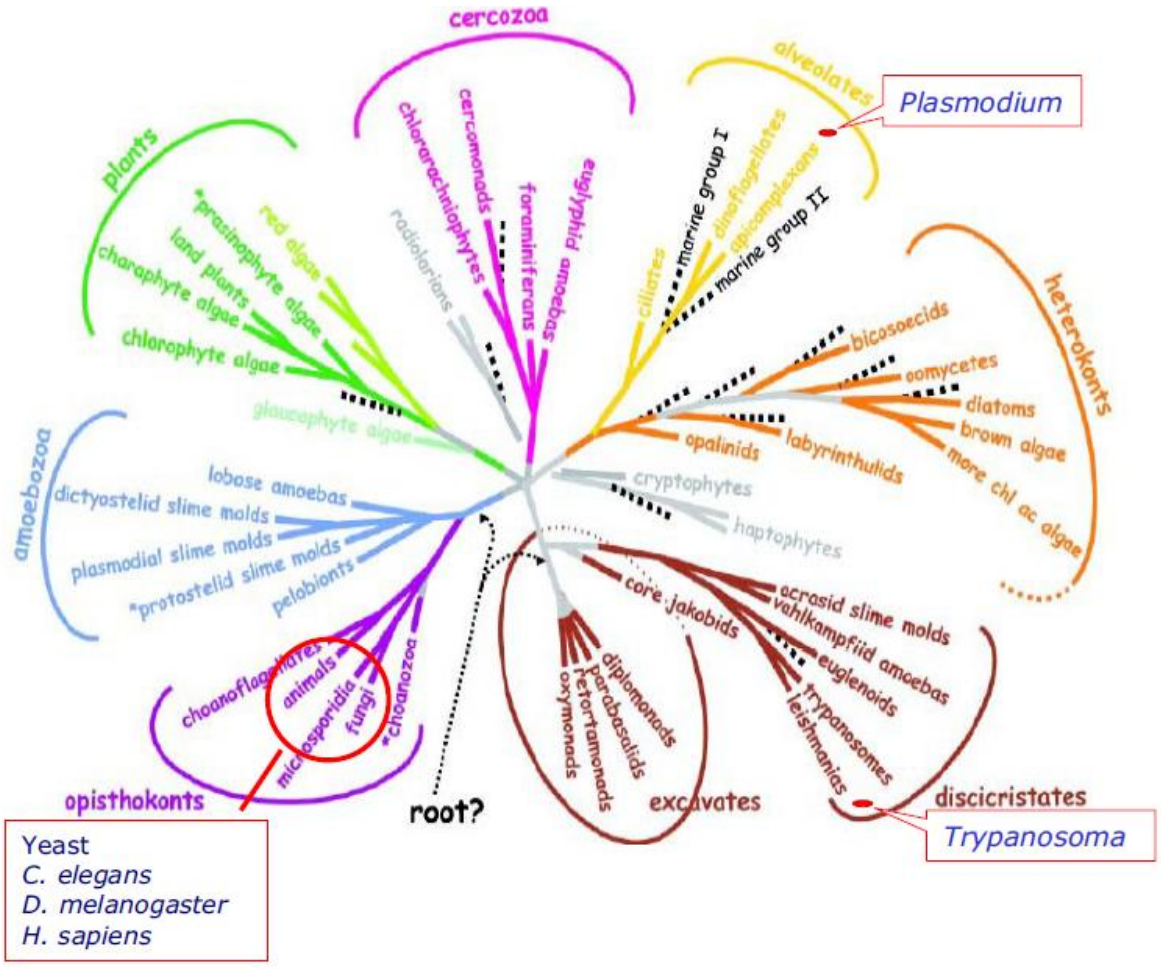


Figure 2 Phylogenetic distance between eukaryotic organisms

The human kinome is composed of more than 500 proteins kinases which represent approximately 2 % of the genome. Except for a few kinases, the large majority of them shares a common amino acid sequence and a similar folding structure and belongs to the eukaryotic proteins kinases (ePKs) whereas kinases that don't fit within this group belong to the atypical proteins kinases family (aPKs). Eukaryotic proteins kinases are divided in seven main clusters, namely:

1. Casein kinase 1 (CK1).
2. CMGC for CDK (cyclin-dependent kinases), MAPK (mitogen-activated protein kinases), GSK3 (glycogen synthase kinase 3) and CLKs (CDK-like kinases).
3. TKL (tyrosine-kinase-like)
4. AGC for PKA (cyclic-adenosine-monophosphate-dependent protein kinase), PKG (cyclic-guanosine-monophosphate-dependent protein kinase) and PKC (Protein kinase C).
5. Camk (calcium/calmodulin-dependent kinases).
6. STE (regulators proteins of MAPKs).
7. TyrK (tyrosine kinases).

Bioinformatics analysis of the *Plasmodium* kinome results in the discovery of 86 or 99 ePKs, depending on the parameters used, and some other proteins which don't fit in ePKs family are classified in the other protein kinases group (OPKs) [13]. Distribution of the *P.falciparum* kinases in the main clusters reveals that most of the kinases are found in the CMGC and CamK clusters. A few ePKs are in the TKL and AGC clusters, and only one is in the CK1 group which is quite surprising since this group contains some proteins in other eukaryote kinomes. No proteins have been found in the STE and TyrK clusters. Some OPKs belong to the NimA (never-in-mitosis A) subgroup and the other proteins do not fit into any group and constitute a satellite cluster [12, 13].

Here we will concentrate on the CMGC family containing containing our proteins of interest in this study.. As mentioned above CMGC group is composed of CDKs, MAPKs, GSK3, and CLKS. In eukaryotes all these proteins are known to be involved in the control of cell proliferation and development.

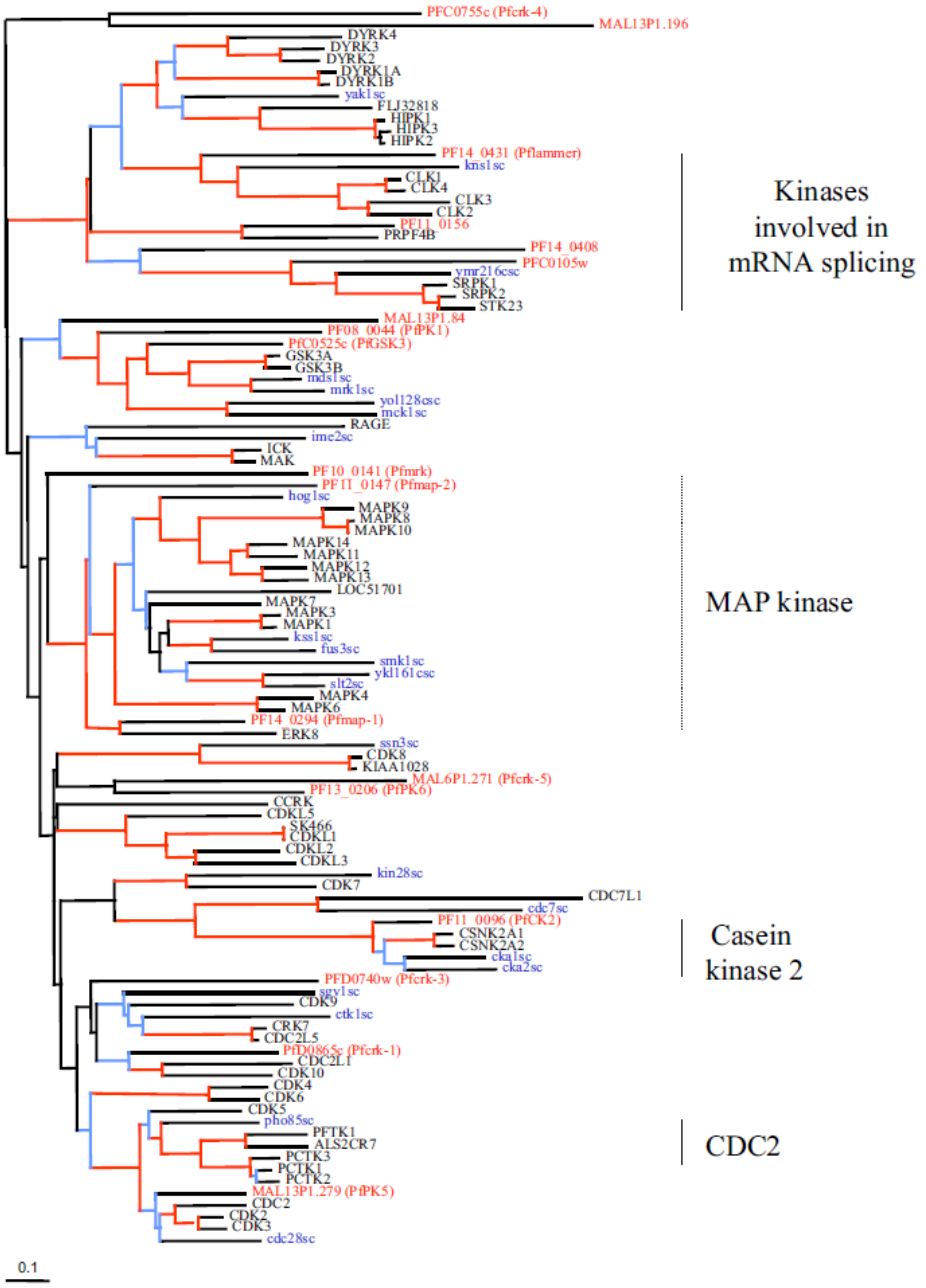


Figure 3 tree-species of CMGC group

Globally CDKs are regulators of cell-cycle progression; MAPKs are crucial transducers of extracellular and intracellular signals. Proteins in the GSK3 family are involved in the regulation of cell proliferation and CLKs have functions in RNA metabolism. In *P.falciparum* the kinome it is the most important group in terms of number of proteins which could be explained by the number of proliferation stages undergone by the parasite during its life cycle[12]. Due to there, expected functions in the cell proliferation of these kinases are being extensively studied. Some studies have already shown that different proteins belonging to this group are essential for the completion of the parasite life cycle. It is actually the case of my proteins of interest *P.falciparum* CK2β subunits and GSK3 [4].

1.4 PfCK2:

Human casein kinase 2 (CK2) is a serine/threonine kinase belonging to the CMGC cluster. CK2 is a holoenzyme composed of catalytic subunit α and regulatory subunits β. The holoenzyme forms a tetrameric structure composed of two α subunits, respectively CK2α and CK2α', forming the active site and a dimer of regulatory subunits CK2β that are needed for the complex formation [14]. CK2 is present in all the eukaryotic cells in a constitutively active manner, despite the fact that the protein is present at a low level, CK2 has a role in various cellular processes including neoplasia, differentiation, cell survival, apoptosis and proliferation. It also has a suspected role in virus infection as well as in different cancers. The kinase has up to 307 substrates. A majority of them has a function in gene expression and syntheses of proteins and are transcriptional factors, translational elements or active elements of DNA/RNA structure [14-16]. Regulation of CK2 is still poorly understood but probably involves interactions with some regulatory proteins [17].

P.falciparum casein kinase 2, the CK2 orthologue exhibits some differences at the structural level. Homology study reveals that instead of having two catalytic subunits, the P.falciparum genome has only one α (PfCK2α) subunit but exhibits two regulatory subunits (PfCK2β1 and PfCK2β2). Nevertheless it has been shown that the protein still possesses a kinase activity and that the three subunits are able to interact at least *in vitro*.

Amino acid sequences analysis reveals that PfCK2α have 65% of its sequence identical to CK2's sequences including the 11 sub domains conserved in all ePKs and the majority of sequences that are conserved in CK2. Alignment between human and *Plasmodium* β subunit also reveals that some features present in CK2β are also present in both PfCK2βs. Sequences present in PfCK2β1 and PfCK2β2 are cystein residues required for zinc-finger formation, a stretch of amino acids needed for the export of CK2 as a hectokinase, a putative signal for nuclear localisation and sequences suspected to be involved in CK2 downregulation and association with the plasma membrane. Furthermore both PfCK2β have a lot of sites that can be phosphorylated in their C-terminal region. Finally, the N-terminal part of PfCK2β2 contains a region with phosphorylable sites and on and an extension predicted to be involved in interactions with partners and substrates proteins.

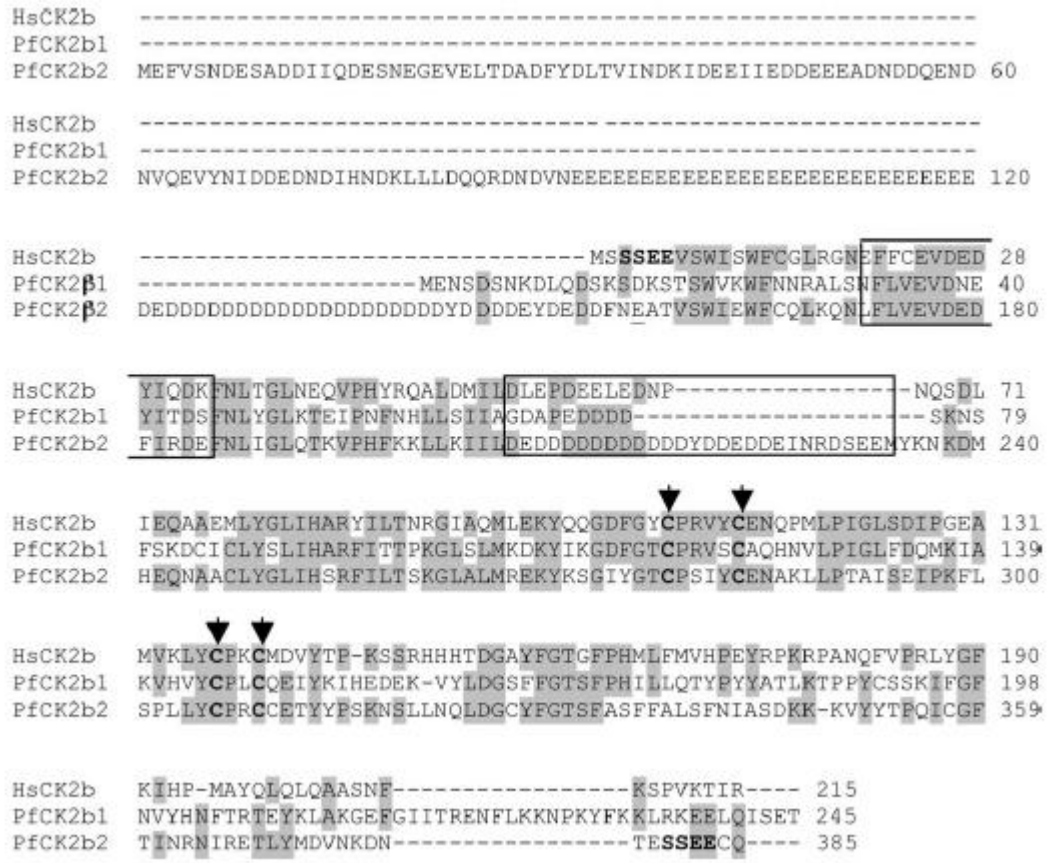


Figure 4 Alignment of both PfCK2 beta and HsCK2 beta. The cysteine residues thought to hold the zinc in place are shown in hold text and with arrowheads. CK2β residues 20 to 33 are responsible for the export of the protein. [18]

Reverse genetics has been used to analyze functional activity of the PfCK2 subunits. Disruption of PfCK2α by single crossover reveals that the 'α' subunit is needed for the completion of the entire asexual life cycle of *P.falciparum* [18]. The same work has been done on both β subunits and leads to the same conclusion (Holland Zoe thesis: *Plasmodium falciparum* Casein Kinase CK2).

1.5 PfGSK3:

The protein of interest is glycogen synthase kinase-3 belonging to the same cluster as CK2. GSK3 is also a serine/threonine kinase expressed ubiquitously, and constitutively active. This enzyme is well known for having a large range of partners in several pathways including metabolism, cell cycle, gene expression, development, inflammation and neuroprotection. It is also involved in diseases like cancers and diabetes [19, 20]. To exercise all these effects, GSK3 interacts with several partners and substrates as, for example, Wnt, Hedgehog, cytokines, growth factors and ligands for G proteins [21]. There are two different genes coding for two isoforms of the enzyme in human called GSK3α and GSK3β. These enzymes have a high level of homology and similar substrates and functions but are not present in a redundant manner in all cells [19]. Regulation of GSK3 is mediated by phosphorylation (reducing its activity), localisation and interactions with other proteins. These mechanisms of regulation imply that GSK3 not only has a lot of substrates but is also a substrate of several other proteins [19, 22].

Genome sequencing of the parasite reveals the presence of a homologue of GSK3, the *P.falciparum* glycogen synthase kinase 3(PfGSK3). Bioinformatics analysis demonstrates that PfGSK3 also possesses the typical 11 domains shared by all kinases. Furthermore PfGSK3 has 62.4%/ 58.9% similarities with GSK3α /GSK3β respectively and finally their central catalytic domain shares more than 70% amino acids.

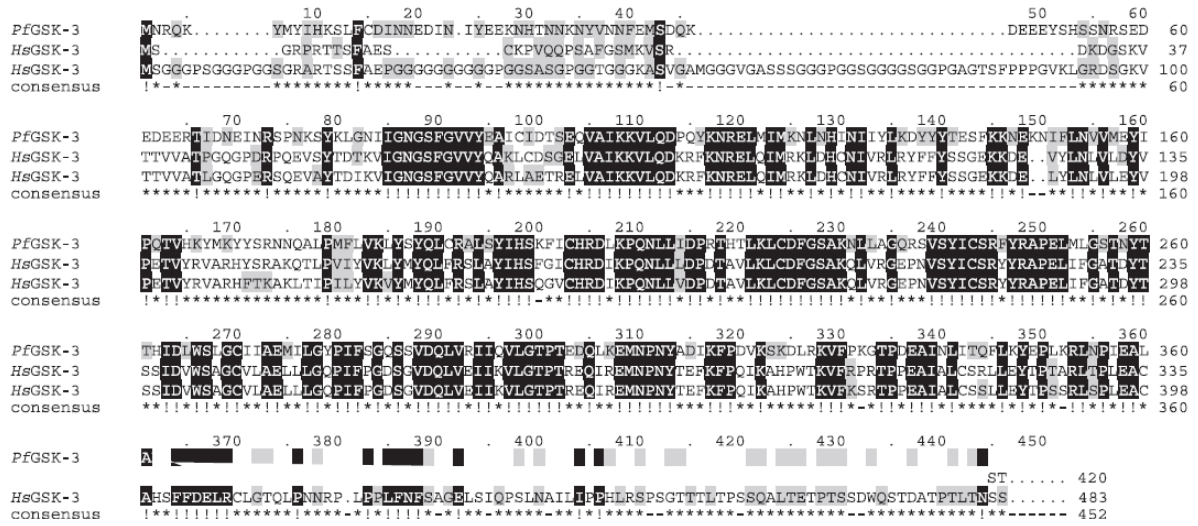


Figure 5 Alignment of PfGSK3 with the two human isoforms of GSK3. [23]

Several other residues are conserved including the ones that are involved in substrate binding, regulation of activity and a region in the N-terminal domain thought to be involved in the excretion of the protein. Investigation of the expression of the protein kinase has been done by using RT-PCR to analyze the level of expression of its mRNA during the life cycle and shows that the protein is expressed throughout all the asexual life cycle but the protein itself is mainly expressed during the early trophozoite stage. Furthermore analysis of the intracellular localisation of the protein suggests that PfGSK3 colocalises with the Maurer's cleft and this colocalisation increases during the passage from rings to trophozoites and schizonts. Finally the use of inhibitors against PfGSK3 also blocks the proliferation of *P.falciparum* at least in vitro [23].

1.6 Reverse genetics study of PfCK2β:

One part of our project is to complete the work of Zoe Holland on PfCK2β. What we are presenting in this discussion is directly taken from her thesis: "*Plasmodium falciparum* Protein Kinase CK2". We give an overview of her results since most of the materials we have used for our project are coming from her work. Their details will be discussed further in the chapter on materials and methods.

Zoe Holland first demonstrated that PfCK2βs locus is recombinogenic. To achieve this goal she transfected parasites with a plasmid pCAM-BSD-HA-PfCK2β1 or pCAM-BSD-HA-PfCK2β2. That plasmid gives the resistance to blasticidin after internalisation and, in case of recombination, adds a haemaglutinin tag at the 3'end of the gene. Parasites were put in culture with blasticidin. Zoe then showed that the locus is recombinogenic by demonstrating that integration arose, using PCR and Southern blot analysis.

Secondly she tried to knockout the PfCK2β genes by transfecting parasites with respectively pCAM-BSD-KOPfCK2β1 and pCAM-BSD-KOPfCK2β2. Integration was then monitored by PCR and Southern blot analysis. Integration did not appear which in turn showed that these genes were essential. Nevertheless to ascertain that the locus can only be disrupted in the presence of a complementation plasmid, Zoe did a complementation experiment by co-transfected parasites with the K.O. plasmid and another plasmid providing with the wild type gene as an episome. Experiment was done in duplicate for both PfCK2βs subunits and integration arose only for one of each co-transfection.

1.7 Project introduction:

CK2 and GSK3 have been extensively studied. We know now that those proteins are involved in several pathways and act with various partners and substrates. Interesting studies on PfCK2 and PfGSK3 have shown that these proteins share homologies with their human counterparts including region involved in interaction, phosphorylation and localisation in the cell. Studies have also revealed that these proteins are essential for the completion of the parasite life cycle. Based on this, we have made the hypothesis that PfCK2 and PfGSK3 are involved in various metabolic pathways of *P.falciparum*. The main part of our project is to use biotechnology tools to get an inside in the interactome of PfCK2β subunits and PfGSK3 in order to get a better understanding of their functions in the parasites. The aim of the project is therefore to use the tagged PfCK2β subunits and PfGSK3 to co-immunoprecipitate interactors and use mass spectrometry to identify them. Another part of our project is to complete the work of Zoe Holland as discussed above. For this side-project parasites will be kept in cultures during few months and integration will be monitored using PCR and Southern blot.

2. Materials and methods

2.1 Biological and chemical reagents

2.1.1 Solutions and buffers

2.1.1.1. Electrophoresis

6X DNA loading buffer: 0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 30% glycerol

4X Laemmli buffer: 40% glycerol, 2% SDS, 20% BME, 250 mM Tris-HCl pH 6.9

1xTAE: Diluted from 10x stock (Invitrogen) in ddH_2O to give 40mM Tris-Acetate, 1mM EDTA

1xTG-SDS: Diluted from 10x stock (BioRad) in ddH_2O to give 25mM Tris, 192mM Glycine, 0.1% SDS, pH 8.3

Coomassie stain: 5 g/L Coomassie Brilliant Blue stain (BioRad), 50% ethanol, 10% acetic acid

Coomassie destain: 45% Methanol, 10% Acetic Acid

2.1.1.2. Western blotting

Running gel: 1.83 mL ddH_2O , 2.33mL 30% acrylamide , 2.73mL Tris pH 8.7 1M, 35 µl SDS 20%, 70 µL ammonium persulfate 10%, 2.8 µL TEMED (for 2 gels).

Stacking gel: 1.41 mL ddH_2O, 330µL 30% acrylamide, 250µL Tris pH 6.9 1M, 10 µl SDS 20%, 20µL ammonium persulfate 10%, 2 µL TEMED (for 2 gels).

PBS: 8g/L NaCl, 40mg/L KCl, 1.15g/L Na_2HPO_4, 328mg/L KH_2PO_4, 0.1g/L $CaCl_2$, 0.1g/L $MgCl_2$, pH7.2, in ddH_2O

Transfer buffer :14.4g/l glycine, 20% methanol and 25mM Tris-HCl pH 8.3

Wash Buffer (PBS-T): PBS with 0.05-0.3% Tween-20 (Sigma)

Blocking buffer: PBS tween 0.1% and Blocking grade Milk 5%

2.1.1.3. Southern blotting

20xSSC buffer :175,3 g NaCl, 88,2g sodium citrate in 800mL ddH^2O, adjusted to pH 7 and complete to 1000mL

Depurination solution: 0.25M NaCl

Denaturation solution: 1.5M NaCl, 0.5M NaOH

Hybridization buffer :NaCl 0.5 M, 4% blocking reagent Amersham

Primary wash buffer :60g urea, 25mL SDS20%, 50mL sodium phospahate (0,5M final) pH 7.0, 4.35 g NaCl, 0.5 mL $MgCl_2$ 1M, 1g blocking reagent, complete to 500mL with ddH_2O

Secondary wash buffer 20x stock: 1M Tris pH base, 2M NaCl adjust to pH 10; dilute 1:20 and add 2mM $MgCl_2$

2.1.1.4. Plasmodium culture and protein extract preparation

Complete RPMI: RPMI 1640 medium completed with 25mM HEPES, 25mM sodium bicarbonate, 2mM L-glutamine, 300 mM hypoxanthine, 10 mg/L gentamycin and 5g/L albumax II, pH 7.2, filter sterilized and kept at +4°C

Phenol solution :Phenol:chloroform:isoanylalcohol(25:24:1) in 10mM Tris pH 8 and 1mM EDTA

Saponin solution: 1.5 g L^{-1} saponin in PBS

Sorbitol solution: 5% sorbitol (w/v) in ddH_2O

Giemsa staining solution: 10% Giemsa, 0.3% Na_2HPO_4 (w/v), 0.06% KH_2PO_4 (w/v) in ddH_2O

Freezing solution: 0.583 g L^{-1} sorbitol, 0.125g L^{-1} NaCl, 28% glycerol

Defrosting solutions: A 12% NaCl (w/v); B 1.6% NaCl (w/v); C 0.2% Dextrose (w/v), 0.9% NaCl (w/v)

RIPA buffer: 30 mM Tris pH8, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 1% NP-40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM PMSF, 10 mM Benzamidine, 1x Roche Complete protease inhibitors

2.1.2 Oligonucleotide primers

The oligonucleotide primers used in this study (custom synthesized by Invitrogen) are listed below. Oligonucleotide primers were designed for site-directed mutagenesis, colony screening PCR, and the amplification of genes for recombinant protein expression and reverse genetics studies.

Table 1: Oligonucleotide primers used in this study. Restriction endonuclease recognition sites are underlined.

CK2b1ForEco	GGGGGAATTCATGGAAAATAGTGATTCGAATAAAGAC
CK2b2RSpe	GGGGACTAGTTCATTGACACTCTTCAGAGGATTCCG
CK2b13primeRev	GCATTAAAATATGAGATGTACAC
CK2b23primeRev	CAAACTATGTCAACTGTTTTGGG
CK2b15primeF	CTTAAGTGTTAATCGG
CK2b25primeF	GGCATAGGAATATTTAAC
pCAMBSDFor	TATTCCTAATCATGTAAATCTTAAA
pCAMBSDRev	CAATTAACCCTCACTAAAG

2.1.3 Antibodies

Primary and secondary antibodies and their dilutions for western blotting are shown in Tables 2 to 4. Antibodies used in this study were bought from commercial suppliers, or commissioned from a custom antibody supplier (BioGenes) and were diluted in blocking buffer

Table 2 Custom primary antibodies from BioGenes (raised in rabbit)

Antibody	Peptide against which antibody was raised	Antibody stock concentration	Dilution for western blotting
Anti-PfCK2b1	DSNKDLQDSKSDKS-amide	0.27 mg/ml	0.38888889
Anti-PfCK2b2	DEINRDSEEMYKNK-amide	0.648 mg/ml	1:750-1:1000
Anti PfGSK3	CDNEINRSPNKSYKL ()	0,98 mg/ml	1/1000

Table 3 Commercial primary antibodies

Antibody (animal species raised in)	Dilution for western blotting	Source
Anti-HA (mouse)	1:1000	Roche

Table 4 Secondary antibodies

Antibody (animal species raised in)	Dilution for western blotting	Source
Anti-rabbit IgG (whole molecule)-Peroxidase (Goat)	1:10,000	Sigma
Anti-chicken IgG (whole molecule)-Peroxidase (Sheep)	1:10,000	Unknown

2.1.4 Parasites lines

During this project, eight different parasites lines were cultured. The wild type strain, 3D7A, and seven others genetically modified strains from the 3D7A strain. These generated lines were :

KOCK2b1 and KOCK2b2 : wild type lines transfected with a plasmid inducing the knockout of the genes coding for PfCK2β1 and PfCK2β2. Further more the plasmid used give also a resistance to blasticidine

KOCK2b1 + comp 2 and KOCK2b2 + comp 1 : these lines have been transfected with two plasmids. The first was the same as before and the second plasmid provided a wild type copy of the gene. These lines have resistance against blasticidin and WR.

PfCK2β1HA, PfCK2β2HA and PfGSK3HA: these lines have integrated a plasmid adding a haemaglutin tag at the 3' end of the gene and provide resistance against blasticidin

Details for the plasmids construction and compositions as well as generation of the lines can be found in Zoe Holland thesis : *Plasmodium Falciparum* casein kinase 2 CK2.

2.2 *Plasmodium Falciparum* cell culture techniques

2.2.1 Malaria parasite culture

Parasites were grown at a 5% haematocrit in complete RPMI medium and at a parasitaemia (PT : number of infected red blood cells / total number of red blood cells) between 0.5% to 10% either in

$25cm^2$ (5ml stocks) or $75cm^2$ (25ml preparative cultures) ventilated flasks. The flasks were kept in a 37^oC incubator with a 5% CO_2 atmosphere. The medium was changed daily and the PT was controlled every two days by Giemsa-stained blood smears. Fresh blood (erythrocytes concentrate washed and kept at 4°C at a concentration of 50% in complete medium) was used to keep the pararasitemia in the right range.

2.2.2 Freezing down cultures

Cultures were centrifuged for five minutes at 2000g at room temperature and the supernatant was removed. One volume of deep-freeze solution was added drop-wise to the pellet. The mix was transferred in cryotubes and immediately frozen in liquid nitrogen.

2.2.3 Defrost parasites cultures

A frozen cryotube containing parasitized red blood cells was thawed at room temperature and transferred into a 15 mL falcon tube. For each mL of blood defrosted, 0.2mL of solution A was added drop-wise, stirred constantly and left at room temperature for 3 minutes. Then for each mL of original culture volume, 10mL of solution B was added drop-wise and the tube was centrifuged at 2000 g for 5 minutes. After removal of the supernatant, solution C was added with an equivalent volume and in the same way as solution B, the tube was centrifuged at 2000 g for 5 minutes and the supernatant discarded. Finally, the pellet was resuspended in complete prewarmed RPMI medium, transferred in a small flask and placed in the incubator for daily culture

2.2.4 Saponin lysis

Saponin lysis was performed to accumulate parasites pellets.

Culture was transferred into a 50 ml Falcon tube and centrifuged for 2 minutes at 2000g at 4°C. The supernatant was discarded and the pellet washed once with 30 ml of 1X of cold PBS. 10mL of saponin (0.15%) was added and solution was resuspended for 10 minutes. When lysis was complete, 45 mL of 1X of cold PBS was added and the tube was centrifuged at 13000g for 5minutes at 4°C.

In the case of proteins preparation, an extra saponin lysis was performed with 2mL of saponin. After the lysis, the tube was centrifuged as described above and the supernatant removed.

Then, the pellet was washed 3 times with 10mL of 1X of cold PBS and finally washed an extra time in 1mL of 1X of cold PBS and transferred into a 1.5ml eppendorf. Once the supernatant was removed, the eppendorf was transferred into a -80°C freezer.

2.2.5 Phenol extraction of DNA

The volume of parasite pellet was resuspended in 100 µl of PBS.1% of the pellet volume of proteinase K and 11% of the pellet volume of SDS20% were added to the pellet and incubated over night at 55°C. DNA was extracted using phenol solution,. 400µl of phenol solution was added in the tube. Pelltes with the phenol were vortexed and centrifuged 5 minutes at 16000g. Then, the supernatant was transferred to a clean Eppendorf. This step was repeated three times. Finally, 2 volumes of ethanol 100% and 1% of the volume of sodium acetate 3M pH 5 were added. The tube was incubated overnight at -20°C.

After 15 minutes of centrifugation at 16000g and removal of the supernatant, the pellet was dried for 5 minutes at room temperature. Subsequently, DNA was resuspended in 50 µl of ddH_2O and DNA concentration was measured using a nanodrop.

2.2.6 DNA extraction for PCR

500 µl of cultures with a parasitemia of at least 4% was centrifuged 3 minutes at 2000g and the supernatant was removed. The pellet was resuspended in 1ml of ddH20 and left for 15 minutes at room temperature. Then, the tube was centrifuged for 3 minutes at 16000g and the supernatant was removed. 200µl of pre-agitated InstaGene Matrix (BioRad) was added, the tube was kept 30 minutes at 56°C, vortexed and kept 8 minutes at 100°C. Finally, the tube was vortexed, centrifuged, the supernatant was transferred into an eppendorf and stored at -20°C.

2.3. Molecular biology methods

2.3.1 Polymerase chain reaction

For DNA amplification we used Phusion polymerase (Finnzyme) for all the analysis. Reactions were set up to contain 1X of Phusion HF reaction buffer (supplied with the polymerase), 1.6µM of primers and 0.2mM of dNTPs. Based on the kit instructions, 0.2 µl of polymerase (2U/µl/250µl), DNA (generally 2µl) and ddH_2O qsp 25µl were added. The PCR cycle was performed under the following conditions:

Initial denaturation: 94°C, 10 minutes
35 cycles:
Denaturation 94°C, 45 seconds
Annealing 50°C, 45 seconds
Elongation 68°C, 50 seconds
Followed by the final elongation 68°C, 10 minutes

2.3.2 Agarose gel electrophoresis

Standard agarose gels were prepared in 1X TAE with 1% agarose and 0.5µg/mL ethidium bromide. For agarose gel electrophoresis, DNA samples were mixed with 6x loading buffer before loading.. The bands were finally visualized either under UV illumination or transillumination linked to a computer.

2.3.3 Restriction endonuclease digestion

Restriction endonuclease digestion was used to fragment DNA before southern blot analysis. 10 µg of DNA was digested in New England Biolabs advised buffer, 1x BSA and 1µl of each enzyme in a final volume of 30µl completed with ddH_2O. The buffer and temperature were chosen based on the neb double digestion finder site (www.neb.com/nebecomm/DoubleDigestCalculator.asp). Digestion took place at 37°C over-night and tubes were pulse-centrifuged and stored at -20°C.

2.3.4 Southern blot analysis

2.3.4.1. Preparation, Migration of digested DNA on Agarose gel:

Southern blot was used to detect plasmid integration. Parasites DNA was extracted using phenol (see section 2.2.5) and DNA concentration and quality was checked using nanodrop and agarose gel electrophoresis (see section 2.3.2). Subsequently, DNA was digested (2.3.3) overnight. DNA samples were loaded on a 0.8%agarose gel (, and run first at 80V for 10 minutes, and then at 23V overnight.The gel was transferred in depurination solution for 35 minutes and washed in ddH_2O followed up by incubation in denaturation solution for 35 minutes.

2.3.4.2. Transfert onto a nylon membrane:

DNA was then transferred on a nylon membrane (Hybond-N+, GE Healthcare) in 20xSSC buffer by means of an overnight capillarity transfer (see figure x).

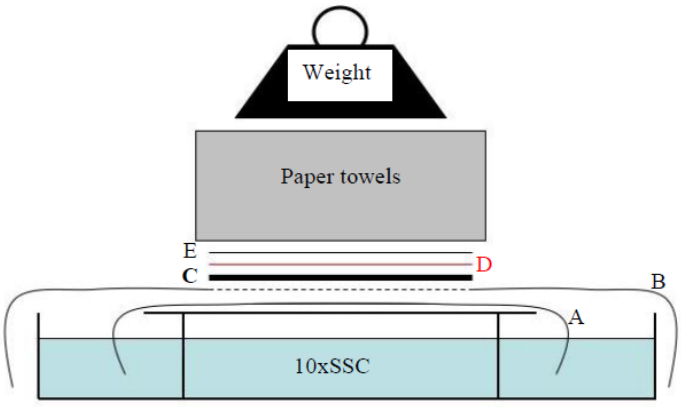


Figure 6 This figure shows how the wicking transfer apparatus was put together. A: Three pieces of Whatman paper, soaked in 20x SSC, and with the ends resting in the buffer reservoir. B: Saranwrap, with a hole the size of the gel cut in it. This will stop evaporation from the reservoir and ensure that the only route for the buffer to get to the absorbent paper is through the gel and membrane. C: The gel, laid to cover precisely the hole in the Saranwrap. D: The H+ Hybond nylon membrane, cut to exactly the same size as the gel. E: Three more pieces of Whatman paper, cut to the same size as the gel, and soaked in 20x SSC.

The membrane was then removed, washed in 100mM Tris pH8, dried in between two pieces of Whatman paper and DNA was crosslinked to the membrane using a UV crosslinker (. The nylon membrane was transferred into a hybridization oven with a prewarmed hybridization buffer, stayed at 55°C over night.

2.3.4.3. Probe preparation:

PCR was performed to amplify BSD fragments and the PCR product was purified using the Qiagen PCR purification product protocol and kit. DNA concentration was monitored using nanodrop. Then, the probe was prepared using an Amersham kit, and diluted from 150 and 100ng in 8µl of H_2O (provided by the kit). The probe was kept 5 minutes at 100°C and directly transferred on ice for 5 minutes. Subsequently 10µl of reaction buffer, 2µl of labelling reagent and 10µl of cross linker worker solution (all provided by the kit) were added. Probes were kept at 37°C during 30 minutes and finally stored at -20°C.

To probe the DNA, the BSD probe was added to the hybridization buffer and left overnight in the oven at 55°C. The solution was removed from the tube and primary wash buffer was added for 10 minutes at 55°C two times. The wash buffer was removed and secondary wash buffer was added for 5 minutes at room temperature two times. 1.5mL of detection reagent (CPN stars) was spread on the membrane and autoradiography was used for revelation.

The membrane was then stripped,60mL of boiling 0.1% SDS was directly added and membrane was incubated 1 hour at 55°C. The membrane was rinsed for 5 to 10 minutes in Tris pH 8.0 100mM and dried between two pieces of Whatman paper and kept dry at room temperature. The re-probing using another probe was then realised as described above.

2.4 Biochemistry methods

2.4.1 Co-immunoprecipitation using HA tag

To avoid degradation of the proteins, all the manipulations were done on ice. Parasites pellet was resuspended in 400µL of ice-cold M-Per mammalian protein extraction buffer (Thermo scientific), Roche protease inhibitors cocktail tablets 1:25 and benzonase 1:500 (Novogen). Samples were kept on ice for 10 minutes, centrifuged for 15 minutes at 11000g at 4°C and supernatant was transferred

into a clean eppendorf. The concentration was measured by spectrometry against a BSA range. The concentration was adjusted for each condition to have the same concentration in all samples.

100 µg of proteins extract (PE) was kept to check the presence of the tagged proteins by western blot.Laemmli 2X was added the PE tubes and the samples were boiled 5 minutes, centrifuged 3 minutes at 16000g and stored at -80°C.

To perform the IP, 6µl of beads linked to anti-Has were transferred in the column (both provided in the kit). Beads were then pulse-centrifugated for 10 s at 500g at 4°C and the flow through discarded. PE was loaded in the column and incubated at 4°C overnight with agitation. Then, a first pulse centrifugation was done and the flow through (FT) was resuspended in Laemmli, boiled, centrifuged and stored as described above. Subsequently, 3 washes were done on the beads using 500µl of TBS buffer and hot Laemmli 4X was added to the column. The column was transferred in an eppendorf, incubated at 100°C for 5 minutes and centrifuged 3 minutes at 16000g. Fifty percent were resuspended in Laemmli 4X to be used as a control for the IP (AfIP) and stored at -80°C. the eighty five other percent were used for mass spectrometry analysis after migration on a 10% polyacrylamide gel.

2.4.2 Western blot

2.4.2.1. Migration:

10% polyacrylamide gels were used to separate proteins. Proteins samples as prepared in 2.4.1 and Pageruler prestained proteins (Fermentas) were loaded in the wells. Migration in the stacking gel was set at 80V for 20 minutes and 180 V for 1 hour in the running gel. One gel was used for Coomassie staining and the other for western-blot.

2.4.2.2. Coomassie staining:

Gels were stained in fresh Coomassie stain for 15min at room temperature with gentle agitation. Then gels were destained in Destain solution for 1h with gentle agitation and dried

2.4.2.3. Gel transfer:

Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (25V, 200mA for 1 hour). The nitrocellulose membrane was blocked overnight at 4°C in blocking buffer and incubated with primary antibody or anti-HA peroxidase for 2 hours at room temperature with agitation. The membrane was washed (3 times for 10min) with cold PBS-T (0.1%) and incubated for 1hour at room temperature with the secondary antibody (not required for anti-HA revelation because the antibody was already coupled to perxidase). The membrane was finally washed three times and signal developed using the chemiluminescence system (ECL, Perkin-Elmer).

2.5 Mass spectrometry analysis

2.5.1 Preparation of gel for mass spectrometry analysis

After immunoprecipation (see section 2.4.3) samples were boiled 5 minutes at 100°C and centrifuged 3 minutes at 16000 g. Then samples were loaded on a 10% polyacrylamide gel for protein separation (see section 2.4.1), migration was done first at 80V for 3 minutes then at 180V until first and last bands were separated by 2.5 to 3cm. The gel was then washed 3 times for 5 minutes with ddH_2O. 50 mL of biosafe Coomassie was added on the gel and tank with the gel and coomassie incubated for 1

hour. Then gel was washed again with excess of ddH_2O for 30 minutes. Finally the gel was stored in 10% ethanol and 1% acetic acid at 4°C and sent to the proteomics platform for further analysis.

2.5.2 Mass spectrometry analysis

Mass spectrometry analysis was performed by Romain Hamelin from the proteomics core facility at EPFL. In summary the gel was scan and spot corresponding to immunoreactive region were excised from the coomassie staining gel. The tryptic peptides were separated after in –gel digestion by nanoflow rpHPLC and analysed on a mass spectrometer. Data search was performed using Mascot 2.2 in Proteome Discoverer against a database of proteins. Finally the result was given via the Scaffold3 protein software.

3. Interactome study of PfCK2β subunits and PfGSK3

3.1 Polyacrylamide gel preparation and result before MS analysis

3.1.1 Introduction

For this experiment we have used proteins having a Haemaglutin tag. This tag was used to immunoprecipitate the proteins using agarose beads coupled with anti-HA. As a controle we have used 3D7A parasite lines which did not expressed proteins with a HA tag.

In order to accumulate enough proteins for interactome studies several rounds of expansion and Saponin lysis were done on unsynchronised *P.falciparum* cultures to allow accumulation of parasites pellets. Before the IP we have adjusted the concentration of proteins in order to load the same amount in all samples. To ascertain that the IP worked, three samples were taken during the process: one before the IP on the protein extract (PE), one was the flow through of the IP (FT) and finally one sample was 15% of the eluted proteins (AfIP). These three samples were loaded on polyacrylamide gels for coomassie staining and western bloting. The coomassie was used to control that the same amount of proteins had been loaded (data not shown) for each samples. One western blot gel was used to control the presence of the kinase of interest using an anti-HA peroxidase antibody and the second one using an antibody specific for the kinase (not used in the case of PfCK2β2 because of the very low efficiency of the antibody). Due to the time scale of this project we have decided that if one control of IP work all the samples would be analysed. Mass spectrometry analyse was performed by Romain Hamelin who scanned the gel to confirm the presence of proteins and further processed the samples as described in materials amd methods part.. Finally the results was given back in a Scaffold3 protein software.

Since there are not many examples of such a study with *P. falciparum* proteins in the literature the quantity of proteins to be loaded was determined based on previous knowledge on mass spectrometry analysis with different organisms (Marc Moniatte and Romain Hamelin) and on parasite (Jean Halbert and Jean-Philippe Semblat).

3.1.2 Experiment 1

For the first experiment we added Nek1 (also has an HA tag) that is a protein studied in the lab by Dominique Dorin-Semblat. Thus in this experiment we used five different lines: PfCK2β1HA, PFCK2B2HA, PFGSK3HA, PfNEK1HA and finally 3D7. For the IP we loaded 1.718 mg of proteins in column with 6 ul of beads, for the control we loaded 20ug for the PE and FT per polyacrylamide gel and 5% of the eluted proteins after IP. Below we presented results for the control western blot revealed with kinase specific antibodies:

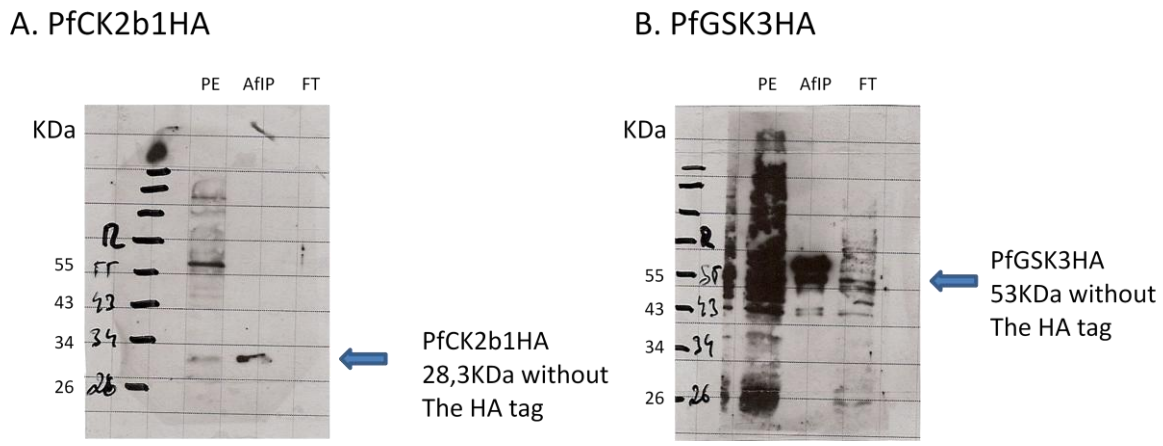


Figure 7: Control results for experiment 1: panel A for PfCK2β1 no bands were saw in the flow through whereas a band appears corresponding to the expected size for the PfCK2b1HA protein in the elution after immunoprecipitation. Panel B: the immunoprecipitation performed wellAfIP band) but there was still some proteins in the flow through.

In this experiment the western blot analysis with anti-HA peroxidase antibodies was not successful, this was also the case for pfnek1ha proteins but this can be explained by the fact that the C-terminal part of the protein is normally processed which leads to the loss of the HA tag (based on discussion with Dominique Dorin-Semblat) but since western blot worked with kinases specific antibodies for PfCK2β1 and pfgsk3 a polyacrylamide gel was prepared and sent for mass spectrometry analysis. As shown in the figure 2 six bands were analyzed for each sample.

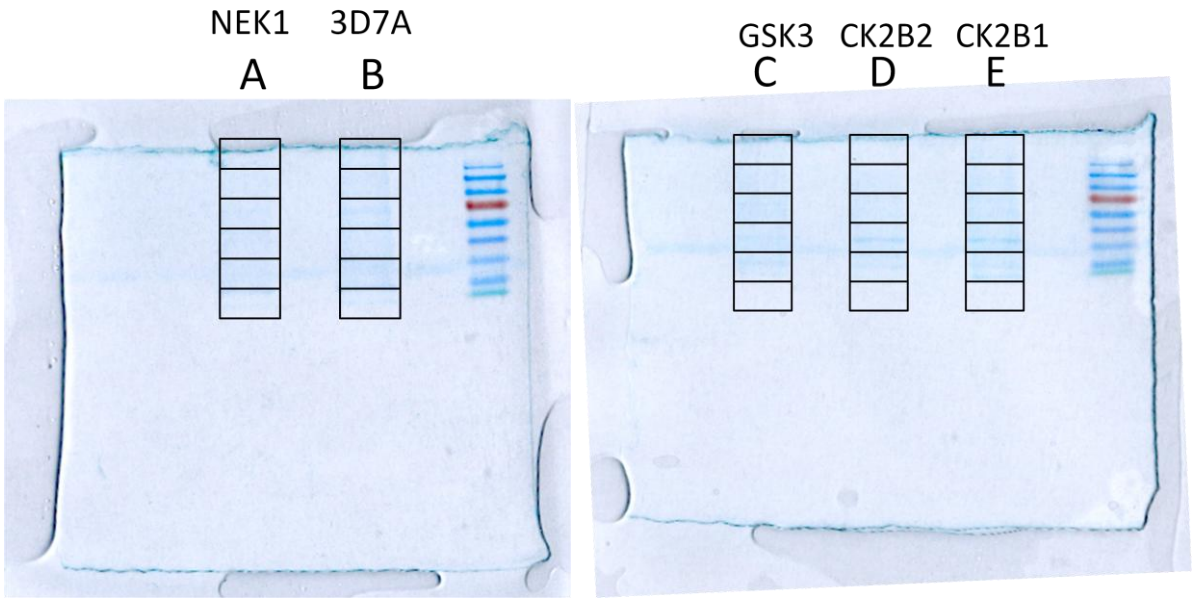


Figure 8 Scan of the gel experiment.1 showing cut bands for each samples of NEK1|3D7A|GSK3|CK2B2|CK2B1

3.1.3 Experiment 2

The same protocol was applied for the second experiment but this time 1.468 mg was loaded for the IP and we only analysed our protein of interest in this project (same proteins except pfnek1).

Unfortunately another difference with the first experiment was the partial loss of PfCK2β2 samples during the process causes by technical problem with the column used for immunoprecipitation.

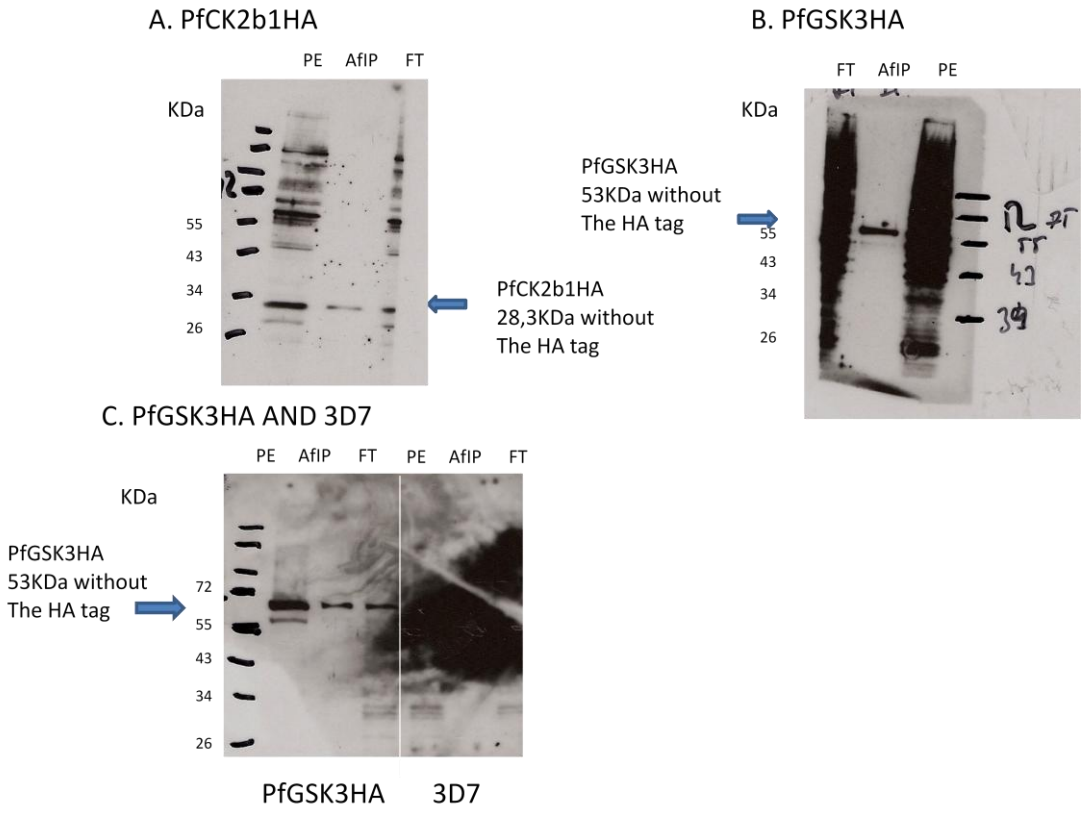


Figure 9 Control results for experiment 2. Panel A and B: western blot analysis with kinase specific antibodies shows the presence of proteins in the immunoprecipitate AfIP. Panel C: shows western blot analysis with the anti-HA peroxidase antibody for PfGSK3HA and also the presence of the proteins in the immunoprecipitate.

Once again the western blot with anti-HA peroxidase antibody did not work for both PfCK2b subunits but shows the presence of the proteins of interest in the three other samples.

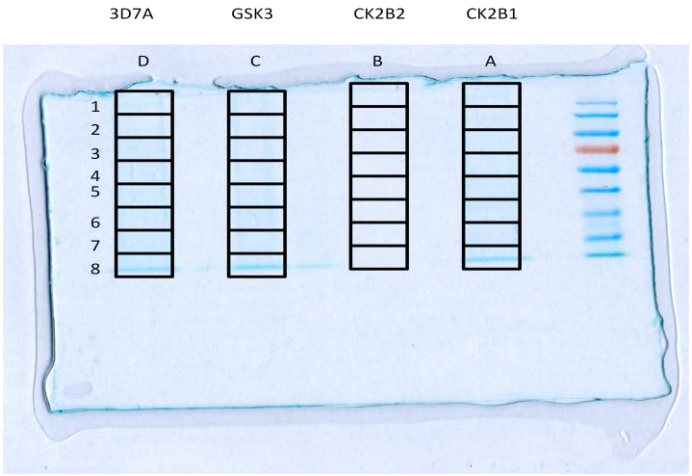


Figure 10 Scan of the gel experiment.2 before in-gel digestion showing the 8 bands cut and analysed. Lane B shows a clearly decreased intensity of coomassie staining.

As mentioned above the quantity of proteins used in the PfCK2β2 IP was smaller compared to the other samples. This was confirmed by looking the coomassie staining of the lane B compared to the

other. Nevertheless few proteins were found in this sample after mass spectrometry analysis. Finally note also that this time we made a longer migration in order to allow a better separation and analysis of the samples.

3.1.4 Experiment 3

For the last experiment we have used a smaller quantity of beads during immunoprecipitation (4ul instead of 6 ul) and the IP was done with 1.716mg of proteins per sample. In this experiment only one control worked. Nevertheless the sample was still sent for MS analysis.

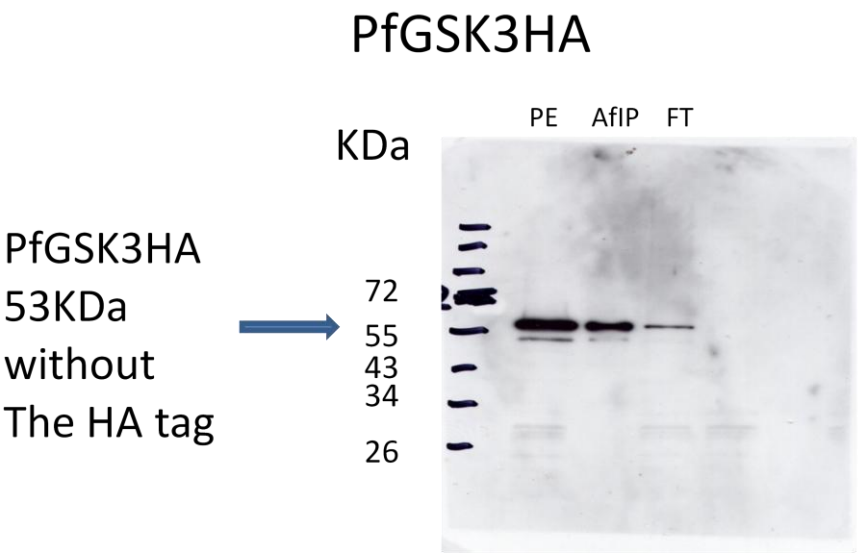


Figure 11 Western blot analysis revealed with anti-HA peroxidase antibody showing presence of PfGSK3HA in the immune precipitate.

The same way as in experiment 2 we allowed the samples to migrate longer resulting in more separated bands which have allowed cutting 8 bands.

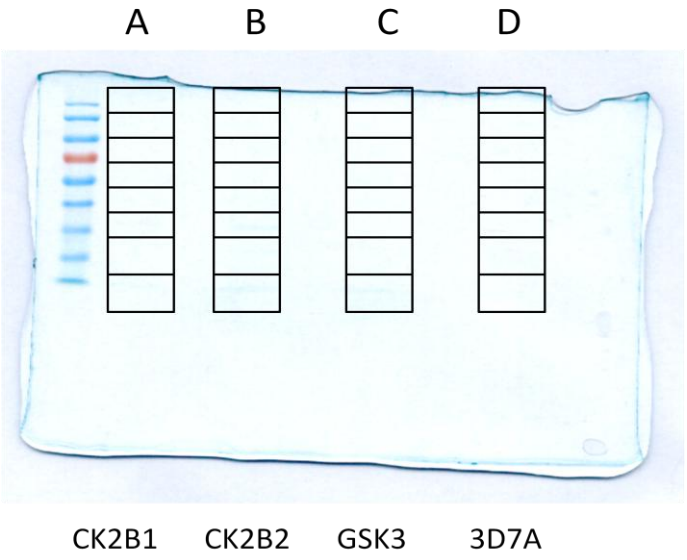


Figure 12 Scan and bands cut for the last gel experiment 3. Biosafe coomassie staining of this gel has been staying shorter than with the other gel which explains the lower intensity observed. As a result, the lower coloration was not due to a smaller quantity of proteins loaded.

3.2 Scaffold 3 results

3.2.1 Methods used to analyse the results

The Scaffold 3 software gives for each protein found in the sample a large range of information including : the number of unique peptides found, how many times a given peptide appeared, the mascot statistic scored for each peptide, the protein ambiguity (in case of high homology with other proteins) etc. In order to analyse data we have chosen three conditions under which we considered a given result as a potential hit:

1. If protein only coimmunoprecipitates with our proteins of interest and not in the 3D7 control
2. If at least 4 different peptides with a probability higher than 95% was found in the samples and no more than 1 peptide with a probability smaller than 95% was found in the control.
3. Finally if more than 10 unique peptides were found in the sample and no more than 1 in the control (all with a probability higher than 95%)

Almost all the values presented further will have a certain probability. Each probability is represented by colour labels and we give below the related table:

Probability Legend:
over 95 %
80 % to 94 %
50 % to 79 %
20 % to 49 %
0% to 19 %

3.2.2 Results for PfCK2β subunits

First to control the efficiency of the IP we investigated the presence of our proteins of interest in the three experiments. Furthermore since it was shown that all pfck2 subunits interact *in vitro* we also investigated the presence of the two other subunits in the immunoprecipitates:

Table1: Illustration of PfCK2α & PfCK2β2 co-immunoprecipitating with PfCK2β1

EXPERIMENT	PROTEIN	NUMBER OF UNIQUE PEPTIDES IN CK2B1 IMMUNOPRECIPITATE	NUMBER OF UNIQUE PEPTIDES IN THE CONTROL	TOTAL NUMBER OF PEPTIDES IN CK2β1 IMMUNOPRECIPITATE	TOTAL NUMBER OF PEPTIDES CK2β1/3D7A	%COVERAGE OF THE PROTEIN
1	PfCK2α	16	1	161	1	57
	PfCK2β1	9	1	31	1	59
	PfCK2β2	13	0	37	0	29
2	PfCK2α	15	5	44	5	21
	PfCK2β1	7	0	14	0	38
	PfCK2β2	9	0	35	0	25
3	PfCK2α	13	0	52	0	51
	PfCK2β1	3	0	9	0	13
	PfCK2β2	8	0	22	0	25

For PfCK2β1 the three subunits were found in each experiment which confirmed the expected result since it has been shown that all subunits interact *in vitro*. In each experiment the higher number of peptides found belong to the PfCK2α subunit, this may be due to higher number of trypsin digestions sites on the protein sequence and, or to the digestion bands. Overall the coverage of proteins was

quite good. The maximum coverage were found for PfCK2β1 in the first experiment with a 59% coverage with surprisingly less unique peptides compared to the other subunit in the same experiment, this can be explained by the fact that PfCK2β1 is a smaller protein (245 amino acids) compared to the other subunits (PfCK2α : 335 amino acids and PfCK2β2 : 385 amino acids). The coverage was also dependent of the peptides size resulting from the trypsin digestion but as before this has not been investigate.

Unfortunately 5 unique pfck2ba peptides were found in the control of the second experiment, but since the difference between the control and the immunoprecipate was quite high in terms of unique peptides (15/5) and of total number of peptides (44/5), we have considered PfCK2α as a hit in this experiment. For the same reason we have excluded the idea that those peptides were due to cross contamination because only 5 peptides were found compared to 161, 44 and 52 found in the immunoprecipitates.

For PfCK2β2 subunits results were quite similar. The three subunits were found in all the immunoprecipitates with a quite similar amount:

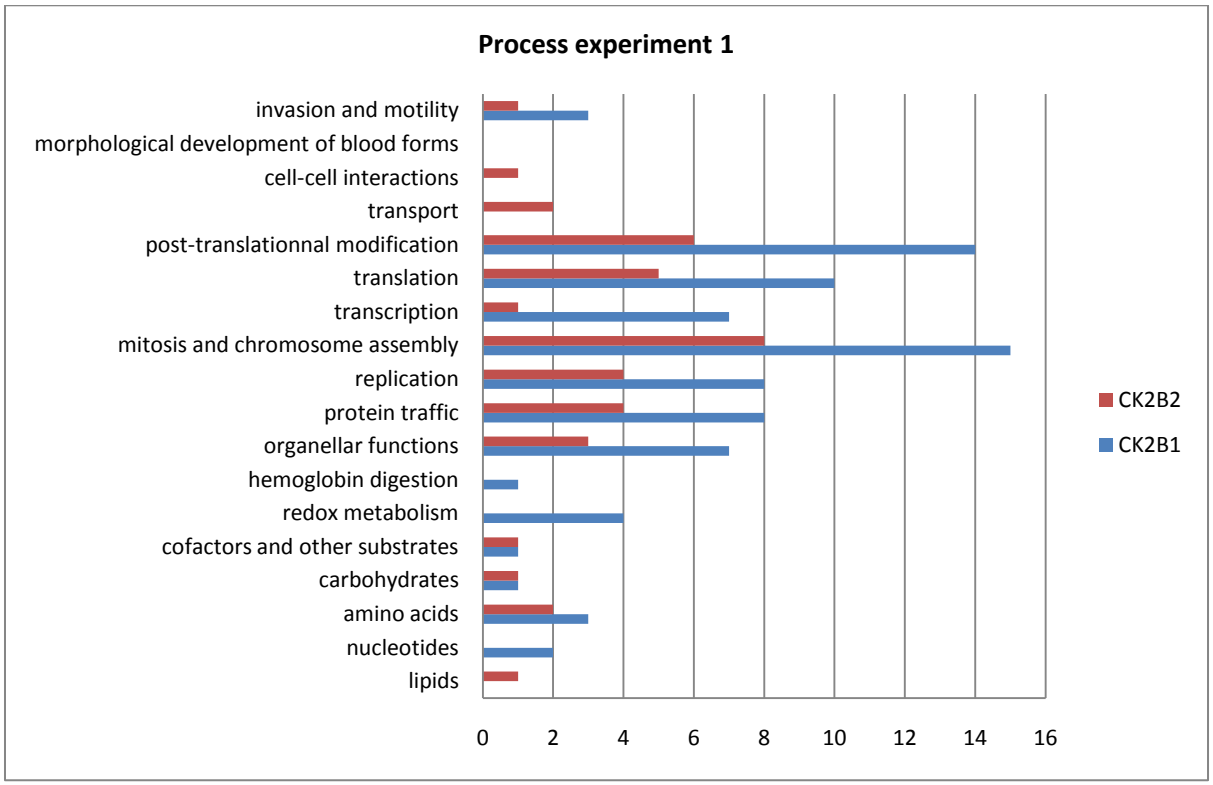
Table 2: Illustration of PfCK2α & PfCK2β1 co-immunoprecipitating with PfCK2β2

EXPERIMENT	PROTEIN	NUMBER OF UNIQUE PEPTIDES IN CK2B2 IMMUNOPRECIPITATE	NUMBER OF UNIQUE PEPTIDES IN THE CONTROL	TOTAL NUMBER OF PEPTIDES IN CK2β2 IMMUNOPRECIPITATE	TOTAL NUMBER OF PEPTIDES CK2β1/3D7A	%COVERAGE OF THE PROTEIN
1	PfCK2α	17	1	188	1	58
	PfCK2β1	11	1	24	1	25
	PfCK2β2	10	0	33	0	63
2	PfCK2α	17	5	59	5	21
	PfCK2β1	9	0	22	0	51
	PfCK2β2	9	0	40	0	26
3	PfCK2α	16	0	105	0	58
	PfCK2β1	7	0	26	0	33
	PfCK2β2	8	0	33	0	25

Interestingly, although a lower amount of proteins was loaded for PfCK2β2 experiment 2, we were quite surprised that the higher number of PfCK2β2 peptides were found in this experiment..

To investigate potential functions of both subunits in the parasite we used malaria parasite metabolic pathway site (http://sites.huji.ac.il/malaria/). After we selected all the potential interactors for both subunits, we searched those proteins on the web sites. Below we presented the number of hits found for each process. Note that we presented the number of different proteins found in each process but some proteins belong to various processes, consequently there were more hits than proteins. Note also that in order to avoid overloading this part the list of selected proteins were listen in the annexe1.

Histogram 1: distribution of potential interactors of PfCK2β1 & PfCK2β2 respectively in blue and red, sorted by metabolic process - experiment 1

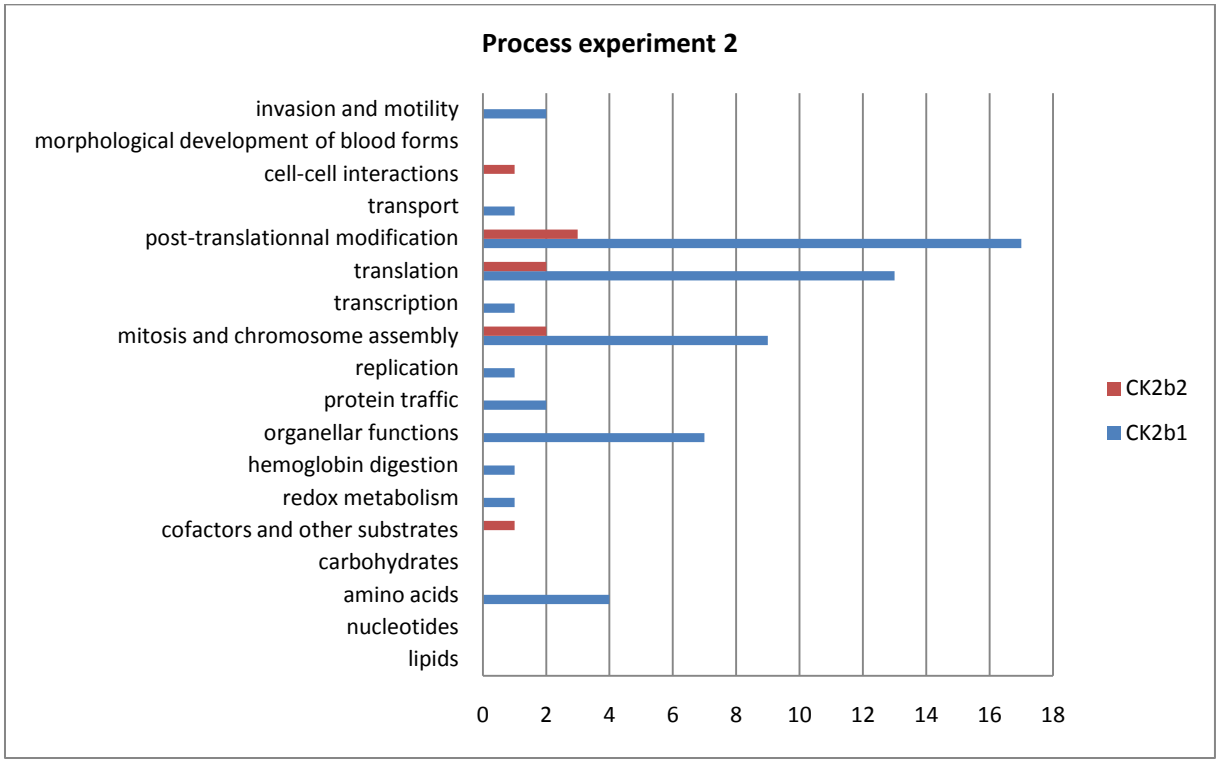


For the first experiment, we identified 83 potential hits for PfCK2β1 and 63 of them fit in process analysed. For PfCK2β2 32 potential interactors out of 41 fit in process described below. Based on first experiment results it seems there was metabolic process involving only one subunit (cell-cell interaction, hemoglobin digestion), there was a lot of processes involving both subunits (post-translationnal modification, translation, mitosis and chromosome assembly), and finally only one process where our proteins of interest were not involved (morphological development of blood forms). Those results were quite different in the second and third experiments (Histograms 2 and 3). In the second experiment only 42 potential interactors were found for PfCK2β1 and 37 distributed in the process. The difference between experience 1 and 2 was mainly due to differences between controls in the two experiments (see annex 2). A lot of proteins selected in the first experiment were found in the control of the second which explained these decreased numbers of hits. For PfCK2β2 only 6 potential hits were found, this very low number of potential interactors was also due to the divergence between controls but also mainly caused by the low amount of proteins loaded for this experiment. Finally for the third experiment we used fewer beads, which may explain that only 12 hits were found for PfCK2β1 (out of 13) and 21 for PfCK2β2 (out of 24). Nevertheless the same processes were highly represented in all the experiments (post –translationnal modification, mitosis and chromosome assembly and translation), there was no proteins belonging to the morphological development of blood form process., finally two pathways contained one of the two beta subunits.

Since the mass spectrometry results were given shortly before the end of the project it was not possible to treat all the processes in details. We decided to treat only the two most represented processes in terms of numbers of potential interactors. The most represented processes were post-translational modification and mitosis and chromosome assembly with respectively 58 and 48 hits. Then for each process we analysed the pathway containing more potential interactors. The first

process treated was mitosis and chromosome assembly. Between the eleven pathways fit in this process, the most represented was nucleosome assembly and regulation.

Histogram 2: distribution of potential interactors of PfCK2β1 & PfCK2β2 respectively in blue and red, sorted by metabolic process - experiment 2



Histogram 3: distribution of potential interactors of PfCK2β1 & PfCK2β2 respectively in blue and red, sorted by metabolic process - experiment 3

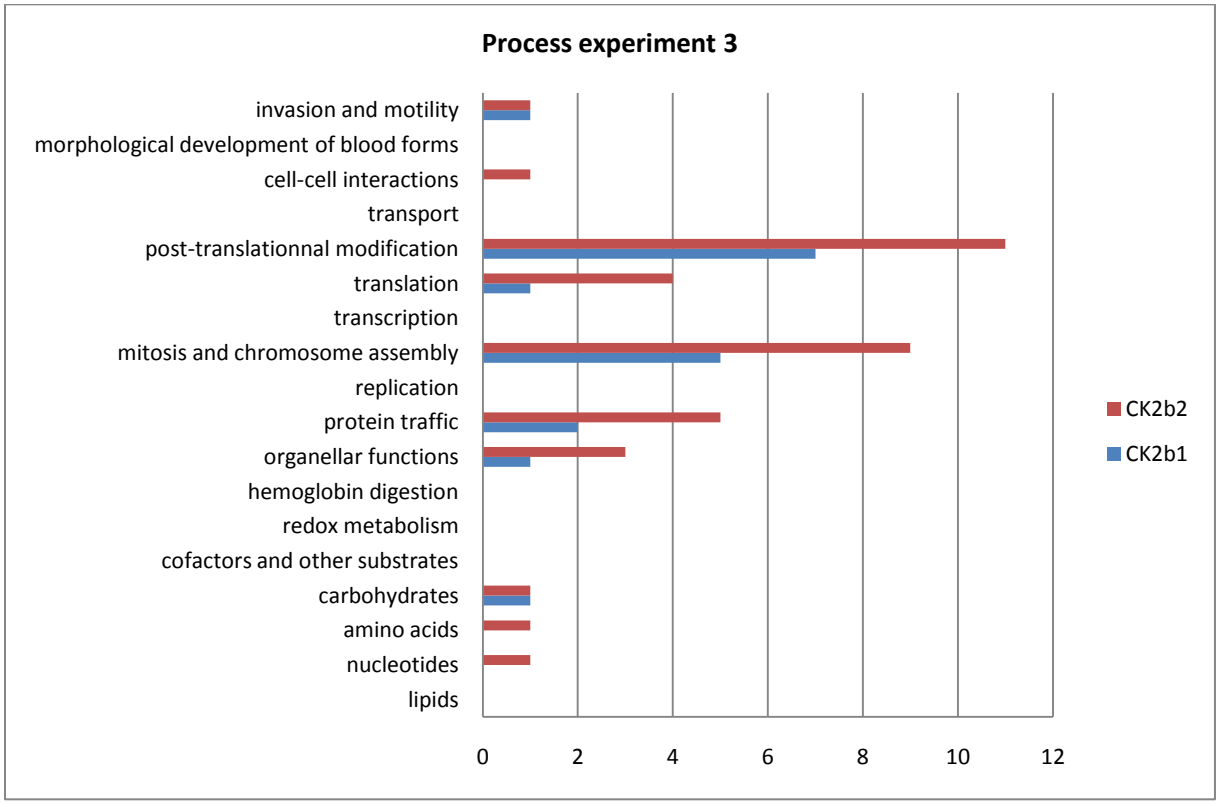


Table 4: Number of proteins observed for each mitosis and chromosome separation pathways sorted by kinases and experimentations and total number of such proteins

MITOSIS AND CHROMOSOME SEPARATION	CK2β1.1	CK2β2.1	CK2β1.2	CK2β2.2	CK2β1.3	CK2β2.3	TOTAL
CENTRIOLE PROTEINS	1	0	1	0	0	0	2
CENTROSOMES PROTEINS	4	3	7	2	3	5	24
CHROMOSOME DYNAMICS IN CELL CYCLE THAT LEADS TO ANAPHASE	0	0	0	0	0	0	0
HISTONE LYSINE METHYLATION	0	0	0	0	0	0	0
KINETOCHORES POWER CHROMOSOME MOVEMENTS IN MITOSIS	1	0	0	0	1	0	2
NUCLEOSOME ASSEMBLY AND REGULATION	10	4	0	0	1	4	19
PROTEINS INVOLVED IN STEPS DURING PASSAGE THROUGH PROPHASE	1	1	0	0	0	0	2
PUTATIVE ORGANIZATION OF THE KINETOCHORE	0	0	0	0	0	2	2
REGULATION OF SPINDLE MICROTUBULE DYNAMICS	1	0	1	0	1	0	3
STRUCTURE AND ORGANIZATION OF CENTROMERIC CHROMATIN	0	0	0	0	0	2	2
STRUCTURE OF THE MITOTIC CENTROSOME	1	0	1	0	0	0	2
THE MITOTIC SPINDLE OF P.FALCIPARUM	0	0	0	0	0	0	0

Interestingly we noticed that potential hits were not present in all the pathways. Unfortunately we also noticed that potential interactors were not nicely distributed in all experiments as it was the case for the three subunits. To assess if proteins found have a good probability to be interactors, we compared the three experiments simultaneously to control that the protein was not only absent in one control but in all controls. Table 5 and 6 give the results obtained for potential PfCK2β1 and PfCK2β2 interactors.

Table 5: Number of unique potential interactors peptides *involved in the nucleosome assembly and regulation pathway* in the case of three PfCK2β1 experimentations as compared to their presence in three control immunoprecipates experimentations. Colours still refer to probabilities (see 3.2.1)

NAME	PLASMO DB	3D7A1	3D7A2	3D7A3	CK2β1 1	CK2β1 2	CK2β1 3
histone H2a	PFF0860c	0	1	0	3	2	1
Smarca related protein	PFF1185w	0	0	0	6	0	0
Histone H2B	PF07_0054	0	2	0	5	3	0
nucleosome assembly protein	PFI0930c	0	3	0	3	5	1
nucleosome assembly protein 1	PFL0185c	0	9	1	3	8	2
chromodomain helicase DNA binding protein1	PF10_0232	0	0	0	2	0	0
high mobility group protein	MAL8P1.72	0	1	1	2	1	1
structure specific recognition protein	PF14_0393	0	0	0	2	0	0
histone H2a variant	PFC0920w	1	3	1	5	4	1
histone H2B variant	PF11_0062	1	4	0	5	5	3

Table 6: Number of unique potential interactors peptides *involved in the nucleosome assembly and regulation pathway* in the case of three PfCK2β2 experimentations as compared to their presence in three control immunoprecipates experimentations. Colours still refer to probabilities (see 3.2.1)

NAME	PLASMO DB	3D7A1	3D7A2	3D7A3	CK2β2 1	CK2β2 2	CK2β2 3
chromodomain helicase DNA binding protein	PF10_0232	0	0	0	5	0	0
nucleosome assembly protein	PFI0930c	0	3	0	2	1	0
nucleosome assembly protein 1	PFL0185c	0	9	1	3	3	1
smarca related protein	PFF1185w	0	0	0	2	0	0
histone H2B	PF11_0062	1	4	0	5	2	4
histoneH4	PF11_0061	2	2	0	4	0	3
histone H3	PFF0510w	2	3	0	4	0	3
histone H2B	PF07_0054	0	2	0	3	0	2

Interestingly Smarca-related protein and chromodomain helicase DNA binding protein were present one time with both beta subunits and absent in all the controls. Furthermore each appeared with at least 3 unique peptides which are criteria of robustness. Nevertheless reproducibility between experiments was highly missing to give a cleared conclusion. Structure specific recognition protein was also only present in one sample and not present in any control, but the reproducibility was still lacking and furthermore there was only 2 peptides. Many of the other potential hits appeared in two samples but also in one or two controls which did not allow us to give any conclusion. Furthermore we noticed that there was a huge difference (already mentioned) between 3D7A2 control and the two others.

The second process we decided to focus on was post-translational modification. Table7 shows the details for the numbers of hits in each pathway for all experiments.

The most represented pathways were protein kinase coding genes and calcium-calmodulin activation of proteins kinases. But since all the PfCK2 subunits fit in those pathways in all experiments there were only 2 proteins in the first one and none in the second that were not one of the subunits.. Based on this we decided to analyse the chaperone assisted protein folding pathway. Tables 8 and 9 show the potential hits for PfCK2β1 and PfCK2β2.

Table7. Number of proteins observed for each post-translational modification pathways sorted by kinases and experimentations and total number of such proteins

POST-TRANSLATIONAL MODIFICATION	CK2B1.1	CK2B2.1	CK2β1.2	CK2β2.2	CK2β1.3	CK2β2.3	TOTAL
14-3-3 PROTEIN	0	0	0	0	0	0	0
ACTIVATION OF eiF5A	0	0	0	0	0	0	0
CALCIUM-CALMODULIN ACTIVATION OF PROTEIN KINASES	3	3	3	3	3	3	18
CHAPERONE-ASSISTED PROTEIN FOLDING	3	0	7	0	2	3	15
COTRANSLATIONAL CLEAVAGE OFN-TERMINAL METHIONINERESUDUES AN N-TERMINAL ACETYLATION	0	0	6	0	0	0	6
GENES CODING FOR CHAPERONES AND THEIR REGULATORS	3	0	5	0	2	4	14
GENES CODING FOR COMPONENTS OF THE PROTEASOME DEGRADATION MACHINERY AND THEIR TIMED TRANSCRIPTION	0	1	0	0	1	0	2
GENES CODING FOR GPI-ANCHORED MEMBRANES PROTEINS	0	0	0	0	0	0	0
HISTONE ACETYLATION	4	0	0	0	1	4	9
HISTONE LYSINE METHYLATION	0	0	0	0	0	0	0
N-MYRISTOYALTION, S-ACYLATION AND PRENYLATION OF PROTEINS	0	0	0	0	0	0	0
PEPTIDASES AND PROTEASES	2	0	4	0	1	0	5
POST-TRANSLATIONAL MODIFICATION-SUMOYLATION	0	0	0	0	0	0	0
PROTEASOME-MEDIATED DEGRADATION OF NON-NATIVE ER PROTEINS	0	0	0	0	0	0	0
PROTEASOME-MEDIATED PROTEOLYSIS OF UBIQUINATED PROTEINS	0	0	4	0	1	0	5
PROTEIN ARGININE METHYLATION	1	0	0	0	0	2	3
PROTEIN KINASE CODING GENES	3	4	4	3	3	3	20
PROTEIN PHOSPHORYLATION	3	1	0	0	0	0	4
ANAPHASE PROMOTING COMPLEX UBIQUITIN-LIGASE	0	0	0	0	0	0	0
SCF (Skp1-Cullin-F-box) UBIQUITINE LIGASE	0	0	0	0	0	0	0

Table 8: Number of unique potential interactors peptides *involved in the chaperone assisted protein folding pathway* in the case of three PfCK2β1 experimentations as compared to their presence in three control immunoprecipates experimentations. Colours still refer to probabilities (see 3.2.1)

NAME	PLASMO DB	3D7A1	3D7A2	3D7A3	CK2β1 1	CK2β1 2	CK2β1 3
ATP dependent CLP protease	PF14_0063	0	0	0	2	0	0
peptidyl-prolyl-cis-transchomerase	PF11_0164	0	11	2	2	11	0
DNA binding chaperon	PFL0815w	0	0	0	2	0	0
FK506 binding protein	PFL2275c	6	0	0	13	4	0
T-complex protein 1 gamma subunit	PFL1425w	9	0	0	9	2	2
p23 co-chaperon	PF14_0510	0	0	0	0	2	0
heat shock 40KDa protein	PFB0595w	0	0	0	0	3	0
TCP1/ cpn 60 chaperonin family	PFC0350c	8	1	0	5	4	0
TCP1/ cpn 60 chaperonin family	PF11_0331	18	1	0	12	4	1
T-complex beta subunit	PFC0285c	12	5	0	5	10	6
endoplasmin homologue precursor	PFL1070c	28	22	1	29	24	6

Table 9: Number of unique potential interactors peptides *involved in the chaperone assisted protein folding pathway* in the case of three PfCK2β2 experimentations as compared to their presence in three control immunoprecipates experimentations. Colours still refer to probabilities (see 3.2.1)

NAME	PLASMO DB	3D7A1	3D7A2	3D7A3	CK2b2 1	CK2b2 2	CK2b2 3
T-complex protein beta subunit	PFC0285c	12	5	0	3	0	3
endopoasmic homologue precursor	PFL1070c	28	22	1	22	0	8

For this pathway most of the selected proteins during a given experiment are found in the control of another experiment excepted for three proteins. Nevertheless these three proteins are not found in more than one experiment thus no conclusion could be drawn.

To conclude this part concerning beta subunit we presented the most '' robust'' result between the hits candidates which have been found to co-immunoprecipitate with PfCK2β2 in all experiments, with sufficient numbers of peptides to be considered as robust results and with a high recognition probability:

Table 10: Number of unique Rifin Peptides observed in the case of three PfCK2β2 experimentations as compared to their presence in three control immunoprecipates experimentations. Colours still refer to probabilities (see 3.2.1)

NAME	PLASMO DB	3D7A1	3D7A2	3D7A3	CK2b2 1	CK2b2 2	CK2b2 3
Rifin	PFD1020c	0	0	0	4	4	3

3.2.3 Results for PfGSK3

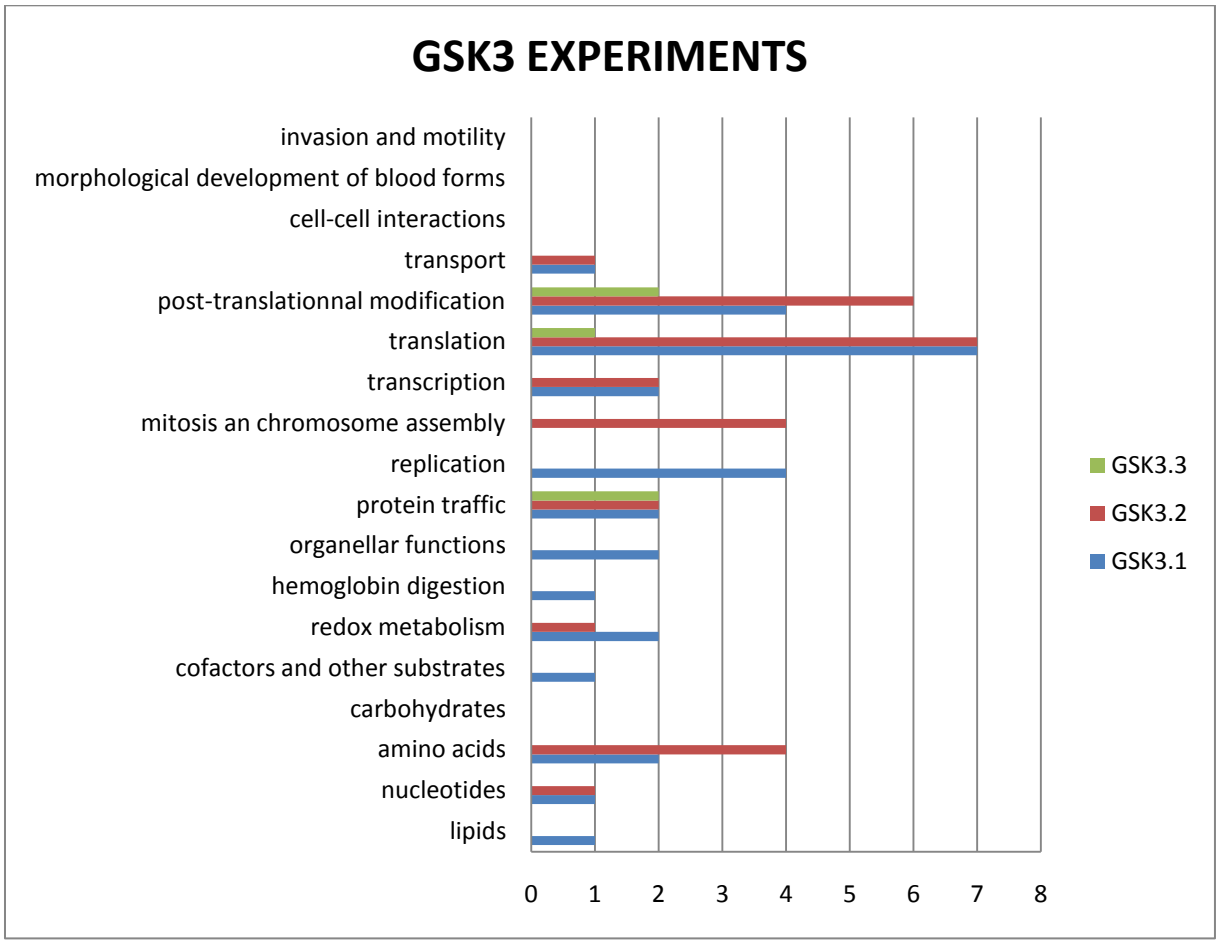
We have first analysed the presence of the proteins in all experiments to control the efficiency of the IP:

Table 10: Number of unique PfGSK3 peptides observed in all immunoprecipitates and in their control

EXPERIMENT	PROTEIN	NUMBER OF UNIQUE PEPTIDES IN GSK3 IMMUNOPRECIPITATE	NUMBER OF UNIQUE PEPTIDES IN THE CONTROL	TOTAL NUMBER OF PEPTIDES IN GSK3 IMMUNOPRECIPITATE	TOTAL NUMBER OF PEPTIDES IN THE CONTROL	%COVERAGE OF THE PROTEIN
1	PfGSK3	25	2	153	3	51
2	PfGSK3	17	1	73	2	39
3	PfGSK3	16	0	79	0	37

PfGSK3 was found in all the samples with numerous unique peptides and a good coverage of the protein. Furthermore proteins peptides were found only at a low level in the control. In the same way as for the analysis of the interactome of the other proteins of interest we have selected potential interactors for each experiment and report them on a histogram to have an overview.

Histogram 4: Distribution of potential interactors of PfGSK3 in the three aforementioned experimentations sorted by metabolic process



There was a huge variation of proteins in the PfGSK3 samples, but based on these preliminary results, it seems that PfGSK3 was not involved in several processes (invasion and motility, morphological development of blood forms, cell-cell interactions and carbohydrates). There were only two processes where potentials proteins were found in every experiment. This was probably due to the 3D7A2 control as for the PfCK2β subunits and to the lower amount of proteins loaded in experiment 3. The experiment 1 analysis resulted in the selection of 28 potentials hits (4 did not belong to any process), 20 in the experiment two and only 5 in the last experiment.

4. Reverse genetics studies of PfCK2β subunits

For this experiment we have used 4 parasites lines. KOCK2β1 + complement 2 and KOCK2β2 + complement 1

The aim here was to assess if both PfCK2β subunits gene could be disrupted in the presence of a complementation plasmid (providing the wilt type gene). Integration was first monitored using PCR then the populations were analysed by Southern blot.

4.1 PfCK2β1

For the PCR, DNA was extracted from KOCK2β1 + complement 2 cultures. Then PCR amplification has been done using oligonucleotides as detailed in table 1.

Table1: oligonigonucleotides used for DNA amplification and detection of integration. Numbers between brackets correspond to numbers in figure 2 showing their localisation on the plasmids and the gene.

Band to be amplified	Forward oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (bp)
Wild type	CK2b15primeF (1)	CK2b13primeRev (2)	934
5' integration	CK2b15primeF (1)	pCAMBSDRev (4)	598
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	604

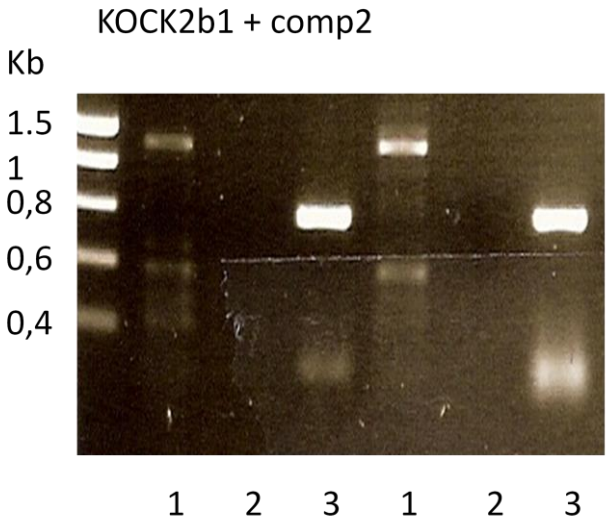


Figure 13 Experiment was done on two different cultures. Lane 1: wild type (primer 1 & 2; size 934bp), lane 2: 5' integration (primer 1 & 4; size 598bp), lane 3: episome (primer 3 & 4; size 604bp).

After several weeks of culture, there was still no sign of 5' integration. This may be due either to a very low level of integration or to premiers' bias. To determine the populations present in the culture we performed a Southern blot. After phenol extraction, DNA digestion was done using enzyme Cla1 and EcoR1. Digestion was performed on the two populations (KOCK2β1 and KOCK2β1 + comp 2). We

used first a BSD probe hybridizing to the episome bands and to the recombinated bands. The membrane was then stripped and probed using a PfCK2β1 probe which hybridizes to the wild type locus band.

Figure 2 Sites for restriction enzyme digestion and expected size of fragments

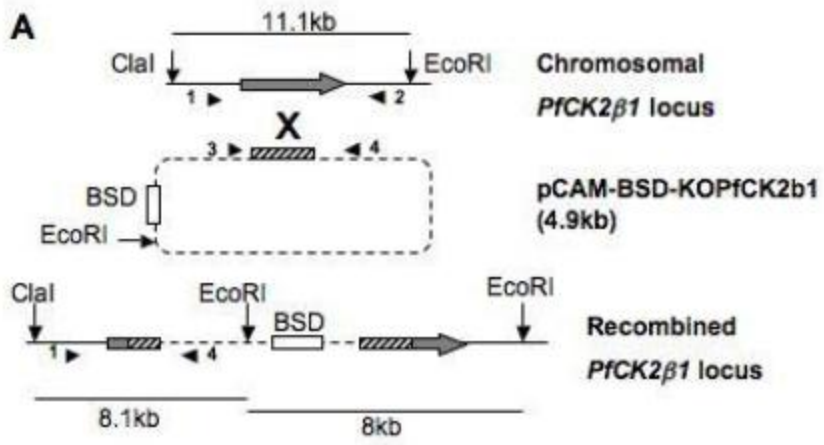


Figure 3 Diagram showing the sites for the restriction enzymes and the oligonucleotides primers used.

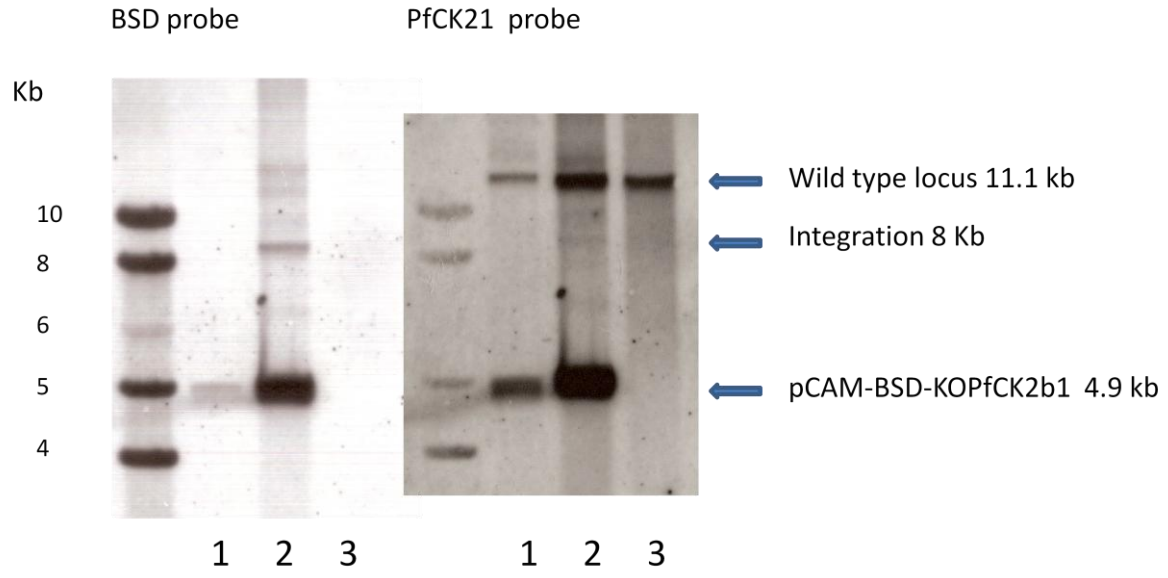


Figure 4 Knockout studies: lane 1: KOCK2β1 + comp2, lane 2: KOCK2β1, lane 3 : 3D7A. A band at the expected size for the integration appeared only in KO lines.

Southern blot analysis revealed a band related to the integration of the koplasmind only in the KOCK2β1 line. This result was not the expected one since Southern blot analysis on the other double transfected line has shown that the integration only appeared in the complemented line. As a result, in order to determine if this band corresponded really to integration a PCR was to be done using the digested DNA to amplify this band. If the PCR worked it would have meant that the gene was not essential. Otherwise this band could be due to a mistake when the gel was loaded or due to an

artefact (unspecific recombination). Finally the decreased intensity of the wild type band in lane 1 was not necessarily caused by the diminution of the WT population, since the amount of digested DNA loaded on this lane was lower than in lane 2 and 3.

4.2 PfCK2β2

An experiment was performed the same way for the second subunit. The primers used for PCR amplification is shown in the table 2 below and the PCR results are presented in figure 4.

Table2: oligonigonucleotides used for DNA amplification and detection of integration. Numbers between brackets correspond to numbers in figure 5 showing the localisation on the plasmids and the gene.

Band to be amplified	Forwards oligonucleotide	Reverse oligonucleotide	Expected size of amplified band (kb)
Wild type	CK2b25primeF (1)	CK2b2RSpe (2)	1.3
5' integration	CK2b25primeF (1)	pCAMBSDRev (4)	1.1
Plasmid	pCAMBSDFor (3)	pCAMBSDRev (4)	1.1

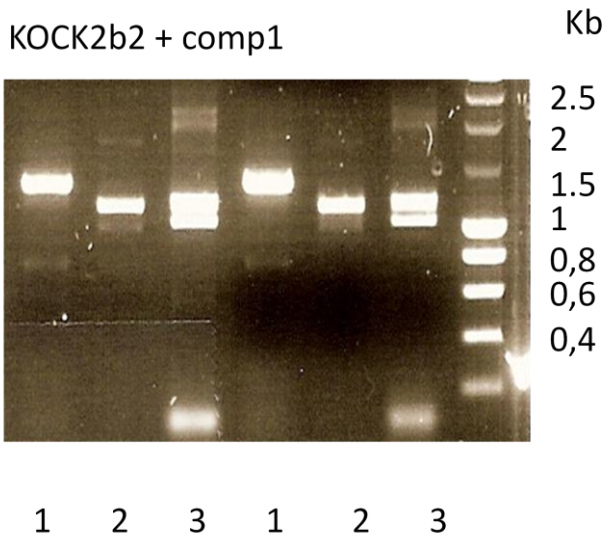


Figure 14: Experiment was done on two different cultures. Lane 1: wild type (primers 1 & 2; size 1.3kb), lane 2: 5' integration (primers 1 & 4; size 1.1kb), lane 3: episome (primers 3 & 4; size 1.1kb).

In this line the integration appeared on the PCR on both lane 2 (see figure 4). We further did a Southern blot to confirm the presence of the integration in our population. DNA was digested with Cla1 and Nco1. The sizes which were expected are presented in figure 5.

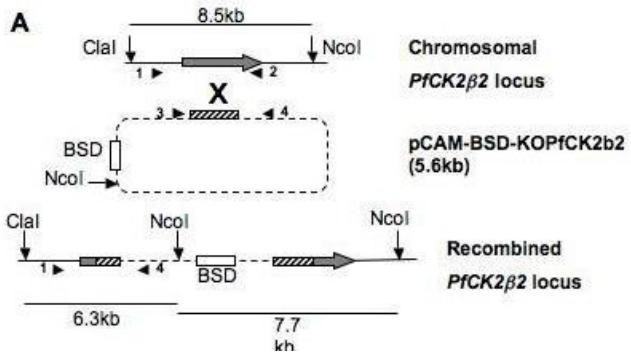


Figure 15: Diagram showing the sites for the restriction enzymes and the oligonucleotides primers used.

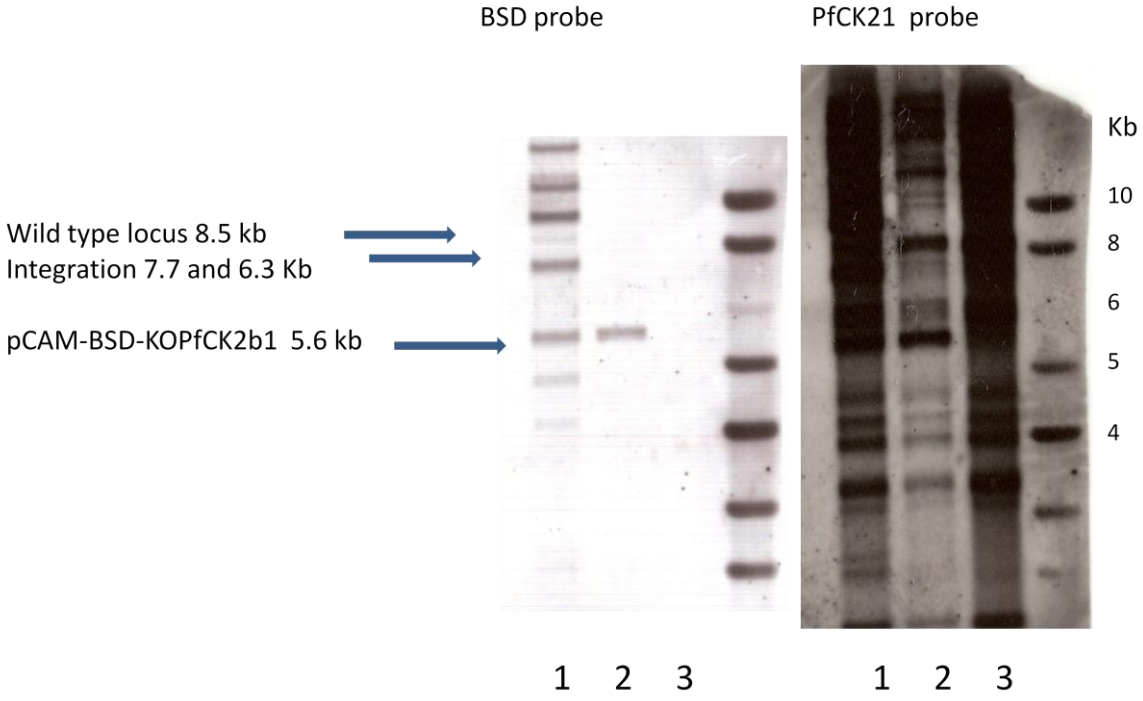


Figure 16: Southern blot result with BSD and PfCK2β2 probe. lane 1: KOCK2β2 + comp1, lane 2: KOCK2β2, lane 3 : 3D7A. A band corresponding to the expected size for integration appeared only in KO lines.

According to the PCR results, we expected to observe integration bands for this line since we saw a PCR 5'integration. But we did not see any bands at the integration size. Furthermore the undisrupted gene was present in the two lines (complemented or not) with the same intensities and the plasmid was still equivalently present in both lines. Thus for this line the cultures had to be followed up

5. Discussion

In this project we have used mass spectrometry analysis to study the interactome of three different proteins: PfCK2β1, PfCK2β2 and PfGSK3. We have shown that our proteins of interest can be efficiently immunoprecipitated using their HA tag. Moreover, in the case of PfCK2β subunits we have obtained the co immunoprecipitation of the three subunits with PfCK2β1 and PfCK2β2. It has already been shown in the past that the three subunits interact in vitro, thus our results add nothing new. However, this approach has to our knowledge never been used on *Plasmodium falciparum* in the past. We would therefore assume based on the preliminary obtained results that this approach can be used to study interactions of *P.falciparum* proteins as it has given the same results as the usual methods.

Our results are preliminary and it is not possible to give an absolute conclusion. However bioinformatics studies have determined that PfCK2β subunits possess a putative signal sequence for the nucleus. Interestingly we found proteins exerting their effect into the nucleus that coimmunoprecipitate only with our proteins of interest (PfCK2β1 and PfCK2β2). On the other side it was also proposed that PfCK2β2 could be exported since these proteins have a putative signal for export. And in our results we found a protein (rifin) which is known to be exported and which has been found in all the PfCK2β2 immunoprecipates. Once again we mention that it is impossible based only on three experimentations to give any definitive conclusion. We showed that this method can be used to identify potential interactors in *P.falciparum.* As mentioned in the project, there was a huge variability between potentials hits from one experiment to another. Furthermore there were many differences between our controls. To limit this variability we need to improve the stringency used. This can be done by increasing the number of washes after the immunoprecipitation. Another solution is to change the method used to elude the proteins after the IP. In this project we used Laemmli who elute the proteins bound to the beads without any specificity. To further remove those unspecific proteins we could use an HA-peptide which is a competitor to the HA epitope, thus proteins eluted would be only the proteins that are bound specifically by the anti HA epitope. Finally in this project the samples were allowed to bind to the beads over night, which may be too long for all the transient interactions, consequently decreasing this incubation time could lead to an increase in the number of hits.

Another important point is that this method can only give potentials interactors, thus in order to select good hits we need to process as many experimentations as possible in order to control that this protein is effectively a potential interactor. Once a protein is selected the result need to be verified by other methods such as fluorescence resonance electron transfer to assess the existence of the interaction.

Finally concerning the reverse genetics studies, we have not yet determined whether the two genes could be disrupted in the presence of a complementation plasmid. Further, it appears that the recombination between the two plasmids and/or between the plasmid(s) and the locus would need

to be analysed. Another possibility might be to apply a complementation plasmid with a synthetic gene while using different codons to avoid this recombination. Based on our results for PfCK2β1 we would have to determine if there has been a recombination between the KO plasmid and the wild type gene in the single knock out plasmid transfected line. In the event that this is not the case, cultures would have to be continuously carried out and continued analysis be done using Southern blot as for PfCK2β2.

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7. Summary

Malaria is the most devastating parasitic infection killing each year between 1 and 3 million of people. Furthermore this decease has a huge socio-economic impact in affected countries. The emergence of resistance against currently used drug makes the development of a new treatment an urgent task. Considering the success of kinase inhibitors in treatment of various cancers and considering the divergence between the parasites and human kinome, proteins kinases have become an attractive potential target. In this project we have used mass spectrometry analysis to study the Interactome of three *P.flaciparum* kinases: PfCK2β1; PfCK2β2 and PfGSK3. The genetically modified lines that we have used possess haemaglutinin tag. We have used this haemaglutinin tag to immunoprecipitate our proteins and their potentials interactors. Samples were then separated on acrylamide gel and analysed at the proteomics core facility at EPFL. Finally we have analysed the potential interactors and study the pathway in which they are involved. Using these methods we have found that the three PfCK2 subunits were immunoprecipitate together. These were already deemed known results since it has already been shown that these subunits interact together. But since there is, to the best of our knowledge, no example of this kind of study in *P.falciparum*, this project tends to demonstrate that mass spectrometry can be used to study Interactome of the parasites proteins. For our three proteins of interest we have identified some potential interactors but our results are too preliminary to conclude that those proteins are real interactors. A better understanding of the parasite life cycle and its metabolic pathway is critical in the development of a treatment against Malaria.