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Identification of Plasmodium falciparum protein kinase substrates and interacting proteins

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IDENTIFICATION OF *PLASMODIUM FALCIPARUM* PROTEIN KINASE SUBSTRATES
AND INTERACTING PROTEINS

by

JESSICA C. YAP

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Molecular Biology and Microbiology
in the Burnett School of Biomedical Sciences
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Orlando, Florida

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Thesis Chair: Dr. Debopam Chakrabarti

ABSTRACT

Malaria is a devastating disease that results in almost one million deaths annually. Most of the victims are children under the age of five in Sub-Saharan Africa. Malaria parasite strains throughout developing countries are continually building resistance to available drugs. Current therapies such as mefloquine, chloroquine, as well as artemisinin are becoming less effective, and this underscores the urgency for therapeutics directed against novel drug targets. In order to identify new drug targets, the molecular biology of the malaria parasite *Plasmodium* needs to be elucidated. *Plasmodium* exhibits a unique cell cycle in which it undergoes multiple rounds of DNA synthesis and mitosis without cytokinesis. Thus, cell cycle regulatory proteins are likely to be promising pathogen-specific drug targets. It is expected that fluctuating activity of key proteins, such as protein kinases, play an essential role in regulating the noncanonical life cycle of *Plasmodium*. Consequently, malarial kinases are a prime target for therapy. One way to better understand the role of malarial kinases in *Plasmodium* cell cycle regulation is to identify putative protein kinase substrates and interacting proteins. Two malarial kinases that have been implicated in regulating malaria parasite cell cycle stages were investigated in this study: *P. falciparum* CDK-like Protein Kinase 5 (PfPK5) and cAMP-Dependent Protein Kinase A (PfPKA). A transgenic *P. falciparum* line was created for the expression of epitope-tagged PfPK5 for pull-down analysis. Phospho-substrate antibodies were used to identify physiological substrates of both PfPK5 and PfPKA. Immunoblotting with these antibodies identified several potential substrates. Identities of the PfPKA physiological substrates were determined from the global *P. falciparum* phosphoproteome dataset that has recently been generated in our laboratory. Characterization of PfPKA and PfPK5 substrates, as well as the proteins they interact with, will help us to develop innovative therapies targeting binding sites.

For the Mind that invented this world and the Word by which it was created.
I am forever grateful to be given the opportunity to explore this exciting world through the eyes of science
...especially through molecular biology.

For my family. Thank you for supporting me. I could not have done it without you.

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INTRODUCTION

In first-world countries, tropical diseases like malaria and typhoid fever receive relatively little attention. However, many infections that are considered archaic devastate underdeveloped countries even today. Malaria, ranking among the top five causes of death in low-income countries, is responsible for close to one million deaths annually. The victims of this deadly disease are primarily children under the age of five in Sub-Saharan Africa. Individuals with vulnerable immune systems are also severely affected and often die within hours [1]. Malaria is often difficult to diagnose due to ambiguity of its early symptoms. Common early manifestations of the infection include fever, chills, increased sweating, headache, back pain, myalgia, vomiting, cough, and diarrhea. If the infection is not quickly diagnosed, it can progress into severe malaria, a stage marked by convulsions, pulmonary and retinal edema, organ dysfunction, and respiratory acidosis. The most severe form of malaria, known as cerebral malaria, occurs when the infection spreads to the capillaries of the brain and causes inflammation. This results in cerebral ischemia, brain damage, and terminal coma [2-4]. Even non-fatal cases of cerebral malaria result in mental dysfunction and paralysis [1].

CHALLENGES FACING ANTIMALARIAL DEVELOPMENT

Human malaria is caused by five eukaryotic parasite species: *Plasmodium malariae*, *P. vivax*, *P. ovale*, *P. knowlesi*, and *P. falciparum*. *P. vivax* and *P. ovale* are notorious for causing chronic malaria, due to their ability to remain dormant in hepatocytes. *P. falciparum* is classified as the most deadly of the *Plasmodium* species [5]. Unfortunately, antimalarial drug resistance is growing and traditional treatments are losing effectiveness. From naturally-derived products such as quinine and artemisinin to synthetic variants like chloroquine, mefloquine, amodiaquine, and artesunate, all efforts to eradicate malaria have

been thwarted by the parasite's ability to adapt to its environment. *P. falciparum* is now resistant to all antimalarials in use, including recently implemented artemisinin-based therapies [6]. In addition to this problem, new generations of treatments are becoming more costly. A major issue facing the development of antimalarial drugs is the unprofitable market. Since the regions most affected by malaria are largely impoverished, ideal treatments need to be inexpensive and this lack of profit is deterrent to both investors and pharmaceutical companies [7].

Despite these challenges, researchers are exploring novel targets by which to eradicate the disease. One of the top five targets of pharmaceutical research and development is a protein family known as kinases [8]. These enzymes phosphorylate a variety of substrates, resulting in either activation or deactivation of a target protein. Because of their ability to influence the activity of other proteins, kinases play key roles in many intracellular signaling cascades. The kinases and pathways that regulate the noncanonical cell cycle of *Plasmodium* are of particular importance in the development of novel antimalarial therapies. Researchers seek pathogen-specific drug targets in order to minimize side effects. Because of this, processes and pathways that are unique to an infectious agent are the most attractive targets for therapy. One of *Plasmodium*'s most unusual features is its complex life cycle, which defies eukaryotic standards.

THE LIFE CYCLE OF *PLASMODIUM*

The transmission of malaria is perpetuated by the *Anopheles* mosquito, which injects *Plasmodium* sporozoites into the host's bloodstream (Fig. 1). These sporozoites then enter the liver and invade hepatocytes. Once inside the hepatocytes, the parasite reproduces asexually to form hundreds of merozoites.

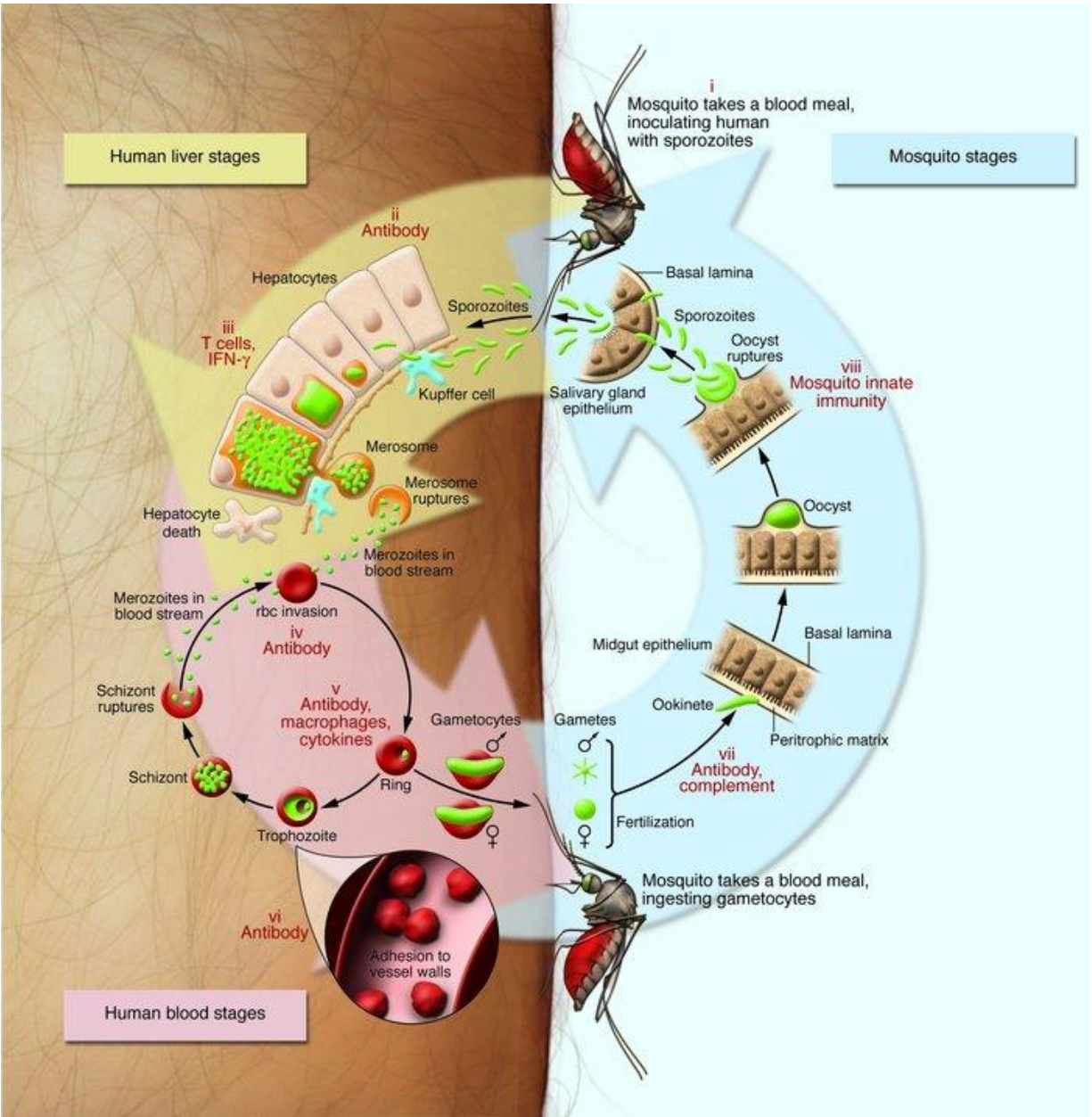


Figure 1: Life cycle of *Plasmodium* [5]

The merozoites rupture the hepatocytes, travel through the blood stream, and infect erythrocytes. *Plasmodium* undergoes an atypical asexual cell cycle inside host erythrocytes (Fig. 2). During its 48-hour intraerythrocytic phase, the parasite goes through three distinct stages: Ring,

Trophozoite, and Schizont [5]. Shortly after ingress, the parasite forms a ring shape within the erythrocyte. While feeding on the nutrients in the red blood cell, *Plasmodium* develops into a larger structure called a trophozoite. It proceeds to destroy the erythrocyte by metabolizing hemoglobin. The toxic by-product, hemozoin, is sequestered. Magnetic synchronization techniques take advantage of the high iron content in this altered form of hemoglobin to separate trophozoites and schizonts from early stage parasites [9]. Beginning in the Trophozoite and proceeding through the Schizont stage, the parasite undergoes multiple rounds of DNA synthesis and mitosis without performing cytokinesis [10-12]. This forms a multinucleated cell called a coenocyte [13]. These nuclei are the beginning form of new merozoites. When the parasite contains more than one nucleus, it is called a schizont. The multinucleated late schizont structure is called a segmenter [10]. The parasite will then rupture both the parasitophorous vacuole membrane that housed it and the erythrocytic plasma membrane to release 8-20 daughter merozoites [5].

Plasmodium's generation of merozoites diverges from the usual eukaryotic cell cycle. Most eukaryotic cells undergo a replication and division cycle consisting of four distinct phases: G1, S, G2, and M. Oscillation of cyclin-dependent protein kinase (CDK) activity drives the progression from one stage to the next [14]. G1 and G2 separate the replication and division stages and contain checkpoints to ensure that the cell is prepared for the next transition. During S phase, the chromosomes are replicated; and in M phase, the cell performs mitosis. Typically, mitosis concludes with cytokinesis and produces two separate daughter cells [15].

In contrast, *Plasmodium* does not appear to utilize distinct gap phases and instead interdigitates mitosis and DNA synthesis. It also does not undergo cytokinesis until multiple nuclei have been generated [16]. Formation of a coenocyte is infrequently observed in other organisms, thereby providing a

Plasmodium-specific basis of study. Identifying the regulatory factors of this process is essential for understanding the uncommon cell cycle of *Plasmodium*.

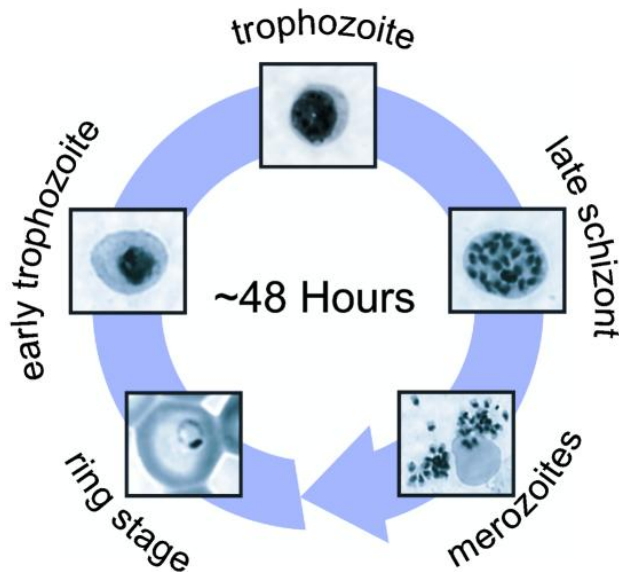


Figure 2: Intraerythrocytic cycle of *Plasmodium* [17]

EUKARYOTIC PROTEIN KINASES AND THE MALARIAL KINOME

Protein kinases are responsible for the reversible phosphorylation that drives a myriad of eukaryotic cellular processes, including reproduction, metabolism, and apoptosis [8, 18]. Seven families of protein kinases exist in eukaryotes: the AGC group, containing calcium-phospholipid- and cyclic-nucleotide-dependent kinases; the calmodulin-dependent kinase group (CaMK); the casein kinase group (CK1), the CGMC group comprised of the cyclin-dependent- (CDK), mitogen-activated- (MAPK), glycogen-synthase- (GSK), and CDK-like kinases; the STE group, containing MAPK-related kinases; the tyrosine kinase (TyrK) group; and the tyrosine kinase-like kinase (TLK) group [19]. *Plasmodium* has sixty-three protein kinases, most of which cluster within these families. Others, like the phenylalanine-isoleucine-lysine-lysine-motif

protein kinase-like enzymes (FIKKs) and the Never in mitosis A-related enzymes (NEKs), exemplify the divergence of *Plasmodium* from conventional eukaryotes [19, 20]. Deviance from eukaryotic standards makes the malarial kinome an attractive source of innovative, pathogen-specific drug targets. Considering the importance and uncommon qualities of the *Plasmodium* cell cycle, understanding cell cycle-related kinases is exceptionally urgent.

***P. FALCIPARUM* PROTEIN KINASE A: A MULTIPURPOSE CAMP-DEPENDENT KINASE**

Protein Kinase A (PKA), also known as cAMP-Dependent Protein Kinase A, is a well-studied regulator of diverse cellular functions. As its name suggests, PKA activity is dependent on cyclic adenosine monophosphate (cAMP). PKA belongs to the AGC family of protein kinases. It is found in a plethora of organisms and its function varies in each. PKA activation is directly related to the G-protein coupled receptor activation of adenylyl cyclase, which in turn converts ATP into cAMP (Fig. 3). Typically, this second messenger binds the two regulatory subunits of PKA, causing them to dissociate from the corresponding catalytic subunits. PKA then interacts with a diverse array of proteins to trigger signaling cascades [21].

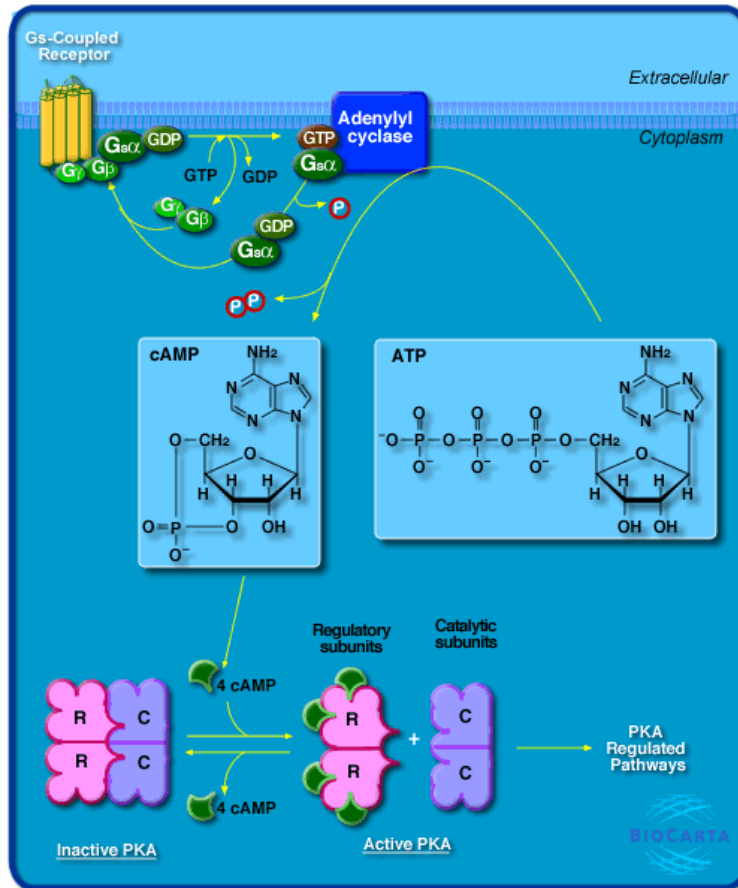


Figure 3: Activation pathway of PKA [22]

Plasmodium falciparum PKA (PfPKA) has been implicated in erythrocyte invasion and permeation pathways [23, 24]. Additionally, it is thought to be involved in cell cycle regulation [25]. However, its substrates and pathways have not all been defined. One known substrate of PfPKA is apical membrane antigen 1 (AMA1) in the formation of tight junctions during erythrocyte invasion [24]. Up-regulation of the regulatory subunits of PfPKA has been shown to slow parasite growth and inhibit formation of new parasite-induced permeation pathways (NPPs) [23]. This illustrates the diversity of processes governed by PfPKA. The kinase has also been linked to regulation of the cell cycle by calcium and

melatonin. *Plasmodium* hijacks the host hormone melatonin to increase the level of cytoplasmic calcium, which then synchronizes the parasite's cell cycle. Evidence suggests that PfPKA amplifies this process. In addition, melatonin has been shown to influence cAMP production and PfPKA activation. Inhibition of PfPKA by PKA inhibitor H89 decreases the effectiveness of melatonin in synchronization [25].

***P. FALCIPARUM* PROTEIN KINASE 5: A CDK-LIKE KINASE**

Plasmodium falciparum Protein Kinase 5 (PfPK5) is a putative cell cycle-regulating kinase that offers a unique window into understanding the life cycle of *Plasmodium*. As with PfPKA, the molecular functions of PfPK5 remain unconfirmed and the substrates phosphorylated by this kinase have yet to be elucidated, illustrating the scarcity of information related to this protein.

PfPK5 is one of the few *P. falciparum* kinases whose structure has been solved. It has proven to be an essential gene, as observed through failed gene knockout efforts (C. Doerig, unpublished data). Because *Plasmodium* is haploid in its asexual stages, knock out of an essential gene will be lethal. PfPK5 is observed in every stage of the intraerythrocytic cycle, but expression and activity peak during the Schizont stage around 36 hours after ingress [11, 26]. PfPK5 has been compared to other cell cycle-related kinases known as cyclin-dependent kinases and has been categorized in the CGMC group of protein kinases. As previously mentioned, CDK activity governs the eukaryotic cell cycle. Their activity is dependent on the binding of cyclins, which are a class of augmenting proteins.

Cyclins bind to cyclin-dependent kinases, thereby activating them and enhancing their activity. In some cases, the binding of a cyclin to a kinase is thought to result in auto-phosphorylation, or self-activation, of the kinase subunit [27]. PfPK5 has been shown to interact with multiple mammalian cyclins *in vitro*, such as cyclin A, cyclin H, and p25; however, *in vivo* binding partners have yet to be identified [27,

28]. The precise function of P/PK5 remains unclear, but it bears high sequence homology to the human cyclin-dependent kinases CDK1, CDK2, and CDK5 [27, 29]. For these reasons, it is classified as a CDK-related kinase [30].

Like P/PK5, CDK1 (human cyclin-dependent kinase 1) is an essential protein. It has been shown to influence mitosis, and inhibition of this kinase results in inappropriate timing of DNA replication and cytokinesis [31]. Careful regulation of these processes is required for the production of merozoites. Since P/PK5 activity peaks during the nuclear division cycles, it is possible that it plays a CDK1-like role in merozoite generation [26].

Both CDK1 and CDK5 share 60% sequence homology with P/PK5. However, the two human kinases influence very different processes. As previously stated, CDK1 has been shown to be involved in M phase of the cell cycle. CDK5 is involved in several pathways including cell migration and adhesion, as well as angiogenesis [32, 33]. Consequently, the function of P/PK5 cannot be deduced by sequence comparison alone. Beyond sequence similarity, P/PK5 and CDK5 share a somewhat conserved PSTAIRE/PSTTIRE cyclin binding motif [28, 30]. Homology of tertiary structure is supported by data showing that p25, a cyclin-related protein that activates CDK5, has been shown to activate P/PK5 *in vitro* [27].

Human cyclin-dependent kinase 2 (CDK2), which demonstrates approximately 58% identity with P/PK5, is a cell cycle-related kinase implicated in the S and G2 phases of the cell cycle [29, 34]. During these stages, Cyclin A associates with CDK2. Cyclin A has also been shown to associate with P/PK5 *in vitro* [28].

Considering the high degree of homology between P/PK5 and the human CDKs, in addition to the interaction of P/PK5 with various classes of human cyclins, it is hypothesized that these kinases will share

similar interacting partners and possibly similar functions [27, 28, 30]. Noting the diverse functions of the previously mentioned human CDKs, it might be assumed that the functions of PfPK5 are divergent as well. However, it has been determined by confocal immunofluorescent microscopy that PfPK5 co-localizes with the DNA stain TOTO 1 during DNA synthesis [26]. This supports the hypothesis that PfPK5, like CDK1 and CDK2, is involved in the nuclear function.

SIGNIFICANCE OF STUDY

There is much to be gained by elucidation of the signaling cascades in which PfPKA and PfPK5 are involved. The understanding of these intracellular processes will help to uncover the role these protein kinases play in cell cycle regulation, as well as identify previously unknown substrates and interacting partners. Although it is known that PfPKA influences multiple processes, most of its substrates have not yet been identified and the pathways are unconfirmed.

The observed similarities between PfPK5 and the CDKs lead to the following conclusions: PfPK5 may influence cell cycle regulation through nuclear activity, and interacting partners are possibly *Plasmodium* cyclins or proteins homologous to the human cyclins tested *in vitro*. Substrates and signaling pathways under the influence of PfPK5 are likely to be unique to *Plasmodium*, providing novel targets for treatment that will not interfere with host cell pathways. Characterization of *P. falciparum* PKA- and PK5-interacting partners and substrates will provide informative insights into the function of these kinases and will guide target-based therapy.

METHODS

CULTURING OF *PLASMODIUM FALCIPARUM*

Two strains of *P. falciparum* were used in this study: wild type 3D7 and chloroquine-resistant Dd2. Unless otherwise specified, both were cultured according to the following procedure. The parasites were grown in human type A+ red blood cells, 4% hematocrit, in RP0.5A (RPMI medium supplemented with 0.5% Albumax). The medium was changed daily and parasitemia was maintained at approximately 6%. Cultures were incubated at 37°C and 5% CO₂. Prior to harvesting for experimental purposes, the culture parasitemia was increased to approximately 15%.

SYNCHRONIZATION OF CELL CULTURES

For studies of the individual intraerythrocytic stages, a 3D7 cell line was synchronized as follows: 5% sorbitol and RP0.5A medium were warmed to 37°C. The parasite culture was confirmed to be predominantly in Ring stage by preparing a Giemsa-stained slide. The culture was resuspended and centrifuged at 2,000 rpm for 7 minutes. After the supernatant was replaced with five times the pellet volume of 5% sorbitol, the culture was incubated at 37°C for 15 minutes and centrifuged again as stated above. The cells were washed thoroughly in RP0.5A and resuspended in the original volume of media.

PREPARATION OF MALARIAL CELL-FREE EXTRACT

Cultures were resuspended and combined in 50-mL conical tubes. In order to isolate the parasites from the red blood cells, they were incubated in a final concentration of 0.1% w/v saponin for 5 minutes at room temperature. Next, the cells were centrifuged at 4,000 rpm for 7 minutes at 4°C. The supernatant was removed and the parasites were washed thoroughly in 1X Dulbecco's Phosphate-buffered Saline

(DPBS). After being resuspended in 1 mL DPBS, the parasites were transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet weight recorded.

IMMUNOBLOT EXPERIMENTS

Inhibitor Treatment to Determine Specificity of Phospho-(Ser/Thr) PKA Substrate Antibody

P. falciparum Dd2 was cultured asynchronously. At approximately 10% parasitemia, the culture was resuspended in RP0.5A with 25 µM H89. This concentration was half the IC90 of the inhibitor [24]. The H89-treated medium was replaced after 24 hours. At 45 hours post-treatment, the culture had a parasitemia of around 25%. The culture was harvested and the pellet was lysed as described above, using an 8 M urea buffer with 1X HALT protease inhibitor and 1X HALT phosphatase inhibitor. 100 µg of protein was resolved on a 4-12% NuPAGE gel as described above. Untreated asynchronous Dd2 was harvested and lysed using Mammalian Protein Extraction Reagent (M-PER) buffer from Pierce Protein Research Products supplemented with 1X HALT protease inhibitor and 1X phosphatase inhibitor. M-PER lysis was utilized due to its compatibility with immunoprecipitation protocols that will be performed. 100 µg of this untreated protein sample was used as a control. The membrane was blocked thoroughly in 1X Tris-Buffered Saline (TBS) with 0.1% Tween-20 and 5% bovine serum albumin (BSA) for 45 minutes at room temperature. A rabbit phospho-(Ser/Thr) PKA substrate antibody from Cell Signaling Technology was used to target proteins with a (K/R)(K/R)X(S*/T*) motif. The antibody was diluted 1:1,000 in TBS with 0.1% Tween-20 and 5% BSA (TBS-T 5% BSA) and incubated with the membrane at 4°C overnight. The membrane was then washed 3 times in TBS-T 5% BSA for 10 minutes each at room temperature. A mouse anti-rabbit antibody was diluted 1:2,000 in TBS-T 5% BSA and incubated with the membrane for 1.5

hours at room temperature. The membrane was washed thoroughly with TBS-T and stored in TBS until it was developed.

Substrate Prediction Using PKA-Phosphorylated Motif Antibody

P. falciparum 3D7 was cultured, synchronized, and harvested as described above. The parasites were harvested at 8 hour intervals for 48 hours. Cell lysis was performed by incubating the pellet in an equal volume of 8 M urea buffer containing 1X HALT protease inhibitor and 1X HALT phosphatase inhibitor. The pellet was incubated on ice for 30 minutes and resuspended every 5 minutes. 100 µg of protein from each time point were separated by SDS-PAGE on a 4-12% gradient NuPAGE gel and blotted onto a polyvinylidene difluoride (PVDF) membrane at 10 V and 4°C overnight. The proteins were blotted onto a PVDF membrane and probed with a phospho-(Ser/Thr) PKA substrate antibody in TBS-T 5% BSA as described above.

Substrate Prediction Using CDK-Phosphorylated Motif Antibody

The same procedure as described for the PKA-phosphorylated motif antibody was followed using a rabbit phospho-(Ser) CDK substrate antibody from Cell Signaling Technology. Proteins with a (K/R)(S*)PX(K/R) motif were targeted.

IMMUNOPRECIPITATION

Asynchronous cultures of Dd2 were harvested and lysed using M-PER buffer with 1X HALT protease inhibitor and 1X HALT phosphatase inhibitor. 500 µg of protein was incubated with a phospho-(Ser/Thr) PKA substrate antibody cross-linked to 20 µL of Protein A/G agarose resin at a 1:100 dilution, according to the protocol provided by Pierce Protein Research Products. This procedure was performed in duplicate. Additionally, a control was prepared using 500 µg of protein and 20 µL of control resin without

antibody. Flow through, washes, and elutions of both immunoprecipitations as well as the control were resolved on 4-12% gradient NuPAGE gels.

SYPRORuby Staining

The SDS-PAGE gels containing the proteins from the control and the first immunoprecipitation were stained according to the SYPRORuby basic protocol provided by BioRad. The gels were fixed in a solution of 50% methanol and 7% acetic acid and stained overnight with SYPRORuby. After being transferred to clean trays, the gels were washed with a solution of 10% methanol and 7% acetic acid. Lastly, the gel was rinsed in ultrapure water prior to imaging on a BioRad molecular imager.

Silver Staining

The second immunoprecipitation SDS-PAGE gel was silver-stained using a mass spectrometry compatible silver staining kit from Pierce Protein Research Products by following the provided protocol. Briefly, the gel was washed in ultrapure water and fixed in a 30% ethanol-10% acetic acid solution overnight. Next, the gel was washed in a 10% ethanol solution and rinsed in ultrapure water. The gel was incubated in the sensitizer solution, rinsed in ultrapure water, and incubated in the silver staining solution. The gel was then incubated in the developing solution and rinsed in ultrapure water. The developing reaction was stopped with a 10% acetic acid solution and the gel was rinsed in ultrapure water prior to imaging.

GENERATION OF TRANSFECTED CELL LINE

Cloning

The P β PK5 gene was generated by reverse transcriptase PCR, tagged with hemagglutinin (HA) and flanked by *Xho*I and *Avr*II restriction sites according to the following program: 42°C for 30 seconds; 95°C for 30 seconds; 5 cycles of 95° for 30 seconds, 46° for 30 seconds, and 68°C for 6 minutes; 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 68°C for 6 minutes. After purification of the PCR product using the Promega Wizard SV gel and PCR clean-up system, the product was ligated into the pGEM-T Easy vector. The construct was amplified in *E. coli* XL10 Gold cells and then verified by dideoxy sequencing at the University of Florida. Next, the construct was digested with *Xho*I and *Avr*II and separated on a 0.8% agarose gel. The band corresponding to P β PK5-HA was excised and the DNA was extracted. Then, the tagged gene was cloned into the *Xho*I and *Avr*II restriction sites of the pDC2-1600crt-1X HA BSD-attP vector and amplified in *E. coli* XL10 Gold cells. This construct was purified and sequence confirmed as stated above.

Episomal Transfection

A Dd2-attB strain of *P. falciparum* was episomally transfected with 100 μ g of purified plasmid DNA (P β PK5-pDC2-1600crt-1X HA BSD-attP) according to the following procedure. 2.5 mL of synchronized Ring stage culture was resuspended and centrifuged at room temperature at 1,500 rpm for 3 minutes. The supernatant was discarded and the pellet washed with 1X Cytomix. The cells were centrifuged under the same conditions as before, and the supernatant was replaced with 500 μ L Cytomix. 100 μ g each of P β PK5-HA and pINT were added to the resuspended cells. After being incubated on ice, the cells were then

transferred to an electroporation cuvette and electroporated at 307 V. 1 mL of RP0.5A medium was added to the cuvette and incubated for 1 minute. The cells were plated in a 6-well plate with 2.5 mL of RP0.5A and 400 μ L of freshly drawn red blood cells diluted 1:1 in RP0.5A. After a 4-hour incubation in RP0.5A, the transfected cells were maintained under triple drug selection (RP0.5A containing blasticidin, WR99210, geneticin). After the first week, fresh blood was added and the media was changed every other day until the parasitemia returned to approximately 10%.

Transfection Verification

The transfected cell line was harvested and lysed with an 8 M urea buffer and 1X HALT protease inhibitor. *P. falciparum* 3D7 was harvested and lysed using the same procedures. 100 μ g of protein from the transfected culture and 100 μ g of protein from the 3D7 control were resolved on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Two blots were performed. The membranes were washed thoroughly with 1X Phosphate-buffered Saline with 0.1% Tween-20 and 5% dry milk (PBS-T 5% milk). Rabbit anti-PK5 was diluted 1:1,000 in PBS-T 5% milk and incubated with one membrane overnight at 4°C. Goat anti-HA was diluted 1:1,000 in PBS-T 5% milk and incubated with the other membrane overnight at 4°C. The membrane was then washed thoroughly in PBS-T 5% milk. Mouse anti-rabbit secondary antibody was diluted 1:2,000 in PBS-T 5% milk and incubated with the anti-PK5 membrane for 1.5 hours at room temperature. Donkey anti-goat secondary antibody was diluted 1:2,000 in PBS-T 5% milk and incubated with the anti-HA membrane for 1.5 hours at room temperature. Both membranes were washed thoroughly in PBS-T and stored in PBS until they were developed.

RESULTS

TREATMENT WITH PKA INHIBITOR H89 DECREASES PHOSPHORYLATION BY P \mathcal{P} PKA

In an effort to determine proteins phosphorylated by P \mathcal{P} PKA, a phospho-(Ser/Thr) PKA substrate antibody was used to probe *P. falciparum* cell extract. Global phosphoproteomic data gathered in our lab was used to identify proteins of similar molecular mass that are phosphorylated at the PKA phosphorylation motif. To prove that the phosphorylation detected by the phospho-(Ser/Thr) PKA substrate antibody was due to phosphorylation by PKA and not caused by non-specific antibody binding, a Dd2 culture was treated with H89, a known inhibitor of PKA. If phosphorylation patterns were unchanged, then it could be inferred that the proteins had not been phosphorylated by P \mathcal{P} PKA. However, if addition of the inhibitor decreased phosphorylation, then P \mathcal{P} PKA could be implicated in the detected proteins' phosphorylation.

H89 inhibits PKA phosphorylation activity by binding to a sequence conserved across a variety of species. Although the inhibitor is not selective for *P. falciparum*, our data supports previous studies, showing that P \mathcal{P} PKA is indeed inhibited by H89 [25]. This inhibition is shown by the decrease in phosphorylation detected in the anti-phospho-(Ser/Thr) PKA Substrate immunoblot of H89-treated and untreated Dd2 (Fig. 4). The bands detected in the control lane (Dd2) at 16 kDa, 33 kDa, 38 kDa, 41 kDa, 45 kDa, 79 kDa, 85 kDa, 130 kDa, and 150 kDa are either absent or show decreased intensity in the inhibitor-treated lane (Dd2+H89). These nine bands, labeled A1-A9, represent putative substrates of P \mathcal{P} PKA. It is interesting to note that A8 is almost entirely inhibited in the H89-treated lane. The intensities of each band relative to A8 were measured in order to compare levels of phosphorylation (Fig. 5).

When the molecular weights were compared to the phosphoproteomic data, the following identities were predicted: protein A1 is a putative ubiquitin carboxyl-terminal hydrolase 2 (Accession number Q8I3U1); A2 is a conserved *Plasmodium* protein potentially involved in transcription (Q8IBU0); A4 is a

putative regulator of chromosome condensation (C0H4M6); and A5 is a putative RNA-binding protein (C0H4V5) (Table 1 and Table 2).

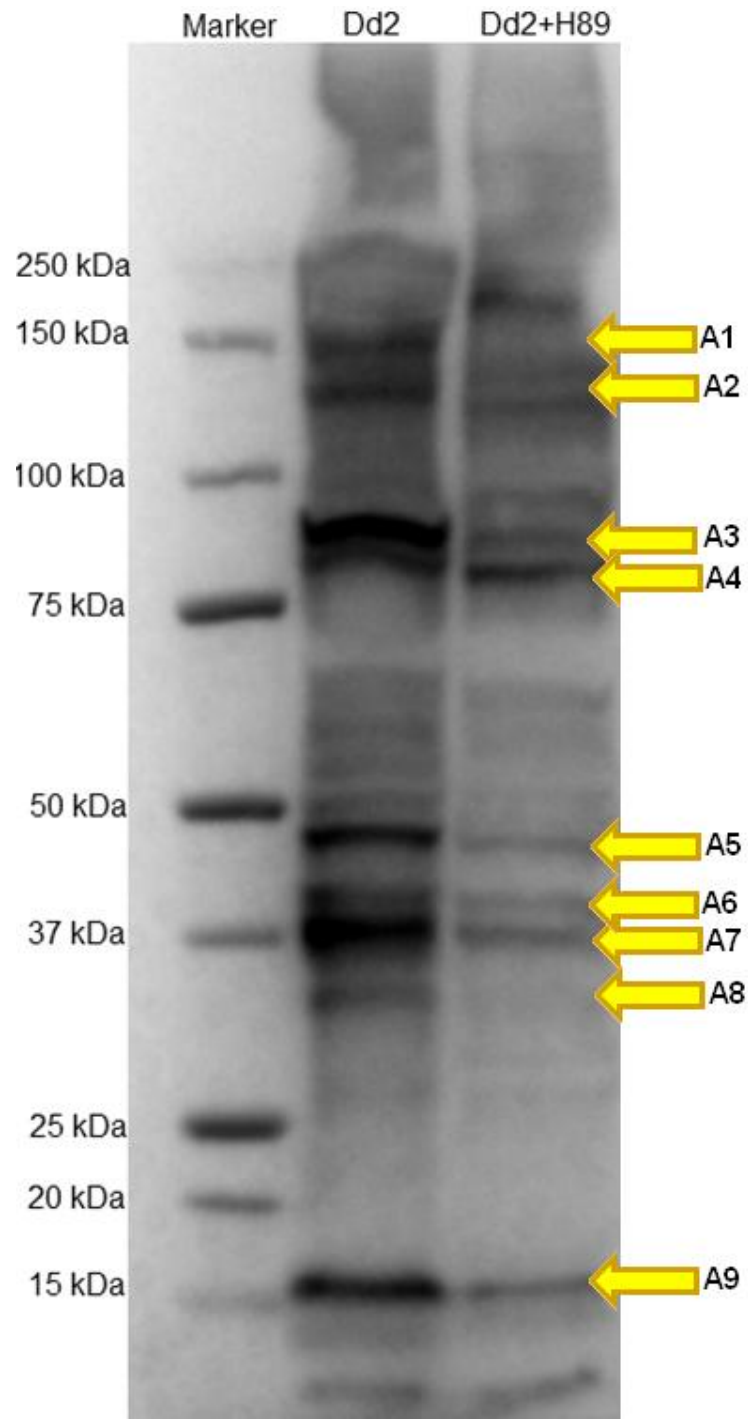


Figure 4: Immunoblot of H89-treated Dd2 lysate using phospho-(Ser/Thr) PKA substrate antibody

Table 1: Phosphoproteomic data

Predicted Molecular Weight (kDa)	Peak Proteomic Stage	Peak Phosphorylation Stage	Annotation	Function	Accession Number
24.876	Global	All	GTP-binding nuclear protein Ran/TC4 [<i>P. falciparum</i> 3D7]	Nucleocytoplasmic Transport	Q7KQK6
45.157	Trophozoite/Schizont	Schizont	RNA binding protein, putative [<i>P. falciparum</i> 3D7]	Hypothetical Uncharacterized	C0H4V5
61.087	Global	Trophozoite	<i>Plasmodium</i> exported protein (PHISTb), unknown function [<i>P. falciparum</i> 3D7]	Unclassified	Q8I3F0
63.62	Schizont	Ring	Protein phosphatase, putative [<i>P. falciparum</i> 3D7]	Hypothetical: Protein Fate	C0H4T6
79.036	Global	Trophozoite/Schizont	Regulator of chromosome condensation, putative [<i>P. falciparum</i> 3D7]	Hypothetical: Cell Cycle and DNA Processing	C0H4M6
92.279	Schizont	Ring	Myosin A [<i>P. falciparum</i> 3D7]	Interaction with the Environment	Q8IDR3
99.311	Ring	Trophozoite/Schizont	Conserved <i>Plasmodium</i> protein, unknown function [<i>P. falciparum</i> 3D7]	Cell Cycle and DNA Processing	Q8I283
106.299	Ring/Trophozoite	Trophozoite/Schizont	RNA binding protein, putative [<i>P. falciparum</i> 3D7]	Hypothetical: Protein with Binding Function or Co-Factor Requirement	Q8IJZ3
110.78	Ring/Trophozoite	Schizont	Ubiquitin-protein ligase E3, putative [<i>P. falciparum</i> 3D7]	Hypothetical: Protein Fate	Q8IC12
123.333	Global	Schizont	Conserved <i>Plasmodium</i> protein, unknown function [<i>P. falciparum</i> 3D7]	Unclassified	C6KSQ8
123.741	Global	Ring	Conserved <i>Plasmodium</i> protein [<i>P. falciparum</i> 3D7]	Cellular Transport, Transport Facilitation, and Transport Routes	Q8IJR3
132.718	Global	All	Conserved <i>Plasmodium</i> protein, unknown function [<i>P. falciparum</i> 3D7]	Transcription	Q8IBU0
156.334	Global	Ring	Ubiquitin carboxyl-terminal hydrolase 2, putative [<i>P. falciparum</i> 3D7]	Hypothetical: Protein Fate	Q8I3U1

Table 2: H89 treatment immunoblot

Label	Estimated Molecular Weight (kDa)	Accession Number of Putative Match	Molecular Weight of Putative Match (kDa)
A1	150	Q8I3U1	156.334
A2	130	Q8IBU0	132.718
A3	85	N/A	N/A
A4	79	C0H4M6	79.036
A5	45	C0H4V5	45.157
A6	41	N/A	N/A
A7	38	N/A	N/A
A8	33	N/A	N/A
A9	16	N/A	N/A

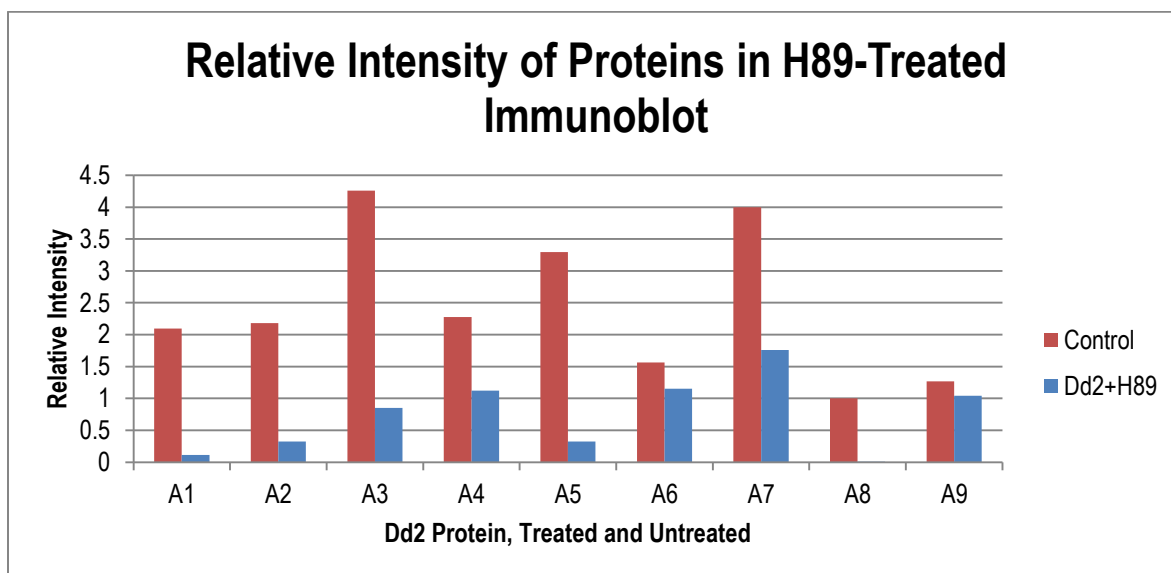


Figure 5: Relative phosphorylation of proteins in H89-treated anti-phospho-(Ser/Thr) PKA substrate immunoblot

PHOSPHORYLATION BY PFPKA FLUCTUATES THROUGHOUT THE INTRAERYTHROCYTIC CYCLE

To further characterize of putative PFPKA substrates, and to confirm the relationship between PFPKA and the *P. falciparum* cell cycle, an immunoblot was performed on resolved time point lysates using

the phospho-(Ser/Thr) PKA substrate antibody. Variation in the phosphorylation of a protein from one intraerythrocytic stage to another implies that its activity fluctuates throughout the asexual cell cycle; therefore, the kinase responsible for phosphorylating the protein may be implicated in cell cycle regulation. Also, peak phosphorylation stages could be compared to the phosphoproteomic dataset.

Fifteen distinct bands can be detected and have been labeled B1-B15 (Fig. 6). These bands, shown at approximately 100 kDa, 90 kDa, 83 kDa, 77 kDa, 46 kDa, 41 kDa, 39 kDa, 36 kDa, 33 kDa, 31 kDa, 27 kDa, 23 kDa, 21 kDa, 17 kDa, and 13 kDa correspond to putative substrates of P ϵ PKA. Some bands remain constant throughout the cell cycle, indicative of a global phosphorylation event. The intensity of other bands increases or decreases drastically from one stage to the next, implying different roles in each stage. The most prominent band, B3 (~83 kDa), intensifies from Late Trophozoite (L. Troph) stage through Schizont, Segmenter, and Early Ring (E. Ring) stages. Abruptly, phosphorylation of this protein drops in Late Ring (L. Ring) stage. In contrast, B6, B7, and B14 (around 41 kDa, 39 kDa, and 17 kDa, respectively) remain of equal intensity throughout the cell cycle. Some bands clearly correspond to proteins detected in the asynchronous inhibitor-treated immunoblot (Fig. 4). For example, bands B3, B4, B5, B6, B7, and B14 appear to be homologous to A2, A3, A5, A6, A7, and A9, respectively.

To gain insight into the putative identities and roles of these proteins, the molecular weights of the fifteen bands were compared to global phosphoproteomic data (Table 1 and Table 3). Comparisons of molecular weight resulted in the following predictions: the identity of Protein B1 is either a conserved *Plasmodium* protein predicted to influence the cell cycle and DNA processing (Accession number Q8I283) or a putative RNA-binding protein (Q8IJZ3); B2 is Myosin A (Q8IDR3); B4 is a putative regulator of chromosome condensation (C0H4M6); B5 is a putative RNA-binding protein (C0H4V5); and B12 is a GTP-binding nuclear protein Ran/TC4 (Q7KQK6).

Relative intensity of each band with respect to B15 was determined in order to compare levels of phosphorylation from stage to stage (Fig. 7). Peak phosphorylation stages were compared to those listed in the global phosphoproteomic dataset. Peak expression stages were included from the dataset in order to further characterize the putative substrates. This analysis supports the prediction of B2 being Myosin A, since both demonstrate highest phosphorylation during Ring stage. Likewise, the peak phosphorylation stages of both B4 and a chromosome condensation regulator (C0H4M6) occur during the Trophozoite/Schizont stages. Lastly, both B12 and the GTP-binding nuclear protein Ran/TC4 demonstrate global phosphorylation. Proteins that match in both molecular weight and peak phosphorylation are highlighted in Table 3, and their relationship to the phosphoproteomic data will be discussed in another section.

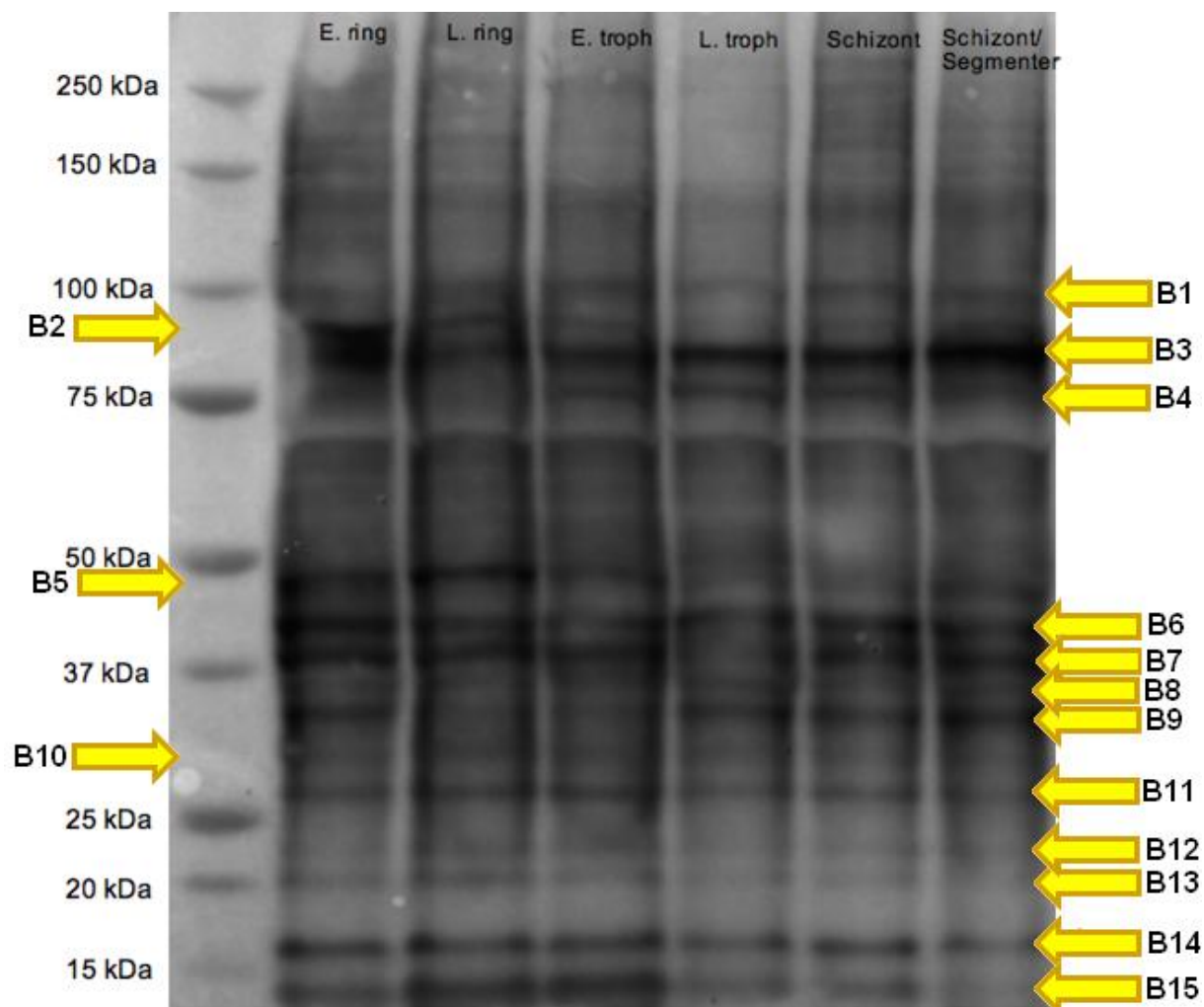


Figure 6: Immunoblot of 8 hour time point lysates using phospho-(Ser/Thr) PKA substrate antibody

Table 3: Anti-phospho-(Ser/Thr) PKA substrate immunoblot

Label	Estimated Molecular Weight (kDa)	Observed Peak Phosphorylation Stage	Accession Number of Putative Match	Molecular Weight of Putative Match (kDa)	Peak Expression of Putative Match	Peak Phosphorylation of Putative Match
B1	100	All	Q8I283 or Q8IJZ3	99.311 or 106.299	Ring or Ring/Trophozoite	Trophozoite/Schizont (both)
B2	90	Early Ring	Q8IDR3	92.279	Schizont	Ring
B3	83	Trophozoite to Early Ring	N/A	N/A	N/A	N/A
B4	77	Trophozoite/Schizont	C0H4M6	79.036	Global	Trophozoite/Schizont
B5	46	Ring	C0H4V5	45.157	Trophozoite/Schizont	Schizont
B6	41	All	N/A	N/A	N/A	N/A
B7	39	All	N/A	N/A	N/A	N/A
B8	36	All	N/A	N/A	N/A	N/A
B9	33	Schizont/Segmenter to Early Ring	N/A	N/A	N/A	N/A
B10	31	All	N/A	N/A	N/A	N/A
B11	27	Late Ring to Early Trophozoite	N/A	N/A	N/A	N/A
B12	23	All	Q7KQK6	24.876	Global	All
B13	21	Late Ring to Early Trophozoite	N/A	N/A	N/A	N/A
B14	17	All	N/A	N/A	N/A	N/A
B15	13	Late Ring to Early Trophozoite	N/A	N/A	N/A	N/A

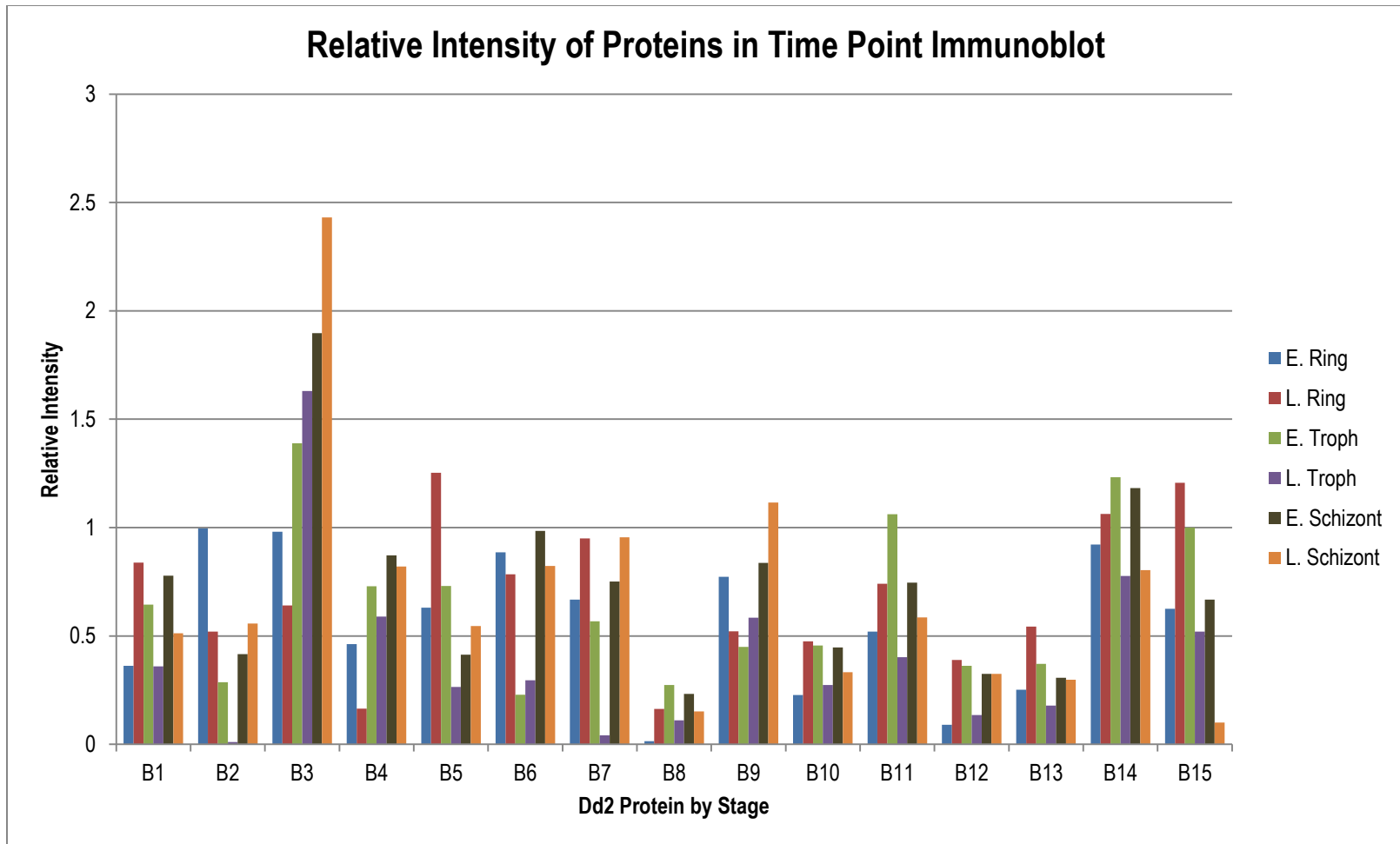


Figure 7: Relative phosphorylation of proteins in time point anti-phospho-(Ser/Thr) PKA substrate immunoblot

IMMUNOPRECIPITATION ISOLATES PUTATIVE PPKA SUBSTRATES

Although *in silico* analysis of molecular weights is good for predicting identities of proteins, mass spectrometry must be employed to reveal a protein's true identity. In order to isolate the proteins that interact with PPKA, an immunoprecipitation was performed in duplicate using the phospho-(Ser/Thr) PKA substrate antibody. Two methods of staining were tested for sensitivity: Mass Spectrometry-Compatible Silver Staining and SYPRORuby staining. While both the silver stain and the SYPRORuby stain were compatible with mass spectrometry procedures, the silver stain proved to be more sensitive (Fig. 10). In the SYPRORuby-stained polyacrylamide gel, only two bands were detected in the elutions (Fig. 9). It is uncertain whether they were bound to and eluted from the antibody or carried over from the flow-through due to insufficient washing. In contrast, the silver-stained gel showed multiple bands in all three elutions. Two bands in the third elution (E3) around 63 kDa and 125 kDa, and one band in the second elution (E2) around 67 kDa, are of particular interest. Since these proteins do not seem to correspond with a thick flow-through band, they are more likely to be putative PPKA substrates. The control elutions contained no detectable bands as was predicted (Fig. 8). Both bioinformatic analysis and mass spectrometry must be performed to determine the identities of the protein in the silver stained gel and determine their relationship, if any, to PPKA.

The most promising bands detected in the silver-stained and the SYPRORuby-stained polyacrylamide gels were labeled C1-C6 (Fig. 9, Fig. 10). Phosphoproteomic analysis matched the molecular weights of C1, C3, and C5. We predict that Protein C1 is Myosin A (Accession number Q8IDR3); C3 is a conserved *Plasmodium* protein (C6KSQ8 or Q8IJR3), possibly involved in cellular transport and transport facilitation (Q8IJR3); and C5 is either *Plasmodium* exported protein PHISTb (Q8I3F0) or a putative protein phosphatase involved in protein fate (C0H4T6) (Table 1, Table 4).

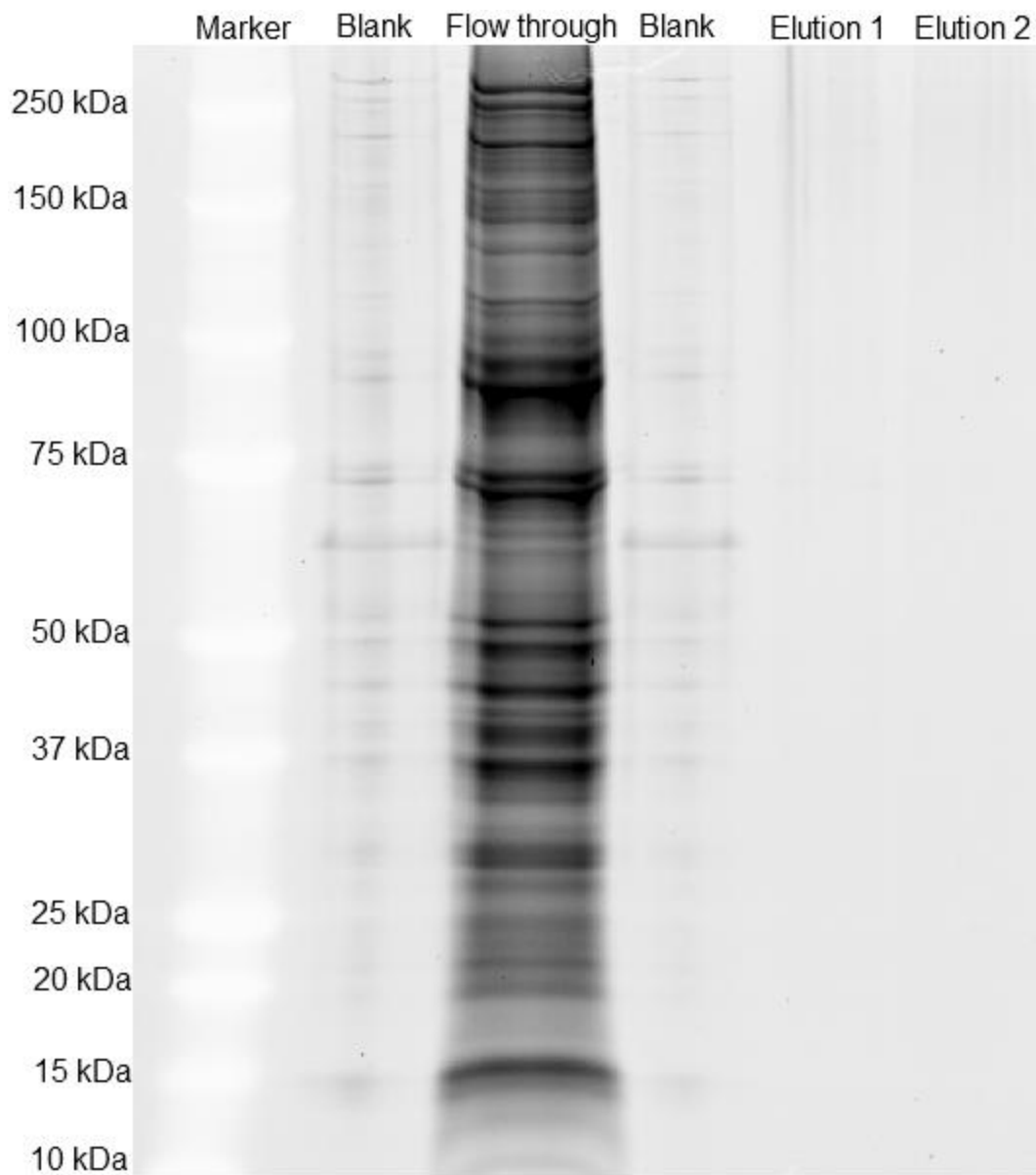


Figure 8: SYPRORuby-stained polyacrylamide gel of immunoprecipitation control elutions

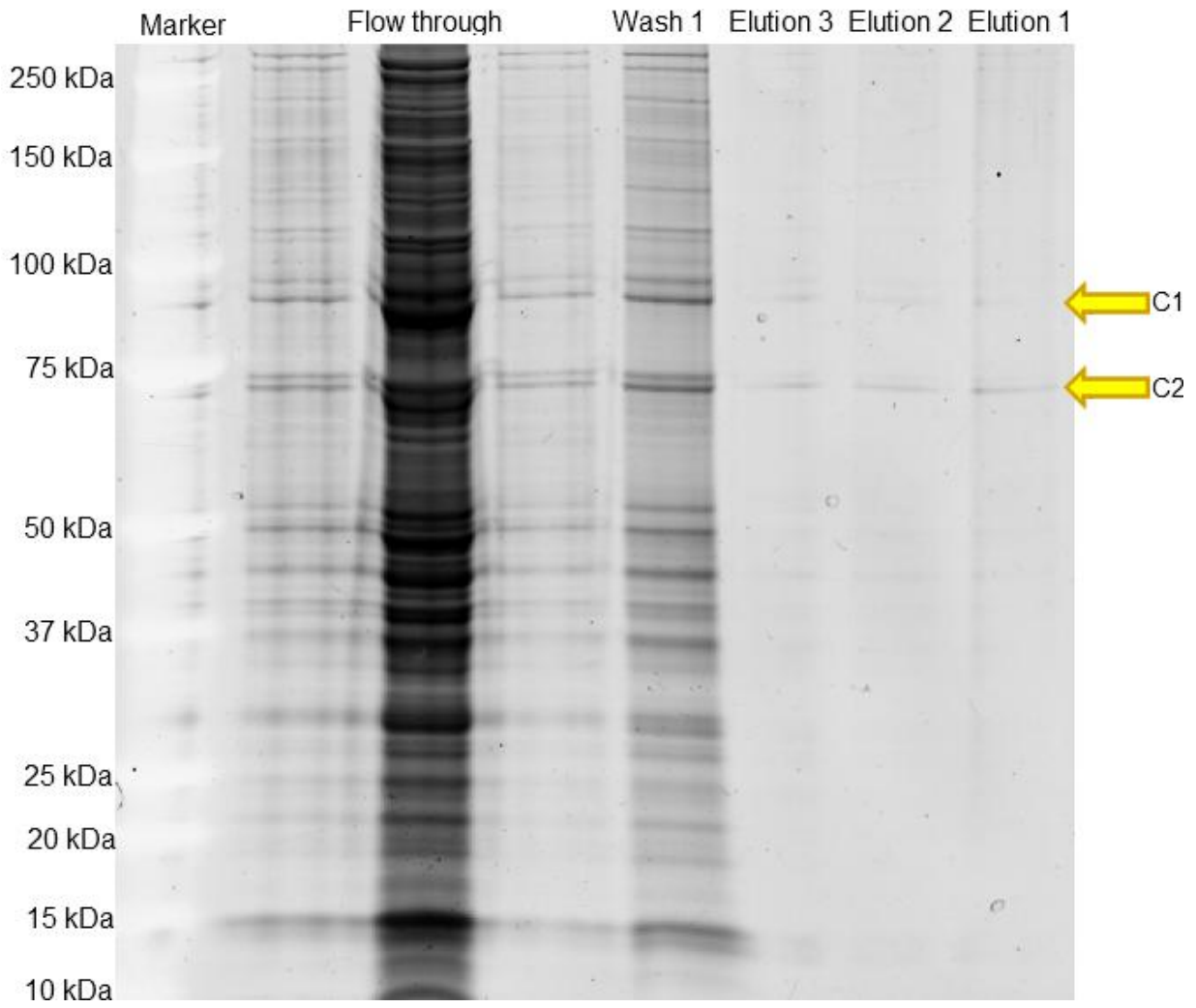


Figure 9: SYPRORuby-stained polyacrylamide gel of immunoprecipitation using phospho-(Ser/Thr) PKA substrate antibody

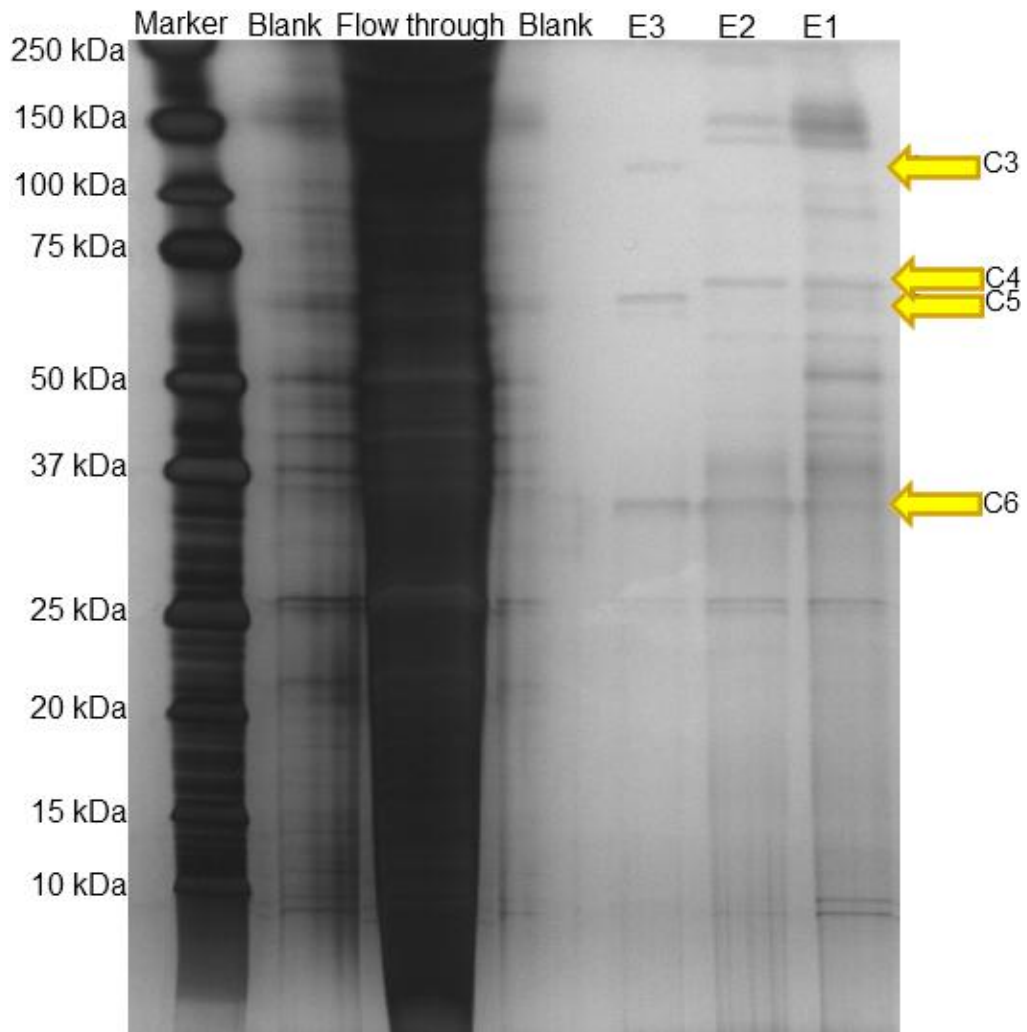


Figure 10: Silver-stained polyacrylamide gel of immunoprecipitation using phospho-(Ser/Thr) PKA substrate antibody

Table 4: Immunoprecipitations

Label	Estimated Molecular Weight (kDa)	Accession Number of Putative Match	Molecular Weight of Putative Match (kDa)	Peak Expression of Putative Match	Peak Phosphorylation of Putative Match
C1	90	Q8IDR3	92.279	Schizont	Ring
C2	74	N/A	N/A	N/A	N/A
C3	125	C6KSQ8 or Q8IJR3	123.333 or 123.741	Global (both)	Schizont or Ring
C4	69	N/A	N/A	N/A	N/A
C5	64	Q8I3F0 or C0H4T6	61.087 or 63.62	Global or Schizont	Trophozoite or Ring
C6	34	N/A	N/A	N/A	N/A

GENERATION OF AN EPITOPE-TAGGED *PLASMODIUM FALCIPARUM* LINE WHERE PFPK5-HA IS EXPRESSED *IN TRANS*

A similar procedure using an anti-PK5 antibody can be used to isolate substrates and other proteins that interact with PfPK5. For a dual confirmation, a tagged target protein can be isolated in an immunoprecipitation using an antibody that binds either the tag or the protein itself. In this case, PfPK5 was constructed with an HA tag. An immunoprecipitation with an anti-PfPK5 antibody would be expected to pull down both PfPK5 and any interacting proteins. An immunoprecipitation with an anti-HA antibody would be expected to have the same results. Thus, generating recombinant PfPK5-HA would provide a means by which the potential interacting partners could be confirmed.

The 863 bp PfPK5 gene was cloned by reverse transcriptase PCR and cloned into the *AvrII* and *XhoI* restriction sites of pDC-1600crt-BSD (Fig. 11). The 29 bp gene encoding the HA tag was added to the 3' end of PfPK5, corresponding to a 1.1 kDa C-terminus HA tag. Finally, the construct was transfected into *P. falciparum* Dd2.

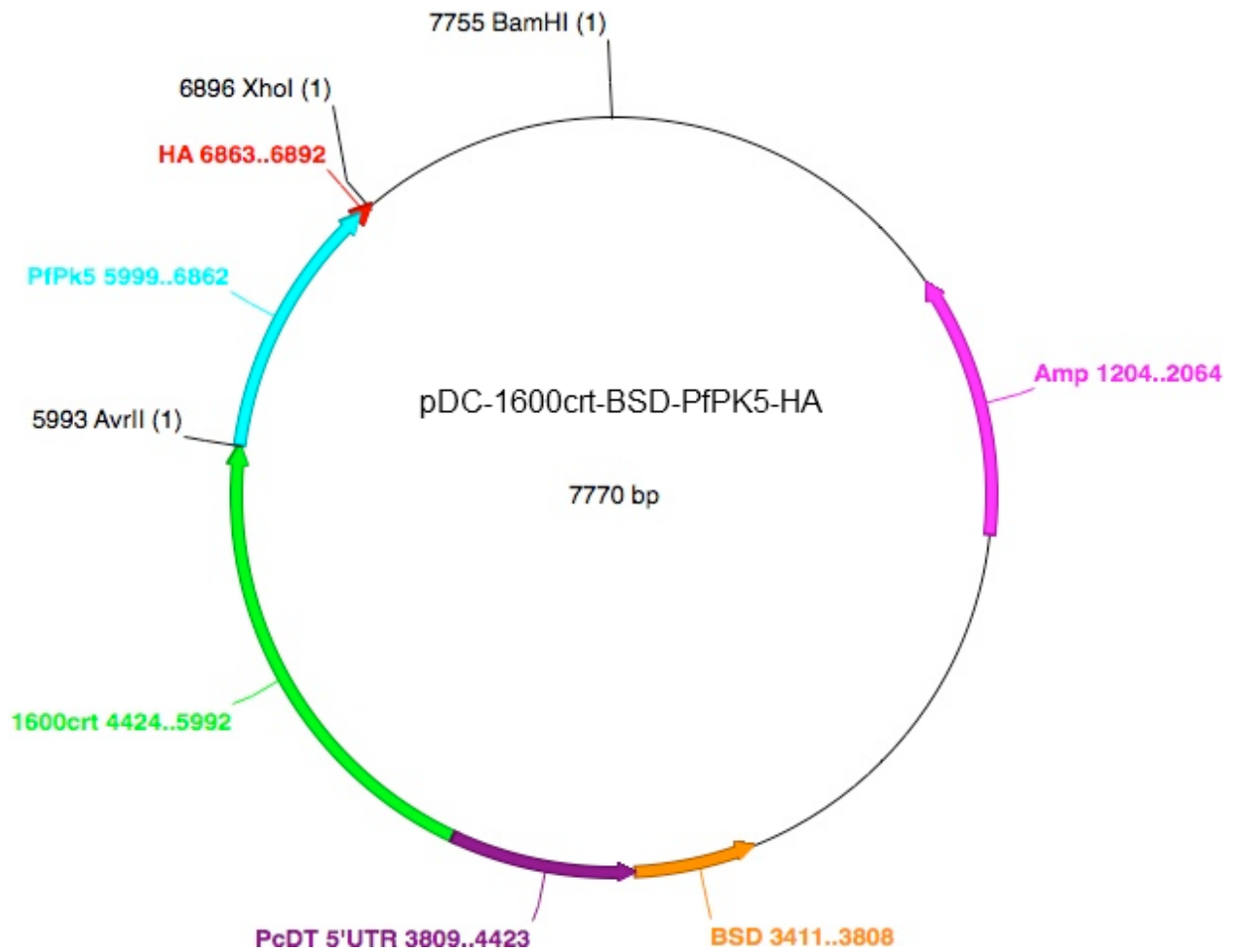


Figure 11: Map of pDC-1600crt-BSD-PfPK5-HA

In order to confirm the transfection, an immunoblot was performed using the transfectant lysate and wild type lysate. Two immunoblots were performed. The first was probed for PfPK5 and the second was probed for the HA tag. The transfectant lysate was expected to produce a signal in both immunoblots, since it contained both PfPK5 and the HA tag. The wild type would react only with the anti-PfPK5 antibody because endogenous PfPK5 will be present but the foreign HA tag would not. PfPK5 is a 33 kDa protein, while its recombinant HA-tagged counterpart is 34 kDa.

The data from the immunoblots confirmed the presence of HA-tagged *PfPK5* in the transfected cell line. Not only is the *PfPK5* band detected around 34 kDa, but it is also more intense than the band in the control lane, which implies over-expression of the protein due to abundance of the gene (Fig. 12). Furthermore, the transfected cell line had a signal in the HA immunoblot demonstrating the presence of the recombinant HA-tagged *PfPK5*, while the control lacked this signal (Fig. 13).

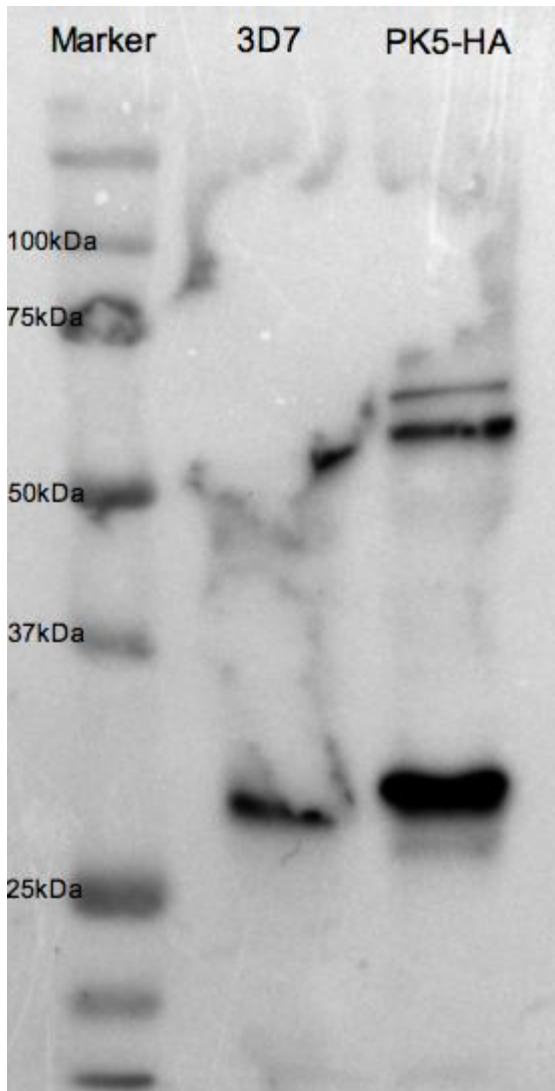


Figure 12: Anti-P/PK5 immunoblot

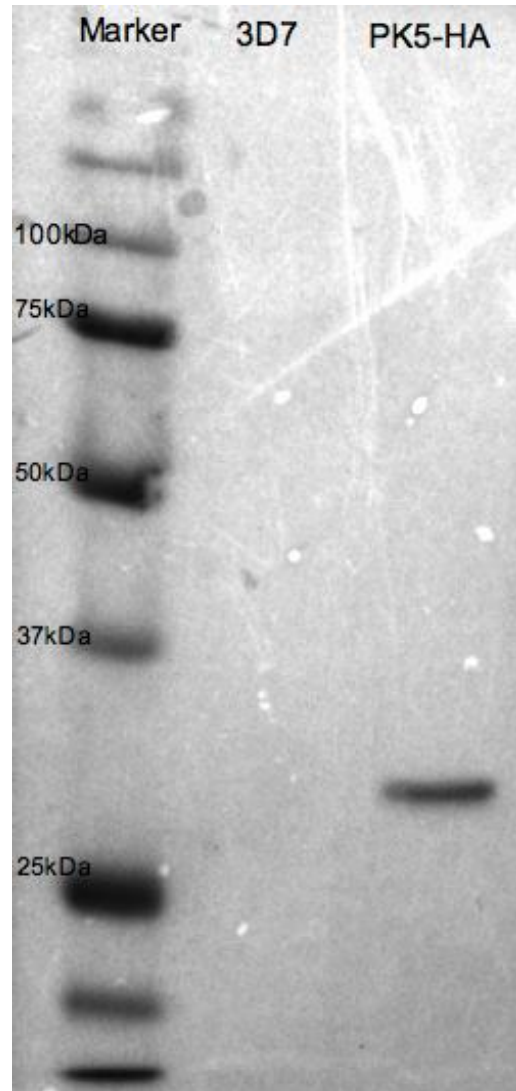


Figure 13: Anti-HA immunoblot

PHOSPHORYLATION BY CDKS FLUCTUATES THROUGHOUT THE INTRAERYTHROCYTIC CYCLE

Considering the central role of CDKs in the cell cycle, it is expected that their level of expression as well as that of their substrates fluctuates from stage to stage. In order to confirm this, an immunoblot was performed using the phospho-(Ser) CDK substrate antibody on 8 hour time point lysates. Given the

similarities between P/PPK5 and the CDKs, it is expected that some of the substrates of P/PPK5 will be among the proteins detected by this antibody.

Nine bands were detected and labeled D1-D9 (Fig. 14). Of these, only one (~43 kDa) maintained a relatively constant intensity. The molecular weights of the bands have been estimated at 27 kDa, 33 kDa, 35 kDa, 39 kDa, 43 kDa, 58 kDa, 62 kDa, 79 kDa, and 116 kDa (Table 5). This data shows that phosphorylation due to CDKs, and presumably CDK-related kinases such as P/PPK5, fluctuates throughout the cell cycle.

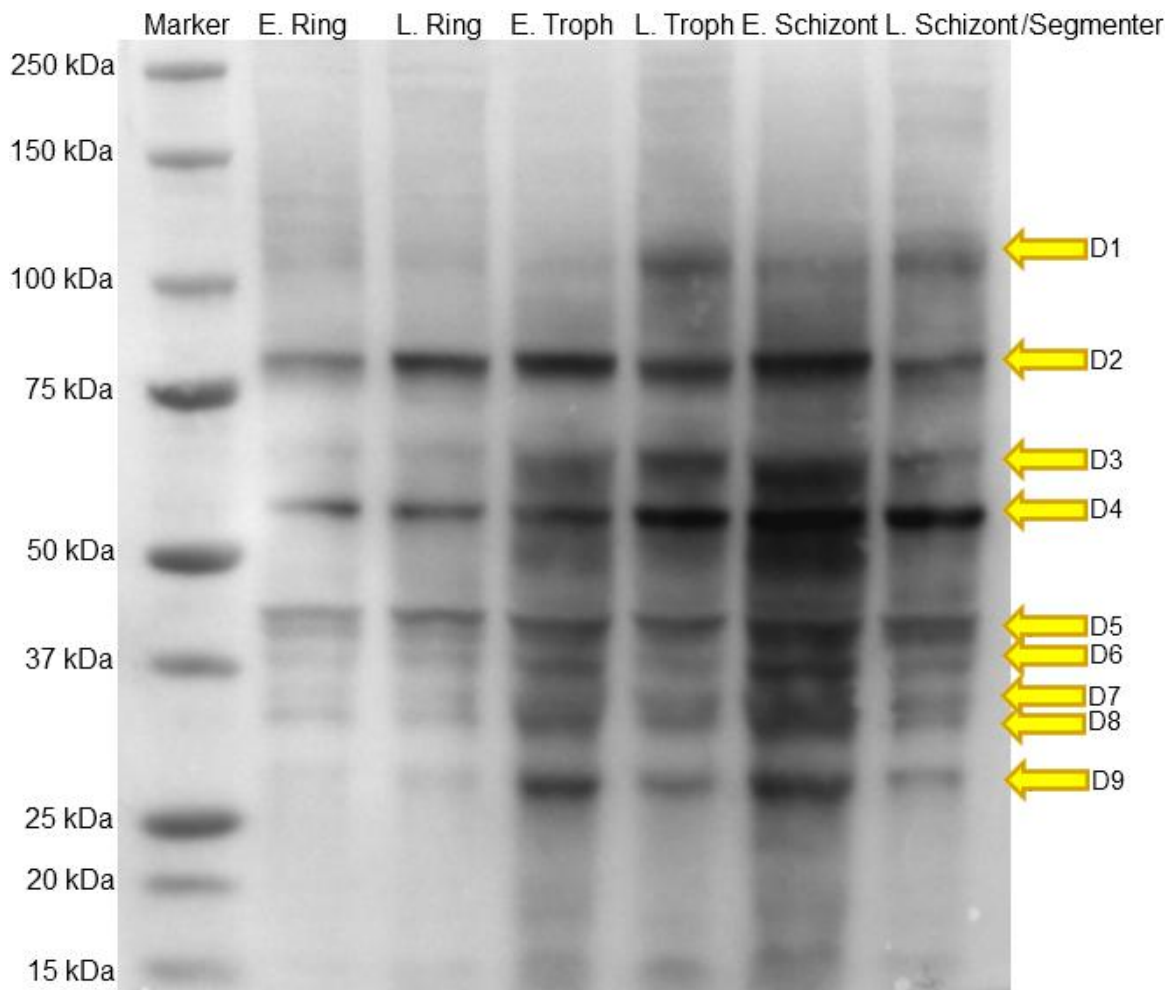


Figure 14: Time point immunoblot using phospho-(Ser) CDK substrate antibody

Table 5: Anti-phospho-(Ser) CDK substrate immunoblot

Label	Estimated Molecular Weight (kDa)	Observed Peak Phosphorylation Stage
D1	115	Trophozoite/Schizont
D2	79	Trophozoite/Schizont
D3	62	Trophozoite/Schizont
D4	58	Trophozoite/Schizont
D5	43	All
D6	39	Trophozoite/Schizont
D7	35	Trophozoite/Schizont
D8	33	Trophozoite/Schizont
D9	27	Early Trophozoite, Early Schizont

DISCUSSION

To summarize, two approaches were taken to identify the substrates and interacting proteins of PfPKA and PfPK5: bioinformatics utilizing phosphoproteomic data and immunoprecipitation (Fig. 15). The global phosphoproteomic dataset was generated in our lab using iTRAQ analysis and mass spectrometry to determine properties of thousands of proteins according to their expression levels and phosphorylation patterns. Changes in phosphorylation during the Ring, Trophozoite, and Schizont stages were quantified in duplicate.

It must be noted that phosphorylation of a protein changes its mass and migration through a gel. For this reason, the estimated molecular weight of each putative PfPKA or PfPK5 substrate may be higher or lower than the actual weight, depending on the structure of the protein. The substrates identified in this study are only putative matches of the proteins listed in the phosphoproteomic dataset and require further investigation.

Phosphoproteomic analysis revealed possible matches for five of the proteins detected in the anti-phospho-(Ser/Thr) PKA substrate time point immunoblot. Of these, three proteins matched the data in both molecular weight and peak phosphorylation stage. Protein B12 demonstrates equal phosphorylation throughout all the stages. The potential match, GTP-binding nuclear protein Ran/TC4, is involved in nucleocytoplasmic transport. Since transport between the nucleus and cytoplasm is an essential function in every stage of the intraerythrocytic cycle, it is very likely that the identity of Protein B12 is Ran/TC4. Protein B4 shows highest phosphorylation in the Trophozoite/Schizont stages. Its putative match is a hypothetical chromosome condenser involved in the cell cycle and DNA processing. As previously described, *Plasmodium* undergoes multiple rounds of DNA synthesis and mitosis beginning in the Trophozoite stage and proceeding through the Schizont stage; therefore it is logical to associate Protein B4

with chromosome condensation during mitosis. Protein B2 demonstrates very high levels of phosphorylation during Early Ring stage. Its putative match, Myosin A, influences *Plasmodium*'s interaction with the environment. Parasites in Early Ring stage are in a new environment, as they are transitioning from being free merozoites to growing inside an erythrocyte. It is a rational conclusion that Protein B2, observed in Early Ring stage, is involved in the parasite's adaptation to the intraerythrocytic environment. Also, considering that the protein phosphorylation is significantly more intense in Early Ring stage than in Late Ring or Late Schizont/Segmenter stage, it can be hypothesized that the protein is also active in the Merozoite stage. Although no match for the molecular weight of B3 was found in the phosphoproteomic dataset, its phosphorylation pattern strongly suggests that it is involved in nuclear activity. The intensity of the band increased steadily from Trophozoite stage through Schizont stage, mirroring the DNA synthesis and mitosis events that occur during those stages. The putative identities of these proteins provide strong evidence that P \mathcal{P} KA is involved in the cell cycle. They also implicate P \mathcal{P} KA in cellular transport and interaction with the environment during the transition from Merozoite to Ring stage. Also, since each of these proteins shows peak phosphorylation at different stages, it can be said that P \mathcal{P} KA exhibits phosphorylation activity in all of the intraerythrocytic stages.

Decreased phosphorylation of the H89-treated culture as compared to untreated culture confirms that H89 is inhibiting P \mathcal{P} KA activity. Additionally, most of the prominent bands detected in the asynchronous control correspond with the strongest bands in the time point immunoblot. This indicates which proteins are most affected by H89 inhibition of P \mathcal{P} KA. Specifically, the data suggests that H89 interferes heavily with the phosphorylation of ubiquitin carboxyl-terminal hydrolase 2, a putative transcription-related protein, a chromosome condensation regulator, and an RNA-binding protein.

Data suggests that a phosphatase involved in protein fate, a putative cellular transport protein, and Myosin A interact with the phospho-(Ser/Thr) PKA substrate antibody strongly enough to be pulled down via immunoprecipitation. Their interaction with the antibody confirms that P ρ PKA influences multiple unrelated pathways throughout the intraerythrocytic cycle. The putative phosphatase is expressed most during Schizont stage and its peak phosphorylation occurs during Ring stage. Phosphorylation can either activate or deactivate a protein; therefore, assuming that the phosphatase is activated by phosphorylation, it may be inferred that this potential substrate of P ρ PKA dephosphorylates proteins involved in erythrocyte invasion or parasite growth and development. If the action of the phosphatase deactivates its target protein, then it is likely that the target protein is no longer necessary and was used in the previous environment. If the action of the phosphatase activates its target protein, then it must be a protein necessary for the parasite to adapt to its new environment within the erythrocyte. The identified putative cellular transport protein is globally expressed, but its phosphorylation peaks in Ring stage. This implies that the protein is involved in transporting nutrients for the growth of the developing parasite. Myosin A, as previously stated, influences the parasite's interaction with its environment, potentially during the transition from Merozoite to Ring stage.

An episomally transfected P ρ PK5-HA cell line was generated and confirmed by immunoblotting. Interestingly, additional bands are observed in the anti-P ρ PK5 immunoblot. This could be caused by the antibody cross-reacting with a similar antigen, such as another CDK-like kinase with high homology to P ρ PK5. It can also be concluded that since P ρ PK5 shares homology with the CDKs, its substrates are likely to be among those detected in the phospho-(Ser) CDK substrate immunoblot. The data supports the hypothesis that the antibody binds P ρ PK5 substrates and allows for prediction of their molecular weights. The peak phosphorylation of most of the detected proteins occurred during the Trophozoite and Schizont

stages, coinciding with the peak expression and activity of PfPK5. The fluctuations of these phosphorylations from one stage to the next are typical of CDK activity, and indicate that the substrates of PfPK5 are involved in cell cycle regulation.

PROSPECTIVE STUDIES

For confirmation of PfPKA substrates, the immunoprecipitation would need to be scaled up using 2-3 times the concentration of malarial lysate and 5 times the concentration of phospho-(Ser/Thr) PKA substrate antibody. This would ensure that there would be enough protein in the elutions to analyze by mass spectrometry. Additionally, new proteins might be isolated by increasing concentrations of antibody and antigen. The proteins would be resolved on a polyacrylamide gel and stained with mass spectrometry-compatible silver stain. Bands of interest could be excised and their protein sequences analyzed by mass spectrometry. This would not only provide the true molecular weight of the protein, but it would also reveal the sequence, allowing for a more detailed study of the protein's structure and its interaction with PfPKA. Another approach would be to perform a co-immunoprecipitation with an antibody against PfPKA itself rather than its phosphorylation motif, and any interacting proteins would be pulled down along with the kinase.

In order to confirm the sequences of PfPK5-interacting proteins, both putative substrates and cyclins, the episomally transfected PfPK5-HA cell line could be used in a series of co-immunoprecipitations: one would use an antibody against PfPK5; a second would use an antibody against the HA tag. If the HA tag is too small to efficiently bind the anti-HA antibody, the recombinant gene could be recloned using a larger tag such as 3X HA or GFP. Because of uneven segregation of plasmids during mitosis, episomally transfected *P. falciparum* provides only a brief window of study before the transfected cell line is lost. For this reason, it would be helpful to integrate the construct into the *Plasmodium* genome. This would require

cloning P f PK5-HA into a plasmid with an attP crossover site. The construct would be transfected along with an integrase plasmid into a cell line containing an attB crossover site, according to the strategy described by Nkrumah et al. [35]. Subcellular localization of integrated recombinant P f PK5 and its endogenous counterpart would be an intriguing comparison.

SIGNIFICANCE OF FINDINGS

The identities of seven different putative P f PKA substrates were predicted using bioinformatics and phosphoproteomic data. Each of the putative substrates varied in role and stage-specificity. There is strong evidence that both P f PKA and P f PK5 are essential in cell cycle-related processes. Though the details of the signaling cascades involving P f PKA and P f PK5 have yet to be elucidated, it is clear that both kinases and the pathways they influence are crucial to the viability of the parasite. Given the divergence of the *Plasmodium* cell cycle from that of other eukaryotes, it is likely that further investigation of P f PKA and P f PK5 will lead to the discovery of novel pathogen-specific targets.

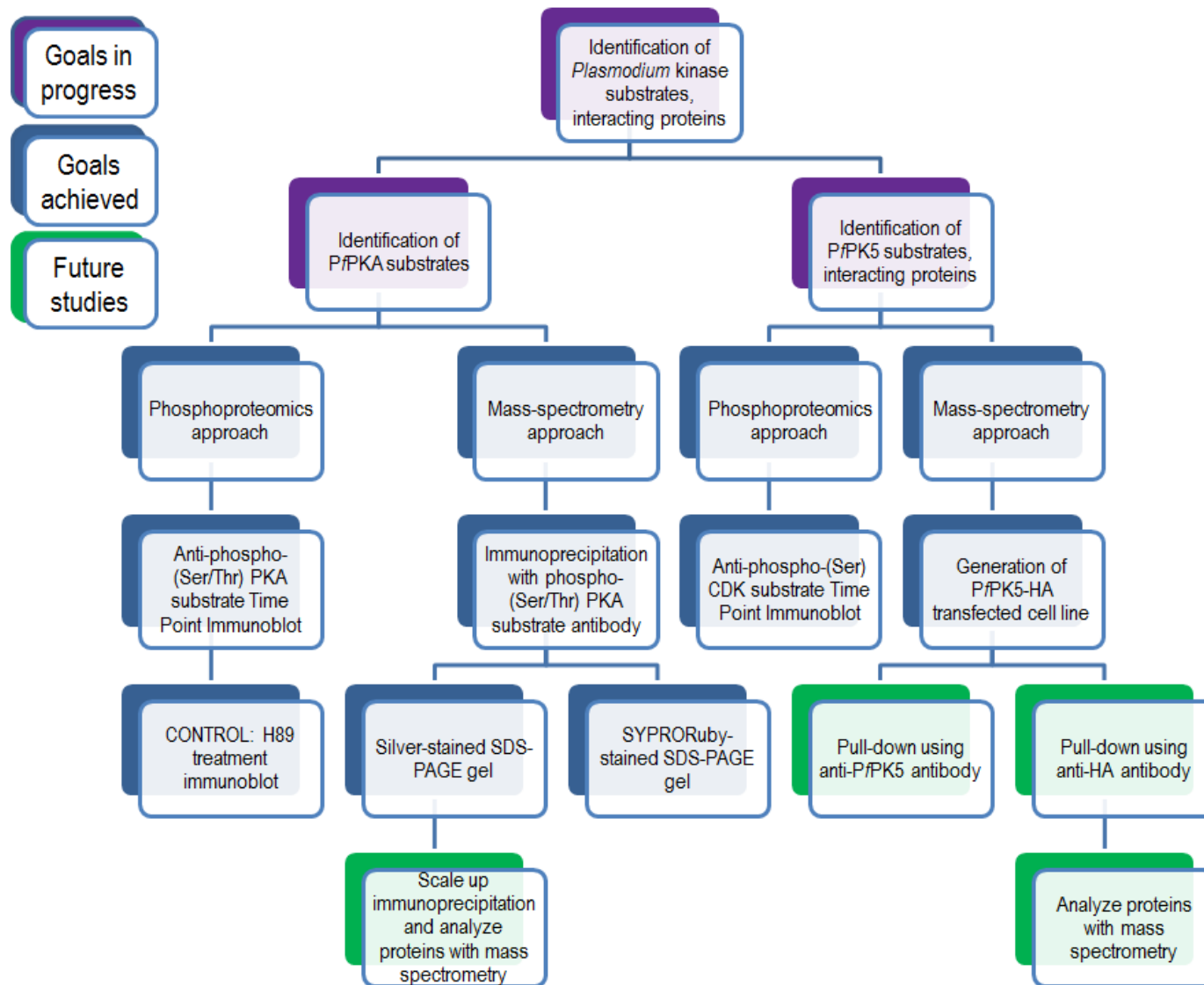


Figure 15: Flow chart of experiments

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