Characterization of PFF1010c, a type IV *Plasmodium falciparum* heat shock protein 40

A thesis submitted in fulfilment of the requirements for the degree

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MASTER OF SCIENCE IN BIOCHEMISTRY

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by

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Abstract

Malaria is caused by protozoa of the genus *Plasmodium.* Malaria accounts for approximately more than half a million deaths yearly. Of the five species of *Plasmodium*, *P. falciparum* accounts for the most deadly form of the disease. *P. falciparum* survives under various physiological conditions during its life cycle. The parasite employs its molecular chaperones machinery particularly heat shock proteins (Hsps) to protect its protein constituents during physiological stress. Hsps are conserved molecules that constitute a major part of the cell's molecular chaperone system. *P. falciparum* Hsps play an important cyto-protective role guaranteeing that the malarial parasite survives under the severe conditions that prevail in the host environment. PFF1010c is a type IV *P. falciparum* heat shock protein 40. PFF1010c is predicted to be expressed only at the gametocyte stage of the malarial parasite's life cycle. The aim of the current study was to investigate the expression PFF1010c by parasites and the gametocyte stage as well as characterize the structure-function features of the protein. PFF1010c was successfully expressed in *E. coli* cells. Despite successful expression of the protein, its purification proved problematic. The attempt to purify PFF1010c was carried out under both native and denaturing conditions. Far Western blot analysis to investigate direct interaction between PFF1010c and PfHsp70-1 was conducted and no interaction was observed. Malarial parasites were harvested at different stages and total protein was isolated. The expression of PFF1010c was confirmed to occur at the gametocyte stage of the parasite's development using Western blot analysis. This study confirmed that PFF1010c is only expressed at the gametocyte stage of the malarial parasite. Furthermore, PFF1010c was not expressed at the asexual stage. Possible interactors of PFF1010c were predicted by STRING, a bioinformatics based tool. The expression of PfHsp90, PfHop and PfHsp70-1 at the gametocyte stage was investigated and confirmed by Western blot analyses.

Declaration

I, the undersigned, declare that this thesis submitted to the University of Venda for the degree of Master of Science in Biochemistry at the Department of Biochemistry in the School of Mathematical and Natural Sciences, is my own unaided work, with exception to citations. This work has not been submitted to any other university in partial or entirely for the award of any degree.

Candidate …………………………………………………………………….

Signature………………………………. Date…………………………

Dedication

This thesis is dedicated to my late parents. May their souls rest in peace.

Acknowledgements

I thank God for giving me the strength to complete the study. I am deeply indebted to my supervisor Prof. Addmore Shonhai for his guidance, expertise and mentorship throughout the project. Your support and encouragement indispensably made it possible for me to finish this study.

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Table of Contents

List of Figures

List of Tables

List of outputs

CONFERENCE PROCEEDINGS

1. **Mutavhatsindi, H.** and Shonhai, A. Characterization of PFF1010c from *Plasmodium falciparum*. University of Venda, Research Open day. 3 March 2015.

List of Symbols

Abbreviations of units	Symbol Interpretation
%	percent
µl	microlitre
A600	absorbance at 600 nanometres
bp	base pair
kDa	kilodalton
µM	micromolar
ºC	degree Celsius
ml	millilitre
l	litres
w/v	weight per volume
v/v	volume per volume
µg	microgram
g	gram
β	beta
α	alpha

Chapter 1

Literature Review

Introduction

1.1 Malaria as a global health burden

Regardless of the scientific advances and efforts which have been made towards the elimination of malaria, it remains one of the leading causes of death, mostly in Africa (WHO, 2015). It was responsible for more than 438 000 deaths in 2015, the most affected being children under 5 years of age (WHO, 2015). Approximately 2 billion people are reported to be at risk of transmission of the malaria parasite (WHO, 2015). The development of drug resistance by malaria parasites continues to surpass efforts to control and eliminate the disease. Resistance to antimalarial drugs was first reported in 1910 in Brazil where *Plasmodium falciparum* parasites were found to be resistant to quinine (Nocht and Werner, 1910). Thereafter, other cases of resistance to antimalarial drugs have been reported (Marfurt et *al*., 2010; Winzeler and Manary, 2014; WHO, 2015). Lately, the use of drug combinations has been proposed in order to overcome resistance to therapy (Winzeler and Manary, 2014; WHO, 2015). For example; artemisinin is an effective alternative when used in combination with other antimalarial drugs such as mefloquine or lumenfantine (Wongsrichanalai *et al*., 2002). Currently artemisinin-based combination therapy (ACT) is recommended for the treatment of *P. falciparum* malaria. Fast acting artemisinin-based compounds are combined with a drug from a different class (WHO, 2015). The companion drugs include lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine, piperaquine and chlorproguanil/dapsone. Artemisinin derivatives include dihydroartemisinin, artesunate and artemether. Implementation of the recommendation to use ACTs is limited by the small number of available and affordable co-formulated anti-malarial drugs, but most countries are now starting to implement this regimen. A co-formulated drug is one in which two different drugs are combined in one formulation (Wongsrichanalai *et al*., 2002; WHO, 2015). Emerging resistance of *P.*

falciparum to artemisinin combination therapy has been reported (Noedl *et al*., 2009; Phyo *et al.,* 2012; Winzeler and Manary, 2014). It has been reported that artemisinin resistance in *P. falciparum* develops at the early ring stage of the parasites (Dondorp *et al*., 2009; Mbengue *et al*., 2015). It is, therefore, important that further research be conducted towards a better understanding of the malaria parasite biology in order to come up with effective disease control and eradication efforts.

1.2 Malaria and life cycle of *Plasmodium*

The malaria parasite vector is the female mosquito of the genus *Anopheles* (Daily *et al*., 2007). The malaria parasites exhibit a heterogeneous life cycle involving a vertebrate host and a phase in an arthropod vector (Figure 1.1; Daily *et al*., 2007).

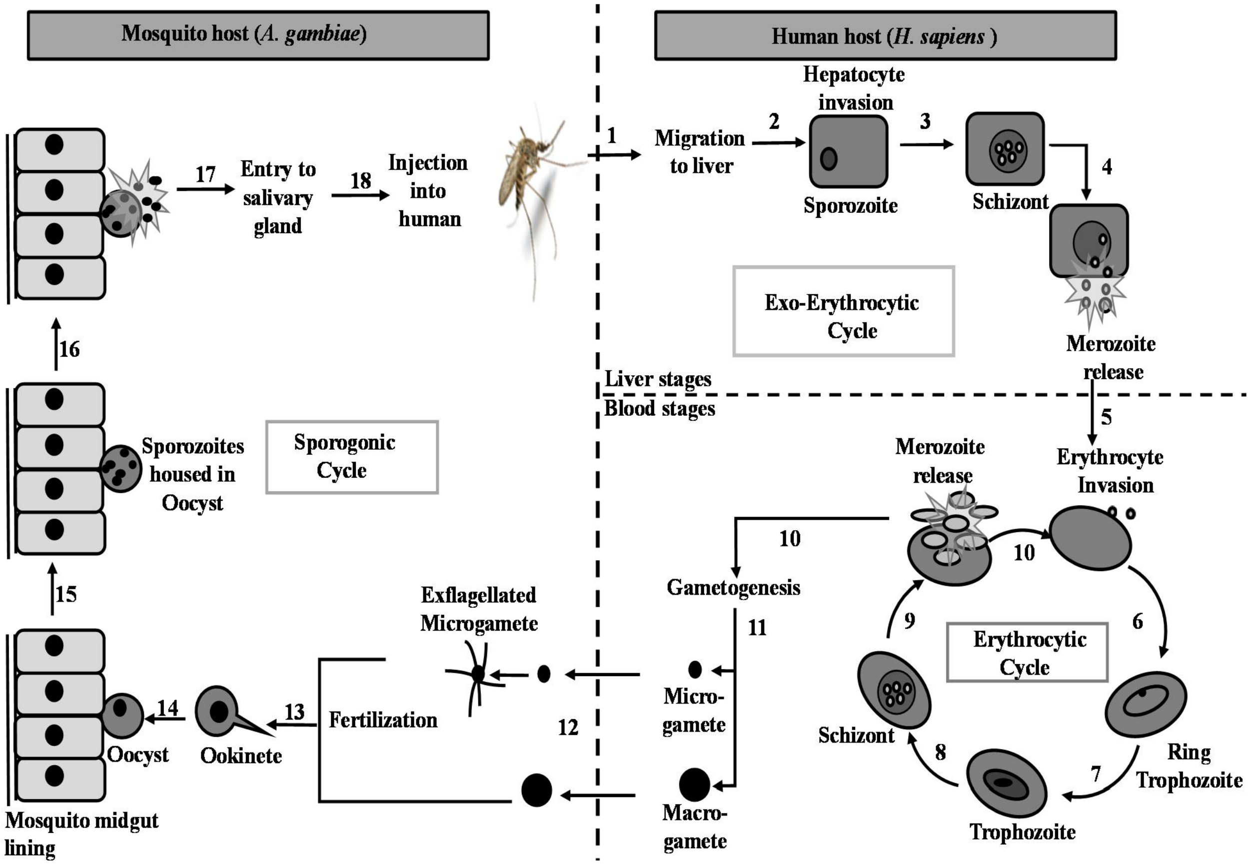


Figure 1.1: Representation of *Plasmodium* life cycle
The schematic representation of *Plasmodium* life cycle showing three distinct stages of malaria progression in the human body, surviving and adapting physiological conditions from two different hosts. Liver stages (1-4) and erthrocytic (blood) schizogony stage (5-11) (human host), sporogonic cycle (12-18) in the mosquito (mosquito host) (Adapted from Fujioka and Aikawa, 2002).

Infection in humans begins with the bite of an infected female *Anopheles* mosquito (Figure 1.1; Stage 1). The sporozoites released from the salivary glands of the mosquito enter the bloodstream during feeding, invading hepatocytes (Figure 1.1; Stage 2). Within the liver cells, the parasite differentiates and then undergoes asexual multiplication, releasing merozoites that burst from the hepatocyte (Figure 1.1; Fujioka and Aikawa, 2002). Individual merozoites invade red blood cells (erythrocytes) and undergo an additional round of asexual multiplication within the schizont (Figure 1.1; Stages 6-10). Some of the merozoites invade uninfected erythrocytes and the erythrocytic cycle continues (Fujioka and Aikawa, 2002). Not all of the merozoites divide into schizonts; some differentiate into sexual forms (male and female gametocytes) (Figure 1.1; Stages 8-10). These gametocytes are taken up by female *Anopheles* mosquitoes during a blood meal (Figure 1.1; Stages 11-12; Fujioka and Aikawa, 2002). Within the mosquito midgut, the male gametocyte undergoes a rapid nuclear division, producing flagellated microgametes that fertilize the female macrogamete. The resulting ookinete traverses the mosquito gut wall and encysts on the exterior of the gut wall as an oocyst (Figure 1.1; Stages 12 -13). Afterwards, the oocyst ruptures, releasing sporozoites, which eventually migrate to the mosquito's salivary glands (Figure 1.1; Stages 14 -18). The clinical manifestations of malaria, fever, and chills are associated with the synchronous rupture of the infected erythrocytes (Fujioka and Aikawa, 2002).

1.3 Gametocyte stage of malarial parasite

The spread of malaria involves the production of gametocytes for transmission from one individual to another *via* a mosquito vector (Figure 1.1; Stage 10; Kuehn and Pradel, 2010). Studies have shown that gametocytes are not efficiently cleared by common antimalarial drugs and hence allow transmission to mosquitoes regardless of the clearance of asexual parasites (Okell *et al*., 2008). Within the human host, erythrocytic stage, parasites can either replicate asexually or differentiate into a single male or female gametocyte (Figure 1.1; Stages 5-11; Eksi *et al*., 2012). For diffusion from human to mosquitoes, a subpopulation of schizonts yield merozoites that attack red blood cells and each differentiate over the course of time into a single male or female gametocyte (Figure 1.1; Stages 2-11; Silvestrini *et al.,* 2000). During the mosquito blood meal, male and female gametocytes are also ingested and gametocytes are stimulated to produce gametes, copulate and develop into sporozoites that are infectious to humans during subsequent blood-feeding, leading to the formation of zygotes which differentiate to form ookinetes (Figure 1.1; Stages 11-14; Lobo and Kumar, 1998; Alano, 2007). *P. falciparum* is comprised of five morphological gametocyte stages (I-V) (Hawking *et al*., 1971). Of the five morphologically distinct gametocyte stages (I to V) (Hawking *et al*., 1991), only one (stage V) is associated with the blood stage (Okell *et al*., 2008).

1.4 Molecular chaperones

Molecular chaperones were first described as nuclear proteins that facilitated disassembly of nucleosomes during amphibian egg formation (Laskey *et al*., 1978). Then Ellis (1987) later extended the term to include proteins that facilitate folding and assembly reactions. Molecular chaperones assist the folding or unfolding and the assembly or disassembly of other macromolecular structures (Mayer and Bukau, 2005; Hartl and Hayer-Hartl, 2009). They facilitate the folding of newly-synthesized proteins (Saibil *et al*., 2013), translocation through membranes, maturation and assembly of proteins, prevention of aggregation, promotion of degradation and they are responsible for most of the cell's response to stress (Eggers *et al*., 1997; Hamman *et al*., 1998; Brodsky and McCracken, 1999; Horváth *et al.,* 2008; Yong, 2009). Most chaperones are heat shock proteins; that is, proteins expressed in response to elevated temperatures or other cellular stresses (Ellis and van der Vies, 1991; Hartl and Hayer-Hartl, 2002). This is because protein folding is severely affected by stress, and therefore some chaperones act to prevent or refold misfolded proteins (Hoffmann *et al*., 2003, Kampinga and Craig, 2010; Burger *et* al., 2014; Zininga *et al*., 2015). Chaperones are also involved in the folding of newly synthesized proteins as they are extruded from the ribosome. Some chaperone systems work as foldases: they support the folding of proteins in an ATP-dependent manner (Hoffmann *et al.,* 2003). Other chaperones work as holdases, and their function is to bind folding intermediates to prevent their aggregation (Hoffmann *et al*., 2003; Kampinga and Craig, 2010). For example, heat shock protein 110 (Hsp110), Hsp40s or small heat shock proteins (sHsps) constitute holdases (Hoffmann *et al*., 2003; Kampinga and Craig, 2010; Burger *et* al., 2014). Molecular chaperones are crucial for the survival of malaria parasites in the human host (Pallavi *et al*., 2010) and their inhibition can result in the disruption of parasite growth (Banumathy *et al* 2003; Dhangadamajhi *et al*., 2010; Shonhai, 2010; Gitau *et al*., 2012; Zininga *et al*., 2015).

1.5 Heat shock proteins

The term heat shock proteins came into use when Ritosa observed that exposure to heat shock led to production of chromosomal puffs in the salivary glands of *Drosophila* (Ritosa, 1962). Some of the most prominent members of Hsps are functionally related and are involved in the folding and unfolding of other proteins (Nathan and Lindquist, 1995). The expression of certain Hsps is up-regulated when cells are exposed to heat stress (De Maio, 1999). The dramatic up-regulation of the Hsps is a key part of the heat shock response. Hsps are found in most living organisms, from prokaryotes to eukaryotes. Hsps are named according to their molecular sizes ranging from 8 – 150 kDa and major subfamilies include the small Hsps, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 which refer to families of Hsps in the order of (40 kDa, 60 kDa, 70 kDa, 90 kDa and 100 kDa) kDa in size, respectively (Raboy *et al*., 1991). Some Hsps are constitutively expressed to maintain cellular homeostasis (Kampinga *et al*., 2009; Pechmann *et al*., 2013). Due to the significant role played by molecular chaperones in maintaining proteome stability, it is not surprising that Hsps also occur in subcellular compartments such as the nucleus, cytosol, mitochondria and the endoplasmic reticulum (ER) (Kampinga *et al*., 2009; Pechmann *et al*., 2013).

1.5.1 Hsp100 proteins

Hsp100, also known as caseinolytic protease (Clp) belongs to the ATPases associated with various cellular activities (AAA) family of proteins (Gottesman *et al*., 1997). These proteins are primarily involved in the disassembly of quaternary structure of polypeptide complexes and are crucial elements required for thermotolerance (Hanson *et al*., 2005; Snider *et al*., 2008). The term "Clp" originated from its role in unfolding proteins before degradation by proteases (Baker and Sauer 2011). Hsp100 has been found to be heat inducible in different systems, for example in bacteria, plants, and parasitic protozoa (Keeler *et al*., 2000). The heat inducibility suggests its cytoprotective role under stressful conditions (Hong and Vierling, 2000; Queitsch *et al*., 2000; Singh *et al*., 2010).

P. falciparum expresses eight Hsp100 genes including PfClpP and PfClpR which lack catalytic serine and histidine amino acid residues (Ramasamy *et al*., 2007; El Bakkouri *et al*., 2010). ClpB was shown to be localized in the parasitophorous vacuole (PV) and is thought to facilitate export of parasite proteins to the infected erythrocyte (Rathore *et al*., 2011). Clp proteins have also been shown to function as heptamer complexes, whose function is essential in apicoplast development (Rathore *et al*., 2010). A typical *P. falciparum* Clp homologue has a general structure consisting of an amino N-terminal domain, two AAA+ modules consisting of conserved sequence known as Walker A and Walker B located within each module (Figure 1.2; El Bakkouri *et al*., 2010). Walker

B is mainly responsible for nucleotide binding, while Walker A is required for ATP hydrolysis (Walker *et* al., 1989). Walker A motif, also identified as the P-loop (phosphate-binding loop) or Walker loop is a motif that is associated with phosphate binding (Henson and Whiteheart, 2005; El Bakkouri *et al*., 2010). Walker A motif is best recognized for its presence in nucleotide-binding proteins, and a variety of proteins with phosphorylated substrates (Walker *et* al., 1989; Ramankrishnan *et al*., 2002). Walker B motif is a motif in most P-loop proteins situated well downstream of the Walker A-motif.

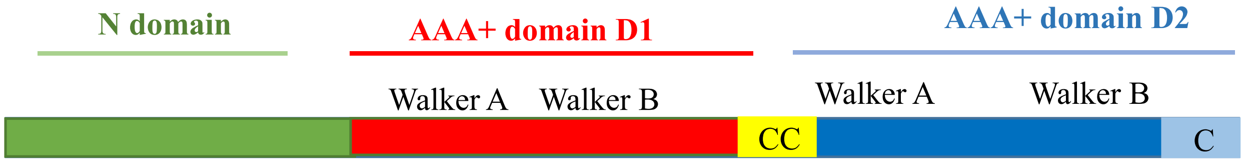


Figure 1.2: The domains of a typical *P. falciparum* Clp homologue. The figure shows the amino N-terminal domain (green) linked to the first AAA+ domain D1 (red) and second AAA+ domain D2 (blue) linked to the first AAA+ domain D1. The two AAA+ domains both consist of a conserved sequence known as walker A and walker B. The D1 domains of ClpB and ClpC chaperones are characterized by the presence of a coiled-coil insertion the middle domain also named "arm" (CC in yellow). D2 domains usually contain a C-terminal module (light blue) (Adapted from El Bakkouri *et al*., 2010).

1.5.2 Hsp90 proteins

The Hsp90 group of chaperones is highly conserved from bacteria to mammals (Csermely *et al* 1998; Louvion 1998). Hsp90 is one of the most abundant families of Hsps (Lindquist and Craig 1988). All known Hsp90s form homodimers (Wegele *et al*., 2004). Hsp90 is found in the cell in a multichaperone complex containing Hsp70 and other co-chaperones like Hop and Hsp40 (Wegele *et al*., 2004). All Hsp90s consist of an N-terminus ATP binding region, a charged linker, a middle domain and a C-terminus dimerization domain with an MEEVED motif (Figure 1.3; Krukenburg *et al*., 2011). Hsp90 has two ATP binding sites located within the N- and C-terminal domains and

ATP hydrolysis is critical for proper functioning of Hsp90 (Tarasawa *et al.,* 2005). The C-terminal domain is specifically responsible for Hsp90 dimerization (Figure 1.3; Nemoto *et al.,* 1995), and all three domains combined are responsible for peptide binding (Figure 1.3; Scheibel *et al* 1999; Sato *et al.,* 2000; Tarasawa *et al.,* 2005).

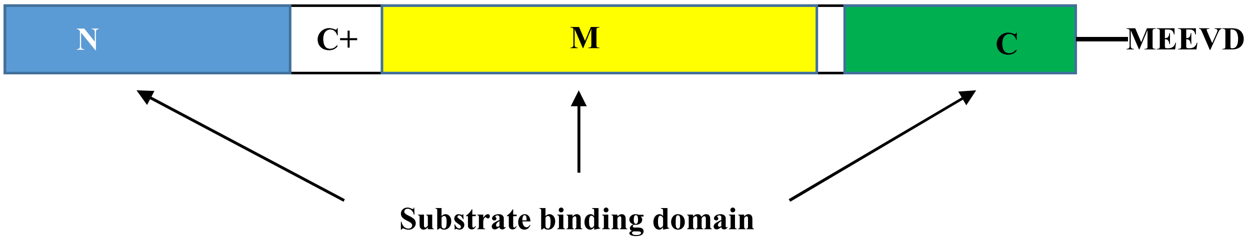


Figure 1.3: Structural domains of Hsp90. Schematic drawing of Hsp90. Hsp90 consists of three domains: an N-terminal ATP-binding domain (N); a middle domain (M); and a C-terminal dimerization domain (C) with the pentapeptide MEEVD sequence. A charged region is located between the N and M domains (C+). All three domains are reported to interact with substrate proteins (Adapted from Tarasawa *et al*., 2005).

Hsp90 modulates functions of native proteins and is a conserved regulator of key protein kinases and nuclear receptors that control the cell cycle and signal transduction events (Lindquist 2009; Taipale *et al.*, 2010). Hsp90 chaperones are crucial in eukaryotes but are not essential at all growth conditions in *E. coli* (Banumathy *et al.*, 2003; Cao *et al.*, 2003; Maynard *et al.*, 2010). Hsp90 group is composed of five sub-families in different cellular locations: cytosolic Hsp90s, ER Hsp90s (Grp94), mitochondrial TRAP1, chloroplast Hsp90 and bacterial Hsp90 HtpG (Jackson *et al.*, 2013). The ADP- bound state of Hsp90 is referred as ‘relaxed’ form which is ideal for client protein loading and the ATP-bound state is referred as ‘closed’ conformation which is capable of retaining the substrate (Csermely *et al.*, 1993). The substrate is released by converting ATP to ADP on Hsp90 (Siligardi *et al.*, 2002). In eukaryotes, function of Hsp90 requires interaction with a network of co-chaperones. This results in a multi-proteins complex that can load specific clients on Hsp90

(Bose *et al*., 1996; Vaughan *et al*., 2006). Several co-chaperones associated with Hsp90 through its tetratricopeptide repeat (TPR) motifs. TPR domains are motifs that consist of highly degenerate repeats of 34 amino acids that are present 1-16 times per domain (Lamb *et al*., 1995).

P. falciparum genome encodes for four Hsp90 homologues (Banumathy *et al*., 2003; Pallavi *et al*., 2010); which are endoplasmic reticulum Grp94 homologue, full length cytosolic Hsp90 homologue, endoplasmic reticulum Grp94 homologue, mitochondrial TRAP1 and a truncated Hsp90 without the cytosolic signal (Kumar *et al*., 2003; Acharya *et al*., 2007). PfHsp90α (PfHsp90) has been shown to function as a dimer and hydrogen bonding could play an important role in the interaction of the two monomers (Kumar *et al*., 2007). PfHsp90 is proposed to play a role in regulating the activities of transcription factors hence translation through its predicted association with proteins such as aspartyl- tRNA synthetase, ribosomal proteins S3A and methionine t-RNA ligase (Banumathy *et al*., 2003; Pavithra *et al*., 2007). According to the bioinformatics based interactome analysis, PfHsp90 is proposed to associate with chromatin assembly factor 1, p55 subunit, histone deacetylase and nucleosome assembly protein (Pavithra *et al*., 2007). PfHsp90 is, therefore, implicated in protein translation (Pavithra *et al*., 2007). Oakley and colleagues (2007) showed that PfHsp90 is highly abundant at the late ring stage of *P. falciparum.* It has been shown to have a crucial function in the survival of *P. falciparum* in the red blood cells especially during the asexual phase (Banumathy *et al*., 2003; Kumar *et al*., 2003). PfHsp90 was upregulated to approximately 2.4 fold when parasites growing *in vitro* were subjected to heat stress at 41 ºC (Oakley *et al*., 2007). PfHsp90 is therefore thought to promote the development of *P. falciparum* particularly during the malaria fever stage (Pavithra *et al*., 2004). The febrile temperature is thought to promote the expression of PfHsp90 which further augments the parasite's growth (Kumar *et al.*, 2007). The expression of Hsp90 in clinical *P. falciparum* isolates has been presented thus suggesting its possible role in the parasite growth and survival in the host cell (Pallavi *et al.*, 2010).

1.5.3 Hop

Heat shock organizing protein (Hop), also known as stress inducible protein 1 (STI1), was first identified in yeast during genetic screening of proteins involved in the heat shock response (Nicolet *et al.*, 1989). Hop facilitates the alliance of Hsp70 and Hsp90 in the folding of kinases and development of steroid hormone receptors (Scheufler *et al.*, 2000; Schmid *et al.*, 2012). Johnson and colleagues (1996) proposed that Hsp70 and Hsp90 do not interact with each other in the absence of Hop, thus Hop is responsible for the constitution of the Hsp70-Hop-Hsp90 complex (Johnson *et al.*, 1996). Hop stimulates Hsp70 ATPase activity therefore serving as a nucleotide exchange factor for Hsp70 (Gross *et al.*, 1996). Hop mediates the interaction of Hsp70 and Hsp90 through specific tetratricopeptide-repeat (TPR) rich (Scheufler *et al.*, 2000). Hop contains three tetratricopeptide repeats (TPR): TPR1, TPR2A and TPR2B (Figure 1.4; Lässle *et al.*, 1997).

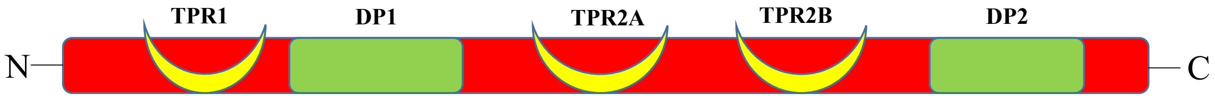


Figure 1.4: Schematic illustrating various domains of Hop. "N" represents the N-terminus and "C" represents the C-terminus. Hop is comprised of three tetracopeptide domains: TPR1, TPR2A, TPR2B and two dipeptide repeats (DP): DP1 and DP2. (Adapted from Zininga *et al.*, 2015).

P. falciparum genome encodes for one Hop homologue (PF3D7_1434300) (Acharya *et al*., 2007). PfHop co-localizes with PfHsp70-1 and PfHsp90 in the parasite cytosol (Gitau *et al*., 2012; Zininga *et al*., 2015). Banumathy and colleagues (2003) observed PfHsp90 and PfHsp70-1 as a functional unit complexed to two species of protein of about 50 and 60 kDa. Gitau and colleagues (2012) reported the existence of PfHop together with PfHsp90 and PfHsp70-1 in a complex based on size exclusion chromatographic analysis. Zininga and colleagues (2015) reported direct interaction of PfHop and PfHsp70-1 based on far Western analyses, they also reported that the interaction of PfHop with PfHsp70-1 occurs *via* TPR1 and TPR2B domains. The TPR2A subdomain of PfHop appears only to mediate interaction with PfHsp90 (Zininga *et al*., 2015).

1.5.4 Hsp60 proteins

Hsp60 chaperonin also known as 60 kDa chaperonin (Cpn60), GroEL/ES, mitochondrial P1 protein (McCallum *et al*., 2000) consists of a family of two proteins: prokaryotic GroEL/ES and eukaryotes t-complex polypeptide 1 (TCP-1) also known as chaperonin containing TCP-1(CCT) localized in the cytosol where it is involved in folding nascent polypeptides such as actin as they exit the ribosome (McCallum *et al*., 2000; Inobe *et al*., 2008). Hsp60 monomers consist of a complex arranged as two stacks of heptameric rings (Hartl and Hayer, 2002). Each heptamer consists of nucleotide-binding domain, a hinge domain and C-terminus substrate binding domain (Inobe *et al*., 2008). Substrate binding is subsequently followed by binding of ATP and Hsp10/GroES which serves as the lid (Hartl and Hayer, 2002). The *P. falciparum* genome codes for two PfHsp60 homologues PF3D7_1015600 and PF3D7_1232100 localized in mitochondria and the apicoplast, respectively (Sato and Wilson, 2004; Sato *et al*., 2005). Although the two isoforms differ in their molecular weight of 62 kDa and 81.6 kDa, they contain a putative mitochondrial targeting sequence (Holloway *et al*., 1994; Syin and Goldman, 1996). The role of PF3D7_1015600 in trafficking of proteins into the mitochondrial matrix has been suggested due to its distinct distribution in the mitochondria of gametocytes (Das *et al*., 1997; Sato *et al*., 2005).

1.5.5 Hsp70 proteins

Hsp70 forms one of the major heat shock protein families. Hsp70s occur in all domains of life archaea, eubacteria and eukaryotes (Lindquist and Craig 1988). In bacteria Hsp70s are referred to as DnaK, in yeast they are named Ssa (Kampinga *et al*., 2009), in mammals they are named as HSPA (Kampinga *et al*., 2009). Generally Hsp70s are induced in response to stress, although some Hsp70 species are constitutively expressed in cells (Bakau and Horwick, 1998). There are four prominent members of Hsp70 family proteins, which includes the constitutively-expressed cytosolic heat shock cognate 70 (Hsc70), heat inducible cytosolic heat shock protein 70 (Hsp70), endoplasmic reticulum heat shock protein 70 (ERHsp70), and mitochondrial heat shock protein 70 (mHsp70). Hsp70's highly conserved structure is made up of two major functional domains: N-terminal ATPase domain (45 kDa) which binds ATP and hydrolyzes it to ADP (Szabo *et al*., 1994; Bukau and Horwich, 1998). The exchange of ATP for ADP drives conformational changes in the other two domains. The substrate binding domain (SBD) (25 kDa) at the C-terminus, separated by a highly conserved hydrophobic linker region (Figure 1.5; Mayer and Bukau, 2005). The C-terminus is further subdivided into a β-sandwich subdomain of 15 kDa with a peptide-binding cleft and a C-terminus lid (α-helical subdomain) (Mayer and Bukau, 2005). The SBD contains a groove with an affinity for neutral, hydrophobic amino acids (Figure 1.5). The groove is long enough to interact with peptides up to seven residues in length (Mayer and Bukau, 2005). The C-terminal domain is rich in alpha helical structure and acts as a 'lid' for the substrate binding domain (Figure 1.5). In the ATP bound state Hsp70 has fast on-off rates resulting in low affinity for substrate and in the ADP bound state there is slow on-off rates that results in overall high affinity for substrate (Figure 1.7). When Hsp70 proteins are bound to ADP, the lid is closed, and peptides are tightly bound to the SBD. Hsp70 binds to peptide substrate, allowing it to refold, followed by a release of the substrate in ATP-expending cycles (Figure 1.7; Szabo *et al*., 1994). Found only in Hsp70 members of the eukaryotic cytosol, at the C-terminus is the EEVD motif (Figure 1.5). The EEVD motif is involved in the binding of Hop and C-terminus of Hsp70 interacting protein (CHIP), which are tetratricopeptide repeat (TPR) cofactors (Scheufler *et al*., 2000). The EEVD motif has been illustrated to play regulatory roles in the functions of Hsp40/Hsp70 (Freeman *et al*., 1995). Hsp70s are divided into two subfamilies: DnaK-like (canonical Hsp70s) and Hsp110 (Mogk *et al*., 1999). DnaK is the bacterial homologue of Hsp70. DnaK refolds misfolded protein and suppresses protein aggregation (Mogk *et al*., 1999). DnaK and DnaK-like Hsp70s from other species are considered to be canonical Hsp70s. Hsp110 members are unique proteins that are structurally different from canonical Hsp70s. Hsp110s inhibit protein aggregation by functioning as substrate holdases (Goeckeler *et al*., 2002; Zininga *et al*., 2015). They have been termed as nucleotide exchange factors (NEF) of Hsp70s (Dragovic *et al*., 2006; Andreasson *et al*., 2008). NEF are proteins that stimulate the exchange (replacement) of nucleoside diphosphates for nucleoside triphosphates bound to other proteins. NEFs actively assist in the exchange of depleted nucleoside diphosphates for fresh nucleoside triphosphates (Johan *et al*., 1998; Zininga *et al*., 2015; Figure 1.7).

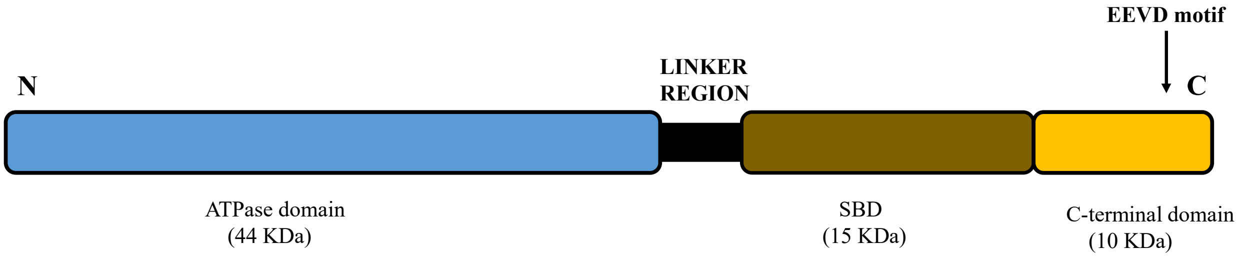


Figure 1.5: Structural domains of Hsp70. The N-terminal consist of ATPase domain and the central substrate binding domain (SBD) are separated by the linker region. The C-terminal domain of cytosolic Hsp70 proteins typically contains a highly conserved EEVD motif. (Adapted from Shonhai *et al.*, 2009).

1.5.6 Hsp40 proteins

Hsp40s also known as DnaJ chaperones, are J domain containing molecular chaperone proteins (Qiu *et al*., 2006). Hsp40 members are proteins that are known to coordinate the protein-folding function of the Hsp70 members (Fan *et al*., 2003). Hsp70 and Hsp40 proteins are often co-localized permitting multiple Hsp40 members to combine with one Hsp70 member forming distinct Hsp70-Hsp40 pairs at different cellular locations (Caplan *et al*., 1992; Ungermann *et al*., 1994). Hsp40s are also known as DnaJ in prokaryotes and consists of a number of unique family members that are classified by the presence of a highly conserved J-domain (Walsh *et al*., 2004; Hennesy *et al*., 2005). They deliver the substrate protein to Hsp70 and modulate the Hsp70 activity by interacting with its substrate-binding and ATPase domains (Young *et al*., 2004). Typically, Hsp40s contain the highly conserved J-domain which is fundamental for successful interactions between Hsp40s and Hsp70s (Walsh *et al*., 2004; Hennesy *et al*., 2005). The J-domain is approximately 70 amino acids long and composed of four α-helices (I-IV) (Hennesy *et al*., 2005). It is characterised by the presence of the highly conserved Histidine-Proline-Arginine (HPD) motif which enables Hsp40s to stimulate the ATPase activity of Hsp70s (Tsai and Douglas, 1996). In addition to the N-terminal J-domain, a canonical Hsp40 possesses a Glycine/Phenylalanine rich region (GF domain), a cysteine-rich zinc-binding domain and a C-terminal substrate-binding domain (Figure 1.6; Cheetham and Caplan, 1998). According to their domain organisation, Hsp40s are classified into four types: type I, type II, type III and type IV. Type I Hsp40s are characterized by all four domains: the J-domain, the glycine/phenylalanine rich domain, the cysteine-repeat region and a C-terminal region. Type II Hsp40s are similar to type I however, they lack the cysteine-rich zinc binding domain. Type III Hsp40 molecules are only characterized by the J-domain, which in this type is not necessarily located at the N-terminus, it can be located anywhere in the molecule. (Figure 1.6; Rug and Maier, 2011; Njunge *et al.*, 2013). Type IV Hsp40s were possess the J-domain with variations within the highly conserved HPD motif (Figure 1.6; Rug and Maier, 2011; Botha *et al.*, 2007; Njunge *et al.*, 2013).

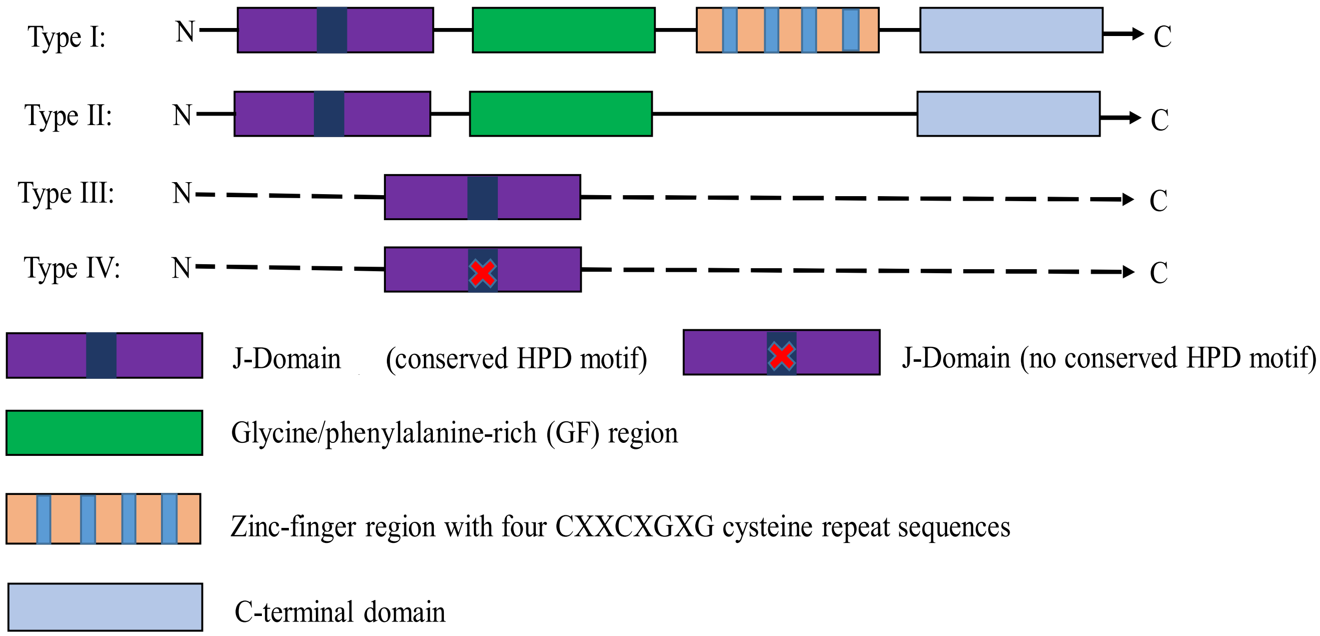


Figure 1.6: Classification of Hsp40 according to their domain organisation
Hsp40 proteins are classified into four subtypes based on the conservation of four domains: the J-domain with a highly conserved His-Pro-Asp (HPD) motif, glycine/phenylalanine-rich region (GF-rich domain), a zinc-finger domain containing four cysteine/glycine repeats and a variable C-terminal substrate binding domain. Type I Hsp40 proteins possess all four domains, type II Hsp40 proteins lack the zinc-finger domain and type III and IV have only the J-domain, with type IV exhibiting variations in the HPD motif (Adapted from Botha *et al.*, 2007).

1.5.7 The Hsp70/Hsp40 partnership

The formation of a complex between Hsp70 and polypeptide substrates is controlled by members of the Hsp40 family and nucleotide exchange factors. Hsp40 regulate complex formation between Hsp70 and polypeptide using three approaches. Hsp40s take advantage of their polypeptide binding domain (PPDs) which bind and transport specific clients to Hsp70, hence regulating the functional specificity of Hsp70 (Cyr *et al*., 1994; Cheetham and Caplan, 1998). Hsp40 stabilizes Hsp70 polypeptide complex by conversion of Hsp70 from its ATP state to ADP form (Langer *et al*., 1992; Landry, 2003; Wittung-Stafshede *et al*., 2003; Jiang *et al*., 2007; Li *et al*., 2009). Hsp40s interact with Hsp70s enabling different Hsp70-Hsp40 pairs within cellular compartments to bind complexes (Brodsky and Schekman, 1993; Cyr and Neupert, 1996; Shen *et al*., 2002). The functional cycle of Hsp70 is conducive to produce folded substrates owing to the fact that when the substrate is bound to the complex, aggregation is prevented. During folding or refolding of substrates, incomplete folding results in the substrate re-entering the cycle (Kampinga and Craig, 2010). The functional partnership of Hsp70/Hsp40 is driven by ATP hydrolysis (Figure 1.7; Patury *et al*., 2009). The Hsp70/Hsp40 functional partnership alternates between the low affinity of the ATP-bound state with rapid substrate-exchange rates, and the high affinity ADP-bound state with low substrate-exchange rates (Figure 1.7; Liberek *et al.,* 1991; Palleros *et al.,* 1993; McCarty *et al.,* 1995; Kampinga and Craig, 2010). The J-domain of Hsp40 is required to excite the ATPase activity of Hsp70 and results in Hsp70-ADP with a high affinity for substrate (Figure 1.7). The functional cycle of Hsp70 is regulated by two co-chaperones Hsp40 and NEFs. After Hsp40 presents the misfolded protein to Hsp70, Hsp70 binds its short peptide region to the protein; the interaction is stabilized by both Hsp40 and a NEF (Hip protein) (Figure 1.7). For the nucleotide binding cleft to be opened, the release of ADP is facilitated by another NEF (Bag-1 protein) (Figure

1.7). The ATPase cycle is completed when ATP binds the ATPase domain of Hsp70 resulting in a conformational change in the substrate binding domain and bound substrates are released (Figure 1.7; Brodsky and Bracher, 2007).

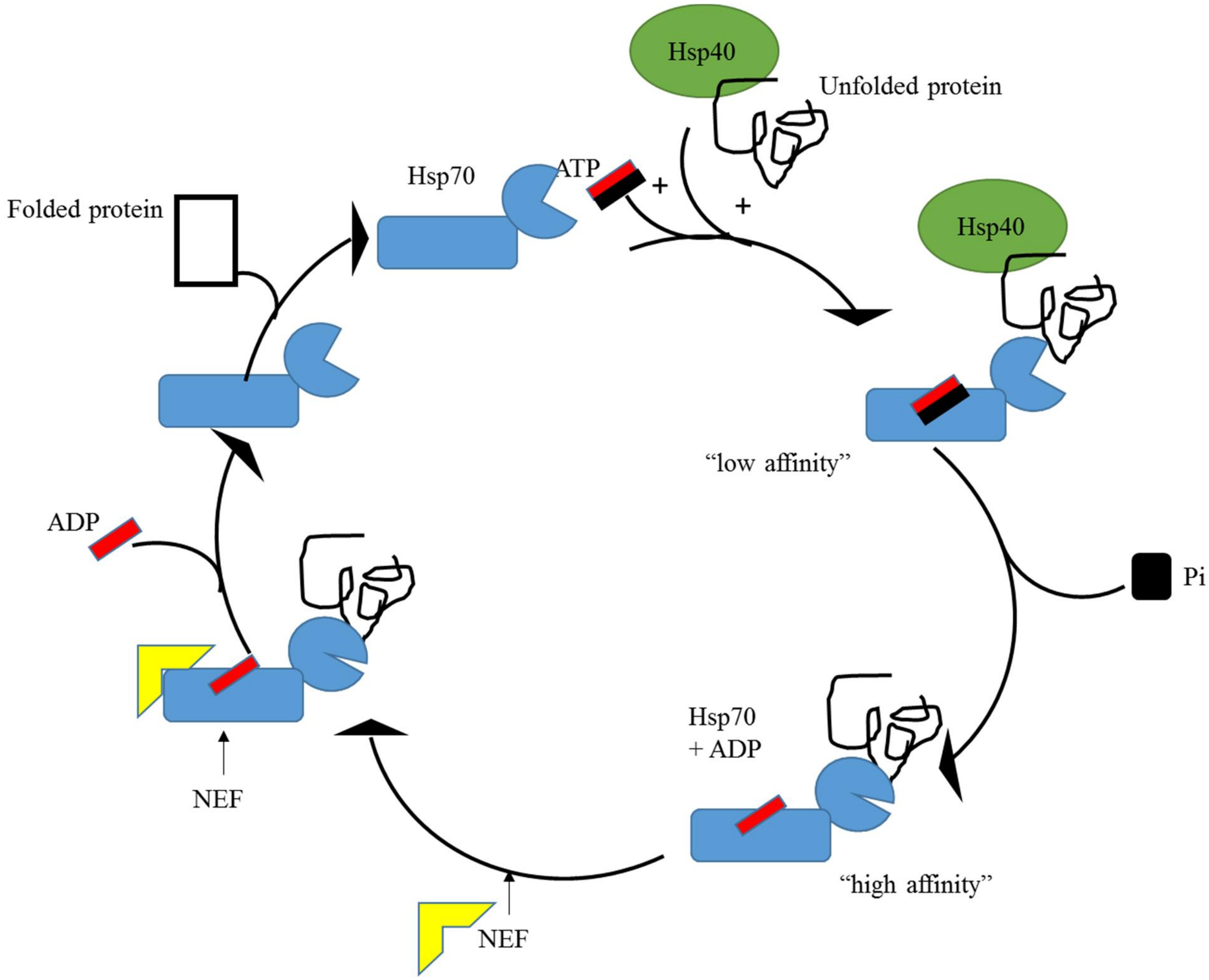


Figure 1.7: The Hsp70 folding cycle

Hydrolysis and exchange of ATP facilitates the Hsp70 folding cycle of substrate binding and release. ATP hydrolysis and nucleotide exchange mediates the Hsp70 folding cycle of substrate release and binding. Substrates are recruited by Hsp40 and they activate ATP hydrolysis which then translates to ADP bound state with high affinity substrate binding, this stage where the substrate is allowed to refold. After which the NEFs exchange ADP for ATP resulting in folded substrate release, adapted from Patury *et al*., (2009).

1.5.8 *P. falciparum* Hsp70 proteins

P. falciparum encodes for six Hsp70 homologues, which are localized in different cellular compartments (Table 1.1; Shonhai *et al*., 2007). These homologues have been implicated in several roles in the parasite, ranging from cyto-protection (Matambo *et al*., 2004; Shonhai *et al*., 2005), to the progression of the exo-erythrocytic schizogony. The distinct localization of Hsp70s serves for role specialization (Table 1.1).

Table 1.1 Classification, localisation and functions of Hsp70s in *P. falciparum*

Features	**Molecular mass**	**Localization**	**Function**
PfHsp70-1[a,f] (PF3D7_0818900)	74	Nucleus, cytosol[i]	Protein folding[h,j,k]
PfHsp70-2[b,c] (PF3D7_0917900)	73	ER[b]	Import of *P. falciparum* protein into ER[g] Protein folding and quality control in the ER[g]
PfHsp70-3[a] (PF3D7_1134000)	73	Mitochondrion[d,e,g]	Import of proteins into the mitochondrial matrix[g,m]
PfHsp70-x[g] (PF3D7_0831700)	76	RBC cytosol and J dots[l]	Export and folding of *P. falciparum* proteins into the infected RBC[i]
PfHsp70-y[g] (PF3D7_1344200)	108	ER[d]	NEF of PfHsp70-2[g]
PfHsp70-z[g] (PF3D7_0708800)	100	Cytosol[n]	NEF of PfHsp70-1[g] Prevents aggregation of a *P. falciparum* Asparagine repeat containing protein[n]

ER- endoplasmic reticulum, RBC- red blood cell, NEF- nucleotide exchange factors. The superscript letters represent the following references; a - Sharma, 1992; b -Kumar *et al*., 1991; c -Nyalwidhe and Lingelbach, 2006; d -Sargeant *et al*., 2006, e -Šlapeta and Keithly, 2004, f -Shonhai *et al*., 2005, g -Shonhai *et al*., 2007, h - Shonhai *et al*., 2008, i - Pesce *et al*., 2008, j -Bell *et al*., 2011, k -Stephens *et al*., 2011, l -Külzer *et al*., 2012 , m - Njunge *et al*., 2013 and n - Muralidharan *et al*., 2012 (Adapted from Shonhai *et al*., 2007).

The most abundant and well-characterised of the Hsp70 homologues from *P. falciparum* include the, PfHsp70-1 protein, which is up-regulated in erythrocytic-stage parasites in response to heat shock (Biswas and Sharma, 1994; Joshi *et al*., 1992). The protein was initially identified as a 75 kDa, protein termed 'p75' on the surface of the merozoite stage of the parasite (Ardeshir *et al*., 1987). Subsequent work has shown that the protein is among the most abundant proteins paralogous to *P. falciparum* (Patankar *et al*, 2001; Le Roch *et al*., 2003) and localizes to the parasite's cytosol, nucleus (Kumar *et al*., 1991) and PV (Nyalwidhe and Lingelbach, 2006). PfHsp70-1 has been shown to occur in complex with PfHsp90, suggesting a related role in the cyto-protection, growth and development of the parasite (Banumathy *et al*., 2003; Gitau *et al*., 2012).

The chaperone capabilities of PfHsp70-1 have been validated experimentally (Matambo *et al*., 2004; Ramya *et al*., 2006; Shonhai *et al*., 2008; Zininga *et al*., 2015) and the protein has been shown to suppress the thermo-sensitivity of a DnaK mutant strain of *E. coli*, consistent with its proposed role in cyto-protection (Shonhai *et al*., 2005). Interestingly, domain-swapping experiments have revealed that the substrate binding domain, but not the ATPase domain, of PfHsp70-1 is functionally equivalent to that of *E. coli* DnaK (Shonhai *et al*., 2005). A 73 kDa homolog of the endoplasmic reticulum Hsp70, BiP (termed PfBiP, PfGrp78 or PfHsp70-2) is conserved in the *Plasmodium* genus (De Oliveira-Ferreira *et al*., 1999) and is reported to be up-regulated in the intra- and exo-erythrocytic stages of *P. falciparum* in response to heat shock (Kumar and Zheng, 1992; Sharma, 1992). Recently, this protein has been implicated in interactions with exported proteins in the stages prior to export (Saridaki *et al*., 2008). Interestingly, both PfHsp70-1 and PfBiP are thought to undergo phosphorylation during the course of the parasite's intraerythrocytic development (Kappes *et al*., 1993). PfHsp70-3 another Hsp70 homolog has similarly been identified in the asexual blood-stages of the parasite (Sharma, 1992) and is suggested to localize to the mitochondria and PV (Nyalwidhe and Lingelbach, 2006; Šlapeta and Keithly, 2004). PfHsp70-3 has been shown to interact with asparagine rich proteins (La Count *et al*., 2005). PfHsp70-x is another close homolog of PfHsp70-1. PfHsp70-x localizes in the PV and is exported to the infected red blood cells (iRBC) cytosol (Külzer *et al*., 2012). PfHsp70-x has been shown to be expressed and exported during the developmental stages of the parasite's blood stage (Külzer *et al*., 2012). The export of parasite proteins to the host red blood cells (RBC) cytosol is thought to be facilitated by the presence of an export signal of the protein sequence (PEXEL/VTS) (Hiller *et al*., 2004; Marti *et al*., 2004). PfHsp70-z is cytosol localized and is involved in the suppression of heat induced aggregation of asparagine rich proteins (Zininga *et al*., 2015).

1.5.9 *P. falciparum* Hsp40 proteins

Bioinformatics analysis has revealed that the *P. falciparum* genome encodes at least 49 Hsp40s (Njunge *et al*., 2013). Interestingly, the parasite Hsp40 machinery exhibits a further Hsp40 type referred to as type IV (Table 1.2; Botha *et al*., 2007). Type IV Hsp40s contain a J-domain like other Hsp40s, but the HPD motif is not conserved (Figure 1.5; Botha *et al*., 2007). However, some Hsp40s are capable of binding to misfolding proteins to prevent their denaturation (Botha *et al*., 2007). Nineteen of these proteins are predicted to be "exported" to the host erythrocyte and are implicated in erythrocyte remodelling (Sargeant *et al*., 2006). The export of parasite proteins to the host red blood cells (RBC) cytosol is thought to be facilitated by the presence of a five amino acid sequence (RxLxQ/E) termed the *Plasmodium* export element (PEXEL) or vacuolar targeting signal (VTS) (Hiller *et al*., 2004; Marti *et al*., 2004). The PEXEL motif was proposed to be processed in the ER, and this cleavage appears to be a requirement for export because mutations in this motif revokes both processing and export of PEXEL-containing proteins (Chang *et al*., 2008; Boddey*et al*., 2009). Some exported proteins, lack the PEXEL/VTS motif (Templeton and Dietch, 2005), and their export signals have not been identified. The export status of certain of these Hsp40 proteins has been verified experimentally (Brown *et al*., 1985; Coppel *et al*., 1988; Hiller *et al*., 2004; Sargeant *et al*., 2006; Njunge *et al*., 2013). Of these four known types of paralogous *P. falciparum* Hsp40s, 2 are type I, 9 are type II, 26 are type III and 12 are type IV (Table 1.2).

Table 1.2 Types of Hsp40s that occur in *P. falciparum*

Types	**Quantity in *P. falciparum***	**Characteristics**	**References**
Type I	2	N-terminal, J-domain, a glycine/phenylalanine-rich (GF-rich) domain, a Zinc-finger domain and a C-terminal substrate binding domain, conserved HPD motif	Watanabe, 1997; Cheetham and Caplan, 1998; Nicoll *et al*., 2007
Type II	9	N-terminal, J-domain, the flanking GF-rich region as well as the C-terminal substrate binding domain and conserved HPD motif	Cheetham and Caplan, 1998; Bhattacharjee *et al*., 2008; Maier *et al*., 2008; Külzer *et al*., 2010
Type III	26	J-domain, which is not necessarily at the N-terminus and a conserved HPD motif	Cheetham and Caplan, 1998; Young *et al*., 2004; Hosoda *et al*., 2010
Type IV	12	J-domain, at a position not necessary N-terminal, that lacks the highly conserved HPD motif	Botha *et al*., 2007; Njunge *et al*., 2013

Type IV Hsp40s were first introduced by Botha and colleagues (2007) who suggested that they contain a variation in the normally conserved HPD motif of the J-domain. This variation would ideally abolish the interaction capacity between Hsp40 and Hsp70, however, these molecules seem to exert their function in a different manner (Figure 1.6; Rug and Maier, 2011; Botha *et al*., 2007; Njunge *et al*., 2013). Type IV PfHsp40s are unique to the parasite as there are no human homologues (Botha *et al*, 2010). Ten out of the twelve type IV PfHsp40s show an export element.

Exported type IV PfHsp40s may play important roles in the remodelling process after parasite invasion (Rug and Maier, 2011). The most studied type IV PfHsp40 is the ring-infected erythrocyte surface antigen (RESA, PFA0110w). It is said to be found in all field isolates, however, it can be disrupted under culture conditions and is believed to play an essential role *in vivo* (Rug and Maier, 2011). RESA is localized in the dense granules in the schizont stage of *P. falciparum*'s life cycle (Foley *et al*., 19990; Da Silva *et al*., 1994; Rug and Maier, 2011). Dense granules are specialized secretory organelles of apicomplexan parasites and play an essential role in host cell invasion (Foley *et al*., 1990; Da Silva *et al*., 1994; Rug and Maier, 2011). RESA is secreted from the dense granules into the forming PV after parasite invasion. It is then exported to the RBC cytoskeleton (Foley *et al*., 1990; Da Silva *et al*., 1994; Rug and Maier, 2011). RESA's role in the parasite is protecting the RBC cytoskeleton from heat-induced damage during febrile episodes. PF11_0509 and PF11_0512 are two molecules that are very similar to RESA, despite the sequence similarities, they seem to display distinct functions (Rug and Maier, 2011). PF10_0381 is also one of the exported type IV PfHsp40s which is said to be associated with red blood cell membrane modifications knob formation. Knobs are membrane protrusions appearing on the infected RBC surface upon maturation of *P. falciparum* parasites (Maier *et al*., 2009). These knobs are characterized by the knob-associated histidine rich protein (KAHRP – PFB0100c), which is linked to various proteins of the RBC cytoskeleton (Rug and Maier, 2011). Amongst other type IV PfHsp40 proteins is PFF1010c, which is yet to be characterized. PFF1010c is thought to be expressed only at the gametocyte stage of the parasite's development cycle (Njunge *et al*., 2013). Type IV Hsp40s are a very promising as potential drug targets, because they have no human homologues. The setbacks are that it is not known how and whether these molecules exert their function as co-chaperones, since they lack the HPD motif. Walsh and colleagues (2004) suggested that a possible function of the nonclassical type III/IV Hsp40s could be to regulate the activity of bona fide Hsp40s. Like other type IV PfHsp40s, PFF1010c also lacks the HPD motif and its hypothesized expression at the gametocyte stage of the parasite suggest it may be essential for the parasites development.

1.6 Study motivation

The type IV *Plasmodium falciparum* Hsp40s represent an interesting family of proteins as they do not have matching human homologues. The subject of the current study is PFF1010c, a type IV Hsp40 that has not been characterized. The predicted expression of PFF1010c at only the gametocyte stage makes this protein an interesting molecule to study. This is because it may represent an essential protein to the parasite. In addition, its expression at this stage of the parasite's development alone presents a bottleneck for its possible inhibition as an antimalarial drug candidate. The partnership between PfHsp70-1 and PfHsp40 molecular chaperones is important host infectivity and subsequent development of malaria parasites. The partnership of plasmodial Hsp70s with their co-chaperones such as the Hsp70/Hsp90 organising protein (Hop) and Hsp40s present potential antimalarial drug targets (Shonhai 2010). This study seeks to investigate the role of this protein in the survival of malaria parasites at the gametocyte stage of development.

1.7 Hypothesis

PFF1010c protein is expressed at the gametocyte stage of *P. falciparum* life cycle and is essential for the survival of the parasite at this stage.

1.8 Objectives

1. Utilise bioinformatics analysis in describing the structural features of PFF1010c

2. To perform composite analysis of PFF1010c amino acid sequence and design of peptide specific antibodies recognizing the protein

3. Express and purify recombinant PFF1010c protein using *E. coli* as expression host

4. Investigate the expression of PFF1010c in malaria parasite at distinct stages of the parasite's life cycle and heat stressed asexual malarial parasites

5. Investigate the expression of select Hsps, other than PFF1010c at the gametocyte stage of the malarial parasites

6. Investigate the interaction of PFF1010c and PfHsp70-1 using far Western blot analysis

Chapter 2

Materials and Methods

2.1 Materials

Reagents used were purchased from Sigma-Aldrich (U.S.A.), Merck Chemicals (Darmstadt, Germany), BioRad (U.S.A.) or ThermoScientific (USA). Nickel beads were purchased from ThermoScientific (USA). The following plasmids were used in this study: pQE30 vector (Qiagen, Germany), pQE30-PFF1010c plasmid expressing PFF1010c (current study) and pQE30-PfHsp70-1 plasmid expressing PfHsp70-1 (Matambo *et al*., 2004; Shonhai *et al*., 2005). The following antibodies were used: the α-PFF1010c antibody produced in rabbit against peptide: KTNELGEKNEKNES, corresponding to residues 9 - 22 of the PFF1010c amino acid sequence was generated by GenScript (USA). Polyclonal full length rabbit raised antibodies specific for PfHsp70-1 that were previously described (Pesce *et al*., 2008) were used to validate the authenticity of recombinant PfHsp70-1 protein. Anti-His antibody was purchased from Sigma-Aldrich (U.S.A.). Asexual and gametocyte stage parasite lysates were a kind donation from Prof LM Birkholtz (University of Pretoria). The heat shocked/stressed parasite lysates were a kind gift from Mr. Tawanda Zininga, a research laboratory colleague. All strains and plasmids used in this study are listed in Table 2.1. The rest of the reagents used in the study are listed in Appendix A.

Table 2.1: Description of strains and plasmids used in this study

Strains & plasmids	**Description**	**Source/Reference**
Strains		
E. coli JM109	*e14*$^-$ (*McrA*$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K–m_K^+) *supE44 relA1* Δ(*lac-proAB*) (*F′ traD36 proAB lacI*^{q} *Z*Δ*M15*).	Thermofisher Scientific, USA
E. coli BL21 (DE3)	F- ompT gal [dcm] [lon] hsdSB λDEs	Prof. LM Birkholtz Studier *et al*., 1990
Plasmids		
pQE30-PFF1010c	pQE30 encoding PFF1010c, Amp^R	Current study
pQE30-PfHsp70-1	pQE30 encoding PfHsp70-1, Amp^R	Matambo *et al*., (2004)

2.2 Methods

2.2.1 Bioinformatics analysis

Plasmodium sequences were obtained from PlasmoDB (www.PlasmoDB.org; Aurrecoechea *et al*., 2009). Other protein sequences were obtained from National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Multiple sequence alignments were generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and shaded using Expasy boxshade (www.expasy.org). The 3D models of proteins were developed using Phyre2 (www.sbg.bio.ic.ac.uk/phyre2; Kelley *et al*., 2015) and visualized using UCSF Chimera version 1.9 (www.cgl.ucsf.edu/chimera; Pettersen *et al*., 2004).

2.2.2 Preparation of competent *E. coli* JM109 and BL21 Star (DE3) cells

A single colony of *E. coli* JM109/BL21 Star (DE3) cells was used to incubate in 5 ml of double strength yeast tryptone [(2 x YT) broth (1 % (w/v) yeast, 1.6 % (w/v) tryptone and 0.5 % (w/v) NaCl] and grown at 37 ˚C overnight in a shaking incubator. The cells were diluted 1/10 in fresh 2 x YT broth and allowed to grow to early log phase (OD_{600nm} between 0.3 – 0.6). The cells were collected by centrifugation at 5000 *g* at 4 ˚C for 5 minutes. The cell pellet was placed on ice and re-suspended in 50 ml ice-cold 0.1 M $MgCl_2$ for 30 minutes. The cells were centrifuged at 5000 *g* for 5 minutes at 4 ˚C and pellet resuspended in 25 ml of ice-cold 0.1 M $CaCl_2$ for 4 hours. After another centrifugation at 5000 *g* for 5 minutes at 4 ˚C, the cells were re-suspended in 3 ml ice-cold $CaCl_2$ and 3 ml 30 % glycerol. The cells were stored in aliquots at -80 ˚C.

2.2.3 Transformation of competent *E. coli* JM109 and BL21 Star (DE3) cells

Competent cells were thawed on ice. The pQE30/PFF1010c plasmid DNA was used to transform *E. coli* JM109/ BL21 Star (DE3) competent cells and pQE30 plasmid was used as a positive control. The cells were incubated on ice for 30 minutes. The cells were heat shocked for 60 seconds at 42 ˚C. After heat shocking the cells were mixed and incubated on ice for 10 minutes. Then 900 µl of fresh 2 x YT broth was added into each tube with transformed cells and incubated at 37 ˚C for 1 hour. After incubation, 100 µl of transformed cells was plated into 2 x YT agar plates [1 % (w/v) yeast, 1.6 % (w/v) tryptone, 0.5 % (w/v) NaCl and 1.5 % (w/v) agar bacteriological] agar plates with ampicillin at 100 µg/ml and incubated at 37 ˚C overnight.

2.2.4 Plasmid DNA extraction

A single colony of PFF1010c transformed cells was inoculated into 5 ml of 2 x YT broth supplemented with 100 µg/ml ampicillin and grown overnight at 37 ˚C with agitation. Plasmid DNA was isolated using Zippy plasmid miniprep kit according to the manufacturer's instructions.

2.2.5 Restriction digestion of plasmid DNA

Fast digest restriction enzymes *Bam*HI and *Hind*III was used to digest the pQE30/PFF1010c plasmid DNA to confirm the integrity of the construct. One reaction remained enzyme-free as the uncut sample. The samples were incubated at 37 ˚C for 15 minutes. Gel loading buffer [0.25 % (w/v) Bromophenol blue in 30 % (v/v) Glycerol] was used to stop the reactions and the samples were analyzed on a 0.8 % (v/v) agarose gel in TAE buffer [45 mM Tris pH 8, 45 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA)]. Agarose gels [0.8 % (v/v)] containing 0.5 µg/ml ethidium bromide were prepared using TAE buffer. Samples were resolved at 120 V. DNA bands were visualized using ultra violet (UV) radiation and imaged using the Chemidoc^{TM} MP Imaging System (BioRad, USA).

2.2.6 Peptide specific anti-PFF1010c antibody design

To design a peptide that was used to develop anti-PFF1010c antibody, the DNASTAR (http://www.dnastar.com) composite analysis was used. The analysis was conducted to determine antigenic regions that are surface exposed and unique to PFF1010c using DNASTAR (Proteone, Proteon3D and Novafold) plugins. The epitope determination analysis included assessment of antigenicity using Jameson-Wolf Index plots (Jameson and Wolf, 1988); charge density analysis using Lehninger plots (Lehninger, 2005); chain flexibility using the Karplus-Schultz plots

(Karplus *et al.,* 1978) and hydrophobicity and hydrophilicity using KyteDoolittle plots (Kyte and Doolitle, 1982). To determine the overall antigenicity the JamesonWolf Antigenic index which combines data from existing protein structural predictions was used (Jameson and Wolf, 1988). PFF1010c and other *Plasmodium* proteins were assessed using BLAST and regions with high antigenicity and lowest similarities to other *Plasmodium* homologues was selected to design peptide specific anti-PFF1010c. The regions selected were characterized by identifying a strongly hydrophilic stretch of amino acids that were fairly unique to the PFF1010c. The epitopic regions corresponding to synthetic peptides: KTNELGEKNEKNES of amino acids 9 - 22 of the PFF1010c sequence and PQSSHRVFGDRISG of amino acids 296 – 309, were commercially generated by GenScript (USA) in rabbit immunized using the peptides.

2.2.7 Expression of PFF1010c recombinant protein in *E. coli* JM109 and BL21 Star (DE3) cells

E. coli JM109 competent cells were transformed with pQE30/PFF1010c DNA was inoculated in 2 x YT broth (50 ml containing 100 µg/ml ampicillin) and incubated overnight at 37 ˚C in a shaker incubator. The overnight culture was diluted into fresh 2 x YT broth (450 ml containing 100 µg/ml ampicillin) (Sigma-Aldrich, USA), and grown to a density of OD_{600} between 0.5 – 0.6. 1 mM Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma- Aldrich, USA) was used to induce protein expression. An aliquot was taken before induction and every hour for four hours after induction. Each aliquot was centrifuged for two minutes at 1500 *g* and supernatant discarded. The pellet was suspended in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.5), in a volume equivalent to $OD_{600}/0.5$ x 150 µl and was treated with SDS-PAGE loading buffer [0.25 % Coomasie Brilliant blue (R250), 2 % SDS, 10 % glycerol (v/v), 100 mM Tris, 1 % β-mercaptoethanol]. The samples were boiled for ten minutes and analyzed by 12 % SDS-PAGE and Western blot analyses. Expression and sample analysis of recombinant PFF1010c protein in *E. coli* BL21 Star (DE3) cells were performed in the same manner.

2.2.8 Determination of protein solubility of PFF1010c

Competent *E. coli* JM109/BL21 Star (DE3) cells transformed with pQE30-PFF010c recombinant plasmid were grown overnight in 10 ml 2 x YT broth containing 10 mg/ml ampicillin. Cultures grown overnight were diluted into fresh 90 ml 2 x YT broth containing 10 mg/ml ampicillin and allowed to grow at 37 ˚C with agitation at 160 rpm until mid-log phase (OD_{600} 0.5–0.6) was reached. Protein expression was induced by the addition of 1 mM IPTG. The cells were harvested by centrifugation at 5000 *g* for 20 minutes at 4 ˚C for 4 hours post induction and thereafter resuspended in 1 ml lysis buffer (10 mM Tris, pH 8.0, 300 mM NaCl and 10 mM imidazole, 1 mM PMSF and 1 mg/ml Lysozyme). The cells were stored at -80 ˚C overnight and thawed the following morning followed by mild sonication at amplitude setting of 50 for 5 cycles with 15 seconds pulse and 30 seconds pause after each cycle. The application of polyethyleneimine (PEI) to solubilize proteins has been reported (Shonhai *et al*., 2008; Marenchino *et al*., 2009; Zininga *et al*., 2015). The PEI precipitates nucleic acids complexed to protein, leaving the target protein in the soluble fraction (Marenchino *et al*., 2009). It is for this reason that PEI has been used to maintain proteins in soluble and native states during the purification (Shonhai *et al*., 2008; Marenchino *et al*., 2009). In the study, cell lysates were sonicated after addition of PEI 0.1 % (v/v), respectively. Cell lysates were subsequently centrifuged at 5000 *g* for 20 minutes at 4 ˚C and the supernatant (soluble fraction) separated from the pellet (insoluble fraction). The pellet was resuspended in 1 ml PBS (pH 7.5). The samples were analyzed by 12% SDS-PAGE and Western blot analysis to determine the solubility of recombinant PFF1010c.

2.2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were treated by boiling in SDS sample loading buffer [0.25 % Coomasie Brilliant blue (R250); 2 % SDS; 10 % glycerol (v/v); 100 mM tris;1 % β-mercaptoethanol] in a ratio of 4:1 for 10 minutes and resolved using 12 % acrylamide resolving gel. The gel was then transferred into the electrophoresis tank and electrophoresis buffer [25 mM Tris, pH 8.3 250 mM Glycine and 0.1 % (w/v) SDS] was added. The boiled samples were loaded in respective wells and PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A) was also loaded. The electrophoresis was performed at 120 volts for an hour.

2.2.10 Western blot analysis

Proteins were separated using 12 % SDS-PAGE and subsequently transferred onto nitrocellulose membrane in Western blotting transfer buffer [25 mM Tris, 192 mM glycine, 20 % (v/v) MeOH] for 1 hour at 100 volts using the TransBlotter. Upon completion, the membrane was removed and rinsed using transfer buffer using a cotton swab. The blot was stained with Ponceau-S stain [0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid] to determine the success of the transfer followed by blocking. The membrane was blocked in 5 % non-fat milk prepared in TBS (50 mM Tris, 150 mM NaCl) for 1 hour on a shaker set at 60 rpm. The membrane was washed 3 times in TBS-Tween [50 mM Tris, 150 mM NaCl, 0.1 % (w/v) Tween 20] in 15 minutes intervals followed by incubation of the membrane with primary antibody in blocking solution at 4 ˚C on a shaker set at 60 rpm for 1 hour. Unbound antibodies were removed by washing the membrane 3 times using TBS-Tween for 15 minutes each wash. This was followed by incubation in secondary antibody and subsequent washing under the same conditions as primary antibody. The bands were visualized using enhanced chemiluminescence (ECL) developing reagents were used and visualization was done using the Chemidoc^{TM} MP Imaging System (BioRad, USA).

2.2.11 Preparation of Ni^{2+} chelating sepharose beads

Sepharose beads were charged with Ni^{2+} according to the manufacturer's instructions (ThermoScientific, USA). The beads were resuspended in 1 ml lysis buffer (10 mM Tris pH 7.5, 300 mM NaCl, 50 mM imidazole, 1 mM PMSF) for native purification and (8 M urea, 300mM NaCl, 100 mM Tris pH 8.0, 10 mM imidazole, 1 mM PMSF) for purification under denaturing conditions.

2.2.12 Purification of PFF1010c recombinant protein under denaturing conditions from *E. coli* JM109 cells

The cells expressing PFF1010c recombinant protein following induction in 1L 2 x YT broth culture containing 100 mg/ml ampicillin for 4 hours at 37 ºC, cells were harvested by centrifugation at 5000 *g* for 20 minutes at 4 ºC and the pellets were re-suspended in denaturing lysis buffer (300 mM NaCl, 10 mM imidazole, 100 mM Tris, pH 8.0, 1 mM PMSF, 1 mM lysozyme and 8 M Urea). The cells were stored at -80 ºC overnight and thawed the following morning followed by mild sonication at amplitude setting of 50 for 5 cycles with 15 seconds pulse and 30 seconds pause after each cycle and centrifuged at 5000 *g* for 20 minutes at 4 ºC. PEI was added to a final concentration of 1 % (v/v). The supernatant containing the recombinant protein was suspended in nickel-chelating sepharose beads. Binding was allowed to occur for 3 hours at 4 ºC with mild agitation. Unbound protein was removed by washing the beads twice in denaturing buffer (300 mM NaCl, 25 mM imidazole, wash I, and 100 mM Tris, pH 8.0 and also 80 mM imidazole, wash II). The bound protein was eluted using Elution buffer (300 mM NaCl, 500 mM imidazole and 100 mM Tris, pH 8.0). The proteins were resolved using 12 % SDS-PAGE and Western blot analysis was used to confirm the presence of the eluted protein. On one occasion a constant concentration of urea (8 M) was used throughout eluting the protein and in another decreasing concentrations of urea was used (8 M, 6 M, 4 M, 2 M, 1 M and 0 M).

2.2.13 Purification of PFF1010c recombinant protein from *E. coli* BL21 Star (DE3) cells

The purification of PFF1010c was conducted as previously described (Zininga *et al*., 2015) with minor modifications. The *E. coli* BL21 Star (DE3) cells expressing PFF1010c recombinant protein were harvested by centrifugation at 5000 *g* for 20 minutes at 4 ºC. The pellet was re-suspended in non-reducing lysis buffer (300 mM NaCl, 10 mM imidazole, 10 mM Tris, pH 8.0, 1 mM PMSF, and 1 mM lysozyme). The cells were stored at -80 ºC overnight and thawed the following morning. Followed by mild sonication at amplitude setting of 50 for seven cycles with 15 seconds pulse and 30 seconds rest after each cycle. Sonication was performed after addition of PEI to a final concentration of 0.1 % (v/v). Cell lysates were subsequently centrifuged at 5000 *g* for 20 minutes at 4 ºC, and the supernatant containing the recombinant protein was suspended in a 50 % (w/v) slurry of nickel-chelating sepharose beads. Binding was allowed to occur for 4 hours at 4 ºC with mild agitation. Unbound protein was removed by washing the beads three times in native buffer (300 mM NaCl, 150 mM imidazole, and 10 mM Tris, pH 7.5). The bound protein was eluted using Elution buffer (300 mM NaCl, 250 M imidazole, and 10 mM Tris, pH 7.5). The proteins were resolved using 12 % SDS-PAGE and Western blot analysis was used to confirm the presence of the eluted protein. Protein concentration was determined using Bradford assay (Appendix C).

2.2.14 Investigation of the interaction between PFF1010c and PfHsp70-1 by far Western analysis

A far Western analysis was conducted as previously described (Wu *et al*., 2007) with slight modifications. Briefly, various concentrations (10 µg, 15 µg, and 20 µg) of PFF1010c cell lysate and 20 µg recombinant PfHsp70-1 protein (positive control for membrane) as well as 20 µg of BSA (negative control) were resolved using 12 % SDS PAGE. The proteins were subsequently transferred onto Hybond ECL nitrocellulose membrane (GE Healthcare, UK). The proteins on the membrane were denatured and renatured by using decreasing concentrations of urea (8 M, 6M, 4M, 2M, 1M, 0.1M and 0 M). The membranes were blocked using 5 % fat free milk in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1 % Tween 20) for 1 hour at room temperature. The blot was then incubated with the ligand protein (20 mg/ml of PfHsp70-1) in protein-binding buffer (100 mM NaCl, 20 mM Tris [pH 7.5], 0.5 mM EDTA, 10 % glycerol, 0.1 % Tween-20, 2 % skim milk powder and 1 mM DTT) (Wu *et al*., 2007) overnight at 4 ˚C. The procedure was repeated in the absence and presence of nucleotides 5 µM ATP and/or ADP. A control blot was incubated in binding buffer lacking ligand. After washing three times in TBS-Tween buffer (0.1 % Tween-20 in Tris-buffered saline pH 7.5), the blots were incubated in 3 % milk (w/v) in the presence of anti-PfHsp70-1 antibodies (1: 2000) for 2 hours at 4 ˚C. After subsequent washing steps, the membranes were incubated with the horseradish peroxidase conjugated secondary antibody for 2 hours at 4 ˚C. Unbound secondary antibodies were removed by washing the membrane twice using TBS-Tween buffer. The membrane bound secondary antibody was detected using ECL chemiluminescence reagent and images were acquired using Chemidoc^{TM} MP Imaging System (Bio-Rad, USA). Protein concentration was determined using Bradford assay (Appendix C).

2.2.15 Total protein isolation from *in vitro P. falciparum* culture

To harvest cells, 25-30 ml of the culture was pelleted by spinning down at 3000 *g* for 5 minutes, the culture media supernatant was poured off. Ice-cold saponin (0.15 % [w/v] dissolved in PBS) was added up to a final volume of 7.5 ml and incubated on ice for 10 minutes. Ice-cold PBS was added up to a final volume of 40 ml and the pellet was re-suspended and spun down at 3000 *g* for 5 minutes. The supernatant was aspirated. The pellet was transferred to micro-centrifuge tubes, and washed three times with 500 µl of PBS at 6000 *g* for 2 minutes each, until the supernatant was clear of visible haemoglobin. The parasite pellet was re-suspended in 1 ml of ice-cold lysis buffer (8 M Urea, 2 M Thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS], 32 mM or 0.5% [w/v] DTT). Samples were pulse sonicated with cooling in between on a Virsonic sonifier with microtip for 20 seconds with alternating pulsing (1 second pulse, 1 second rest) at 3 W output. The samples were cooled on ice for 1 minute between cycles. The cycle was repeated 7 times for each sample. Samples were centrifuged at 16 000 *g* for 1 hour at 4 ˚C. The supernatant was transferred into a clean microfuge tube and stored at -80 ˚C for further use and the pellet was discarded.

2.2.16 Investigation of the expression of PFF1010c by parasites at distinct growth stages

PFF1010c's expression by parasites at distinct growth stages was investigated by Western blot analysis using anti-PFF1010c antibody. Asexual stage parasites used were of a mixed population of rings and trophozoites at 4.2 % parasitaemia and 5 % haematocrit. The early gametocyte stage parasites were at 1.3 % gametocitaemia and also 5 % haematocrit and the late gametocyte stage parasites were at 1 % gametocitaemia and 5 % haematocrit as well. The parasites were harvested and lysate prepared as described (section 2.2.18). The expression of PFF1010c by parasites at distinct stages was confirmed by Western blot analysis using anti-PFF1010c antibody. PFF1010c lysate was used as a positive control for anti-PFF1010c antibody. Chemiluminescent detection was carried out on the Western blots using ECL developing reagents and images were acquired using the Chemidoc^{TM} MP Imaging System (BioRad, USA).

2.2.17 Investigation of the expression of PFF1010c by heat stressed asexual parasites

PFF1010c's expression by heat stressed asexual stage parasites was investigated by Western blot analysis using anti-PFF1010c antibody. Heat stressed asexual stage parasites were prepared as previously described (Zininga *et al*., 2015). Samples of parasites grown at 37 ˚C and those grown at 42 ˚C (heat stressed) were resolved using 12 % SDS-PAGE. Uninfected red blood cells were used as a negative control. PFF1010c lysate was used as a positive control for anti-PFF1010c antibody. The expression of PFF1010c by heat stressed asexual stage parasites was investigated by Western blot analysis using anti-PFF1010c antibody. Chemiluminescent detection was carried out on the Western blots using ECL developing reagents and images were acquired using the Chemidoc^{TM} MP Imaging System (BioRad, USA).

2.2.18 Investigation of the expression of select heat shock proteins by parasites at the gametocyte stage

The expression of PfHsp90, PfHop and PfHsp70-1 at the gametocyte stage was investigated using Western blot analysis and anti-PfHsp90, anti-PfHop and anti-PfHsp70-1 respectively. Both early gametocyte stage parasites were at 1.3 % gametocitaemia and also 5 % haematocrit. The parasites were harvested and lysate prepared as described (Section 2.2.15). Chemiluminescent detection was carried out on the Western blots using ECL developing reagents and images were acquired using the Chemidoc^{TM} MP Imaging System (BioRad, USA).

Chapter 3

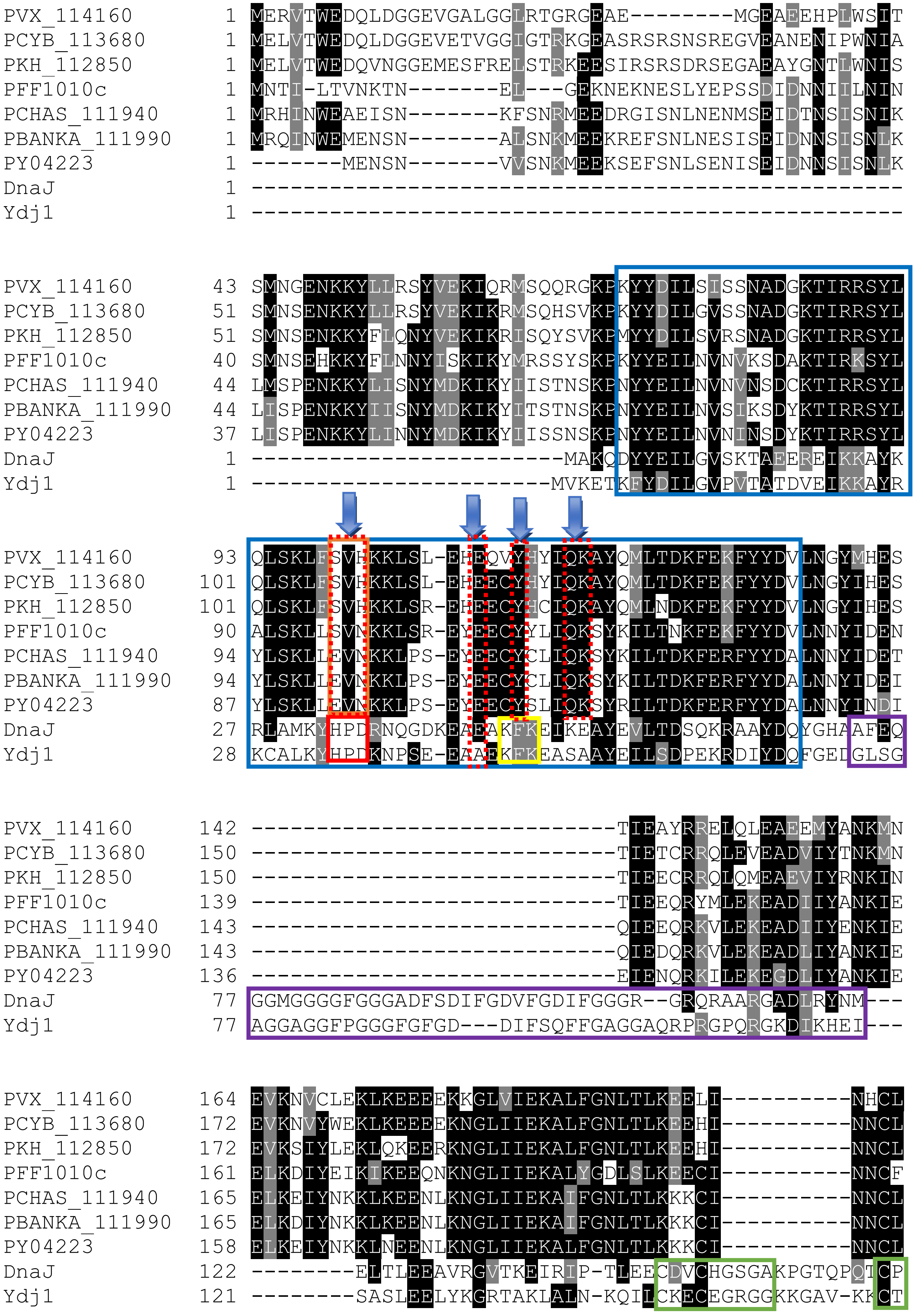
Results

3. Results

3.1 Bioinformatics

3.1.1 Protein sequence alignments

The amino acid sequence of PFF1010c was aligned with that of DnaJ, an *E. coli* type I Hsp40; Ydj1, a *S. cerevisae* type I Hsp40 as well as with those of its *Plasmodium* type IV homologues (Figure 3.1.1). PFF1010c shares a sequence identity of above 50 % with its *Plasmodium* homologues PVX_114160, PCYB_113680, PKH_112850, PCHAS_111940, PBANKA_111990 and PY04223 (53.6 %, 57.1 %, 57.6 %, 64.4%, 64.2 % and 63.1 %, respectively). *E. coli* DnaJ and *S. cerevisiae*, do not share high sequence identities (8.5 % and 7.1 %, respectively) with PFF1010c. The ClustalW alignments show the J domain of PFF1010c, it's *Plasmodium* type IV homologues, DnaJ and Ydj1 (Figure 3.1.1; blue box). The J domain in PFF1010c and its *Plasmodium* type IV homologues is not necessarily situated at the N-terminal of the protein, whereas that of DnaJ and Ydj1 is situated at the N-terminus (Figure 3.1.1 red box). Within the J domain of DnaJ and Ydj1 is the highly conserved HPD motif (Figure 3.1.1; red box; Cheetham and Caplan, 1998; Rug and Mair, 2011). However, PFF1010c and its *Plasmodium* type IV homologues do not possess the HPD motif (Botha *et al*., 2007), they either have [S/E]V[H/N], residues in place of the HPD motif (Figure 3.1.1; orange box). Residues important for interaction with Hsp70 are highly conserved in PFF1010c and its *Plasmodium* type IV homologues (Figure 3.1.1; red dashed boxes with blue arrow). DnaJ and Ydj1 also possess the KFK motif, on the other hand PFF1010c and its *Plasmodium* homologues possess a [V/C]Y[H/Y/S] (Figure 3.1.1; yellow box). PFF1010c shows high amino acid residue conservation in comparison to its *Plasmodium* type IV homologues (Figure 3.1.1; shaded black). Whereas there is evidence of a high degree of variation between PFF1010c and the two type I *E. coli* and *S. cerevisae.* Botha and colleagues (2007) discussed that type IV Hsp40s only possess the J domain with a corrupted HPD. The multiple sequence alignments showed the presence of an insertion on DnaJ and Ydj1 the G/F rich region (Figure 3.1.1; purple box) which is absent in PFF1010c and its *Plasmodium* homologues, the G/F rich region is a disordered region that has been suggested to provide flexibility to these proteins (Karzai and MaMacken, 1996). Cysteine-rich repeats are also shown in the alignments (Figure 3.1.1; green box) which are only conserved in DnaJ and Ydj1, and absent in PFF1010c and its *Plasmodium* homologues.



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PVX_114160       1 MERVTWEDQLDGGEVGALGGLRTGRGEAE--------MGEAEEHPLWSIT
PCYB_113680      1 MELVTWEDQLDGGEVETVGGIGTRKGEASRSRSNSREGVEANENIPWNIA
PKH_112850       1 MELVTWEDQVNGGEMESFRELSTRKEESIRSRSDRSEGAEAYGNTLWNIS
PFF1010c         1 MNTI-LTVNKTN-------EL---GEKNEKNESLYEPSSDIDNNIILNIN
PCHAS_111940     1 MRHINWEAEISN-------KFSNRMEEDRGISNLNENMSEIDTNSISNIK
PBANKA_111990    1 MRQINWEMENSN-------ALSNKMEEKREFSNLNESISEIDNNSISNLK
PY04223          1 -------MENSN-------VVSNKMEEKSEFSNLSENISEIDNNSISNLK
DnaJ             1 --------------------------------------------------
Ydj1             1 --------------------------------------------------

PVX_114160      43 SMNGENKKYLLRSYVEKIQRMSQQRGKPKYYDILSISSNADGKTIRRSYL
PCYB_113680     51 SMNSENKKYLLRSYVEKIKRMSQHSVKPKYYDILGVSSNADGKTIRRSYL
PKH_112850      51 SMNSENKKYFLQNYVEKIKRISQYSVKPMYYDILSVRSNADGKTIRRSYL
PFF1010c        40 SMNSEHKKYFLNNYISKIKYMRSSYSKPKYYEILNVNVKSDAKTIRKSYL
PCHAS_111940    44 LMSPENKKYLISNYMDKIKYIISTNSKPNYYEILNVNVNSDCKTIRRSYL
PBANKA_111990   44 LISPENKKYIISNYMDKIKYITSTNSKPNYYEILNVSIKSDYKTIRRSYL
PY04223         37 LISPENKKYLINNYMDKIKYIISSNSKPNYYEILNVNINSDYKTIRRSYL
DnaJ             1 ------------------------MAKQDYYEILGVSKTAEEREIKKAYK
Ydj1             1 -----------------------MVKETKFYDILGVPVTATDVEIKKAYR

PVX_114160      93 QLSKLFSVHKKLSL-EHEQVYHYIQKAYQMLTDKFEKFYYDVLNGYMHES
PCYB_113680    101 QLSKLFSVHKKLSL-EHEECYHYIQKAYQMLTDKFEKFYYDVLNGYIHES
PKH_112850     101 QLSKLFSVHKKLSR-EHEECYHCIQKAYQMLNDKFEKFYYDVLNGYIHES
PFF1010c        90 ALSKLLSVNKKLSR-EYEECYYLIQKSYKILTNKFEKFYYDVLNNYIDEN
PCHAS_111940    94 YLSKLLEVNKKLPS-EYEECYCLIQKSYKILTDKFERFYYDALNNYIDET
PBANKA_111990   94 YLSKLLEVNKKLPS-EYEECYCLIQKSYKILTDKFERFYYDALNNYIDEI
PY04223         87 YLSKLLEVNKKLPS-EYEECYSLIQKSYRILTDKFERFYYDALNNYINDI
DnaJ            27 RLAMKYHPDRNQGDKEAEAKFKEIKEAYEVLTDSQKRAAYDQYGHAAFEQ
Ydj1            28 KCALKYHPDKNPSE-EAAEKFKEASAAYEILSDPEKRDIYDQFGEDGLSG

PVX_114160     142 ----------------------------TIEAYRRELQLEAEEMYANKMN
PCYB_113680    150 ----------------------------TIETCRRQLEVEADVIYTNKMN
PKH_112850     150 ----------------------------TIEECRRQLQMEAEVIYRNKIN
PFF1010c       139 ----------------------------TIEEQRYMLEKEADIIYANKIE
PCHAS_111940   143 ----------------------------QIEEQRKVLEKEADIIYENKIE
PBANKA_111990  143 ----------------------------QIEDQRKVLEKEADLIYANKIE
PY04223        136 ----------------------------EIENQRKILEKEGDLIYANKIE
DnaJ            77 GGMGGGGFGGGADFSDIFGDVFGDIFGGGR--GRQRAARGADLRYNM---
Ydj1            77 AGGAGGFPGGGFGFGD---DIFSQFFGAGGAQRPRGPQRGKDIKHEI---

PVX_114160     164 EVKNVCLEKLKEEEEKKGLVIEKALFGNLTLKEELI----------NHCL
PCYB_113680    172 EVKNVYWEKLKEEEEKNGLIIEKALFGNLTLKEEHI----------NNCL
PKH_112850     172 EVKSIYLEKLQKEERKNGLIIEKALFGNLTLKEEHI----------NNCL
PFF1010c       161 ELKDIYEIKIKEEQNKNGLIIEKALYGDLSLKEECI----------NNCF
PCHAS_111940   165 ELKEIYNKKLKEENLKNGLIIEKAIFGNLTLKKKCI----------NNCL
PBANKA_111990  165 ELKDIYNKKLKEENLKNGLIIEKAIFGNLTLKKKCI----------NNCL
PY04223        158 ELKEIYNKKLNEENLKNGLIIEKALFGNLTLKKKCI----------NNCL
DnaJ           122 --------ELTLEEAVRGVTKEIRIP-TLEECDVCHGSGAKPGTQPQTCP
Ydj1           121 --------SASLEELYKGRTAKLALN-KQILCKECEGRGGKKGAV-KKCT
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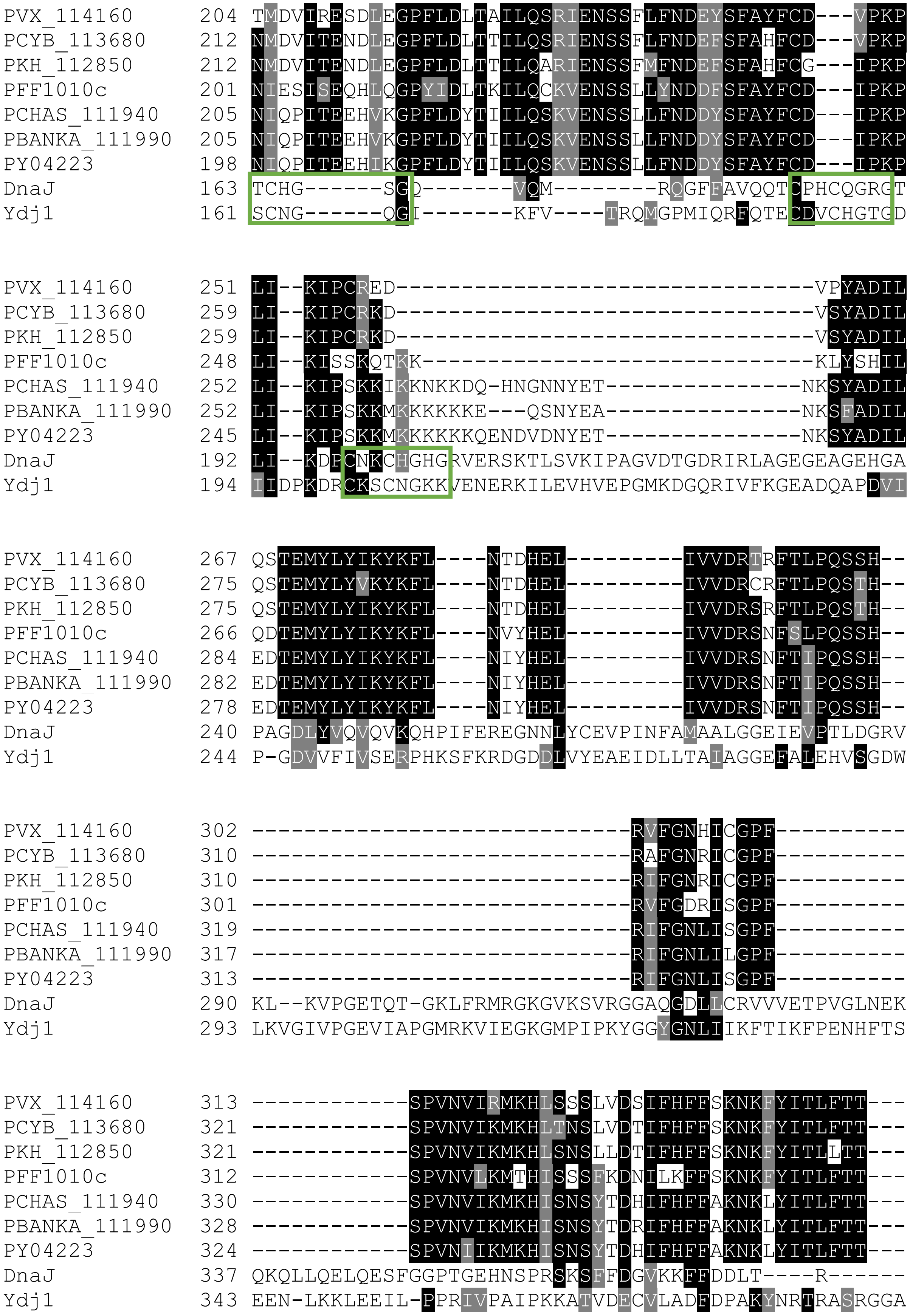

```
PVX_114160     204 TMDVIRESDLEGPFLDLTAILQSRIENSSFLFNDEYSFAYFCD---VPKP
PCYB_113680    212 NMDVITENDLEGPFLDLTTILQSRIENSSFLFNDEFSFAHFCD---VPKP
PKH_112850     212 NMDVITENDLEGPFLDLTTILQARIENSSFMFNDEFSFAHFCG---IPKP
PFF1010c       201 NIESISEQHLQGPYIDLTKILQCKVENSSLLYNDDFSFAYFCD---IPKP
PCHAS_111940   205 NIQPITEEHVKGPFLDYTIILQSKVENSSLLFNDDYSFAYFCD---IPKP
PBANKA_111990  205 NIQPITEEHVKGPFLDYTIILQSKVENSSLLFNDDYSFAYFCD---IPKP
PY04223        198 NIQPITEEHIKGPFLDYTIILQSKVENSSLLFNDDYSFAYFCD---IPKP
DnaJ           163 TCHG------SGQ-------VQM--------RQGFFAVQQTCPHCQGRGT
Ydj1           161 SCNG------QGI-------KFV----TRQMGPMIQRFQTECDVCHGTGD

PVX_114160     251 LI--KIPCRED--------------------------------VPYADIL
PCYB_113680    259 LI--KIPCRKD--------------------------------VSYADIL
PKH_112850     259 LI--KIPCRKD--------------------------------VSYADIL
PFF1010c       248 LI--KISSKQTKK------------------------------KLYSHIL
PCHAS_111940   252 LI--KIPSKKIKKNKKDQ-HNGNNYET---------------NKSYADIL
PBANKA_111990  252 LI--KIPSKKMKKKKKKE---QSNYEA---------------NKSFADIL
PY04223        245 LI--KIPSKKMKKKKKKQENDVDNYET---------------NKSYADIL
DnaJ           192 LI--KDPCNKCHGHGRVERSKTLSVKIPAGVDTGDRIRLAGEGEAGEHGA
Ydj1           194 IIDPKDRCKSCNGKKVENERKILEVHVEPGMKDGQRIVFKGEADQAPDVI

PVX_114160     267 QSTEMYLYIKYKFL----NTDHEL---------IVVDRTRFTLPQSSH--
PCYB_113680    275 QSTEMYLYVKYKFL----NTDHEL---------IVVDRCRFTLPQSTH--
PKH_112850     275 QSTEMYLYIKYKFL----NTDHEL---------IVVDRSRFTLPQSTH--
PFF1010c       266 QDTEMYLYIKYKFL----NVYHEL---------IVVDRSNFSLPQSSH--
PCHAS_111940   284 EDTEMYLYIKYKFL----NIYHEL---------IVVDRSNFTIPQSSH--
PBANKA_111990  282 EDTEMYLYIKYKFL----NIYHEL---------IVVDRSNFTIPQSSH--
PY04223        278 EDTEMYLYIKYKFL----NIYHEL---------IVVDRSNFTIPQSSH--
DnaJ           240 PAGDLYVQVQVKQHPIFEREGNNLYCEVPINFAMAALGGEIEVPTLDGRV
Ydj1           244 P-GDVVFIVSERPHKSFKRDGDDLVYEAEIDLLTAIAGGEFALEHVSGDW

PVX_114160     302 -----------------------------RVFGNHICGPF----------
PCYB_113680    310 -----------------------------RAFGNRICGPF----------
PKH_112850     310 -----------------------------RIFGNRICGPF----------
PFF1010c       301 -----------------------------RVFGDRISGPF----------
PCHAS_111940   319 -----------------------------RIFGNLISGPF----------
PBANKA_111990  317 -----------------------------RIFGNLILGPF----------
PY04223        313 -----------------------------RIFGNLISGPF----------
DnaJ           290 KL--KVPGETQT-GKLFRMRGKGVKSVRGGAQGDLLCRVVVETPVGLNEK
Ydj1           293 LKVGIVPGEVIAPGMRKVIEGKGMPIPKYGGYGNLIIKFTIKFPENHFTS

PVX_114160     313 ------------SPVNVIRMKHLSSSLVDSIFHFFSKNKFYITLFTT---
PCYB_113680    321 ------------SPVNVIKMKHLTNSLVDTIFHFFSKNKFYITLFTT---
PKH_112850     321 ------------SPVNVIKMKHLSNSLLDTIFHFFSKNKFYITLLTT---
PFF1010c       312 ------------SPVNVLKMTHISSSFKDNILKFFSKNKFYITLFTT---
PCHAS_111940   330 ------------SPVNVIKMKHISNSYTDHIFHFFAKNKLYITLFTT---
PBANKA_111990  328 ------------SPVNVIKMKHISNSYTDRIFHFFAKNKLYITLFTT---
PY04223        324 ------------SPVNIIKMKHISNSYTDHIFHFFAKNKLYITLFTT---
DnaJ           337 QKQLLQELQESFGGPTGEHNSPRSKSFFDGVKKFFDDLT----R------
Ydj1           343 EEN-LKKLEEIL-PPRIVPAIPKKATVDECVLADFDPAKYNRTRASRGGA
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PVX_114160     348 -------------ILLCAQSVKSG-----
PCYB_113680    356 -------------ILLCAQS---------
PKH_112850     356 -------------ILLCAQSVKSA-----
PFF1010c       347 -------------IVLCAQSIKIKMNKLE
PCHAS_111940   365 -------------IMLCVQSMKSMCT---
PBANKA_111990  363 -------------IMLCVQSMKSMCA---
PY04223        359 -------------IMLCVQSMKSMCA---
DnaJ               -----------------------------
Ydj1           391 NYDSDEEEQGGEGVQCASQ---------
```

Figure 3.1.1 Sequence alignment of PFF1010c and its homologues as well as DnaJ and Ydj1. The alignment was generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). PFF1010c was aligned with its homologues obtained from PlasmoDB and with DnaJ a type I Hsp40 from *E. coli* and Ydj1 which is a type I Hsp40 from *S. cerevisiae*. The J-domains are highlighted by the blue solid box, the G/F rich region is highlighted by the purple solid box; and the cysteine-rich repeats are highlighted by green solid boxes. The HPD motif (Cheetham and Caplan, 1998) within the J-domain which is corrupted in PFF1010c and its homologues is highlighted by a red solid box. The KFK motif is highlighted by a yellow solid box.

3.1.2 Comparison of 3 dimensional models of PFF1010c, DnaJ and Ydj1

The 3D model of PFF1010c, DnaJ and Ydj1 were generated using Phyre2 and visualized using UCSF Chimera (www.cgl.ucsf.edu/chimera; Pettersen *et al*., 2004). The 3D model of PFF1010c was compared using match maker tool on Chimera with those of DnaJ and Ydj1 (Figure 3.1.2). The 3D-models show the alpha helix regions, the beta sheets, and coil regions on the proteins (Figure 3.1.2). The models reveal that PFF1010c possesses more alpha helices (44 %) than DnaJ (23 %) and Ydj1 (17 %). PFF1010c appears to be in a more compact conformation than DnaJ and Ydj1 which are in a more relaxed conformation (Figure 3.1.2). DnaJ possesses more beta sheets (26 %) than PFF1010c (15 %) and Ydj1 (23 %), beta sheets are associated with substrate binding, these suggest that DnaJ binds to substrate abundantly. Ydj1 possesses more disordered/coil regions (46 %) than PFF1010c (44 %) and DnaJ (38 %). The J-domain models of PFF1010c, DnaJ and Ydj1 were compared by both multiple sequence alignment and also by their 3D models (Figure 3.1.3). Sequences of PFF1010c, DnaJ and Ydj1 were aligned and an extract of multiple sequence alignment highlighting the J-domain is represented by blue solid box (Figure 3.1.3A). The 3D models show the J-domains in red, the HPD motif of DnaJ and Ydj1 are shown as ball and stick in green and the SVN the residues in PFF1010c corresponding to the HPD motif of DnaJ and Ydj1 are shown as ball and stick in yellow (Figure 3.1.3B). DnaJ and Ydj1 J domain contain four alpha helices, the HPD motifs are situated at the loop/coil between helix II and III whereas PFF1010c's J domain is characterized by 5 alpha helices and the SVN residues that substituted HPD motif on PFF1010c is situated partially on the loop between helix II and III and partly on helix III (Figure 3.1.3B). There are several residues that are conserved between PFF1010c, DnaJ and Ydj1's J domain (Figure 3.1.3; purple dashed boxes). The conserved residues are; Y, V, I, L, D, K, and E, with tyrosine being the most conserved amongst the rest.

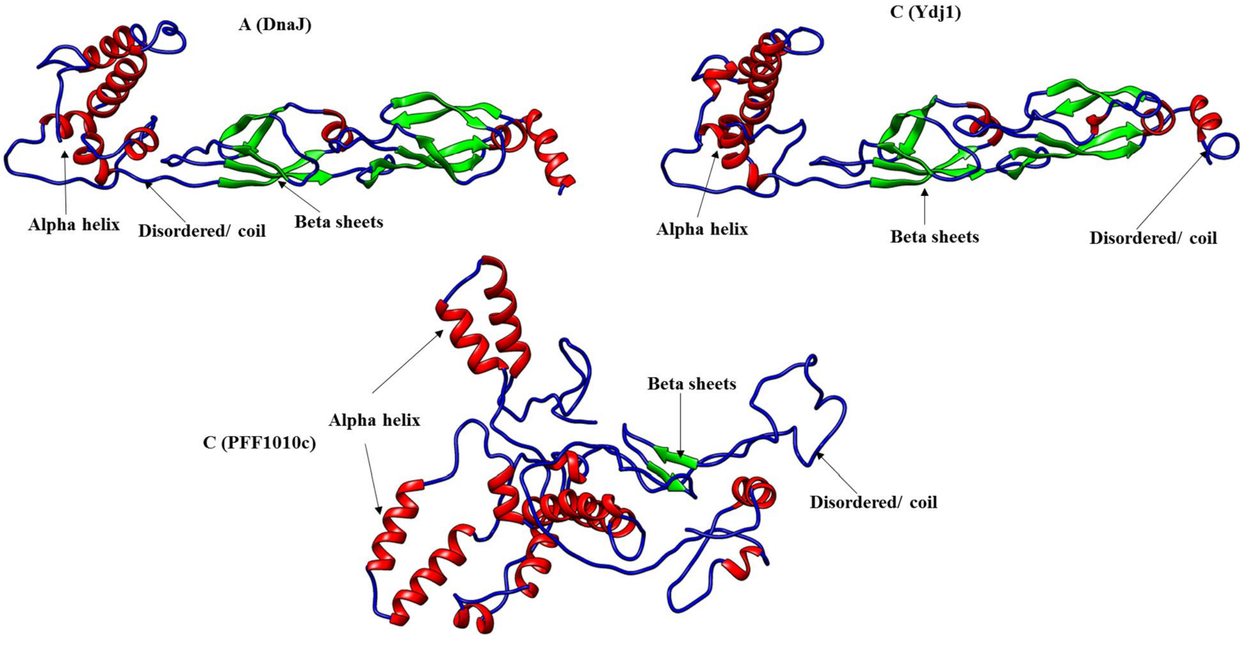

Figure 3.1.2 Comparison of 3 dimensional models of PFF1010c, DnaJ and Ydj1. (A), **(B)** and **(C)** represent 3 dimensional structures of DnaJ, PFF1010c and Ydj1 respectively. The 3D models shows the disordered/coil region in blue, the beta sheets in green and the alpha helix in red. The models were generated by Phyre2 (www.sbg.bio.ic.ac.uk/phyre2) and visualized using Chimera (www.cgl.ucsf.edu/chimera; Pettersen *et al*., 2004).

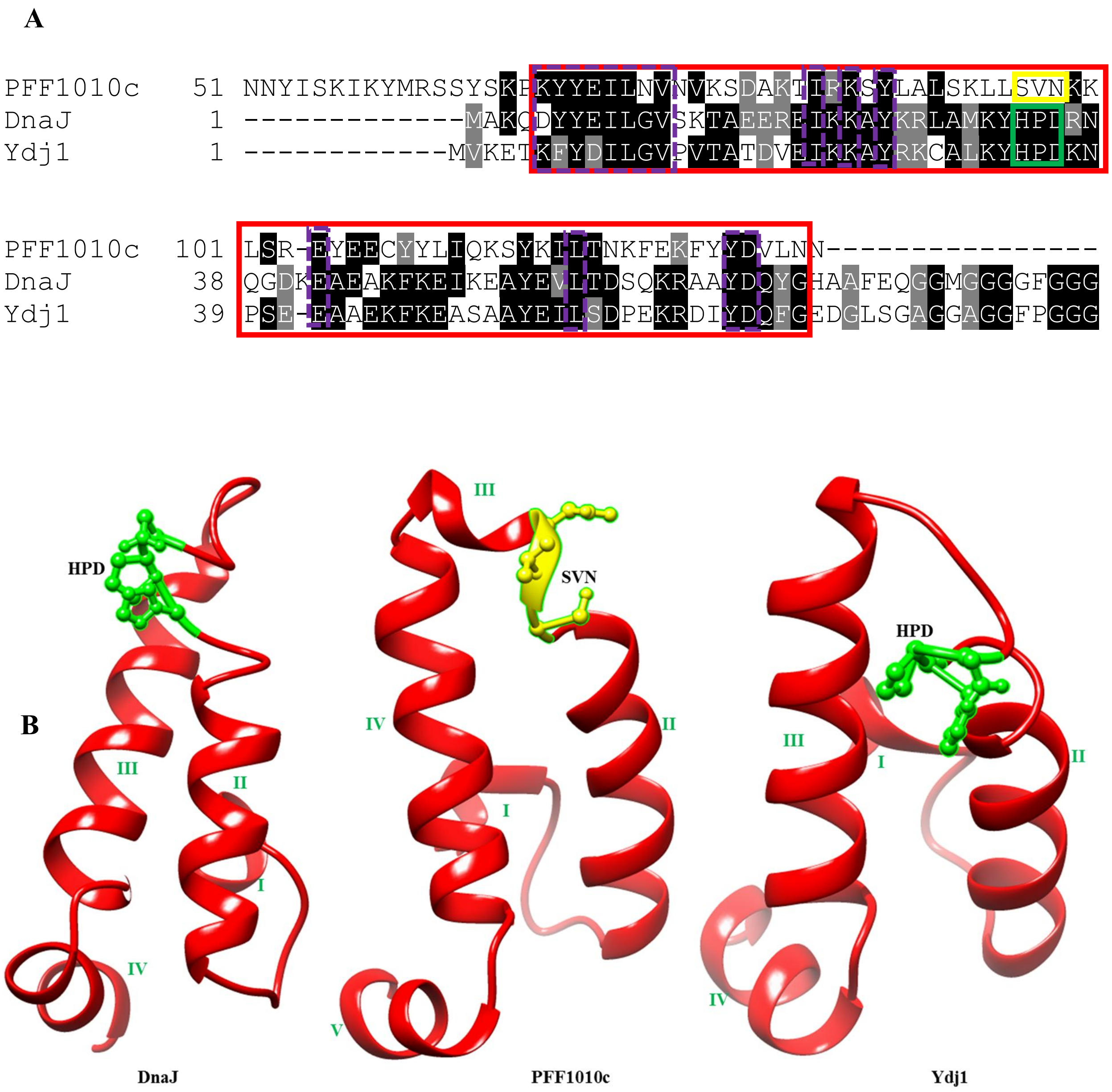

Figure 3.1.3 Comparison of J-domain models from PFF1010c, DnaJ and Ydj1. (A) An extract of a multiple sequence alignment showing the J-domains of PFF1010c, DnaJ and Ydj1 (highlighted by the blue solid box) (Hennessy *et al*., 2005). The HPD motif is highlighted by the red solid box and the residues (SVN) that replaced HPD in PFF1010c is highlighted in the yellow solid box. Conserved residues within the J domains (highlighted by purple dashed boxes). **(B)** 3D models of DnaJ, PFF1010c and Ydj1 with the HPD motif in DnaJ and Ydj1 represented by ball and stick in green and SVN of PFF1010c represented also in ball and stick yellow. The helices are indicated on the figure. The models were generated by Phyre2 (www.sbg.bio.ic.ac.uk/phyre2) and visualized using Chimera (www.cgl.ucsf.edu/chimera; Pettersen *et al*., 2004).

3.1.3 Prediction of PFF1010c's interaction partners

PFF1010c network data was retrieved from STRING (www.string-db.org; Jensen *et al*., 2009) and analyzed using Cytoscape (Shannon *et al*., 2003). STRING is a biological database and web resource of known and predicted protein interactions (Szklarczyk *et al*., 2015). Cytoscape is an open source bioinformatics software platform for visualizing molecular interaction networks and integrating with gene expression profiles and other state data (Shannon *et al*., 2003; Bell and Lewitter, 2006). The predicted functional partners of PFF1010c are shown (Figure 3.1.4). The predicted functional network is based on co-expression data (Szklarczyk *et al*., 2015). The interaction of these proteins are yet to be experimentally investigated. The predicted functional network shows that PFF1010c possibly interacts with these proteins in the following order, based on decreasing co-expression scores; PFE1105c (PF3D7_0522100), PFD0487c (PF3D7_0409900), PF11_0243 (PF3D7_1123200), PF14_0246 (PF3D7_1426700), PFI1560c (PF3D7_0932100), PFD0190w (PF3D7_0403900), MAL1391.228, PFB0435c (PF3D7_0209600), PF10_0308, and PFD0530c (PF3D7_0410700) (Figure 3.1.4). A table summarizing the predicted functional partners of PFF1010c, their description and co-expression scores of the prediction (Table 2). Co-expression scores represent predicted association between genes based on observed patterns of simultaneous expression of genes, the higher the score, the better the chance of protein-protein interaction. STRING recognizes the old annotation hence the use of both the old and new annotation of the proteins.

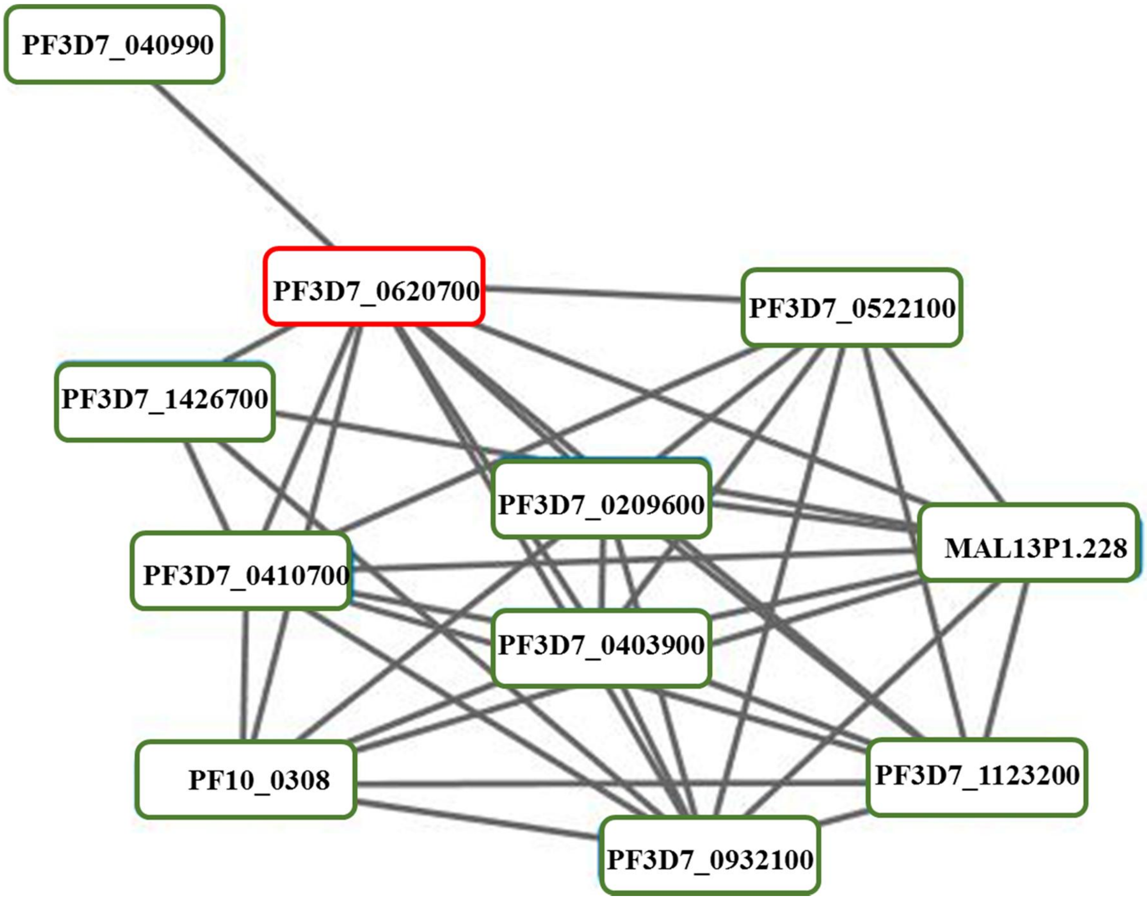

Figure 3.1.4 Network of PFF1010c and its predicted functional partners. PFF1010c (PF3D7_0620700) highlighted by a red box and its predicted functional partners. The accession names and corresponding numbers of the predicted interactors are : PFF1010c (PF3D7_0620700) putative DnaJ protein, PFE1105c (PF3D7_0522100) putative uncharacterized protein, PFD0487c (PF3D7_0409900) putative actin-like protein, PF11_0243 (PF3D7_1123200) leucine-rich repeat protein 11 (LRR11), PF14_0246 (PF3D7_1426700) phosphoenolpyruvate carboxylase (PEPC), PFI1560c (PF3D7_0932100) putative MAM3 protein, PFD0190w (PF3D7_0403900) putative SET domain protein, MAL1391.228 putative uncharacterized protein, PFB0435c (PF3D7_0209600) putative transporter protein, PF10_0308 putative OUT-like cysteine protease, and PFD0530c (PF3D7_0410700) ribosome biogenesis GTPase A, putative (RbgA). The interaction of these proteins is based on their co-expression scores. The interaction network was predicted using STRING (www.string-db.org; Jensen *et al.*, 2009) and analyzed using Cytoscape (Shannon *et al.*, 2003).

Table 2: Predicted functional interactors of PFF1010c, their description and co-expression score

Predicted interactor	**Description**	**Co-expression score**
PFE1105c (PF3D7_0522100)	putative uncharacterized protein	0.795
PFD0487c (PF3D7_0409900)	putative actin-like protein	0.748
PF11_0243 (PF3D7_1123200)	leucine-rich repeat protein 11 (LRR11)	0.738
PF14_0246 (PF3D7_1426700)	phosphoenolpyruvate carboxylase (PEPC)	0.737
PFI1560c (PF3D7_0932100)	putative MAM3* protein	0.735
PFD0190w (PF3D7_0403900)	putative SET* domain protein	0.717
MAL1391.228	putative uncharacterized protein	0.713
PFB0435c (PF3D7_0209600)	putative transporter protein	0.707
PF10_0308	putative OUT*-like cysteine protease	0.707
PFD0530c (PF3D7_0410700)	ribosome biogenesis GTPase* A, putative (RbgA)	0.703

*SET (Su(var), Enhancer of zeste,Trithorax) domain, OTU - ovarian tumour proteases, MAM domain is an evolutionary conserved protein domain, it is an extracellular domain found in many receptors, GTPases (singular GTPase) are a large family of hydrolase enzymes that can bind and hydrolyze guanosine triphosphate (GTP).

3.2 Confirmation of pQE30/PFF1010c plasmid

Restriction digest of pQE30/PFF1010c was carried out to verify the integrity of the construct (Figure 3.2). The pQE30/PFF1010c plasmid map is shown (Figure 3.2A), and the agarose gel electrophoresis of the digestion products (Figure 3.2B). Figure 3.2 B, lane M represents the DNA molecular mass marker (ThermoScientific, USA). The base pairs (bp) sizes of selected bands are indicated on the left. The pQE30/PFF1010c construct was linearized with restriction enzymes *Bam* HI and *Hind* III and a double digest was performed using both *Bam* HI and *Hind* III (Figure 3.2). *Bam* HI and *Hind* III digested at restriction sites 146 bp and 1240 bp respectively. Digestion with *Bam* HI produced a single linearized DNA fragment that was approximately 4513 bp (Figure 3.2) and a single digestion with *Hind* III (Figure 3.2) resulted in a fragment of the same size. A double digest with *Bam* HI and *Hind* III (Figure 3.2) resulted in two DNA fragments. The larger fragment observed in lane 4 was approximately 3424 bp and the smaller fragment was just above 1089 bp, these fragments were thus of the expected bp sizes. Thus the restriction analysis successfully verified the integrity of the pQE30/PFF1010c construct.

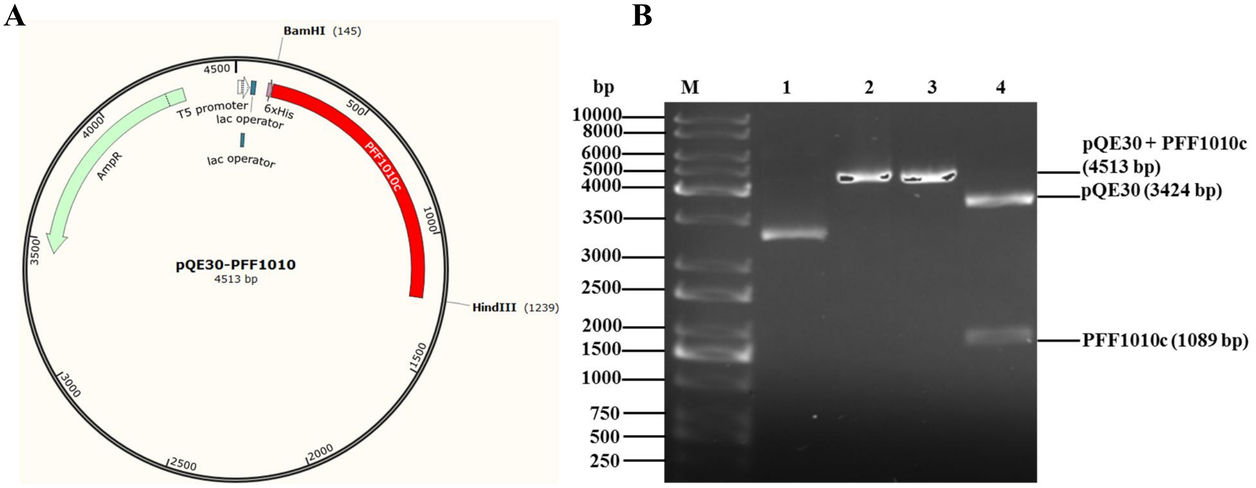

Figure 3.2 Restriction digest of pQE30/PFF1010c construct. (A) Plasmid map of pQE30/PFF1010c indicating the enzymes *Bam* HI and *Hind* III used for the restriction digest. (**B)** Agarose gel electrophoresis of pQE30/PFF1010c whereby the DNA samples were loaded onto the gel in the following order: M, molecular weight maker; lane 1, unrestricted pQE30/PFF1010c; lane 2, pQE30/PFF1010c restricted with *Bam* HI; lane 3, pQE30/PFF1010c restricted with *Hind* III; lane 4, pQE30/PFF1010c restricted with *Bam* HI and *Hind* III.

3.3 Expression of PFF1010c recombinant protein

Protein expression studies of PFF1010c was conducted using *E. coli* JM109 and BL21 Star (DE3) cells at 37 ºC. Protein expression was induced using 1 mM IPTG and 12 % SDS-PAGE and Western blot analysis were used to confirm the expression of PFF1010c at a molecular weight of 43 kDa (Figure 3.3). PFF1010c is expressed as a poly-histidine-tagged protein, in this study Western blot analysis using anti-His antibody was used to confirm expression of PFF1010c (Figure 3.3). In addition rabbit raised anti-PFF1010c was used to validate the expression of PF1010c

(Figure 3.3). Two *E. coli* strains were used in order to optimize expression of PFF1010c. The over expression of PFF1010c in *E. coli* JM109 cells was successful, (Figure 3.3 A). pQE30 was used as a negative control (Figure 3.3A; Lane C). Pre-induction sample does not show the expression of PFF1010c on the SDS-PAGE (Figure 3.3A; Lane 0; upper panel). However, Western blot analysis using anti-PFF1010c antibody detected PFF1010c in the pre-induction sample (Figure 3.3A; lower panel) which reveals leaky expression. The expression of PFF1010c in *E. coli* JM109 cells is observed to increase 1-4 hours post-induction (Figure 3.3A, upper panel). Western blot analyses using peptide specific anti-PFF1010c antibody also detected a second band below PFF1010c that is most likely break-down product and not due to non-specific binding as the band was not present in the control, or pre-induction samples, respectively (Figure 3.3A, lower panel). The expression of PFF1010c in *E. coli* BL21 Star (DE3) cells was also successful, however, protein levels appeared to remain constant over the course of 4 hours post-induction (Figure 3.3B; upper panel). Western blot analysis using anti-His and anti-PFF1010c revealed leaky expression as PFF1010c was detected in the pre-induced sample (Figure 3.3B; Lane 0; lower panel).

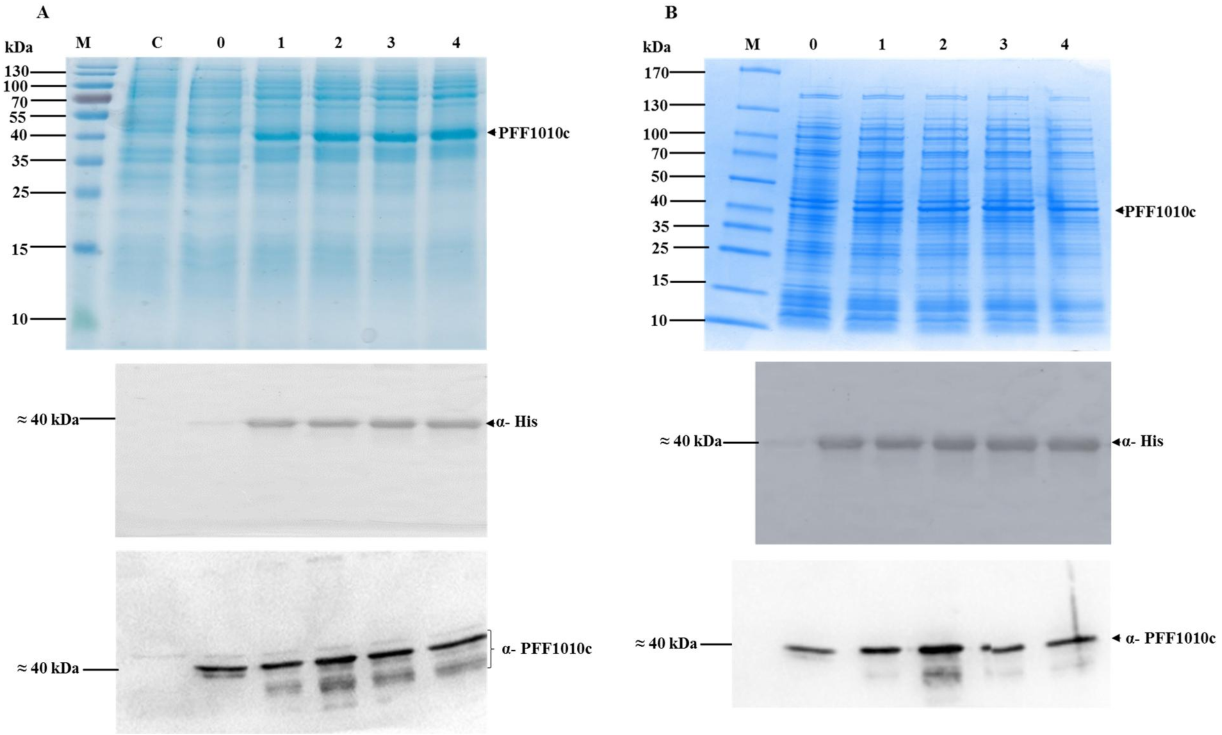

Figure 3.3 Analyses of the expression of PFF1010c in *E. coli*. (A) and **(B)** PFF1010c expressed in *E. coli* JM109 and BL21 Star (DE3) cells transformed with pQE30/PFF1010c . SDS-PAGE (upper panel), and Western analyses of (lower panel) the expression of PFF1010c using anti-His antibodies and anti-PFF1010c antibodies, respectively. Lane M – PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A) in kDa is shown on the left hand side; lane C – the total extract for cells transformed with pQE30 plasmid; lane 0 – total extract for cells transformed with pQE30/PFF1010c prior to IPTG induction; lanes 1, 2, 3, and 4 – total cell lysate obtained 1, 2, 3, and 4 hours post induction.

3.4 Solubility studies of PFF1010c recombinant protein

Proteins can be purified under either native or denaturing conditions (Bornhost and Falke, 2010). The recombinant PFF1010c protein was highly insoluble (most of the protein was recovered in the pellet fraction) (Figure 3.4A) when expressed in *E. coli* JM109 cells. Most of the protein was recovered in the insoluble fraction despite the addition of polyethyleneimine (PEI) (Section 3.4; Figure 3.4). PEI has been reported to aid the solubilisation of insoluble proteins, by precipitating nucleic acids complexed to the proteins leaving the target protein in the soluble fraction (Shonhai *et al*., 2008; Marenchino *et al*., 2009). The poor resolution observed in the SDS-PAGE images occurs as a result of the dense protein content and inclusion bodies associated with the material used in the solubility study (Figure 3.4). Protein purification was performed in batch, under denaturing conditions in the presence of PEI for protein solubilisation as previously described by Botha and colleagues (2010). When expressed in *E. coli* BL21 Star (DE3) cells PFF1010c recombinant protein was partially soluble as some protein was recovered in the soluble fraction and some also recovered from the pellet fraction (Figure 3.4B). PFF1010c was more soluble in *E. coli* BL21 Star (DE3) cells compered to *E. coli* JM109 cells (Figure 3.4). Therefore, nickel affinity purification of PFF1010c was performed under denaturing conditions for *E. coli* JM109 and native conditions for *E. coli* BL21 Star (DE3) cells.

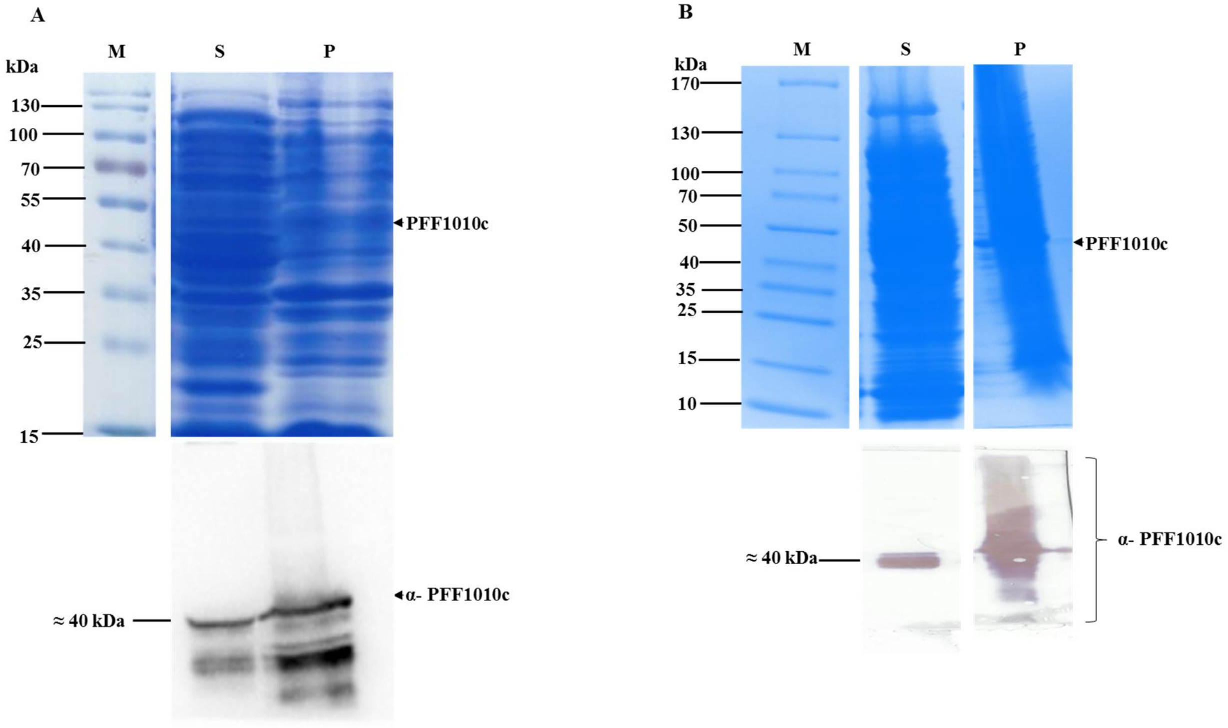

Figure 3.4 Analyses of the solubility of recombinant PFF1010c protein expressed in *E. coli* cells. (A) and **(B)** represents SDS-PAGE (upper panel) and Western blot analyses (lower panel) for the solubility of recombinant PFF1010c expressed in JM109 cells and *E. coli* BL21 Star (DE3) cells using anti-PFF1010c antibodies . Samples were collected after 4 hours of induction using IPTG. Lane M represents PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A) (kDa). Lanes S and P represent soluble and pellet fractions samples, respectively.

3.5 Purification of PFF1010c recombinant protein

Protein expression and induction using 1 mM IPTG at 37 ˚C was performed and the optimal level of expression was found to be 4 hours post-induction. Most of the PFF1010c expressed in *E. coli* JM109 was recovered in the pellet (insoluble) fraction (Section 3.4; Figure 3.4A). Nickel affinity chromatography was performed under denaturing conditions, using urea as the denaturing agent, for PFF1010c expressed in *E. coli* JM109 cells. SDS-PAGE was used to analyze the purification of PFF1010c (Figure 3.5 A and B; upper panel). It was observed on SDS-PAGE that a lot of protein did not bind to the beads as it remained in the flow through (Figure 3.5 A and B; Lanes FT; upper panel). Protein was also lost in the washing steps as depicted on the SDS-PAGE (Figure 3.5 A and B; Lanes W1 and W2; upper panel). Eluted protein in the denaturing purification was barely visible on the SDS-PAGE (Figure 3.5 A and B; Lanes E1 and E2; upper panel). However, Western blot analyses using peptide anti-PFF1010c confirmed the presence of PFF1010c (Figure 3.5 A and B; lower panel).

PFF1010c protein expressed in *E. coli* BL21 Star (DE3) cells was partially recovered in the soluble fraction (Section 3.4; Figure 3.4B) therefore purification was done under native conditions. SDS-PAGE was used to analyze the purification of PFF1010c (Figure 3.5C; upper panel). SDS-PAGE reveals that some protein was lost in the flow through (Figure 3.5C; Lane FT; upper panel). The protein was also lost during the washing steps (Figure 3.5C). The eluted protein came down with contaminants as there are bands of different molecular weight sizes (Figure 3.5C; Lane E; upper panel). Western blot analysis using peptide anti-PFF1010c confirmed the presence of PFF1010c (Figure 3.5C; lower panel). The typical yield of PFF1010c was ≈14.27 mg/L (Figure 3.5C).

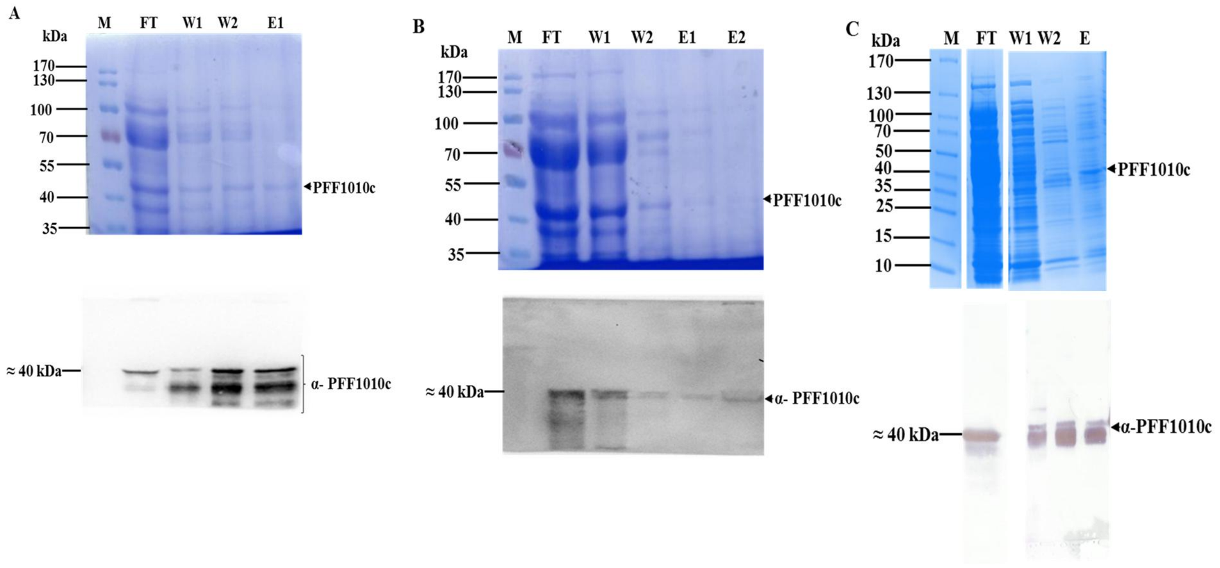

Figure 3.5. Analyses of the purification of recombinant PFF1010c. PFF1010c was purified under denaturing and native conditions. **(A)** SDS-PAGE (upper panel) and Western blot analyses (lower panel) for the purification of PFF1010c in *E. coli* JM109 cells using anti-PFF1010c, 8M urea was used throughout the purification process. **(B)** SDS-PAGE (upper panel) and Western blot analyses (lower panel) for the purification of PFF1010c in *E. coli* JM109 cells using anti-PFF1010c, purification performed with gradually decreasing urea concentration (8 M lysis buffer, 4M wash 1, 2 M wash 2 and no urea for elution 1 and 2). **(C)** SDS-PAGE (upper panel) and Western blot analyses (lower panel) for the purification of PFF1010c in *E. coli* BL21 Star (DE3) cells using anti-PFF1010c. Lane M - PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A) (kDa) is shown on the left hand side, lane FT - Flow through, lanes W1 and W2 - Washes 1 and 2, and lane E the Elution.

3.6 Detection of the PFF1010c-PfHsp70-1 partnership using far Western analysis

In order to determine whether recombinant PFF1010c protein directly interacted with recombinant PfHsp70-1 protein, far Western analysis was conducted (Figure 3.6). A blot containing PFF1010c lysate at various concentrations (10 µg, 15 µg, and 20 µg) and controls (BSA, PfHsp70-1 and PfHop) was probed using α-PfHsp70-1 antibody which detected only PfHsp70-1 (Figure 3.6A). This confirmed that the α-PfHsp70-1 antibody differentiates between PfHsp70-1 and PFF1010c. A similar blot was overlaid with PfHsp70-1 protein and then probed using α-PfHsp70-1 antibodies. A protein band was only observed in the lane representing PfHsp70-1 protein (positive control) (Figure 3.6; B). The assay was repeated in the presence of ADP and ATP (Figure 3.6 C and D respectively) and once more a protein band was only observed in the lane representing PfHsp70-1 protein (positive antibody control) also. No direct interaction between PFF1010c and PfHsp70-1 was observed in the absence or presence of nucleotides (Figure 3.6 panel B, C and D). However, direct interaction was observed for PfHop and PfHsp70-1 as previously reported (Gitau *et al.*, 2012) (Figure 3.6 panel B, panel C and panel D: lane C). There are some bands visible in lanes 3-5 which may be due to non-specific binding of anti-PfHsp70-1 to PFF1010c lysates. This may also be a result of insufficient blocking.

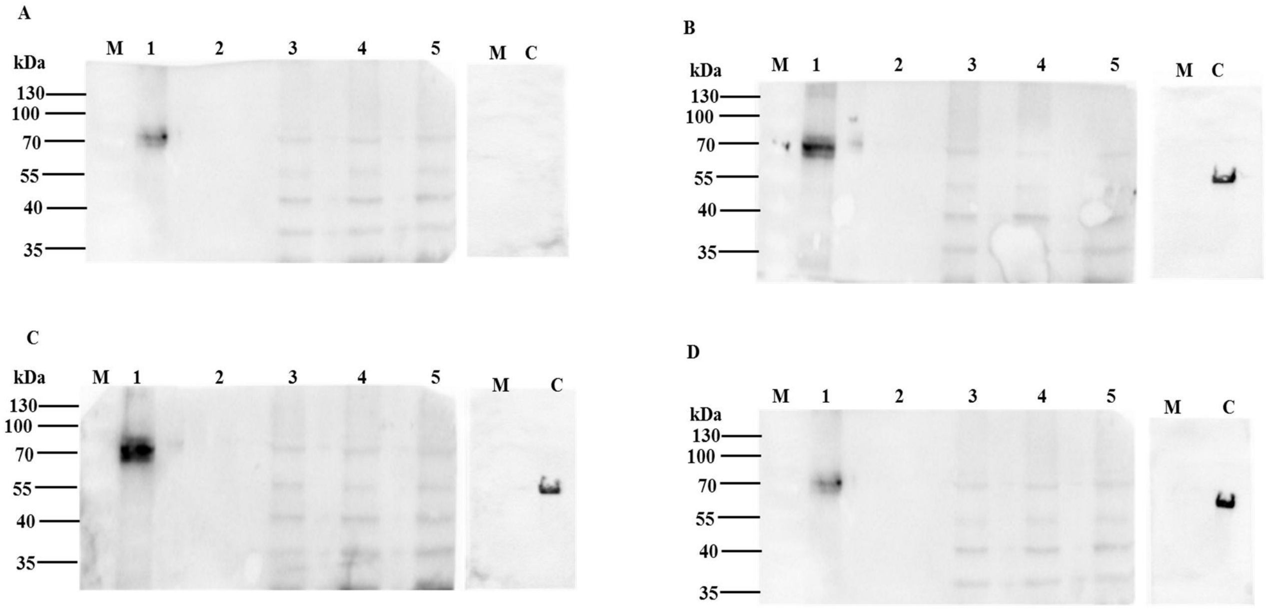

Figure 3.6 Far Western analysis of the interaction between lysate of PFF1010c and PfHsp70-1. Increasing concentrations of PFF1010c (10, 15 and 20 µg/ml) were run on 12 % SDS PAGE as the prey protein. BSA (20 µg/ml) was used as a negative control, PfHsp70-1 (20 µg/ml) as the antibody control and PfHop (20 µg/ml) was used as the positive control. The proteins were transferred to nitrocellulose membrane and overlaid with 20 µg/ml recombinant PfHsp70-1 protein in the presence of ATP and ADP. **(A)** Blot analyzed using α-PfHsp70-1 antibody. No overlaying of bait protein was done. **(B)** Overlaid with PfHsp70-1 protein in the absence of either ATP or ADP **(C)** Overlaid with PfHsp70-1 protein in the presence of ADP and **(D)** Overlaid with PfHsp70-1 protein in the presence of ATP. Anti-PfHsp70-1 antibody was used to detect the presence of PfHsp70-1 protein on the blots. Samples were loaded as follows: Lane M: PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A), Lane 1: PfHsp70-1 protein, Lane 2: BSA, lane 3: 10 µg PFF1010c, Lane 4: 15 µg PFF1010c, Lane 5: 20 µg PFF1010c lysate, and lane C: 20 µg/ml PfHop.

3.7 Investigating the expression of PFF1010c by parasites at distinct growth stages

Proteins were isolated from parasites at different stages of the parasite's life cycle; asexual stage, early gametocyte stage and the late gametocyte stage as previously described (Zininga *et al.*, 2015).

Asexual stage parasites used for protein isolation were of a mixed population of rings and trophozoites (Figure 3.7.1) at 4.25 % parasitaemia and 5 % haematocrit. The early gametocytes were at 1.3 % gametocitaemia and also 5 % haematocrit (Figure 3.7.1) and the late gametocytes were at 1 % gametocitaemia and also 5 % haematocrit (Figure 3.7.1).

Parasite lysates at these stages were loaded onto an SDS-PAGE to investigate whether PFF1010c was expressed at the gametocyte stages (Figure 3.7.2A). A Western blot analysis was conducted using peptide anti-PFF1010c antibody to confirm the expression of PFF1010c at the early and late gametocyte stages of the malarial parasite's life cycle (Figure 3.7.2B). Lane C, PFF1010c lysate (expressed in *E. coli* BL21 Star (DE3) cells) was used as positive control (Figure 3.7.2 A and B). PFF1010c was observed at the early and late gametocyte stages (Figure 3.7.2B). PFF1010c is shown to be poorly expressed as indicated by the intensity of the Western blot analysis (Figure 3.7.2B). This experiment was performed twice due to time constrains, however confirming a similar result. Western blot analysis using peptide anti-PFF1010c confirmed the expression of PFF1010c at the early and late gametocyte stages. Very low PFF1010C protein levels were detected at the early and late gametocyte stages possibly due to samples that may have degraded over long term storage (Figure 2; Appendix D).

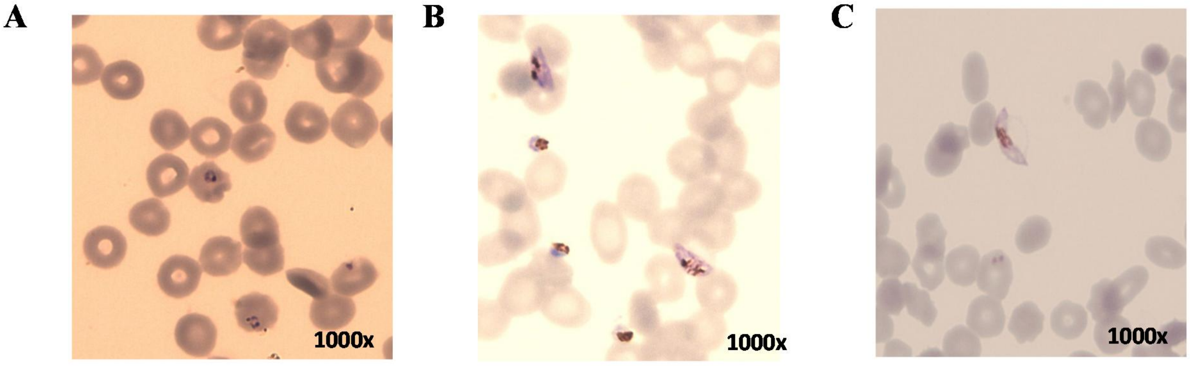

Figure 3.7.1 Light microscope images of PF3D7 parasite cultures. (A) Shows asexual parasites, ring and trophozoite stages. **(B)** Shows early gametocyte stage parasites. **(C)** Shows late gametocyte stage parasites.

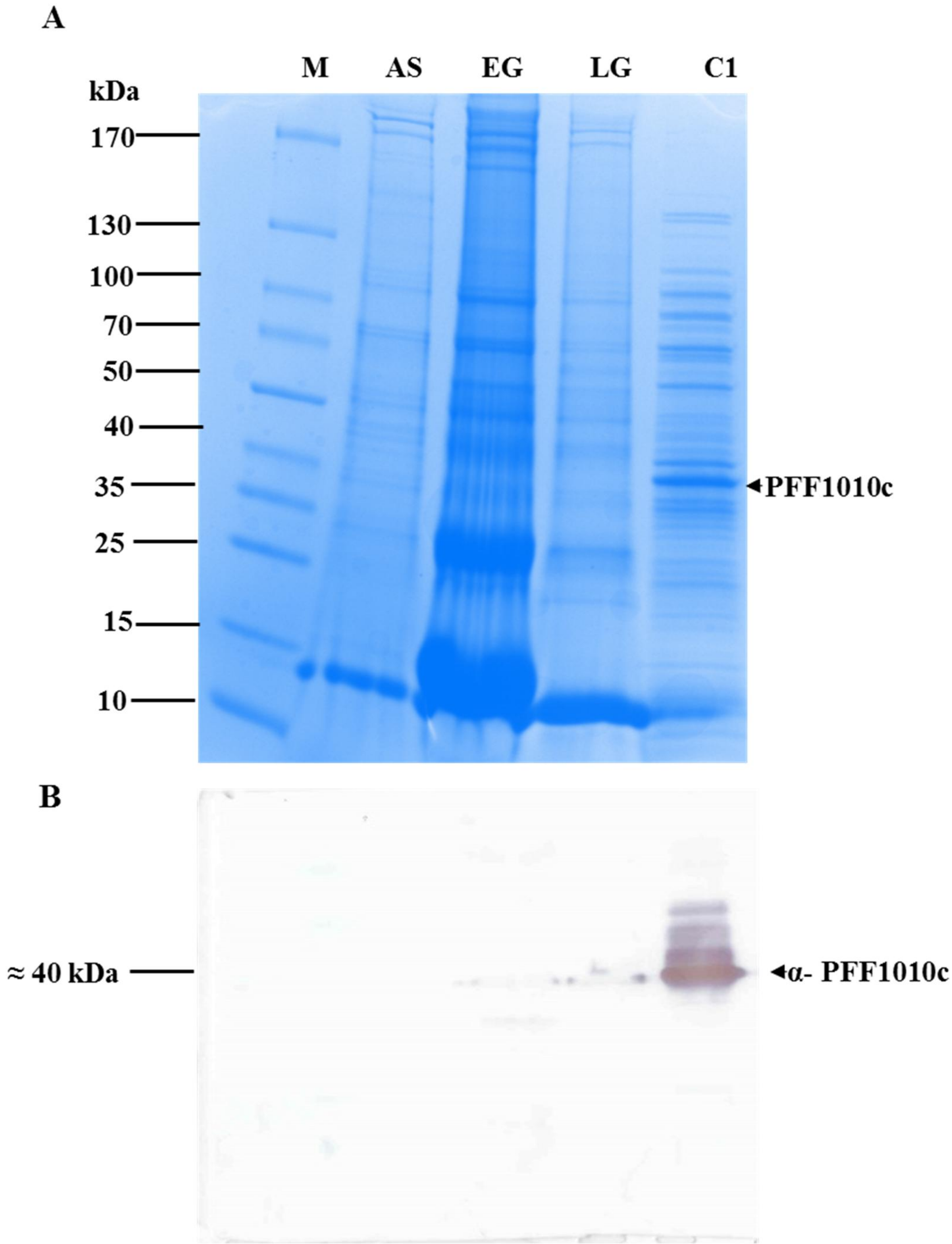

Figure 3.7.2 Detection of PFF1010c expression by *Plasmodium*. (A) 12 % SDS-PAGE of the analysis of PFF1010c expression malarial parasites at different stages of the parasite's life cycle. The samples were loaded as follows: Lane M: PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A), Lane AS: Proteins isolated from asexual parasites (negative control), Lane EG: Proteins isolated from early gametocyte parasites, Lane LG: Proteins isolated from late gametocyte parasites, Lane C: Total cell lysate extract of *E. coli* BL21 Star (DE3) cells transformed with pQE30/PFF1010c 4 hours post IPTG induction (positive control). **(B)** Western blot analysis using peptide anti-PFF1010c (1: 2000) as the primary antibody and HRP conjugated anti-rabbit (1:2000) as the secondary antibody.

3.8 Investigating the expression of PfHsp90, PfHop and PfHsp70-1 by parasites at the gametocyte stage

PFF1010c's expression at the gametocyte stage was reported in Section 3.7 (Figure 3.7). Bioinformatics analysis of PFF1010c failed to predict the interaction of this protein with any chaperone due to it being a novel and as of yet completely uncharacterized protein. To investigate the possibility of PFF1010c functioning as a promiscuous PfHsp40 we sought to study its possible interaction with canonical proteins PfHsp70-1, PfHsp90 and PfHop. Firstly, the expression of these canonical proteins at the gametocyte stage were investigated by Western blot analysis using anti-PfHsp90, anti-PfHop and anti-PfHsp70-1 antibodies. Isolated proteins from parasites at the early and late gametocyte stage were resolved using SDS-PAGE to investigate the expression of PfHsp90, PfHop and PfHsp70-1 at the gametocyte stage (Figure 3.8A). PfHsp70-1 (70-1), PfHop (Hop) and PfHsp90 (90) were used as positive controls (Figure 3.8A). The positive controls were barely visible on the SDS-PAGE (Figure 3.8A; 70-1, Hop and 90, respectively). However, Western blot analysis using corresponding antibodies detected all the positive controls (Figure 3.8 B, C and D). PfHop was detected as a doublet which is a result of protein degradation (Figure 3.8C). PfHsp70-1 (74 kDa) was observed to be expressed at both the early and late gametocyte stage. High yield of PfHsp70-1 was observed at the late gametocyte stage (Figure 3.8B). PfHop (66kDa) was observed to be expressed at the early gametocyte stage and no expression of PfHop was observed at the late gametocyte stage (Figure 3.8C). PfHsp90 (86 kDa) like PfHop was also observed to be expressed at the early gametocyte stage and no expression of PfHsp90 was observed at the late gametocyte stage (Figure 3.8D).

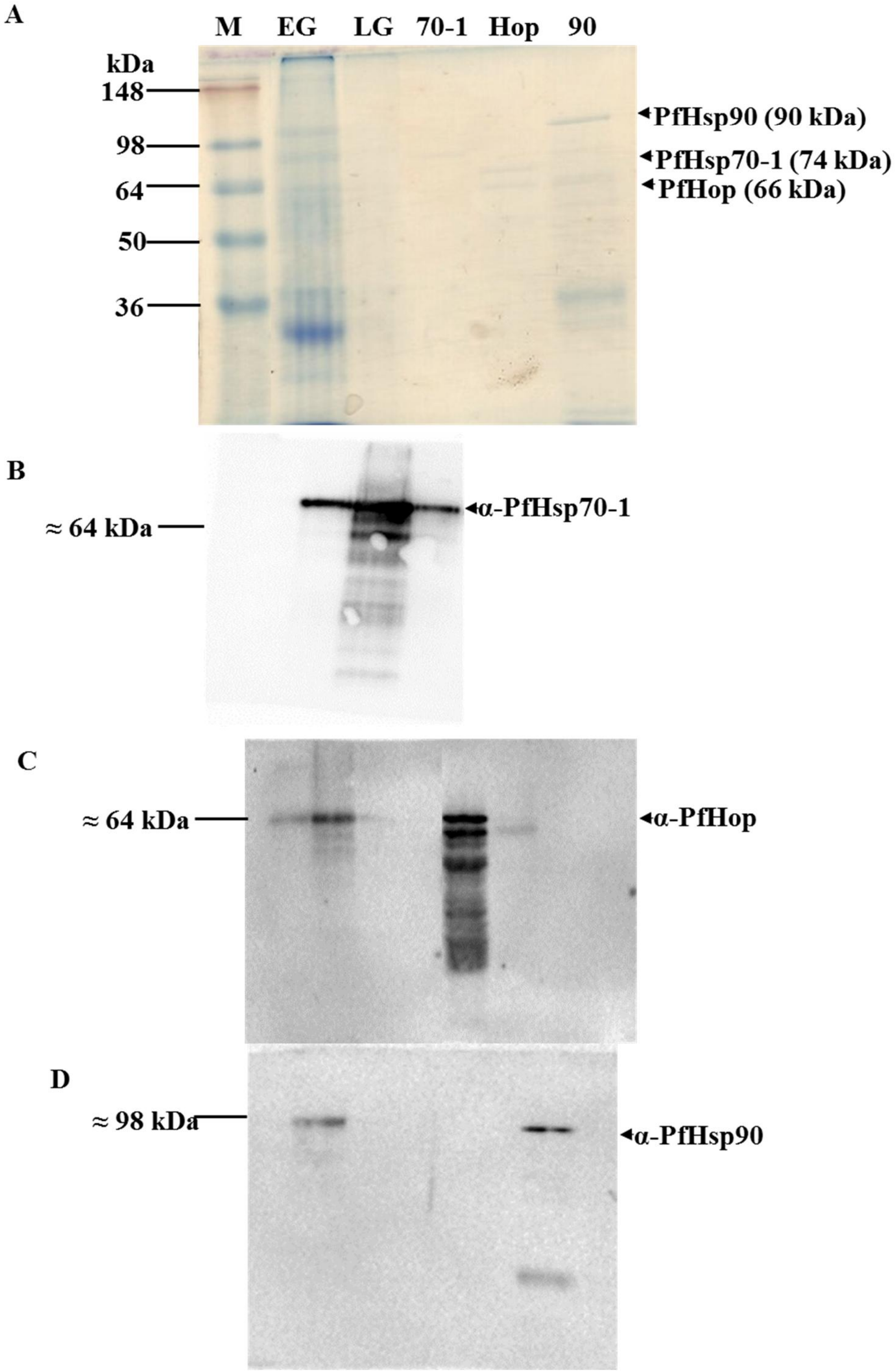

Figure 3.8 Expression of PfHsp90, PfHop and PfHsp70-1 by parasites at the gametocyte stage. (A) 12 % SDS-PAGE of expression of PfHsp90, PfHop and PfHsp70-1 at the *Plasmodium* gametocyte stage. Lane M: SeeBlue® Plus2 Pre-Stained Standard, Lane EG: Proteins isolated from early gametocyte parasites, Lane LG: Proteins isolated from late gametocyte parasites, Lane 70-1: purified PfHsp70-1, Lane Hop: purified PfHop and Lane 90: purified PfHsp90. **(B)** Western blot analysis using anti-PfHsp70-1 antibody. **(C)** Western blot analysis using anti-PfHop antibody. **(D)** Western blot analysis using anti-PfHsp90 antibody.

3.9 Assessment of PFF1010c's expression at the *Plasmodium* asexual stage

The development of malaria parasites at the blood stage is coupled with periodic fever conditions (Pallavi *et al*., 2010). These fever conditions are known to induce select Hsps which are required for maintaining the development of the parasite (Pallavi *et al*., 2010). In addition, the upregulation of some of the Hsps during fever conditions supplements parasite infectivity (Pallavi *et al*., 2010). Parasite lysates were obtained from and prepared as previously described by Zininga and colleagues (2015). To determine whether PFF1010c is an essential protein, the *in vivo* expression of PFF1010c under conditions of thermal stress was investigated. The heat stressed parasite lysate was subjected to Western blot analysis to investigate the expression of PFF1010c under thermal stress using PFF1010c antibodies (1:2000) (Figure 3.9) and HRP-conjugated anti-rabbit secondary antibodies (1: 2000) (Thermoscientific, USA). Detection of PFF1010c using Western blot analysis indicated its absence in uninfected red blood cells, as expected (lane UR). However, PFF1010c was also shown to be absent in parasites cultured at 37 ˚C and those subjected to heat stress (42 ˚C) (Figure 3.9).

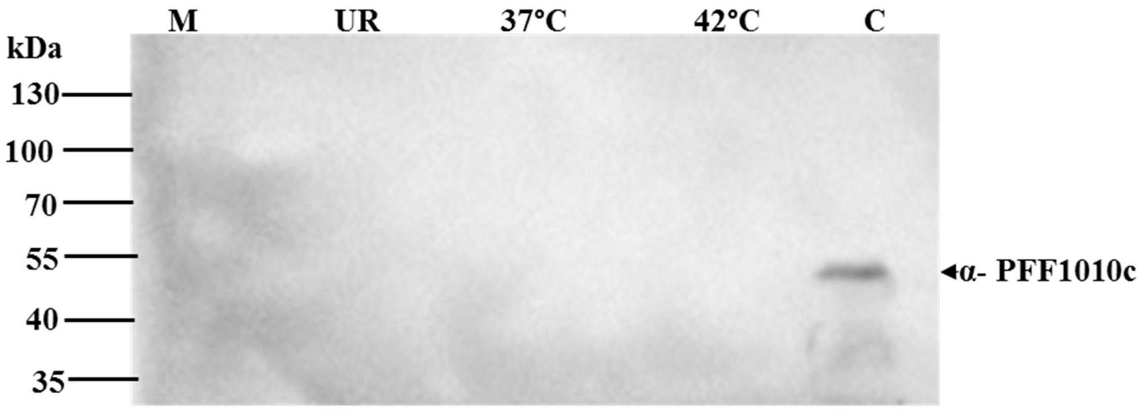

Figure 3.9 Analysis of PFF1010c expression by heat stressed asexual stage parasites. Western blot analysis for the expression of PFF1010c by parasite lysates under different temperatures. The samples were loaded as follows M is molecular marker, UR is the negative control which are uninfected RBCs, 37 ˚C represents the parasite lysates that were grown at 37 ˚C, 41 ˚C represents parasite lysates that were grown at 42 ˚C and C represents total cell lysate extract of *E. coli* BL21 Star (DE3) cells transformed with pQE30/PFF1010c collected 4 hours post induction. Anti-PFF1010c was used as the primary antibody and HRP conjugated anti-rabbit was used as the secondary antibody.

Chapter 4

Discussion and conclusive remarks

4.1 Discussion and conclusive remarks

This is the first report on the successful expression and purification of a previously uncharacterized type IV Hsp40 named PFF1010c (Njunge *et al*., 2013). Even though the type I's, II's and III's are typically well conserved across many species, the type IV Hsp40's are known to be abundant in the malaria parasite, *P. falciparum*, and are also known to occur in *Trypanosoma brucei* (Botha *et al*., 2007). Type IV Hsp40s have no homologs in higher eukaryotes including humans (Botha *et al*., 2010; Rug and Maier, 2011).

In the current study recombinant PFF1010c was successfully expressed in two *E. coli* strains. Despite the successful expression of the protein, its purification was problematic. In spite of this set-back bioinformatics analysis coupled with biochemical analysis revealed that Hsp40 type IV PFF1010c is expressed at the gametocyte stage of *P. falciparum*. The expression of this protein only at this stage suggests that it may play a crucial role in the development of malaria parasite at this stage.

In order to gain understanding of the structure-function features of PFF1010c, bioinformatics analysis were conducted. Upon its retrieval, PFF1010c's amino acid sequence was aligned with other type IV Hsp40 homologues from the *Plasmodium* species as well as the canonical and well characterized type I Hsp40s *E. coli* DnaJ and *S. cerevisiae* Ydj1. The HPD motif, located in the J-domain, is essential for interaction between Hsp40s and Hsp70s (Cheetham and Caplan, 1998). Due to the highly unstructured and unknown sequences of type IV proteins, excepting their J-domains, an alignment including type Is would serve as a form of orientation in unknown territory.

All the aligned protein sequences in this study possesses the J-domain (Figure 3.1.1). The alignment confirmed that PFF1010c and its homologues do not possess the HPD motif typical of J-domains (Botha *et al.,* 2007). Its absence in PFF1010c does not necessarily mean there is no interaction between Hsp70 and PFF1010c as there are other residues that were shown to be essential for interaction with Hsp70 (section 3.1; Figure 3.1.1). It is known that the mutation of the HPD motif causes an abrogation in binding of Hsp40 to Hsp70 (Botha *et al.,* 2007; Rug and Maier, 2011). It is however, proposed that type IV Hsp40s exert their function *via* different mechanisms to that of the type I and type III Hsp40s (Botha *et al*., 2007). Walsh and colleagues (2004) proposed that proteins containing J-domains with non-conservative substitutions in their HPD motifs imitate conserved HPD-motif containing J-domains, which results in a more complex regulation of Hsp70s (Walsh *et al*., 2004).

Comparison of the 3D structures of PFF1010c, Ydj1 and DnaJ revealed that the two type I Hsp40s share very similar protein structures. However in comparison PFF1010c showed a unique conformation to that observed for Ydj1 and DnaJ (Figure 3.1.2). Furthermore, the comparison also revealed that PFF1010c possesses more alpha helices and less beta sheets than observed for Ydj1 and DnaJ. The 3D structures indicated DnaJ to possess the highest number of beta sheets and Ydj1 had the most coiled regions (Figure 3.1.2). DnaJ and Ydj1 have a more relaxed or open conformation whereas PFF1010c is comprised of a more clustered conformation. Alpha helices have particular significance in DNA binding motifs, including helix-turn-helix motifs, leucine zipper motifs and zinc finger motifs (Ackbarow *et al.,* 2007). Alpha helices are also strongly associated with proteins that are harboured by biological membranes (transmembrane protein). This is because the helical structure can satisfy all backbone hydrogen-bonds internally, leaving no polar groups exposed to the membrane if the side chains are hydrophobic (Ackbarow *et al.,* 2007).

E. coli is known to be a reliable host for the expression of recombinant proteins, however not all recombinant proteins are produced successfully in this system (Martinez-Alonso *et al.,* 2010). *E. coli* is widely preferred due to cost considerations, speed, ease of use and genetic manipulation (Flick *et al*., 2004; Birkholtz *et al*., 2008). However, the quality of many proteins expressed in *E. coli* has not been satisfactory. In many cases, the recombinant proteins are either expressed as truncated forms or precipitate in insoluble inclusion bodies in the bacterial cells (Pandey *et al*., 2002; Sigh *et al*., 2003; Flick *et al*., 2004). Although protocols have been developed to obtain correctly folded proteins from these inclusion bodies, the process of refolding cannot be successfully applied to all proteins (Pandey *et al*., 2002; Singh *et al*., 2003).

The heterologous expression of *P. falciparum* proteins in *E. coli* is often challenging since the *P. falciparum* genome is AT rich, making it difficult for malarial proteins to be produced in *E. coli* (Flick *et al*., 2004). The high content of A/T repeats in the mRNA template is a reason for early translation termination and results in heterogeneity of recombinant proteins (Coppel and Black, 1998; Flick *et al*., 2004). Being aware of the potential difficulties of expression of a malarial protein in a bacterial host, this study employed two *E. coli* strains [JM109 and BL21 Star (DE3) cells] for the expression of PFF1010c. PFF1010c was successfully expressed as a species of approximately 43 kDa in both *E. coli* JM109 and BL21 Star (DE3) cells (Figure 3.3). Western blot analysis using α-PFF1010c against both strains of *E. coli* cells transformed with PFF1010c confirmed the presence of PFF1010c in the pre-induction sample indicative of "leaky" protein expression (Figure 3.3 A and B; Saluta and Bell, 1998; Saida *et al*., 2006). A major problem with *E. coli* expression is leaky expression of some proteins that could be toxic. This leads to plasmid loss/rearrangement, poor cell growth and poor protein expression (Saida *et al*., 2006). Leaky expression of the *lac* promoter can be minimized by introducing a catabolite such as glucose into the growth medium (Saluta and Bell, 1998). The introduction of a catabolite in the medium is termed catabolite repression. In order to minimize leaky expression it is recommended that preparations of transformations, stocks and overnight starter culture be done in the presence of glucose (Saluta and Bell, 1998). The solution for leaky expression could be to switch to a tightly regulated (i.e. non-leaky) expression systems such as the IPTG-induced *trc* promoter (Ptrc) (Amann *et al*., 1988) and the arabinose-induced *araBAD* promoter (P_{BAD}) systems (Guzman *et al*., 1995; Saida *et al*., 2006). *E. coli* JM109 cells yielded the best PFF1010c expression 4 hours post IPTG induction over *E. coli* BL21 Star (DE3) cells.

Solubility study of PFF1010c in both *E. coli* strains yielded partially soluble protein, but to different degrees (Figure 3.4). *E. coli* BL21 Star (DE3) yielded more soluble protein than *E. coli* JM109 cells (Figure 3.4). Insolubility of protein in *E. coli* is mainly due to protein accumulating in the so called inclusion bodies (Flick *et al*., 2004; Birkholtz *et al*., 2008). Some factors that may contribute to aggregate formation include lack of posttranslational modification (especially glycosylation), lack of access to chaperones, and enzymes catalyzing folding (e.g., cis-trans isomerase) and the very high concentration of protein coupled with limited solubility of folding intermediates (Palmer and Wingfield, 2004)

PFF1010c expressed in *E. coli* BL21 Star (DE3) cells was purified natively (Figure 3.5C). Purification under native conditions was employed because the protein was partially recovered in the soluble fraction after separation of the soluble and insoluble fraction by centrifugation (Figure 3.4B). The native purification of PFF1010c was unsuccessful as the eluted protein contained some contaminating bands of which most were not detected by peptide anti-PFF1010c during the Western blot analysis of the natively purified samples (Figure 3.5C). Contamination of the purified protein with *E. coli* host cell proteins is an indication that sonication may have been too severe (Haper and Speicher, 2013).

PFF1010c expressed in *E. coli* JM109 was successfully purified under denaturing conditions. However, the eluted proteins in both the denaturing purifications (using 8 M urea throughout the purification process and using gradually decreasing urea concentrations) was of very low yields as they were barely visible on the SDS-PAGE (Figure 3.5 A and B). Western blot analysis confirmed the presence of PFF1010c (Figure 3.5 A and B). This was mainly because large amounts of the protein was lost in the flow through and washing steps, which in most cases is the result of high concentrations of imidazole in the washing buffers. However, in this study this was not the case as the wash buffers either contained no imidazole or very low concentrations of the chemical. The little eluted protein was lost during attempts to remove the denaturant (urea) by dialysis/ buffer exchange in order to use the protein for further analysis. This may be due to proteases, because the dialysis process was lengthy and possibly the concentration of protease inhibitors used was insufficient. Matambo and colleagues (2004) and Ramya and colleagues (2006) showed that proteins successfully purified under denaturing conditions could regain their functional activity once the denaturant has been removed.

Expression data of PFF1010c assayed by oligonucleotide microarray analysis suggests that the protein is expressed at the gametocyte stage of the parasite's life cycle (www.PlasmoDB.org; Aurrecoechea *et al*., 2009). However, expression of the protein at the gametocyte stage has not been validated to date. PFF1010c's expression at the gametocyte stage was confirmed by Western blot analysis using peptide anti-PFF1010c (Figure 3.7.1.2). This confirmed that PFF1010c is only expressed only at the gametocyte stage of the parasites. It is known that PfHsp70-1, PfHop and PfHsp90 are expressed at the asexual stage of malaria parasite (Gitau, *et* al., 2012; Zininga *et al*., 2015). However, expression of these proteins at the gametocyte stages had not yet been validated. The investigation of the expression of these proteins at the gametocyte stage was carried out so as determine which ones are co-expressed with PFF1010c. PFF1010c's interaction partners was predicted using STRING (www.string-db.org; Jensen *et al*., 2009). In the current study, Far Western blot did not show an interaction between PfHsp70-1 and PFF1010c. Hence the next step was to look at canonical Hsps that are co-expressed with PFF1010c. The co-expression of these proteins with PFF1010c may be used as a guideline to investigate direct interaction between PFF1010c and these chaperones. This study investigated the expression of these proteins at the early and late gametocyte stages (Figure 3.8). PfHsp70-1 was confirmed to be expressed at both the early and late gametocyte stages of the parasite whereas PfHop and PfHsp90 were confirmed to be expressed only at the early gametocyte stage of the parasite's development (Figure 3.8). Further studies such as Dot blot, far Western blot and Surface plasmon resonance (SPR) need to be carried out to determine whether these proteins interact with PFF1010c.

Far Western analysis was conducted in order to determine whether PFF1010c would interact with PfHsp70-1 despite its variations in the highly conserved HPD motif. The bioinformatics suggested that the residues that substitute the HPD motif in PFF1010c have some degree of chemical similarity to the HPD motif. Far Western analysis was performed using recombinant PFF1010c present in *E. coli* lysate. The positive control (PfHsp70-1) only revealed the specificity of the antibody. The far Western blot analysis was successful as the positive control (PfHop) showed interaction with PfHsp70-1 in the absence and presents of nucleotides (ADP and ATP) (Gitau, 2014). The fact that PFF1010c does not have an HPD motif may also be the reason no interaction was observed as it is said to be the primary mode of interaction between Hsp70 and Hsp40 (Shonhai and Blatch, 2014). However, the lack of interaction between PFF1010c and PfHsp70-1 observed in this study may also be due to Far Western not detecting transient interactions (Bowman *et al*., 2015).

It was further investigated whether PFF010c is essential by investigating it's *in vivo* expression under conditions of heat stress using asexual malarial parasites as a negative control (Figure 3.7.2). The expression of PfHsp70-1, PfHop and PfHsp90 at the gametocyte stage by *P. falciparum* was investigated and confirmed by Western blot analyses. PFF1010c was well expressed in both *E. coli* JM109 and *E. coli* BL21 Star (DE3) cells, however it was mostly recovered in the insoluble fraction. Partial protein was also recovered in the soluble fraction in both strains, with *E. coli* BL21 Star (DE3) having more soluble protein than that recovered in *E. coli* JM109. Despite the good expression of PFF1010c, the purification of this protein was problematic. Which in turn was a limitation to performing activity assays using the pure protein. For future work, it would be interesting to investigate whether the expression of PFF1010c by gametocyte stage parasites is upregulated by heat stress. Optimization of native and denaturing purification of PFF1010c. PFF1010c could also be produced in a Gram-positive expression strain, the *Bacilli* strains. *Bacilli* strains are attractive hosts due to their ability to express heterologous protein inside cultured cells and secrete the expressed protein into the extracellular medium (Westers *et al*., 2004; D'Urzo *et al*., 2013). The *Bacilli* expression system thus offers an attractive alternative to the problematic expression of low protein expression levels in *E. coli* (Takara-Bio, Japan). The *Brevibacillus choshinensis* strain also possess a mutated intracellular protease gene (*imp*) and extracellular protease gene (*emp*) to further protect the structural integrity of expressed protein. Thus the purification of PFF1010c may be successful and activity assays can be conducted for the protein thereafter.

In conclusion, this study has confirmed that PFF1010c is expressed at the early and late stages of the gametocyte phase of parasite growth. Furthermore, PFF1010c's expression is restricted to the gametocyte stage as the protein is not expressed at the blood stages of parasite growth. PfHsp70-1, PfHop and PfHsp90 were also shown to be expressed at the gametocyte stages, suggested their possible direct or indirect cooperation with PFF1010c. These preliminary findings indicated that even though these proteins are both expressed at the gametocyte stages, PFF1010c does not interact with PfHsp70-1. This suggests a crucial role for this protein at the gametocyte stage. The expression of PFF1010c at the gametocyte stage suggests a crucial role for this protein. Attempts to purify the recombinant protein were unsuccessful, thereby impeding further biochemical characterisation of the protein. Protocols to improve the production and purification of the recombinant protein need to be developed towards facilitating further biochemical characterization of this apparently important molecule. Considering PFF1010c's expression is tied to one end of the malarial parasite's development, characterization of this protein and identification of possible partnerships with molecular chaperones could lead to development of antimalarial drug targets.

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Appendix A

Special chemical reagents

Name of reagents	Vendor/Supplier
Agarose	Merck
Ammonium persulphate	Sigma-Aldrich
Ampicillin	Calbiochem
Chemiluminescence Western blotting kit	Amarsham
2-mercaptoethanol	Merck
Bovine serum albumin	Melford
Bromophenol blue	Merck
Calcium chloride	Merck
Coomasie brilliant blue R250	Merck
Ethidium bromide	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	Merck
Imidazole	Merck
Lambda DNA	Fermantas Life sciences
Lysozyme	Merck
Methanol	Merck
Magnesium chloride	Merck
Monoclonal mouse anti-His antibody	Merck
Phenylmethylsufonyl fluoride	Merck
Polyacrylamide	Merck
Ponceau S	Sigma-Aldrich
Restriction enzymes	Merck
Sodium chloride	Merck
Sodium dodecyl sulphate	Merck
Sodium hydroxide	Merck
TEMED	Sigma-Aldrich
Tris	Merck
Tryptone	Oxoid, England
Tween 20	Radchem
Yeast	Merck
Broth	Merck
Nutrient agar	Merck
Glucose	Merck
SeeBlue® Plus2 Pre-Stained Standard	Thermo Scientific
PagerRuler Prestained Protein Ladder	Thermo Scientific

Appendix B

Preparation of solutions

PBS buffer (pH 7.4)

137 mM sodium chloride, 27 mM potassium chloride, 4.3 mM sodium hydrophosphate and 1.4 mM potassium hydrophosphate were dissolved in ddH_20 and autoclaved.

TE buffer (pH 8.0)

0.1211 g Tris and 0.0370 g EDTA 100 ml were mixed together with distilled water and autoclaved.

Sodium hydroxide

115 g of sodium hydroxide was dissolved in 250 ml of distilled water.

SDS (10%)

1 g of SDS was dissolved in 10 ml of distilled water

30% glycerol

30 ml of glycerol was mixed together with 70 ml of distilled water to make 100 ml of 30% glycerol.

IPTG

1 g of IPTG was dissolved in 4.2 ml of distilled water and filtered through filter into 1 ml eppendorf tube then stored at -20 ^{0}C

10X TAE buffer (pH 7.4)

48.4 g of Tris, 20 ml of 0.5M EDTA and 11.42 ml of glacial acetic acid were mixed together with distilled water and made up to 1000 ml.

Ampicillin

1 g of ampicillin was dissolved in 10 ml of distilled water and filtered through a filter unit into eppendorf tube100 µg/ml

Ampicillin agar plates

1.6 g of pancreatic tryptone, 1.5 g nutrient agar, 0.5 g of sodium chloride and 1 g of yeast were mixed together with distilled water and the volume was made up to 100 ml in a flask and autoclaved. The mixture was allowed to cool and 10 µl of ampicillin was added and poured into Petri dishes and allowed to solidify.

2xYT broth

1.6 g of pancreatic tryptone, 0.5 g of sodium chloride and 1 g of yeast were mixed together with distilled water and the volume was made up to 100 ml in a flask and autoclaved.

Ammonium persulphate (10%)

1 g of Ammonium persulphate was dissolved in 10 ml of distilled water.

Acrylamide stock solution

300 ml of distilled water was placed in a beaker and 150 g of acrylamide was added to distilled water while stirring and 4.0 g of bisacrylamide was added while stirring and allowed to dissolve. The volume of the solution was made up to 500 ml with distilled water. The

acrylamide/Bisacrylamide solution was filtered with a cloth and placed in a dark container and stored in the fridge.

Ethanol 70%

70 ml of alcohol was mixed with 30 ml of distilled water and the volume was made up to 100 ml

0.1 M Calcium chloride

1.47 g of calcium chloride was dissolved in 100 ml of distilled water

0.1 M Magnesium chloride

2.0 g of magnesium chloride was dissolved in 100 ml of distilled water

Ethidium bromide (10 mg/ml)

1 g of ethidium bromide was dissolved in 100 ml of distilled water and stored in a bottle covered with aluminium foil

Tris

1.5M Tris (pH 8.8)

36.3 g of Tris was dissolved in 200 ml of distilled water

0.5M Tris (pH 6.8)

12.11 g of Tris was dissolved in 200 ml of distilled water

Coomassie destaining solution

800 ml of distilled water, 7.5 ml glacial acetic acid and 100 ml of ethanol were mixed together and the volume was made up to 1000 ml

Appendix C

Protein concentration determination

Materials:

Protein serial dilution (200, 100, 50, 25, 12.5, 6.25 µg/ml)

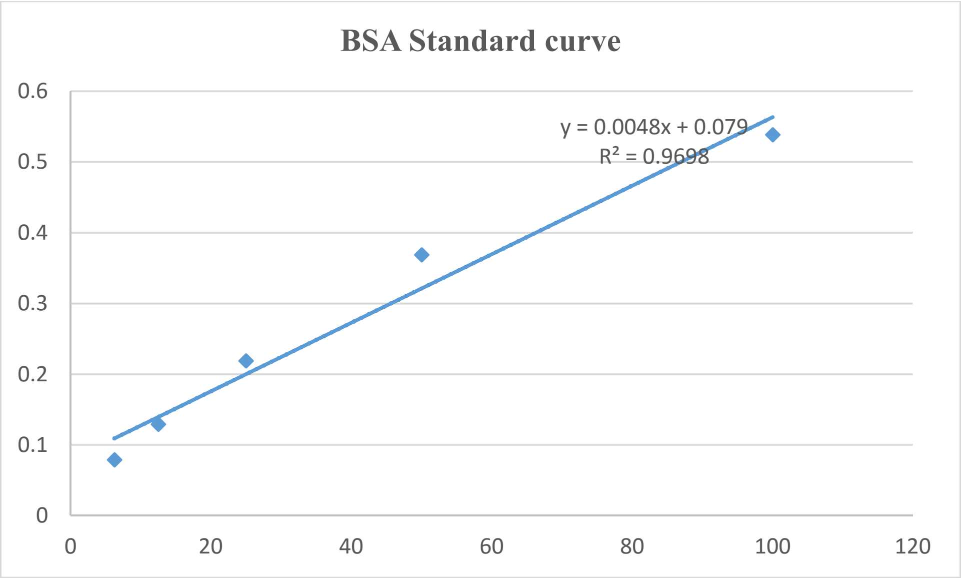
Bradford

Elisa plate

Needle

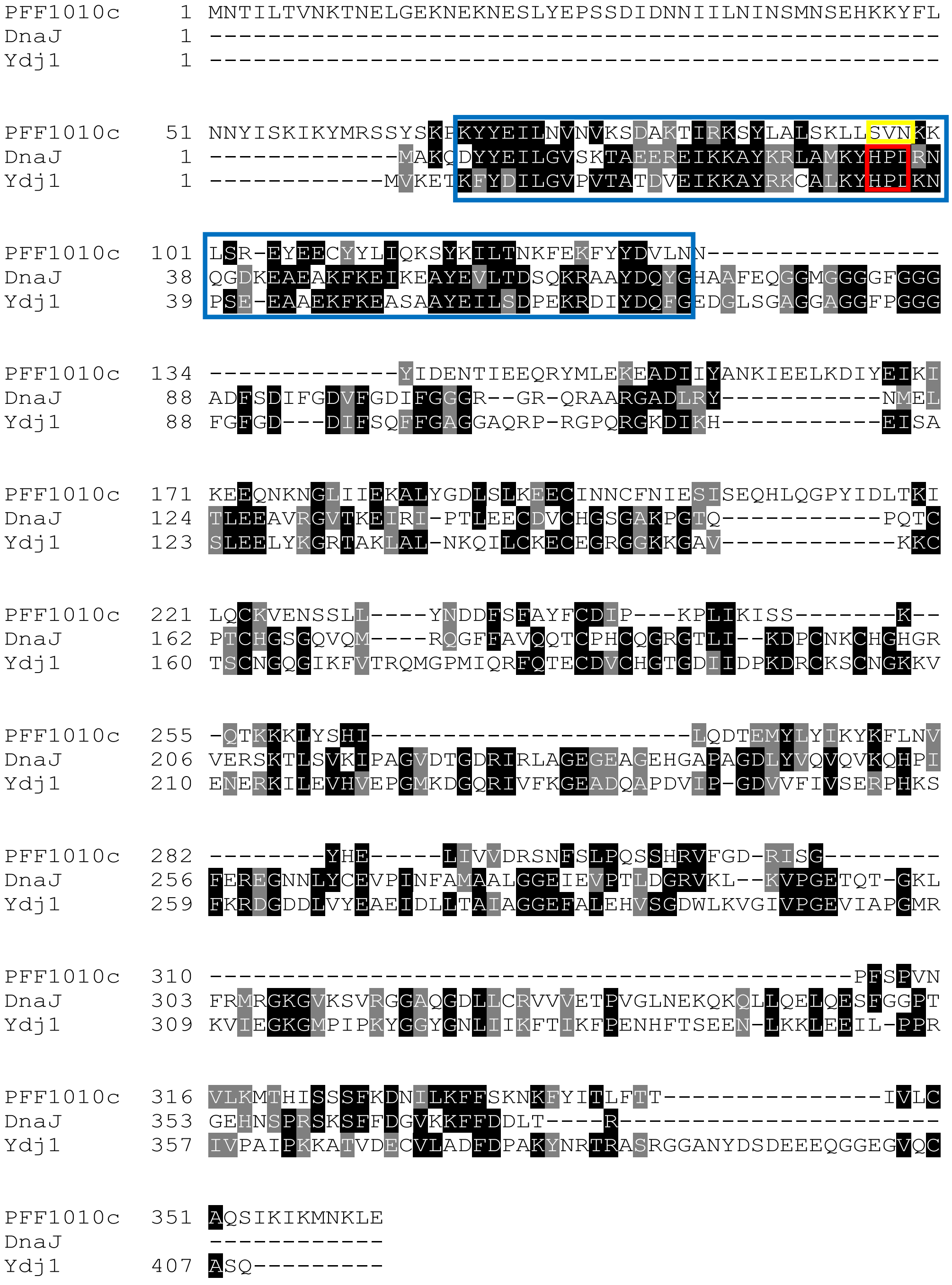
Method:

(1) Load 150 µl Bradford
(2) Load 50 µl serial dilution and blank in duplicate
(3) Load 50 µl protein samples in duplicate
(4) Burst bubbles with needle
(5) Incubate for 15’
(6) Read absorbance at 595 nm



Appendix D Supplementary data

D1 Full length multiple sequence alignment of PFF1010c, DnaJ and Ydj1



```
PFF1010c     1 MNTILTVNKTNELGEKNEKNESLYEPSSDIDNNIILNINSMNSEHKKYFL
DnaJ         1 --------------------------------------------------
Ydj1         1 --------------------------------------------------

PFF1010c    51 NNYISKIKYMRSSYSKPKYYEILNVNVKSDAKTIRKSYLALSKLLSVNKK
DnaJ         1 -------------MAKQDYYEILGVSKTAEEREIKKAYKRLAMKYHPDRN
Ydj1         1 ------------MVKETKFYDILGVPVTATDVEIKKAYRKCALKYHPDKN

PFF1010c   101 LSR-EYEECYYLIQKSYKILTNKFEKFYYDVLNN----------------
DnaJ        38 QGDKEAEAKFKEIKEAYEVLTDSQKRAAYDQYGHAAFEQGGMGGGGFGGG
Ydj1        39 PSE-EAAEKFKEASAAYEILSDPEKRDIYDQFGEDGLSGAGGAGGFPGGG

PFF1010c   134 --------------YIDENTIEEQRYMLEKEADIIYANKIEELKDIYEIKI
DnaJ        88 ADFSDIFGDVFGDIFGGGR--GR-QRAARGADLRY-----------NMEL
Ydj1        88 FGFGD---DIFSQFFGAGGAQRP-RGPQRGKDIKH-----------EISA

PFF1010c   171 KEEQNKNGLIIEKALYGDLSLKEECINNCFNIESISEQHLQGPYIDLTKI
DnaJ       124 TLEEAVRGVTKEIRI-PTLEECDVCHGSGAKPGTQ-----------PQTC
Ydj1       123 SLEELYKGRTAKLAL-NKQILCKECEGRGGKKGAV------------KKC

PFF1010c   221 LQCKVENSSLL----YNDDFSFAYFCDIP---KPLIKISS-------K--
DnaJ       162 PTCHGSGQVQM----RQGFFAVQQTCPHCQGRGTLI--KDPCNKCHGHGR
Ydj1       160 TSCNGQGIKFVTRQMGPMIQRFQTECDVCHGTGDIIDPKDRCKSCNGKKV

PFF1010c   255 -QTKKKLYSHI----------------------LQDTEMYLYIKYKFLNV
DnaJ       206 VERSKTLSVKIPAGVDTGDRIRLAGEGEAGEHGAPAGDLYVQVQVKQHPI
Ydj1       210 ENERKILEVHVEPGMKDGQRIVFKGEADQAPDVIP-GDVVFIVSERPHKS

PFF1010c   282 --------YHE-----LIVVDRSNFSLPQSSHRVFGD-RISG--------
DnaJ       256 FEREGNNLYCEVPINFAMAALGGEIEVPTLDGRVKL--KVPGETQT-GKL
Ydj1       259 FKRDGDDLVYEAEIDLLTAIAGGEFALEHVSGDWLKVGIVPGEVIAPGMR

PFF1010c   310 --------------------------------------------PFSPVN
DnaJ       303 FRMRGKGVKSVRGGAQGDLLCRVVVETPVGLNEKQKQLLQELQESFGGPT
Ydj1       309 KVIEGKGMPIPKYGGYGNLIIKFTIKFPENHFTSEEN-LKKLEEIL-PPR

PFF1010c   316 VLKMTHISSSFKDNILKFFSKNKFYITLFTT---------------IVLC
DnaJ       353 GEHNSPRSKSFFDGVKKFFDDLT----R----------------------
Ydj1       357 IVPAIPKKATVDECVLADFDPAKYNRTRASRGGANYDSDEEEQGGEGVQC

PFF1010c   351 AQSIKIKMNKLE
DnaJ           ------------
Ydj1       407 ASQ---------
```

Figure D1. Full length multiple sequence alignment of PFF1010c, DnaJ and Ydj1 showing the J-domains (Highlighted by the blue solid box). The HPD motif is highlighted by the red solid box and the residues (SVN) that replaced the HPD in PFF1010c are highlighted in the yellow solid box.

D2 Analysis of PFF1010c expression by parasite lysates

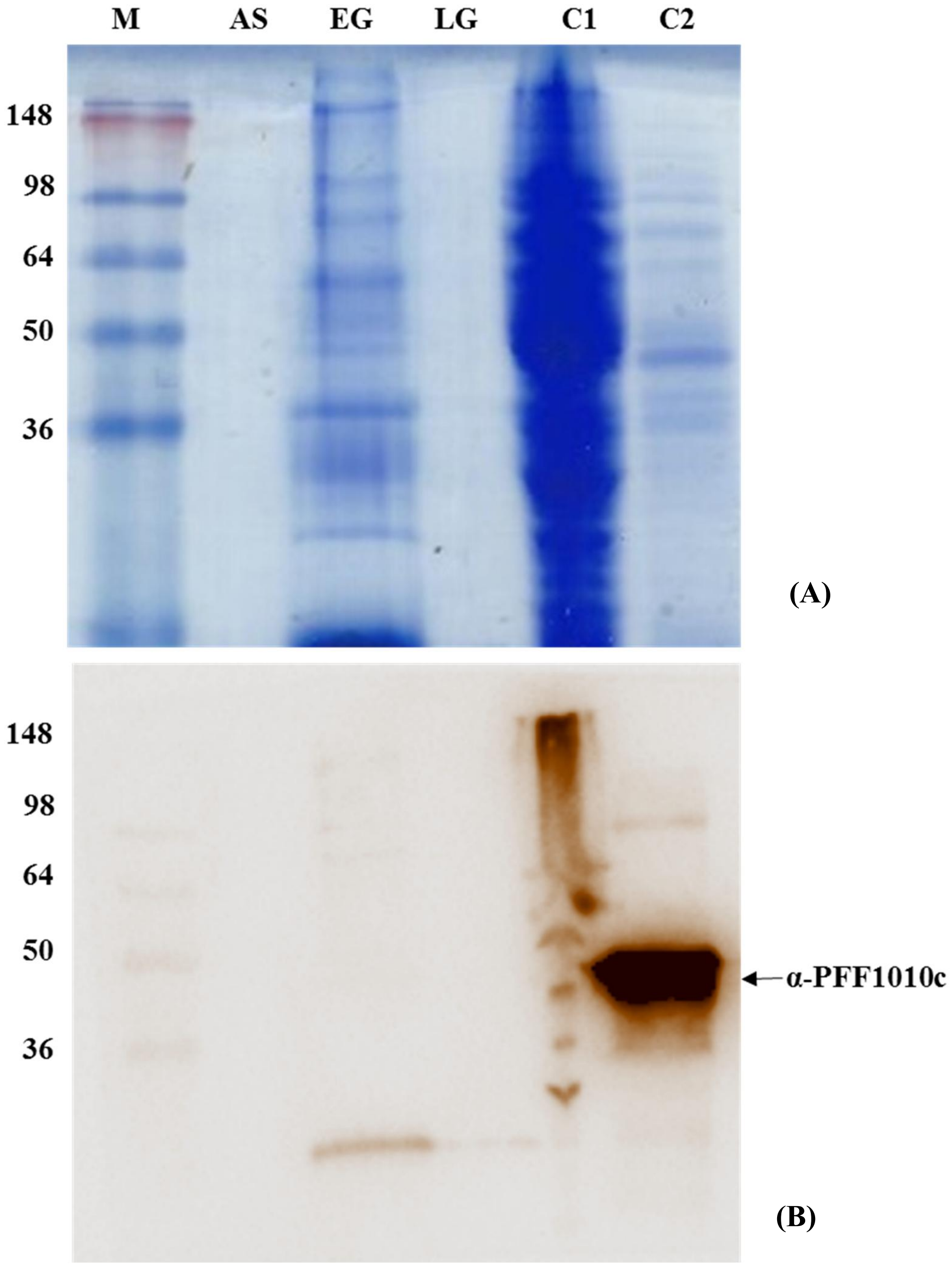


Figure D2. Analysis of PFF1010c expression by parasite lysates. (A) SDS-PAGE of the analysis of PFF1010c expression malarial parasites at different stages of the parasite's life cycle. **(B)** Western blot of the SDS-PAGE using anti-PFF1010c as the primary antibody and HRP conjugated anti-rabbit as the secondary antibody. The samples were loaded as follows: Lane M: Protein marker, Lane AS: Proteins isolated from asexual parasites (negative control), Lane EG: Proteins isolated from early gametocyte parasites, Lane LG: Proteins isolated from late gametocyte parasites, Lane C1: Soluble fraction of the expressed PFF1010c after harvesting and Lane C2: Total cell lysate of PFF1010c collected 4 hours post induction with IPTG.

D3 Predicted PFF1010c peptide epitopes

No	Start	Antigenic Determinant	Length	Antigenicity/Surface/Hydrophilicity	Disordered Score	Coil	Amphipathic	Synthesis
1	9	KTNELGEKNEKNESC	14	3.18/1.00/1.15	0.1657	Y	Y	N
2	296	CPQSSHRVFGDRISG	14	1.79/0.71/0.15	0.0867	Y	Y	N
3	243	CDIPKPLIKISSKQT	14	1.61/0.64/0.37	0.0926	Y	Y	N
4	166	CYEIKIKEEQNKNGL	14	1.51/0.71/0.83	0.0985	Y	Y	N
5	23	LYEPSSDIDNNIILC	14	1.51/0.64/0.13	0.0936	Y	Y	N
6	138	CNTIEEQRYMLEKEA	14	1.48/0.64/0.85	0.0627	Y	Y	N
7	58	KYMRSSYSKPKYYEC	14	1.22/0.79/0.25	0.0614	Y	Y	N
8	75	VNVKSDAKTIRKSYC	14	1.09/0.64/0.52	0.0611	Y	Y	N
9	349	CLCAQSIKIKMNKLE	14	1.01/0.64/0.16	0.1165	Y	N	N
10	257	CKKKLYSHILQDTEM	14	0.81/0.57/0.09	0.0600	Y	Y	N

Table legend. An extra "C" (highlighted as green) is added to the C terminus (or N terminus) to facilitate conjugation. Positive charged residues (K, R, H) are in blue. Negative charged residues (D, E) are in red. Synthesis: "N" means this peptide is easy to synthesize. "Y" means it has difficulty to synthesize.

D4 Hydrophobicity/Hydrophilicity plots of PFF1010c

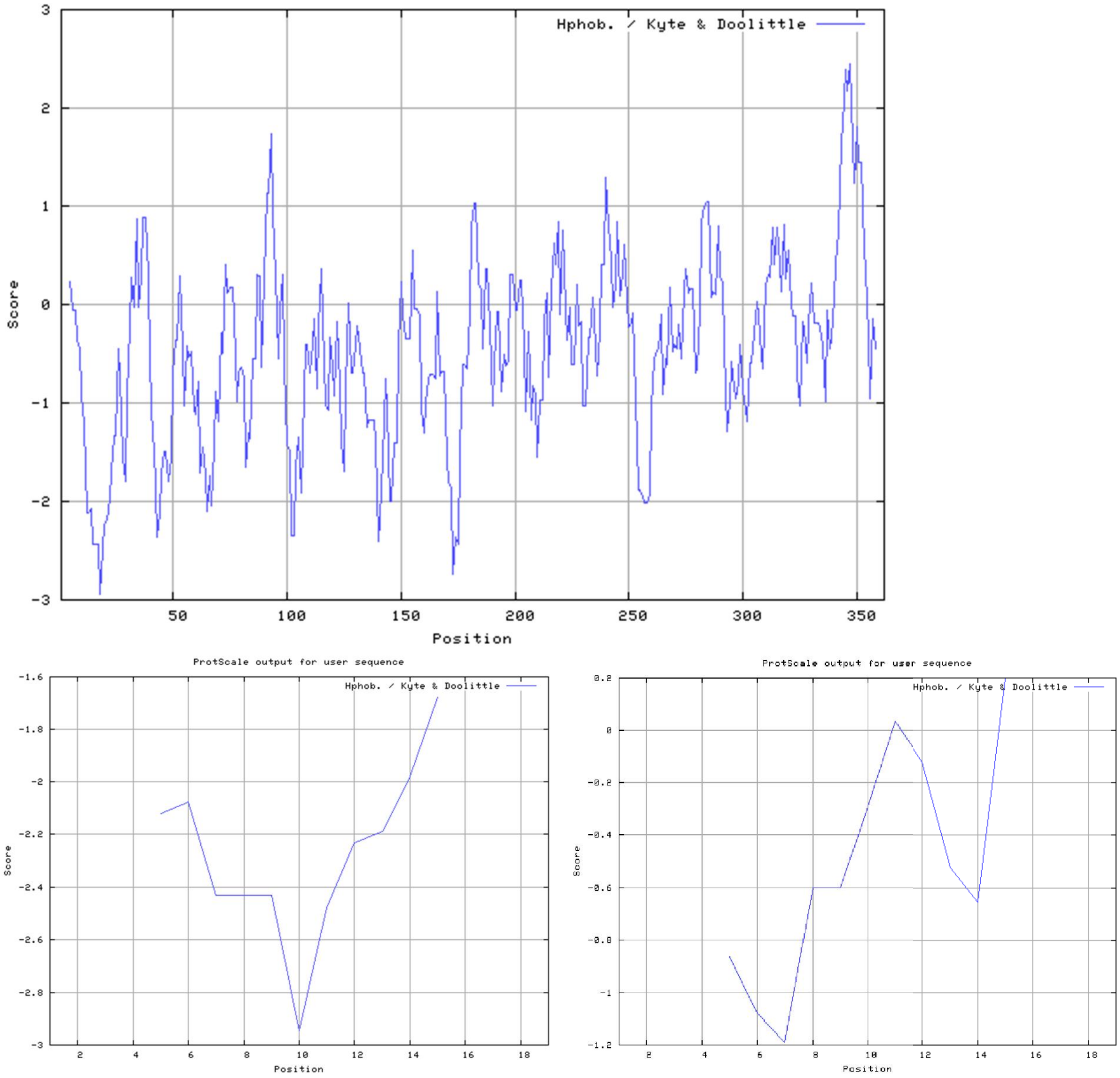


Figure D4. Hydrophobicity/Hydrophilicity plots. Upper panel depicts hydrophobicity plot of overall PFF1010c and lower panel shows hydrophobicity plot of the selected regions. Below 0 is hydrophilic and above 0 is hydrophobic.

D5 Positive control of far Western blot analysis

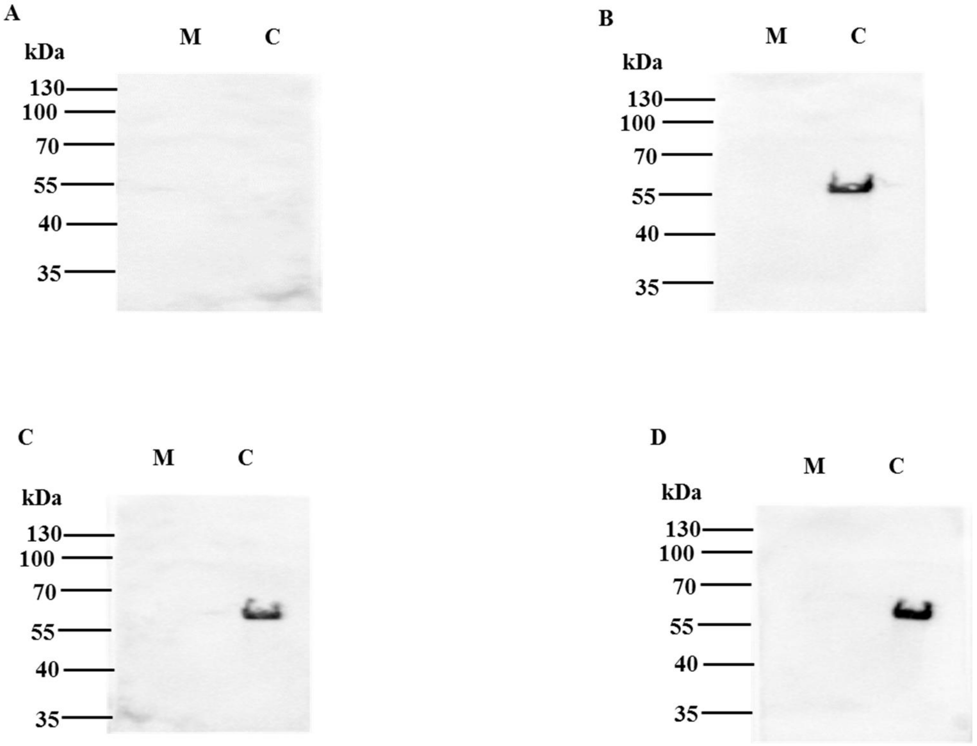


Figure D5. Positive control of the far Western blot analysis. PfHop was used as prey protein and PfHsp70-1 was used as bait **(A)** Blot analyzed using α-PfHsp70-1 antibody. No overlaying of bait protein was done. **(B)** Overlaid with PfHsp70-1 protein in the absence of either ATP or ADP **(C)** Overlaid with PfHsp70-1 protein in the presence of ADP and **(D)** Overlaid with PfHsp70-1 protein in the presence of ATP. Anti-PfHsp70-1 antibody was used to detect the presence of PfHsp70-1 protein on the blots. Samples were loaded as follows: Lane M: PagerRuler Prestained Protein Ladder (Thermo Scientific, U.S.A),