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A destabilisation domain approach to define the *in vivo* functional importance of *Pf*Hsp70-1 and *Pf*Hsp40 in the intraerythrocytic life cycle of *Plasmodium falciparum*

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I. Abbreviations

°C	Degree Celsius
1º	Primary
2º	Secondary
A	Adenine
A+	Rhesus factor positive blood group with A antigen on the surface
ACT	Artemisinin- based combination therapy
APS	Ammonium peroxodisulphate
AR	Androgen receptor
ATc	Anhydrotetracycline
bp	Basepair
BSD	Blasticidin-S-deaminase (BSD)-selectable marker
C	Cytosine
C-terminus	COOH-terminus/ carboxyl-terminus
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CIP	alkaline phosphatase
ClpB	Caseinolytic peptidase B protein
cm	Centimeters
CO_2	Carbon dioxide
CPD	Citrate phosphate dextrose solution
CRR	Cysteine rich region
CRT	Calreticulin
CsA	Cyclosporine A
CTD	C-terminal domain
D	Aspartic acid
dd-ecDHFR	*E. coli* dihydrofolate reductase derived destabilization domain
dd-FKBP	Human FK506 binding protein derived destabilization domain
DD/ dd	Destabilization domain
DD29	FKBP derived triple mutant destabilization domain
ddH_2O	Double distilled water
dhfr/ DHFR	Dihydrofolate reductase
dhps	Dihydropteroate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DnaJ	Hsp40 homologue in *E. coli*
DnaK	Hsp70 homologue in *E. coli*
dNTP	Deoxyribonucleotide triphosphate
DSG	15-deoxysperguallin
DTT	Dithiothreitol
E	Glutamic acid
E. coli	*Escherechia coli*
E. histolytica	*Entamoeba histolytica*
ecDHFR	*E. coli* dihydrofolate reductase
ECL	Enhanced chemiluminescence
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
EtOH	Ethanol
F	Phenylalanine
F	Forward
FCS	Fetal calf serum
Fig	Figure
FKBP	FK506 binding protein
FRT	FLP recognition target
G	Guanine

G. intestinalis	*Giardia intestinalis*
G6PD	Glucose-6-phosphate dehydrogenase
GA	Geldanamycin
GF	Glycin-phenylalanine
GFP	Green Fluorescent Protein
GOI	Gene of interest
H_2O_2	Hydrogen peroxide
HA	Hemagglutinin
hDHFR	Human dihydrofolate reductase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFF	Human Foreskin Fibroblast
HOP	Hsp70-Hsp90 organising protein
HPD	Histidine Proline Aspartic acid
HPN	Histidine Proline Asparagine acid
Hsp	Heat shock protein
HT	Host targeting signal
HX	Hypoxanthine-xanthine
IFA	Immunofluorescence assay
K	Lysine
kb	Kilobases
KBr	Potassium bromide
KCl	Potassium chloride
KO	Knock-out
kV	Kilovolt
LacZ	β-galactosidase codin gene
LB	Luria-Bertani
M	Molar
mA	Milliampere
MCS	Multiple cloning site
MD	Middle domain
MDH	Malate dehydrogenase
MetOH	Methanol
Mg	Magnesium
MgCl2	Magnesium chloride
MgSO4	Magnesium sulphate
ml	Millilitre
MLC1	Myocin light chain 1
mM	Millimolar
Mut	Mutant
N	Asparagine
N-terminus	NH2-terminus/ amine terminus
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NEF	Nucleotide exchange factor
ng	Nanogram
nM	Nano molar
nm	Nanometer
NTD	N-terminal ATP binding domain
O^+	Rhesus factor positive blood group with no A or B antigens on the surface
O_2	Oxygen
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEXEL	*Plasmodium* Export Element
Pf	*Plasmodium falciparum*
*Pf*CRT	chloroquine resistance transporter
*Pf*EMP-1	*P. falciparum* Erythrocyte Membrane Protein-1
PfMSP2	*Plasmodium falciparum* Merozoite surface protein 2
*Pf*UB	*Plasmodium falciparum* polyubiquitin

pH	Negative logarithm of the activity of the (solvated) hydronium ion
Pi	Phosphate ion
PIC	Protease inhibitor cocktail
PM1	Plasmepsin 1
Pmol	Picomole
PMSF	Phenylmethanesulfonylfluoride
POI	Protein of interest
polyQ	polyglutamine
PPIase	Peptidylprolyl isomerase
PPM	parasite plasma membrane
PR	Progesterone receptor
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuolar membrane
R	Reverse
RBC	Red blood cell
RESA	Ring-infected erythrocyte surface antigen
Rh	Rhesus factor
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Rotation Per Minute
Rpn6	Proteosomal 19S regulatory particle subunit 6
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
SBD	Substrate binding domain
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sHsps	Small heat shock proteins
SOB	Super Optimal Broth
SOC	Super Optimal broth with Catabolite repression
TAE	Tris-acetate-EDTA
TATi1	Trans-activator trap identified 1
TATi2	Trans-activator trap identified 2
TCP1	t-complex polypeptide-1
TE	Tris-EDTA
TEMED	NNN′N-tetramethylethylenediamine
TetO	Tet operator
TetRep	Tetracyclin Repressor
TetRep	Tetracycline repressor
Tg	*Toxoplasma gondii*
TMP	Trimethoprim
TRAD	TetRep and putative Activating Domain
Tris-HCl	Tris-Hydrochloride
U	Unit
UTR	Untranslated region
V	Valine
V	Volt
v/v	Volume / volume
w/v	Weight/ volume
WHO	World Health Organization
WT	Wild type
X	Times
YFP	Yellow Fluorescent Protein
α	anti
µF	Microfarad
µg	Microgram
µl	Microliter
µM	Micromolar
Ω	Ohm

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1. Introduction

1.1. Malaria: the disease and the parasite

1.1.1. Global disease burden

Malaria is a life-threatening infectious disease caused by protozoan parasite, *Plasmodium falciparum* (Miller et al., 2002). It is responsible for the highest amount of morbidity caused by any infectious disease, therefore qualifies as the most significant parasitic disease. According to very recent WHO (World Health Organization) statistics, malaria caused an estimated death of 660,000 people in 2010, majority of which are children from Africa (WHO, 2013) . Noteworthy, in sub Saharan Africa malaria causes death of 1-3 million people per year, over 75% of which are children (Breman, 2001, Snow et al., 1999). In Africa 400-900 million children under 5 years of age living in endemic areas are the major victims of the disease (Breman, 2001, Miller et al., 2002). Overall, this trend raises serious concerns about the impact of malaria and the failure of control strategies.

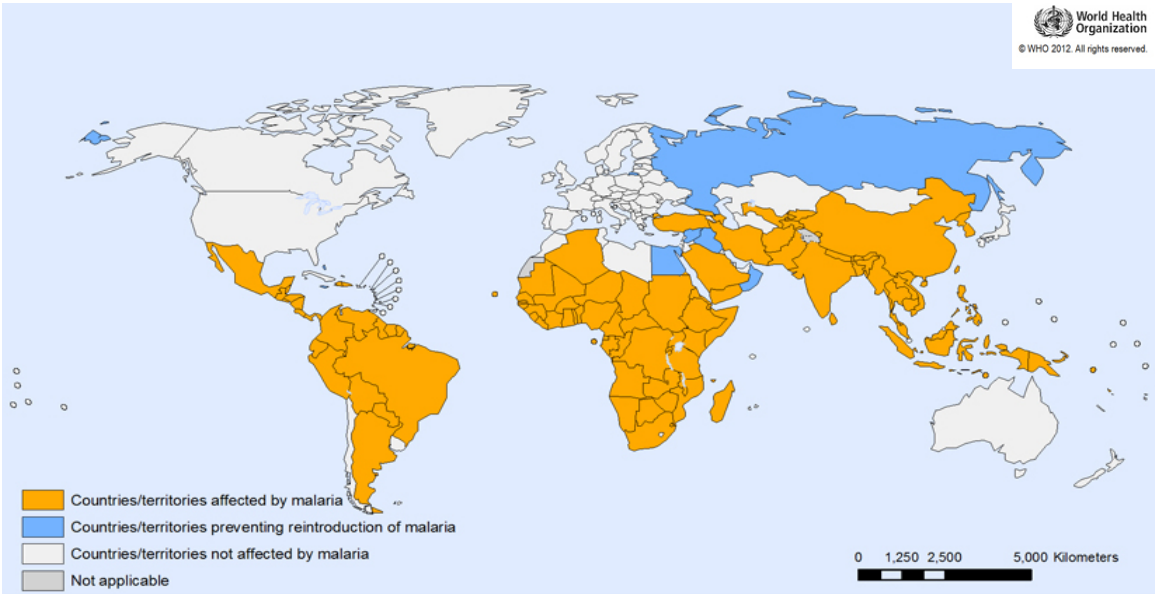

Figure 1.1 World map representing countries and territories affected by malaria, 2010.

Orange areas represent the high risk regions affected by malaria. The zones in white represent malaria free areas. Territories in blue indicate the countries taking preventive measures against the reintroduction of the disease. Retrieved from (WHO, 2012).

1.1.2. Current drugs and emerging drug resistance

Once detected with malaria, the individual should be immediately treated with antimalarial drugs within 24 hours. Delay in effective treatment can lead to the development of severe form of the disease causing death of the patient.

Several anti-malarial drugs have been in practice so far. Methylene blue was the first synthetic drug with slight antimalarial activity. It could strongly inhibit the formation of hemozoin crystals in the food vacuole of the parasite. Combination therapy using methylene blue even proved to be efficacious for children below the age of five years in Africa (Schirmer et al., 2011). But, methylene blue can cause haemolysis in the Glucose-6-phosphate dehydrogenase (G6PD) deficient individuals. G6PD deficiency is a disorder of the human erythrocyte. This mutation is prevalent among the people in the areas where malaria is or has been endemic (Peters and Van Noorden, 2009). For this reason, methylene blue was never acknowledged as a commercially available therapy for malaria and later discontinued.

Quinine was also among the very first drugs developed in 17^{th} century against malaria. This continued for long time as a therapy until 1940s, when chloroquine was introduced with lesser side effects and replaced the former (Toovey, 2004).

Among the most effective and long used drugs for malaria are chloroquine and sulfadoxine-pyrimethamine. Since its discovery in 1934, chloroquine has been extensively used in different parts of the world as one the most effective solution for treating uncomplicated malaria in patients. Choloroquine inhibits the parasites ability to form non-toxic hemozoin crystal from heme. As a result, the increasing accumulation of the toxic heme in the food vacuole causes death of the parasite (Roepe, 2009). After extensive use over a decade resistance against chloroquine had emerged in several parts of the world around 1980s (Uhlemann and Krishna, 2005). This resistance is acquired by multiple mutations in the *Pf*CRT (chloroquine resistance transporter) protein that functions as a transporter on the parasite digestive vacuole membrane (Wellems and Plowe, 2001). The resistant parasites efflux chloroquine from the food vacuole and thus inhibits its function (Wellems and Plowe, 2001, Lim et al., 1990). In many parts of the world chloroquine is no more prescribed as an effective antimalarial (Hempelmann, 2007).

Pyrimethamine–sulfadoxine combination was introduced instead of chloroquine as an antimalarial drug since 1993. This combination works by inhibiting the function of dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) in *Plasmodium falciparum*. Although this combination initially proved to be effective, resistance has already started growing in Southeast Asia and South America (Sibley et al., 2001). Therefore, the effectiveness of this combination drug is rapidly decreasing (Jelinek et al., 1999, Jelinek et al., 1998, Nzila et al., 2000). Point mutation in the dhfr and dhps genes of the parasite is the cause behind such acquired resistance (Sibley et al., 2001).

The morbidity and mortality due to malaria has increased rapidly due to loss of effectiveness of several antimalarial drugs. In the past few years this situation has improved by the introduction of artemisinin - based combination therapy (ACT) (Ecker et al., 2012, Smith and Doerder, 1992, Kokwaro, 2009). In this therapy artemisinin is usually provided in combination with other antimalarials such as lumefantrine, mefloquine, amodiaquine, or sulfadoxine/pyrimethamine (WHO, 2012). Other possible combination is usually piperaquine and dihydroartemisinin (Keating, 2012, WHO, 2012). Currently, this therapeutic approach is the most effective way of treating uncomplicated *P. falciparum* malaria. ACT inhibits the development of resistance to any single drug component in the given combination (Kokwaro, 2009). In spite of this, there are several reports indicating emerging drug resistance or loss of effectiveness of ACT since 2008 (Noedl et al., 2008, Dondorp et al., 2009, Phyo et al., 2012).

Therefore, the constantly emerging resistance of the parasite against the previously effective antimalarial drugs makes the eradication of the disease highly challenging (Sachs and Malaney, 2002, Ridley, 2002, Goldberg, 2002, Kokwaro, 2009) and is disastrous on public health. Hence, development of new effective antimalarial therapy demands extensive amount of research attention. This will help us to have better understanding of the increasing resistance that in turn would facilitate the development of new efficacious antimalarial drugs.

1.1.3. *Plasmodium*: the malaria parasite

Although it was believed that only four different species of malaria parasites (*P. falciparum*, *P. malarie*, *P. ovale* and *P. vivax*) are capable of infecting human, *Plasmodium knowlesi* that was known to infect long-tailed (*Macaca fascicularis*) and pig-tailed (*Macaca nemestrina*) macaques is a recent addition onto the list (Sabbatani et al., 2010, Vythilingam et al., 2008, Ng et al., 2008, Cox-Singh and Singh, 2008). Nevertheless, *Plasmodium falciparum* is still

considered as the most virulent species causing the deadliest form of the disease known as ‘malaria tropica’ that includes malignant malaria or cerebral malaria (Gupta et al., 1994).

1.1.4. Life cycle of human malaria parasite

Plasmodium falciparum has a complex life cycle divided into two phases: ‘sexual phase’ taking place in the mid-gut of its vector female anopheles mosquito followed by the ‘asexual phase’ that occurs in the human host. Infection with *Plasmodium falciparum* begins with the bite of an infected female Anopheles mosquito during its blood meal (Miller et al., 2002). Sporozoites from the salivary gland of the mosquito are released into the subcutaneous tissue of the infected person (rarely direct into the blood stream), from where they migrate to the liver hepatocytes (Miller et al., 2002). Research findings indicate that sporozoite travels through random hepatocytes before it reaches and invades a particular hepatocyte where it undergoes replication. Therefore, the silent infection phase lasts around 14 days (Mota et al., 2001, Prudencio et al., 2011). Inside the hepatocyte the parasite divides and develops into tens of thousands of exo-erythrocytic merozoites (Cowman et al., 2012). Once the hepatocyte bursts the merozoites are released into the blood stream of the individual where each free living merozoite attaches and invades an erythrocyte. Inside the red blood cells (RBC) the merozoites rapidly grow and develop into ring stage (0-10 hours post invasion) followed by trophozoite stage (10-36 hours post invasion) and finally the schizont stage that consist of 12-16 merozoites. The length of the intraerythrocytic stage varies among different species. For example, to complete a full life cycle *P. falciparum* needs 48 hours, establishing a non-synchronous infection in the host. Similarly, *P. vivax,* and *P. ovale* also take 48 hours whereas *P. malariae* needs approximately 72 hours (Sylvie Manguin, 2008). Synchronous rupture of the infected erythrocyte leads to the clinical manifestation of the disease that is associated with fever and chills. During these febrile temperature episodes body temperature of the host can rise up to 41 ° C (Pavithra et al., 2004). The released merozoites invade additional erythrocyte where they undergo further rounds of replication. Amongst this pool of merozoites some escape and differentiate into sexual forms (female and male gametocytes). These gametocytes are further taken up by female Anopheles mosquito during its blood meal from an infected individual. They mature into male and female gametes in the midgut of the mosquito. Following fertilization, the zygotes develop into ookinete and oocyst finally forming sporozoites. The sporozoites migrate to the salivary gland and continue infecting healthy individuals and thus, the cycle continues (Cowman et al., 2012).

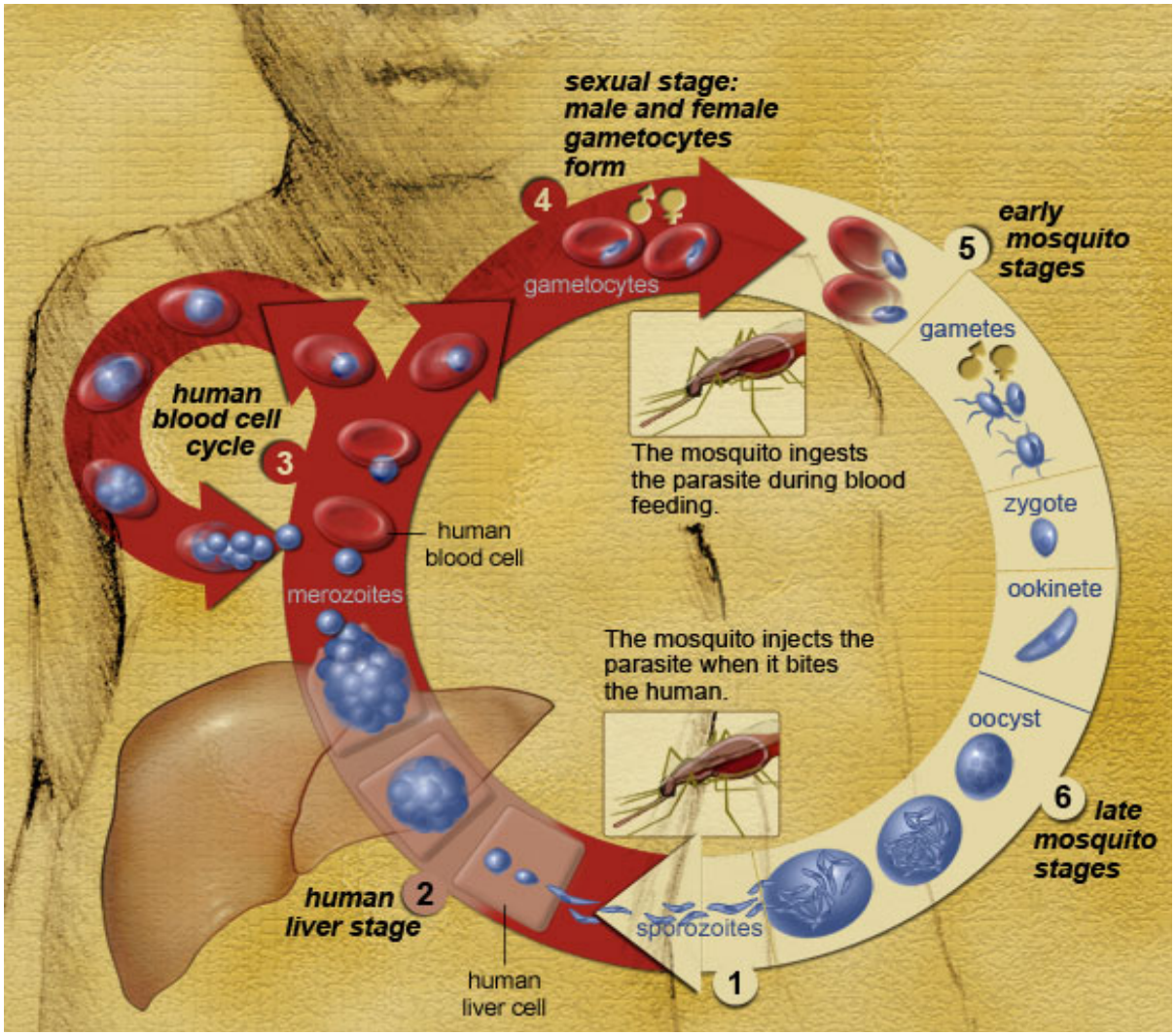

Figure1.2 Life cycle of human malaria parasite.

During the blood meal by the female anopheles mosquito the sporozoites are injected into the human body where they first migrate to the liver hepatocytes. After reaching the hepatocytes, the parasites undergo amplification producing tens and thousands of merozoites. The merozoites are released from the hepatocytes into the blood stream where they invade human RBC and undergo development into several stages, finally forming schizonts containing 14-16 merozoites. The RBC bursts open releasing these merozoites into the bloodstream that in turn infect new RBC. Some merozoites escape this cycle and develop into male and female gametocytes that are ingested by mosquito during consecutive blood meal from an infected individual and develop into gametes in the midgut. After fertilization following ookinete and oocyte formation the zygote develop into sporozoites that reach the salivary gland and can infect new individual. Retrieved from (NIAD, 2012).

1.2. Cell Biology of the *Plasmodium falciparum* infected erythrocyte

Once hijacked by *P. falciparum*, the red blood cell undergoes profound morphological and structural changes that end up in altering the normal circulation and physical properties of the cell (Maier et al., 2009). The human RBC loses nucleus, mitochondria, ribosome,

endoplasmic reticulum (ER), and Golgi apparatus. Therefore, is completely devoid of protein synthesis and trafficking (Blanc et al., 2005). But surprisingly, in the RBC the parasite carries out different functions including replication, acquisition of nutrients, protein trafficking, and evasion of the host immune response (Pasini et al., 2013, Przyborski and Lanzer, 2005). In the intraerythrocytic stage the parasite resides inside a vacuole termed as 'Parasitophorous Vacuole' or PV. The formation of PV is initiated by the parasite during invasion (Lingelbach and Joiner, 1998). The Parasitophorous Vacuolar Membrane (PVM) works as a novel semipermeable membrane between the erythrocyte cytoplasm and the parasite through which ion exchange and nutrient acquisition take place (Ansorge et al., 1996, Joiner, 1991). *Plasmodium* actively transports a range of proteins through this vacuole and beyond the PVM into the host RBC and vice versa (Haldar et al., 2005, Lingelbach and Przyborski, 2006). For example, *P. falciparum* exports Erythrocyte Membrane Protein-1 (*Pf*EMP-1), a major key virulence protein to the surface of the infected RBC. *Pf*EMP1 facilitates the adhesion of the parasite to the vessel endothelium thereby avoiding splenic clearance (McMillan et al., 2013, Cowman et al., 2012). These modifications of the human RBC largely contribute to the severe complications of *Plasmodium falciparum* malaria that includes fever, anaemia and in most severe cases coma, resulting in death of the patient (Cowman et al., 2012, Miller et al., 2002). The proteins destined for the RBC contain a highly conserved pentameric transport sequence known as host targeting signal (HT) or plasmodium export element (PEXEL) (Ansorge et al., 1996, Marti et al., 2005, Baumeister et al., 2001, Hiller et al., 2004). Although protein transport is actively mediated by the parasite, it is not quite clear how in a molecular mechanistic level the proteins are trafficked through the PV into the RBC. Previously, it was believed that there is an establishment of a close contact between the parasite plasma membrane (PPM) and PVM that together function as a bilayer. Proteins destined for the RBC follows a classical secretory pathway involving vesicular transport for their membrane passage (Elmendorf and Haldar, 1993, Gormley et al., 1992). In contrary, recent research in this field proposes a 'two-step model' of secretion. According to this model, proteins emerging from ER as vesicle are first released into the lumen of PV from where they are transported across the PVM possibly by the involvement of protein conducting channels. This model to be functional requires the presence of chaperone in the PV and also in the host erythrocyte. The chaperone present in the PV lumen would unfold the protein and hold them in a 'translocation competent state' to pass through the possible translocon. On the other hand, the chaperone present in the RBC would further facilitate proper folding of this protein into their native functional forms (Ansorge et al., 1996). In

agreement with this hypothesis, using a model protein it was experimentally shown that proteins trafficked to the host cytoplasm are indeed first unfolded in the lumen of the PV and then translocated through the PVM into the RBC. Therefore, it justifies an important role of chaperones involved in trafficking (Gehde et al., 2009).

Interestingly, *Plasmodium* genome encodes a significant amount of Hsps (Sargeant et al., 2006). By a previous approach around 27 vacuolar proteins were identified, among which 34 % were mostly chaperones (Nyalwidhe and Lingelbach, 2006). To date parasite encoded several Hsp40 co-chaperones have been reported to be present in the RBC (Rug and Maier, 2011) but evidence of the presence of Hsp70 homologue was lacking. Recently, an Hsp70 homologue known as *Pf*Hsp70-x was found to localise in the PV and RBC (Kulzer et al., 2012). In addition, several other heat shock proteins are constitutively expressed and found be localized in the parasite cytoplasm, possibly involved in housekeeping. These facts indicate the presence of diverse chaperone machinery facilitating an evolutionary adaptation for better intracellular parasitism (Nyalwidhe et al., 2003). Further research is required to investigate the essential function of these chaperones for intraerythrocytic survival of *Plasmodium falciparum*.

1.3. Molecular Chaperone as Heat shock proteins: the protein folding catalyst of the cell

Protein folding in the cell is a very important biological process by which nascent polypeptide chain emerging from ribosome obtains its three dimensional native functional conformation (Alberts Bruce, 2007). The dogma known as ‘Anfinsen Dogma’ was put forward suggesting that the native structure of the protein is determined by its amino acid sequences (Anfinsen, 1972). In normal condition, spontaneous folding of protein occurs if the energy status and the size of the protein favour the folding (Dinner et al., 2000).

But, highly crowded cellular environment and other stress often impose challenge to the normal protein folding process. These stress factors include heat shock (hot or cold), free oxygen radicals, hyperthermia, pH shift, degrading enzymes, heavy metals, ethanol, and clinically adverse situations like reperfusion or ischemia. Stress causing malfunction of the protein folding machinery results in the accumulation of toxic protein aggregates in the cell. These aggregates are associated with numerous diseases states like Alzheimer's disease, Cystic Fibrosis etc (Ellis, 2001, Luheshi et al., 2008, Dobson, 2003). To overcome these

obstacles cells appoint a diverse network of highly specialised group of chaperones named as 'Heat shock proteins' (Hsps) (Hartl et al., 2011). This family of proteins was first noticed by Ritossa (1962). He observed that the introduction of heat shock resulted in an enlargement of special segment of *Drosophila* chromosome (heat shock puffs) and rapid synthesis of a new set of RNA. Indeed this was one of the biggest observations of the effect of environmental changes on gene expression (Ritossa, 1996, Ritossa, 1962) This observation eventually led to the discovery of Hsps much later in 1974 (Schlesinger, 1990, Tissieres et al., 1974). Hsp coding genes are highly conserved from yeast to human (Schlesinger, 1990). Hsps inhibit the formation of protein aggregates and facilitate protein folding into their native state.

Proteins are highly dynamic in nature therefore the involvement of Hsp is crucial to maintain protein homeostasis of the cell (Hartl et al., 2011, Ellis, 2001, Smith et al., 1998, Feldman and Frydman, 2000). This involves a specialized machinery that determines the concentration of unfolded protein accumulated in cytosol or different cellular locations including extracytoplasmic compartments (McMillan et al., 1994). The concentration of unfolded proteins in these compartments induces Hsp expression (Parsell and Sauer, 1989). Cells recruit diverse range of heat shock proteins of different sizes. The small Hsps first get involved by shielding the hydrophobic region of the protein and prevent them from aggregation and degradation (Yon, 2001). Whereas, large Hsps or Hsp complexes comes into play at a later stage and capture the misfolded proteins in a cavity. This provides the protein an isolated environment allowing their unfolding and further correct folding into native functional structures (White et al., 2012, Ranford et al., 2000). There exists a highly orchestrated cross talk between different Hsps and various components of the cell signalling pathways that altogether controls the normal development and proliferation of a cell (Nollen and Morimoto, 2002).

1.4. Common classes of Heat shock proteins (Hsps)

1.4.1. Hsp 100

In eukaryotic organisms Hsp100 group of molecular chaperones are known for their distinct property to reactivate proteins during stress by resolubilizing the aggregates (Zolkiewski et al., 2012). In *E. coli,* Hsp100 is known as 'Caseinolytic peptidase B' protein (ClpB) (Squires et al., 1991). In a cell, Hsp100s are detected with basal ATPase activity that is triggered by the induction of protein substrate delivered by co-chaperone Hsp70 (DnaK in prokaryotic

organisms) during cellular stress (Zeymer et al., 2013, Bosl et al., 2006, Goloubinoff et al., 1999).

ClpB functions via a highly specialised mechanism. Two models of action have been proposed so far. According to the first model, ClpB6 directly interacts with the protein aggregates (Barnett et al., 2005, Bosl et al., 2006). Exchange of nucleotides by nucleotide exchange factor (NEF) provides the necessary energy upon hydrolysis of ATP to ADP. Energy mediated conformational change in the structure of the ClpB6 ring results in the shearing of protein aggregates making its hydrophobic region highly exposed (fig 1.3) (Bosl et al., 2006).

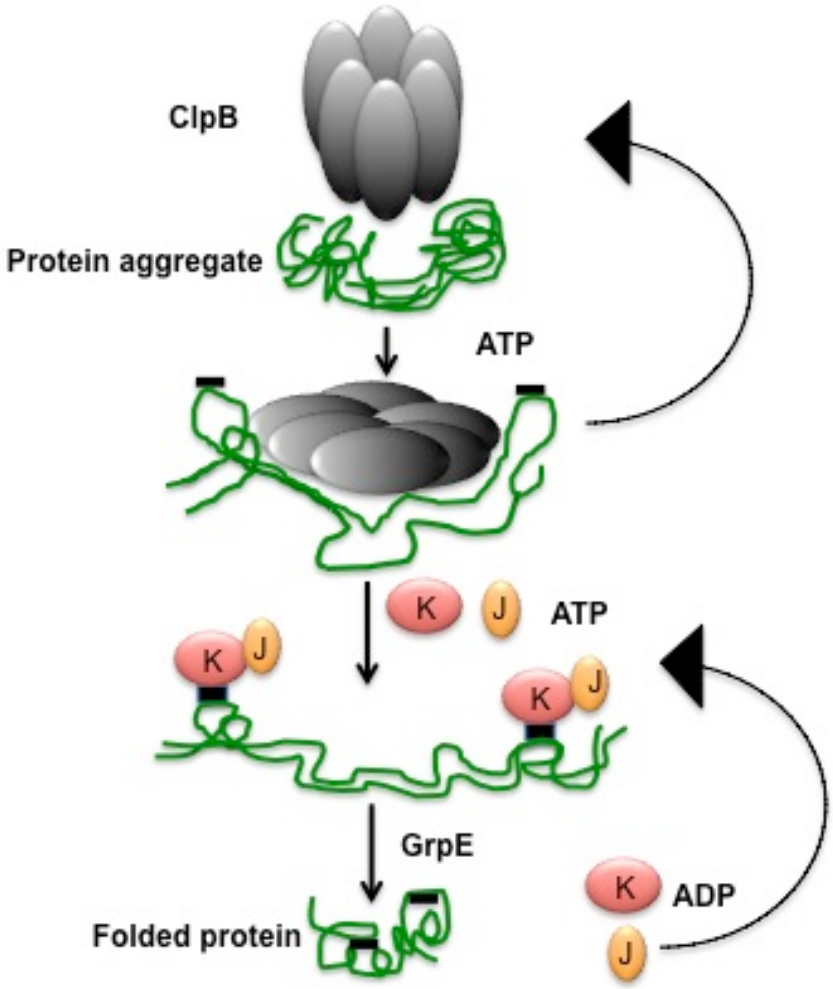

Figure1.3 Model of action of ClpB in collaboration with DnaK-DnaJ-GrpE

ClpB recognises and binds to protein aggregates. Hydrolysis of ATP to ADP provides the required energy for the structural change of the ClpB6 complex. This results in the exposure of new hydrophobic sites in ClpB-bound aggregates. Finally, DnaK-DnaJ-GrpE binds to the aggregates containing exposed hydrophobic domains. DnaK-DnaJ-GrpE mediated disaggregation of the aggregates, unfold and refold them into native functional forms. Adapted from (Goloubinoff et al., 1999).

The other model either functions by 'crowbar and ratchet' mechanisms or by 'threading mechanism'. According to the first mechanism, hydrolysis of ATP exerts a mechanical force on the bound protein aggregate resulting in their disruption (fig 1.4a) (Glover and Tkach, 2001), (Bosl et al., 2006). Whereas, the later claims energy dependent extraction of individual polypeptide chains from the protein aggregates following passage through the pore of the chaperone central channel of the hexamer (fig 1.4b) (Lum et al., 2004, Bosl et al., 2006).

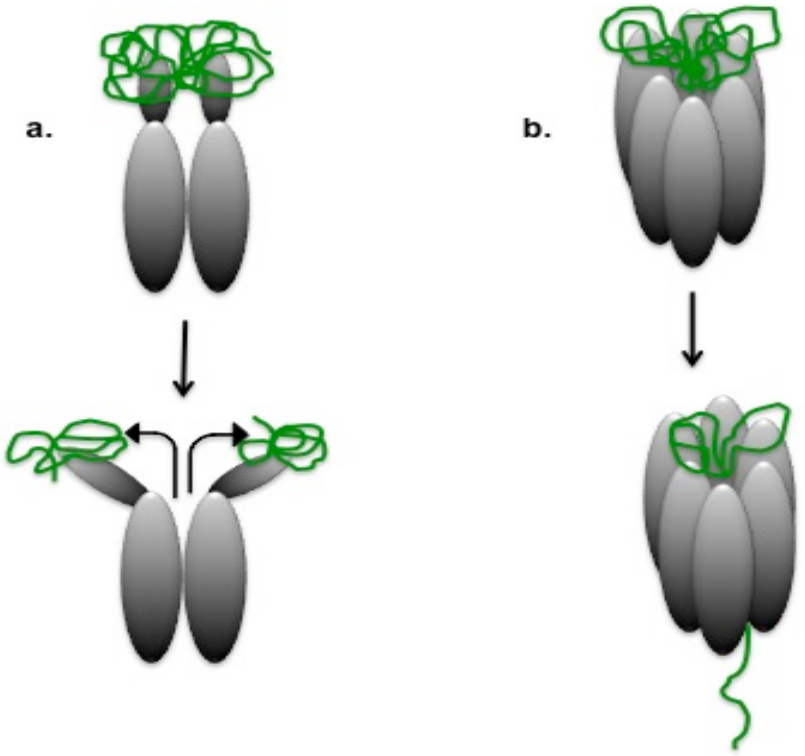

Figure 1.4 Model of Hsp100/ ClpB complex mediated disaggregation of protein aggregates.

(a) In the 'crowbar and rachet' model disaggregation is mediated by the mechanical force on the aggregate induced by the conformational movement of the ClpB complex. (b) In the 'threading mechanism' first individual polypeptides are extracted from the aggregates, which are further released through the central axial pore of the chaperone hexamer. Modified from (Bosl et al., 2006).

In both of these models released polypeptides are recognised by DnaK mediated chaperone machinery (DnaK-DnaJ-GrpE) by the exposed hydrophobic region on the polypeptides. DnaK system, further folds the polypeptides into their native states (Bosl et al., 2006, Goloubinoff et al., 1999) (fig.1.3).

1.4.2. Hsp90

Hsp90 is an important group of chaperone, highly conserved across all organisms and counts towards 1-2 % cellular proteins (Csermely et al., 1998). In eukaryotes it is found in cytosol,

nucleus and other organelles. Hsp90 is involved in many processes important for guarding imbalance in cell homeostasis during stress (Kaplan and Li, 2012, Dezwaan and Freeman, 2008, Taipale et al., 2010). In addition, Hsp90 mediates many fundamental cellular processes including hormone signalling, cell survival, and cell cycle control (Wandinger et al., 2008, Zhao et al., 2005, Borkovich et al., 1989, Richter et al., 2010, Young et al., 2001). Most importantly many oncogenic proteins are client of Hsp90 qualifying it as a potential drug target (Mahalingam et al., 2009, Audisio et al., 2010).

Hsp90 contains ~ 25 kD N-terminal ATP binding domain (NTD), a ~ 40 kD middle domain (MD) followed by a ~ 12 kD C-terminal domain (CTD), responsible for the dimerization of the protein. The C-terminal domain has MEEVD motif that facilitates co-chaperone recruitment (fig 1.5) (Pearl and Prodromou, 2000, Scheufler et al., 2000, Prodromou et al., 1999, Chen et al., 1998, Ali et al., 2006).

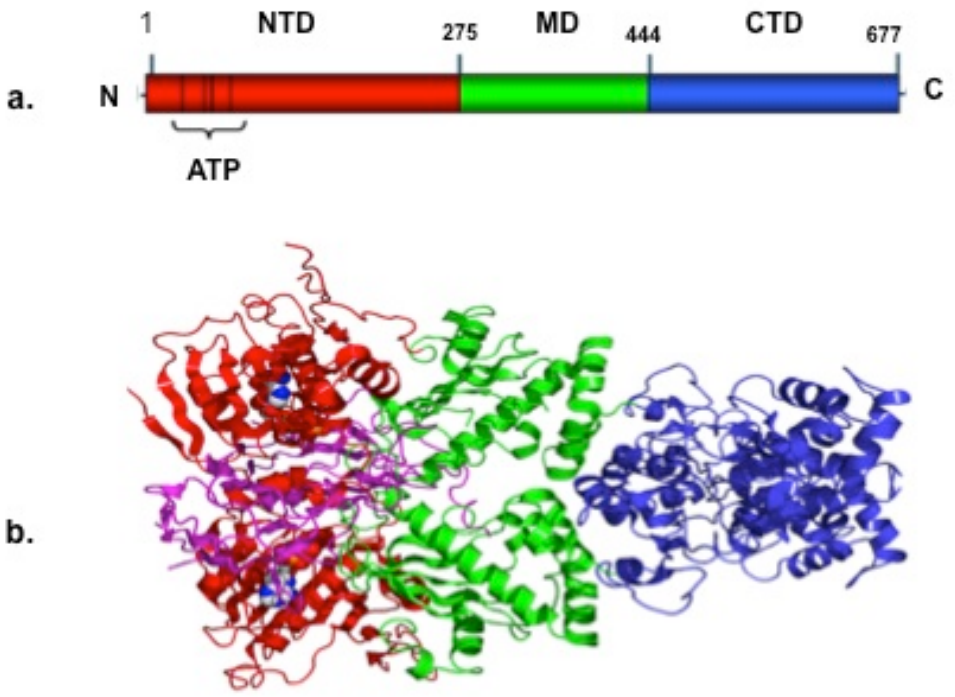

Figure1.5 Structure of Hsp90.

(a) Schematic representation of the domain organisation of Hsp90, showing N-terminal domain (NTD), middle domain (MD) and C-terminal domain (CTD). (b) Crystallographic structure of Hsp90 dimer. Bound ATP molecules are depicted by space filling spheres. Adapted from (Ali et al., 2006)

Hsp90 functions in collaboration with other co-chaperones like Hsp70 and Hsp70-Hsp90 organising protein (HOP) to form large multi-chaperone complex (Wiech et al., 1993, Smith,

1993, Pratt and Toft, 2003). The chaperone cycle of Hsp90 progresses through three complex formations involving co-chaperones and substrates (Smith, 1993). The early complex is formed by the recognition and binding of Hsp70/Hsp40 to the substrate protein (Smith et al., 1992, Hernandez et al., 2002, Cintron and Toft, 2006, Li et al., 2012). The cycle progresses with the dissociation of Hsp40 and the association of Hsp70 facilitated by adaptor co-chaperone HOP leading to the formation of 'intermediate complex' where the client protein is transferred from Hsp70 to Hsp90 via HOP/Sti1. P23/Sba1 stabilizes the closed complex resulting in the dissociation of HOP and Sti1 (Smith, 1993, Chen and Smith, 1998, Johnson et al., 1998, Wegele et al., 2006). The final complex in the cycle is known as 'late complex' where another peptidylprolyl isomerase (PPIase) associates with Hsp90, p23 and Sba1. During this complex formation Hsp90 adapts an ATPase active close conformation allowing client protein a chance to refold (Pratt and Toft, 2003, Johnson et al., 1994, Johnson and Toft, 1995, McLaughlin et al., 2006, Forafonov et al., 2008). At the end of the cycle with the hydrolysis of ATP to ADP, p23, Sba1 and client proteins are released from the complex (Li et al., 2012).

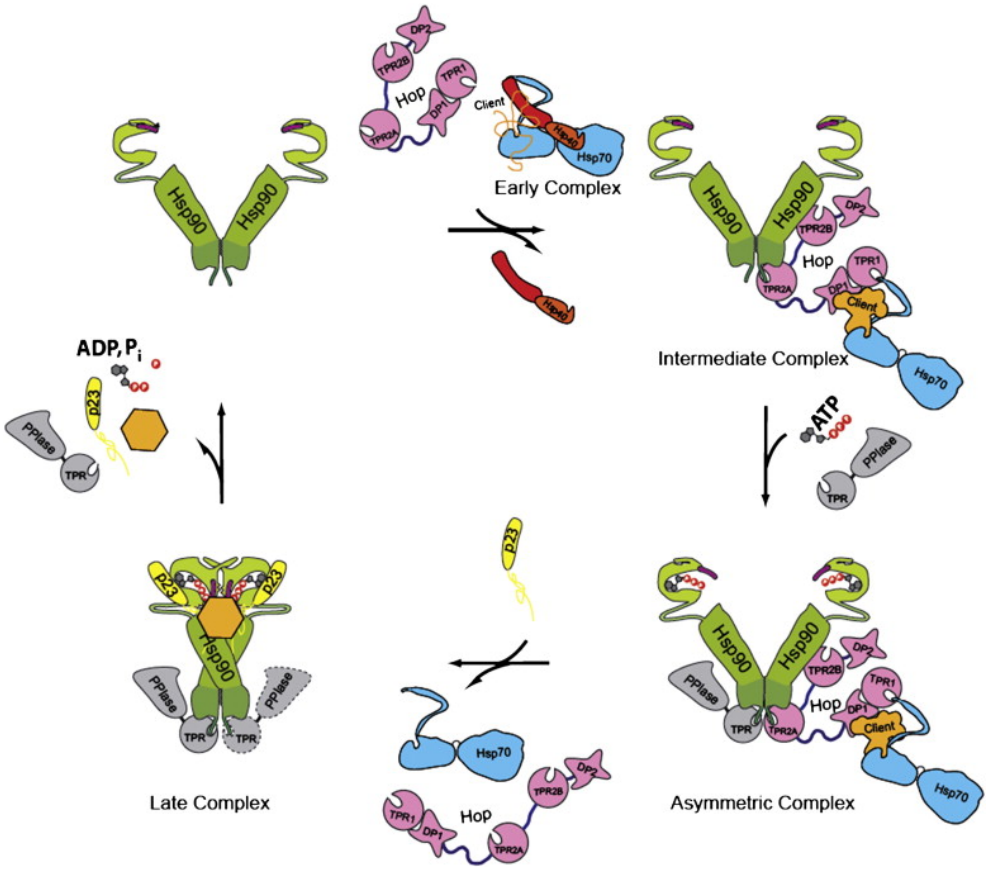

Figure1.6 Schematic representation of Hsp90 chaperone cycle.

The cycle progresses through the formations of several intermediate stages. 'Early complex' formation is mediated by the association of the progesterone receptor (PR) and Hsp70/40 that in turn interacts with client

protein. In the 'intermediate complex' the substrate is transferred to Hsp90 via the involvement of the adaptor protein HOP/Sti1. As the cycle progresses HOP, Hsp70 dissociates following the stabilization of the complex by p23/Sba1. In the 'late complex' Hsp90 adapts a closed conformation bound to the p23, capturing the client protein inside. Hydrolysis of ATP to ADP occurs following the release of p23/Sba1 at the end of the cycle (Li et al., 2012).

1.4.3. Hsp60

Hsp60 is one of the most abundant eukaryotic proteins involved in the folding and assembly of a specific group of proteins in the cell (Johnson et al., 1989). This group of protein plays essential role during stress by their involvement in folding of ~15-30% cellular proteins (Johnson et al., 1989). Apart from its chaperone activity, Hsp60 also plays an important role in the transport and maintenance of mitochondrial DNA (Itoh et al., 2002). In eukaryotes it is known as eukaryotic t-complex polypeptide-1 (TCP1 complex). TCP1 complex binds to short nascent polypeptides, among which cytosolic protein actin and tubulin are the distinct ones (McCallum et al., 2000, Melki et al., 1997). In bacteria, Hsp60 homologue is called as 'GroES' (Viitanen et al., 1990). Structure and function of this group of Hsp is highly conserved from human to bacteria (Johnson et al., 1989).

Mode of action of Hsp60 has been much studied and explained in light of GroES. GroES mediated protein folding involves the participation of GroEL to form a multi-chaperone complex to fold large polypeptides (Viitanen et al., 1990). This complex is a double ring structure containing 14 subunits forming a large central cavity that accommodates unfolded protein via hydrophobic interaction. Protein folding in the cavity of GroEL is in agreement with the "Anfinsen cage" hypothesis that explains the folding of protein in an isolated cellular environment (Ellis, 1994).

Each GroEL subunits have three domains: an apical domain; accommodating GroES and substrates, an equatorial domain; providing the ATP binding site and contacts for ring binding, and an intermediate domain; connecting the two rings (Ranson et al., 1998).

Recently, a 'two timer' model was proposed towards the action of GroEL/GroES chaperone complex (Ueno et al., 2004). According to this model, the GroEL-substrate protein complex binds ATP and GroES to form the *cis*-ATP* complex. In this initial complex the substrate is caged, but the folding is still in an arrested stage. The *cis*-ATP* complex is further converted to the *cis*-ATP complex. Following this transition several events take place. Firstly, the substrate is converted to a folding competent state. The hydrolysis of ATP to ADP releases

phosphate (Pi). Folding of substrate polypeptide continues to the *cis*-ADP* complex. The *cis*-ADP* complex is further transformed to the *cis*-ADP complex. The transition of *cis*-ADP* to *cis*-ADP takes the longest time in the cycle allowing folding of protein substrate in the cavity. The newly formed *cis*-ADP complex can accommodate new substrate and ATP to its *trans* ring. Binding of new substrate to the *trans* ring of the GroEL leads to the release of folded native protein from the *cis* ring along with the dissociation of GroES and ADP from the complex (fig 1.7) (Rye et al., 1999, Ueno et al., 2004).

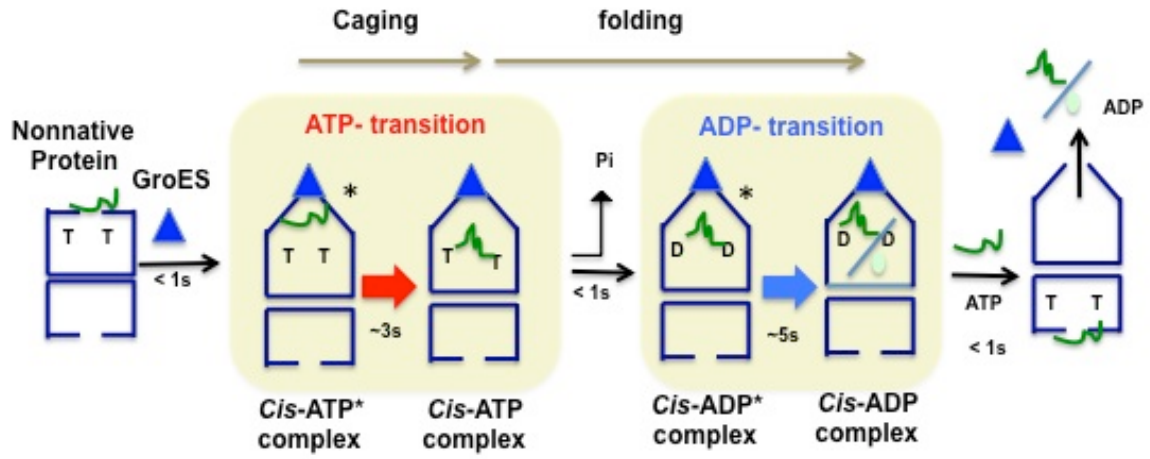

Figure 1.7 Two-timer model of GroEL/GroES chaperone cycle.

Two-timer model represents two rate-limiting steps, shown by arrows (colored red and blue). GroES (blue filled triangle) binds to the GroEL-substrate protein-ATP complex forming the *cis*-ATP* complex. Transition of the *cis*-ATP* complex to the *cis*-ATP complex is followed by the release of the substrate in the cavity and the initiation of protein folding. ATP hydrolysis converts *cis*-ATP to *cis*-ADP* complex. Protein folding occurs between *cis*-ATP and *cis*-ADP complex. Upon transition from *cis*-ADP* to the *cis*-ADP complex and attachment of ATP and substrate to the *trans* GroEL ring leads to the release of the folded native protein. Modified from (Ueno et al., 2004).

1.4.4. Hsp40

Hsp40 is one of the large protein families in the cell that function as co-chaperones of Hsp70 for protein folding during normal and stress conditions. Hsp40 (DnaJ in prokaryotes) is defined by highly conserved J domain (Laufen et al., 1999, Kakkar et al., 2012) and are classified in four major types depending on the domain organizations (fig 1.8) (Cheetham and Caplan, 1998, Rug and Maier, 2011). These include: type I, II, III and IV. Type I Hsp40 is identical in domain structure with that of DnaJ. It contains a signature J domain with its highly conserved HPD motif (~70 amino acids), followed by glycin-phenylalanine (GF) rich region, cysteine rich region (CRR) and C-terminal domain. Type II Hsp40 is similar in

domain structure when compared to type I, with only exception of CRR being absent. The type III and IV both contains the J domain but varies in the length and position of the HPD motif (fig 1.8) (Rug and Maier, 2011).

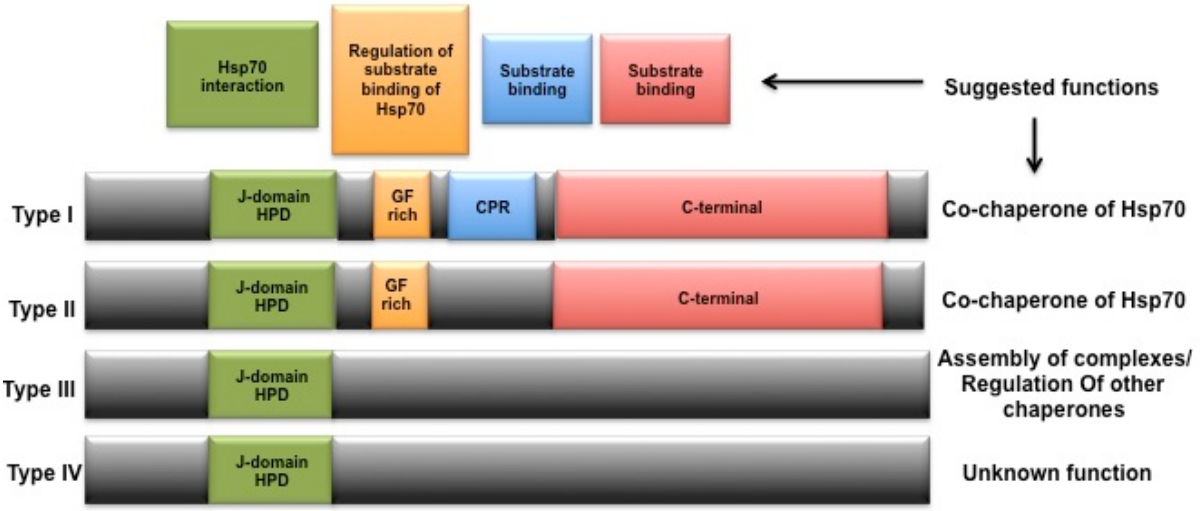

Figure 1.8 Schematic representation of the domain organisations of different types of Hsp40.

Type I DnaJ contains the signature J-domain with a conserved HPD motif (70 amino acids). The glycine/phenylalanine-rich region (GF-rich) (~30 amino acids) is present followed by the J domain. The cysteine-rich region forms a zinc finger domain. At the C-terminus it contains a less conserved substrate-binding domain (SBD) (~120–170 amino acids). The type II DnaJ molecules are structurally and functionally similar to the type I with only cysteine-rich region being absent. Type III DnaJ contains the J domain and it can be present anywhere on the protein molecule. Like Type III, Type IV DnaJ molecules contain the J-domain but with variations in the HPD motif. Possible cellular functions for the individual domains and DnaJs are mentioned. Modified from (Rug and Maier, 2011).

As mentioned earlier Hsp40 functions as a co-chaperone of Hsp70 and facilitates complex formation between Hsp70 and the client proteins. Both the type I and II Hsp40s come under this group. Hsp40 establishes an electrostatic interaction by forming an interface between the J domain and ATPase domain of Hsp70, thereby accelerating the ATPase activity of the later (Greene et al., 1998). This is an important rate limiting step leading to the stabilization of Hsp70-client protein complex (Fan et al., 2003, McCarty et al., 1995, Sterrenberg et al., 2011). The Hsp40/Hsp70 chaperone partnership is governed by ATP expanding cycles (Njunge et al., 2013) involving several steps (fig 1.10) (Liberek et al., 1991, Cyr et al., 1992, Langer et al., 1992). It is documented that Hsp40 also acts as a co-chaperone of Hsp90 chaperone machinery and are also independently involved in many cellular processes (Tarcsa and Fesus, 1990) including inhibition of protein aggregation under stress (Schroder et al., 1993). Recently, it has been shown that Hsp40 has a potential therapeutic role in the

prevention of polyglutamine (polyQ) diseases such as Huntington's disease (HD) (Popiel et al., 2012).

The number of different types of Hsp40 in a cell is much higher than that of its chaperone partner Hsp70 (Ohtsuka and Hata, 2000). Further it has been hypothesised that Hsp70 is capable of interacting with variety of Hsp40s. Therefore, Hsp70 can establish a diversified functional partnership with different types of Hsp40 to carry out functions including protein folding, trafficking, and post translational membrane integration (Kampinga and Craig, 2010, Hegde and Keenan, 2011, Kulzer et al., 2012).

1.4.5. Small heat shock proteins

Small heat shock proteins (sHsps) are characterised by their relatively small size between 16-30 kDa and the presence of the α-crystallin domain at the C-terminus (Shemetov et al., 2008). They are predominantly found in placenta, skeletal, heart, spleen and smooth muscles (Kappe et al., 2001) and form oligomeric complexes within the cell (van de Klundert et al., 1998). Small heat shock proteins might be involved in combating the stress produced in muscle tissues (Sugiyama et al., 2000). sHsps functions as molecular co-chaperone facilitating folding of client protein by larger chaperone like Hsp90 (Jakob et al., 1993, Chua et al., 2010). Malfunction of sHsps, result in various diseases like neuromuscular disease, congenital disease etc (Shemetov et al., 2008, Datskevich et al., 2012).

1.4.6. Hsp70

Heat shock protein 70, also known as Hsp70 is highly essential molecular chaperones and folding catalyst in a cell. They are involved in a large variety of protein folding machinery. This is facilitated by the transient interaction of the Hsp70 with the substrate coupled with ATPase activity (Mayer and Bukau, 2005).

Hsp70 has a molar mass of approximately 70kDa and represents ubiquitously expressed heat shock protein family, highly conserved among all living organisms. Some Hsp70s are constitutively expressed in the cell and are important for the housekeeping protein folding machinery whereas others come into play during cellular stress (Tavaria et al., 1996, Morano, 2007, Silver and Noble, 2012). Hsp70s are found to be present in different cellular compartments to carry out some specific functions. Depending on the function and compartmentalisation, Hsp70s are classified into four subfamilies: cytosol, endoplasmic

reticulum, mitochondria, and chloroplast (Krenek et al., 2013). Several homologues of Hsp70 proteins can be present in a cell that enable them to carry out a large variety of functions like, folding of nascent polypeptide chain, degradation, inhibit proteins from forming aggregates, intracellular signalling, and facilitating protein transport through membranes (da Silva and Borges, 2011). Prokaryotic version of Hsp70 is called DnaK. DnaK is expressed in the cell constitutively and also during stress periods (VanBogelen et al., 1997).

E. coli without DnaK function are sensitive to heat stress and as a result are associated with a variety of physiological defects: defective chromosomal segregation, temperature sensitivity, poor maintenance of plasmid, and defects in cellular metabolism (Paek and Walker, 1987, Kusukawa and Yura, 1988, Bukau and Walker, 1989). In *E. coli*, chaperone function of DnaK occurs in collaboration with co-chaperones such as, DnaJ (prokaryotic counterpart of Hsp40), GrpE (nucleotide exchange factor for DnaK) (Georgopoulos and Welch, 1993, Straus et al., 1990). Interestingly, DnaK and DnaJ function as modulators of heat shock response in the cell in association with the transcription activator sigma32 subunit of RNA polymerase. Small increase or decrease in the level of DnaK/DnaJ controls the switch on and off of the heat shock response via the controlled activity of the sigma32. Therefore, DnaK/DnaJ functions as primary sensors in the cell detecting protein misfolding (Tomoyasu et al., 1998).

1.4.6.1. Domain organisation of Hsp70

As mentioned earlier, despite of their diverse function in the cell, Hsp70 molecular chaperones have highly conserved amino acid sequence and domain organisation (Strub et al., 2002, Bukau and Horwich, 1998). Hsp70 consists of three domains:

N-terminal ATPase domain: The 45 kDa ATPase domain located at the N-terminus is responsible for the ATPase activity of Hsp70. ATP binds to this domain and the hydrolysis of ATP to ADP is promoted by nucleotide exchange factor (NEF). This results in important conformational changes that in turn get transferred to the substrate binding domain (SBD) which finally determines the binding or release of the substrate from Hsp70 (fig 1.9a and fig 1.9b) (McCarty et al., 1995, Strub et al., 2002, Bukau and Horwich, 1998).

Linker region: A highly conserved linker region is present following the ATPase domain that functions as a hinge between the ATPase domain and the substrate binding domain. The linker region facilitates inter-domain movement (fig 1.9a) (Strub et al., 2002).

Substrate binding domain (SBD): Hsp70 has a 25kDa substrate binding domain for the interaction of client proteins. This domain provides the groove with the affinity of neutral or hydrophobic amino acids to accommodate client proteins. The SBD is further sub divided into a 15kDa β-sandwich subdomain and a C-terminal α-helical subdomain (Mayer and Bukau, 2005). The C-terminal α-helices serve as a "lid" for the substrate binding domain. In an ATP bound state, the "lid" is open and allows the binding of substrates to the SBD whereas, in the ADP bound state the "lid" is tightly closed capturing the substrate in the groove (fig 1.9a and 1.9c).

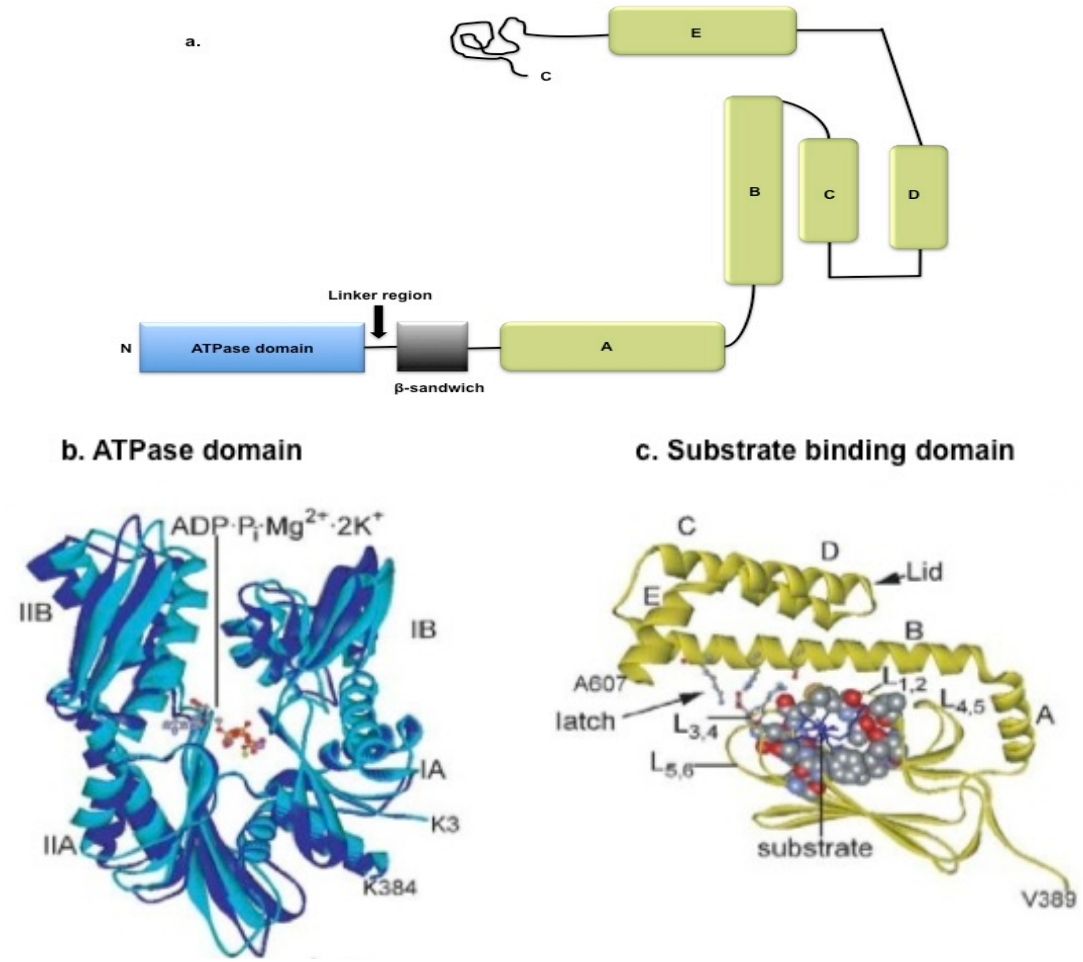

Figure 1.9 Domain organisation of Hsp70.

(a) Schematic representation of the domain organisation of *E.coli* DnaK. The ATPase domain present on the N-terminal end of the Hsp70 is connected to the substrate binding domain via the highly conserved linker region. The C-terminal substrate binding domain is further divided into helical subdomains (A-E). These subdomains form the β sandwich and lid segment. (b) ATPase domain of bovine Hsc70. Schematic representation of the secondary structure of the ATPase domain. Hsc70 in associated with inorganic phosphate, two potassium ions

and ADP. (c) Schematic representation of the model of substrate binding domain. Substrate binding domain of *E. coli* DnaK is associated with the client protein substrate at the hydrophobic core. H-bonds forming "latch" and salt bridge along with the presence of "lid" structure are indicated. (b) and (c) adapted from (Mayer and Bukau, 2005).

1.4.6.2. Hsp70 chaperone cycle: an overview

The Hsp70 multistep chaperone cycle involves co-chaperone Hsp40 and NEF. The steps are as follows: 1) The Hsp70 chaperone cycle starts with the transient interaction of client protein with Hsp40 by their exposed hydrophobic patches. The efficiency of Hsp40 to enhance ATPase activity of Hsp70 is induced by the establishment of such interactions (Kelley, 1998, Kelley, 1999). In certain occasion the cycle can begin with the direct interaction of Hsp70-ATP complex with the substrate. 2) In the next step, the substrate is transferred from Hsp40 to Hsp70. This process requires two steps, the first step is characterised by the transient interaction of the Hsp70-ATP complex with the J domain of the Hsp40 via the highly conserved HPD motif. The second step involves the subsequent transfer of the substrate from the Hsp40-substrate complex to the open substrate binding pocket of the Hsp70-ATP complex. These steps are needed to couple the ATPase activity of Hsp70 with substrate binding, therefore stabilizing the Hsp70-substrate complex. During substrate bound forms, Hsp40 accelerates the ATPase activity of Hsp70 thus inhibiting ATP hydrolysis on the free form of Hsp70. 3) Once the substrate is transferred to the Hsp70 and the ATP is hydrolysed to ADP, the affinity of Hsp70 for Hsp40 co-chaperone reduces due to the reduction in electrostatic interaction. As a result, Hsp40 dissociates from the complex. This indicates that Hsp40 works as a catalyst to transfer the client protein to Hsp70. Hydrolysis of ATP-ADP results in a conformational change in the ATPase domain of the Hsp70 that get transduced to the substrate binding domain. The 'lid' of the substrate binding domain obtains a 'close conformation' and tightly entraps the substrate into the cavity. 4) The cycle ends by the replacement of ADP with ATP by the NEF. ATP bound form of Hsp70 has high exchange rate and low affinity for substrate. As a result the substrate dissociates from the complex and the Hsp70 obtains 'open conformation'. This is the initial state where it is bound to ATP and can further accommodate new client proteins to start another cycle (Bukau and Horwich, 1998, Richter et al., 2010, Theyssen et al., 1996, Karzai and McMacken, 1996).

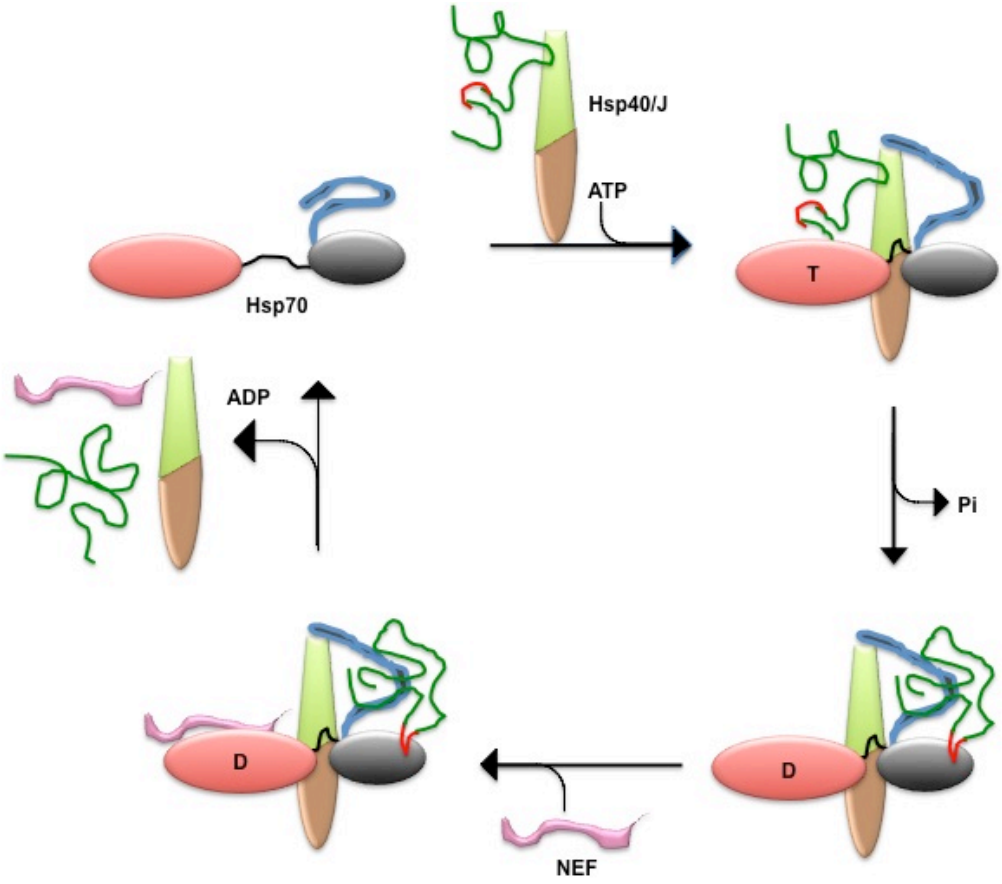

Figure1.10 Schematic representation of Hsp70 chaperone cycle.

The Hsp70 chaperone cycle involves the participation of Hsp70 and its co-chaperone Hsp40 and the NEF. Hsp40 interacts with client protein via its exposed hydrophobic residues. Hsp40 further establishes a transient interaction with Hsp70 via its highly conserved HPD motif on the J domain. After establishment of the electrostatic interaction Hsp40 delivers the substrate to Hsp70 and induces the ATPase activity of Hsp70 resulting in the stabilization of the Hsp70-substrate complex. The NEF replaces ADP with ATP and thus, the ADP, Hsp40, and the substrate dissociates from the complex. Hsp70 returns to its ATP bound initial stage and can accommodate new substrate. Adapted from (Richter et al., 2010).

1.4.6.3. Hsp70: the central player

Hsp70 plays an important role as a central chaperone to maintain the protein homeostasis of the cell during normal physiological condition and under stress. Hsp70 carry out its biological functions in the cell either independently or in association with other co-chaperones (Mayer and Bukau, 1998, Borges and Ramos, 2005).

Recently, by a quantitative proteomic approach it was demonstrated that DnaK interacts with ~ 700 cytosolic protein, among which ~180 are aggregation prone and requires DnaK for its proper folding (Calloni et al., 2012). In *E. coli*, DnaK functions in collaboration with other partners like DnaJ, GrpE, GroEl/GroES. The fact is well evidenced that DnaK can bind to protein aggregates and can facilitate their correct folding into the native form in collaboration

with DnaJ/GrpE. But, in addition it has been documented that DnaK can efficiently fulfil its function only if the aggregates are formed early and are relatively smaller in size. For much bigger aggregates either the binding site of the DnaK system needs to be highly exposed or it involves the catalytic activity of ClpB (Hsp100 homologue in bacteria) and ATP (Diamant et al., 2000). Therefore, the association of DnaK and ClpB machinery helps in disaggregating protein aggregates and their further folding in native forms (Rosenzweig et al., 2013). ClpB and DnaK chaperone system and the ratio between their cellular levels have an important role in inhibiting protein aggregates during heat shock (Kedzierska and Matuszewska, 2001).

In the eukaryotic system the client proteins are passed from Hsp40 to Hsp70. Hsp40 as discussed earlier (section 1.4.6.2), also helps in the accelerating the ATPase activity of Hsp70, a step that is crucially important for the chaperone activity of Hsp70 (Bukau and Horwich, 1998). Despite of the fact that Hsp70 and Hsp90 can function independently in the cell, the transfer of client protein from Hsp70 to the Hsp 90 has been well studied. The functional interaction between Hsp70 and Hsp90 is mediated via HOP. Therefore, HOP works as an adaptor molecule bridging the gap between Hsp70 and Hsp90 (Smith et al., 1993, Li et al., 2012). Once the substrate is transferred to Hsp90, Hsp70, Hsp40, and HOP dissociate from the chaperone complex and further folding of the client protein takes place via the participation of Hsp90 and p23 (fig 1.6) (Morishima et al., 2003).

Therefore, it can be concluded that in both prokaryotic and eukaryotic systems the involvement of Hsp70/DnaK is crucial to establish functional link between individual chaperone pathways. Hsp70 plays an intersecting role by receiving substrate from one co-chaperone partner (Hsp40/DnaJ) and delivering it to the other co-chaperone for the final folding (Hsp90/ClpB) (Richter et al., 2010).

1.4.7. Heat Shock proteins in health and disease

The fact whether heat shock proteins are the potential therapeutic agent or targets for potential drugs in several pathogenic diseases remains highly controversial (Pockley, 2001). Significant amount of study is being undertaken to understand involvement of heat shock proteins in disease development or prevention.

In recent years, Hsps are highly acknowledged as therapeutic candidate and their induction show significant promise against several diseases. For instance, the role of heat shock

proteins in the pathogenesis of neurogenerative disorders is being extensively studied. Several such disorders like Alzheimer's disease, Parkinson's disease and Huntington's disease etc, are the outcomes of Hsp dysfunction. It has been documented that the overexpression of several heat shock proteins including Hsp70 provided protection in model organism against such diseases (Mayer et al., 1991). Age related neurogenerative disorders known as 'proteinopathies' leads to the deposition of protein in several tissues. Since, deposition is an outcome of protein unfolding or misfolding in the event of stress, the involvement of heat shock protein is becoming more prominent and qualifies them as potential therapeutic candidates (Macario and Conway de Macario, 2000). Another example is 'Vitiligo'. It is an autoimmune disorder causing the destruction of melanocytes, leading to progressive depigmentation. In a recent study using mouse model it has been shown that the delivery of iHsp70, a modified head inducible Hsp70 could attribute to significant inhibition of depigmentation (Mosenson et al., 2013). Aging is also related to the loss of Hsp function, and induction of Hsp70 proved to be effective in increasing life expectancy in model organisms like yeast or *Drosophila* (Tatar et al., 1997). Induction of Hsp70 leads to the efficient recovery from a large number of other cases including diabetes and ischemic heart disease (Soti et al., 2005).

In contrast to all these facts, Hsps are also exploited for development of several types of cancers, where the inhibition of their function is necessary for therapy. Besides the protein folding role, heat shock proteins are largely involved in protein trafficking and regulation of transcription. Crucial cytoprotective role of heat shock proteins makes them essential for the survival of cancer cells. Androgen receptor (AR), a transcription factor has a significant contribution towards the development of several cancers, most importantly prostate cancer. ARs are found to be associated with several Hsps and are upregulated during cancers (Hessenkemper and Baniahmad, 2013). For instance, Hsp90 plays an important role in signal transduction via its interaction with several transcription factors and protein kinases (Rutherford and Zuker, 1994). Hsp90 maintains AR at a high affinity conformation and the loss of function of the former in the cell renders AR less active (Fang et al., 1996). Therefore, many emerging drugs against prostate cancer target Hsp90 and AR associated Hsps (Hessenkemper and Baniahmad, 2013). Several other Hsps like Hsp70 and Hsp27 are present at very high levels in cancer cells and further increased by cancer chemotherapy. It has been postulated that these group of Hsps are involved in the development of cancer and confer

protection to cancer cells against chemotherapy. Therefore, inhibition of these Hsps has also emerged as a novel anticancer therapy (Garrido et al., 2006).

Additionally, Hsps are often exploited during the onset of pathogenesis caused by invading microorganisms. Molecular chaperones are often used by several disease causing bacteria for their adaptation in the host. Many of these expose Hsps on their cell surface that induces a huge immune response during several diseases like leprosy, tuberculosis, Chagas's disease, legionnaire's disease, chlamydial infections, lyme disease, and Q fever. Unfortunately, in many occasions due to the high level of conservation between Hsps from host and bacterial origin, cells develop autoimmunity causing diseases like rheumatoid arthritis, lupus erythematodes, multiple sclerosis, diabetes etc (Lamb et al., 1989, Pockley, 2002). Several pathogens produce heat shock proteins during the invasion of host cells that have become targets for drug and vaccine development (Newport, 1991). The role of heat shock proteins in the development of infection and parasitic diseases is being highly scrutinised (Macario, 1995). Parasite exploits a range of heat shock protein for better adaptation and infectivity within the host cell. They produce Hsps in response to stress and exploit them for various purposes ranging from adaptation, differentiation and protein trafficking to establish pathogenicity (Silva et al., 1998). For example, apicomplexan parasite *Toxoplasma gondii* expresses Hsp70 for the development of bradyzoites to tachyzoites. Hsp70 as a vaccine was shown to protect experimental animal model from *Toxoplasma* challenge (Makino et al., 2011). On the other hand Hsp90 function in *Leishmania* is essential for the morphological differentiation of the parasite (Wiesgigl and Clos, 2001). Hsp90 is also important for adaptation of *Toxoplasma gondii* and *Trypanosomatids* in their hosts (Angel et al., 2012).

Plasmodium falciparum expresses a wide range of heat shock proteins for better adaptation in the host. It has recently been documented that chaperones of parasitic origin are upregulated in malaria patients and that the expression profile is correlated with different stages of parasite life cycle (Pallavi et al., 2010). Cytoplasmic Hsp90 has been reported to be indispensible for the survival of *Plasmodium* in the human RBC (Banumathy et al., 2003). Elevated temperature during the course of fever has been shown to augment parasite development and infectivity. This is mostly occurs due to the upregulation of Hsp90 and its co-chaperone complex. Inhibition of Hsp90 by geldanamycin (GA) inhibited temperature dependent development of the intraerythrocytic parasite (Pavithra et al., 2004). Hsps in the parasite might be involved in interesting roles, ranging from the protection against stress in

the host cell (elevated temperature, oxidative stress, degrading enzymes, immune system) to the establishment of virulence (Polla, 1991). It has been hypothesised that *Plasmodium* codes different chaperones (Hsp70/Hsp40 homologues) for the trafficking of parasite virulence protein (*Pf*EMP1) to the RBC membrane (Kulzer et al., 2012). It can be speculated that the challenges encountered by *Plasmodium* has led to the evolutionary selection of a diverse group of chaperone for better adaptation (Shonhai et al., 2011). Therefore, the different chaperone networks are important weapons for the parasite to combat insults encountered in the host. Nevertheless, the essential role of chaperone in the establishment of adaptation and virulence qualifies them as potential drug candidates. Since heat shock proteins are highly conserved among different organisms, cautious assessment needs to be taken into consideration for the development of new drugs to avoid cross reactivity between the pathogen and the host (Newport, 1991).

1.5. Molecular chaperones: role in the adaptation of *Plasmodium falciparum* within the human host

The lifecycle of *Plasmodium,* as explained earlier is divided into two phases; sexual phase, that takes place in the poikilothermic mosquito vector and an asexual phase, occurring in the warm blooded human host (fig 1.2). *Plasmodium falciparum* during its life cycle comes across a wide range of environmental challenges that is associated with its migration from the mosquito vector to the human host. During the passage from poikilothermic mosquito vector to the warm blooded human host it encounters a temperature shift from 25 ° C to 37 ° C. Most importantly the body temperature of the host can rise up to 41° C during the course of fever episodes which is a characteristic of human malaria. As mentioned earlier such elevated temperature facilitates parasite development inside the human RBC, a key stage in *Plasmodium* life cycle. This observation supports the notion that temperature is an important environmental factor for the development and infectivity of the malaria parasite in human (Pavithra et al., 2004).

In almost all organisms, heat shock proteins provide defence to the cell under stressful conditions. It is therefore suspected that heat shock proteins play an important role in the adaptation of the parasite in the human host. *Plasmodium falciparum* codes for many heat shock proteins belonging to the families of Hsp90, Hsp70, Hsp60 and Hsp40 (Watanabe, 1997, Das et al., 1997, Bonnefoy et al., 1994, Kumar et al., 1991, Banumathy et al., 2003).

For example, one of the *P. falciparum* Hsp70s known as *Pf*Hsp70-1 has been shown to supress thermosensitivity of *E. coli* DnaK. This indicates a possible cytoprotective role of this protein in the human malaria parasite (Shonhai et al., 2005). Parasite growth is terminated by blocking *Pf*Hsp90 and qualifies it as an essential protein for parasite survival (Banumathy et al., 2003, Kumar et al., 2003). On the other hand, an Hsp40 homologue known as the 'ring-infected erythrocyte surface antigen' (RESA) has been found to be involved in conferring resistance to the parasite during elevated temperature in the host (Silva et al., 2005). Therefore, it is interesting to note that *Plasmodium* adapts to several challenges encountered within the host and establishes pathogenesis by recruiting diverse chaperone network (Pavithra et al., 2007). In many aspects direct biochemical evidences, supporting the importance of these chaperones are still missing. Therefore, further research on several parasitic chaperones would help us to better understand the parasite biology in order to design efficacious antimalarial drugs.

1.6. Major heat shock proteins in *Plasmodium falciparum*

1.6.1. *Plasmodium falciparum* Hsp90 (*Pf*Hsp90)

Plasmodium codes only one *Pf*Hsp90 (PF3D7_0708400), expressed throughout all intraerythrocytic stages of the life cycle (Bonnefoy et al., 1994, Khachane et al., 2005, Acharya et al., 2007). *Pf*Hsp90 has retained a conserved domain structure and shares 64 % sequence identity to its human homologue. N-terminal ATP binding domain is highly conserved in *Pf*Hsp90 (Acharya et al., 2007, Shonhai, 2010). The dimerization domain helps in the establishment of functional dimeric structure of the protein. It also contains EEVD motif at its C- terminus, characteristic of cytosolic chaperones and might be involved in the interaction with co-chaperones (i.e. Hsp70 and HOP) (Kumar et al., 2007). In fact, due to its essential role for the intraerythrocytic survival, it is the most well studied chaperone in *P. falciparum.*

Figure 1.11 Schematic representation of domain organisation of *Pf*Hsp90.

*Pf*Hsp90 contains a highly conserved ATPase domain at the N-terminus (pink). In addition to the linker region (red) it also possesses calmodulin binding domain (brown), leucine zipper motif (green) and a dimerization domain (yellow) present towards the C- terminus. At the extreme C-terminus it contains a highly conserved EEVD motif (blue) characteristic of cytosolic Hsp90. This motif plays important role in the interaction with Hsp70/HOP. Adapted from (Acharya et al., 2007).

As previously mentioned Hsp90 is essential for the development of the parasite in the mature human erythrocyte. Inhibition of *Pf*Hsp90 pathway by geldanamycin arrested the growth of the parasite at the ring stage (Pavithra et al., 2004, Banumathy et al., 2003). Geldanamycin (Hsp90 inhibitor and a benzoquinone ansamycin antibiotic) functions by inhibiting the interaction of Hsp90 with its co-chaperone Hsp70 and thereby, rendering the *Pf*Hsp90 machinery non-functional. Therefore, the arrest of parasite development resulting from the inhibition of Hsp90 function indicates the passive or active involvement of other co-chaperones (Banumathy et al., 2003). In agreement with this notion, *Pf*Hsp90 has been found to associate with other molecular co-chaperones *in vitro* or *in vivo* (Acharya et al., 2007). The co-precipitation of *Pf*Hsp90 with *Pf*Hsp70-1 indicates functional partnership between both (Banumathy et al., 2003). A p23 homologue was identified in *Plasmodium*, which is highly conserved among this species and a relatively abundant cytosolic protein expressed through ring to trophozoite stages of the parasite. Therefore, it might be involved in the replication of the parasite within the human RBC in collaboration with other chaperones like *Pf*Hsp90 (Wiser, 2003, Kumar et al., 2003). A HOP homologue was recently characterised in *Plasmodium* and was found to co-localise and co-precipitate with *Pf*Hsp90 indicating a functional interaction between them similar to other eukaryotic system (Gitau et al., 2012). *Pf*PP5 (a co-chaperone of Hsp90) was also co-immunoprecipitated with *Pf*Hsp90 (Dobson et al., 2001, Dobson et al., 1999, Acharya et al., 2007). Calcineurin is a protein that is needed by

Hsp90 for folding. In *Plasmodium,* calcineurin was found to be closely associated with *Pf*Hsp90 and the interaction could be strongly inhibited by cyclosporine A (CsA) and probably explains the reason behind the CsA sensitivity to malaria (Dobson et al., 1999). Association with all these co-chaperones indicates an obvious involvement of Hsp90 machinery in the intraerythrocytic stages of *Plasmodium*.

Therefore, it can be concluded that *Pf*Hsp90 is a potential drug target and inhibition of *Pf*Hsp90 function could overcome the resistance of *Plasmodium* to current antimalarial drugs (Acharya et al., 2007). Presently, drugs against *Pf*Hsp90 are going through phase III clinical trial (Angel et al., 2012).

1.6.2. *Plasmodium falciparum* Hsp60 (*Pf*Hsp60)

There is very limited amount of information available about Hsp60 homologue in *Plasmodium falciparum*. *Pf*Hsp60 (PF3D7_1015600) is expressed in all intraerythrocytic stages (Das et al., 1997). A study reported, a heat dependent increase in the cellular mRNA level for Hsp60 (Syin and Goldman, 1996). Although a three-fold increase was observed in the total mRNA level of *Pf*Hsp60, there was no increase in the total protein content, indicating a possible involvement of *Pf*Hsp60 in the translational level. *Pf*Hsp60 was also found to co-immunoprecipitate with several other proteins suggesting possible interactions (Das et al., 1997).

1.6.3. *Plasmodium falciparum* small heat shock proteins

As previously described, sHsps plays an important role in the disaggregation and proper folding of denatured proteins in the cell (Jakob et al., 1993). *Plasmodium falciparum* genome possesses only two genes coding for small heat shock proteins (PF3D7_0816500 and PF3D7_1304500). PF3D7_0816500 codes for a small heat shock protein of 20kDa (Hsp20). The gene expression profile reveals that there is not much stage dependent change in the expression level of this protein in intraerythrocytic stages of *P. falciparum*. Although the parasite specific role of this protein is yet to be determined, there are predictions that this could be a possible Bag1 co-chaperone of *P. falciparum* as it shares homology with the Bag1 gene from its apicomplexan relative, *T. gondii*. On the other hand, PF3D7_1304500 gene codes for a 25kDa putative protein. The expression profile of the gene in the intraerythrocytic stage of the parasite demonstrates several fold increase in the protein level during the

trophozoite stage. But, the actual role of this protein in the parasite is still unknown (Aurrecoechea et al., 2009).

1.6.4. *Plasmodium falciparum* Hsp40 (*Pf*Hsp40)

Plasmodium genome codes for 43 Hsp40 genes in total. Very few Hsp40s from *Plasmodium falciparum* have human homologue except for those involved in fundamental cellular processes (Botha et al., 2007). The pool of Hsp40 from *Plasmodium* is further classified into different types according to the DnaJ canonical classification system. Out of the 43 Hsp40s, 31 Hsp40s contain a signature J domain with a conserved HPD motif. We further classify the Hsp40s in two subgroups: exported and non-exported Hsp40.

1.6.4.1 Exported *Pf*Hsp40s

Amongst the 43 Hsp40s, 18 contain a *Plasmodium* export element 'PEXEL' motif and can be exported. But, only five of them were found to be exported to the host cell till date (Hiller et al., 2004, Silva et al., 2005, Bhattacharjee et al., 2008, Kulzer et al., 2010). PF3D7_0113700, PF3D7_0201800, PF3D7_0501100.1 code for exported type II *Pf*Hsp40s, (Rug and Maier, 2011). Both the PF3D7_0113700 and PF3D7_0501100.1 *Pf*Hsp40s were found to localize in the infected RBC and associates with mobile structures in the RBC cytosol named as 'J dots' (Kulzer et al., 2010). PF3D7_0113700 was proved to be essential for the survival of *Plasmodium* (Maier et al., 2008). Several Type IV exported *Pf*Hsp40s are trafficked to the RBC cytosol, thereby could be involved in the remodelling of erythrocyte. RESA is one of the most extensively studied exported type IV *Pf*Hsp40s (PFA0110w) from *Plasmodium* origin. RESA is initially localized in the dense granule and plays an important role during invasion. After being released in the forming PV it gets transported through PVM into the RBC cytosol and finally gets associated with spectrin present on the RBC membrane (Foley et al., 1990, Da Silva et al., 1994). There is a huge amount of conflicting data about the timing and extent of RESA influencing RBC cytoskeleton. But, the important role of this protein in the protection of cytoskeleton during high temperature fever episodes remains unquestioned (Rug and Maier, 2011). Another highly important exported type IV *Pf*Hsp40 is PF3D7_1039100. It is thought to be involved in the knob formation on the infected erythrocyte. Knobs are formed as a result of membrane remodeling induced by the parasite, and provide anchoring point for the major virulence factor *Pf*EMP-1. Knockout of PF3D7_1039100 from the parasite genome largely affects the ability of the parasite to form

knobs (Maier et al., 2008) impairing their ability to adhere to the endothelium (LaCount et al., 2005).

1.6.4.2. Non-exported *Pf*Hsp40s

We predict that the non-exported cytosolic Hsp40s might be involved in house keeping function and cytoprotection of the cell. They function either independently or in association with Hsp70 chaperone machinery (Rug and Maier, 2011). Indeed heat shock mediated upregulation of the mRNA and protein level was observed for a type I cytosolic *Pf*Hsp40 (PF3D7_0409400) (Watanabe, 1997). The J domain from this *Pf*Hsp40 could reverse the thermosensitivity of *E. coli* strain lacking its DnaJ by establishing a possible interaction with DnaK (Nicoll et al., 2007). In addition, it could facilitate the chaperone activity of *Pf*Hsp70-1 (cytosolic, heat inducible Hsp70 from *Plasmodium*) indicating its possible interaction with the later *in vivo* (Misra and Ramachandran, 2009). Another type I cytosolic Hsp40 (PF3D7_1437900) was also found to be heat inducible and co-localized with *Pf*Hsp70-1 in the cytosol of the parasite upon heat shock (Botha et al., 2011). This also indicates a housekeeping and cytoprotective role *in vivo*. This Hsp40 candidate was also able to enhance ATPase activity of *Pf*Hsp70-1, which could further be inhibited by small molecules. On the other hand PfJ4 (PF3D7_1211400), a heat inducible type II *Pf*Hsp40 co-localized, co-immunoprecipitated with *Pf*Hsp70-1 and demonstrated thermosensitivity (Nicoll et al., 2007, Pesce et al., 2008). Another type II cytosolic *Pf*Hsp40 (PF3D7_0213100) is likely to be involved in cytoprotection. Interaction of this protein with *Pf*Hsp70-1 has been predicted (Botha et al., 2007). Although, this prediction indicates a possible co-chaperone partnership of this particular *Pf*Hsp40s with *Pf*Hsp70-1 to provide cytoprotection to the parasite, direct research data supporting this notion is still lacking.

The facts mentioned above, all together indicate a very important role of a diverse group of *Pf*Hsp40s either in the cytoprotection or pathogenesis of the parasite. The PEXEL positive Hsp40 crosses the parasite plasma membrane (PPM), PV, PVM and contribute mostly to the establishment of pathogenesis in the host. Whereas, the non-PEXEL cytosolic ones might be involved in the cytoprotection of the parasite during thermal stress period. Therefore, all these functions qualify *Pf*Hsp40s as potential drug candidates.

1.6.5. *Plasmodium falciparum* Hsp70 (*Pf*Hsp70)

Plasmodium genome codes for 6 Hsp70 genes and cellular localisations of these proteins are either already shown or predicted (Table 1) (Shonhai, 2010).

Amongst all, *Pf*Hsp70-1 (PF3D7_0818900) is highly abundant and constitutively expressed during all intraerythrocytic stages of the parasite. *Pf*Hsp70-1 is localised in the parasite cytosol (Kumar et al., 1991). In fact it is the most extensively studied candidate from all the Hsp70s identified so far (Refer to section 1.7.4.1 for details).

*Pf*Hsp70-2 (PF3D7_0917900) is a homologue of the ER resident Grp78 and BiP. *Pf*Hsp70-2 has conserved nuclear localization signal and is localized to ER like structures in *Plasmodium*, mediated by N-terminal leader sequence and C-terminal ER-retention signal (Kumar et al., 1991, Lanzer et al., 2006, Shonhai et al., 2007, Shonhai, 2010). It has also been found to be present in the Maurer's cleft (Vincensini et al., 2005).

*Pf*Hsp70-3 (PF3D7_1134000) is a mitochondrial homologue and also found in Maurer's cleft (Lanzer et al., 2006, Shonhai, 2010, Vincensini et al., 2005). It has a conserved nuclear localization signal (Shonhai, 2010). This Hsp70 homologue was also detected in the PV (Nyalwidhe and Lingelbach, 2006, Shonhai, 2010).

*Pf*Hsp70-x (PF3D7_0831700) shares high sequence identity with *Pf*Hsp70-1 and contains an EEVN domain suggesting its role as a cytosolic chaperone (Shonhai, 2010). But, in contrast to this prediction recent work has demonstrated the trafficking of *Pf*Hsp70-x to the PV and cytosol of the infected erythrocyte mediated by a short segment of eight amino acid sequences present directly after the predicted signal sequence. This is the first report of a parasite encoded Hsp70 to be trafficked to the host cell. This suggests a possible involvement of *Pf*Hsp70-x in trafficking of a number of parasite encoded proteins favoring pathogenesis. In addition, this indicates a possible collaboration of *Pf*Hsp70-x with parasite coded Hsp40s either in the host cytosol or in the PV (Kulzer et al., 2012).

The last two Hsp70 homologues *Pf*Hsp70-y (PF3D7_1344200) and *Pf*Hsp70-z (PF3D7_0708800) have a relatively conserved ATPase domain, but the substrate binding domains do not show any conservation. These Hsps do not have nuclear localization signal. They neither have the threonine residue like the DnaK, crucial for phosphorylation nor a proper linker region. Therefore, these two *Pf*Hsp70s might be involved in functions that is

not typical of Hsp70. *Pf*Hsp70-y might be involved in functioning as an ER-based NEF of the Hsp110/Grp170 family of protein whereas, *Pf*Hsp70-z might act as cytosolic Hsp110/Grp170 (Shonhai, 2010).

Name/PlasmoDB annotation	Molecular weight (kDa)	Signal sequence/special features	Predicted cellular localization	Expression phase
***Pf*Hsp70-1/ PF3D7_0818900**	74	C-terminal EEVD motif, GGMP motif	Cytoplasm	Erythrocytic stage
***Pf*Hsp70-2/ PF3D7_0917900**	73	Carboxy terminal ER sequence/homologue of Bip/Grp78	ER	Exo-erythrocytic stage
***Pf*Hsp70-3 / PF3D7_1134000**	73	Mitochondrial transit sequence/ homologue of mtHsp70	Mitochondria (not confirmed)	Unknown
***Pf*Hsp70-x/ PF3D7_0831700**	76	Predicted signal peptide followed by eight amino acid sequence	Parasite cytosol/PV/RBC cytosol	Erythrocytic stage
***Pf*Hsp70-y/ PF3D7_1344200**	108	Carboxy terminal ER sequence	ER (not confirmed)	Unknown
***Pf*Hsp70-z/ PF3D7_0708800**	100	Homologue of cytoplasmic hsp105	Cytoplasm (not confirmed)	Unknown

Table 1. Six different Hsp70s from *Plasmodium falciparum*.

This table summarizes molecular weight, special features, cellular localizations, and expression phases of individual *Pf*Hsp70 of *P. falciparum*. Adapted from (Shonhai et al., 2007, Kulzer et al., 2012).

1.6.5.1. *Plasmodium falciparum* Hsp70-1 (*Pf*Hsp70-1): findings, chaperone properties and physiological importance

*Pf*Hsp70-1 has received an extensive amount of research interest in the last decades. As mentioned earlier, cytosolic *Pf*hsp70-1 is one of the most abundant proteins expressed by *Plasmodium* and is expressed throughout all intraerythrocytic stages (Shonhai, 2010). Stress introduced to cultured parasite in human red blood cell induced upregulation in the level of *Pf*Hsp70-1, indicating a cytoprotective role (Kumar et al., 1991). *Pf*Hsp70-1 could reverse the thermosensitivity of *E. coli* having compromised DnaK function, therefore suggesting its *in vivo* chaperone function (Matambo et al., 2004, Shonhai et al., 2005). *Pf*Hsp70-1 has conserved substrate binding domain allowing it to interact with protein aggregates or degradation prone substrates. In support to this hypothesis it has been demonstrated that

*Pf*Hsp70-1 could efficiently inhibit the heat induced aggregation of malate dehydrogenase (MDH) *in vitro* (Shonhai et al., 2008).

In other biological systems Hsp70 functions in collaboration with Hsp90 where Hsp90 receives substrate from Hsp70. *Pf*Hsp70-1 possess a C-terminal EEVD motif, responsible for the establishment of interaction with Hsp90 via the adaptor co- chaperone HOP (Shonhai et al., 2007). Therefore, such a functional interaction between Hsp70 and Hsp90 in *Plasmodium* system cannot be overlooked. In fact, *Pf*Hsp70-1 was detected in a complex with *Pf*Hsp90 during co-immunoprecipitation and proposes a possible functional partnership between them (Banumathy et al., 2003, Banumathy et al., 2002). In a recent study, a HOP homologue has been identified and characterised in *Plasmodium*. The *Pf*HOP (PF3D7_1434300) co-localised with *Pf*Hsp70-1 and *Pf*Hsp90 and was detected in a common complex (Gitau et al., 2012). Hence, these data suggests a functional partnership between *Pf*Hsp70-1 and *Pf*Hsp90 via *Pf*HOP. Since, *Pf*Hsp90 is essential for parasite survival, the interaction of *Pf*Hsp70-1 with the former demands high research attention. It also indirectly implicates *Pf*Hsp70-1 as a potential drug target.

Another important co-chaperone of Hsp70 is Hsp40. As described earlier *Plasmodium* codes for 43 Hsp40 homologues among which type I and type II members possess J domain with highly conserved HPD motif and therefore qualify as a co-chaperones of cytosolic *Pf*Hsp70-1. In support to this hypothesis several *Pf*Hsp40 were found to interact with *Pf*Hsp70-1 (as detailed under section 1.7.4).

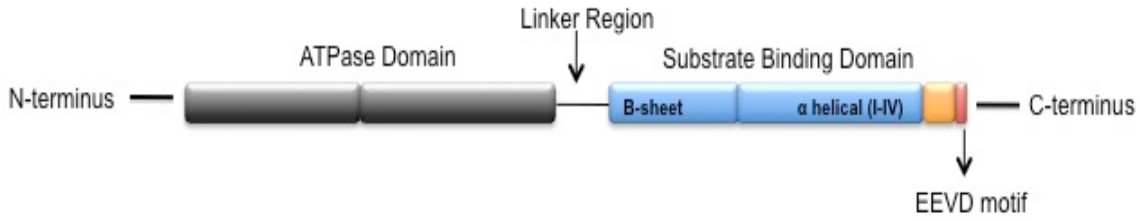

Figure 1.12 Schematic representation of the domain organisation of *Pf*Hsp70-1.

*Pf*Hsp70-1 is highly conserved in its domain organisation. It possesses a 45kDa N-terminal ATPase domain (black) divided into two subdomains. The ATPase domain is connected to the substrate-binding domain by a highly conserved linker region (black line). The substrate binding domain (blue) is formed of β and α-helices. The β provides the hydrophobic pocket for the substrate binding whereas α-helices work as lid entrapping the substrate. *Pf*Hsp70-1 has a C-terminal EEVD motif (green) characteristic of cytosolic Hsp70s to interact with co- chaperones. Modified from (Fan et al., 2003).

In addition to the mentioned Hsp40s another putative type II non PEXEL Hsp40 from *Plasmodium* (PF3D7_0213100) is predicted to be the cytosolic co-chaperone of *Pf*Hsp70-1

(Pavithra et al., 2007), but till date there is no direct biochemical evidence supporting this partnership (Rug and Maier, 2011).

Small molecule inhibitor for the interaction of *Pf*Hsp70-1 with either its potential co-chaperones (Hsp40/Hsp90) or substrate could emerge as a promising approach towards drug development against malaria.

1.6.5.2. *Pf*Hsp70-1 as a potential drug candidate

Hsp70 brings different chaperone networks into functional collaboration in a cell. A small molecule inhibitor, immunosupressant 15-deoxysperguallin (DSG) has been proposed as an antimalarial agent that targets *Pf*Hsp70-1 and is undergoing phase II clinical trial. DSG is known to bind *Pf*Hsp70-1 (Ramya et al., 2007) and is thought to inhibit its function in two possible ways. Firstly, DSG attaches to the EEVD motif at the C-terminus of the protein and thus inhibits the interaction with *Pf*Hsp90. Since, *Pf*Hsp90 is predicted to receive substrate from *Pf*Hsp70-1 like in other eukaryotic organisms, this inhibition of interaction render *Pf*Hsp90 chaperone machinery non-functional, leading to the death of the parasite (Shonhai, 2010). The other possible way that DSG could function is by interrupting the interaction between *Pf*Hsp70-1 and its co-chaperone, *Pf*Hsp40 via the C-terminal EEVD motif (Demand et al., 1998). By a recent approach several molecules were screened to assess their potential to inhibit chaperone function of *Pf*Hsp70-1. A few promising candidates emerged from this screen that could inhibit the protein aggregation suppression activity of purified *Pf*Hsp70-1 *in vitro* in a concentration dependent manner. These small molecules were also found to inhibit parasite growth. Therefore, this compound justifies the potential of *Pf*Hsp70-1 as a drug target. On the other hand, they could prove to be important molecular probes for the study of *Pf*Hsp70-1 chaperone function (Cockburn et al., 2011). As previously mentioned, the functional interaction of *Pf*Hsp70-1 in the parasite with either *Pf*Hsp40 or *Pf*Hsp90 co-chaperone is predicted. Such partnership might be involved in cytoprotection and trafficking of proteins to establish pathogenesis and virulence. Therefore, the functional partnership between *Pf*Hsp70-1 and their chaperone counterpart is crucial for the stress management of the cell and is of high priority (Botha et al., 2007). The inhibition of such crucial function might interrupt several processes including cytoprotection, protein trafficking, and actin polymerisation (Tardieux et al., 1998). Although Hsp70s are conserved between organisms, *Pf*Hsp70-1 has some unique features. The SBD of *Pf*Hsp70-1 shares very less amino acid identity with that of its human homologue (Hsc70). In addition, *Pf*Hsp70-1 has high basal

ATPase activity in comparison to that of Hsc70. Therefore the *Pf*Hsp70-1 function can be inhibited by limiting the cellular level of ATP, without affecting the function of Hsc70 (Matambo et al., 2004). Due to the growing resistance of *Plasmodium* to commercially available antimalarials, further screening and identification of small molecule inhibitors of *Pf*Hsp70-1 co-chaperone/substrate interaction could prove to be a promising approach towards the development of new efficacious drugs against malaria.

1.7. Strategy to generate *Pf*Hsp70-1 and *Pf*Hsp40 knockdown cell lines using dominant negative protein combined with destabilization domain approach

The study of essential genes by classical knockout approaches is hindered with several challenges in *Plasmodium* system. Firstly, the intraerythrocytic stage of *plasmodium* is haploid therefore knocking out essential gene would result in parasite death. RNA interference (RNAi) is another frequently used approach for gene knockout in other parasitic organism like *Trypanosoma brucei* in combination with Tetracyclin Repressor (TetRep) based system (Wirtz and Clayton, 1995, Bastin et al., 2000, Pino et al., 2012). But, *P. falciparum* does not code for the crucial enzymes of the RNAi pathways and previous attempt to establish RNAi in *Plasmodium falciparum* failed (Baum et al., 2009). This indicates that the effective application of RNAi is yet to be proven in *Plasmodium falciparum* (Agop-Nersesian et al., 2008). Another inducible gene expression system, where the Tet Repressor (TetRep) is fused to a nonendogenous activating domain (TATi) was established in close apicomplexan *Toxoplasma gondii* (Meissner et al., 2002). This tool was found to be highly effective to study the functional importance of several essential candidate genes in *T. gondii* (Meissner et al., 2002, Buguliskis et al., 2010, Plattner et al., 2008). This artificial transactivator was also be used in *P. falciparum* to regulate gene expression from episomal plasmid (Meissner et al., 2005). Unfortunately, this system also failed to generate conditional knockout of genes because of less transactivation activity (Pino et al., 2012). FLP recognition target (FRT)-specific/FLP recombinase system has been reported to be used with some success at specific stages of the parasite, but this system is irreversible (O'Neill et al., 2011, van Schaijk et al., 2010, Combe et al., 2009).

Hence, during this study we used a different strategy to identify the functional importance of *Pf*Hsp70-1 in the intraerythrocytic stage of the *Plasmodium*. We wanted to inhibit the

function of the endogenous *Pf*Hsp70-1/Hsp40 by episomal over expression of the dominant negative (DN) version of the protein. Therefore, we expected the dominant negative proteins to shield the effect of wild type endogenous ones in a competitive manner by capturing all possible co-chaperone and client substrates. DN version can bind to all interacting co-chaperone/substrate proteins without being catalytically active. This would result in blocking the access of the endogenous *Pf*Hsp70-1/Hsp40 to their respective substrates, rendering their cellular function silent. Similar work was carried out in *E. coli* by introducing a dominant negative mutation in the DnaK, resulting in visible phenotypic outcome (Buchberger et al., 1994). This strategy has been extensively been adapted to study the function of a number of protein families. Nevertheless, it is most applicable for proteins that require interaction with several ligands within the cell to form protein multimers to be functional (Sheppard, 1994). This approach has been established in *Toxoplasma gondii* (Herm-Gotz et al., 2007, Kremer et al., 2013) and also in *Plasmodium falciparum* (Jain et al., 2013, Bongfen et al., 2012, DeSimone et al., 2009) to study the function of several genes.

We further combined this approach with destabilization domain strategy. In this system the protein of interests (POIs) are further fused to destabilisation domains. There are several dds reported till date (dd-FKBP, dd-ecDHFR), (Armstrong and Goldberg, 2007, Dvorin et al., 2010, Iwamoto et al., 2010), but their mode of action is somehow similar. These domains are unstable when expressed in the cell and target the whole fusion protein to proteosomal degradation pathway. Upon addition of a small cell permeable ligand (either shield-1 or trimethoprim (TMP) depending on the dd used) the protein is stabilised and can carry out its biological function (fig 1.13). Thus, a tuneable dose dependent regulation of the protein level is possible *in vivo*. Recently, the destabilization domain strategy has been successfully applied to assess the importance of a number of proteins in different organisms including *Trypanosoma cruzi, Leishmania major*, *Toxoplasma gondii, Plasmodium falciparum* and also in murine model (Ma et al., 2012, Madeira da Silva et al., 2009, Herm-Gotz et al., 2007, Jain et al., 2013, Dvorin et al., 2010, Armstrong and Goldberg, 2007, Iwamoto et al., 2010).

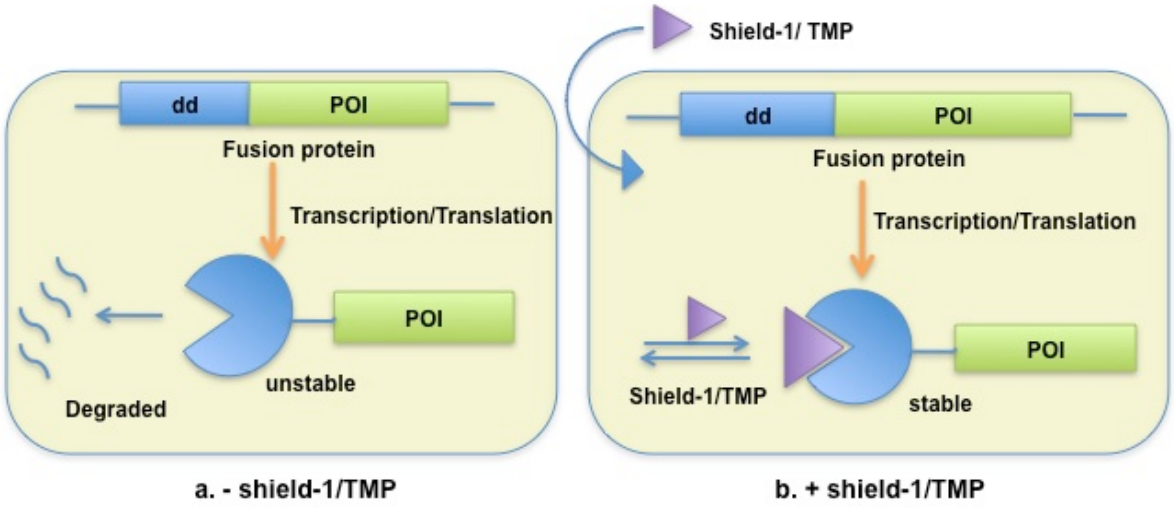

Figure 1.13 Schematic representation of dd based control of protein stability by shield-1/TMP.

(a) In absence of the ligand the fusion protein is unstable and rapidly degraded by the proteasome. b) Cell permeable ligand binds to the dd domain and stabilises the fusion protein that can carry out its function.

1.8. Objective

Plasmodium falciparum is the causative agent of malaria and responsible for the death of approximately 6 million people worldwide. It mostly affects children under five years of age. The severity of the disease is an outcome of the replication of the parasite in the human erythrocyte. Interestingly, research findings indicate an enhanced replication and infectivity of the parasite during the febrile temperature episodes, that is characteristic of malaria. The heat-mediated development is facilitated by molecular chaperones. This specialised group of heat shock proteins (Hsps) provide cytoprotection to the parasite during its diverse life cycle from mosquito to human. Hsps play essential role in maintaining protein homeostasis during stress and other cellular insults encountered in the host.

There is growing evidence that *Pf*Hsp70-1, a heat inducible, cytosolic chaperone is important for the intraerythrocytic survival of *Plasmodium*. In other organisms, Hsp70 works as a central chaperone and accomplish its role in tight collaboration with other co-chaperones like Hsp40 and Hsp90. Thus, existence of such a partnership cannot be overlooked in *Plasmodium*. But, till date there is very limited direct *in vivo* biochemical evidence available to prove this notion. In fact, our knowledge about the parasite specific function of *Pf*Hsp70-1 in the intraerythrocytic life cycle of *Plasmodium falciparum* is scarce.

Therefore, the aim of this project was to further elucidate the crucial parasite specific importance of *Pf*Hsp70-1. For this purpose, we wanted to generate *Pf*Hsp70-1 and *Pf*Hsp40 knockdown parasite lines to assess the contribution of these chaperones towards the intraerythrocytic life cycle of *P. falciparum*. We aimed to accomplish this by overexpression of the dominant negative versions of the candidate proteins, fused to ligand-controlled destabilisation domains (Armstrong and Goldberg, 2007, Iwamoto et al., 2010). By this approach we wanted to establish a tunable conditional knockdown of the endogenous wild type proteins by inhibiting their cellular functions. To our knowledge, this is the first attempt to use such a system to study Hsps in *P. falciparum*. The outcome would have helped us to define the essential role of *Pf*Hsp70-1 and *Pf*Hsp40 during the intraerythrocytic life cycle of the parasite. The findings would have finally contributed towards the validation of these candidate proteins as potential drug targets for malaria in the future.

2. Materials & Methods

2.1. Materials

2.1.1. Instrumentation

Instrument	Manufacturer
5810 R refrigerated centrifuge	Eppendorf
Agarose gel chambers	Gibco BRL
Analytical balance 2414	Sartorius
Autoclave (Varioklav)	Thermo scientific
Biofuge fresco centrifuge	Heraeus
Blotting chambers	Phase
Film developing cassette	Rego
Gel doc system	Intas
Gene pulser II	Biorad
Ice machine (AF-20)	Scotsman
Incubator B5060-EC	Heraeus
Incubator C16	Labotect
Shaking incubator (G25)	New Brunswick Scientific Co
Laminar flow Herasafe	Heraeus
Mikro 22R refrigerated centrifuge	Hettich
pH meter	Greisinger electronic GmbH
Power Pac basic power supply	Biorad
Precision balance 1205 MP	Satorius
Roller mixer	NeoLab
T-personal PCR cycler	Biometra
Thermoblock	Stuart Scientific
Thermomixer 5436	Eppendorf
Vista viosion microscope	VWR
Vortexer vortex-genie 2	Scientific industries
Waterbath	Köttermann

2.1.2. Consumables

Material	Provider
Centrifuge tubes PC	Beckman
Cryotubes	Sarstedt
Culture flasks	Greiner
Electroporation cuvettes	BioRad
SDS sample loading tips	VWR
Glass ware	Schott
Medical X-ray films RX NIF	Fuji
Microscope slides	VWR
Nitrocellulose membrane	Schleicher & Schuell
Pasteur pipettes	Brand GmBH
Petri dishes	VWR/ Greiner
Pipette tips	Sarstedt / Greiner
Plastic consumables	Sarstedt / Eppendorf / Greiner
Reaction tubes (0.2 ml, 0.5 ml, 1.5ml, 2.0 ml, 15 ml, 50 ml)	Sarstedt / Eppendorf
Whatman paper	Schleicher & Schuell

2.1.3. Chemicals / reagents

Chemicals	Provider
1kb^{+} DNA ladder	Invitrogen
1,4-Dithio-DL-threitol (DTT)	Roth
Agar	Roth
Agarose (molecular biology grade)	Eurogentec / Peqlab
Ammonium peroxodisulphate (APS)	Roth
Ampicillin	Roth
Bovine serum albumin fraction V (BSA)	PAA
Bromophenol blue	Amersham Biosciences
Calcium chloride	Roth
Chloroform	Roth
Citrate phosphate dextrose solution (CPD)	Sigma
Dimethyl sulphoxide (DMSO)	Roth
Deoxyribonucleotide Mix (peq Gold dNTP mix)	Peqlab

Ethanol p.a. (EtOH)	Roth
Ethidium bromide (EtBr)	Sigma
Ethylendiamintetra-acetic acis (EDTA)	Roth
Glacial acetic acid	Roth
Glycerol anhydrous	Applichem
Hoechst 3325	Molecular probes
Hydrochloric acid (37%)	Roth
Hydrogen peroxide (H_2O_2)	Merck
Isopropanol	Mercl
LB-agar (Lennox)	Roth
Luminol	Applichem
Magnesium chloride ($MgCl_2$)	Roth
Magnesium sulphate ($MgSO_4$)	Roth
Methanol (MetOH)	Roth
NNN′N-tetramethyleneethylenediamine (TEMED)	Roth
o-cresolsulfonephthalein (Cresol Red)	Sigma Aldrich
p-Coumaric acid	Roth
PageRuler Prestained Protein Ladder (10-170kDa) (Cat# 26616)	Thermo Scientific
Pepton	Roth
Phenylmethylsulfonylfluoride (PMSF)	Serva
Protease Inhibitor Cocktail Set III	Calbiochem
Rotiphorese ®Gel 30	Roth
Shield-1	Clontech
Skimmed milk powder	Roth
SOB – medium	Roth
Sodium acetate ($C_2H_3NaO_2$, NaOAc)	Roth
Sodium chloride (NaCl)	Roth
Sodium docecyl sulphate (SDS)	Applichem
Sodium hydroxide (NaOH)	Merck
Sucrose	Roth
Trimethioprim (TMP)	Sigma
Tris	Applichem

Water (sterile, pyrogen-free)	Roth
Yeast extract	Roth

2.1.4. Enzymes

Enzymes	**Provider**
Alkaline phosphatase, Calf intestinal (CIP)	New England Biolabs
DNase	Applichem
KOD Hot start DNA Polymerase	Novagen
RNase	Applichem
SuperScript^{TM}III one-step RT-PCR system	Invitrogen
T4 DNA Ligase	Invitrogen
Taq DNA polymerase	New England Biolabs

2.1.5. Molecular biology kits

Kits	**Provider**
Gel extraction kit	Seqlab
PCR purification kit	Seqlab
Peq GOLD plasmid miniprep kit I	Peqlab
Plasmid Maxi kit	Qiagen

2.1.6. Cell culture materials

Materials	**Provider**
AlbuMAXII	Invitrogen
Blasticidin S	Invitrogen
D-Sorbitol	Roth
Gelafundin	B. Braun
Gentamycin	PAA
Giemsa	Merck
Human erythrocyte concentrate A (Rh+) and O (Rh+)	Blood bank, University Hospital Marburg
Human Plasma A (Rh+)	Blood bank, University Hospital Marburg
Hypoxanthine	CC Pro
Neomycin solution	Sigma
RPMI 1640	Gibco

RPMI 1640	PAA
WR99210	Jacobus Pharmaceuticals

2.1.7. Antibodies

Primary antibodies	
Mouse α-GFP monoclonal	Roche Diagnostic
Mouse α-Glycophorin A/B monoclonal	Sigma Aldrich
Mouse α-*Pf*Hsp70-1 monoclonal	T. Blisnick, Pasteur Institute, Paris, France
Rabbit α-*Pf* Aldolase polyclonal	This working group
Rabbit α-HA polyclonal	Rockland
Rabbit α-FKBP12 polyclonal	Abcam
Secondary antibodies	
Goat α-mouse HRP	DAKO
Goat α-Rabbit HRP	DAKO

2.1.8. Cells and organisms

Strain	**Genotype**	**Reference**
***E. coli* TOP 10**	DH10B™	Invitrogen
***P. falciparum* clone 3D7**	The clone was obtained by limiting dilution of the original NF54 (Isolated near Schipol Airport, Amsterdam, Netherlands)	The Walter and Eliza Institute of Medical Research, Melbourne, Australia

2.1.9. Buffers and solutions

2.1.9.1. Used for protein work

Buffers and solutions	**Composition**
Ammonium peroxodisulphate (APS)	10% in ddH_2O
Blocking solution- IFA	3% bovine serum albumin in PBS pH7.4
Blocking solution-western blot	3% skim milk powder in PBS pH7.4

Developer (X-Ray film)	6.4 mM Metol 80 mM Hydroquinone 571 mM sodium sulfite (Na_2SO_3) 452 mM sodium carbonate (Na_2CO_3) 34 mM Potassium bromide (KBr)
ECL developing solution	5 mM luminol 0.8 mM p-coumaric acid 200 mM Tris-HCl pH8.5
Electrophoresis buffer (5X)	124 mM Tris 960 mM glycine 0.5 % SDS
Phosphate Buffer Saline (PBS)	140 mM Sodium chloride (NaCl) 2.7 mM Potassium chloride (KCl) 1.4 mM mono-potassium phosphate (KH_2PO_4) 0.8 M di-sodium phosphate (Na_2HPO_4)
SDS sample buffer (2X)	100 mM Tris/HCl pH6.8 5mM EDTA 20% Glycerol (v/v) 4% SDS 0.2 % bromophenol blue 100 mM DTT
Seperating buffer (4X)	1.5 mM Tris-HCl pH8.8 0.4% SDS (w/v)
Stacking buffer (4X)	500 mM Tris-HCl pH6.8 0.4% SDS (w/v)
Western blot transfer buffer	48 mM Tris-HCl pH9.5 39 mM glycine 0.04% SDS (w/v) 20 % MetOH (v/v)

2.1.9.2. Molecular biology solutions

Buffers and solutions	**Composition**
Colony mix (6ml)	1ml Cresol Red solution 600 µl reaction buffer (10X) 120 µl dNTPs (10mM) 4.28 ml sterile water
DNA extraction buffer A	100mM Sodium Chloride (NaCl) 50mM Sodium Acetate ($C_2H_3NaO_2$, NaOAc) 1 mM EDTA

DNA loading dye (6X)	1 % bromophenol blue 30% glycerol (v/v) 50 mM Tris-HCl pH8.0 5 mM EDTA
TAE (50X)	2 M Tris 2 M acetic acid 50 mM EDTA

2.1.9.3. Bacteriological media

Media	Composition
LB (Luria-Bertani) agar	35 gm / LB agar
SOC media	SOB medium 20 mM glucose
SOB medium	20 gm /L peptone 5 gm /L yeast extract 10mM sodium chloride (NaCl) 2.5mM potassium chloride (KCl) 20mM magnesium Chloride ($MgCl_2$)
Superbroth Medium, pH7.0	35 gm/ L tryptone 20 gm / L yeast extract 5 gm / L sodium chloride (NaCl) 5 ml / L sodium hydroxide (NaOH)

2.1.9.4. *Plasmodium* cell culture solutions

Blasticidin S Hydrochloride	Stock solution (10 mg /ml) was diluted to a working concentration (5 µg / ml)
Cytomix (100 ml)	6 ml 2M potassium chloride (KCl) 7.5 µl 2M calcium chloride ($CaCl_2$) 1 ml stock solution III 10 ml stock solution II 500 µl 1M magnesium Chloride ($MgCl_2$)
Cytomix stock solutions	I. 10M potassium hydroxide (KOH) II. 250 mM HEPES, 20mM EGTA, pH7.6 (5.96 gm/100 ml HEPES, 0.76 gm /100 ml EGTA, pH7.6 with stock solution I) III. 1 M phosphate buffer pH7.6 (8.66 ml 1M dipotassium phosphate, 1.34 ml 1M monopotassium phosphate)

Freezing solution	28 % glycerol (v/v) 3% d-sorbitol (w/v) 0.65 % sodium chloride (NaCl) (w/v)
Thawing solutions	I. 12 % sodium chloride (w/v) solution II. 1.6 % sodium chloride (w/v) solution III. 0.9 % sodium chloride (w/v) solution
WR 99210	20 mM stock (8.6 mgs/1ml DMSO) diluted to a working concentration of 20µM with RPMI 1640 Final working concentration 2.5nM

2.1.10. Basic Plasmids used in this study

Plasmid	Provider
pARL2-GFP mut2	This working group
pARL-BSD	This working group
pCHD-3/4-Hsp86-FKBP-YFP	Prof. Daniel E. Goldberg, (Armstrong and Goldberg, 2007)
pT8-dd-Myc*Tg*MLC1-HX	Prof. D. Soldati-Fabre
pJDD41	Prof. Thomas J Wandless

2.1.11. Synthetic oligonucleotides

All oligonucleotides were synthesized by Europhins MWG operon. Restriction sites are underlined.

Vector Primers		
pARL_F	5	CGT TAA TAA TAA ATA CAC GCA G
pARL_R	5	CAG TTA TAA ATA CAA TCA ATT GG
GFP+54_R	5	GTG CCC ATT AAC ATC ACC ATC

Primers to amplify the wild type (WT) and dominant negative (DN) *Pf*Hsp70-1		
Hsp70_F	5	CC GCG CGC ATG GCT AGT GCA AAA GGT TCA AAA C
Hsp70_R	5	CC CCC GGG TTA ATC AAC TTC TTC AAC TGT TGG TC

Hsp70_E187K_F	5	GAG AAT TAT TAA TAA ACC TAC TGC AGC TGC
Hsp70_E187K_R	5	GTA GGT TTA TTA ATA ATT CTC ATA AC
Hsp70_TA_F	5	CC GCG CGC TA ATG GCT AGT GCA AAA GGT TCA AAA C
Hsp70_323_R	5	CCT GAT TTA ACA GTG AAT GGC C
Hsp70_878_R	5	GCT TGT GTA GAT GAT GAT AAA GTA CG
Hsp70_1508_R	5	GCC GTA ACG TTT AAG ATA CCG

Primers to amplify the WT and DN *Pf*Hsp40		
Hsp40_F	5	CC GCG CGC ATG GGG AAG GAT TAT TAT TCA ATA TTA GG
Hsp40_R	5	CC CCC GGG TTA GAA TGT ATT TGC CAA TGT TTC TC
HSP40_D34N_F	5	GAT GTG GCA TCC TAA TAA ACA TAA TGA C
HSP40_D34N_R	5	GAT TTC TCG TCA TTA TGT TTA TTA GGA TGC
Hsp40_ AvrII_F_P	5	CC CCT AGG ATG GGG AAG GAT TAT TAT TCA ATA TTA GG
Hsp40_BssHII_R_2	5	CC GCG CGC GAA TGT ATT TGC CAA TGT TTC TC

Primers to amplify 3XHA		
3XHA_F	5	CC CTC GAG ATG TAC CCG TAC GAC GTC C
3XHA_R	5	CC CCT AGG GAG CTC GGC ATA ATC TGG AAG

Primers to amplify GFP		
GFP_AvrII_F	5	CC CCT AGG ATG AGT AAA GGA GAA GAA CTT TTC
GFP_BssHII_R	5	CC GCG CGC TTT GTA TAG TTC ATC CAT GCC
GFP_BssHII_TA_F_P	5	CC GCG CGC TA ATG AGT AAA GGA GAA GAA CTT TTC
GFP_XmaI_R_P	5	CC CCC GGG TTT GTA TAG TTC ATC CAT GCC

Primers to amplify dd-FKBP		
FKBP_F	5	CC CCT AGG ATG ACT GCA GGA GTG CAG GTG
FKBP_R	5	CC GCG CGC TTC CGG TTT TAG AAG CTC CAC ATC

Primers to amplify ecDHFR		
ecDHFR_AvrII _F_P	5	CC CCT AGG ATC AGT CTG ATT GCG GCG TTA GC
ecDHFR_BssHII_R_P	5	CC GCG CGC GCA ATA GCT GTG AGA GTT CTG C
ecDHFR_BssHII_F_P	5	CC GCG CGC ATC AGT CTG ATT GCG GCG TTA GC
ecDHFR_XmaI_R_P	5	CC CCC GGG GCA ATA GCT GTG AGA GTT CTG C

Primers to amplify DD29		
DD29_AvrII_F_P	5	CC CCT AGG ATG GGA GTG CAG GTG G
DD29_BssHII_R_P	5	CC GCG CGC TCA TTC CAG TTT TAG AAG CTC
DD29_BssHII_F_P	5	CC GCG CGC ATG GGA GTG CAG GTG G
DD29_KpnI_R_P	5	CC GGT ACC TCA TTC CAG TTT TAG AAG CTC

Primers for the cloning of *Pf*Hsp70-1 native promoter		
Hsp70_5'_F1	5	GATGCTATTAAAAGCGGAATATTTTAC
Hsp70_5'_R1	5	GGTTTTGAACCTTTTGCACTAGCC
Hsp70_5'_NotI_F2	5	CC GC GGCC GC TTT ATA TAT ATA CGC AAA ATT ATG AAA ATC CAC
Hsp70_5'_XhoI_R2	5	GG CTC GAG TTT TTC TTA ATT CTT TTG

2.1.12. Softwares and databases

Software	Provider
PlasmoDB	http://plasmodb.org/plasmo/ PlasmoDB: A genomic for *Plasmodium falciparum* (version 9.1)
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/ (New England BioLabs)
Oligoproperties	http://www.basic.northwestern.edu/biotools/oligocalc.html
Blast	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Clustlw	http://www.ebi.ac.uk/Tools/msa/clustalw2/
4peaks	Mekentosj.com

2.2. Methods

2.2.1. Molecular biology techniques

2.2.1.1. DNA based techniques

2.2.1.1.2. Quantification of DNA samples

Concentration of DNA was measured using NanoDrop (NanoDrop Technologies, Wilmigton, USA). For this 1 µl of DNA was loaded onto the nanodrop and the quantification was done following manufacturers protocol. The ratio of absorbancse at 260 nm and 280 nm was calculated to assess the purity of DNA (~ 1.8 for pure DNA). TE or dH_2O served as blank.

2.2.1.1.3. *In vitro* amplification of DNA and cDNA

DNA templates were amplified *in vitro,* either from genomic DNA or from pre-existing plasmid by Polymerase Chain Reaction (PCR) (Mullis et al., 1986). For the *in vitro* amplification of the DNA template, either *Taq* polymerase or Hot start KOD polymerase were used following the manufacturer's protocol. Amplification of cDNA was carried out by Reverse Transcription PCR (RT-PCR) using ribonucleic acid (RNA) as template. First one step RT-PCR was performed using Superscript III reverse transcriptase kit (Invitrogen) as per manufacturer's instruction. Further standard PCR was carried out using the resulting cDNA as a template to achieve double stranded DNA. The reaction set up and cycle condition for

the PCR was optimized depending on the length of the DNA to be amplified and the melting temperatures of the primers.

2.2.1.1.3.1. Standard PCR reaction setup

Reagents & buffers	Amount
Template DNA	1.0 µl (taken directly from the genomic or plasmid DNA stock)
Forward primer (50 pmol/ µl)	1.0 µl (final concentration 1.0 pmol/µl)
Reverse primer (50 pmol/ µl)	1.0 µl (final concentration 1.0 pmol/µl)
KOD Hot Start DNA Polymerase (1U/µl)	1.0 µl (1U)
10X PCR Buffer for KOD Hot Start DNA Polymerase	5.0 µl (final concentration 1X)
dNTPs (10 mM each)	5.0 µl (final concentration 0.2 mM)
$MgSO_4$ (25 mM)	3.0 µl (final concentration 1.5 mM)
PCR Grade Water	33.0 µl
Total Volume	**50.0 µl**

2.2.1.1.3.2. Standard colony PCR cycling conditions

Initial denaturation	94°C	5 minutes
35 cycles	94°C 50°C 68°C	30 seconds 50 seconds 30 seconds per kb
Final extension	68°C	10 minutes
Storage	4°C	Hold

2.2.1.1.4. Generation of dominant negative mutations

2.2.1.1.4.1. Dominant negative mutation of *Pf*Hsp70-1

To generate dominant negative version of *Pf*Hsp70-1 (PF3D7_0818900), we replaced the Glutamic acid (E) at amino acid position 187 with a lysine (K) residue by the method of overlapping extension PCR as explained under section 2.2.1.1.5. The E187K mutation should inhibit the coupling of the substrate binding with the ATPase activity of the *Pf*Hsp70-1. Although the mutated protein binds and occupies the possible substrates of the endogenous

*Pf*Hsp70-1, cannot accomplish its function in the cell, and is therefore dominant negative. It has already been shown in *E. coli* that E171 is essential for the coupling of substrate binding and ATPase activity of DnaK (Hsp70 homologue in *E. coli*). Mutation to K (Lysine) compromises the chaperone function of DnaK, (Buchberger et al., 1994). Alignment of the amino acid sequences of DnaK and *Pf*Hsp70-1 was done (fig S.1).

2.2.1.1.4.2. Dominant negative mutation of *Pf*Hsp40

The highly conserved HPD motif in the J domain of *Pf*Hsp40 (PF3D7_0213100) was mutated to the HPN (D34N) motif. It is well known that the conservation of the HPD motif is highly important for the interaction of the Hsp40 with Hsp70. Mutation of this domain abolishes the interaction with Hsp70, thereby inhibiting the stimulation of its ATPase activity by Hsp40 (Cheetham and Caplan, 1998, Hennessy et al., 2000). It has been experimentally shown that DnaJ (Hsp40 homologue in *E. coli*) with aspartic acid (D) in the HPD motif mutated to Asparagine (N) could no longer bind to DnaK with comparable affinity between wild type DnaJ and DnaK. Therefore, confirmed that such mutation disrupts the train for a functional Hsp70-Hsp40 interaction (Hennessy et al., 2005). During this work the desired mutation was incorporated into the HPD motif of *Pf*Hsp40 by the method of overlapping extension PCR as explained under section 2.2.1.1.5.

2.2.1.1.5. Overlapping Extension PCR

Overlapping extension PCR method was used to incorporate point mutation into a DNA template. The DNA template was first amplified in two parts using two different sets of primers. The 3′ fragment of the DNA was amplified using two primers; one annealing to the extreme 3′ end and other annealing at the middle of the template DNA (fig 2.1c). Whereas, the 5′ fragment of the DNA was amplified using another set of primers; one annealing at the middle and the other annealing at the extreme 5′ end of the template DNA (fig 2.1b). Both the primers that annealed to the opposite strands at the middle of the template in these two individual PCR reactions share few complimentary nucleotide bases therefore complimentary to each other. Among the complimentary region one nucleotide base is altered in the primer compared to that of the original template (fig 2.1f). This nucleotide base was altered depending on the mutation required, and will be incorporated in the resulting final PCR product. Mutagenesis in a nucleotide base will alter the codon that will eventually end up in coding for a different amino acid. After the PCR reaction both the resulting 3′ and 5′ fragments were purified by gel extraction and were used together as templates for the final

overlapping extension PCR (fig 2.1d and fig 2.1e). In the denaturation step of the overlapping extension PCR both the strands from each template denature. The single strand resulting from both the 3′ and 5′ fragments have a complimentary region as mentioned earlier. Therefore the strand anneal to each other utilizing the complimentary overhangs (fig. 2.1f). Furthermore, polymerase adds on dNTP to complete the strands towards the 3′ and 5′ ends and results in the full length double stranded DNA. Once the double stranded DNA is formed the PCR cycle progresses as normal, amplifying copies of the DNA carrying the desired mutation (fig 2.1g).

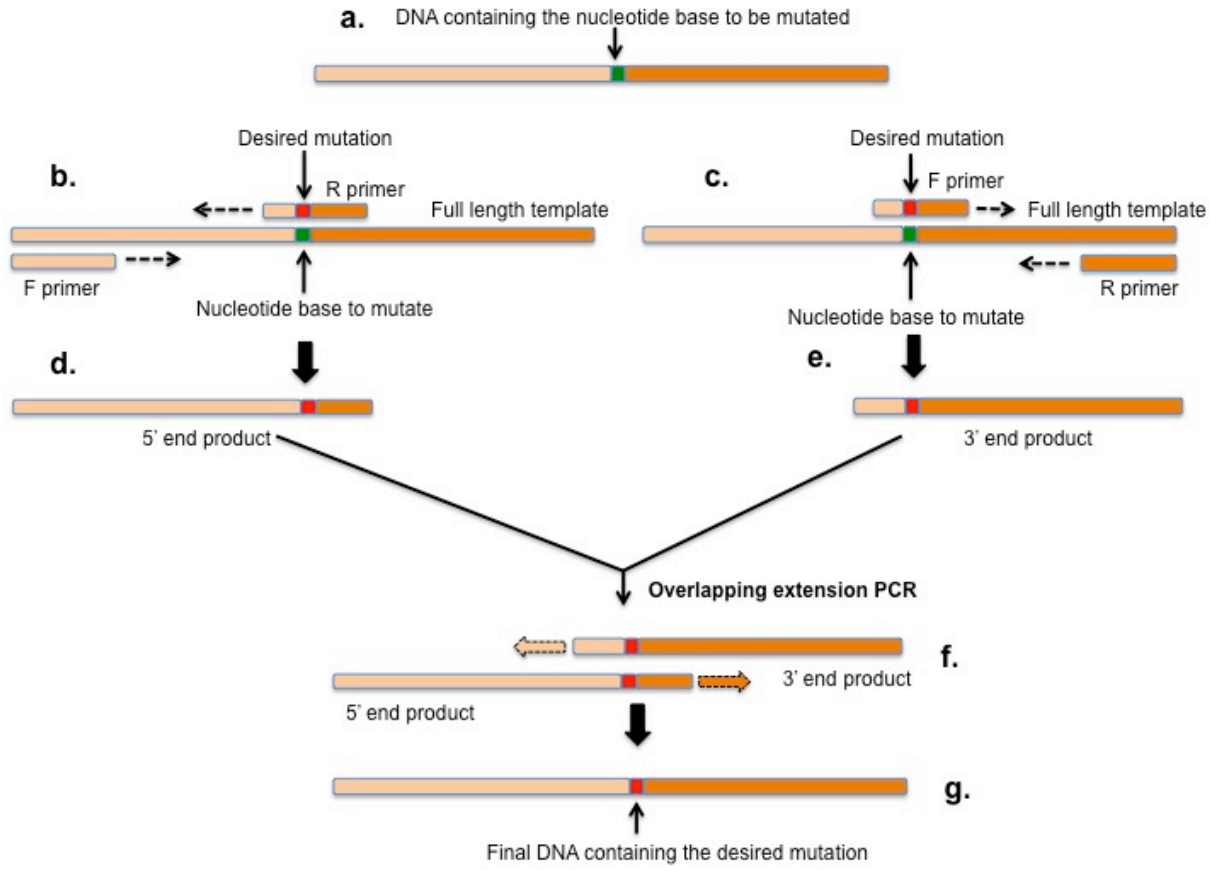

Figure 2.1 Schematic illustration of overlapping extension PCR.

(a) The wild type template containing the base to be mutated (shown in green). (b) PCR for the amplification of the 5' part of the DNA. The reverse primer binding in the middle of the template contains the desired mutation (shown in red). (c) PCR for the amplification of the 3' part of the DNA. The forward primer binding in the middle of the template contains the desired mutation (shown in red). (d) Amplified 5' part of the DNA containing the desired mutation (shown in red). (e) Amplified 3' part of the DNA containing the desired mutation (shown in red). (f) Overlapping extension PCR, where both 5' and 3' products obtained from individual PCR are mixed. After initial denaturation both the templates anneal to each other using the complementary regions. DNA polymerase fills the gap on both direction and completes the formation of the full

length DNA molecule. Finally, 5' forward and 3' reverse primers amplify the DNA template containing the desired mutation. (g) Final product carrying the desired mutation (shown in green).

2.2.1.1.6. Agarose gel electrophoresis for the separation of nucleic acid

DNA and RNA molecules were separated depending on their respected sizes via agarose gel electrophoresis as described by Sambrook *et al.,* (2001) (Sambrook J, 2001) . Concentration of the agarose gels varied from 0.8-1.5% depending on the size of the nucleic acids to be separated. For larger DNA or RNA lower percentage of agarose was used allowing the molecule to pass through the relatively bigger pores in the agarose matrix. Whereas, for much smaller nucleic acids high percentage of agarose was used leading to the formation of small pore size in the matrix that serves as better sieve for the small molecules. Agarose gels were prepared by dissolving the appropriate amount of agarose in 1XTAE buffer. Ethidium Bromide (EtBr) was added to a final concentration of 0.3ng/ml to the melted agarose. Ethidium bromide is extensively used to make DNA or RNA molecule visible. It intercalates into the major groove of nucleic acids and fluoresces when the samples are exposed to the UV light. Once the gel was casted samples were mixed with 6X loading dye to a 1X final concentration and were loaded onto the wells followed by electrophoresis at 90V in 1X TAE buffer. $1KB^{+}$ DNA ladder (Invitrogen) was loaded alongside for size determination of the separated bands resulting from the samples. Images were captured using the IX Imager (INTAS, Gottingen).

2.2.1.1.7. Extraction and purification of DNA

To purify DNA from contaminants like enzyme and buffers, the bands were carefully excised from the agarose gel post electrophoresis. Further, the DNA was extracted and purified from the gel using Gel Extraction Kit (Seqlab) following the manufacturer's protocol. After purification the DNA was eluted from the column using appropriate amount of TE or autoclaved dH_2O. After the PCR, if a single specific DNA band was observed in the gel, the product was directly purified from the reaction mixture using PCR purification kit (Seqlab) and was eluted with appropriate amount of TE or autoclaved dH_2O.

2.2.1.1.8. Ethanol precipitation of DNA

Ethanol precipitation was carried out to purify and concentrate DNA samples. Therefore, the DNA samples were mixed with 0.1 volume of 3M NaAc (pH5.2) and 3 volume of 100 % EtOH following storage at -20 °C for the duration of 1 hour to overnight for better precipitation. The following day the samples were centrifuged at 13000 rpm for 30 minutes at

4 °C. Following centrifugation the supernatant was carefully discarded and the pellet was washed with 500 µl of 70% EtHO followed by another centrifugation at 13000 rpm for 10 minutes at 4 °C. The resulting pellet was air dried and the DNA was redissolved in appropriate volume of TE or autoclaved dH_2O.

2.2.1.1.9. Restriction digestion of DNA

Digestion of DNA was carried out using specific restriction endonucleases. Restriction endonucleases recognise specific cleavage sites on the DNA, also known as restriction sites. The size of the restriction sites usually varies from 4-8 nucleotide bases depending on individual enzyme. Digestion of templates or plasmid DNAs were carried out using 10U of enzymes/~ 0.2-3.0ng of DNA in an appropriate buffer as recommended by the manufacturer. The optimum temperature for the reaction was determined depending on the enzyme used. Usually, 2 hours were allowed for the digestion of linear DNA molecules, whereas 6-8 hours were allowed plasmids. Two different restriction enzymes were selected for digestion of individual template and plasmid DNA. Primers to amplify these templates were designed in such a way that they contained these restriction sites and therefore the restriction sites were incorporated at the 5′ and 3′ ends of the resulting DNA. Use of two different restriction sites guided orientation specific ligation of these inserts into the multiple cloning sites (MCS) of the plasmids. In order to avoid re-circularization of the plasmid DNA, the digested vectors were treated with 1µl of alkaline phosphatase (CIP) to remove the terminal phosphate groups at the 5′ end of the linearized plasmids.

2.2.1.1.10. Ligation

Prior to ligation insert and vector were digested using compatible restriction enzymes in the appropriate buffer and temperature. Although, a molar ratio of 3:1 (insert: plasmid) was used to set up ligation, some exception were done and the molar ratio was adjusted according to the size, concentration and quality of the purified products (insert and vector). Usually, ligation was carried out in a total volume of 30 µl with 1U of T4 DNA ligase in a suitable buffer as recommended by the manufacturer. Ligation was set up overnight at 16 °C water bath. Negative controls were set up alongside using two different ligation reactions; one without the insert and the other without the T4 ligase. Following the overnight ligation the DNA from each reaction was precipitated using 100% EtOH and 3M NaAc as explained earlier under section 2.2.1.1.8. The precipitated DNA was re-dissolved in 10 µl of water and was used for transformation into electrocompetent *E. coli* cells.

2.2.1.1.11. Plasmid preparation

Extraction and purification of plasmid DNA was carried out using Mini or Midiprep kit (Seqlab, Qiagen) according to the manufacturer's protocol. Maxi preparation was done with an initial bacterial culture of 400 ml, whereas for miniprep initial volume of culture was 3-6 ml. The protocol for the maxi preparation was slightly optimized. Basic principle of the extraction and purification of plasmid DNA underlines the alkaline lysis of bacteria using ionic detergent SDS in the lysis buffer (buffer II in the miniprep kit). SDS with a high pH lyses the bacteria and denatures proteins and contaminating chromosomal DNA. Plasmid DNA is also released as a result of lysis, but is non-separable because of its circular nature. Plasmid DNA binds to the ion exchange resin of the column provided with the kit. The column was washed several times with wash buffer following elution of the bound DNA with high salt buffer. The eluted DNA was precipitated using isopropanol and was further washed with 70% ethanol. After the final ethanol wash the DNA pellet was air-dried and re dissolved in 1.5 ml of TE buffer. In contrast, during mini preparation the DNA binds to silica matrix in the column in the presence of high salt buffer. After several washes the bound plasmid DNA was eluted with appropriate amount of TE buffer.

2.2.1.1.12. Sequencing of DNA

After purification, plasmid DNA samples were sequenced by Seqlab to ensure correct ligation and also absence of any mutation in the coding nucleotide regions. For this purpose 600-700ng plasmid DNA mixed with 200 pmol appropriate primer was sent to Seqlab. For each construct one set of primers (forward and reverse primer) was used: one binding to the insert and the other to the vector. Sometimes depending on the length of DNA multiple primers were used to cover the entire length of the insert DNA. After obtaining the sequencing report from Seqlab the sequence was aligned with the original sequence using EMBL-EBL ClustalW2 online software.

2.2.1.2. Bacteriological methods

2.2.1.2.1. Preparation of electrocompetent *E. coli* cells

TOP 10 cells were inoculated from the stock into 10 ml superbroth and were allowed to grow overnight at 37 º C shaking incubator. The following morning 6 ml from the overnight culture was added to 600 ml of superbroth and was allowed to grow for another 4 hours and 30 minutes in the 37 º C shaking incubator. Following incubation, the culture was cooled down

on ice and was centrifuged at 6000 rpm for 10 minutes. The pellet was washed three times with 600 ml of autoclaved ddH_2O and three times with 600 ml of 10 % glycerol (v/v in ddH_2O). Centrifugation between each wash was carried out at 6000 RPM for 15 minutes at 4 º C. After the final wash the pellet obtained from 600 ml initial culture was dissolved in 1.5 ml of 10 % glycerol (v/v in ddH_2O) on ice in the cold room. The cell suspension was aliquoted in several 1.5 ml eppendorf tubes (100 µl in each) and shock frozen in liquid nitrogen. The cells were finally stored at -80 º C for future use.

2.2.1.2.2. Liquid and solid bacteria cultures

Electrocompetent *E. coli* TOP10 cells transformed with plasmid DNA were grown in super broth containing appropriate antibiotic at 37°C shaking incubator overnight. For plating the bacterial culture on solid media, LB agar (Roth) was made with appropriate antibiotics.

2.2.1.2.3. Transformation of *E. coli*

For transformation of plasmid DNA electrocompetent *E. coli* Top10 cells were used unless stated otherwise. As mentioned earlier, the DNA from ligation was precipitated and was redissolved in 10 µl of dH_2O. For retransformation of plasmid DNA 3-10 µl of DNA was used depending on the concentration of the samples. The DNA was mixed well with 50µl of TOP10 cells on ice and immediately transferred to chilled electroporation cuvettes. Electroporation was carried out at 2kV, 25µF and 200Ω. Immediately after electroporation the cells were mixed with 1ml of prewarmed SOC medium following incubation for 45 minutes at 37°C shaking incubator. The cultures were then plated on several dilutions ranging from 100 µl to 900 µl on LB agar plate containing appropriate antibiotics. The plates were further transferred to 37°C incubator and incubated overnight for the growth of bacterial colonies.

2.2.1.2.4. Colony PCR

Since blue-white selection using LacZ could not be used to identify the recombinant bacterial colonies, colony PCR method was adapted. After allowing overnight incubation of the bacterial agar plate at 37°C, single colonies were picked up from the plate and were first streaked on a prewarmed LB agar replica plate (numbered at the back). The same colony was further inoculated in 25µl colony mixture. The colony mixture contained appropriate primer pair along with premixed *Taq* polymerase, *Taq* reaction buffer (including Mg), dNTPs and loading dye. The duration of the extension step of the PCR was adjusted according to the

length of the template to be amplified. Following colony PCR samples were loaded on agarose gel to detect the amplified DNA product of the expected size in order to confirm successful cloning of the insert into the vector.

2.2.1.2.4.1. Standard colony PCR reaction setup

Reagents & buffers	Amount
Colony Mix	23.5 µl
Forward primer	0.5 µl (final concentration 1.0 pmol/µl)
Reverse primer	0.5 µl (final concentration 1.0 pmol/µl)
Taq polymerase	0.5 µl
Colony	Inoculate with a pipette tip
Final volume	**25 µl**

2.2.1.2.4.2. Standard colony PCR cycling conditions

Initial denaturation	94°C	5 minutes
35 cycles	94°C 50°C 72°C	30 seconds 50 seconds 60 seconds per kb
Final extension	72°C	10 minute
Storage	4°C	Hold

2.2.2. Generation of transfection plasmids for this study

2.2.2.1. Generation of dd-FKBP based constructs (Armstrong and Goldberg, 2007)

2.2.2.1.1. Generation of *Pf*Hsp70-1 (WT/DN) and GFP control constructs

The coding sequence of the WT *Pf*Hsp70-1 (PF3D7_0818900) was amplified using Hsp70_F and Hsp70_R primers from 3D7 genomic DNA for the generation of the wild type Hsp70-1 construct. Whereas, for the generation of the dominant negative constructs DN*Pf*Hsp70-1 insert was amplified using Hsp70-1_F, Hsp70_E187K_R, Hsp70_E187K_F and Hsp70_R, primers by the method of overlapping extension PCR using 3D7 genomic DNA as the initial template as explained earlier under section 2.2.1.1.5. Following successful amplification and purification, the inserts were digested by BssHII and XmaI restriction enzymes. Digested and

purified fragments were cloned into pARL2-GFP vector pre-digested with BssHII and XmaI. Plasmids resulting from this ligation were amplified by miniprep and confirmed by test digestion and sequencing. The same plasmids were further used to subclone the dd-FKBP or control GFP on the N-terminus of the *Pf*Hsp70-1 (WT/DN) ORFs between AvrII and BssHII restriction sites. For this reason the ORF of FKBP was amplified from pre-existing plasmid (contained the FKBP insert) as template by FKBP_F and FKBP_R primers. The control GFP template was amplified from pARL2-GFP plasmid using GFP_AvrII_F_P and GFP_BssHII_R_P. Following PCR amplification and purification, both the FKBP and GFP templates were digested with AvrII and BssHII restriction enzymes. Purified templates were cloned between AvrII and BssHII sites into the predigested plasmids (already containing the WT/DN *Pf*Hsp70-1 insert). The constructs resulting from this ligation were amplified by miniprep and confirmed by test digestion and sequencing. The generated constructs were used to clone the 3XHA tag at the N-terminus of FKBP or GFP between XhoI and AvrII restriction sites. The 3XHA tag was amplified using a pre-existing plasmid (contained the 3XHA tag) as template by 3XHA_F and 3XHA_R primers. Purified templates were cloned between XhoI and AvrII sites into the predigested plasmids (already containing the FKBP/GFP and WT/DN *Pf*Hsp70-1 inserts). The final transfection construct resulting from this ligation were amplified by miniprep and were confirmed by test digestion and sequencing. Finally, plasmids were amplified by maxiprep and were used for transfection. All the resulting plasmids are schematically illustrated in fig 2.2.

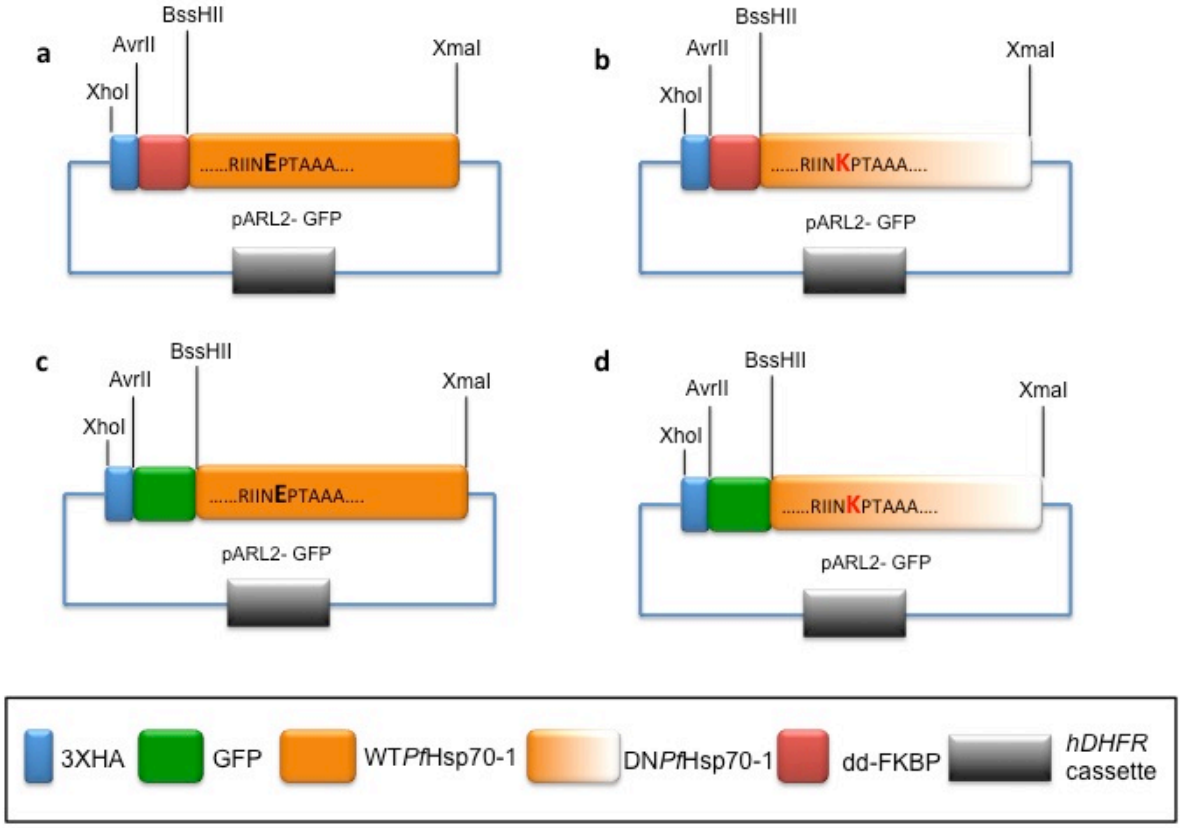

Figure 2.2 Schematic illustration of dd-FKBP based WT/DN *Pf*Hsp70-1 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp70-1 fussed to the dd-FKBP domain at the N-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp70-1 (E187K) fussed to dd-FKBP at the N-terminus. (c) Construct coding for WT*Pf*Hsp70-1, where the dd-FKBP at the N-terminus is replaced with control GFP. (d) Construct coding for DN*Pf*Hsp70-1, where dd-FKBP at the N-terminus is replaced with control GFP. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of detection of the fusion proteins. All constructs were generated using pARL2-GFP plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.1.2 Generation of *Pf*Hsp40 (WT/DN) and GFP control constructs

The Hsp40 (PF3D7_0213100) gene contains several introns. Therefore, reverse transcriptase PCR was carried out using RNA. The coding region of the WT*Pf*Hsp40 was amplified using Hsp40_F and Hsp40_R primers from the cDNA. On the other hand, for the generation of dominant negative *Pf*Hsp40 constructs, the DN*Pf*Hsp40 insert was amplified using Hsp40_F, Hsp40_D34N_R, Hsp40_D34N_F and Hsp40_R primers by the method of overlapping extension PCR using wild type *Pf*Hsp40 as a template as detailed earlier under section 2.2.1.1.5. Following successful amplification and purification of the templates, digestions were carried out using BssHII and XmaI restriction enzymes. Digested and purified fragments were cloned into pARL2-GFP vector predigested with BssHII and XmaI. Constructs resulting from this ligation were amplified by miniprep and confirmed by test

digestion and sequencing. The construct was further used to subclone the dd-FKBP or control GFP on the N-terminus of the *Pf*Hsp40s (WT/DN) between AvrII and BssHII restriction sites. For this reason the ORF of FKBP was amplified from pre-existing plasmid (contained the FKBP insert) as template by FKBP_F and FKBP_R primers. The control GFP template was amplified from pARL-2GFP plasmid using GFP_AvrII_F_P and GFP_BssHII_R_P. Following PCR amplification and purification, both the FKBP and GFP templates were digested with AvrII and BssHII restriction enzymes. Purified templates were then cloned between AvrII and BssHII sites into the predigested plasmid (already containing the WT/DN *Pf*Hsp40 inserts). The constructs resulting from this ligation were amplified by miniprep and confirmed by test digestion and sequencing. The generated constructs were further used to clone the 3XHA tag at the N-terminus of FKBP or GFP between XhoI and AvrII restriction sites. The 3XHA tag was amplified from pre-existing plasmid (contained the 3XHA tag) as template by 3XHA_F and 3XHA_R primers. Purified templates were cloned between XhoI and AvrII sites into the pre-digested plasmid (already containing the FKBP/GFP and WT/DN *Pf*Hsp40 inserts). The final transfection constructs resulting from this ligation were amplified by miniprep and were confirmed by test digestion and sequencing. After confirmation plasmid DNAs were amplified by maxi preparation and used for transfection. All the resulting plasmids are schematically illustrated in fig 2.3.

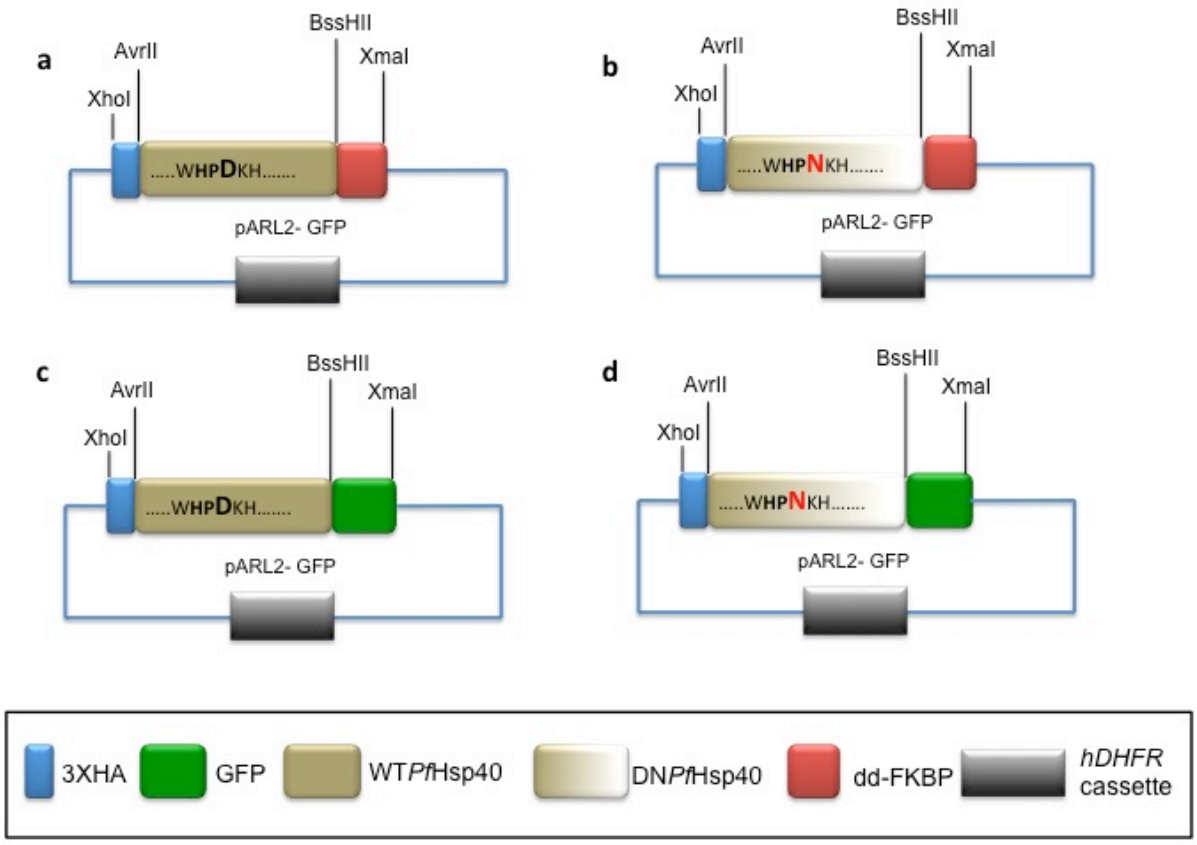

Figure 2.3 Schematic illustration of dd-FKBP based WT/DN *Pf*Hsp40 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp40 fussed to the dd-FKBP domain at the C-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp40 (D34N) fussed to dd-FKBP at the C-terminus. (c) Construct coding for WT *Pf*Hsp40, where the C-terminus dd-FKBP is replaced with control GFP. (d) Construct coding for DN *Pf*Hsp40, where dd-FKBP at the C-terminus is replaced with control GFP. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of detection of the fusion proteins. All constructs were generated using pARL2-GFP plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.2. Generation of dd-ecDHFR based constructs (Iwamoto et al., 2010)

2.2.2.2.1. Generation of *Pf*Hsp70-1 (WT/DN) and GFP control constructs

For the generation of the dd-ecDHFR based constructs, the 3XHA tag was amplified from a pre-existing plasmid (contained the 3XHA tag) as a template using 3XHA_F and 3XHA_R primers. Digested and purified 3XHA tag was cloned between XhoI and AvrII sites into the predigested pARL-BSD plasmid. The construct resulting from this ligation was amplified by miniprep and confirmed by test digestion and sequencing. The resulting plasmid containing the 3XHA tag was further used for further cloning of dd-ecDHFR. Therefore, ORF of dd-ecDHFR was amplified by ecDHFR_AvrII_F_P and ecDHFR_BssHII_R_P from a plasmid containing the dd-ecDHFR domain kindly provided by Prof Thomas J Wandless. After amplification the dd-ecDHFR insert was digested with AvrII and BssHII, following

purification and cloning into pARL-BSD (containing the HA tag) predigested with the compatible restriction endonucleases. The resulting constructs were amplified by miniprep and was confirmed by test digestion and sequencing. The sequencing revealed two bases missing at the end of the dd-ecDHFR insert. Since the template ends with a pair of 'GC' and followed by a 'GCGCGC' (BssHII restriction site), BssHII recognised GC of the template as the start of the restriction site and the other GC at the middle of the actual restriction site as the end instead of the last GC of the BssHII site. As a consequence the resulting construct had a pair of 'GC' missing at the end. This ends up in shifting the reading frame of the multiple cloning sites. The inserts that needed to be cloned to the C-terminus of the dd-ecDHFR ORFs are GFP and WT/DN *Pf*Hsp70-1. Therefore, to solve this frame shift an extra 'TA' was planned to be incorporated between the BssHII restriction sites and start codon (ATG) of the following inserts. Therefore, the forward primers to amplify GFP or WT/DN *Pf*Hsp70 were designed accordingly to incorporate two extra nucleotide bases TA at the beginning of the ORF. WT/DN *Pf*Hsp70-1 was amplified from pre-existing construct (fig. 2.2a and fig. 2.2b) using Hsp70_TA_F and Hsp70_R primers. Likewise, the GFP fragment was amplified from a pre-existing construct (fig. 2.2c) using GFP_BssHII_TA_F and GFP_XmaI_R_P. After successful amplification, the inserts were digested with appropriate enzymes and were cloned between the BssHII and XmaI restriction sites of the pre-digested vector (containing 3XHA-dd-ecDHFR) for the generation of the final transfection construct. The resulting constructs were amplified by miniprep and was confirmed by restriction digestion and sequencing. Sequencing revealed that the ORF was back in frame by the successful insertion of the additional TA before the open reading frame (ORF) of the WT/DN *Pf*Hsp70-1 and GFP. These final constructs were further propagated by maxi preparation and were used for transfection. All the resulting plasmids are schematically illustrated in fig 2.4.

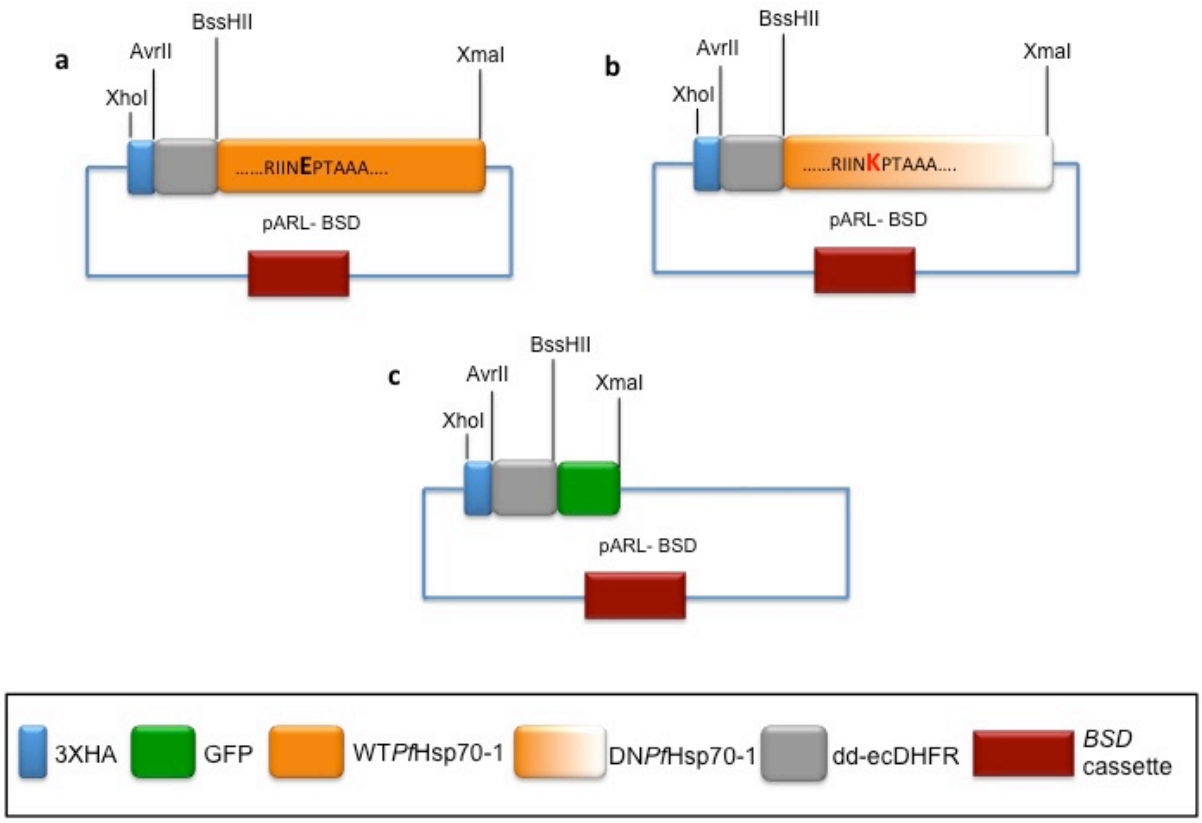

Figure 2.4 Schematic illustration of dd-ecDHFR based WT/DN *Pf*Hsp70-1 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp70-1 fussed to the dd-ecDHFR domain at the N-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp70-1 (E187K) fussed to dd-ecDHFR at the N-terminus. (c) Control construct coding for GFP, fussed to dd-ecDHFR at the N-terminus. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of the fusion proteins. All constructs were generated using pARL-BSD plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.2.2. Generation of *Pf*Hsp40 (WT/DN) and GFP control constructs

For the generation of the dd-ecDHFR based WT/DN *Pf*Hsp40 and control GFP construct WT/DN *Pf*Hsp40 ORFs were amplified from two pre-existing plasmids (fig. 2.3a and fig.2.3b) using Hsp40_AvrII_F_P and Hsp40_BssHII_R_P. Whereas, the ORF of the GFP was amplified from another construct (fig. 2.2c) using GFP_AvrII_F and GFP_BssHII_R primers. The amplified inserts were purified and digested with AvrII and BssHII restriction endonucleases. The digested and purified inserts were then cloned into digested pARL-BSD plasmid (containing the 3XHA between XhoI and AvrII) between AvrII and BssHII restriction sites. The constructs resulting from this ligation were amplified by miniprep and confirmed by test digestion and sequencing. Unlike the dd-ecDHFR based *Pf*Hsp70-1 constructs as mentioned earlier, the dd-ecDHFR was cloned to the C-terminus of the protein of interests (POIs) in these constructs. Therefore, dd-ecDHFR was amplified from a plasmid

(fig. 2.4a) using ecDHFR_BssHII_F_P and ecDHFR_XmaI_R_P. Following digestion with the appropriate enzymes and purification, the dd-ecDHFR insert was cloned between BssHII and XmaI restriction sites of the predigested pARL-BSD (containing the 3XHA-WT/DN*Pf*Hsp40 or GFP). The resulting constructs were amplified by miniprep and were confirmed by restriction digestion and sequencing. The resulting plasmids were multiplied and purified by maxi preparation and were used for transfection. All the resulting plasmids are schematically illustrated in fig 2.5.

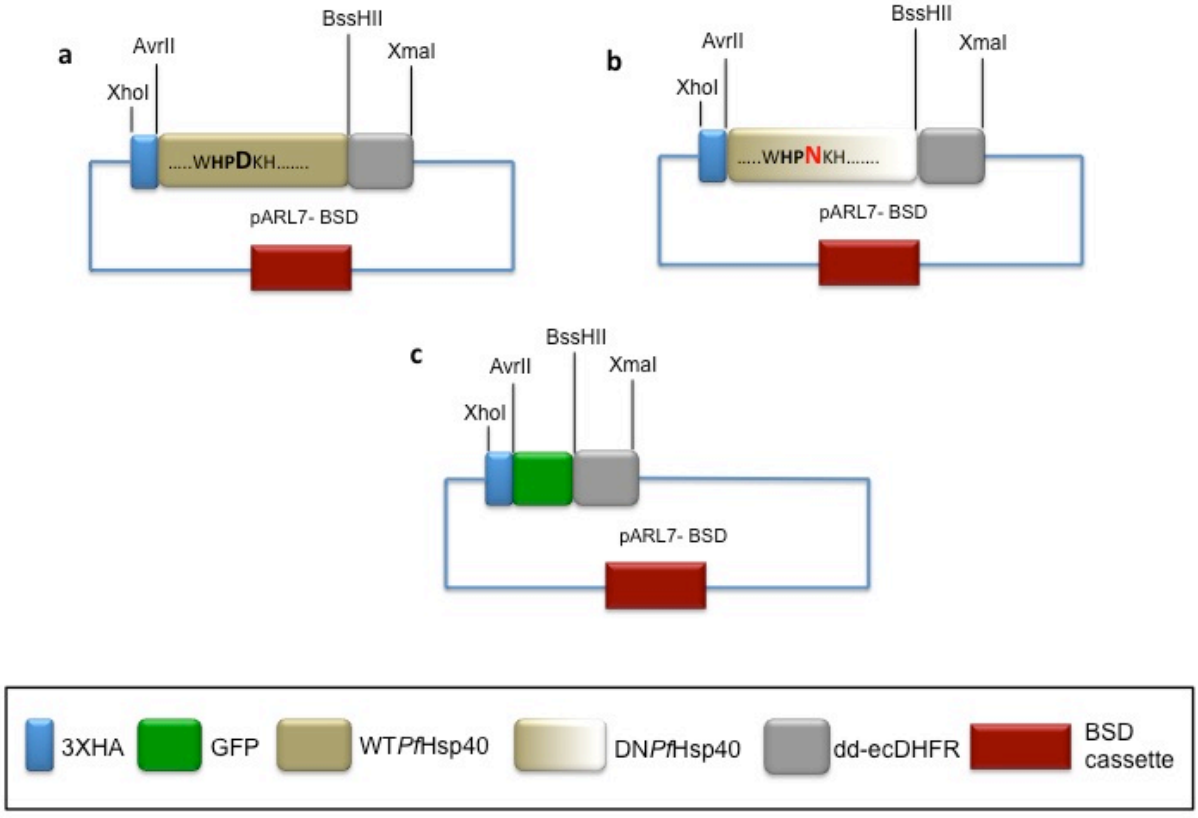

Figure 2.5 Schematic illustration of dd-ecDHFR based WT/DN *Pf*Hsp40 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp40 fussed to the dd-ecDHFR domain at the C-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp40 (D34N) fussed to dd-ecDHFR at the C-terminus. (c) Control construct coding for GFP, fussed to dd-ecDHFR at the C-terminus. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of detection of the fusion proteins. All constructs were generated using pARL-BSD plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.3. Generation of DD29 based constructs (Chu et al., 2008)

2.2.2.3.1. Generation of *Pf*Hsp70-1 (WT/DN) and GFP control constructs

For the generation of the DD29 based WT/DN*Pf*Hsp70-1 constructs pre-existing dd-FKBP_WT/DN transfection plasmids (fig. 2.2a and fig. 2.2b) were used. These plasmids had hDHFR cassette in the backbone. The plasmids also contained an N-terminus 3XHA

(between XhoI/AvrII) followed by dd-FKBP (between AvrII/BssHII) and WT or DNHsp70-1 (between BssHII and XmaI) as mentioned earlier. DD29 ORF was amplified by DD29_AvrII_F_P and DD29_BssHII_R_P from PJDD41 plasmid (containing the DD29 coding region) kindly provided by Prof Thomas J Wandless. Following amplification, the DD29 insert was digested by AvrII and BssHII restriction endonucleases. The plasmids coding for 3XHA-dd-FKBP_WT/DN*Pf*Hsp70-1 (fig.2.2a and fig.2.2b) were digested with AvrII and BssHII restriction endonucleases to swap the dd-FKBP with DD29 insert. After digestion the plasmids were purified. DD29 was cloned between the AvrII and BssHII restriction sites of the digested plasmids. The resulting plasmids from this ligation were amplified by mini preparation and were confirmed by test digestion and sequencing. As the dd_FKBP in these two plasmids was replaced successfully with DD29 the resulting plasmids had 3XHA at the N-terminus (between XhoI/AvrII) followed by DD29 (between AvrII/ BssHII) and WT or DN*Pf*Hsp70-1 (between BssHII/XmaI) (fig.2.6a and fig.2.6b). Therefore, the DD29 based WT/DN*Pf*Hsp70-1 transfection was generated by this one step ligation. For the generation of the control GFP construct a different strategy was employed. In neither of the *Pf*Hsp70-1 constructs (DN and WT), the *Pf*Hsp70-1 insert could be swapped with GFP insert using BssHII and XmaI. The reason being that DD29 contained an XmaI restriction site within its ORF. For this reason, pARL2-GFP plasmid was used for the generation of 3XHA-DD29-GFP plasmid. The 3XHA was first amplified from one of the pre existing plasmids using 3XHA_F and 3XHA_R primers. Purified insert was digested using XhoI and AvrII restriction endonucleases and purified. Digested and purified 3XHA tag was cloned between the XhoI and AvrII restriction sites in predigested and purified pARL2-GFP plasmid. Construct resulting from this ligation was amplified by miniprerp and confirmed by test digestion and sequencing. Further, ORF coding for DD29 was amplified from PJDD41 plasmid using DD29_AvrII_F_P and DD29_BssHII_R_P. The primers deleted the stop codon at the end of the amplified insert. After PCR amplification the insert was digested with AvrII and BssHII restriction enzymes following purification. The purified DD29 insert was cloned in frame to the C-terminus of 3XHA in the pARL2-GFP plasmid in between the AvrII and BssHII restriction sites. The resulting construct was amplified and was confirmed by test digestion and sequencing. An advantage of using the pARL2-GFP plasmid for cloning was that it already contained a GFP in frame to the C-terminus of the cloned DD29 between KpnI and XmaI restriction sites. Therefore, the construction 3XHA-DD29-GFP plasmid was complete. The plasmids were further amplified following purification by maxi preparation and used for transfection. All the resulting plasmids are schematically illustrated in fig 2.6.

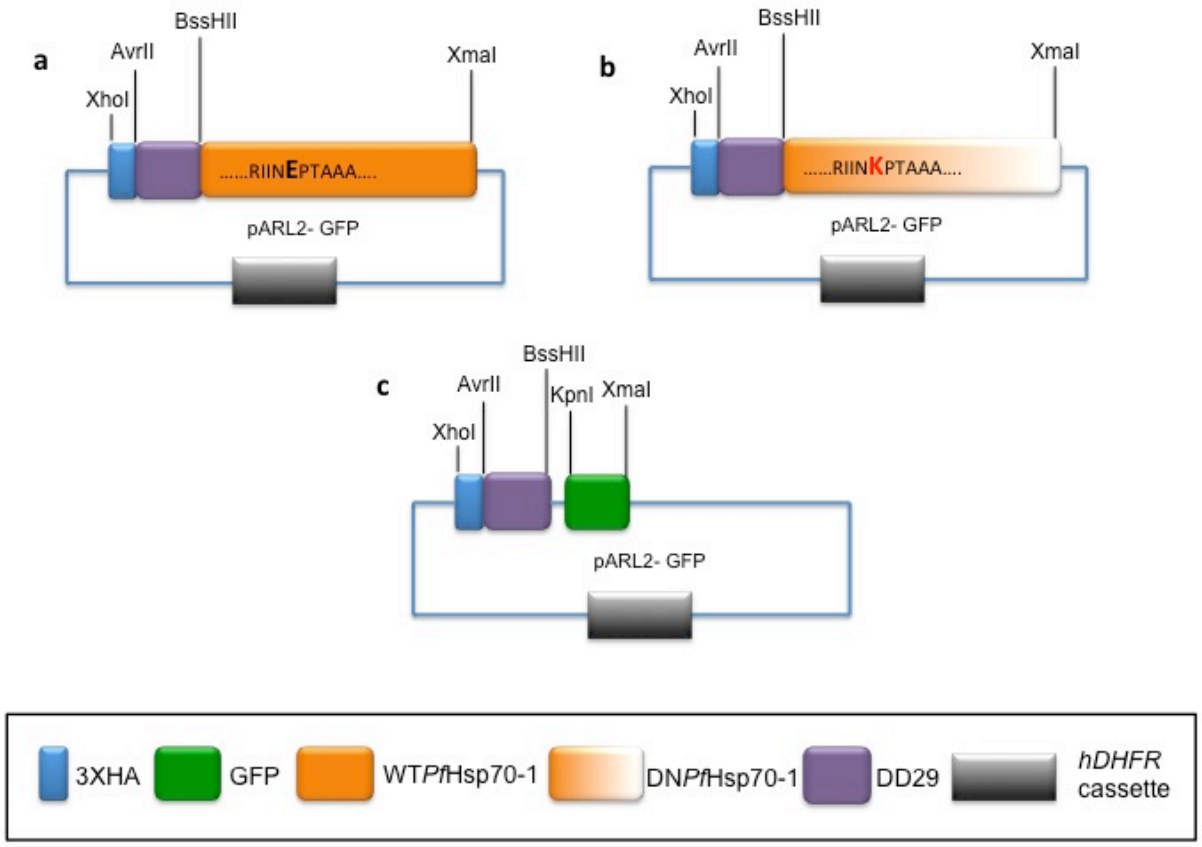

Figure 2.6 Schematic illustration of DD29 based WT/DN *Pf*Hsp70-1 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp70-1 fussed to the DD29 domain at the N-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp70-1 (E187K) fussed to DD29 at the N-terminus. (c) Control construct coding for GFP fussed to the DD29 domain at the N-terminus. Unlike in the WT and DN *Pf*Hsp70-1 coding constructs, GFP was cloned between KpnI and XmaI restriction sites. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of the detection of the fusion proteins. All constructs were generated using pARL2-GFP plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.3.2. Generation of *Pf*Hsp40 (WT/DN) and GFP control constructs

For the generation of the DD29 based WT/DN*Pf*Hsp40 constructs a pre-existing plasmid kindly provided by Sabrina Heiny was used. This plasmid unlike pARL2-GFP had a blasticidin (BSD) selection cassette instead of hDHFR. The DD29 was amplified from PJDD41 plasmid using DD29_BssHII_F_P and DD29_KpnI_R_P. The PCR product was purified and the insert was digested with BssHII and KpnI restriction enzymes. The purified insert was cloned between BssHII and KpnI restriction sites in the predigested plasmid (obtained from Sabrina Heiny). After successful ligation the resulting plasmid was amplified following confirmation by test digestion and sequencing. This plasmid already contained an ORF of GFP in frame at the N-terminus of the cloned DD29 between AvrII and BssHII restriction sites. Therefore, to clone the 3XHA tag the plasmid was further digested with

XhoI and AvrII restriction endonucleases. Simultaneously, the 3XHA insert was amplified from pre-existing plasmid using 3XHA_F and 3XHA_R primers. After PCR the insert was digested with XhoI and AvrII restriction enzymes following purification. The purified insert was cloned in frame to the N-terminus of the GFP between XhoI and AvrII restriction sites in the predigested plasmid (already containing GFP and DD29). The resulting construct from this ligation was amplified following confirmation by test digestion and sequencing. This construct was used as GFP-dd-ecDHFR control transfection construct. The GFP construct was further used for the cloning of WT and DN*Pf*Hsp40 inserts. The ORF of the WT*Pf*Hsp40 (PF3D7_0213100) and DN*Pf*Hsp40 was amplified from pre-existing dd-FKBP based WT/DN-Hsp40 constructs (fig.2.3a and fig.2.3b) using Hsp40_AvrII_F and Hsp40_BssHII_R_P primers. The inserts were digested using AvrII and BssHII restriction endonucleases. The purified inserts were cloned between the AvrII and BssHII restriction sites in the predigested GFP control construct (fig.2.7c), thus replacing the GFP by either WT*Pf*Hsp40 or DN*Pf*Hsp40. Both the resulting plasmids were amplified following confirmation by test digestion and sequencing. Finally, all the dd-ecDHFR based WT/DN*Pf*Hsp40 and control GFP plasmids were amplified and used for transfection. All the resulting plasmids are schematically illustrated in fig 2.7.

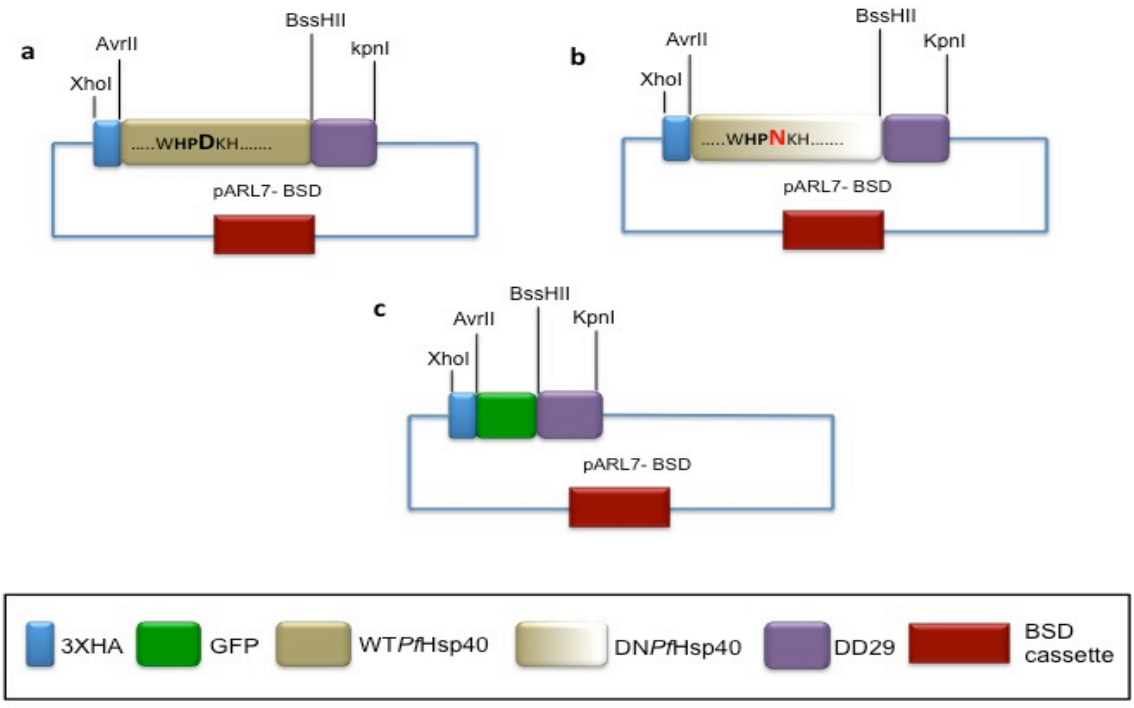

Figure 2.7 Schematic illustration of DD29 based WT/DN *Pf*Hsp40 and GFP control plasmids.

(a) Construct coding for wild type (WT) *Pf*Hsp40 fussed to the DD29 domain at the C-terminus. (b) Construct coding for dominant negative (DN) *Pf*Hsp40 (D34N) fussed to DD29 at the C-terminus. (c) Control construct

coding for GFP fussed to the DD29 domain at the C-terminus. 3XHA tag was cloned in all constructs at the extreme N-terminus between XhoI and AvrII for the purpose of detection of the fusion proteins. All constructs were generated using pARL-BSD plasmid backbone. Colour codes representing each coding region are shown in the inset. Relative sizes of different inserts are not in scale.

2.2.2.4. Importance of tagging the destabilisation domains (dds) to the N - terminus of the *Pf*Hsp70-1 whereas, the same is ligated to the C-terminus of the *Pf*Hsp40

In all the plasmids coding for either wild type of dominant negative *Pf*Hsp70-1 the GFP/FKBP/dd-ecDHFR/DD29 was fused to the N-terminus of the protein to be studied. Since, the C-terminal EEVD motif of *Pf*Hsp70-1 is important for interaction with partner proteins (Shonhai et al., 2007) we avoided tagging the C-terminus that could interfere with the *in vivo* normal functional interaction of the fusion proteins.

On the other hand, in all the plasmids coding for either wild type or dominant negative *Pf*Hsp40 the GFP/FKBP/dd-ecDHFR/DD29 was ligated to the C-terminus of the candidate proteins. *Pf*Hsp40 contains a highly conserved HPD motif located in the J domain towards the N-terminus. The HPD tripeptide is the signature domain of the Hsp40 family of proteins and facilitates interaction with the ATPase domain of Hsp70 by binding to an acidic cleft (Fan et al., 2003). Therefore, we avoided tagging at the N-terminus that could interfere with the *in vivo* normal functional interaction of *Pf*Hsp40 with *Pf*Hsp70-1.

2.2.2.5. Cloning of *Pf*Hsp70-1 promoter into pARL2-GFP vector

During this study pARL2-GFP plasmid was mostly used as a backbone to generate all plasmids for transfection. pARL2-GFP possesses a CRT promoter, which is weaker in nature when compared to the native Hsp70 promoter of the *Plasmodium falciparum*. Therefore, CRT promoter was replaced with Hsp70 promoter to generate a pARL2-GFP plasmid, where expression of the fusion protein could be driven from Hsp70-1 promoter. This provides the opportunity to place *Pf*Hsp70 insert in frame next to its native promoter. This makes comparative study with transgenic parasite line more realistic as both the endogenous wild type and episomal dominant negative *Pf*Hsp70-1 is under the control of their native promoter. Promoter region is always highly AT rich making it challenging to design primer for this region. Therefore, first the larger fragment of the 5 ′ promoter region was amplified by the initial PCR reaction using 3D7 genomic DNA as template. For this PCR, Hsp70_5'_F1 forward primer (annealing 1749 bp upstream the 5′ UTR of the *Pf*Hsp70-1 ORF) and Hsp70_5'_R1 primer (annealing 2 bp downstream from the start codon of Hsp70_1 ORF) were used. At the end of the PCR reaction the PCR product was found to be at the right size

on agarose gel. The desired DNA band was excised from the agarose gel following purification by the gel extraction kit (following manufacturer's protocol). The amplified product from the initial PCR reaction was further used as template for the second PCR reaction. This minimizes the chances of unspecific annealing of the primers as a single isolated template is used, instead of the whole 3D7 genomic DNA. Hsp70_5'_NotI_F2 forward primer (annealing 1520 bp upstream the 5 ′ UTR) and Hsp70_5'_XhoI_R2 reverse primer (annealing 18 bp upstream the 5 ′ UTR) were used. The PCR product was found to be at the right size on agarose gel and was further purified by gel extraction kit (following manufacturer's protocol). The purified insert was digested with NotI and XhoI restriction endonucleases. The digested insert was purified by PCR purification kit (following manufacturer's instructions). Simultaneously, pARL2-GFP plasmid was also digested with NotI and XhoI restriction endonucleases that resulted in the excision of the CRT promoter. The digested plasmid was loaded on agarose gel following purification by gel extraction kit (following manufacturer's protocol). The insert (Hsp70-1 promoter) was finally cloned between the NotI and XhoI restriction sites in the pre-digested pARL2-GFP plasmid. The resulting plasmid was amplified following confirmation by test digestion and sequencing. Upon confirming the successful swapping of the CRT promoter with the *Pf*Hsp70-1 promoter the plasmid was amplified and used for transfection.

2.2.3. Parasite cultivation

2.2.3.1. Routine culture of *Plasmodium falciparum*, synchronisation and enrichment of the infected erythrocytes

Plasmodium falciparum was cultured in human A^+ blood and was maintained at a haematocrit of 4% in RPMI cell culture media. The RPMI was supplemented with 10% A^+ human serum, 20μg/ml gentamycin or neomycin (0.1mg/ml), and 200μg hypoxanthine as described by Trager and Jensen (1976) (Trager and Jensen, 1976). Culture of the parasite was either maintained in a T75 cell culture flask or plates with 35 ml of RPMI at 37°C gas incubator with a constant supply of 5% CO_2 and 5% O_2.

D-sorbitol was used to synchronise parasites at ring stage (Lambros and Vanderberg, 1979). Late stage parasite (trophozoite or schizont) leads to increased permeability of the erythrocytes. Therefore, treatment with 5% d-sorbitol for 10 minutes at 37°C results in an osmotic imbalance in the late stage infected erythrocyte that finally leads to cell lysis. Whereas, RBCs infected with ring stage of *Plasmodium falciparum* do not have such

increased permeability and therefore can survive this treatment. After treatment the pellet (which should contain only ring infected erythrocytes) was washed 1X with cell culture RPMI media and was immediately plated with 35ml of media.

Enrichment of trophozoite - infected erythrocytes was achieved by the method of gelafundin flotation as described by Pasvol *et al*., (1978) (Pasvol et al., 1978). Once the infected blood pellet was prepared from the whole culture, it was incubated with 7 ml of gelafundin solution for 10 minutes at 37°C. Following incubation the supernatant was carefully collected in a new tube and was centrifuged at 3000 rpm for 2 minutes. The pellet was further mixed with 10 fold volume of RPMI cell culture media and a slide was made from the cell suspension in order to calculate the parasitemia. Following the determination of the parasitemia, the culture was diluted by the addition of fresh blood to achieve the required parasitemia. 35 ml of RPMI was added and the culture was returned to 37°C incubator.

2.2.3.2. Culture of *Toxoplasma gondii*

The RH strain of *Toxoplasma Gondii* was cultured in Human Foreskin Fibroblast (HFF) with Dulbecco's Modified Eagle Medium (DMEM) at 37 °C gas incubator (5% CO_2). The parasites grow and replicate intracellular. Upon rupture of the HFF cells, the free tachyzoites (extracellular parasites) invade new host cells. Once all the HFFs are utilised by the parasites, appropriate amount of media (containing the free tachyzoites) was transferred to a new flask containing HFFs. Thus, the free tachyzoites could further invade new host cells and the culture could be continued.

2.2.4. Culture of Human Foreskin Fibroblast (HFF) cell line

The HFFs are adherent cells that can be visualized under a 10X objective of a light microscope. The media was changed twice a week. The cells were first washed 1X with DMEM medium (without FCS) following supplementation with fresh DMEM. The HFFs were diluted once they were confluent. For dilution and subculture of the HFFs, first the supernatant was discarded following 1X wash with DMEM (without FCS). The HFFs were detached from the surface of the flask by trypsinisation. For this, the cell monolayer was incubated shortly with 1X trypsin/EDTA solution. After this short incubation period trypsin was removed and the flask containing the HFFs was incubated for additional 10 minutes at 37 °C for efficient detachment of the cells. Detachment of the cells was confirmed by checking flask under the microscope. The cells were collected by centrifugation. Finally appropriate

amount of HFFs were seeded into a new flask supplemented with DMEM and transferred to the 37 °C gas incubator (5% CO_2).

2.2.4. Transfection of parasite lines

2.2.4.1. Transient transfection of *Toxoplasma gondii* (Soldati and Boothroyd, 1993)

For Transient transfection of *Toxoplasma gondii* 50-100µg of plasmid DNA was linearized by NotI endonuclease one day prior to the transfection. The DNA was precipitated overnight with 100 % EtOH and 3M NaAc as described previously. On the day of transfection the precipitated DNA pellet was further washed with 70% EtOH and dried on 50 °C. The dried DNA pellet was resuspended in 50 µl of cytomix and incubated at 50 °C until the pellet was completely dissolved. Simultaneously, 3 X 10^7 extracellular parasites (Tachyzoites) were collected by centrifugation. The parasite pellet obtained, was dissolved in 500 µl of cytomix following the addition of the plasmid DNA. The mixture was transferred to electroporation cuvette and electroporated at 2000 V, 25 Ω and 50µF. Post pulse the mixture was immediately transferred to a 6 well plate containing high density of HFFs and further incubated for one day at 37 °C gas incubator (5% CO_2). On the next day Xanthin (50µg/ml) and Mycophenolic acid (25µg/ml) was added to the well for selection. Medium supplemented with Xanthin (50µg/ml) and Mycophenolic acid (25µg/ml) was changed on alternative days. Once the parasites were extracellular they were used for the study.

2.2.4.2. Transfection of *Plasmodium falciparum* (Crabb et al., 1997)

Plasmodium falciparum was cultured in human O^+ RBC and supplemented with gibco cell culture medium 24 hours prior to transfection. For transfection approximately 100µg plasmid DNA was precipitated overnight using 100 % EtOH and 3M NaAc as explained earlier. On the day of transfection the DNA pellet obtained from precipitation was washed 1X with 70% EtOH and was dried at 50 °C. The dried DNA pellet was redissolved in 30 µl of TE buffer (pH7.6) at 50 °C. Simultaneously a thin blood smear was prepared from the culture in order to determine the stage and parasitemia. Transfection of *Plasmodium* was mostly carried out at ring stage with a parasitemia of 5-10 %. After determination of the desired stage and optimal parasitemia, the whole culture was centrifuged at 3000 rpm for 2 minutes. The pellet obtained from the parasitized RBC was used for transfection. Prior to transfection 385 µl of cytomix and 200 µl of iRBC (from the RBC pellet) was mixed with the DNA suspension (30 µl) by gentle tapping. The entire mixture was transferred to an electroporation cuvette (0.2 cm gap)

and was electroporated at 0.310kV and 950µF (high capacitance). Usually the time constant was found to vary between 8-12 msec. Post pulse the cells were transferred to a T25 cell culture flask or plate containing 12 ml of pre-warmed gibco cell culture medium (supplemented with 0.25% AlbuMAX II, 5% human serum, hypoxanthine and gentamycin / neomycin). In addition, 400 µl of fresh O^+ human erythrocyte was added to the medium and the cells were transferred to the 37°C gas incubator. 4 hours post transfection appropriate selection drug was added to the culture. During this work, WR 99210 was added to a final concentration of 2.5 nM, whereas Blasticidin (BSD) was used at a final concentration of 4µg/ml. From the following day a thin slide was made from the blood and was observed under 100X objective of the light microscope. The media was changed daily until no live parasite could be seen. Once all parasites were found to be dead, only twice a week used medium was replaced with fresh medium supplemented with appropriate drug. The culture was passaged with 200µl fresh O^+ human erythrocyte after 14 days. Once the parasite started to reappear at least 3-4 vials of transfected parasite were immediately frozen as back up (following the freezing protocol) and were stored at -80°C. Once few such vials were stored experiments were carried out with the transgenic lines.

Noteworthy, all the plasmids coding for the fusion proteins tagged to dd-ecDHFR were transfected into Plasmepsin I (PM1 KO) cell lines during this work. PM1 KO 3D7 line contained a human DHFR (hDHFR) integrated into the gene of the parasite (Liu et al., 2005). The stabilising ligand, TMP used in this study is toxic to wild type 3D7, but the presence of hDHFR confers resistance to the parasite against TMP.

2.2.5. DNA extraction from infected erythrocyte

To confirm that the transgenic parasite line is expressing the right plasmid, the plasmid DNA was extracted from each transgenic line. In order to carry out DNA extraction the whole culture was lysed by 0.1% saponin solution in a total volume of 10 ml with PBS (PIC and PMSF supplemented) as mentioned under section 2.2.8.1.1. After lysis the parasite pellet was dissolved in 200 µl PBS (pH7.4) following the addition of 200µl 2x sample buffer A. 100 µl 20 % SDS was added to the sample and mixed by inverting the tube. The DNA was further extracted by phenol-chloroform treatment. Following the phenol-chloroform treatment the top most layer containing the DNA was collected carefully in a separate tube and was precipitated by ethanol. The ethanol precipitated DNA was re-dissolved in 500 µl of TE buffer (pH8.0) and the phenol-chloroform extraction was repeated. After this treatment the

top most layer was collected and was subjected to chloroform extraction to remove residual phenol. The resulting top phase was further carefully transferred to a new tube and plasmid DNA was precipitated by ethanol precipitation. The DNA pellet was finally dissolved in appropriate amount (50-100 µl) of TE buffer (pH8.0). Finally the plasmid DNA was digested with appropriate restriction endonucleases and visualised on agarose gel against 1 Kb^+ DNA ladder.

2.2.6. Cryopreservation

In order to maintain long-term storage of transgenic parasite line, parasite cultures at ring stage with at least 3% parasitemia, was centrifuged at 3000 RMP for 2 minutes. The pellet was mixed with equal amount (volume/volume) of freezing solution. The mixture was immediately transferred to a labelled cryopreservation tube and was shock frozen at liquid nitrogen for 10 minutes. Following freezing the tubes were stored at -80 °C for few days before being transferred to liquid nitrogen.

2.2.7. Thawing of cryopreserved infected RBC

In order to thaw frozen transgenic parasite line and bring them back to culture, the frozen cells were taken out from the liquid nitrogen and were first allowed to thaw at room temperature (RT). Once the frozen aliquots were completely thawed, they were transferred to a 15ml falcon tube. To this 200 µl of thawing solution I (200µl/1ml of frozen iRBC) was added drop-wise with constant shaking following incubation for 3 minutes at RT. After 3 min of incubation 5ml of the thawing solution II was added drop-wise with constant shaking followed by an additional incubation for 3 minutes at RT. At last 5 ml of thawing solution III was added drop-wise with shaking and was incubated for 3 minutes at RT. The mixture was finally centrifuged at 3000 rpm for 2 minutes. The pellet was washed 1X with gibco cell culture media (supplemented with 0.25 % AlbuMAXII, 5 % human serum, hypoxanthine). Post washing the pellet was plated in a T25 cell culture flask/ plate with 12 ml gibco media and fresh A^+/O^+ human RBC (300-400 µl). The following day appropriate selection drug was added. The cells were observed regularly under the microscope for the reappearance of the live parasite.

2.2.8. Methods with infected erythrocytes

2.2.8.1. Protein biochemical methods

2.2.8.1.1. Saponin lysis of infected erythrocytes

To gather pool of free parasite with intact parasite membrane saponin lysis of infected erythrocyte was carried out. Saponin is a non-ionic detergent and believed to interact with cholesterol in lipid bilayers and liposomes (Beaumelle et al., 1987). During treatment with saponin the erythrocyte plasma membrane along with the parasitophorous vacuolar membrane (PVM) are permeabilise, but the parasite membrane stays intact. Infected blood pellet (mixed or gelafundin enriched culture) was treated with 0.1% of saponin solution in a total volume of 10 ml PBS (pH7.4, supplemented with PIC and PMSF) for 6 minutes on ice. After the incubation period the intact parasites were collected by centrifugation at 5000 rpm for 4 minutes at 4 °C. The pellet was washed with PBS until no haemoglobin was left and the supernatant turned colourless. Supernatant was discarded (contained soluble content of the PV and the host RBC cytosol) and the pellet contained the intact parasites. Since during this thesis work cytosolic protein contents from the parasite were studied, we were not interested in the content of the RBC and PV. Further, the parasite pellet was dissolved in appropriate amount of 2X SDS sample buffer (PIC and PMSF supplemented) and boiled at 95 °C for 10 minutes prior to be analysed by SDS-PAGE. In some cases depending on the experiments the resulting pellet from the saponin lysis was further lysed by Tris lysis buffer and centrifuged at 18000 rpm for 20 minutes at 4 °C. The pellet resulting from the centrifugation contained the parasite plasma membrane whereas the supernatant contained the cytosolic protein from the parasite. The supernatant was carefully transferred to a new tube. The supernatant (cytosolic fraction) and the pellet (membrane fraction) were mixed separately with appropriate amount of 2X SDS sample buffer and boiled at 95 °C for 10 minutes. Finally, the samples were analysed by SDS-PAGE.

2.2.8.1.2. Experiment with Shield-1 or TMP

To determine whether shield-1 or TMP has any effect on the stabilization of the fusion protein, appropriate transgenic parasite lines were treated without or with different concentration of shield-1 or TMP. Individual *Plasmodium falciparum* transgenic line was treated with D-sorbitol and synchronised at ring stage. Under the light microscope the resulting parasitemia of the culture was determined. The parasetimia was further adjusted to 0.4 % by adding fresh A^+ human blood. 200 µl of infected blood (0.4 % parasetimia) was

seeded in each well with 5 ml of gibco cell culture media supplemented with appropriate selection drug. Individual well was treated without or with increasing concentration of shield-1 (0.5-1.5 µM) / TMP (5.0-15.0 µM). TMP stock was maintained at a concentration of 100mM in 100 % EtOH, which was further diluted by adding cell culture media to the desired working concentration. The shield-1 was diluted directly into the cell culture media to obtain the desired working concentration. The media was changed every day supplemented with drug and +/- shield-1/TMP until 72 hours. After 72 hours the parasitemia of the culture from individual well was determined. Following determination of the parasitemia cells from each well was pelleted and was treated with saponin separately as explained earlier. The pellets obtained from saponin lysis were dissolved in appropriate amount of 2X SDS sample buffer and boiled for 10 minutes at 95 °C prior to SDS-PAGE. Equal amount of parasite equivalence were loaded in each well of the SDS-PAGE for comparative study.

Transgenic *Toxoplasma gondii* was treated with +/- shield-1. Shield-1 was added to a final concentration of 0.5 µM into the media in a T25 cell culture flask on free tachyzoites. At the same time another T25 flask with free tachyzoites was maintained without shield-1 as negative control. After 24 hours of incubation +/- shield-1, parasites from both the flasks were collected in 15 ml falcon tubes and 10 µl of cell suspension was loaded onto haemocytometer chamber for counting. The suspensions were centrifuged at 3000 RPM for 5 minutes to obtain cell pellet. The pellets were finally dissolved in appropriate amount 2X SDS sample buffer (supplemented with PIC and PMSF) to achieve similar cell equivalence. Finally, $3x10^7$ parasite equivalence was loaded in each well of the SDS-PAGE for comparative analysis.

2.2.8.1.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis or SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out to separate individual protein according to their molecular weight from a pull of cytosolic protein. Proteins are usually neutrally charged but the application of SDS imparts an overall negative charge to the protein molecules. As a result, the negatively charged proteins run towards the positive pole under electric field and get separated according to their molecular weight. Presence of dithiothreitol (DTT) to the SDS PAGE sample buffer reduces disulphide bonds thereby supporting protein denaturation. During electrophoresis the gel matrix serves as a molecular sieve. The smaller proteins more easily pass through the pores,

therefore migrate longer distance in the gel. Whereas, the larger ones cannot migrate easily hence remain close to the points of their origin. Therefore, the proteins would have differentially migrated according to their sizes that result in the separation of different size proteins at different levels in the gel matrix. During this work prior to analysis the samples were mixed with appropriate amount of 2X SDS-PAGE sample buffer (supplemented with fresh DTT) and boiled for 10 minutes at 95 °C. The samples were loaded onto the gel alongside a prestained protein marker (Thermo Scientific) for the determination of the size of individual protein band. The percentage of the SDS-PAGE separating gel was determined depending on the expected size of protein. Usually 10-12% gel was used. Concentration of the gel was increased further if small molecular weight protein was being studied.

2.2.8.1.4. Semi-dry immunoblotting

Semi-dry immunoblotting (Towbin et al., 1979) method was used for the detection of specific proteins using antibody directed against them. For this reason first proteins needed to be transferred onto nitrocellulose membrane from the gel matrix after SDS-PAGE. Following SDS-PAGE the gel unit was carefully dismantled and the stacking gel was cut out. The separating gel was then carefully placed on an equally sized nitrocellulose membrane pre soaked in transfer buffer (supplemented with fresh 20% MetOH). 3 soaked whatman papers cut to the same size was also placed on each side of the gel. The sandwich was then placed carefully onto the chamber and any air bubble formed between the layers was removed by pressing the sandwich with a roller in order to confirm efficient transfer. The chamber was closed and transfer was carried out at a constant current of 1mA/cm^2 for 1 hour. Following transfer, the membrane was removed from the chamber. The transfer of protein was confirmed as the prestained protein marker got transferred onto the nitrocellulose membrane. The membrane was further blocked with 3% milk in PBS (pH7.4) for 1 hour at room temperature. After blocking, the membrane was incubated with 1ºantibody solution (diluted accordingly in 3% blocking solution) overnight at 4 ºC. The following day blot was washed 3 times with PBS (pH7.4) followed by the incubation in the 2ºantibody solution (diluted accordingly in 3% blocking buffer) for 2 hours at room temperature. After two hours of incubation the membrane was washed 3 times with PBS (pH7.4). The blot was finally incubated for 60 seconds in ECL solution (supplemented with 0.02% H_2O_2) followed by exposer to the medical X-ray film in the dark room and developed.

2.2.8.2. Fluorescence Microscopy

2.2.8.2.1. Live cell imaging

1 ml of cell culture suspension containing GFP expressing transgenic parasite, was directly taken from the cell culture plate and was incubated shortly with Hoechst 33258 (10µg/ml) following imaging under the microscope. Images of the GFP parasites were obtained using a Zeiss Axio Observer inverse epifluorescence microscope system. Appropriate filter sets were used for imaging which was equipped with Axiovision 4 software. In order to minimise bleaching exposure time of the samples were adjusted accordingly allowing proper visualisation of the details.

2.2.8.2.2. Image restoration

After capturing individual image from the live parasites under different channels of the microscope, the images were imported to Image J64 (version 1.39u: available at http://rsb.info.nih.gov/ij). The images were further converted to 8-bit greyscale following background substraction, and overlay with ImageJ plugin RGB merge. To create figures for the data TIF files were imported to Microsoft powerpoint. After making the final images they were saved as JPEG files. During this work no image was ever subjected to gamma adjustment. At the same time all data presented in this work are in agreement with the recommendation of Rossner and Yamada (2004) (Rossner and Yamada, 2004).

3. Results

Several postulations towards the function of *Pf*Hsp70-1, (PF3D7_0818900) in *Plasmodium falciparum* has been put forward till date, but direct biochemical evidence supporting the essential role of this protein for intraerythrocyitc survival of the parasite is still missing. At the same time, it was also predicted that a cytosolic Hsp40 homologue, *Pf*Hsp40 (PF3D7_0213100) might be a possible co-chaperone of *Pf*Hsp70-1 (Rug and Maier, 2011, Botha et al., 2007, Shonhai et al., 2007) but, no study has so far documented any *in vivo* experimental evidence of such partnership. Therefore, our aim was to define the essential roles of these two proteins by their conditional knockdown in blood stage *P. falciparum*. Classical knockout of essential genes in *Plasmodium* during intraerythrocytic haploid stage is difficult to achieve. Therefore, we sought to adapt dominant negative system combined with destabilization domains (dd) strategy to obtain regulatable overexpression of dominant negative alleles of the endogenous proteins. The dd system has recently been developed where a destabilisation domain (dd) is fused either to the N- or C- terminus of the proteins of interest. The destabilisation domain tagged fusion protein is unstable and therefore should be detected by the cellular machinery leading to the proteosomal degradation. The stability can be reverted by the addition of stabilising ligands and thereby degradation is mitigated. The system has been successfully adapted to study function of several proteins in *Plasmodium* and apicomplexan relative *Toxoplasma gondii* (Banaszynski et al., 2006, Armstrong and Goldberg, 2007, Herm-Gotz et al., 2007).

During this study, we tried to use the system to overexpress dominant negative versions of *Pf*Hsp70-1 and *Pf*Hsp40 driven from episomal plasmid, keeping the wild type genes intact at their respective loci. We expected the dominant negative versions to override the native endogenous protein functions in a competitive manner by occupying their cellular ligands. Although the fusion proteins interacts with the cellular ligands, they cannot carry out the necessary biological functions in the cell as they are catalytically dead. This would have helped us to establish a conditional knockdown of the endogenous proteins by inhibiting their essential cellular function. Further, the resulting phenotype could be studied to assess the parasite specific importance of *Pf*Hsp70-1 and *Pf*Hsp40 for intraerythrocytic survival of *Plasmodium*.

3.1 dd-FKBP based approaches

3.1.1 Dominant negative system coupled with dd-FKBP to establish conditional knockdown of *Pf*Hsp70-1 in intraerythrocytic *Plasmodium falciparum*

3.1.1.1 Validation of the episomal expression of the correct fusion proteins

Plasmids coding for WT*Pf*Hsp70-1 (PF3D7_0818900), DN*Pf*Hsp70-1 were generated as explained under section 2.2. Post transfection with the plasmids, the parasites were cultured under drug pressure (WR99210) for 2-3 weeks for selection of plasmid-carrying transgenic parasite lines. To confirm the expression of the fusion proteins, free parasites were isolated from infected RBCs (iRBC) by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). SDS-PAGE and western blot were carried out to validate the expression of the right fusion proteins in the parasites. The signals were detected at the correct molecular mass with anti-HA antibody and thereby confirmed the episomal expression of the right fusion proteins (fig 3.1b and fig 3.1d). Noteworthy, during this initial experiment all transgenic parasite lines were maintained without shield-1.

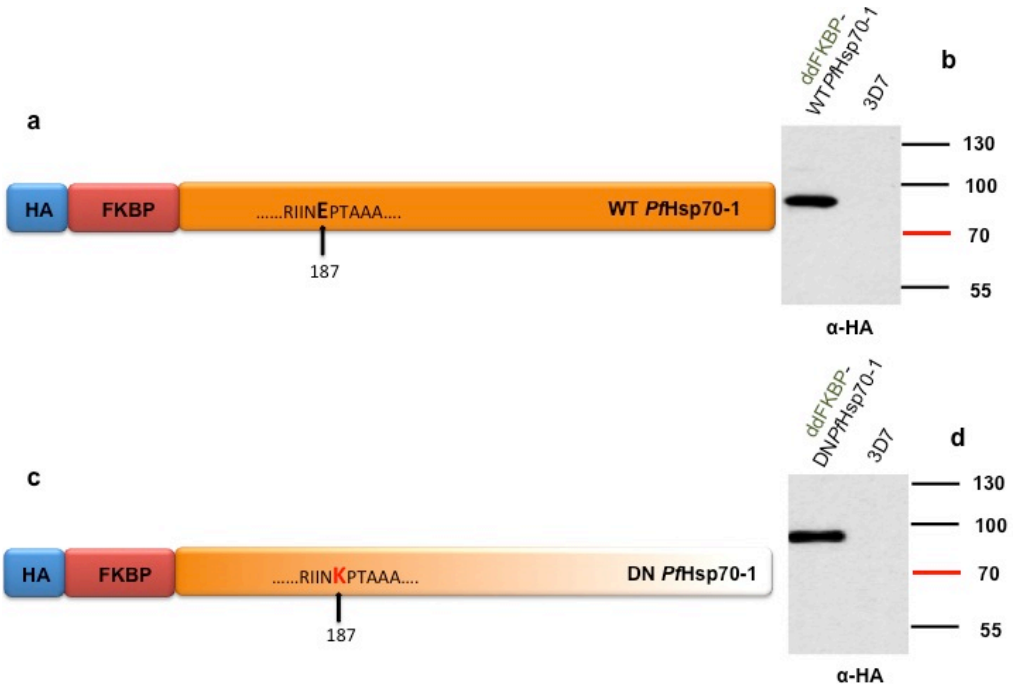

Figure 3.1 Schematic illustrations and detection of of WT/DN*Pf*Hsp70-1 fusion proteins.

(a) Schematic illustration of the wild type (WT) *Pf*Hsp70-1 fusion protein. Full length WT*Pf*Hsp70-1 is shown in dark orange and the N-terminal dd-FKBP domain is shown in red. 3XHA tag on the extreme N-terminus is shown in blue. (b) Western blot with anti-HA antibody on the whole cell extract from parasite transfected with

the dd-FKBP-WT*Pf*Hsp70-1 plasmid shows the expression of the correct molecular mass fusion protein at ~ 92kDa. (c) Schematic illustration of the dominant negative (DN) *Pf*Hsp70-1 fusion protein. Full length DN*Pf*Hsp70-1 is shown in light orange with gradient and contains the E187K mutation. N-terminal dd-FKBP and 3XHA tag are shown in red and blue simultaneously. (d) Western blot with anti-HA antibody on the whole cell extract from parasite transfected with the dd-FKBP-DN*Pf*Hsp70-1 plasmid shows the expression of the fusion protein at right molecular mass of ~ 92kDa. No protein was detected in the 3D7 (negative control) lanes (3.1b and 3.1d). $1x10^7$ parasite cell equivalents were used for analysis.

3.1.1.2 Wild type *Pf*Hsp70-1 could be destabilised by N-terminal fusion of dd-FKBP and addition of shield-1 yielded further increase in the fusion protein level in the intraerythocytic stages of *P. falciparum*

Cell lines expressing dd-FKBP-*Pf*Hsp70-1 (fig 3.2a) were cultured with or without 1.0µM shield-1 for 72 hours. Post treatment with or without shield-1 free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of shield-1 on the stabilization of the fusion proteins.

In the extracts from cells expression dd-FKBP-*Pf*Hsp70-1, the fusion protein could be detected at the expected size of ~92kDa in the absence of the shield-1 by western blot with rabbit anti-HA and mouse anti-*Pf*Hsp70-1 antibodies (fig 3.1b and fig 3. 2b). This indicated that dd-FKBP domain could not mediate complete degradation of the fusion protein since some protein could still be detected in the absence of shield-1. As expected, on the other hand in the presence of 1.0µM shield-1 there was a noticeable increase in the level of the fusion protein as shown by anti-*Pf*Hsp70 (fig 2b) and anti-HA antibody (fig 2c). Noteworthy, in the western blot with anti-*Pf*Hsp70-1 antibody from cell lines expressing WT*Pf*Hsp70-1, the level of endogenous *PfH*sp70-1 was much higher in magnitude in comparison to that of the fusion proteins in both the lanes (+/- shield-1) (fig 3. 2b). Western blot with anti-glycophorin antibody served as loading control (fig 3. 2d).

To conclude, we could see shield-1 dependent regulation of the episomally expressed WT*Pf*Hsp70-1 protein but the level of the fusion protein was lower by several orders of magnitude compared to that of the endogenous *Pf*Hsp70-1. We further wanted to test whether level of the dominant negative *Pf*Hsp70-1 can be controlled in the same way.

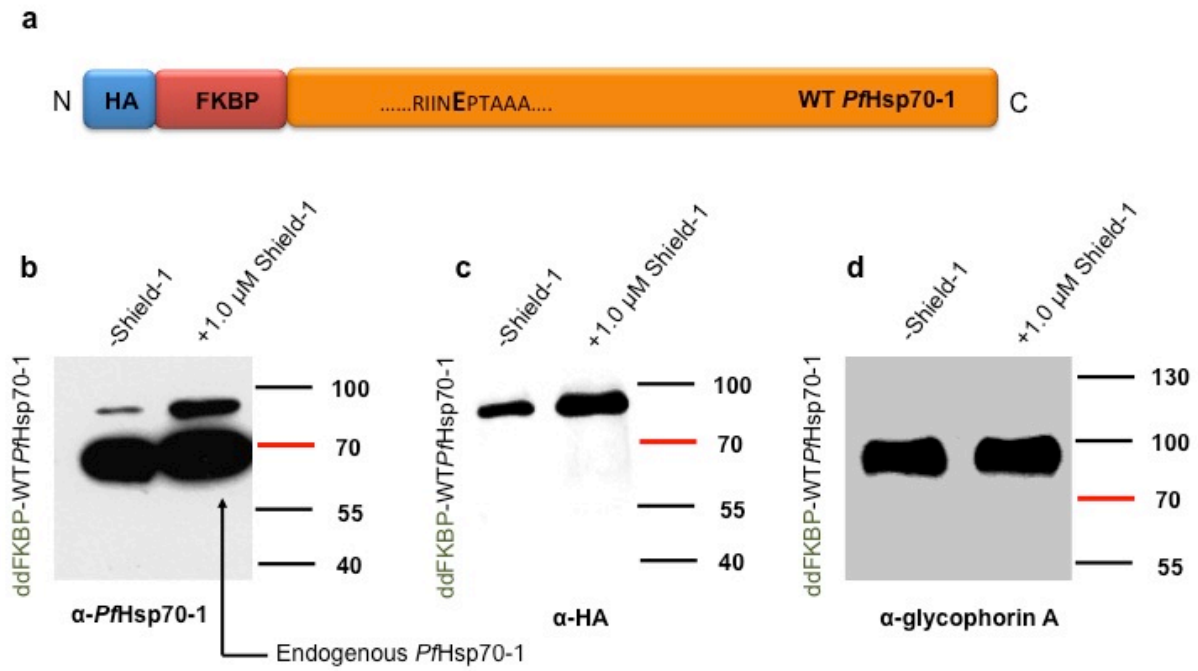

Figure 3.2 Analysis of dd-FKBP mediated destabilisation and the effect of Shield-1 on the suppression of the destabilisation of *Pf*Hsp70-1 fusion protein.

(a) Schematic representation of dd-FKBP-*Pf*Hsp70-1 fusion protein. (b) and (c) Western blot analysis from the whole cell extract of cells expressing WT*Pf*Hsp70-1 N-terminally tagged to dd-FKBP, that grew with or without 1.0µM shield-1 for 72 hours. (b) Without shield-1 the fusion protein could be detected at the correct size of ~92 kDa with anti-*Pf*Hsp70 antibody. Addition of 1.0µM of shield-1 to the same parasite line shows increase in the level of the fusion protein as detected with stronger signal intensity compared to that without shield-1. Endogenous *Pf*Hsp70-1 was detected with much stronger signal intensity than compared to that of the fusion proteins in both the lanes (+/- shield-1). (c) The fusion proteins were detected from the same samples at the expected molecular mass of ~92 kDa with anti-HA antibody. The intensity of the band was shown to be at least two time stronger in the cells treated with shield-1 that the untreated ones. (d) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in both the lanes (+/- shield-1). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.1.1.3 Dominant negative *Pf*Hsp70-1 could not be destabilised by N-terminal fusion of dd-FKBP and addition of shield-1 did not yield any further increase in the fusion protein level in the intraerythocytic stages of *P. falciparum*

Here we wanted to assess whether shield-1 dependent regulation of the level of DN*Pf*Hsp70-1 can be obtained. For this reason, cell lines expressing dd-FKBP-DN*Pf*Hsp70-1 (fig 3.3a) were cultured with or without 1.0µM shield-1 for 72 hours. Post treatment with or without shield-1, free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of shield-1 on the stabilization of the fusion proteins.

In the absence of the shield-1 fusion protein could be detected with anti-*Pf*Hsp70-1 antibody at the correct molecular mass of ~ 92 kDa, representing the fact that dd-FKBP failed to mediate degradation of dd-FKBP-DN*Pf*Hsp70-1 fusion protein (fig 3.1d, fig 3.3b and fig 3.3c). Further, we wanted to find out whether addition of shield-1 has any effect on protein level. Therefore parasites were treated with 1.0µM and 1.5µM shield-1 for 72 hours. No further increase in the level of fusion proteins was seen from the whole cell extract by western blot with anti-*Pf*Hsp70-1 and anti-HA antibody (fig 3.3b and fig 3.3c). Noteworthy, the level of endogenous *Pf*Hsp70-1 was found to be higher in comparison to that of the dominant negative fusion proteins in both the lanes (fig 3.3a). The cells expressing DN*Pf*Hsp70-1 exhibited normal developmental stages indicating the absence of any dominant negative effect in the parasites. Western blot with anti-glycophorin A antibody served as loading control (fig 3.3d).

Therefore, from this experiment we could conclude that shield-1 dependent regulation of the episomally expressed DN*Pf*Hsp70-1 protein level was not possible. At the same time the level of dominant negative protein was lower than compared to that of the wild type. In addition, expression of the dominant negative *Pf*Hsp70-1 did not affect normal growth and development of the parasite. Being unsuccessful to establish a dominant negative phenotype in the parasite line, we further wanted to try this system for *Pf*Hsp40 (PF3D7_0213100).

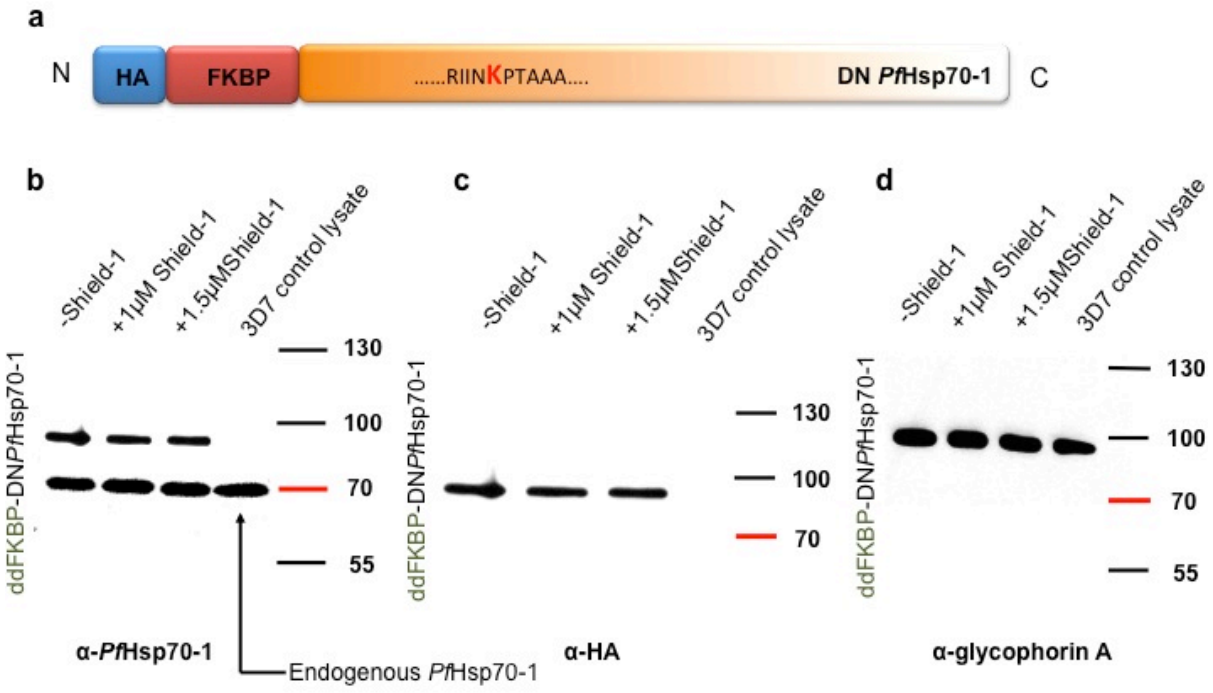

Figure 3.3 Analysis of dd-FKBP mediated destabilisation and the effect of Shield-1 on suppression of the destabilisation of DN*Pf*Hsp70-1 fusion protein.

(a) Schematic representation of dd-FKBP-DN*Pf*Hsp70-1 fusion protein. The dominant negative *Pf*Hsp70-1 contains an E187K mutation. (b) and (c) Western blot analysis from the whole extract of cells expressing DN *Pf*Hsp70-1 N-terminally tagged to dd-FKBP, that grew with or without shield-1 for 72 hours. (b) Without shield-1 the fusion protein could be detected at the correct size of ~92 kDa with anti-*Pf*Hsp70-1 antibody. Maintenance of the same parasite line in shield-1 ranging upto 1.5µM does not yeild any increase in the level of fusion proteins. Fusion protein not detected in the 3D7 control lane confirming the specificity of the antibody. Endogenous *Pf*Hsp70-1 was detected at least with tow time stronger signal intensity than compared to that of the dominant negative protein in all the lanes (+/- shield-1). (c) The fusion proteins were detected from the same samples at the expected size of ~92kDa with anti-HA antibody and no increase in the level of the fusion protein was observed by shield-1 treatment (1.0-1.5µM). (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicated equal loading of samples in all the lanes (+/- shield-1 and control lane). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.1.2 Dominant negative system coupled with dd-FKBP to establish conditional knockdown of *Pf*Hsp40 in intraerythrocytic *Plasmodium falciparum*

*Pf*Hsp40 (PF3D7_0213100) was predicted to be a potential cytosolic co-chaperone of the *Pf*Hsp70-1. Therefore, we aimed to overexpress a dominant negative (DN) version of *Pf*Hsp40 to define its importance in the parasite. In the DN*Pf*Hsp40 the conserved HPD motif in the J domain is mutated to HPN motif (fig 3.4b) (explained under section 2.2). Such mutagenesis hinders the functional interaction with *Pf*Hsp70-1(Hennessy et al., 2005). The loss of such interaction should inhibit the transfer of client protein and stimulation of ATPase activity of *Pf*Hsp70-1 by *Pf*Hsp40. This should adversely effect the chaperoning of substrate by *Pf*Hsp70-1. In our belief, this should be reflected by the defective phenotype of the parasite during the intraerythrocytic stages, considering *Pf*Hsp40 plays an essential role as a co-chaperone.

3.1.2.1 Validation of the episomal expression of the fusion proteins

Plasmids coding for *Pf*Hsp40 and DN*Pf*Hsp40 were generated as explained under section 2.2. Post transfection of 3D7 line with these plasmids the parasites were cultured under drug pressure (WR99210) for 2-3 weeks for selection of plasmid-carrying transgenic parasite lines. To confirm the expression of the fusion proteins, free parasites were isolated from infected RBCs (iRBC) by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). SDS-PAGE and western blot were carried out to validate the expression of the right fusion proteins in the parasites. The signals were detected at the correct molecular mass with anti-HA antibody and

thereby confirmed the episomal expression of the right fusion proteins (fig 3.4b and fig 3.4d). Noteworthy, during this initial experiment all transgenic parasite lines were maintained without shield-1.

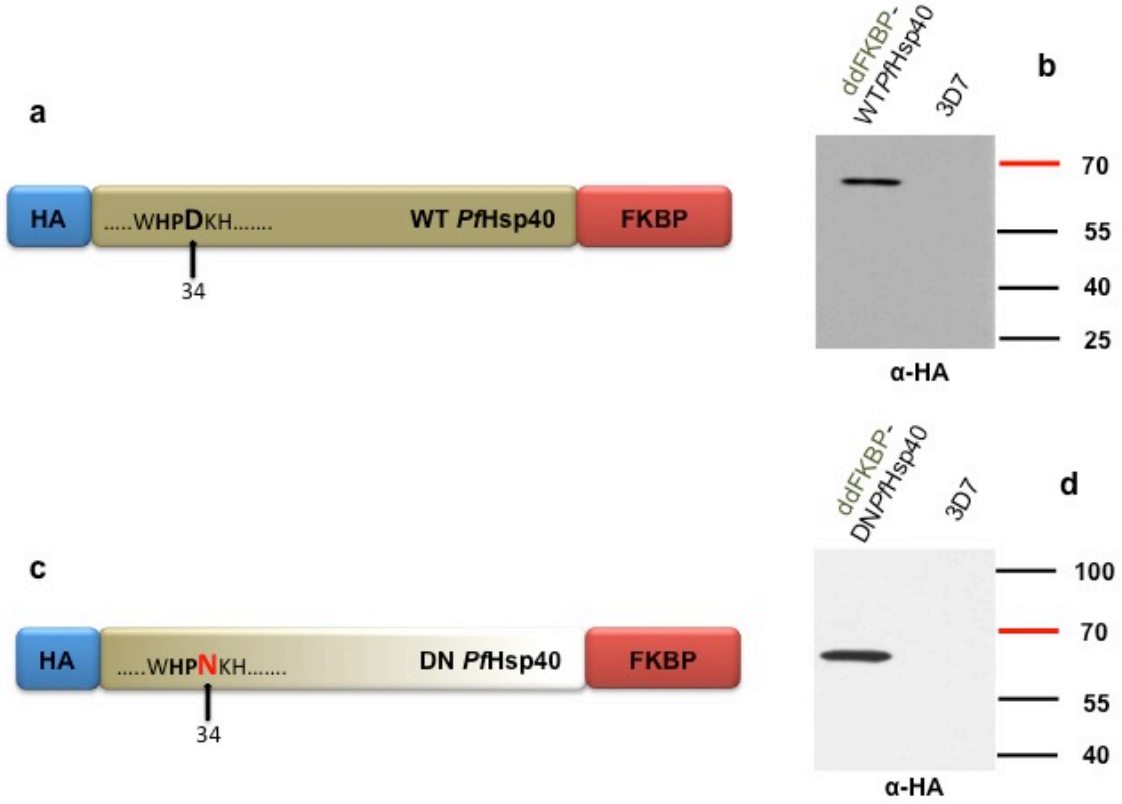

Figure 3.4 Schematic illustrations and the detection of WT/DN*Pf*Hsp40 fusion proteins.

(a) Schematic illustration of the wild type (WT) *Pf*Hsp40 fusion protein. Full length WT*Pf*Hsp40 is shown in dark olive green and the N-terminal 3XHA tag is shown in blue. The dd-FKBP domain is fused to the C-terminus of the protein and shown in red. (b) Western blot with anti-HA antibody on the whole cell extract from parasite transfected with the dd-FKBP-WT*Pf*Hsp40 plasmid shows the expression of the right molecular mass fusion protein at ~ 56kDa. (c) Schematic illustration of the dominant negative (DN) *Pf*Hsp40 fusion protein. Full length DN*Pf*Hsp40 is shown in light olive green with gradient and possess a D34N. The N-terminal 3XHA tag is shown in blue. The dd-FKBP domain is fused to the C-terminus of the protein and shown in red. (d) Western blot with anti-HA antibody on the whole cell extract from parasite transfected with the dd-FKBP-DN*Pf*Hsp40 confirms the expression of the right molecular mass fusion protein at ~ 56kDa. No protein was detected in the 3D7 (negative control) lanes (3.4b and 3.4d). $1x10^7$ cell equivalents were used for all the western blot analysis.

3.1.2.2 Wild type *Pf*Hsp40 could not be destabilised by N-terminal fusion of dd-FKBP and addition of shield-1 did not yield any further increase in the fusion protein level in the intraerythocytic stages of *P. falciparum*

We started with WT*Pf*Hsp40 to assess whether protein level can be regulated in a shield-1 dependent manner. For this, cell lines expressing dd-FKBP-WT*Pf*Hsp40 (fig 3.5a) were cultured with or without increasing concentrations of shield-1 ranging upto 1.0µM for 72

hours. Post treatment with or without shield-1 free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of shield-1 on the stabilization of the fusion proteins.

In the absence of shield-1 the wild type fusion protein could be detected at expected molecular mass of ~56 kDa by western blot with anti-HA antibody from the whole extract obtained from WT*Pf*Hsp40-dd-FKBP expressing parasite line (fig 3.4a and fig 3.5b). On the other hand by the addition of either 0.5 µM or 1.0 µM shield-1 no further stabilization of fusion protein could be seen since the level of the fusion protein remained same with no noticeable increase (fig 3.5b). Due to the unavailability of a specific anti-*Pf*Hsp40 antibody, the level of endogenous *Pf*Hsp40 could not be determined. This restricted us to compare the level of episomally expressed WT*Pf*Hsp40 with that of the endogenous version. Western blot with anti-glycophorin antibody served as loading control and confirmed equal loading of samples in all the lanes (fig 3.5c).

Hence, from this experiment we could summarise that fusion of dd-FKBP to *Pf*Hsp40 failed to direct fusion protein to degradation. Additionally, shield-1 dependent regulation of the episomally expressed WT*Pf*Hsp40 fusion protein was not possible. Therefore, we further wanted to test whether the stabilization of dominant negative *Pf*Hsp40 could be regulated by shield-1.

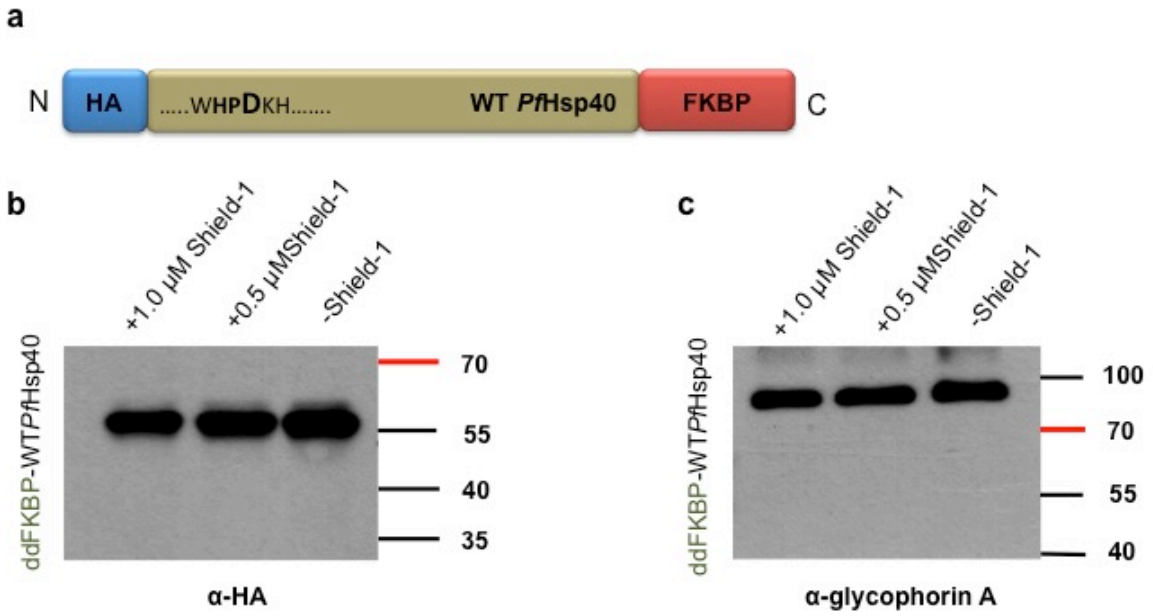

Figure 3.5 Analysis of dd-FKBP mediated destabilisation and the effect of Shield-1 on suppression of the destabilisation of WT*Pf*Hsp40 fusion protein.

(a) Schematic representation of WT*Pf*Hsp40-dd-FKBP fusion protein. (b) Western blot analysis from the whole extract of cells expressing WT *Pf*Hsp40, C- terminally tagged dd-FKBP maintained with or without shield-1 for 72 hours. Without shield-1 the fusion protein was detected with anti-HA antibody at the correct molecular mass of ~56kDa with strong signal intensity. Maintenance of the same parasite line in shield-1 ranging from 0.5-1.0µM did not yield any increase in the level of the fusion proteins (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- shield-1). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.1.2.3 Dominant negative *Pf*Hsp40 could not be destabilised by N-terminal fusion of dd-FKBP and addition of shield-1 did not yield further increase in the fusion protein level in the intraerythocytic stages of *P. falciparum*

We further wanted to figure out whether dominant negative *Pf*Hsp40 fusion protein could be regulated in a shield-1 dependent manner. For this reason, cell line expressing dd-FKBP-DN*Pf*Hsp40 (fig 3.6a) was cultured with or without increasing concentrations of shield-1 ranging upto 1.0µM for 72 hours. Post treatment, with or without shield-1 free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of shield-1 on the stabilization of the fusion proteins.

In the absence of shield-1 the dominant negative fusion protein could be detected at expected molecular mass of ~56 kDa by western blot with anti-HA antibody from the whole extract obtained from DN*Pf*Hsp40-dd-FKBP expressing parasite line (fig 3.4d and fig 3.6b). On the other hand by the addition of either 0.5µM or 1.0µM shield-1 no further stabilization of fusion protein could be achieved since the level of the fusion protein remained same with no noticeable increase (fig 3.6b). The cells expressing DN*Pf*Hsp40 exhibited normal developmental stages indicating the absence of any dominant negative effect in the parasites. As mentioned earlier due to the unavailability of a specific anti-*Pf*Hsp40 antibody, the level of endogenous *Pf*Hsp40 could not be determined. This restricted us to compare the level of episomally expressed DN*Pf*Hsp40 with that of its endogenous counterpart. This is why we could not determine whether the level of the endogenous protein is higher than that of the episomal dominant negative version, responsible nullifying any dominant negative effect. Western blot with anti-glycophorin antibody served as loading control and confirmed equal loading of samples in all the lanes (fig 3.6c).

Altogether, from this experiment we could summarise that dd-FKBP could not mediate degradation of the fusion proteins. In addition, regulation of DN*Pf*Hsp40 protein level by

shield-1 was also not possible and the expression a dominant negative version of the *Pf*Hsp40 did not affect the normal growth and development of the parasite.

From the experiments carried out so far we could only regulate the level of dd-FKBP-WT*Pf*Hsp70-1 fusion protein. Other than this, none of the dd-FKBP tagged candidate proteins could be regulated by shield-1. Therefore, at this point we wanted to validate the efficacy of the system by using a positive control.

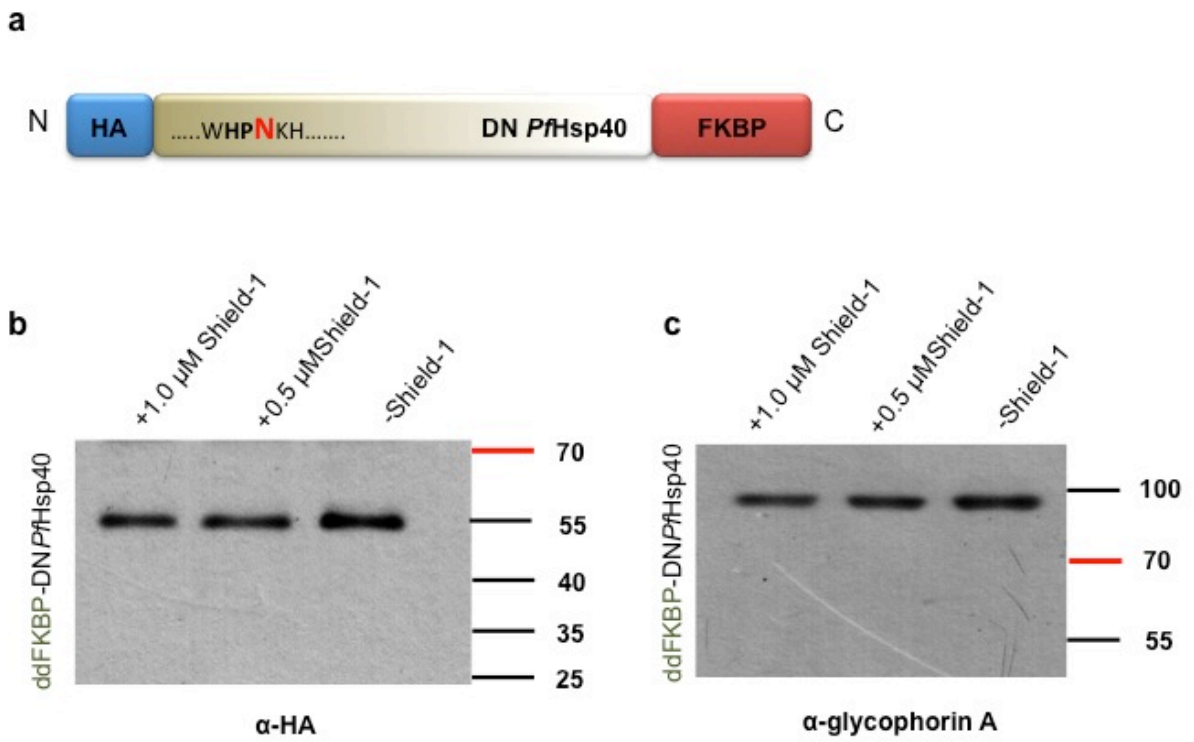

Figure 3.6 Analysis of dd-FKBP mediated destabilisation and the effect of Shield-1 on suppression of the destabilisation of DN*Pf*Hsp40 fusion protein.

(a) Schematic representation of DN*Pf*Hsp40-dd-FKBP fusion protein. (b) Western blot analysis from the whole extract of cells expressing DN*Pf*Hsp40 tagged to the dd-FKBP on the C-terminus that grew with or without shield-1 for 72 hours. Without shield-1 the fusion protein could be detected with anti-HA antibody at the correct molecular mass of ~56 kDa with strong signal intensity. Maintenance of the same parasite line in shield-1 ranging upto 0.5-1.0µM does not yield any increase in the level of the fusion proteins. (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and confirmed equal loading of samples in all the lanes (+/- shield-1). $1x10^7$ cell equivalents were used for all the western blot analyses. Similar results were obtained in three independent experiments.

3.1.3 Robust destabilisation of *Toxoplasma gondii* myosin light chain 1 (MLC1) could be achieved, when fused dd-FKBP on the N-terminus and stability could be reverted by the addition of shield-1

Since we failed to regulate the level of almost all dd-FKBP tagged candidate proteins (except wild type *Pf*Hsp70-1) by shield-1, we wanted to further test the system by using a positive control. This would help us to figure out any technical or handling error involved. At the same time effectiveness of the shield-1 could be tested, as it is susceptible to degradation by repeated freeze thaw and inappropriate handling. For this reason, a plasmid (DD-*Myc*-*Tg*MLC1-HXGPRT) coding for *Toxoplasma gondii* Myocin light chain (MLC1) fused to dd-FKBP on the N-terminus (fig 3.7a) was used. The plasmid was kindly provided by Prof. Dominique Soldati-favre. It served as a positive control, since the level of DD-*Myc*-*Tg*MLC1 fusion protein could be efficiently regulated by shield-1 at Prof. Soldati-favre's group.

For this reason, we transfected *T. gondii* with the reporter plasmid (described under section 2.2.4.1). Post transfection, parasites expression the MLC1 tagged to dd-FKBP were treated with or without 0.5µM shield-1 for 24 hours. Post treatment with or without shield-1, free parasite pellets were collected from each individual experiments. Following several washes of the parasite pellets, soluble protein extracts were obtained by boiling the pellets in SDS-PAGE sample buffer (described under section 2.2). Finally, samples were analysed by SDS-PAGE and western blot to determine the effect of shield-1 on the stabilization of the fusion proteins.

In the absence of shield-1 the fusion protein could barely be detected by western blot with anti-FKBP antibody from the whole cell extract obtained from DD-*Myc*-*Tg*MLC1 expressing parasite line (fig 3.7b). Longer exposure of the same resulted in stronger signal depicting residual amount of fusion protein in the absence of the shield-1 (fig 3.7c). However, on the other hand, treatment of the parasites with 0.5µM shield-1 resulted in a clear and robust increase in the level of fusion protein as detected at the expected molar mass of ~40kDa (fig 3.7b and fig 3.7c). Amount of actin served as loading control and confirmed equal loading of samples in both the lanes (fig 3.7d).

This experiment confirmed that the episomal level of *Tg*MLC1 fused to dd-FKBP could be efficiently regulated by shield-1. This proved that the dd-FKBP domain can mediate efficient

degradation of the fusion protein and that can further be supressed by the addition of shield-1 in our experimental set up. At the same time the outcome also validated the effectiveness of shield-1 used for previous experiments.

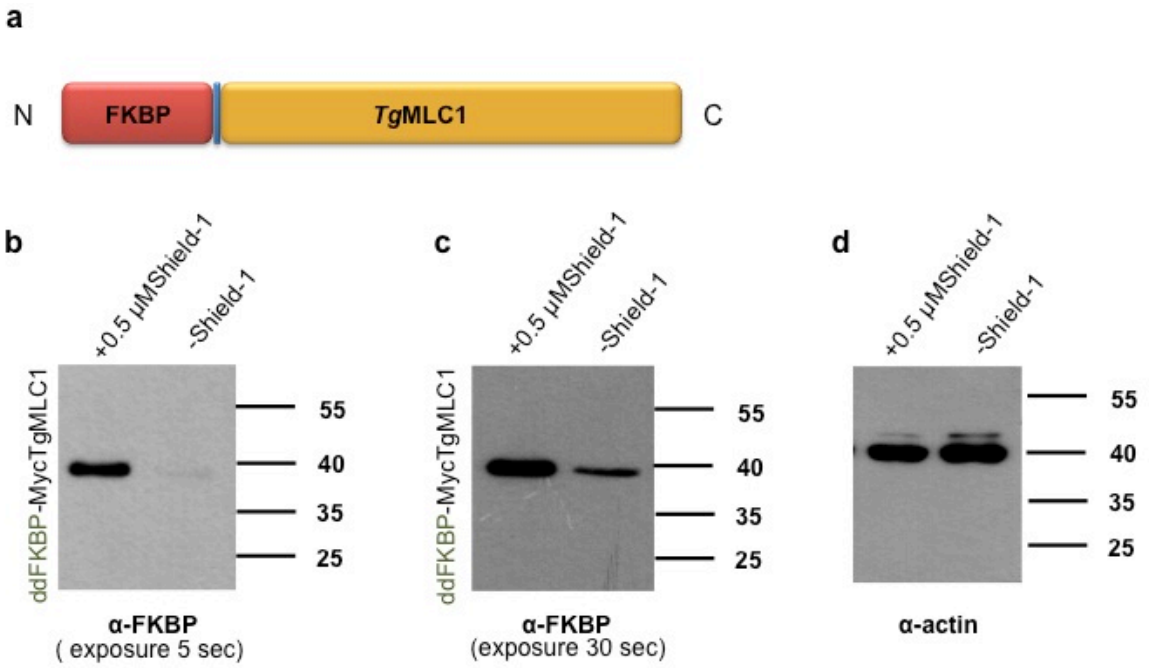

Figure 3.7 Analysis of dd-FKBP mediated destabilisation and the effect of Shield-1 on further suppression of the destabilisation of *Myc*MLC1 fusion protein.

(a) Schematic representation of dd-FKBP-Myc-MLC1 fusion protein. *Toxoplasma gondii* MLC1 is shown in dark yellow and fused to the *myc* tag on the N-terminus (shown in blue). The dd-FKBP domain is shown in red and is fused to the N-terminus of *myc*-MLC1 (b) Western blot with anti-FKBP antibody from the whole extract of parasites expressing MLC1 tagged to dd-FKBP on the N-terminus, that grew with or without shield-1 for 24 hours. Without shield-1 the fusion protein was almost completely degraded and could barely be detected. Maintenance of the same parasite line in shield-1 at a concentration of 0.5µM shows rapid increase in the protein level by several magnitudes (c) same blot exposed for a duration of 30 seconds, shows residual amount of protein without shield-1. With shield-1 protein level is significantly increased. (d) Actin level was assessed as loading control and confirmed equal loading of samples in both the lanes (+/- shield-1). $5x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

We also attempted to test a positive control of the dd-FKBP system in *Plasmodium falciparum*. For this study, the control plasmid was kindly provided by Prof. Daniel E. Goldberg. It contained a yellow fluorescent protein (YFP) fused the dd-FKBP to the N-terminus under strong *hsp86* promoter. The stabilization of the level of the fusion protein could be efficiently controlled by Shield-1 as documented earlier (Armstrong and Goldberg, 2007). Therefore, the plasmid was transfected into 3D7 parasite line (as explained under section 2.2). Unfortunately, no transgenic parasite line could be generated inspite of repeated transfection during the time frame of this work.

Therefore, taking into account the outcomes from all the dd-FKBP based approaches we can summarize that dose dependent regulation of almost no dd-FKBP tagged fusion proteins could not be achieved. Only wild type *Pf*Hsp70-1 fusion protein level could be regulated by shield-1. At the same time, the level of *Toxoplasma gondii* MLC1 (positive control) could be regulated (Table 2). Therefore, we further sought to employ a different destabilization domain derived from *E. coli* dihydrofolate reductase (ecDHFR) (Iwamoto et al., 2010) to achieve conditional tuneable regulation of protein level in the parasite.

Construct	Regulation by shield-1
HA- dd-FKBP- *Pf*WTHsp70-1	✔
HA- dd-FKBP- *Pf*DNHsp70-1	✗
HA- WT*Pf*Hsp40- dd-FKBP	✗
HA- WT*Pf*Hsp40- dd-FKBP	✗
dd-FKBP-*Myc*-TgMLC1 (control)	✔

Table 2. Summary of all the candidate and control proteins studied and their regulation conferred by shield-1

3.2 dd-ecDHFR based approaches

Recently it was reported that reported that *E. coli* dihydrofolate reductase (ecDHFR) derived destabilization domain (dd-ecDHFR) confers better instability to a tagged protein, leading to the rapid degradation by proteasome in the absence of a stabilizing ligand (Iwamoto et al., 2010). Trimethoprim (TMP) is cell permeable high-affinity ligand for ecDHFR and stabilizes fusion proteins in a dose-dependent manner.

Although, ecDHFR-derived dd functions in a similar fashion as compared to that of the dd-FKBP, there are some significant advantages of using this system (Rakhit et al., 2011). The most noteworthy advantage is that ecDHFR based dd, when fused to the C-terminus of the protein, is more efficient in destabilizing the fusion protein in compared to that of the dd-FKBP. In addition, the ligand TMP is commercially available, inexpensive, and possesses good pharmacological characteristics. Therefore, due to these advantages we further adapted this system to establish conditional knockdown of *Pf*Hsp70-1 and *Pf*hsp40.

3.2.1 Dominant negative system coupled with dd-ecDHFR to establish conditional knockdown of *Pf*Hsp70-1 in intraerythrocytic *P. falciparum*

3.2.1.1 Generation of cell lines expressing WT/DN *Pf*Hsp70-1 and GFP, N-terminally fused to dd-ecDHFR

Plasmids coding for *Pf*Hsp70-1, DN*Pf*Hsp70-1 and GFP fused to the dd-ecDHFR on the N-terminus, were generated as explained under section 2.2. Unlike in the dd-FKBP approach, the transfection of these plasmids was carried out into Plasmepsin I knockout (PM1 KO) parasite line where human DHFR (hDHFR) is integrated into the endogenous locus. Presence of hDHFR in 3D7 (PM1 KO) line confers resistance against TMP, which is otherwise toxic to the wild type 3D7. This parasite line was always cultured in the presence of 10.0nM WR99210. Post transfection, parasites were cultured under drug pressure (BSD) for 2-3 weeks for selection of plasmid carrying parasites.

3.2.1.2 Wild type *Pf*Hsp70-1 could not be destabilised by N-terminal fusion of dd-ecDHFR and addition of TMP did not yield further increase in the level of fusion protein in the intraerythocytic stages of *P. falciparum*

Cell lines expressing dd-ecDHFR-*Pf*Hsp70-1 (fig 3.8a) were cultured without or with increasing concentrations of TMP ranging upto 15.0µM for 72 hours. Post treatment, with or without TMP free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the fusion protein.

In the absence of the TMP, the fusion protein could be detected with anti-*Pf*Hsp70-1 antibody at the correct molecular mass of ~ 95kDa represents the inefficiency of dd-ecDHFR to direct degradation of DN*Pf*Hsp70-1 fusion protein (fig 3.8b). Further, we wanted to verify whether addition of TMP had any effect on fusion protein level. Therefore, same parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0µM for 72 hours. Unfortunately, no further increase in the level of fusion proteins were seen from the whole cell extract by western blot with anti-*Pf*Hsp70-1 antibody (fig 3.8b). At the same time, an additional weak signal was detected in al the lanes at ~ 80kDa. This could be a possible degradation product generated during sample preparation. Noteworthy, the endogenous

*Pf*Hsp70-1 level was found to be higher than compared to that of the fusion proteins in all the lanes (fig 3.8b). Western blot with anti-glycophorin A antibody served as loading control (fig 3.8c).

Therefore, from this experiment we could conclude that dd-ecDHFR could not mediate degradation of the WT*Pf*Hsp70-1 fusion protein and further TMP dependent regulation of protein level was not possible. At the same time, the level of episomal fusion protein was lower than that of its endogenous version. Therefore, we further tried to use this system for the dominant negative version of the *Pf*Hsp70-1 and to assess whether a TMP dependent regulation could be achieved.

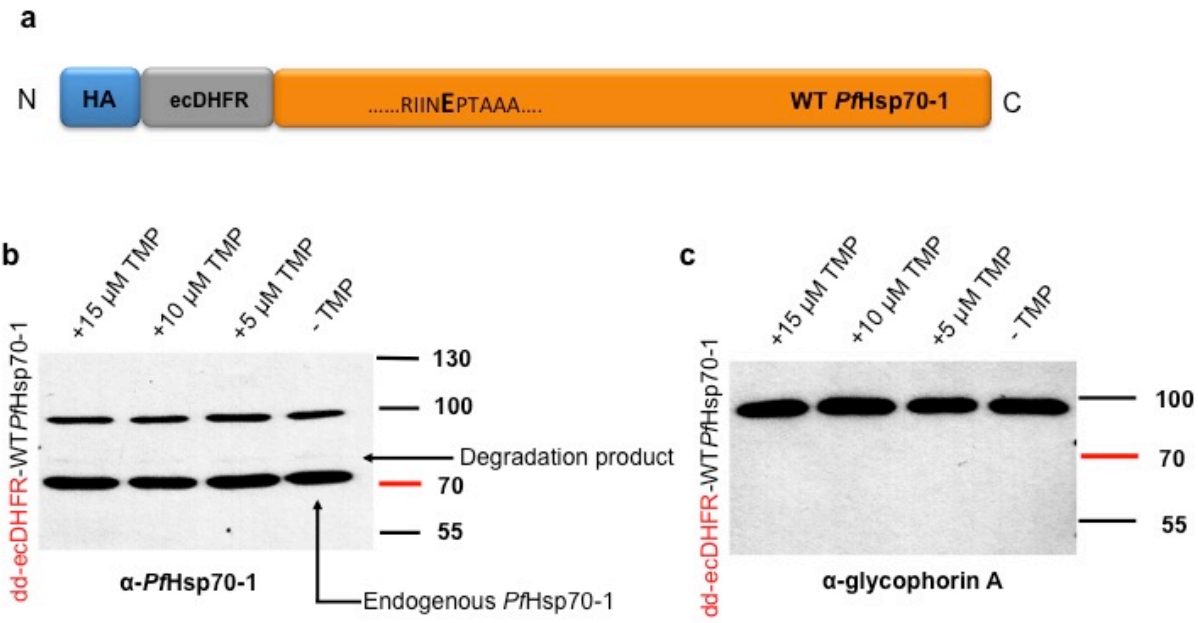

Figure 3.8 Analysis of dd-ecDHFR mediated destabilisation and the effect of Shield-1 on further suppression of the destabilisation of WT*Pf*Hsp70-1 fusion protein.

(a) Schematic representation of dd-ecDHFR-WT*Pf*Hsp70-1 fusion protein. The wild type *Pf*Hsp70-1 is shown in dark orange. The dd-ecDHFR domain is shown in grey and is fused to the N- terminus of the candidate protein. The HA tag is shown in blue at the extreme N-terminus of the fusion protein (b) Western blot analysis from the whole extract of cells expressing WT*Pf*Hsp70-1 tagged to dd-ecDHFR on the N-terminus, that grew with or without TMP for 72 hours. Without TMP the fusion protein could be detected at the expected molecular mass of ~95kDa with anti-*Pf*Hsp70-1 antibody. Maintenance of the same parasite line with TMP ranging upto 15.0 µM did not yield any increase in the level of the fusion proteins. Endogenous *Pf*Hsp70-1 was detected at least with equal or tow time stronger signal intensity than compared to that of the fusion proteins in all the lanes (+/- TMP). A weak signal is detected at ~80kDa that can be a possible degradation product (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments

3.2.1.3 Dominant negative *Pf*Hsp70-1 could not be destabilised by N-terminal fusion of dd-ecDHFR and addition of TMP did not yield further increase in the level of fusion protein in the intraerythocytic stages of *P. falciparum*

To further assess whether shield-1 dependent regulation could be achieved for DN*Pf*Hsp70-1, cell lines expressing dd-ecDHFR-DN*Pf*Hsp70-1 (fig 3.9a) were cultured without or with increasing concentrations of TMP ranging upto 15.0μM for 72 hours. Post-treatment with or without TMP, free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the fusion proteins.

In the absence of the TMP, the fusion protein could be detected with anti-*Pf*Hsp70-1 antibody at the correct molecular mass of ~ 95kDa indicating inefficiency of dd-ecDHFR to mediate degradation (fig 3.9b). Further we wanted to find out whether addition of TMP has any effect on the stabilisation of protein level. Therefore, parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0μM for 72 hours. No further increase in the level of fusion proteins were seen from the whole cell extract by western blot with anti-*Pf*Hsp70-1 antibody (fig 3.9b). In addition, a possible degradation product of the fusion protein could be detected at ~ 80kDa. Noteworthy, the endogenous wild type *Pf*Hsp70-1 level was found to be higher than compared to that of the dominant negative fusion proteins in all the lanes (fig 3.9b). The cells expressing DN*Pf*Hsp70-1 exhibited normal developmental stages indicating the absence of any dominant negative effect in the parasites. Western blot with anti-glycophorin A antibody served as loading control (fig 3.9c).

Therefore, from this experiment it could be concluded that dd-ecDHFR mediated degradation and TMP dependent regulation of DN*Pf*Hsp70-1 fusion protein was also not possible. At the same time, the level of dominant negative fusion protein was lower than that of the endogenous wild type protein. Hence the expression of the dominant negative *Pf*Hsp70-1 did not affect normal growth and development of the parasite. Therefore, we further attempted to try this system to regulate eplisomal expression of GFP as a control.

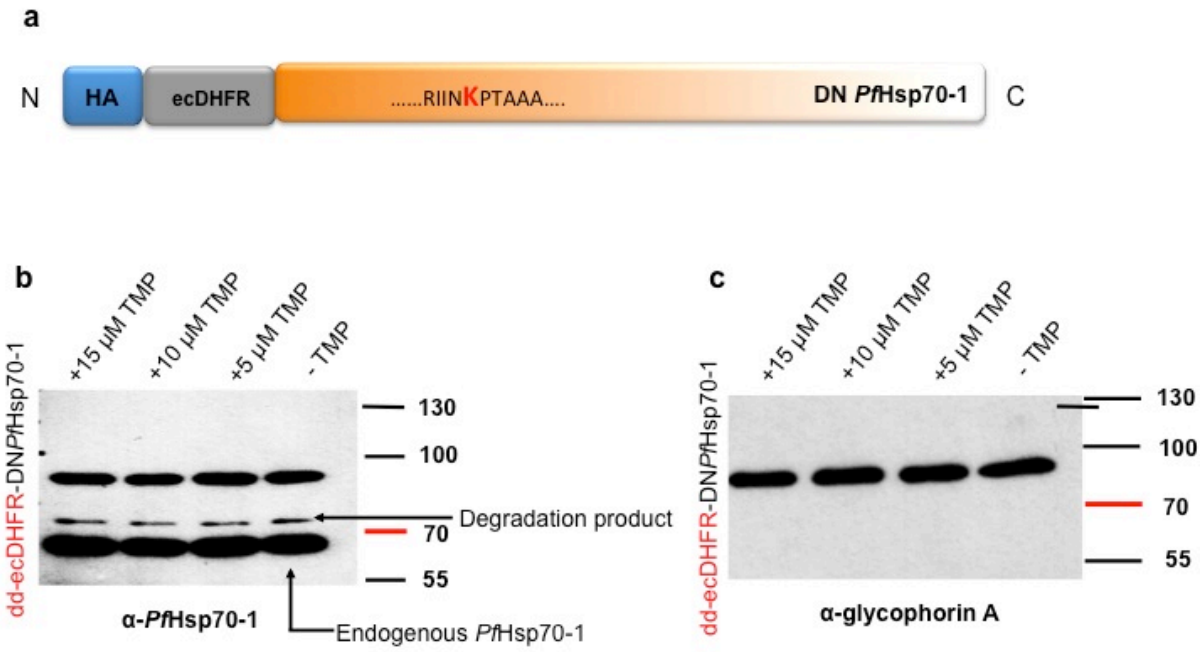

Figure 3.9 Analysis of dd-ecDHFR mediated destabilisation and the effect of Shield-1 on further suppression of the destabilisation of DN*Pf*Hsp70-1 fusion protein.

(a) Schematic representation of dd-ecDHFR-DN*Pf*Hsp70-1 fusion protein. The dominant negative *Pf*Hsp70-1 shown in dark orange with gradient and contains an E187K mutation. The dd-ecDHFR domain is shown in grey and is fused to the N- terminus of the candidate protein. The HA tag is shown in blue at the extreme N-terminus of the fusion protein (b) Western blot analysis from the whole extract of cells expressing *Pf*Hsp70-1 N-terminally tagged to dd-ecDHFR that grew with or without TMP for 72 hours. Without TMP the fusion protein could be detected at the correct molecular mass of ~95 kDa with anti-*Pf*Hsp70-1 antibody. Maintenance of the same parasite line with TMP ranging upto 15.0µM did not yield any increase in the level of the fusion proteins. An additional band at ~80kDa indicates to possible degradation product. Endogenous wild type *Pf*Hsp70-1 was detected at least with tow time stronger signal intensity compared to that of the dominant negative fusion proteins in all the lanes (+/- TMP). (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ parasite cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.2.1.4 Control GFP could not be destabilised by N-terminal fusion of dd-ecDHFR and addition of TMP did not yield any further increase in the level of fusion protein in the intraerythocytic stages of *P. falciparum*

Since we failed to achieve a TMP dependent regulation of both the wild type and dominant negative version of the *Pf*Hsp70-1 we wanted to validate the system using a GFP control. Parasite line expressing GFP tagged to dd-ecDHFR on the N-terminus (fig 3.10a) was used to study dd-ecDHFR mediated destabilization of candidate protein. At the same time, the effect of TMP on the suppression of destabilization was also tested.

For this purpose cell lines expressing dd-ecDHFR-GFP were cultured without or with increasing concentrations of TMP ranging upto 15.0µM for 72 hours in a similar way as

followed for the candidate proteins studied earlier. Post-treatment with or without TMP, free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts from the parasite were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the GFP fusion proteins.

In the absence of the TMP fusion protein could be detected with anti-HA antibody at the expected molecular mass of ~ 49kDa. This showed that dd-ecDHFR could not lead to degradation of the GFP fusion protein (fig 3.10b). Further we wanted to verify whether addition of TMP has any effect on the stabilisation of GFP level. Therefore parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0µM for 72 hours. No further increase in the level of fusion proteins were seen from the whole cell extract by western blot with anti-HA antibody (fig 3.10b). Western blot with anti-glycophorin A antibody served as loading control (fig 3.10c).

Hence, it could be concluded that dd-ecDHFR could not mediate the degradation of control GFP fusion protein. At the same time the level of GFP could not be regulated in a TMP dependent manner.

In previous studies it has been reported that C-terminal fusion of ec-DHFR has stronger destabilisation effect (Rakhit et al., 2011). Therefore we further wanted to obtain TMP dependent regulation of parasite Hsp40 homologue (PF3D7_0213100), where the ec-DHFR is fused to the C-terminus.

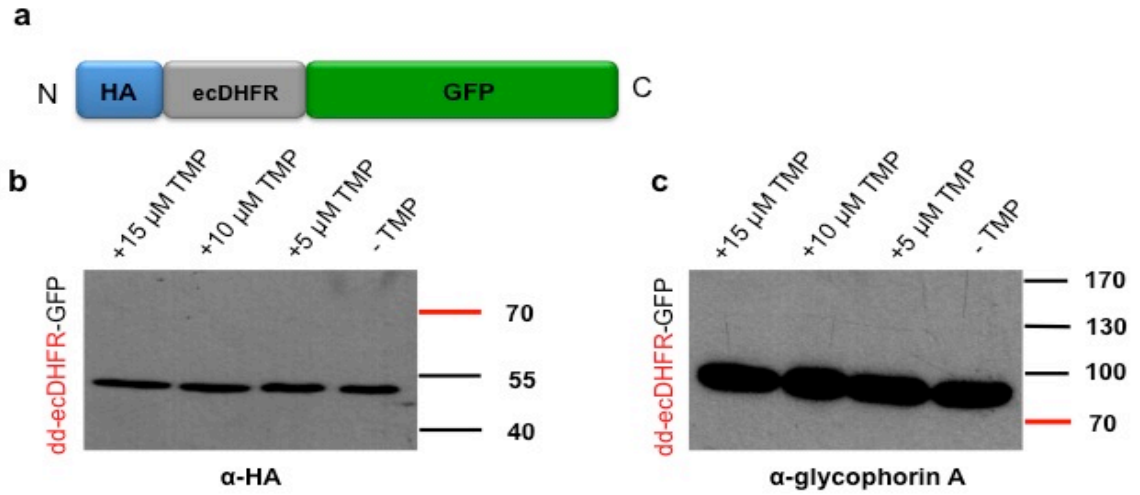

Figure 3.10 Analysis of dd-ecDHFR mediated destabilisation and the effect of Shield-1 on further suppression of the destabilisation of GFP fusion protein

(a) Schematic representation of dd-ecDHFR-GFP fusion protein. The GFP is shown in green. The dd-ecDHFR domain is shown in grey and is fused to the N-terminus of the GFP. The HA tag is shown in blue at the extreme

N-terminus of the fusion protein (b) Western blot analysis from the whole extract of cells expressing GFP tagged to dd-ecDHFR on the N-terminus, that grew with or without TMP for 72 hours. Without TMP the fusion protein could be detected at the correct molar mass of ~49 kDa with anti-HA antibody. Maintenance of the same parasite line with TMP ranging from 5.0 -15.0µM did not yield any increase in the level of the fusion proteins as protein bands were detected with same signal intensity. (c) Glycophorin level was assessed as loading control with anti- glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.2.2 Dominant negative system coupled with dd-ecDHFR to establish conditional knockdown of *Pf*Hsp40 in intraerythrocytic *P. falciparum*

3.2.2.1 Generation of cell lines expressing WT/DN *Pf*Hsp40 and GFP, C-terminally fused to dd-ecDHFR

Plasmids coding for *Pf*Hsp40 (PF3D7_0213100), DN*Pf*Hsp40 and GFP fused to the dd-ecDHFR on the C-terminus, were generated as explained under section 2.2. The transfection of these plasmids was carried out into 3D7 (PM1 KO) parasite line as used earlier for the transfection of dd-ecDHFR-*Pf*Hsp70-1 coding plasmids. Post transfection, cells were cultured under drug pressure (BSD) for 2-3 weeks for selection of plasmid-carrying parasites.

3.2.2.2 Wild type *Pf*Hsp40 could not be destabilised by C-terminal fusion of dd-ecDHFR and addition of TMP did not yield further increase in the level of fusion protein in the intraerythocytic stages of *P. falciparum*

Cell lines expressing *Pf*Hsp40-dd-ecDHFR (fig 3.11a) were cultured without or with increasing concentrations of TMP ranging upto 15.0µM for 72 hours. Post treatment, with or without TMP free parasites were isolated by saponin lysis. Following several washes of the parasite pellet, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the fusion proteins.

In the absence of the TMP fusion protein could be detected with anti-HA antibody at the correct molecular mass of ~ 59kDa (fig 3.11b). This indicated that the dd-ecDHFR was not efficient in mediating the degradation of wild type *Pf*Hsp40 when fused to the C-terminus. Further, we wanted to determine whether addition of TMP had any effect on the fusion protein level. Therefore, parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0µM for 72 hours. Unfortunately, no increase in the level of fusion proteins was seen from the whole cell extract by western blot with anti-HA antibody (fig

3.11b). The endogenous *Pf*Hsp40 level could not be determined and compared due to unavailability of a specific anti-*Pf*Hsp40 antibody. Western blot with anti-glycophorin A antibody served as loading control (fig 3.11c).

Therefore, from this experiment we could conclude that inspite of previous reports, C-terminus tagging of dd-ecDHFR could not lead to the destabilisation of *Pf*Hsp40. In addition, TMP dependent regulation of the episomal WT*Pf*Hsp40 protein level was not possible. Hence, we further wanted to try this system for the dominant negative version of the *Pf*Hsp40 and to assess whether ec-DHFR mediated degradation was achievable and could be regulated by TMP.

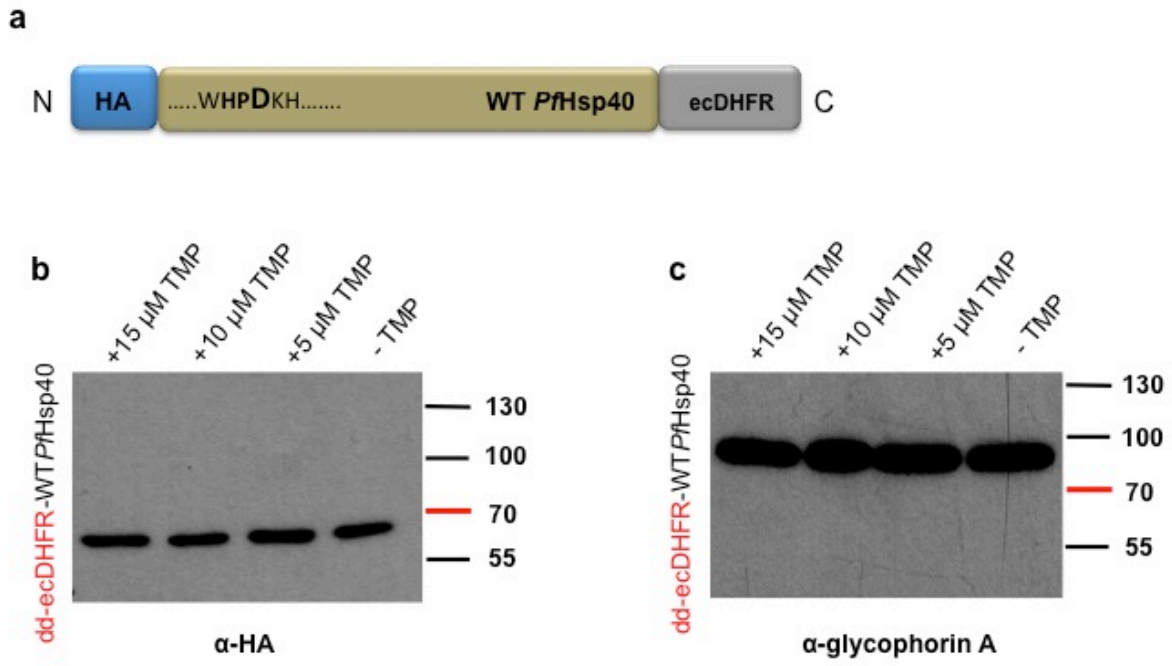

Figure 3.11 Analysis of dd-ecDHFR mediated destabilisation and the effect of TMP on further suppression of the destabilisation of WT*Pf*Hsp40 fusion protein.

(a) Schematic representation of WT*Pf*Hsp40-dd-ecDHFR fusion protein. The WT*Pf*Hsp40 is shown in olive green. The dd-ecDHFR domain is shown in grey and is fused to the C-terminus of the candidate protein. The HA tag is shown in blue at the extreme N-terminus of the fusion protein (b) Western blot analysis from the whole extract of cells expressing WT*Pf*Hsp40, C-terminally tagged to dd-ecDHFR maintained with or without TMP for 72 hours. Without TMP the fusion protein could be detected at the correct molecular mass of ~59kDa with anti-HA antibody. Maintenance of the same parasite line with TMP ranging upto 15.0µM does not show any increase in the level of the fusion protein. (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.4.2.3 Dominant negative *Pf*Hsp40 could not be destabilised by C-terminal fusion of dd-ecDHFR and addition of TMP did not yield further increase in the level of fusion protein in the intraerythocytic stage of *P. falciparum*

Cell lines expressing DN*Pf*Hsp40-dd-ecDHFR (fig 3.12a) were cultured without or with increasing concentrations of TMP ranging upto 15.0µM for 72 hours. Post treatment with or without TMP free parasites were isolated by saponin lysis. Following several washes of the parasite pellets, soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the fusion protein.

In the absence of the TMP fusion protein could be detected with anti-HA antibody at the correct molecular mass of ~ 59 kDa that (fig 3.12b). This indicated that the dd-ecDHFR was not efficient in mediating the degradation of the dominant negative version of *Pf*Hsp40 when fused to the C-terminus. Further, we wanted to determine whether addition of TMP has any effect on the protein level. Therefore, parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0µM for 72 hours. No increase in the level of fusion proteins was seen from the whole cell extract by western blot with anti-HA antibody (fig 3.12b). Although, the endogenous *Pf*Hsp40 level could not be determined and compared due to unavailability of a specific anti-*Pf*Hsp40 antibody, the parasites exhibited normal developmental stages indicating the absence of any dominant negative effect in the parasites. Western blot with anti-glycophorin A antibody served as loading control (fig 3.12c).

Therefore, we could conclude that similar to the WT*Pf*Hsp40, C-terminus tagging of dd-ecDHFR to the DN*Pf*Hsp40 also could not lead to the destabilisation of the fusion proteins. At the same time, TMP dependent regulation of the DN*Pf*Hsp40 protein level was not possible. In addition, expression of a dominant negative version of the endogenous *Pf*Hsp40 did not affect the normal growth and developmental cycle of the parasite. Therefore, we further attempted to test the applicability of the system towards the regulation of the level of control GFP.

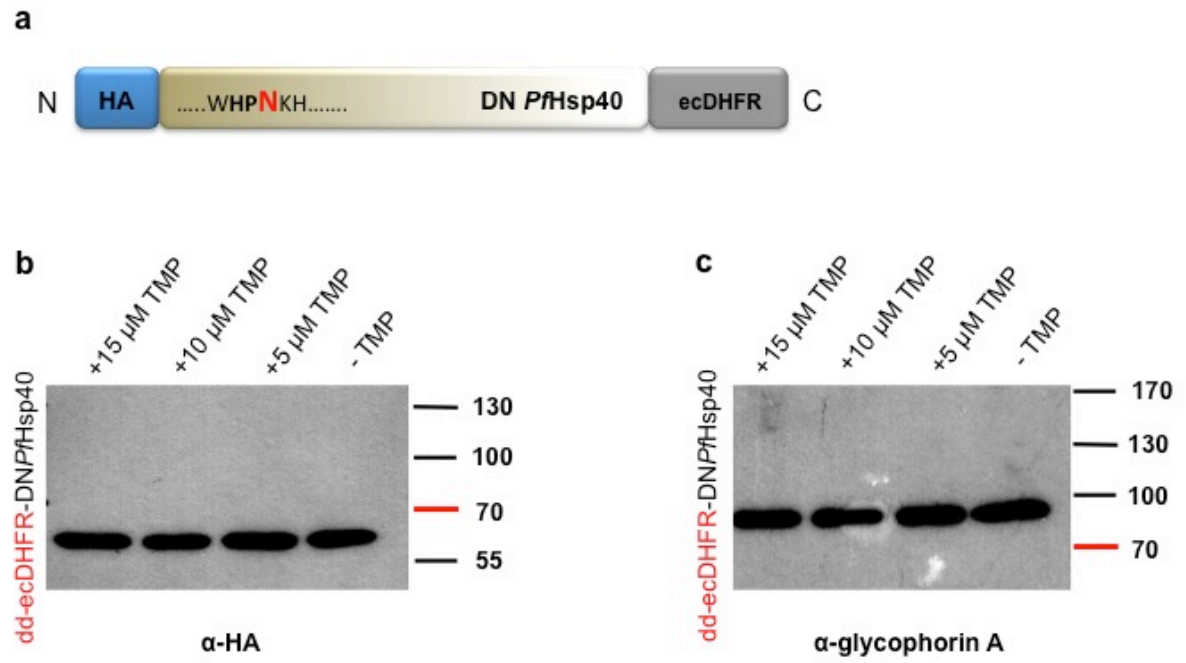

Figure 3.12 Analysis of dd-ecDHFR mediated destabilisation and the effect of Shield-1 on further suppression of the destabilisation of DN*Pf*Hsp40 fusion protein

(a) Schematic representation of DN*Pf*Hsp40-dd-ecDHFR fusion protein. The DN*Pf*Hsp40 is shown in olive green with gradient containing a D34N mutation. The dd-ecDHFR domain is shown in grey and is fused to the C-terminus of the candidate protein. The HA tag is shown in blue at the extreme N-terminus of the fusion protein (b) Western blot analysis from the whole extract of parasites expressing DN*Pf*Hsp40, C-terminally tagged to dd-ecDHFR, maintained with or without TMP for 72 hours. Without TMP the fusion protein could be detected at the expected molecular mass of ~59kDa with anti-HA antibody. Maintenance of the same parasite line with TMP ranging upto 15.0µM did not yield any increase in the level of the fusion proteins. (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

3.4.2.4 Control GFP could be efficiently destabilised by C-terminal fusion of dd-ecDHFR and addition of TMP yielded a robust dose dependent stabilisation of fusion protein in the intraerythocytic stages of *P. falciparum*

Since we failed to achieve a TMP dependent regulation of both the wild type and dominant negative version of the *Pf*Hsp40 when tagged to the C-terminus, we wanted to validate the system using a GFP control. Parasite line expressing GFP tagged to dd-ecDHFR on the C-terminus (fig 3.13a) was used to study dd-ecDHFR mediated destabilization and degradation. At the same time, the effect of TMP on the suppression of destabilization was tested.

For this reason, cell lines expressing GFP-dd-ecDHFR were cultured without or with increasing concentrations of TMP ranging upto 15.0µM for 72 hours in a similar way as explained for the candidate proteins studied earlier. Post treatment with or without TMP free parasites were isolated by saponin lysis. Following several washes of the parasite pellets,

soluble protein extracts were obtained by cell lysis (described under section 2.2). Samples were analysed by SDS-PAGE and western blot to determine the effect of TMP on the stabilization of the fusion protein.

In the absence of the TMP, GFP fusion protein was efficiently degraded leaving behind residual amount that could barely be detected with anti-HA antibody (fig 3.13b). Further we wanted to verify whether addition of TMP had any effect on the stabilisation. Therefore, parasites were treated with increasing concentrations of TMP ranging from 5.0 - 15.0µM for 72 hours. This resulted in a robust increase in the level of episomal fusion protein. At the same time the regulation conferred by TMP was clearly dose dependent. The fusion protein could barely be detected in the absence of TMP and the level reached to the maximum at a TMP concentration of 15.0µM (fig 3.13b). Western blot with anti-glycophorin A antibody served as loading control (fig 3.13c).

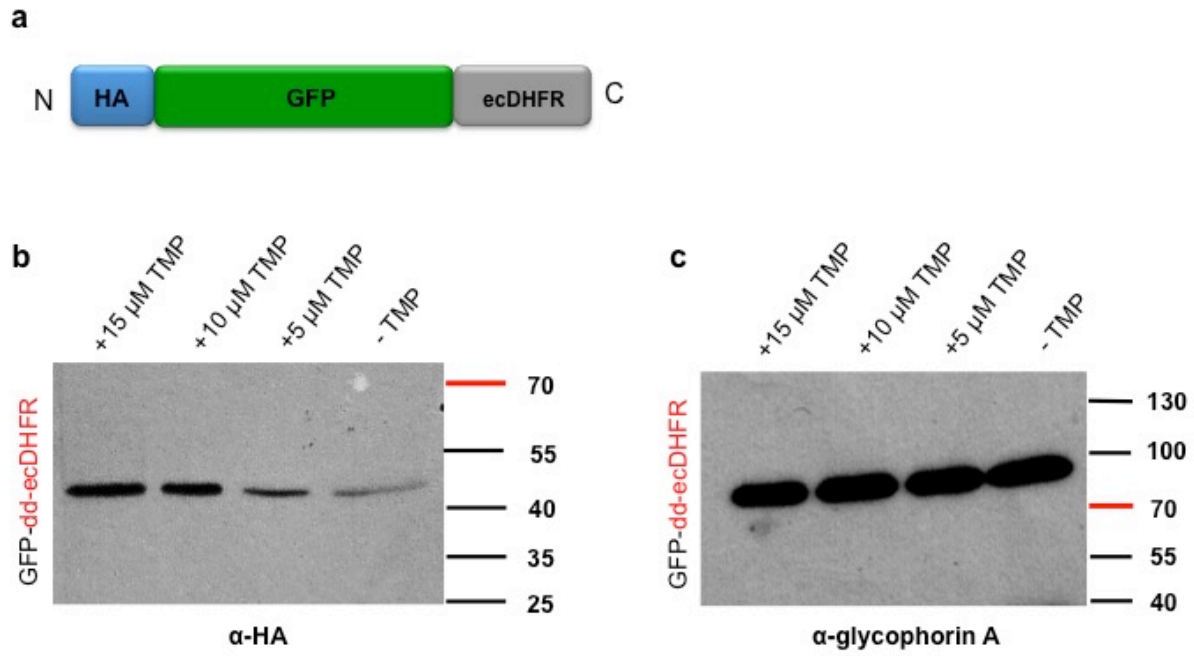

Figure 3.13 Analysis of dd-ecDHFR mediated destabilisation and the effect of TMP on further suppression of the destabilisation of GFP fusion protein.

(a) Schematic representation of GFP-dd-ecDHFR fusion protein. The GFP is shown in green. The dd-ecDHFR domain is shown in grey and is fused to the C-terminus of the candidate protein. The HA tag is shown in blue at the extreme N-terminus of the fusion protein (b) Western blot analysis from the whole extract of cells expressing GFP, C-terminally tagged to dd-ecDHFR and maintained with or without TMP for 72 hours. Without TMP the fusion protein could barely be detected at the correct size of ~49kDa with anti-HA antibody. Maintenance of the same parasite line with TMP ranging from 5.0 µM upto 15.0 µM shows a dose dependent increase in the level of the fusion proteins (c) Glycophorin level was assessed as loading control with anti-glycophorin A antibody, and indicted equal loading of samples in all the lanes (+/- TMP). $1x10^7$ cell equivalents were used for all the western blot analysis. Similar results were obtained in three independent experiments.

After confirming the TMP dependent regulation of GFP-dd-ecDHFR by western blot we wanted carry out a secondary verification of the observation. Hence, live parasites expressing GFP-dd-ecDHFR, were treated with or without TMP (15.0µM) for 24 hours as mentioned above. Post treatment live parasites were visualized under the fluorescence microscope as explained under section 2.2. Consistent with the data form SDS-PAGE, the residual GFP signal could barely be detected from parasite line cultured in the absence of TMP (fig 3.14a). Whereas, robust and significant increase in the cytoplasmic GFP level was observed in the parasite cultured in the presence of 15.0µM TMP (fig 3.14b) indicating efficient stabilization.

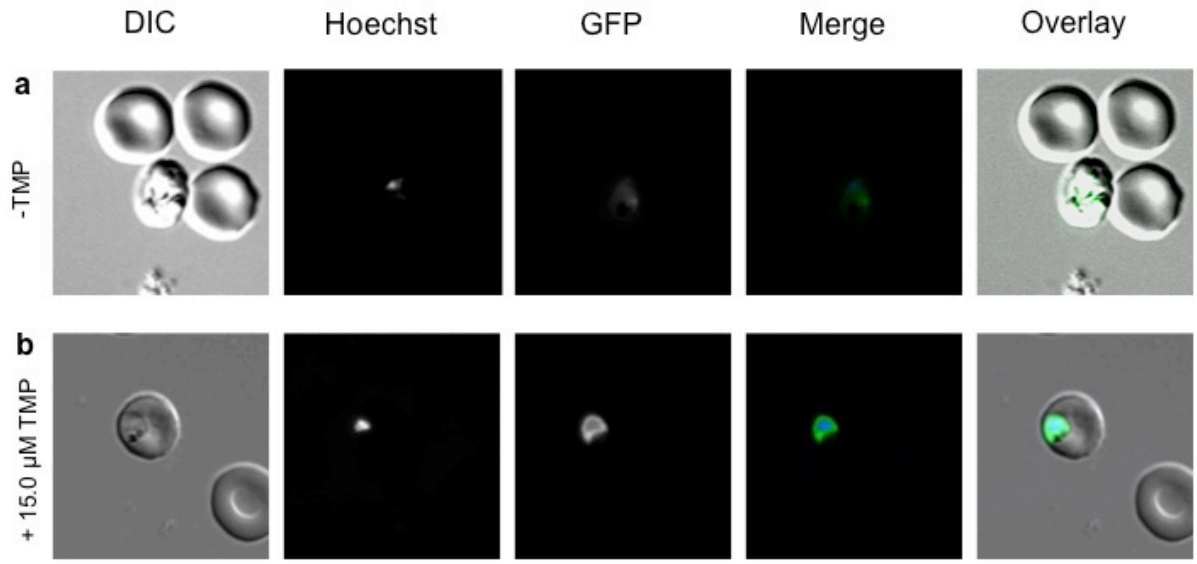

Figure 3.14 Fluorescent microscopy of live parasites treated without or with TMP.

(a) TMP untreated parasites labelled with hoechst showing barely detectable residual GFP signal in the cytoplasm. (b) 15.0 µM TMP treated parasites labelled with hoechst showing strong recovery of GFP signal in the cytoplasm.

Therefore, from this experiment it could be concluded that unlike the N-terminal fusion, C terminal fusion of dd-ecDHFR to the GFP leads to almost complete degradation of the fusion protein. Furthermore the stability can be efficiently reverted in a dose dependent manner by the addition of TMP.

To conclude, we could not establish a dose dependent regulation of almost all episomally expressed dd-ecDHFR tagged candidate proteins. The level of control GFP fused to dd-ecDHFR on the C-terminus could only be regulated by TMP amongst all proteins studied (table 3).

Construct	Regulation by TMP
HA- dd-ecDHFR- *Pf*WTHsp70-1	✗
HA- dd-ecDHFR- *Pf*DNHsp70-1	✗
HA- dd-ecDHFR- GFP	✗
HA- WT*Pf*Hsp40 - dd-ecDHFR	✗
HA- WT*Pf*Hsp40 - dd-ecDHFR	✗
HA- GFP - dd-ecDHFR	✔

Table 3. Summary of all the candidate and control proteins studied and their regulation conferred by TMP

4. Discussion

Plasmodium falciparum is the causative agent of the most lethal form of malaria resulting in death of millions of people worldwide. Growing resistance of the parasite against effective and available drugs is the biggest concern towards the treatment and successful eradication of the disease. Therefore, further understanding of essential genes and their mode of action at different stages of the parasite life cycle will strengthen our knowledge of the parasite biology. This in turn holds enormous potential for the development of efficacious antimalarial drugs and vaccines.

Unfortunately, the experimental validation of the function of essential gene in blood stage *Plasmodium falciparum* is posed with several challenges (Meissner et al., 2007). *Plasmodium falciparum* haploid genome during intraerythrocytic stage has only allowed mutagenesis to be carried out only for non-essential genes (Maier et al., 2008, Cowman and Crabb, 2006). For example, erythrocyte invasion of the parasite has typically been studied via the classical reverse genetics methods. Since invasion of erythrocyte by the parasite involves multiple pathways and ligands, none of which are completely essential (Cowman and Crabb, 2006) mutation of one of the ligands does not prove to be lethal for the parasite. Essential genes required during the different stages of life cycle other than the blood stage could be successfully studied by introducing a mutation in the gene at the blood stage (where it is not expressed) and then passing it through the mosquito and liver stage (where it is expressed) to establish phenotype (Ishino et al., 2005, van Dijk et al., 2005, van Dijk et al., 2001). Nevertheless it is the blood stage where the manifestation of the disease occurs, giving rise to the symptoms of malaria. Till date the function of several essential proteins during the blood stage has been severely hampered due to various reasons.

In modern experimental biology the function of an essential gene is often determined by perturbation of the gene of interest with several methods following the observation and analysis of the resulting phenotype. This is often achieved by RNA interference (RNAi) that has become a widely acceptable and favourable approach to determine the function of putative proteins and pathways (Medema, 2004, Banaszynski et al., 2006). Among parasitic protozoa RNAi is extensively used with success in *Trypanosoma brucei* to assess the function of several proteins and their pathways. From direct experimentation it is becoming prominent

that the RNAi pathway is also likely to be functional in *T. gondii, E. histolytica* and *G. intestinalis* and *Leishmania* (Ullu et al., 2004, Kolev et al., 2011, McCarthy et al., 2013).

Extensively used gene silencing approaches including RNAi are yet to be validated in *Plasmodium* (Agop-Nersesian et al., 2008). Specifically RNAi appears to be of limited use since *Plasmodium* does not code for many essential protein for the RNAi approach to be functional (Baum et al., 2009).

On the other hand, one by one gene knockout approach has often been exploited to assess the functionality of such genes in blood stage malaria parasite but, yielded more general information on the protein function (Cowman and Crabb, 2006). Since the intraerythrocytic stage of *Plasmodium* is haploid, gene knockout by homologous recombination proved to be simple and straightforward. But, if the gene is essential for the parasite and indispensible for the blood stage growth knockout of such gene could prove to be lethal. Therefore, to overcome this challenge there has been huge amount of studies to develop genetic tools that can conditionally downregulate the expression level of the gene or can lead to mutagenesis (Collins et al., 2013, Meissner et al., 2007).

One such technique known as Tetracyline inducible gene expression or TetR has been used to study essential genes in *Plasmodium*. Commonly used tetracycline sensitive transactivator does not activate minimal promoter in apicomplexan parasite like *Plasmodium falciparum* (Meissner et al., 2001, Collins et al., 2013). An artificial transactivation domain (TATi), fused to TetR has been used in distant apicomplexan *Toxoplasma gondii* with great success to obtain conditional knockout (Meissner et al., 2002). This system has been applied with some success in *Plasmodium* to achieve regulatable expression of genes (Meissner et al., 2005, Gilson et al., 2008).

Site-specific recombination event using Cre recombinase is widely used and Cre was found to be active and working in *Plasmodium falciparum* (O'Neill et al., 2011). But, unfortunately this system cannot be regulated in a robust manner. The most noteworthy disadvantage is that, this system is irreversible and hence, cannot be applied for the study of essential genes. In this approach the excision of the gene using Cre recombinase would have lethal effect on the growth of the parasite making it impossible to pursue further study.

For the limitations mentioned above we employed an alternative strategy to analyse the importance of heat shock protein, *Pf*Hsp70-1 and *Pf*Hsp40 which we predicted to have an

essential role in the intraerythrocyitc stage of the parasite. Functional assesment of an essential gene is often carried out by the overexpression of a catalytically dead dominant negative version of the endogenous protein. This method involves introducing a mutation into the protein of interest that results in the abolishment of its function and at the same time inhibits the function of the wild type endogenous protein. The phenotypic consequences resulting from such expression of the dominant negative version not only helps to determine the importance of the gene of interest but also shed light on the pathways where the protein might be involved. The functions of a number of proteins have been determined using this approach. But, this method is most effective for elucidating the function of proteins that requires forming multimers in the cell to be functional for example, Heat shock proteins (Hsps) in our case. Dominant negative studies using mutant proteins have already been used as a successful tool to determine the function of a number of protein families including oncogenes, growth factor receptors, hormone receptors etc (Sheppard, 1994). This system has been already applied successfully to determine the function of several proteins in *Toxoplasma gondii* (Herm-Gotz et al., 2007), and blood stage *Plasmodium falciparum* (Russo et al., 2010).

To determine the essential role of the *Pf*Hsp70-1 and *Pf*Hsp40 we generated and transfected expression constructs expressing either dominant negative version or wild type form (control) of the candidate proteins episomally under the control of a *crt* promoter. *crt* promoter ensures the expression of the fusion proteins throughout all the stages of asexual life cycle. We further coupled this approach with destabilisation domain strategy. This is a recently developed conditional genetic tool to control gene expression in an exogenous manner. This is a system where we fused the candidate proteins to the C- or N-terminus with destabilisation domains (either FKBP derived L106P or ecDHFR) (Armstrong and Goldberg, 2007, Herm-Gotz et al., 2007, Iwamoto et al., 2010). Generally, such domain leads to the degradation of the entire fusion protein by the proteasome. But, the reversible binding of the synthetic ligands (shield-1 or TMP) should stabilize the protein, allowing it to carry out its biological function. Thus, a dose dependent regulation of protein level by this method should be possible. This approach has already been applied with some success to study the function of essential proteins in *Leishmania, Toxoplasma gondii* and *Plasmodium falciparum* (Madeira da Silva et al., 2009, Armstrong and Goldberg, 2007, Dvorin et al., 2010, Gershlick, 1990, Herm-Gotz et al., 2007).

4.1. dd-FKBP mediated degradation of episomally expressed fusion protein?

During this study our observations have confirmed several limitations of the dd-FKBP system to efficiently regulate the level of episomally expressed fusion protein. Therefore, such limitations hindered the conditional knockdown of the candidate proteins.

We expressed both the dominant negative *Pf*Hsp70-1 and *Pf*Hsp40 tagged to dd-FKBP domain under the control of *crt* promoter. We expected an episomal overexpression of the dominant negative *Pf*Hsp70-1 in the parasite line. In normal condition, the dd-FKBP should lead to the destabilization of the entire fusion protein resulting in proteasome mediated rapid degradation, leaving none or insignificant amount of fusion protein behind. Similar observation was also expected for dominant negative *Pf*Hsp40-dd-FKBP fusion. It was reported previously that dd-FKBP fusion protein is completely degraded in the absence of the stabilizing ligand and therefore certified the applicability of this system for studying the function of native plasmodial proteins (Armstrong and Goldberg, 2007, Herm-Gotz et al., 2007). In fact, L106P mutant of dd-FKBP, used for our study was claimed to be one of the most potent (Destabilisation domain) DDs identified during initial screening (Chu et al., 2008).

Since, *Pf*Hsp70-1has been predicted to interact with *Pf*Hsp90 or *Pf*HOP and many other substrate proteins via the long C-terminus EEVD motif, we tagged both the wild type and dominant negative *Pf*Hsp70-1 on the N-terminus with the dd-FKBP domain to avoid interrupting the native interaction of the *Pf*Hsp70-1 in the cell (Gitau et al., 2012, Banumathy et al., 2003, Banerjee et al., 2012) . Whereas, the *Pf*Hsp40 was tagged at the C-terminus of the destabilisation domain to avoid interfering with the possible HPD mediated interaction with partner *Pf*Hsp70-1 (Fan et al., 2003). According to previous publications, dd-FKBP domain when fused to the N-terminus of the *Pf*Hsp70-1 should establish stronger rapid degradation kinetics. It was also mentioned that the efficiency of L106P mutant of dd-FKBP to degrade YFP and other fusion proteins from the N-terminus was much higher compared to that from the C-terminus (Chu et al., 2008, Banaszynski et al., 2006). But in contrast, during our study we could detect significant amount of wild type and dominant negative *Pf*Hsp70-1 fusion protein in the absence of the shield-1 (fig.3.1b, 3.1d, fig.3.2b, 3.2c and fig.3.3b, 3.3c). Similar observation was also made for *Pf*Hsp40 expressing transgenic lines where strong

protein level was detected from the cells in the absence of the shield-1 (fig.3.4b, 3.4d, and 3.5b, 3.6b).

Several possible interpretations can be put forward to explain the contrasting observation in our work. Firstly, the rapid degradation kinetics of this system has been reported in several literatures. But, at the same time such a system failed to efficiently degrade some candidate proteins. Therefore, the success of this strategy remains greatly protein dependent (Armstrong and Goldberg, 2007, Herm-Gotz et al., 2007). Although destabilisation domain has been used to test the function of several essential proteins this was the first attempt to tag parasite Hsps. Secondly, Hsps play an important role in stabilisation of unfolded precursor polypeptide, the maintenance of protein homeostasis in the cell in unfavourable conditions, and represent an important class of protein family for parasites. Therefore, destabilisation and degradation of such an important cytosolic protein might prove to be more challenging (Bell et al., 2011, Hartl et al., 1992). Our data reflects that the destabilization of Hsps in *Plasmodium* is not efficient compared to previous studies with other candidate proteins. In a recent study it was suggested that detection of fusion protein in the absence of shield-1 might be a direct impact of the less active protein degradation in *Plasmodium* (de Azevedo et al., 2012). *Pf*Hsp70-1 is one of the most abundant and essential proteins constitutively expressed in the parasite. As suggested by de Azevedo *et al*., (2012) at an optimum level the proteasome might not be capable of complete degradation of the fusion protein as it might be burdened with a pool of fusion proteins and other proteins from the cell. Hence, a significant amount of the fusion protein is left behind. In our study, study we synchronised transfected 3D7 parasite line expressing the WT or DN *Pf*Hsp70-1/ *Pf*Hsp40 to ring stage and then maintained them with or without shield-1. Since the *crt* promoter mediated episomal expression of the protein is constitutive, the residual amount of *Pf*Hsp70-1 (fig.3.2b, 3.2c and fig.3.3b, 3.3c) and *Pf*Hsp40 (fig. 3.5b and 3.6b) escaping proteosomal degradation might have been accumulated which was detected with western blot from the whole parasite lysate.

While we are assuming that a significant amount of protein was either not destabilised or escaped proteasomal degradation, we cannot rule out the possibility of the dd-FKBP domain having no effect altogether on the destabilisation of the tagged protein. In such an event the amount of protein detected from the whole parasite lysate with western blot at a particular time point is not a part but the whole pool of fusion protein expressed episomally by the plasmid. Since we know that destabilisation domain mediated degradation of the fusion

protein is proteasome dependent, therefore blocking the proteasome by several proteasome inhibitor should compromise the dd-FKBP mediated degradation. This will shed light on very important data whether or not dd-FKBP is engaged in the degradation of the fusion protein to any extent (Gantt et al., 1998, Czesny et al., 2009, Kreidenweiss et al., 2008). If the dd-FKBP domain is involved to some extent in directing the tagged fusion protein to proteasome mediated degradation, the inhibition of the protesome should lead to the blockage of the degradation which should be reflected by an increase in the level of fusion protein in the cell. At the same time we expect to see further increase in the reporter level after adding shield-1.

In addition, one of the initial experiments carried out during our study could provide some information about the involvement of dd-FKBP in degradation of the candidate proteins. We generated several control plasmids where we replaced the dd-FKBP domain with GFP (fig. S2 and S3). We loaded equal amount of parasite equivalence from both transgenic line expressing the same candidate proteins tagged to either dd-FKBP or GFP. In SDS PAGE we did not see any noticeable difference in the level of the fusion protein expression without shield-1 between two transgenic lines. If the dd-FKBP was involved in mediating degradation of the fusion protein, we would have expected to see a lower level of fusion protein detected from the transgenic parasite line where candidate protein was fused to dd-FKBP compared to the one where protein was tagged to GFP. Since we do not see any significant difference in the level of fusion protein we can argue that dd-FKBP might not be efficiently involved in mediating degradation. But, these experiments also have some drawbacks. Firstly, since the fusion protein under the control of dd-FKBP and GFP were collected from two separate parasite line, therefore the level of the fusion protein was not really comparable because the rate of expression of the episomal plasmid in the two different transgenic lines might differ. Secondly, equal level of fusion protein indicates that protein might not be efficiently degraded by the proteasome, but the dd-FKBP domain might be still destabilising the fusion protein. This can be further clarified using a proteosome inhibitor as explained earlier.

Although the destabilization domain confers instability to the protein when tagged to either N or C-terminus, N-terminal tagging establishes more destabilizing effect when compared to that of C-terminal fusion. This might indicate that the degradation machinery of the cell can recognize the protein domain more efficiently when the dd-FKBP is fused to the N –terminus (Chu et al., 2008). Since in all our plasmids we had a 3x hemagglutinin (HA) tag fused to the extreme N-terminus of the fusion protein we speculate whether this interrupts the dd-FKBP

mediated destabilization finally leading to degradation. In our study, *Pf*Hsp70-1 (WT/DN) expressing plasmids have the HA tag at the N-terminus followed by the FKBP domain and then the candidate protein at the C-terminus (fig.2.2a and fig.2.2b) unlike published data where the dd-FKBP domain is present at the extreme N- or C-terminus of the fusion protein (Armstrong and Goldberg, 2007, de Azevedo et al., 2012). Therefore, the position of the dd-FKBP might have affected efficient destabilization. But we do not see dd-FKBP dependent degradation of the fusion protein for the *Pf*Hsp40 fusion constructs where the dd-FKBP tag is present at the extreme C-terminus of the fusion protein (fig.2.3a and fig.2.3b). Therefore, it rules out the possibility that the presence of HA tag is responsible for the inefficiency of the dd-FKBP domain to direct degradation. As we did not see any significant degradation of the fusion protein by tagging it to dd-FKBP, one can assume that the destabilization effect of the dd-FKBP might be strengthened by tagging several destabilization domains to the N- or C-terminus of the candidate proteins. But it has already been shown by Chu *et al*., (2008) that fusion of a series of destabilizing domains on either end of the protein of interest decreases rather than increasing the effectiveness of the system (Chu et al., 2008). To conclude, it has been shown by a recent systematic analysis of the FKBP derived DD domains by Azevedo *et al*., (2012) that regulation offered by DD domain is generally low in *Plasmodium* (de Azevedo et al., 2012).

4.2. Dominant negative variants of *Plasmodium* Hsps do not establish negative phenotypes in ligand dependent manner?

The destabilization domain strategy attracted us to exploit the system to investigate protein function due to its claimed applicability to specifically block the native *in vivo* function of a protein in a reversible fashion. This aim can be achieved by two possible approaches: 1) conditional knockdown of the endogenous protein leading to the expression of the endogenous protein in relatively low non function level; or 2) small molecule regulated episomal over expression of a dominant negative variant of the endogenous protein that can interfere and inhibit the function of native protein by targeting and occupying its ligand and substrates in the cell. We adapted the later approach for our study.

As already discussed we wanted to determine the essential role of the two abundantly expressed cytosolic heat shock proteins in *Plasmodium*; *Pf*hsp70-1 and *Pf*Hsp40. Dominant

negative mutation in the *Pf*Hsp70-1 was generated by substituting an important amino acid (E) in the ATPase domain (refer to section 2.2.1.1.4.1 and fig. S1). This was based on a similar mutational analysis in *E. coli* DnaK (Buchberger et al., 1994). As mentioned earlier, the E187K mutation is predicted to interfere with the interdomain movement of the *Pf*Hsp70-1 required for the coupling of the ATPase activity with the substrate binding that is important for proteolytic activity of the protein. On the other hand, *Pf*Hsp40 was mutated in the signature J domain. D34N mutation in the highly conserved HPD motif (refer to section 2.2.1.1.4.2) should disrupt the interaction of *Pf*Hsp40 with *Pf*Hsp70-1 (provided they interact and function together in the cell) leading to the development of a dominant negative phenotype. But in contrast to our predictions, episomal expression of both the dominant negative versions of the *Pf*Hsp70-1 and *Pf*Hsp40 under the control of *crt* promoter (fig.3.3b and 3.6b) does not affect the normal phenotype, growth or the progression of the development stages of the transgenic parasites. There could be several possible reasons that could contribute to our inability to establish a dominant negative effect in the parasite lines. In the three following sections possible interpretations will be discussed in detail.

4.2.1. Shield-1 mediated suppression of destabilisation is not sufficient

The point behind using the destabilisation domain approach was that the protein level can be regulated by adding a small ligand known as shield-1 to the cell. In a previous study, using mammalian cells, and apicomplexan like *Toxoplasma* and *Plasmodium* dd-FKBP tagged proteins could be efficiently regulated by the addition of shield-1 (Chu et al., 2008, Herm-Gotz et al., 2007, Armstrong and Goldberg, 2007). In an ideal situation one would expect to see no or negligible amount of protein in the absence of the shield-1 that should increase by several folds upon addition of shield-1. But, during this study a significant amount of fusion proteins (both dominant negative and wild type) was detected even in the absence of the shield-1 as explained earlier. Now if we consider that dd-FKBP mediated degradation of fusion protein is active, then we must have seen some increase in the level of fusion protein in the cell after the addition of shield-1. The only observation that is in agreement with this expectation came for the experiment with the wild type *Pf*Hsp70-1 fused to dd-FKBP (refer to fig.3.3.b and fig.3.2.c). The fusion protein could be stabilised to some level by the addition of 1.0 μM shield-1. Firstly there was only one fold increase in the level of the fusion protein and secondly the level of the endogenous *Pf*Hsp70-1 was higher by several folds than that of

the fusion protein. We did not expect to see a dominant negative effect in this transgenic line since the parasites were expressing the wild type fusion protein. But, the most disappointing observations were made in the parasite line expressing either of DN*Pf*Hsp70-1, WT*Pf*Hsp40 and DN*Pf*Hsp40 (fig.3.3b, 3.3c, fig.3.5b, and fig.3.6b simultaneously). Treatment of the transfectant lines with as high as 1.5μM shield-1 for 72 hours did not yield any further increase in the level of the fusion protein. There was no effect on the normal phenotype of the cell expressing either of dominant negative *Pf*Hsp70-1 or *Pf*Hsp40. We need to consider that in our experimental model we have kept the endogenous protein intact therefore upon addition of shield-1 we needed to obtain a level of the dominant negative version that could override the function of the endogenous protein. But, if we compare the level of endogenous with that of the dominant negative fusion protein after the addition of shield-1 we observed that the level of episomally expressed dominant negative protein is lower than that of the endogenous one (fig.3.3b). In previous studies it has been shown that only 0.5μM shield-1 could supress the destabilisation of fusion protein in *Plasmodium* (Armstrong and Goldberg, 2007). If we consider that the fusion protein was unstable, the inability to supress the destabilisation could be an outcome of highly strong destabilisation of the fusion protein. In fact, experiments with different FKBP derived dd domain was carried out by de Azevedo *et al*., (2012). In this study from transient and stable transfectants it was shown that the entire FKBP derived dd domain including L106P was quite sufficient in the destabilization. But the drawback is that none of these dd could be stabilized efficiently by the addition of as high as 1.0μM shield-1. Therefore, the reporter activity could not be reverted. This confirmed that the instability of the several dd domains could not be supressed by adding the ligand. The authors also commented that the efficiency of destabilisation and the robust range of induction in *Plasmodium* are much lower in comparison to other cell types. While 1.0μM shield-1 could increase protein level to 20 fold in other cell types, only 4-5 fold increase in the protein level was observed in *Plasmodium*. In the same study it was also shown that longer induction with shield-1 does not have any significant effect. Higher shield-1 concentration was tried too with no improvement. While increasing the shield-1 concentration it needs to be considered that shield-1 is toxic for the parasite. Although shield-1 concentration at or higher than 1.0 μM does not affect the growth yet it delays the development of the parasite. This needs to be considered while interpreting the resulting phenotype. Therefore, shield-1 concentration cannot be increased further to supress stabilization as it could prove to be lethal for the parasite (de Azevedo et al., 2012). At the same time a rapamycin sensitive FKBP homologue of *Plasmodium* is constitutively expressed (Kumar et al., 2005) but whether it is sensitive to

rapamycin derivative shield-1 is not known (de Azevedo et al., 2012). As shield-1 is sensitive to repeated freeze and thaw so before each experiment we freshly diluted shield-1 stock in the cell culture media to obtain desirable concentrations. To rule out the possibility that shield-1 has lost its effectiveness we used it to supress the destabilisation of control *T. gondii* MLC1. Incubation with only 0.5 μM shield-1 for 24 hours induced robust suppression of destabilisation of the dd-FKBP-MycTgMLC1, resulting in several fold increase in the level of fusion protein (fig.3.7b and fig.3.7c).

4.2.2. N- or C-terminal tagging render the fusion proteins non functional

In modern biology, we often use N- or C-terminal tags for various purposes. Protein tags are peptide sequences that are fused either to the N- or C-terminus of the protein of interests. Tags are used for several purposes like purification, detection, trafficking in the cell, and localization etc. One criterion that needs to be considered is that all proteins do not tolerate N- or C- terminal tagging. During our work we have fused dd-FKBP tag either to the N- or C-terminus depending on the protein. In addition, we have also fused the HA tag on the protein of interest for the purpose of detection. Since we could not achieve a dominant negative effect in the parasite line by episomal expression of the dominant negative alleles of *Pf*Hsp70-1 and *Pf*Hsp40, we speculate whether this could be due to the compromised function of the protein of interests *in vivo* due to tagging with the HA and dd-FKBP. As already discussed earlier, the whole idea of using dominant negative approach is the episomal expression of a catalytically dead protein. This competitively occupies the interaction partner for endogenous protein rendering the protein functionally silent. Although the proteins are catalytically dead in this approach yet the overall native structure of the episomally expressed fusion protein needs to be conserved in order to allow them to interact and block the ligands of the endogenous protein. As we could not obtain overexpression of the endogenous protein to a certain level for establishing dominant negative phenotype, we cannot rule out the possibility of the proteins not behaving as 'dominant negative' at all in the cell. For both the *Pf*Hsp70-1 and *Pf*Hsp40 we do not know whether the tagging of the protein with the dd-FKBP affects their overall folding into native functional structure and therefore hinders binding to the native substrate. In fact this would explain why expression of a certain amount of dominant negative *Pf*hsp70-1 and *Pf*Hsp40 do not have even slightest effect on the normal growth and phenotype of the parasite. The mutations have been constructed in *Pf*Hsp70-1 and

*Pf*Hsp40 in agreement with previous work that should render the proteins 'dominant negative' (Buchberger et al., 1994, Hennessy et al., 2005) (refer to section 2.2.1.1.4 and 7.2). But, we do not know whether the fusion of the 15kDa destabilisation domain onto the protein adversely affects the overall native folding of the protein and therefore the fusion protein cannot compete with the endogenous version. Hence, significant alteration of the native folding of the fusion protein might compromise its interaction with the endogenous ligands that would in turn dissatisfy the purpose of the study. But in two independent studies from our group, the endogenous copies of *Pf*Hsp40 and also episomal *Pf*Hsp70x could be tagged to GFP without an effect on the protein function (Kulzer et al., 2010, Kulzer et al., 2012). One of the *Pf*Hsp40s is likely to be essential therefore the tagging cannot have affected the protein function. Inspite of the conservation of several amino acids, between members of *Pf*Hsp70 and *Pf*Hsp40s, we do not know whether there are some significant differences in folding among individual homologues. As a result, the function of one might be affected by tagging whereas the other is unaffected. For the same reason, we constructed control plasmids expressing the candidate proteins tagged to both GFP and HA in order to avoid an influence of the tag on protein function.

Nevertheless, this problem has already been reported as one of the important drawbacks of using this system. Recently it was reported by Herm-Götz *et al*., (2007) that some proteins are no longer functional when tagged to the dd-FKBP domain, (Herm-Gotz et al., 2007, Armstrong and Goldberg, 2007). The point, that parasite proteins might not tolerate the fusion of dd-FKBP domain, has been raised by study in *Plasmodium* as well (Armstrong and Goldberg, 2007, de Koning-Ward and Gilson, 2009). Chu *et al*., (2008) has addressed this challenge and therefore tested the efficiency of dd-FKBP to confer destabilization of the fusion protein when spliced into the middle of a protein. They tethered the L106P using long linkers between certain amino acids of yellow fluorescent protein (YFP) that tolerate insertion of additional peptide keeping the overall folding topology intact. This fusion protein showed robust strong destabilization kinetics that could further be reverted very efficiently by the addition of shield-1 (Chu et al., 2008). Therefore, an alternative approach would be to introduce the destabilisation domain inside *Pf*Hsp70-1 and *Pf*Hsp40. Since these proteins achieve their function via several domains present throughout the protein, finding a site where these will tolerate additional peptide insertion without affecting the function and overall folding of the protein, could be challenging.

4.2.3. Episomal expression driven by *crt* promoter does not lead to sufficient overexpression of fusion proteins

Although we could detect wild type and dominant negative fusion protein expressed episomally under *crt* promoter, the level of expression was much lower compared to the endogenous protein (fig.3.2b and fig.3.3b). Since the whole idea of this approach was to override the function of the endogenous protein by dominant negative alleles in a competitive manner, the success partly lies in the sufficient overexpression of the dominant negative version. Till date, this system has been applied in different organisms with success but in all these cases the protein has been placed under the control of a strong constitutive promoter or native promoter of the gene. For example in *Toxoplasma gondii,* functional analysis of essential parasite protein was carried out using this approach where the fusion protein was either placed under the control of strong constitutive p5RT70 promoter or stable transfection was carried out using the plasmid coding for dominant negative protein (Herm-Gotz et al., 2007). In a similar study, in *Plasmodium falciparum,* episomal expression of the protein of interest fused to dd-FKBP was driven from a strong hsp86 promoter. In addition to that the plasmid was transfected into a clone lacking the endogenous gene (Armstrong and Goldberg, 2007). This makes the assessment of the function of the protein much simpler in comparison to our work where the wild type gene coding for the endogenous protein has been kept intact. Since in our work the endogenous *Pf*Hsp70-1 and *Pf*Hsp40 are present intact to carry out their function unlike in the work of Amstrong *et al*., (2007) where episomal overexpression of the dominant negative versions by several fold under a native or strong constitutive promoter is a 'must' for the success of the strategy. Another study in *Plasmodium* showed that the protein of interests fused to destabilisation domain were either expressed under strong *Pfhsp86* promoter or integrated into the endogenous locus (de Azevedo et al., 2012).

In our study, we attempted to clone the *Pf*Hsp70-1 native promoter in front of the 3XHA-dd-FKBP-*Pf*Hsp70-1 fusion protein by replacing the *crt* promoter of the transfection plasmid (refer to section 2.2.2.5). By this we wanted to assess whether the expression of the fusion protein under its native promoter is much higher in comparison to the much weaker *crt* promoter. At the same time we could compare the level of the endogenous *Pf*Hsp70-1 and episomally expressed *Pf*Hsp70-1 fusion protein under native promoter. Unfortunately, due to low transfection efficiency in *Plasmodium* we could not generate any successful transgenic parasite line.

In order to bypass this challenges associated with poor expression of the fusion protein another straightforward possible approach would be to integrate the destabilization domain by either single or double crossover recombination at the 3' end of the endogenous coding region. In this manner the protein of interest is expressed as a fusion protein tagged to destabilization domain at its C-terminus. At the same time the gene is maintained under the control of the endogenous promoter. *Plasmodium falciparum* protein kinase (Dvorin et al., 2010) and calpain (Russo et al., 2009) have been reported to be conditionally knocked down with this approach of C-terminal tagging of the destabilization domain. But, among several proteins studied only these two essential proteins could be successfully tagged by this method. The reason behind low success rate could be explained by two possible reasons. Firstly, the protein might become nonfunctional by the C-terminal tagging of the destabilization domain therefore cannot carry out essential function for the survival of the organism. Secondly, the small ligand (shield-1) might not be effective to suppress the destabilization, reverting back the function of the protein necessary for the cellular function (de Azevedo et al., 2012). Even if the protein accepts C-terminal destabilization domain tagging the success of achieving a knockdown of the protein might be obstructed. This is because the leakiness of the destabilization domain cannot lead to complete degradation of the target protein to establish a detectable phenotype. This challenge can be possibly overcome by using other triple mutant of the dd-FKBP derived destabilization domains DD24 (E31G-R71G-K105E) or DD29 (D79G-P93S-D100R) (Chu et al., 2008) that have much stronger destabilization effect in comparison to that of dd-FKBP (L106P) used in our study (refer to section.4.3.1). DD29 (DD_{TM}) has been successfully used to generate conditional knockdown of *Plasmodium falciparum* calcium dependent protein kinase domain (Dvorin et al., 2010).

4.2. Alternative ecDHFR-derived dd failed to regulate protein level in the parasite by ligand TMP

Being unable to control protein level tagged to the FKBP derived destabilization domain we further attempted to use another *E. coli* dihydrofolate reductase (ecDHFR) derived destabilization domain recently characterized by Iwamoto *et al*., (2010). This domain was engineered to successfully confer its instability to the fused protein. Therefore the previously used dd-FKBP and dd-ecDHFR function in similar fashion. But, as claimed by the authors, dd-ecDHFR had several improved properties compared to the previous dd-FKBP (Iwamoto et

al., 2010). Therefore, by using dd-ecDHFR we aimed to overcome several drawbacks of the dd-FKBP system. Firstly, it was reported that dd-ecDHFR is much more efficient in destabilising the level of the fusion protein when tagged to the C-terminus of the protein of interest unlike the L106P dd-FKBP where negligible protein was detected in absence of the ligand (Chu et al., 2008, Iwamoto et al., 2010). We expected that the level of *Pf*Hsp40 could be more efficiently regulated when tagged to the dd-ecDHFR at the C-terminus (fig. 3.11a and fig. 3.12a). Secondly, trimethoprim (TMP), the stabilizing ligand of the dd-ecDHFR is commercially available, inexpensive, and does not have any effect on the growth or normal developmental cycle of the TMP resistant parasite when used at a concentration as high as 15.0 μM. On the other hand, dd-FKBP delays the growth cycle of the parasite when used at a concentration higher than 1.0 μM (de Azevedo et al., 2012).

Unfortunately, the tagging of the dd-ecDHFR also failed to regulate protein level when expressed episomally. All the wild type and dominant negative *Pf*Hsp70-1 and control GFP when fused to the dd-ecDHFR at the N-terminus (fig. 3.8a, 3.9a and 3.10a) resulted in a significant amount of fusion protein in the absence of TMP (fig.3.8b, 3.9b and 3.10b). Further, there was no upregulation in the level of protein on increasing the concentration of TMP upto 15.0 μM (fig.3.8b, 3.9b and 3.10b). This observation suggested that dd-ecDHFR is also not efficient to regulate parasite heat shock proteins and the GFP level. At the same time since the level of the endogenous protein were much higher than the dominant negative *Pf*Hsp70-1, we could not obtain a dominant negative effect in the parasite (fig 3.9b). In addition, the failure to establish a dominant negative effect in the parasite by expressing DN*Pf*Hsp70-1 can be due to several reasons that is, either inefficient stabilisation by TMP, the loss of native interaction by N-terminal tagging and the expression under weak *crt* promoter as mentioned earlier (refer to section. 4.2.1, 4.2.2 and 4.2.3). As previously mentioned that C-terminal tagging of the dd-ecDHFR is claimed to be more effective in regulation, we thought that tagging of these candidate protein at the N-terminus (fig. 3.8a, 3.9a, and 3.10a) might be the reason for poor regulation by this domain.

If this reasoning is right, and the N-terminal tagging is less effective in regulating protein level, then we expected to see better regulation of the level of wild type and dominant negative *Pf*Hsp40 and GFP where the dd-ecDHFR was fused to the C-terminus (fig. 3.11a, 3.12a and 3.13a). But, once again we did not witness any sign of regulation of the wild type and dominant negative *Pf*Hsp40 fusion protein level by dd-ecDHFR. In the absence of TMP,

high level of fusion proteins was detected. Increasing TMP concentration up to 15.0 μM for the *Pf*Hsp70-1 fusion protein did not show any visible increase in the protein level (fig. 3.11b and 3.12b). In contrast, on addition of TMP the GFP level was regulated in a robust and dose dependent manner. GFP fusion protein was barely detected in the absence of the TMP, while increasing concentration of TMP up to 15.0 μM resulted in clear dose dependent stabilization and increase in the protein level (fig. 3.13b). This observation is in agreement with previous studies carried out with dd-ecDHFR fused to YFP and GFP (Muralidharan et al., 2011, Iwamoto et al., 2010, Rakhit et al., 2011). On the other hand, our inability to regulate parasite protein level by this approach once again suggests that the success rate of the destabilisation domain approach in *Plasmodium* is low and tagging of essential plasmodium protein is highly challenging.

4.2.1. Applicability of dd-ecDHFR domain is not universal and success is protein dependent

Although, the dd-ecDHFR domain has been gaining research attention as an alternative approach to FKBP derived destabilisation domain, the success rate depends on several factor and is purely dependent on the organism and protein to be studied. This domain was first characterised and used to regulate the stability of several proteins in mammalian central nervous system to successfully regulate some transmembrane protein that was difficult to be controlled by the dd-FKBP (Iwamoto et al., 2010). Since then the dd-ecDHFR has been used to regulate the level of essential kinase in *S. cerevisiae*, where the authors wanted to evaluate the efficiency of dd-FKBP and dd-ecDHFR. The authors reported that the dd-ecDHFR tagged proteins were rapidly degraded and stabilization was obtained with the addition of TMP, whereas dd-FKBP was unstable under all conditions (Rakhit et al., 2011). Very recently the dd-ecDHFR has been used in *Plasmodium falciparum* to successfully generate conditional knockout of a proteasome lid subunit 6 (Rpn6) (Muralidharan et al., 2011). In this study the conditional knockdown of the Rpn6 could be efficiently obtained in a TMP dependent manner and therefore the author claimed the applicability of the dd-ecDHFR to study essential genes in *Plasmodium falciparum*. Noteworthy, during this work dd-ecDHFR along with the reporter protein was fused to the 3' end of the endogenous Rpn6 gene via single cross over homologous recombination. Hence, the success of this work greatly relied on the possibility to regulate the endogenous Rpn6 by dd-ecDHFR under the control of the native promoter (Muralidharan et al., 2011). Unlike our study where we wanted to establish

dominant negative phenotype by episomal overexpression of the dominant negative protein fused to dd-ecDHFR under the control of weaker *crt* promoter instead of knocking out the gene of interests. At the same time to our knowledge Rpn6 is the only *Plasmodium* protein studied so far by this system with success. During our study, we were able to accomplish a clear dose dependent regulation of GFP, whereas several *Plasmodium* heat shock proteins (*Pf*Hsp70-1 and *Pf*Hsp40) could not be controlled under the same condition. Considering the fact that applicability of the destabilisation domain strategy in *Plasmodium* is much lower when compared to other organisms (de Azevedo et al., 2012), regulation of more essential plasmodial protein needs to be studied in order to claim the universal application of this system.

To conclude, inspite of several reports of successful application of FKBP and ecDHFR derived destabilisation domain strategy in *Plasmodium falciparum*, the success of the approach is dependent on several factors. One major factor is to either integrate the destabilisation domain into the gene locus or to drive episomal expression of the wild type and dominant negative protein under strong *hsp86* like promoter in a knockout cell line. Again, both of these factors could further be posed with several challenges. As mentioned earlier, integration into the gene locus might render the protein nonfunctional whereas, the endogenous or episomal destabilisation might be leaky. If we cannot regulate the protein level, this approach would not be of much help. Therefore, these factors need to be carefully considered and the right approach should be determined depending on individual protein candidate. Although, our attempt to study the essential role of *Pf*Hsp70-1 and *Pf*Hsp40 in the parasite by establishing a dominant negative phenotype using this approach failed, several combinations with other systems can be tried in future in order to ensure success of the method. In fact, to our knowledge conditional over expression of protein of interest to establish dominant negative phenotypes has not yet been published for *P. falciparum* (de Azevedo et al., 2012).

4.3. Possible alternative future approaches that can be tested to ensure success

Several alternative strategies that can be tested either individually or in combination with the other, to generate conditional knock down of the essential proteins during asexual blood stage of *Plasmodium falciparum* will be discussed in the following sections. Nevertheless, it needs

to be seriously considered that none of these techniques are of a "gold standard" and therefore has advantages as well as drawbacks. Hence, a careful selection of the approach that is most suited for the protein of interest needs to be determined.

4.3.1. Triple mutant of dd-FKBP conferring greater instability to protein of interest

We used L106P mutant of dd-FKBP that failed to regulate the level of episomally expressed protein. The dd-ecDHFR domain also proved to be ineffective inspite of being successfully used for other proteins in rat, *S. cerevisiae* and *Plasmodium falciparum*. The L106P dd-FKBP was among the very first potent destabilisation domain identified during the initial screening carried out by Banaszynski *et al*., (2006) (Banaszynski et al., 2006). This dd-FKBP possesses only single point mutation (F36V). But, there were also several possible drawbacks of the L106P mutant. For example, the fusion protein was unstable when L106P was tagged to the C-terminus. At the same time this single mutant was not dominant to endogenous degrons (Banaszynski et al., 2006). Realising this drawbacks Chu *et al*., (2008) identified two addition triple mutant dd-FKBP known as either DD24 (E31G-R71G-K105E called FKBP) or DD29 (D79G-P93S-D100R). After comparing the efficiency of these two domains with L106P mutant, it was revealed that these were capable of conferring better instability to the fusion protein and obtained complete stabilization on addition of shield-1 in a dose dependent manner (Chu et al., 2008).

DD29 has already been used in *Plasmodium falciparum* with great success for generating conditional knock down of a protein kinase and calpin to assess their importance during the blood stage (Dvorin et al., 2010, Russo et al., 2009). The applicability of DD24 to regulate the level of *Plasmodium* Ring-Infected Erythrocyte Surface Antigen (RESA) has also been tested in a recent study while doing systematic analysis of the efficacy of all destabilization domains (de Azevedo et al., 2012). The same study also showed that DD29 is the most efficient destabilisation domain, capable of reducing reporter protein level down to 6% in the absence of shield-1.

During the course of our study, we have generated all the transfection plasmids for *Pf*Hsp70-1, *Pf*Hsp40 and GFP by using triple mutant DD29, fused to either N- or C- terminus depending on the protein of interest (*Pf*Hsp70-1 or *Pf*Hsp40). In future we could express the protein of interest under strong *hsp86* promoter instead of the weaker *crt* promoter as used

earlier (fig. 4.1). Therefore, we expect to override the level and function of the endogenous candidate Hsps by the overexpression of dominant negative version fused to more efficient destabilisation domain under the control of a much stronger promoter.

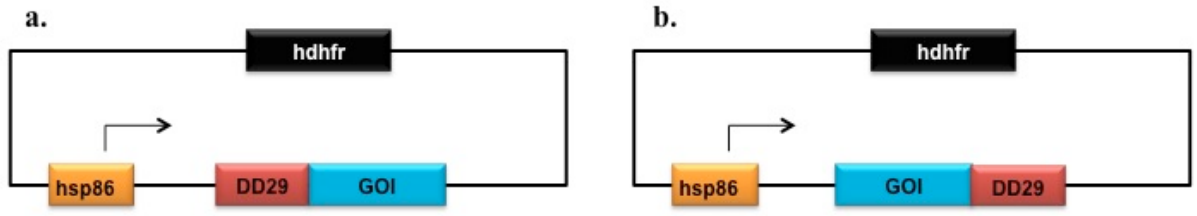

Figure 4.1 Schematic illustration of a transfection plasmid for conditional episomal expression of the protein of interest.

The coding region of the gene of interest (GOI) is fused to the dd triple mutant (DD29) either at the N-terminus (a) or C-terminus (b). Expression is driven under the control of a strong *hsp86* promoter.

Nevertheless this strategy might have few disadvantages. It has already been published that although the triple mutant of the dd-FKBP has stronger destabilizing ability the problem lies in poor suppression of destabilization by the addition of shield-1. Therefore, the expression and function of the fused protein cannot be efficiently reverted (de Azevedo et al., 2012). This is also in agreement with the study of Chu *et al*., (2008), where 3.0µM shield-1 was needed to obtain full stabilization (Chu et al., 2008).

Considering the fact that the destabilisation domain is in some part greatly organism and protein dependent, in future the ability to regulate the episomal level of one of the candidate heat shock proteins need to be checked to assess the applicability of this system. This way one can save time and resources. Since, we claim that the system is partly protein dependent there is no real "control" as the outcome from the study of one candidate protein will not be of much help in deciphering the success of another.

4.3.2. Application of a recently characterised tertracyline repressible transactivator system for *Plasmodium falciparum*

An inducible gene expression system was characterized and established by Meissner *et al*., (2002) for *Toxoplasma gondii*. The transactivator domain contained a Tet repressor (TetRep) fused to a non-endogenous activating domain (TATi1) (Meissner et al., 2002). Since characterization, this domain has been severely adapted to study the function of several essential genes in *T. gondii* (Meissner et al., 2002, Huynh and Carruthers, 2006, Plattner et al., 2008, Buguliskis et al., 2010).

TATi2 identified from *Toxoplasma* qualified to be functional in *Plasmodium* yet when expressed from multicopy episomal plasmid (Meissner et al., 2005). But, it failed to generate conditional knockouts of essential gene in *P. falciparum*. This could be an outcome of the poor transactivation activity. Therefore, the best possible way would have been to design a transactivation system depending on the transcriptional activation pathway of the parasite (Pino et al., 2012).

Very recently highly efficient transactivator domains have been identified and characterized for their utility in *Plasmodium berghei* and *Plasmodium falciparum*. This robust Tet-repressible transactivator system has been efficiently and successfully applied to study the function of essential gene in *Plasmodium berghei* when integrated into the genome by homologous recombination. Nevertheless, it has also been shown that TRAD4 (one out of the few identified transactivator domains) was highly efficient in mediating the derivative anhydrotetracycline (ATc) dependent conditional expression of reporter protein when expressed from an episomal plasmid in human malaria parasite. The mode of function of this system is based on 'Tet on and off' system. In the absence of the tetracycline derivative anhydrotetracycline (ATc) transactivator interact with the tet operator (*TetO*) sequence via the Tet repressor (*TetR*) and initiate transcription. Whereas when ATc is added, there is a dramatic reduction in the affinity of the *TetR* for the *TetO* that results in switching off the transcription (Bujard, 1999). Therefore, using this system we could try to achieve the conditional episomal expression of our candidate Hsps by ATc dependent episomal expression of the candidate protein (Fig. 4.2).

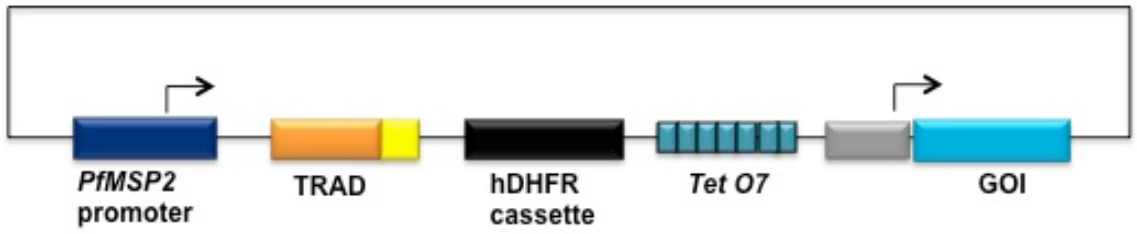

Figure 4.2 Schematic illustration of a transfection plasmid for conditional episomal expression of the protein of interest.

TRAD4 is placed under the control of a *PfMSP2* promoter. The gene of interest (GOI) is under the control of a *tet* transactivator-responsive promoter. This promoter contains seven *tet* operator sites. Adapted from (Pino et al., 2012).

This system is much more leak proof, where a tight regulation of protein expression is possible compared to that offered by destabilization domain. On the other hand, it is a reversible system compared to Di-Cre recombinase system reported recently for *P. falciparum* (Pino et al., 2012, Collins et al., 2013). But, the disadvantage is that this system is not yet fully optimised for use in *Plasmodium falciparum*. And, copy numbers of plasmid under same conditions greatly vary between individual parasites. Therefore, it needs to be investigated whether TRAD4 is functional and effective when integrated into the genome for obtaining more homogenous and reproducible response to ATc (Pino et al., 2012).

4.3.3. Integration of the destabilisation domain to the 3' end of the endogenous coding region

If we find DD29 mutant of dd-FKBP to be much stronger and robust in achieving conditional expression of protein level upon addition of shield-1, another straightforward strategy would be to genetically fuse this domain to the C-terminus of the coding region of the gene. This approach has previously been tried successfully in two individual studies to elucidate the essential role of protein kinase and calpin in intraythrocytic stage of *P. falciparum* (Dvorin et al., 2010, Russo et al., 2009). Therefore, after the validation of the effectiveness of the DD29 mutant we can try to integrate it to the C-terminus of the candidate Hsps by homologous recombination. This way the expression level of the endogenous protein could directly be controlled by the addition or withdrawal of shield-1. In absence of the shield the level of expressed protein should be significantly reduced resulting in a conditional knockdown and therefore the resulting phenotype could further be analysed (fig. 4.3).

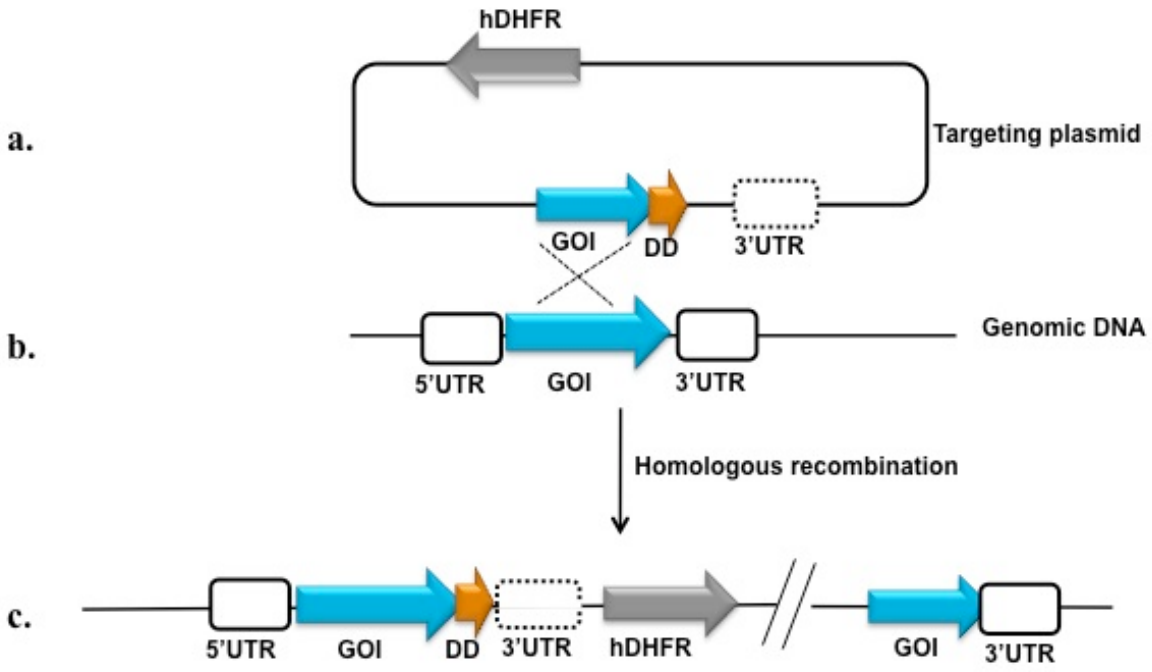

Figure 4.3 Schematic illustration of homologous recombination event at C-terminal end of an endogenous coding region.

a. Targeting plasmid to integrate the gene of interest fused to the destabilization domain (DD) at the 3' end of the endogenous coding region. b. Structure of the gene locus containing the gene to be targeted (GOI). c. Integrated GOI fused to DD at the C-terminus coding region of the endogenous locus resulting from homologous recombination. Adapted from (Dvorin et al., 2010), (supplementary documents).

This method has the advantage of directly regulating the expression level of endogenous proteins. But at the same time this strategy has several disadvantages and uncertainties. As previously mentioned, at the very first stage it needs to be assured whether there is complete stabilisation of the protein level by the addition of shield-1 as it has already been noticed that the destabilisation cannot be efficiently supressed upon shield-1 addition (de Azevedo et al., 2012). Since the endogenous protein is directly tagged to the destabilisation domain, stabilisation ensuring essential protein function for parasite survival is mandatory. Secondly, the shield-1 is highly expensive therefore the application of this strategy that need the parasite to be maintained all the time under shield-1, demands huge financial investment. Thirdly, since the parasites are selected and always maintained under shield-1, they might become non responsive after its removal (Pino, 2013). There have been several reports where the fusion of the destabilisation domain to the N- or C-terminus interferes with the protein function rendering the protein non-functional (Herm-Gotz et al., 2007). Therefore the last and most importantly, the tagging to the C-terminus of the endogenous coding region should be able to tolerate fusion.

In recent years there has been a significant increase in the amount of diverse technologies to target essential gene during intraerythrocytic life stage of *Plasmodium falciparum*. Noteworthy, inspite of these progresses only less than ten genes have been successfully studied by either of these approaches. This indeed reflects the challenges and difficulties involved in obtaining knockdown of essential gene during blood stage malaria parasite. As mentioned earlier all the possible approaches explained herein along with other available ones have huge drawbacks. Therefore, identification of a reliable strategy to generate reversible conditional knockdown of essential genes will be a significant step forward. Needless to say, this will help in further investigation and validation of potential vaccine and drug targets to reduce the global burden of malaria.

5a. Summary

The apicomplexan malaria parasite, *Plasmodium falciparum* is capable of invading red blood cells and causes the most virulent form of malaria. The life cycle of *P. falciparum* involves the migration from the poikilothermic mosquito vector to warm-blooded human host and *vice versa*. Such transition introduces radical differences between the cellular environments where the parasite resides, imposing physiological stress. The diverse environmental insults in addition to the febrile fever episodes imparts challenge on the proteostasis, resulting in the evolutionary selection of a diverse network of molecular chaperones. In fact, some molecular chaperones are essential for the survival of *Plasmodium*. Due to the developing resistance of *Plasmodium* against currently available drugs, heat shock proteins have received extensive research attention as antimalarial targets in recent years.

Plasmodium codes for one Hsp90 homologue and a constitutively expressed heat inducible cytosolic Hsp70 known as *Pf*Hsp70-1. In general, Hsp70 interacts with co-chaperone Hsp40 initiating the protein folding machinery that finally interacts with Hsp90 to maintain proteostasis in a cell. *Pf*Hsp90 has been found to be essential for the intraerythrocytic development of *P. falciparum*. Although there have been some *in vitro* studies on the biology of *Pf*Hsp70-1, the information on the *in vivo* essential function of *Pf*Hsp70-1 and its interaction with *Pf*Hsp40 is limited.

In this study, we wanted to identify the *in vivo* biological importance of *Pf*Hsp70-1 and one of its predicted co-chaperones, *Pf*Hsp40 by the overexpression of the dominant negative alleles tagged to recently characterised destabilisation domain (dd) to regulate protein level. We expressed a dominant negative *Pf*Hsp70-1 possessing a point mutation (E187K), severely affecting normal domain movement important for its function. *Pf*Hsp40 was mutated in the conserved HPD motif (D34N) necessary for establishing interaction with *Pf*Hsp70-1. Unfortunately, we could not obtain sufficient overexpression of the episomal dominant negative versions to override the function of the endogenous proteins in a competitive manner. The cellular levels of endogenous proteins were higher by several folds compared to that of the episomally expressed dominant negative alleles. The destabilisation strategy has been reported to be successful for studying certain plasmodial proteins. But in contrast, during our work the level of almost none of the candidate chaperones could be controlled by either FKBP or *E. coli* DHFR derived destabilisation domains in a ligand dependent fashion. Although, the level of wild type *Pf*Hsp70-1 could be regulated by this strategy, the dominant negative version with only one amino acid substitution made it non responsive to dd tagging

and further ligand treatment. At the same time, the level of control proteins could be efficiently regulated by stabilising ligands. Recently, success of destabilization domain strategy for conditional knockdown of several genes has been reported. But in contrast, our observations in this study unravel the possible drawbacks. We assume that the success of such an approach is greatly protein dependent. Based on the several reports initially this approach appeared to be the most beneficial system. But, the failure to successfully implement this strategy demands careful consideration in selecting an alternative future approach to study the function of essential genes in *Plasmodium*.

5b. Zusammenfassung

Der apikomplexe Parasit *Plasmodium falciparum* ist in der Lage, Erythrozyten zu invadieren und verursacht die virulenteste Form der Malaria. Der Lebenszyklus von *P. falciparum* umfasst den Übergang vom poikilothermen Moskito Vektor zum warmblütigen, menschlichen Wirt und zurück. Ein solcher Wechsel bedeutet gleichzeitig einen radikalen Wechsel der zellulären Umgebung, in welcher der Parasit sich befindet und letzten Endes physiologischen Stress. Die unterschiedlichen Stressfaktoren durch die Umgebung stellen zusätzlich zu den Fieberepisoden eine Herausforderung bezüglich der Proteostase dar, was zur evolutionären Selektion eines Netzwerks unterschiedlicher molekularer Chaperone geführt hat. Tatsächlich sind einige dieser molekularen Chaperone für das Überleben von *Plasmodium* unabdinglich. Aufgrund der immer weiter fortschreitenden Ausbildung von Resistenzen gegenüber den verfügbaren Medikamenten kam den Hitzeschock Proteinen (Hsp) große Beachtung bezüglich der möglichen Verwendung als Angriffspunkte für neue Antimalaria Therapien zu.

Plasmodium kodiert für ein Hsp90 Homolog sowie ein konstitutiv exprimiertes, hitzeinduziertes zytosolisches Hsp70 namens *Pf*Hsp70-1. Grundsätzlich interagiert Hsp70 mit dem Co-Chaperon Hsp40 und initiiert die durch die Interaktion mit Hsp90 vervollständigte Proteinfaltungsmaschinerie, welche die Proteostase in der Zelle aufrechterhält. *Pf*Hsp90 hat sich als essentiell für die intraerythrozytäre Entwicklung von *P. falciparum* erwiesen. Obwohl bezüglich der Biologie von *Pf*Hsp70-1 einige *in vitro* Studien durchgeführt wurden, existiert wenig Information bezüglich der essentiellen Funktion der Interaktion von *Pf*Hsp70-1 und *Pf*Hsp40 in vivo.

Im Zuge der hier vorliegenden Arbeit sollte *in vivo* die biologische Bedeutung von *Pf*Hsp70-1 und einem seiner vorhergesagten Co-Chaperonen, *Pf*Hsp40, untersucht werden. Dazu sollte mit Hilfe von Überexpression der dominant negativen Allele, welche an die kürzlich charakterisierte Destabilisierungsdomäne (dd) gebunden wurden, der intrazelluläre Proteinlevel reguliert werden. Dabei wurde eine dominant negative Mutante von *Pf*Hsp70-1 exprimiert, welche eine Punktmutation (E187K) trägt, die die für die normale Funktion des Proteins wichtige Beweglichkeit der Domänen stark beeinflusst. *Pf*Hsp40 wurde im konservierten HPD Motiv (D34N) mutiert, welches wichtig für die Ausbildung der Interaktion mit *Pf*Hsp70-1 ist. Eine ausreichende Überexpression der episomalen dominant negativen Versionen, welche notwendig ist, um die Funktion der endogenen Proteine kompetitiv auszuschalten, war nicht möglich. Hierbei lagen die zellulären Level der

endogenen Proteine um viele Stufen höher als die der episomal exprimierten dominant negativen Allele. In der Literatur wurde der Destabilisierungsansatz als erfolgreich bei der Untersuchung vieler Plasmodienproteine beschrieben. Im Gegensatz dazu konnte im Zuge dieser Arbeit bei nahezu keinem der ausgewählten Chaperone der Proteinlevel durch die Liganden FKBP oder DHFR (eine aus *E. coli* genutzte Destabilisierungsdomäne) kontrolliert werden. Obwohl der Level von Wildtyp *Pf*Hsp70-1 über diese Strategie reguliert werden konnte, war die dominant negative Version des Proteins mit einer mutierten Aminosäure gegenüber dem dd-Tag und Zugabe des Liganden resistent. Gleichzeitig war es möglich, die als Kontrolle fungierenden Proteine effektiv durch die stabilisierenden Liganden zu regulieren.

Kürzlich konnte die erfolgreiche Nutzung des Destabilisierungsdomänen Ansatzes für konditionelle Knock-Outs verschiedener Gene gezeigt werden. Die in der vorliegenden Arbeit dargestellten Ergebnisse weisen im Gegensatz dazu aber auch auf die möglichen Nachteile dieser Technik hin. Wir vermuten dabei, dass der Erfolg dieses Ansatzes stark vom jeweiligen Protein abhängig ist. Die Wahl dieses Ansatzes bei der vorliegenden Arbeit basierte auf verschiedenen Veröffentlichungen, welche auf eine erfolgreiche mögliche Nutzung hindeuteten. Die Tatsache jedoch, dass es nicht möglich war, diese Strategie erfolgreich zu implementieren, verlangt ein vorsichtiges Vorgehen bei der Wahl zukünftiger Ansätze zur Untersuchung der Funktion essentieller Gene.

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7. Supplementary Data

7.1 List of sequence

7.1.1 Coding sequence for *Pf*Hsp70-1 (PF3D7_0818900)

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   1 ATGGCTAGTGCAAAA GGTTCAAAACCAAAT TTACCAGAATCCAAT ATCGCTATTGGAATT GATTTAGGTACTACT
   1  M  A  S  A  K   G  S  K  P  N   L  P  E  S  N   I  A  I  G  I   D  L  G  T  T
  76 TATTCTTGTGTTGGT GTATGGAGAAATGAA AATGTAGATATTATT GCTAATGACCAAGGT AATAGAACAACCCCA
  26  Y  S  C  V  G   V  W  R  N  E   N  V  D  I  I   A  N  D  Q  G   N  R  T  T  P
 151 TCTTATGTTGCTTTC ACCGATACCGAAAGA TTAATTGGAGATGCT GCTAAAAACCAAGTA GCTAGGAATCCAGAA
  51  S  Y  V  A  F   T  D  T  E  R   L  I  G  D  A   A  K  N  Q  V   A  R  N  P  E
 226 AATACAGTATTTGAT GCTAAGAGATTAATT GGTAGAAAATTTACA GAATCATCAGTACAA AGTGATATGAAACAT
  76  N  T  V  F  D   A  K  R  L  I   G  R  K  F  T   E  S  S  V  Q   S  D  M  K  H
 301 TGGCCATTCACTGTT AAATCAGGTGTTGAT GAGAAACCAATGATT GAAGTTACCTATCAA GGAGAAAAGAAATTA
 101  W  P  F  T  V   K  S  G  V  D   E  K  P  M  I   E  V  T  Y  Q   G  E  K  K  L
 376 TTCCATCCAGAAGAA ATTTCTTCTATGGTA TTACAAAAAATGAAA GAAAATGCTGAAGCA TTTTTAGGAAAATCT
 126  F  H  P  E  E   I  S  S  M  V   L  Q  K  M  K   E  N  A  E  A   F  L  G  K  S
 451 ATAAAGAATGCTGTC ATTACCGTTCCAGCT TATTTTAACGATTCA CAAAGACAAGCTACT AAAGATGCTGGTACA
 151  I  K  N  A  V   I  T  V  P  A   Y  F  N  D  S   Q  R  Q  A  T   K  D  A  G  T
 526 ATTGCAGGATTAAAT GTTATGAGAATTATT AATGAACCTACTGCA GCTGCTATTGCATAT GGTTTACACAAAAAA
 176  I  A  G  L  N   V  M  R  I  I   N  E  P  T  A   A  A  I  A  Y   G  L  H  K  K
 601 GGAAAAGGTGAAAAG AACATTTTAATTTTC GACTTAGGAGGAGGT ACATTTGATGTATCA TTATTAACTATTGAA
 201  G  K  G  E  K   N  I  L  I  F   D  L  G  G  G   T  F  D  V  S   L  L  T  I  E
 676 GATGGTATTTTTGAA GTAAAAGCTACTGCT GGTGATACTCATTTA GGTGGTGAAGATTTC GATAACAGATTAGTA
 226  D  G  I  F  E   V  K  A  T  A   G  D  T  H  L   G  G  E  D  F   D  N  R  L  V
 751 AATTTCTGTGTTGAA GATTTCAAAAGAAAA AACAGAGGTAAAGAT TTATCAAAAAATAGT AGAGCCTTAAGAAGA
 251  N  F  C  V  E   D  F  K  R  K   N  R  G  K  D   L  S  K  N  S   R  A  L  R  R
 826 TTAAGAACACAATGT GAAAGAGCAAAACGT ACTTTATCATCATCT ACACAAGCTACAATT GAAATAGATTCCTTA
 276  L  R  T  Q  C   E  R  A  K  R   T  L  S  S  S   T  Q  A  T  I   E  I  D  S  L
 901 TTTGAAGGTATTGAT TACAGTGTTACTGTA AGTAGAGCAAGATTT GAAGAATTATGTATC GACTATTTCCGTGAT
 301  F  E  G  I  D   Y  S  V  T  V   S  R  A  R  F   E  E  L  C  I   D  Y  F  R  D
 976 ACTTTAATTCCAGTA GAAAAAGTTTTAAAA GATGCTATGATGGAT AAAAAAAGTGTACAT GAAGTTGTTTTAGTT
 326  T  L  I  P  V   E  K  V  L  K   D  A  M  M  D   K  K  S  V  H   E  V  V  L  V
1051 GGTGGTTCTACAAGA ATTCCAAAAATCCAA ACTTTAATAAAAGAA TTCTTTAATGGTAAA GAAGCATGCAGATCA
 351  G  G  S  T  R   I  P  K  I  Q   T  L  I  K  E   F  F  N  G  K   E  A  C  R  S
1126 ATTAACCCAGATGAA GCTGTTGCATATGGT GCAGCTGTACAAGCA GCCATTTTATCTGGT GACCAATCAAATGCT
 376  I  N  P  D  E   A  V  A  Y  G   A  A  V  Q  A   A  I  L  S  G   D  Q  S  N  A
1201 GTCCAAGATTTATTA TTATTAGATGTTTGC TCCTTATCATTAGGT TTAGAAACTGCTGGT GGTGTTATGACCAAA
 401  V  Q  D  L  L   L  L  D  V  C   S  L  S  L  G   L  E  T  A  G   G  V  M  T  K
1276 TTAATTGAAAGAAAC ACAACCATACCTGCT AAAAAGAGTCAAATC TTTACTACTTATGCT GATAACCAACCAGGT
 426  L  I  E  R  N   T  T  I  P  A   K  K  S  Q  I   F  T  T  Y  A   D  N  Q  P  G
1351 GTCTTAATTCAAGTA TATGAAGGTGAAAGA GCCTTAACCAAAGAT AACAATTTATTAGGA AAATTTCACTTAGAT
 451  V  L  I  Q  V   Y  E  G  E  R   A  L  T  K  D   N  N  L  L  G   K  F  H  L  D
1426 GGTATTCCACCTGCA CCAAGAAAGGTACCA CAAATCGAAGTTACA TTCGATATCGATGCT AACGGTATCTTAAAC
 476  G  I  P  P  A   P  R  K  V  P   Q  I  E  V  T   F  D  I  D  A   N  G  I  L  N
1501 GTTACGGCTGTAGAA AAATCCACTGGTAAA CAAAACCATATTACA ATTACCAACGACAAA GGAAGATTATCTCAA
 501  V  T  A  V  E   K  S  T  G  K   Q  N  H  I  T   I  T  N  D  K   G  R  L  S  Q
1576 GATGAAATTGATCGT ATGGTTAATGATGCT GAAAAATACAAAGCA GAAGATGAAGAAAAC AGAAAAAGAATCGAA
 526  D  E  I  D  R   M  V  N  D  A   E  K  Y  K  A   E  D  E  E  N   R  K  R  I  E
1651 GCAAGAAACAGCCTT GAAAATTACTGCTAT GGAGTTAAAAGCTCA TTAGAAGACCAAAAA ATTAAAGAAAAATTA
 551  A  R  N  S  L   E  N  Y  C  Y   G  V  K  S  S   L  E  D  Q  K   I  K  E  K  L
1726 CAACCAGCTGAAATT GAAACATGTATGAAA ACTATTACAACCATA CTTGAATGGTTAGAA AAAAACCAACTTGCT
 576  Q  P  A  E  I   E  T  C  M  K   T  I  T  T  I   L  E  W  L  E   K  N  Q  L  A
1801 GGAAAAGATGAATAT GAAGCCAAACAAAAA GAAGCAGAATCGGTT TGTGCTCCAATTATG TCTAAAATCTATCAA
 601  G  K  D  E  Y   E  A  K  Q  K   E  A  E  S  V   C  A  P  I  M   S  K  I  Y  Q
1876 GATGCTGCTGGTGCA GCCGGTGGTATGCCA GGAGGTATGCCCGGT GGAATGCCCGGTGGA ATGCCAGGTGGTATG
 626  D  A  A  G  A   A  G  G  M  P   G  G  M  P  G   G  M  P  G  G   M  P  G  G  M
1951 AATTTCCCAGGAGGT ATGCCCGGAGCAGGA ATGCCAGGAAATGCC CCAGCTGGAAGTGGA CCAACAGTTGAAGAA
 651  N  F  P  G  G   M  P  G  A  G   M  P  G  N  A   P  A  G  S  G   P  T  V  E  E
2026 GTTGATTAA
 676  V  D  *
```

7.1.2 Coding sequence for *Pf*Hsp40 (PF3D7_0213100)

```
  1 ATGGGGAAGGATTAT TATTCAATATTAGGT GTTAGTAGAGACTGT ACAACAAATGATTTA AAAAAAGCGTATAGG
  1  M  G  K  D  Y   Y  S  I  L  G   V  S  R  D  C   T  T  N  D  L   K  K  A  Y  R
 76 AAGCTAGCCATGATG TGGCATCCTGATAAA CATAATGACGAGAAA TCAAAAAAAGAAGCA GAAGAAAAATTTAAG
 26  K  L  A  M  M   W  H  P  D  K   H  N  D  E  K   S  K  K  E  A   E  E  K  F  K
151 AATATTGCTGAAGCA TATGATGTTTTAGCA GATGAGGAAAAAAGG AAAATTTATGATACA TATGGAGAAGAAGGA
 51  N  I  A  E  A   Y  D  V  L  A   D  E  E  K  R   K  I  Y  D  T   Y  G  E  E  G
226 TTAAAAGGTTCAATA CCAACAGGTGGAAAT ACATATGTCTATAGT GGTGTTGATCCTTCA GAATTATTTAGTAGA
 76  L  K  G  S  I   P  T  G  G  N   T  Y  V  Y  S   G  V  D  P  S   E  L  F  S  R
301 ATATTTGGTTCGGAT GGACAATTTTCTTTT ACCTCAACTTTTGAT GAGGACTTTTCTCCC TTTTCCACTTTTGTC
101  I  F  G  S  D   G  Q  F  S  F   T  S  T  F  D   E  D  F  S  P   F  S  T  F  V
376 AACATGACTTCTAGA AAATCTAGACCATCC ACAACAACAAATATT AATACGAACAATTAT AACAAACCAGCCACA
126  N  M  T  S  R   K  S  R  P  S   T  T  T  N  I   N  T  N  N  Y   N  K  P  A  T
451 TACGAGGTGCCTCTT TCTTTATCCCTAGAA GAATTGTACAGTGGT TGTAAGAAAAAATTA AAAATAACGAGAAAG
151  Y  E  V  P  L   S  L  S  L  E   E  L  Y  S  G   C  K  K  K  L   K  I  T  R  K
526 AGATTTATGGGTACA AAAAGTTATGAAGAT GATAATTATGTAACA ATCGATGTAAAGGCA GGATGGAAAGATGGC
176  R  F  M  G  T   K  S  Y  E  D   D  N  Y  V  T   I  D  V  K  A   G  W  K  D  G
601 ACAAAAATAACTTTT TATGGAGAAGGGGAT CAATTATCTCCTATG GCACAACCAGGAGAT TTAGTTTTTAAAGTA
201  T  K  I  T  F   Y  G  E  G  D   Q  L  S  P  M   A  Q  P  G  D   L  V  F  K  V
676 AAAACCAAAACACAT GATAGATTCCTAAGA GACGCTAATCATTTA ATATATAAATGTCCT GTACCTTTAGATAAA
226  K  T  K  T  H   D  R  F  L  R   D  A  N  H  L   I  Y  K  C  P   V  P  L  D  K
751 GCTTTAACAGGATTC CAATTTATTGTTAAA TCATTAGATAATAGA GATATTAATGTAAGG GTAGATGATATTGTT
251  A  L  T  G  F   Q  F  I  V  K   S  L  D  N  R   D  I  N  V  R   V  D  D  I  V
826 ACTCCTAAATCAAGG AAAATTGTAGCAAAA GAAGGTATGCCTTCT TCCAAATACCCAAGC ATGAAAGGGGATCTC
276  T  P  K  S  R   K  I  V  A  K   E  G  M  P  S   S  K  Y  P  S   M  K  G  D  L
901 ATTGTAGAATTTGAT ATTGTCTTTCCAAAA AGTTTAACCAGTGAA AAAAAAAAAATTATA AGAGAAACATTGGCA
301  I  V  E  F  D   I  V  F  P  K   S  L  T  S  E   K  K  K  I  I   R  E  T  L  A
976 AATACATTCTAA
326  N  T  F  *
```

7.1.3 Coding sequence for dd-FKBP

ATGACTGCAGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTC
CCCAAGCGCGGCCAGACCTGTGTGGTGCACTACACCGGGATGCTTGAAGATGGA
AAGAAAGTCGATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGC
AAGCAGGAGGTGATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGG
TCAGAGAGCCAAACTGACTATATCTCCAGATTATGCCTATGGTGCCACTGGGCAC
CCAGGCATCATCCCACCACATGCCACTCTCGTCTTCGATGTGGAGCTTCTAAAAC
CGGAATAA

7.1.4 Coding sequence for dd-ecDHFR

ATCAGTCTGATTGCGGCGTTAGCGGTAGATCACGTTATCGGCATGGAAACCGTCA
TGCCGTGGAACCTGCCTGCCGATCTCGCCTGGTTTAAACGCAACACCTTAAATAA
ACCCGTGATTATGGGCCCCATACCTGGGAATCAATCGGTCGTCCGTTGCCAGGAC
GCAAAAATATTATCCTCAGCAGTCAACGAGTACGGACGATCGCGTAACGTGGGT
GAAGTCGGTGGATGAAGCCATCGCGGCGTGTGGTGACGTACCAGAAATCATGGT
TATTGGCGGCGGTCGCGTTTATGAACAGTTCTTGCCAAAAGCGCAAAATGTATCT
GACGCATATCGACGCAGAAGTGGAAGGCGACACCCATTTCCCGGATTACGAGCC
GGATGACTGGGAATCGGTATTCAGCGAATTCCACGATGCTGATGCGCAGAACTC
TCACAGCTATTGC

7.1.5 Coding sequence for DD29

ATGGGAGTGCAGGTGGAAACCATCTCCCCAGGAGACGGGCGCACCTTCCCCAAG
GCCAGACCTGCGTGGTGCACTACACCGGGATGCTTGAAGATGGAAAGAAAGTTG
ATTCCTCCCGGGACAGAAACAAGCCCTTTAAGTTTATGCTAGGCAAGCAGGAGT
GATCCGAGGCTGGGAAGAAGGGGTTGCCCAGATGAGTGTGGGTCAGAGGCCAA
ACTGACTATATCTCCAGGTTATGCCTATGGTGCCACTGGGCACCCAGGCATCATC
CCATCACATGCCACTCTCGTCTTCCGTGTGGAGCTTCTAAAACTGGAATGA

7.1.6 Coding sequence for 3XHA

ATGTACCCGTACGACGTCCCGGACTACGCTGGCTATCCCTATGATGTGCCCGATT
ATGCGTATCCTTACGATGTTCCAGATTATGCCGAGCTC

7.2 Amino acid sequence alignments of *P. falciparum* Hsp70-1 with *E. coli* DnaK

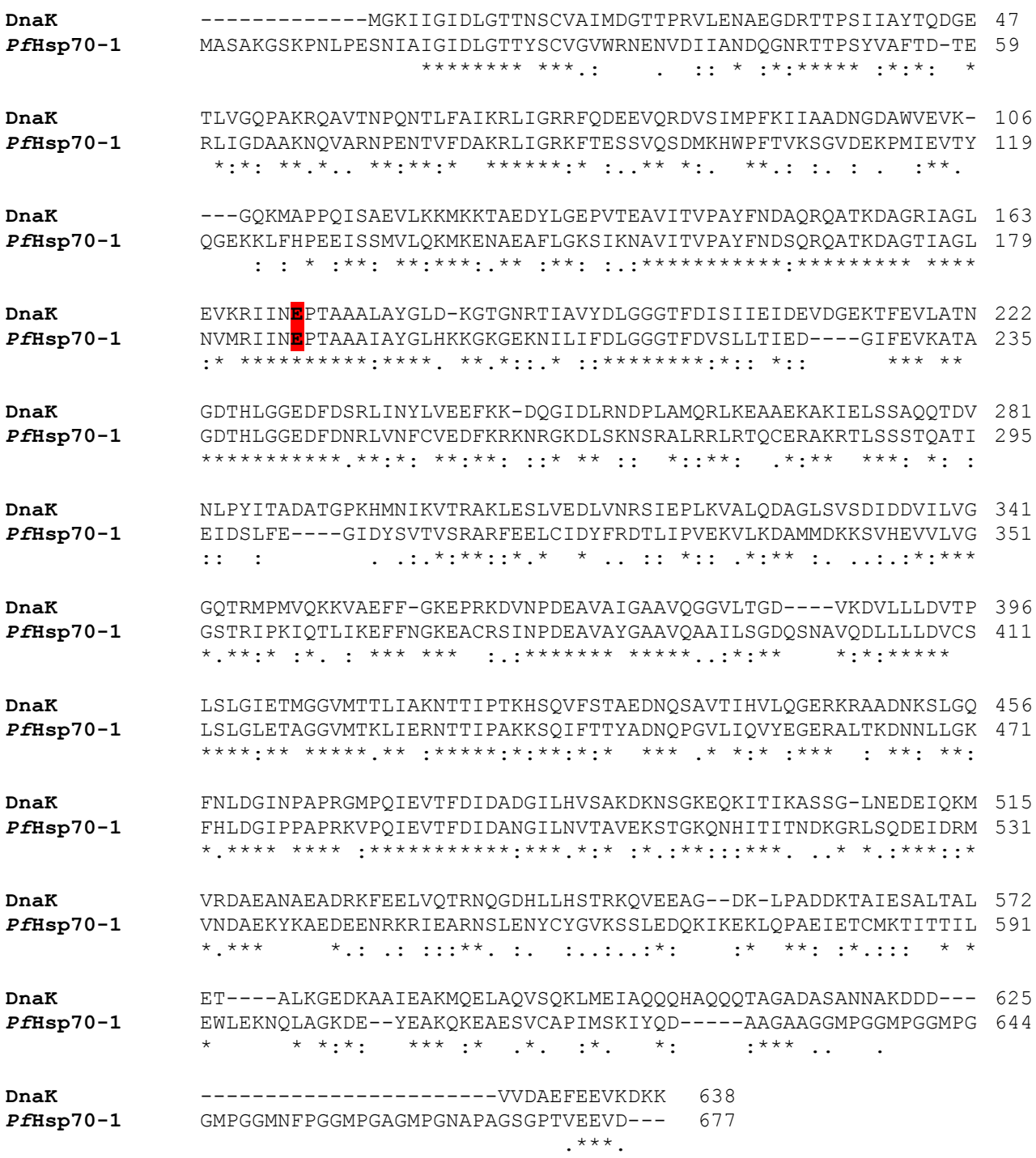

Figure S.1 Amino acid sequence alignments of *P. falciparum* Hsp70-1 with *E. coli* DnaK.

Based on the DnaK sequence E171 residue, important for the coupling of ATPase activity with substrate binding has been shown in *Pf*Hsp70-1 at the corresponding amino acid position 187 (Shown in red).

7.3 Episomal expression of WT/DN *Pf*Hsp70-1 and *Pf*Hsp40 where N- or C- terminus dd-FKBP is replaced with control GFP

7.3.1 Episomal expression of WT/DN *Pf*Hsp70-1 fused to GFP on the N-terminus

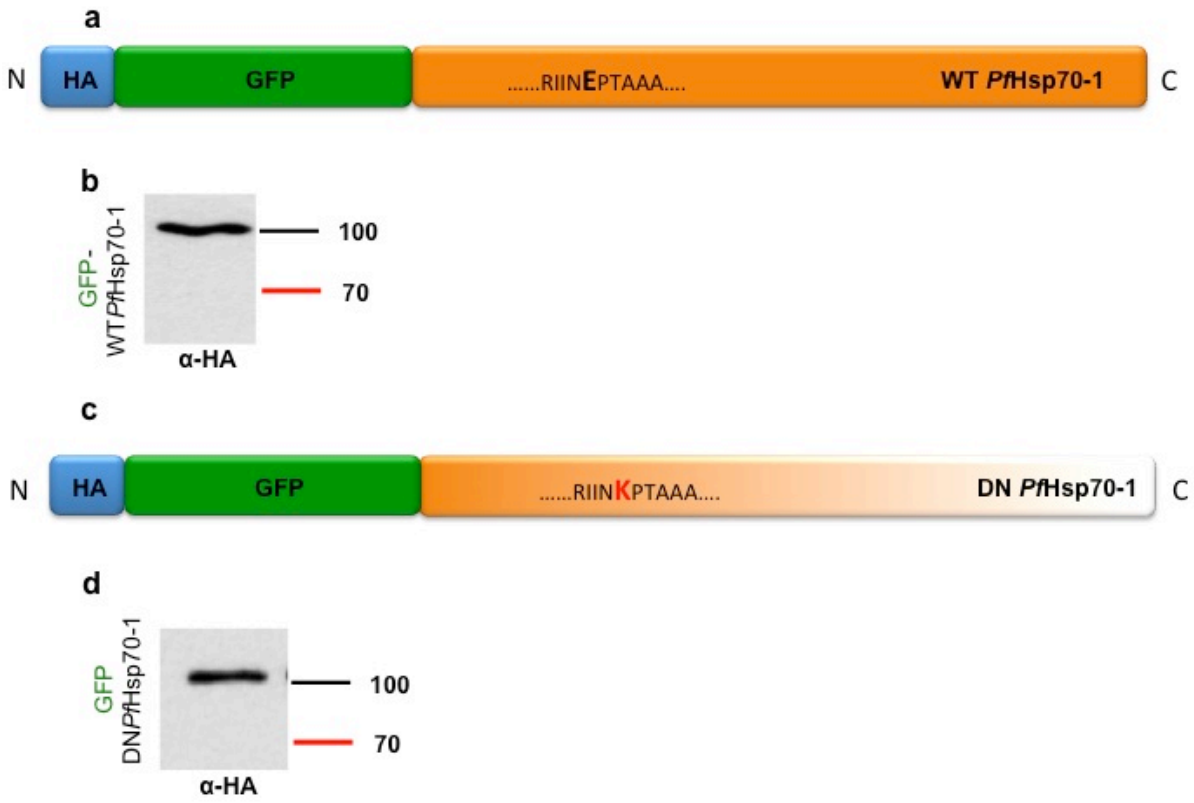

Figure S.2 Episomal expression of WT/DN *Pf*Hsp70-1 fused to GFP on the N-terminus.

(a) Schematic illustration of GFP-WT*Pf*Hsp70-1 fusion protein where the dd-FKBP is replaced with GFP. (b) Western blot analysis from the whole cell extract of cells expressing WT*Pf*Hsp70-1 N-terminally tagged to GFP. The fusion protein could be detected at the correct size of ~102 kDa with anti-HA antibody. (c) Schematic illustration of GFP-DN*Pf*Hsp70-1 fusion protein where the dd-FKBP is replaced with GFP. (b) Western blot analysis from the whole cell extract of cells expressing DN*Pf*Hsp70-1 N-terminally tagged to GFP. The fusion protein could be detected at the correct size of ~102kDa with anti-HA antibody. $1x10^7$ cell equivalents were used for all the western blot analysis.

7.3.2 Episomal expression of WT/DN *Pf*Hsp40 fused to GFP on the C-terminus

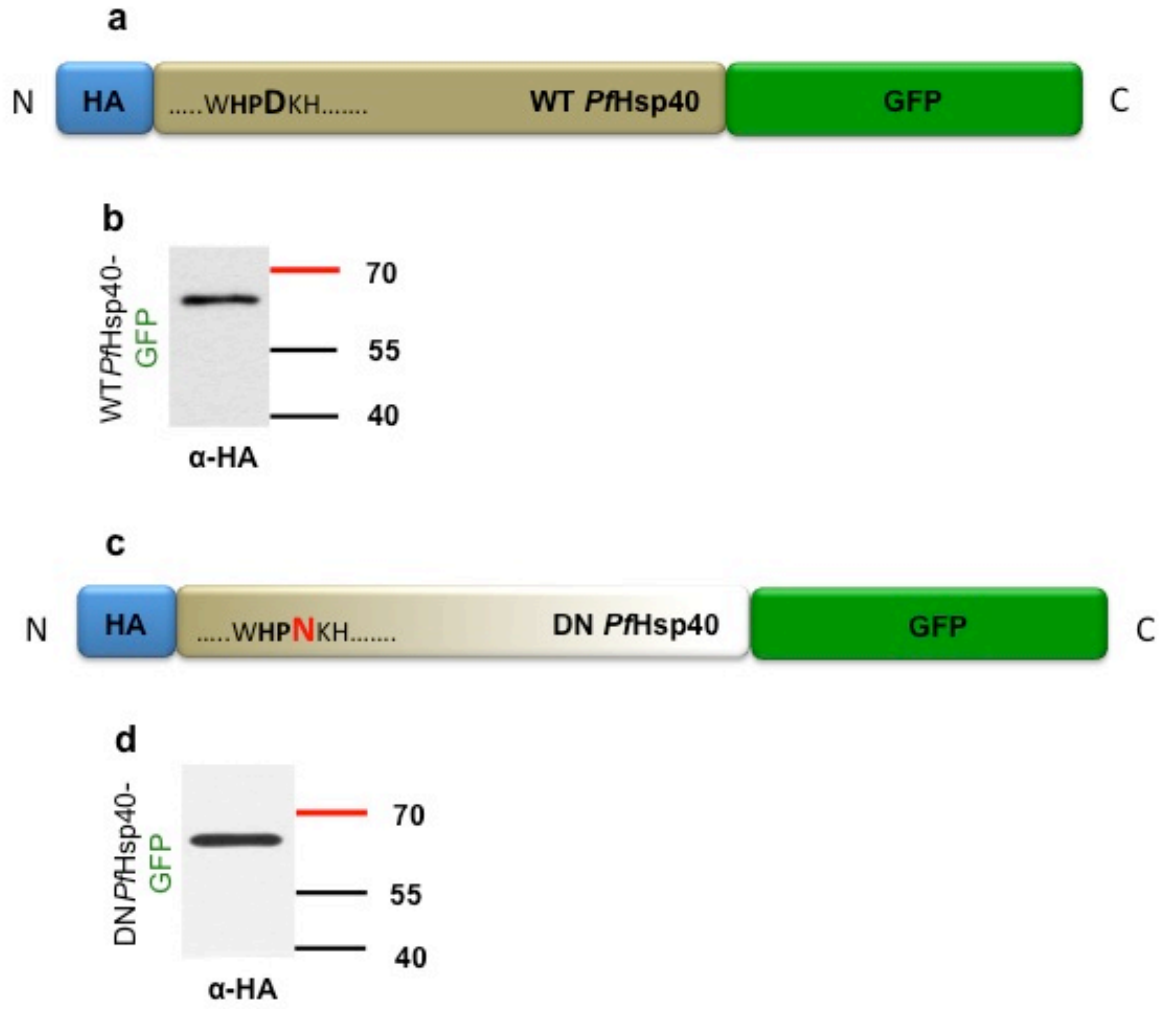

Figure S.3 Episomal expression of WT/DN *Pf*Hsp40 fused to GFP on the C-terminus.

(a) Schematic illustration of GFP-WT*Pf*Hsp40 fusion protein where the dd-FKBP is replaced with GFP. (b) Western blot analysis from the whole cell extract of cells expressing WT *Pf*Hsp40 C-terminally tagged to GFP. The fusion protein could be detected at the correct size of ~67kDa with anti-HA antibody. (c) Schematic illustration of GFP-DN*Pf*Hsp70-1 fusion protein where the dd-FKBP is replaced with GFP. (d) Western blot analysis from the whole cell extract of cells expressing DN*Pf*Hsp40 C-terminally tagged to GFP. The fusion protein could be detected at the correct size of ~67kDa with anti-HA antibody. $1x10^7$ cell equivalents were used for all the western blot analysis.

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Curriculum vitae

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Erklärung

Ich versichere hiermit, dass ich meine Dissertation

"A destabilisation domain approach to define the *in vivo* functional importance of *Pf*Hsp70-1 and *Pf*Hsp40 in the intraerythrocytic life cycle of *Plasmodium falciparum"*

Selbständig, ohne unerlaubte Hilfe und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den Pradipta Mandal