CLONING OF DDI (DNA DAMAGE INDUCIBLE) GENE IN PfCENV3/V4 TRANSECTION VECTORS AND PRODUCTION OF RECOMBINANT ATG7 PROTEINS FOR GENERATION OF ANTIBODIES

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Declaration by the Candidate

I, hereby, declare that the project report entitled **"Cloning of DDI (DNA damage inducible) gene in pfCENV3/V4 transfection vectors and production of recombinant Atg7 proteins for generation of antibodies"** submitted by me, Bhagawat Majhi (Reg. No.5-3-28-52-2013), to Tribhuvan University, Kirtipur, Nepal with supervision of Prof. Dr. Krishna Das Manandhar for partial fulfilment of requirement for the degree of M.Sc in Biotechnology is a record of bonafide research work carried out by me. This work was carried out under the guidance of Dr. Puran Singh Sijwali at the Centre for Cellular and Molecular Biology, Hyderabad, India during January-July 2016. I further declare that the work reported in this thesis has not been submitted elsewhere, either in part or in full, for the award of any other degree or diploma.

Date: ……. Signature of the candidate

Dedicated to my PARENTS and Science

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I.LIST OF ABBREVIATIONS AND SYMBOLS

S. No.	ABBREVIATION	FULL FORM
1	DDI	DNA damage inducible
2	UBA	Ubiquitin-associated
3	UBL	Ubiquitin-like
4	RVP	Retro-viral protease
5	Atg7	Autophagy- related protein 7
6	PCR	Polymerase chain reaction
7	°C	Degree centigrade
8	Amp	Ampicillin
9	APS	Ammonium per-sulfate
10	βME	Beta- mercaptoethanol
11	EDTA	Ethylenediaminetetraacetic acid
12	SDS	Sodium dodecyl sulfate
13	Tris	Tris(hydroxyl-methyl)aminomethane
14	TBE	Tris–borate EDTA
15	TGS	Tris-Glycine SDS
16	dNTPs	Deoxynucleotide triphosphate
17	*E. coli*	*Escherichia coli*
18	IPTG	Isopropyl β-D thiogalactoside
19	LB	Luria Bertani media
20	LA	LB Agar
21	kbp	Kilobase pair

22	kD	Kilodalton
23	mM	Millimolar
24	ng	nanogram
25	µg	microgram
26	µl	microlitre
27	DTT	Dithiothreitol
28	Ni-NTA	Nickel–nitrilotriacetic acid
29	PAGE	Polyacrylamide Gel Electrophoresis
30	RPM	Revolutions per minute
31	*Taq*	*Thermus aquaticus*
32	TEMED	N,N,N',N'- tetramethylethylenediamine
33	WT	Wild type
34	MT	Mutant type
35	Pb	*Plasmodium berghei*
36	Pf	*Plasmodium falciparum*
37	BCA	Bicinchoninic acid
38	PBS	Phosphate buffer saline
39	UPS	Ubiquitin proteasomal system
40	PQC	Protein Quality Control
41	GTX	Gliotoxin
42	Pfptm	Phosphoethanolamine methyltransferase
43	LPV	Lopinavir

44	RTV	Ritonavir
45	CCM	Confirmed clinical malaria
46	PIs	Protease inhibitors
47	BS	Blood smears
48	NNRTI	Non-nucleoside reverse transcriptase inhibitors
49	PfHT	Plasmodium glucose transporter
50	HDAC6	Histone deacetylase 6
51	ER	Endoplasmic reticulumn
52	UPR	Unfolded Protein response
53	MTOC	Microtubule organizing center
54	ATP	Adenosine Triphosphate
55	AMP	Adenosine Monophosphate
56	DUBs	Deubiquitinase
57	NEB	New England Biolabs
58	GFP	Green Fluorescent Protein
59	AMQ	Autoclaved MilliQ Water
60	CFA	Complete Freund Adjuvant
61	IFA	Incomplete Freund Adjuvant
62	ELISA	Enzyme Linked Immunosorbent Assay
63	PVDF	Polyvinylidene fluoride
64	DNA	Deoxyribonucleic acid
65	CVt	Cytoplasm to vacuole transport

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ABSTRACT

Researchers are inclined towards analysing protein synthesis especially in studying transcriptional and translational signals but the protein degradation process has been overlooked. The two major pathways of degradation are Ubiquitin proteasomal system (UPS) and autophagy. DNA damage inducible (DDI) acts as an ubiquitin receptor and has a role in degradation of ubiquitylated substrates through UPS. It has three domains: Ubiquitin-like (Ubl), Retroviral Protease (RVP) and Ubiquitin-associated (Uba). Ubl domain plays a role in binding to proteasome, Uba binds to ubiquitylated substrates and RVP domain codes for a retro-viral aspartic protease and is required for protein homo-dimerization. Our work mainly focuses on cloning of *Plasmodium falciparum* wild (WT) and mutant (MT) DDI genes in transfection vectors PfCENV3 and PfCENV4. The mutant DDI has catalytic aspartate residue at the position 220 substituted with alanine. The plasmid (pSSPF2-wtDDI and pSSPF2-mutDDI) was extracted from the bacterial culture and was then digested with restriction endonuclease Xho I and Bgl II to release DDI gene (1.2kbp) and pSSF2 backbone. The DDI coding gene both WT and MT were ligated in linear plasmid vector pfCENV3 as well as pfCENV4 backbone with T4 DNA ligase. The DNA was added to the competent cells and the true transformants were screened by colony PCR. The plasmid from the confirmed colonies were sequenced, analysed and its glycerol stocks were made for further analysis. The RPN11 is the important regulatory component of the proteasome so the RPN11 was cloned in PGT backbone which formed PGT-PbRPN11 vector. The autophagy is executed by concerted action of over 35 Autophagy-related protein 7 (Atg7). The presence of atypical autophagy system has been shown in malarial parasites in recent years, but the functions of autophagy in *Plasmodium* remain to be identified. The main focus of present work is Atg7, is E1-like activating enzyme that is essential for autophagy. The recombinant Atg7 was produced using bacterial expression system and antibodies against the recombinant protein were generated in rats. PET32a-Atg7 plasmid was isolated from BL21DE3 cells and transformed into rosetta-gami cells for the expression analysis and were cultured in large scale. The vector pET32a was added a 6X-His tag to the N-terminus of Atg7 and the resultant recombinant Atg7 was purified by Nickel-NTA affinity chromatography and refolded by dialysis. The anti-Atg antibodies were produced in rats and were used in ELISA and western blotting. The recombinant Atg7 was found to be immunogenic with enough production of antibodies against native Atg7. Purified antibodies can be useful in understanding the role of Atg7 in autophagy in *Plasmodium* and the recombinant antibodies can be assessed for therapeutic significance against malaria.

Keywords: -DDI, plasmid, autophagy, recombinant Atg7, transfection, antibodies.

CHAPTER I

INTRODUCTION

Researchers are inclined towards analysing protein synthesis especially in studying transcriptional and translational signals but the protein degradation process has been overlooked. During recent years, it was shown that proteasome is a valid drug target for sleeping sickness and malaria. Proteasomes has role in biology and virulence of the parasite and appears as a chemotherapeutic target.

Recombinant antibodies are slowly displacing the natural polyclonal antibodies in research, diagnostics and therapy because of its high specificity which almost nullify the exaggerated immune response. Nowadays, the most widely accepted approach to meet the feat is the through molecular cloning. Though cloning of genome is tedious, costly and has several limitations in itself but the outcome of the final product has benefits worth every penny. The synergistic effect of several genes makes difficult to understand the correct mechanism and sort out the correct effects. So, in order to understand the role of a particular genome, it is necessary to pick particular genome for the expression study. The autophagy process is more complex in human and eukaryotes with a large set of genes involved in complex mechanism so a detailed understanding of simple autophagy in the organism which is medically very important will throw light on the complex autophagic system of human. An understanding of molecular mechanism of autophagy in response to various stimuli will help to manipulate the pathways for therapeutic goals as well as help in pharmaceutical development of novel treatment methods for several diseases like malaria, HIV etc.

1.1 Malaria

Malaria is one of the most common infectious diseases with high mortality and morbidity rates. The World malaria report released on November 2018 showed that there were 219 million cases of malaria in 2017 up from 217 million cases in 2016 with an estimated number of deaths from malaria to be 435,000 in 2017. It is an endemic disease prevalent mostly in tropical and sub-tropical regions with high shares of global malaria burden in WHO African region. The causative agent of the disease is the parasite of *Plasmodium* species transmitted by the bite of the female *Anopheles* mosquito causing symptoms like fever, sweats, chills, headache, muscle aches, fatigue, and nausea.

The malaria epidemiology depends on multiple factors which include the density of mosquito vector, the optimised temperature, suitable environmental conditions, the

movements of population, altitude, parasitemia rates of the endemic populations and the species of the *Anopheles* mosquito (Anthony PC**,** Lennox A 2017). The five known *Plasmodium* species responsible to cause malaria in humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodim knowlesi* and of these *P. falciparum* and *P. vivax* possess greatest threat (Malaria, WHO, 27th March 2019). Malaria can be treated using chloroquine, primaquine, pyrimethamine, sulfadoxine and artemisinin. However, the emergence of drug resistant parasite against the drugs and unavailability of vaccine pose a challenge to malaria control effects.

1.1.1 *Life cycle of Plasmodium falciparum*

The life cycle of *Plasmodium falciparum* is complex and involves 2 hosts: A female *Anopheles* mosquito host as definite host and human host as secondary host.

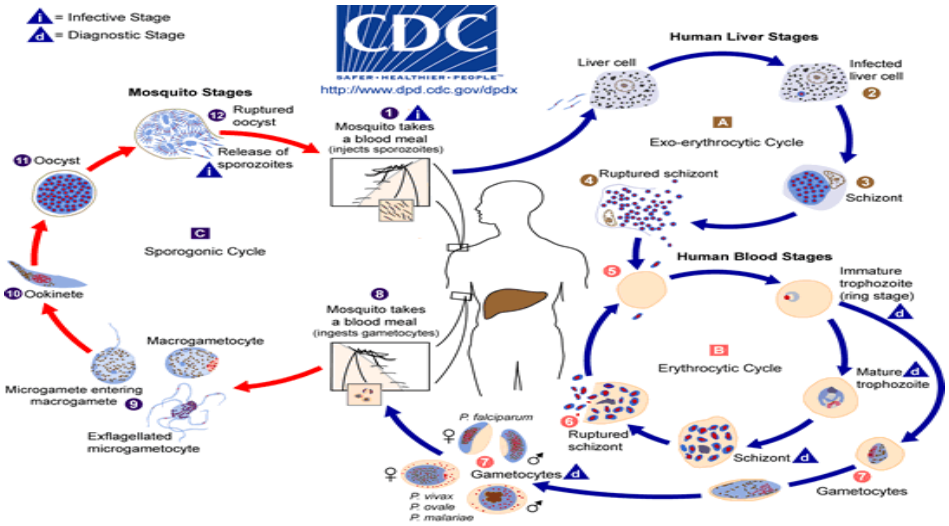

Fig 1.1 Life cycle of *Plasmodium falciparum*

(Reference: https://www.cdc.gov/dpdx/malaria/index.html)

When an infected female *Anopheles* mosquito bites a human, the sporozites are injected into the blood stream, initiating the infection. Sporozoites reach liver, invade liver cells and multiply inside the cell forming schizont in a process called exo-erythrocytic schizony. The schizont ruptures, releasing merozoites into the blood circulation, which invade RBCs, develop through ring-trophozoite-schizont stages. The schizont produces merozoites upon rupture, which invade fresh red blood cells, thus beginning a new round of infection. This phase of development is known as erythrocytic schigony and is responsible for disease. Some merozoites infect fresh RBCs and differentiate into gametocytes. When mosquito ingest gametocytes, each develops into a female macrogamete or eight male microgametes. The fusion of gametes results a zygote, which develops into a motile ookinete that penetrates the midgut wall and forms an

oocyst. Oocyst undergoes nuclear division followed by cytokinesis, forming hundreds of sporozoites, which are released in the hamocoel upon rupture of oocyst. Sporozoites make their way to the salivary gland of mosquito and are ready for new infection.

1.2 Protein Degradation

Researchers are mainly inclined towards analysing protein synthesis especially in studying transcriptional and translational signals but the protein steady-state degradation has been overlooked. The pioneering studies started from 1940s and two major pathways of degradation have been described for most cellular proteins in eukaryotic cells i.e. the ubiquitin proteasome system (UPS) and autophagy. Either UPS (during acute starvation) or autophagy (during chronic starvation) degrades the proteins into small polypeptides, maintains amino acid pools and energy balance, for which the de novo synthesis of amino acids is not effective. UPS degrades majority of proteins (80-90%) includes regulated, short-lived, abnormal, denatured, or, in general, damaged proteins while autophagy degrades most long lived or aggregated proteins and cellular organelles (mitochondria, peroxisomes, ribosomes, infectious organisms) and on disruption of either pathway contributes to pathology of many diseases. Both pathways maintain cellular homeostasis. These two degradative systems not only degrade proteins into small polypeptides, but help maintain amino acid pools and energy balance, either during acute starvation for the UPS, or in the course of chronic starvation for autophagy. Indeed, as de novo synthesis of amino acids carries high energy costs, recycling of amino acids is important for its contribution to maintenance of cellular homeostasis with less energy expenditure. Moreover, these catabolic pathways constitute essential components of the cellular control of protein quality (PQC), which senses misfolded or damaged proteins, tags them, and, finally, degrades them. Therefore, studying these systems is essential for developing a better understanding of protein homeostasis.

1.2.1 Ubiquitin Proteasomal System (UPS)

Proteasomes are protein complexes found in nucleus and cytoplasm in all eukaryotes. The function of UPS is to degrade damaged or unneeded proteins through proteolysis by the enzyme proteases. It regulates the concentration of the particular protein. The proteasomes perform several roles in many cellular pathways by degrading proteins to enforce quality control and regulate many cellular processes. The 20S proteasome (catalytic heart) is highly conserved in bacteria, yeast, and humans. The role of proteasomes in parasite biological processes such as cell differentiation, cell cycle, proliferation, and encystation has been confirmed by several reports. The key steps in

host colonization are proliferation and cell differentiation. The UPS has been strongly suggested as a viable parasitic therapeutic target by several evidences. Proteasome as a valid drug target for sleeping sickness and malaria has been shown by various researches in the recent years (Munoz et.al.,2015), so proteasomes are a key organelle in parasite biology and virulence which appears to be an attractive new chemotherapeutic target.

The ubiquitin modifies protein in three successive steps and are mediated by three enzymes namely; the activating enzyme (E1), the conjugating enzyme (E2), and the ubiquitin ligase (E3). The protein modification is reversible and can be done by specific deubiquitinating enzymes.

1.2.2 *Plasmodium* proteasome

The inhibition of the in-vitro development of exoerythrocytic forms of *Plasmodium berghei* was done by the lactacystin which showed stage specific inhibition having reduced parasitemia , which suggests the proteasome as a promising chemotherapeutic target. The cloning of genes that encode the β subunits of *P. falciparum* 20S proteasome has been done that has a key role in protein degradation process. The proteasome inhibitor bortezomib mediates transcriptional regulation and proteasome degradation in *Plasmodium* by blocking choline-mediated Phosphoethanolamine methyltransferase (Pfpmt) degradation (Witola et. al.,2007). Gliotoxin (GTX) is a fungal metabolite that has activity against *P. falciparum* with significant lower cytotoxicity against normal liver cell lines, and it also blocks chymotrypsin-like activity in the *P. falciparum* proteasome (Hatabu et.al., 2006). Thus, proteasome inhibitors suggest that *Plasmodium* proteasome could be used as potential antimalarial drug and validating the proteasome as a viable antimalarial-drug target (Munoz et.al.2015).

1.2.3 Autophagy

Autophagy is a lysosome mediated catabolic process in which cellular constituents are enclosed in double membrane vesicles, which then fuse with lysosome and the contents are degraded. The autophagy involves in diverse cellular functions such as adaptation to starvation, cell differentiation, quality control of proteins and organelles, aging, and degradation of invading microbes, so the study and implication of autophagy in human diseases such as cancer, inflammatory diseases, and neurodegeneration is increasing. Macroautophagy, microautophagy and chaperone-mediated autophagy are three different forms of autophagy, which differ on the mechanism of cargo selection.

There are as many as 35 Atg genes in yeast and mammalian cells that play key roles during autophagy. It has role in maintaining homeostasis, removing damaged organelles

and protein aggregates, balancing energy, antigen presentation and protein transport etc., and has crucial roles in development and disease. Starvation conditions enhance the formation of vesicles, whereas the amino acids inhibit their formation. So, autophagy appeared as a strategy to allow cell survival in low-nutrient conditions but is now clear that it involves in different diverse processes such as neurodegeneration, immune function, cancer, ageing and development (Focusing on autophagy , 2010, nature cell biology)**.** The malarial parasite, *Plasmodium* has conserved some of autophagic machinery whose purpose remained a mystery for a long.

1.3 Rationale of the study

The regulatory control mechanism of the *Plasmodium* parasite pathogenesis/physiology has not been fully elucidated till date. DNA damage inducible (DDI) has role in proteasomal degradation of specific proteins but its role has not been explored in case of malarial parasites. The role of autophagy is not known much in case of *Plasmodium* and the cloned genes of DDI can be used in transfection for episomal expression of these genes in the parasite and will also help in assessing if DDI as the target of malarial protease inhibitors similar to HIV protease inhibitors.

The role of Autophagy-related protein 7 (Atg7) in autophagy of *Plasmodium* has not been understood. The aim of this project was to generate antibodies against PfAtg7, which will be used as a marker for elucidating the endosomal trafficking pathway in *Plasmodium*. Anti-PfAtg7 antibodies will also serve as a marker to investigate association of autophagy and endosomal trafficking pathways. Particularly, the use of anti-PfAtg7 antibodies as an endosomal marker and anti-Atg8 antibodies as an autophagy marker will likely offer insights into the role of autophagy in cytoplasm to vesicular transport (CVt) during the development of *Plasmodium*. The purified recombinant antibodies if successful would have tremendous scope to be used as a therapeutic agent against malaria and characterization of Atg7 in *Plasmodium*.

1.4 Objectives

1.4.1 General Objective

Molecular cloning of DDI gene in plasmid vector and production of recombinant Atg7 gene to produce antibodies

1.4.2 Specific Objective

1. Cloning of DDI gene into transfection vector pfCENV3 and pfCENV4
2. Cloning of PGT-PbRPN11
3. Expression of Atg7 protein in large quantity and its titration
4. Purification of antibodies against Atg7 protein
5. Functional Characterisation of purified antibodies

1.5 Research Hypothesis

1.5.1 Null hypothesis (H0):-

- DDI is a target for retroviral protease inhibitors and have same role in *Plasmodium* pathogenesis.
- The recombinant protein PfAtg7 obtained from pET32a is more immunogenic than the native PfAtg7 in 3D7 parasite lysate.

1.5.2 Alternative hypothesis (H1):-

- DDI is a target for retroviral protease inhibitors but do not have same role in Plasmodium pathogenesis.
- The recombinant protein PfAtg7 is not as immunogenic as the native PfAtg7.

CHAPTER 2

LITERATURE REVIEW

2.1 DNA damage inducible (DDI) gene

DNA Damage inducible 1(DDI1) protein was found in yeast as a gene induced in response to a variety of genotoxic stresses (Liu and Xiao, 1997). DDI belongs to ubiquitin receptor family, transfers ubiquitinated substrates to the proteasome. DDI contain an amino-terminal ubiquitin-like (UBL) domain, a carboxy-terminal ubiquitin-associated (UBA) domain, and a retroviral aspartyl protease domain (RVP). DDI1 is the only representative of *Plasmodium falciparum* in which the UBL and UBA domains flank an aspartyl protease-like domain (Sirkis et al.,2006). The UBL domain of DDI1 shares 16% identity with ubiquitin (Bertolaet et. al*.,* 2001b) and was shown to bind to the proteasome (Kaplun et. al., 2005; Gabriely et.al., 2008).

2.1.1 Putative Functions of the DDI protein

a) Aspartic protease function

DDI1 is a putative aspartic protease, which has aspartic acid at its active site. Mutation of the aspartic acid with alanine has been carried out in retroviral aspartic proteases to study the role of particular function of the amino acid in protease activity. These proteases are crucial for proteolytic processing of the retroviral polyprotein and are targets proteinase inhibitor, such as HIV protease inhibitors.

b) Transfer of substrate to proteasome

The UBA domain of DDI1 interacts with the ubiquitinated protein that is delivered to the proteasome through interactions of the UBL domain (Nowicka et al.,2016). In yeast, the UBA-UBL protein of DDI1 is specifically required for Ho endonuclease degradation. Ho endonuclease initiates sexual mating type interconversion by making a site specific double-strand break at the mating type locus in late G1-phase just before entering the S-phase. The double stranded break is followed by unidirectional gene conversion where Ho endonuclease is required for switching of the mating type. After mating type switch, Ho endonuclease must be degraded. DDI1 recruits ubiquitylated Ho endonuclease to the proteasome for degradation. In the absence of DDI1, Ho endonuclease is stabilized and accumulates in the cytoplasm.

c) Negative regulation of protein secretion

DDI1 has been shown to negatively regulate external secretory pathway. Mutation in DDI1 or knockout of DDI1 enhances secretion onto the extra cellular media in yeast. Protein secretion of DDI1 appears to be mediated via interaction with the SNARE protein (Saccharomyces Genome Database- SGD, DDI1).

d) DDI1 domain participates in homodimerization

DDI1 shows ablility to homodimerize (Bertolaet et al., 2001a) like other UBL-UBA family members in *Saccharomyces cerevisiae.* Presence of RVP domain in DDI1 is well conserved throughout evolution and was shown to be active when present in dimeric form (Miller et al., 1989), which make it possible for homodimerization. This RVP domain of DDI1 mediates homodimerization in vivo, like in other conserved aspartyl proteases, suggesting a possible involvement in proteolysis (Krylov and Koonin, 2001). DDI also heterodimerizes with other members of UBL-UBA family proteins

2.1.2 Component and Structure of DDI1

DDI1 is composed of 385 amino acid residue in *Plasmodium bergei* and 382 amino acids in *Plasmodium falciparum*. A DDI1 homologue is found in all eukaryotes. The sequence identity of the central domain (*Saccharomyces cerevisiae* residues 198–324) is 55% with human orthologue and 50% with plant ortholog, indicating that the domain has been highly conserved throughout the evolution.

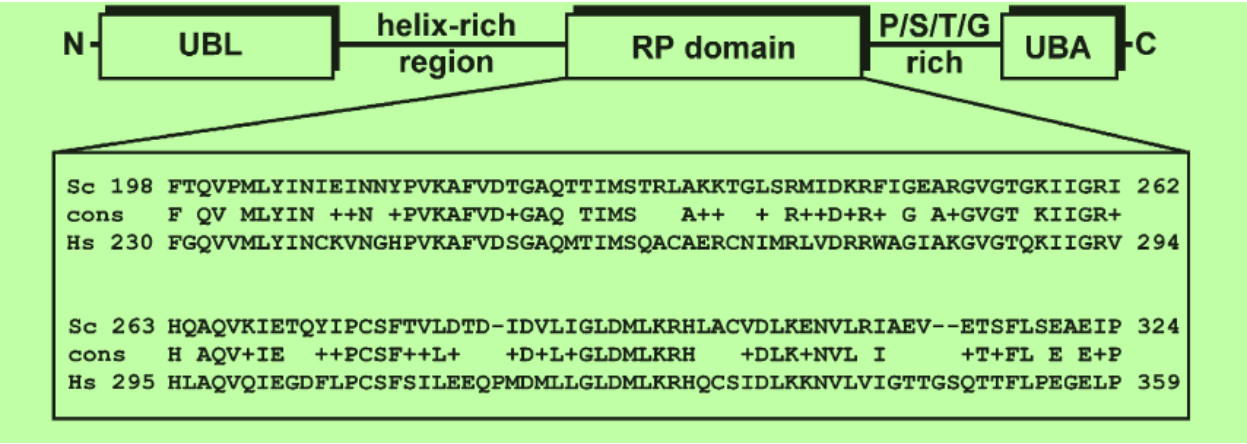

Fig 2.1Domain organization of DDI1 and conservation between yeast and human orthologues.

Fig 2.2.Domain architecture of DDI1 of Leishmania major, RVP, UBA and UBL domain (PMCID: PMC6120238PMID: 30186740)

DDI1 shares a fold and active-site structure with retroviral proteases

The RVP domain of DDI1 resembles the retroviral proteases (HIV-protease) in structure and may be a functional aspartyl protease involved in proteasome-mediated protein turn over. DDI1 shows similarity to retroviral protease in a manner of the arrangement of the active site.

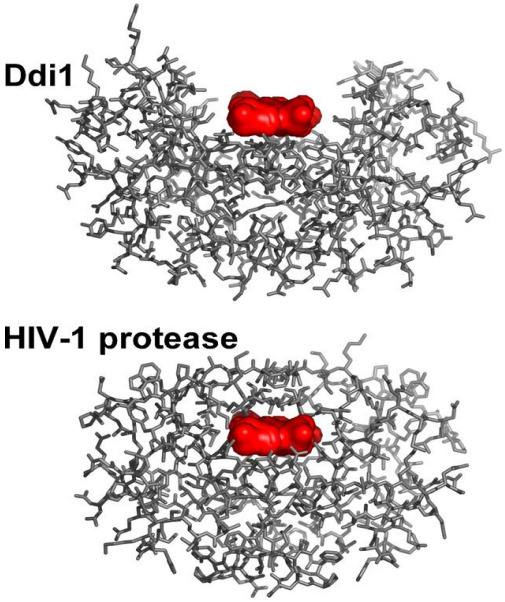

Fig 2.3 shows the modeling of peptides into the cavity of DDI1 and HIV-1 protease. Its red volume is a superposition of space filling models of peptides crystallized in the central cavity of DDI1 and HIV-1 protease.

Malaria and Human immunodeficiency virus (HIV) share some cross reactivity among the patients. The HIV protease inhibitors show activity against *Plasmodium falciparum* in vitro. The study conducted in 2012 showed that anti-retroviral drugs given to the HIV patients suffering from malarial infection have a lower propensity of suffering from a second round of infection (Achan et.al.,2012). This study gives the idea that anti-retroviral drugs with protease inhibitors have some effect on the *Plasmodium* pathogenesis. The study conducted among HIV-infected children in Uganda showed that a lopinavir–ritonavir–based antiretroviral regimen reduced the incidence of malaria by 41%. The HIV protease inhibitors show antimalarial effects which is supported by in-vitro studies of lopinavir activity in humans, in vivo studies showing activity against *P. chabaudi*, activity against clinical isolates of *P. falciparum* and *P. vivax*, in vitro activity against *P. falciparum* in serum samples from patients receiving protease inhibitors, and action against *P. falciparum* cytoadherence and phagocytosis. It is seen that HIV and malaria geographically overlap and the HIV protease inhibitors, lopinavir –ritonavir (LPV-RTV) used were in combination with malaria treatment showed lower risk of recurrent positive blood smear (BS) but not confirmed clinical malaria (CCM) in HIV-infected

children. The HIV protease inhibitor is supposed to exhibit killing activity for *Plasmodium* species because of inhibition of aspartyl protease enzymes. The reduced frequency of positive BS accounts malaria treatment while reduced episodes of CCM could be because of direct drug killing or pharmacokinetic interactions. The HIV protease inhibitors (PIs) associates with reduction in malaria for children on LPV-RTV ART. This finding was partly attributed to an interaction between the ritonavir of LPV-RTV and lumefantrine component of the artemisinin-combination regimen used to treat clinical malaria (Hobbs et.al.,2016). The lopinavir was the most potent protease inhibitor compound which was active against the *Plasmodium falciparum* (IC_{50}) at lower concentration. The antimalarial action of HIV1 protease inhibitors is due to the inhibition of plasmepsins. There are 10 plasmepsins in *P. falciparum*. The availability of antiretroviral therapy is most to HIV-infected individuals in malaria-endemic regions and also the use of protease inhibitors is likely to increase as NNRTI resistance is rising and protease inhibitor regimens are being simplified. The clinical trials for testing the hypothesis that HIV-1 protease inhibitors conferring protection against malaria are under the process (Parikh et. al., 2005). The growth and survival of *Plasmodium* is through plasmodium glucose transporter PfHT because it needs constant supply glucose as their primary source of energy. The HIV protease inhibitors (PIs) are shown to be the antagonists to the mammalian glucose transporters. The lopinavir (PI) has antimalarial activity and it blocks glucose uptake into isolated malaria parasites at therapeutically relevant drug levels. The lopinavir is inhibitor of malaria hexose transporter PfHT which provides strong rationale and molecular basis in antimalarial development by targeting the protein (Kraft et. al., 2015).

The *Plasmodium falciparum* is growing resistance to the available drugs nowadays for which the search for novel antimalarial drug targets is urgent, where the proteases can serve as an attractive antimalarial targets as they have indispensable roles in parasite infection and development. The proteases have role in host erythrocyte rupture (invasion) and hemoglobin degradation. The limited number of proteases have been identified and characterized in *Plasmodium* species. The 92 putative proteases in the *P. falciparum* genome have been identified using an extensive sequence similarity search, which includes calpain, metacaspase, and signal peptidase I. There are eighty three putative proteases which are transcribed actively in the intra-erythrocytic stage, and only sixty-seven are translated actively in the life cycle stage. The study represents an essential proteases as targets for inhibitor-based drug design (Yimin et. al., 2003).

2.2 Atg7 gene

Atg proteins are partially conserved in protozoa where as in eukaryotes they are highly conserved. All protozoa has Atg8 conjugation system with factors, Atg3, Atg4, Atg7, and

Atg8 for the unique organelle, the apicoplast. *Plasmodium falciparum* has partial Atg protein sets conserved (Kitamura et.al 2012).

A

S.cerevisiae	*H.sapiens*	*P.falciparum*
Atg1	ULK1/2	–
Atg13	Atg13	–
Atg17	FIP200	–
Atg29		
Atg31	Atg101	
Atg9	Atg9A/B	–
Vps34	Vps34	Vps34 (PFE0765W)
Vps15	Vps15	–
Vps30/Atg6	Beclin 1	–
Atg14	Atg14(L)	–
–	AMBRA1	–
Atg2	Atg2A/B	–
Atg18	WIPI1/2/3/4	Atg18 (PF10_0126)
Atg12	Atg12	Atg12 (PF14_0667a)
Atg7	Atg7	Atg7 (PF11_0271)
Atg10	Atg10	–
Atg5	Atg5	Atg5? (PF14_0283)
Atg16	Atg16L1	–
Atg3	Atg3	Atg3 (PFI0280c)
Atg4	Atg4A/B/C/D	Atg4 (PF14_0171)
Atg7	Atg7	Atg7 (PF11_0271)
Atg8	LC3A/B/B2/C GABARAP/L1/L2/L3	Atg8 (PF10_0193)

B

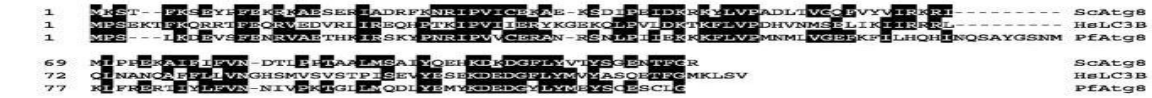

Fig 2.4 (A) List of Atg proteins in *S. cerevisiae*, *Homo sapiens* and *P. falciparum*.
(B)Alignment of the full sequences of *S. cerevisiae* Atg8, *H. sapiens* LC3B (one of the Atg8 homologs), and *P. falciparum* Atg8 (Kitamura et.al 2012)

The role of autophagy in *Plasmodium falciparum* is not known much. Apicoplast biogenesis in Plasmodium involves Atg8 conjugation system whose members (Atg7, Atg3, Atg8, and Atg4) were detected. Atg7 genes encodes Atg7 protein and the gene is related to Gsa7, Apg7Land Apg7-like which is essential in cell degradation and recycling, also is required for fusion of peroxisomal and vacuolar membranes. Atg7 acts as an E1 like enzymes on Atg12 and Atg8 (UBL proteins). Atg7 helps UBL proteins during autophagy initiation by binding to them and activating their transfer to an E2 enzyme. The Atg7 plays role in autophagy specific UBL systems, thus acts an essential regulator of autophagosome assembly. Atg7 involves in cytoplasm to vacuole transport (CVt) and autophagy. Atg7 activates Atg12 to conjugate with Atg5 which activates Atg8 to conjugate with phosphatidylethanolamine.These systems are essential for the Atg8 association to Cytoplasm to Vacuole transport (CVt) vesicles and auto-phagosomes membranes (Kitamura et.al 2012).

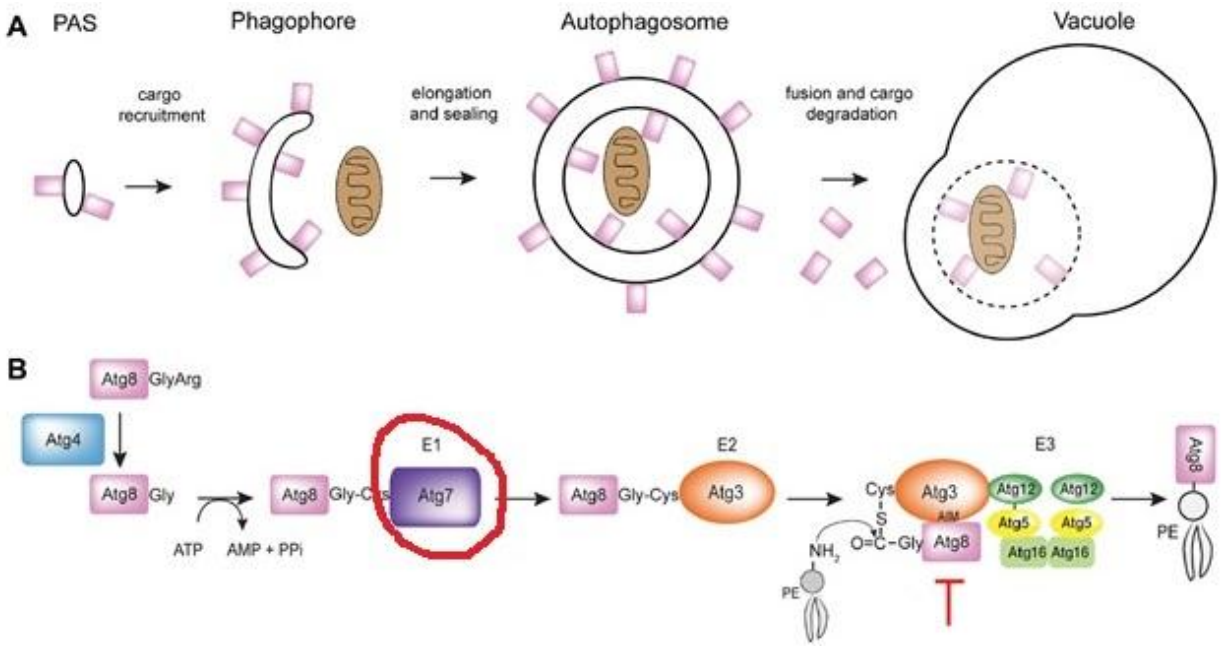

Fig 2.5.Overview of Atg8 Conjugation pathway in autophagy.

(A) Visual representation of the localization of Atg8 in autophagy. Atg8 (represented by the rectangle) is essential to the elongation of the autophagosomal membrane.

(B) Generic conjugation pathway shown for yeast system. In Plasmodium, Atg8 is synthesized with a C-terminal glycine that does not require proteolytic processing for activation. The red line indicates step of pathway targeted by our inhibitors. (Hain et al., 2014)

The study analysing the Atg8-conjugation pathway in the life cycle of *Plasmodium falciparum* and only the liver stage of *Plasmodium berghei* was done. The transgenic *P. falciparum* strain was engineered which expressed mCherry-PfAtg8 in human liver cells and erythrocytes as well as in the midgut and salivary glands of female *Anopheles* mosquitoes. It was shown that mCherry-PfAtg8 was localized to tubular structures in all stages. Also the electron microscopy and co-localization studies showed the association of PbAtg8 on the apicoplast in *Plasmodium berghei*. PbAtg7, PbAtg3, and PbAtg8 are co-transcribed in all stages of parasite. PbAtg3, PbAtg7, and PbAtg8 are constitutively transcribed throughout the parasite life cycle. The preferential distribution of PbATG8 and PfAtg8 is on the outermost membranes of the apicoplast (Jayabalasingham et. al, 2014).

The E1 type ligase Atg7, an E2 type ligase Atg3, and a cysteine protease Atg4 are required in Atg8 lipidation in yeast. During the parasite's erythrocytic stages, the four PfAtg genes (Atg7, Atg8, Atg3 and Atg4) gets transcribed. The PfAtg7 is conserved and activtes PfAtg8. There was rapid PfAtg7 and parasite growth reduction performed in attenuation experiments that demonstrated the necessity of PfAtg7 for parasite growth. The parasites upon PfAtg7 attenuation did not die which may be due to insufficient PfAtg7 attenuation or possibly the parasite is able to survive in its absence for which the

further experiment is needed. Thus, it suggests that PfAtg7 can be a druggable target to treat malaria (Walker et. al.,2013).

The generation of the conditional knockout mice of Atg7 showed that the deficiency of Atg7 led to multiple cellular abnormalities like deformed mitochondria and accumulation of ubiquitin-positive aggregates that indicates the importance of autophagy in starvation response and the quality control of proteins and organelles. The autophagy plays important role that includes cellular remodeling during differentiation and development stages of multicellular organisms. Atg12 is activated by Atg7 (E1 like enzyme) and transferred to Atg10 (E2-like enzyme) and then finally conjugates to Atg5. Atg8 is another ubiquitin-like protein that conjugates to phosphatidylethanolamine. Atg8 is activated by Atg7, which is common to the Atg12 conjugation system, and is transferred to Atg3 (E2-like enzyme).The Atg7 deficient mice were born at Mendelian ratio and had normal histology of major organs but their body weight was reduced and died within one day after birth. Atg7 deficient mice had lower amino acid level. There was no autophagosome formation in the Atg7-deficient liver and showed that the degradation of proteins and organelles under fasting condition was largely impaired. This suggests that the rapid proteins and organelles reduction upon fasting is largely dependent on Atg7 and autophagosome formation.

The degradation of long-lived protein in mutant hepatocytes was inhibited and also the presence of concentric membranous structure and accumulation of deformed mitochondria were observed in Atg7-deficient hepatocytes. Atg7 deficiency showed hepatomegaly in the Atg7-deficient cells. The detection of protein aggregates in Atg7-deficient liver reports that ubiquitin positive protein aggregate formation leads to proteasome inhibition and their elimination is largely dependent on the autophagic process. The aggregation of these proteins is predicted to be involved in the pathogenesis of several diseases such as Huntington's disease, Parkinson's disease and peripheral neuropathies so the role of the autophagy is very important in cure of these diseases (Komatsu et.al.,2005). The Apg12p–Apg5p conjugating system in *Saccharomyces cerevisiae* is essential for autophagy. The conjugation reaction requires Apg7p as Apg12p is unable to conjugate with Apg5p in the *apg7/cvt2* mutant which shows Apg7p is similar to ubiquitin-activating enzyme (Uba1p). Apg7p (similar to Atg7) is Apg12p -activating enzyme that has indispensable role in the yeast autophagy. A human Apg12p homologue have been identified and observed that the Apg12p homologue also conjugates with a human Apg5p homologue (Tanida et.al., 1999).

Alignment of Homologous Atg7 of:-

I. ***Saccharomyces cerevisiae* Atg7 (ScAtg7)**

MSSERVLSYAPAFKSFLDTSFFQELSRLKLDVLKLDSTCQPLTVNLDLHNIPKSADQVPL
FLTNRSFEKHNNKRTNEVPLQGSIFNFNVLDEFKNLDKQLFLHQRALECWEDGIKDINKC
VSFVIISFADLKKYRFYYWLGVPCFQRPSSTVLHVRPEPSLKGLFSKCQKWFDVNYSKWV
CILDADDEIVNYDKCIIRKTKVLAIRDTSTMENVPSALTKNFLSVLQYDVPDLIDFKLLI
IRQNEGSFALNATFASIDPQSSSSNPDMKVSGWERNVQGKLAPRVVDLSSLLDPLKIADQ
SVDLNLKLMKWRILPDLNLDIIKNTKVLLLGAGTLGCYVSRALIAWGVRKITFVDNGTVS
YSNPVRQALYNFEDCGKPKAELAAASLKRIFPLMDATGVKLSIPMIGHKLVNEEAQHKDF
DRLRALIKEHDIIFLLVDSRESRWLPSLLSNIENKTVINAALGFDSYLVMRHGNRDEQSS
KQLGCYFCHDVVAPTDSLTDRTLDQMCTVTRPGVAMMASSLAVELMTSLLQTKYSGSETT
VLGDIPHQIRGFLHNFSILKLETPAYEHCPACSPKVIEAFTDLGWEFVKKALEHPLYLEE
ISGLSVIKQEVERLGNDVFEWEDDESDEIA

The underlined sequence is the ATP-binding pocket. The region in blue is N-terminal domain (NTD) that binds to Atg3. The region in red is the C-terminal domain (CTD) or the adenylation domain, which is required for dimerization and binds to Atg8.

II. ***Homo sapiens* Atg7 (HsATG7)**

MAAATGDPGLSKLQFAPFSSALDVGFWHELTQKKLNEYRLDEAPKDIKGYYYNGDSAGLP
ARLTLEFSAFDMSAPTPARCCPAIGTLYNTNTLESFKTADKKLLLEQAANEIWESIKSGT
ALENPVLLNKFLLLTFADLKKYHFYYWFCYPALCLPESLPLIQGPVGLDQRFSLKQIEAL
ECAYDNLCQTEGVTALPYFLIKYDENMVLVSLLKHYSDFFQGQRTKITIGVYDPCNLAQY
PGWPLRNFLVLAAHRWSSSFQSVEVVCFRDRTMQGARDVAHSIIFEVKLPEMAFSPDCPK
AVGWEKNQKGGMGPRMVNLSECMDPKRLAESSVDLNLKLMCWRLVPTLDLDKVVSVKCLL
LGAGTLGCNVARTLMGWGVRHITFVDNAKISYSNPVRQPLYEFEDCLGGGKPKALAAADR
LQKIFPGVNARGFNMSIPMPGHPVNFSSVTLEQARRDVEQLEQLIESHDVVFLLMDTRES
RWLPAVIAASKRKLVINAALGFDTFVVMRHGLKKPKQQGAGDLCPNHPVASADLLGSSLF
ANIPGYKLGCYFCNDVVAPGDSTRDRTLDQQCTVSRPGLAVIAGALAVELMVSVLQHPEG
GYAIASSSDDRMNEPPTSLGLVPHQIRGFLSRFDNVLPVSLAFDKCTACSSKVLDQYERE
GFNFLAKVFNSSHSFLEDLTGLTLLHQETQAAEIWDMSDDETI

III. *Plasmodium falciparum* Atg7 (PfAtg7)

MKKKFEEENKPSYILKHNNNEFKIDISYFTQLHEHKINIYKLQSNYVNLCSSTYVNKIKL
GFKYKLLNRYLIEFAHPFIHVRTIEINKKSFLKYENFDNEDEKNNMEPNDCTKTIENERN
HINNINDGNKKVQKIWYIMNNYRNNYLGVLLNFNTLEEFLKCNKDDHINYTLEPLKCYIN
NEKNDICKDMNLYIHDNIYDDTFWEYKENCLTVLEKINKYVILSFFDLKKYICYYSIANP
IIKPKDNYYKLIKNSTRYFFYIDSKYVYINTENRHINIIDIFYLSYKIDDYFNNYKMFLN
TNIFLLLKFDNIPLHTMNNQDYYDEYINKLYTNIECEEDQKSKKEFYQINSFYKLFEYLK
LNDISQNSYHPMGNKSFNNHYNNNSSMLHKNYDMVILPINALSELKEDIKNSKDKILRYI
KKDFFDLYICFIDINYIFNSLSWDFRNLLYCLTLKYKLYDFQIDVLAFRDISLLRQQYVG
TFKSQEGFIWSYPKVVMKRGSINNPRNYNDEDKNNDNNNYDDKNNDNNNYDDSHNNNYDD
KNNDNNNYDDSHNNNYEDSHNNNYDDSDLHKDIDMDKDKNNSFHYNPINNCLSHQDVSFC
SVTKMCKVNYNSIKDCKNDWRDDLTNEYSHDMNPIHEDIEHSSSQYENNMSVNNTYKKDN
RNIKHNHNNIYHNHLVKYILNSSLFQVTVPDKVHFIYDNGSNYVDINLNGKKDDSLNKQD
IHILEKKKEGDTCIINSYLKSFSDEKKNDCIDVSSNLGFSINIRKEDNHFTTRVKYKDEE
MDVLHISEGDENENNMNNATNNNINNNIIKNYKTFCCDNKVYDILCGWKYYEDKKKEKKS
IISIINLNDFINKDTIQRISLELNIKLIKWKILKDLKFEHIKKLKILIIGLGTLGCMVAR
NCVSWGIQHYTFVDNSRVSFSNISRQYLYTLEDAEKYGNIGEYKCVAAKKNLLKICPDLN
ITAKVMDIPMPGHLNYLNENLEDTINELDNLINNHDVVFLLTDSKESRYFPCLMIAEKQY
NSLKELQESVNHNNNNNNNNNSSSSSSGSNKFRKGDNVLCEEENMITHEYIENIKCTKIM
DKSLNNILLYEQNNNIYKSLNNIHMYDRYQEIFYNNILTSVKRLCKMPPLGITVAISFDS
FVVLRHSYLYFKGACYFCNDMHCPSDSLSYRTLDEKCTVTRCGISNISSSIATELLLALT
QHPLYFFAPHIDRDQYIYNYDNDMNQKKNSDISNIFTSCLGATPHIMNFNLANFTIKKIF
CEPFEKCMCCSERVILKYQEDKMDFIRNVIRDSSILERITNMDQLKVEENDVIILE

Sequence Length: 1316 aa

Alignment of PfAtg7 with ScAtg7 and HsAtg7

```
ScAtg7          -MSSER------VLSYAPAFKSFLDTSFFQELSRLKLDVLKLDSTCQPLTVNLDLHNIPK 53
HsATG7          -MAAATGDPGLSKLQFAP-FSSALDVGFWHELTQKKLNEYRLDEAPKDIKG--YYYNGDS 56
PfAtg7          MKKKFEEENKPSYILKHNNNEFKIDISYFTQLHEHKINIYKLQSNYVNLCSSTYVNKIKL 60
                            :      .  :* .:: :* . *::  :*:.    :       :

ScAtg7          ----------------------SADQVPLFLTNRSFEKHNNKRTNEVP----------- 79
HsATG7          ----------------------AGLPARLTLEFSAFDMSAPTPARCCP----------- 82
PfAtg7          GFKYKLLNRYLIEFAHPFIHVRTIEINKKSFLKYENFDNEDEKNNMEPNDCTKTIENERN 120
                                        *    *:    .
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```
ScAtg7          -------------------------LQGSIFNFNVLDEFKNLDK-----------QLFLH 103
HsATG7          -------------------------AIGTLYNTNTLESFKTADK-----------KLLLE 106
PfAtg7          HINNINDGNKKVQKIWYIMNNYRNNYLGVLLNFNTLEEFLKCNKDDHINYTLEPLKCYIN 180
                                          * : * *.*:.* . :*          :  :.

ScAtg7          QRALECWEDG--------------IKDINKCVS------FVIISFADLKKYRFYYWLGVP 143
HsATG7          QAANEIWES---------------IKSGTALENPVLLNKFLLLTFADLKKYHFYYWFCYP 151
PfAtg7          NEKNDICKDMNLYIHDNIYDDTFWEYKENCLTVLEKINKYVILSFFDLKKYICYYSIANP 240
                :   :  :.                . .          :::::* *****  ** :  *

ScAtg7          CFQRPSSTVLHVR------------------------------------------------ 156
HsATG7          ALCLPESLPLIQG------------------------------------------------ 164
PfAtg7          IIKPKDNYYKLIKNSTRYFFYIDSKYVYINTENRHINIIDIFYLSYKIDDYFNNYKMFLN 300
                 :   ..

ScAtg7          ------------------PEPSLKGLFSKCQKWFDVNYS---------------------- 177
HsATG7          ------------------PVGLDQRFSLKQIEALECAYDNLC------------------- 188
PfAtg7          TNIFLLLKFDNIPLHTMNNQDYYDEYINKLYTNIECEEDQKSKKEFYQINSFYKLFEYLK 360
                                .    *    ::   .

ScAtg7          -----------------KWVCILDADDEIVNYDKCII---------------RKTKVLAI 205
HsATG7          -----------------QTEGVTALPYFLIKYDENMVLVSLLKHYSDFFQGQRTKITIGV 231
PfAtg7          LNDISQNSYHPMGNKSFNNHYNNNSSMLHKNYDMVILPINALSELKEDIKNSKDKILRYI 420
                                :           :**  ::              : .    :

ScAtg7          R-----------DTSTMENVPSALTKNFLSVLQYDVP-DLIDFKLLIIR----------- 242
HsATG7          Y-----------DPCNLAQYPGWPLRNFLVLAAHRWSSSFQSVEVVCFRDR--------- 271
PfAtg7          KKDFFDLYICFIDINYIFNSLSWDFRNLLYCLTLKYKLYDFQIDVLAFRDISLLRQQYVG 480
                           *   : :  .   :*:*           ...:: :*

ScAtg7          ------------------------------------------------------------
HsATG7          ------------------------------------------------------------
PfAtg7          TFKSQEGFIWSYPKVVMKRGSINNPRNYNDEDKNNDNNNYDDKNNDNNNYDDSHNNNYDD 540


ScAtg7          ------------------------------------------------------------
HsATG7          ------------------------------------------------------------
PfAtg7          KNNDNNNYDDSHNNNYEDSHNNNYDDSDLHKDIDMDKDKNNSFHYNPINNCLSHQDVSFC 600


ScAtg7          ------------------------QNEGSFALNATFASIDPQSSSSNPDMKVSGWERNVQ 278
HsATG7          ----------------------TMQGARDVAHSIIFEVKLPEMAFSPDCPKAVGWEKNQK 309
PfAtg7          SVTKMCKVNYNSIKDCKNDWRDDLTNEYSHDMNPIHEDIEHSSSQYENNMSVNNTYKKDN 660
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ScAtg7          GKLAP---------------------------------------------------- 283
HsATG7          GGMGP---------------------------------------------------- 314
PfAtg7          RNIKHNHNNIYHNHLVKYILNSSLFQVTVPDKVHFIYDNGSNYVDINLNGKKDDSLNKQD 720
                  :

ScAtg7          ------------------------------------------------------------
HsATG7          ------------------------------------------------------------
PfAtg7          IHILEKKKEGDTCIINSYLKSFSDEKKNDCIDVSSNLGFSINIRKEDNHFTTRVKYKDEE 780

ScAtg7          ------------------------------------------------------------
HsATG7          ------------------------------------------------------------
PfAtg7          MDVLHISEGDENENNMNNATNNNINNNIIKNYKTFCCDNKVYDILCGWKYYEDKKKEKKS 840

ScAtg7          --RVVDLSSLLDPLKIADQSVDLNLKLMKWRILPDLNLDIIKNTKVLLLGAGTLGCYVSR 341
HsATG7          --RMVNLSECMDPKRLAESSVDLNLKLMCWRLVPTLDLDKVVSVKCLLLGAGTLGCNVAR 372
PfAtg7          IISIINLNDFINKDTIQRISLELNIKLIKWKILKDLKFEHIKKLKILIIGLGTLGCMVAR 900
                   :::*.. ::   :   *::**:**: *:::  *.:: : . * *::* ***** *:*

ScAtg7          ALIAWGVRKITFVDNGTVSYSNPVRQALYNFEDC------GKPKAELAAASLKRIFPLMD 395
HsATG7          TLMGWGVRHITFVDNAKISYSNPVRQPLYEFEDCLGG---GKPKALAAADRLQKIFPGVN 429
PfAtg7          NCVSWGIQHYTFVDNSRVSFSNISRQYLYTLEDAEKYGNIGEYKCVAAKKNLLKICPDLN 960
                  :.**::: *****. :*:**  ** ** :**.      *: *.  *   * :* * ::

ScAtg7          ATGVKLSIPMIGHKL----VNEEAQHKDFDRLRALIKEHDIIFLLVDSRESRWLPSLLSN 451
HsATG7          ARGFNMSIPMPGHPVNFSSVTLEQARRDVEQLEQLIESHDVVFLLMDTRESRWLPAVIAA 489
PfAtg7          ITAKVMDIPMPGHLN----YLNENLEDTINELDNLINNHDVVFLLTDSKESRYFPCLMIA 1016
                  .  :.*** **         *  .  .:.*  **:.**::*** *::***::*.::

ScAtg7          IENKTVINAALGFDSYLVMRHGNRDEQSS------------------------------ 480
HsATG7          SKRKLVINAALGFDTFVVMRHGLKKPKQQGAGDLCPNH---------------------- 527
PfAtg7          EKQYNSLKELQESVNHNNNNNNNNNSSSSSSGSNKFRKGDNVLCEEENMITHEYIENIKC 1076
                 :.   ::      ..   .:. .. ...

ScAtg7          ------------------------------------------------------------
HsATG7          ------------------------------------------------------PVAS 531
PfAtg7          TKIMDKSLNNILLYEQNNNIYKSLNNIHMYDRYQEIFYNNILTSVKRLCKMPPLGITVAI 1136
```

```
ScAtg7          --------------KQLGCYFCHDVVAPTDSLTDRTLDQMCTVTRPGVAMMASSLAVELM 526
HsATG7          ADLLGSSLFANIPGYKLGCYFCNDVVAPGDSTRDRTLDQQCTVSRPGLAVIAGALAVELM 591
PfAtg7          SFDSFVVLRHSYLYFKGACYFCNDMHCPSDSLSYRTLDEKCTVTRCGISNISSSIATELL 1196
                              : .****:*: .* **   ****: ***:* *:: ::.::*.**:

ScAtg7          TSLLQT-----------------KYSG------------SETTVLGDIPHQIRGFLHNFSI 558
HsATG7          VSVLQH-----------------PEGGYAIASSSDDRMNEPPTSLGLVPHQIRGFLSRFDN 635
PfAtg7          LALTQHPLYFFAPHIDRDQYIYNYDNDMNQKKNSDISNIFTSCLGATPHIMNFNLANFTI 1256
                 :: *                   ..              .: **  ** :.  * .*

ScAtg7          LKLETPAYEHCPACSPKVIEAFTDLGWEFVKKALEHP-LYLEEISGLSVIKQEVERLGND 617
HsATG7          VLPVSLAFDKCTACSSKVLDQYEREGFNFLAKVFNSSHSFLEDLTGLTLLHQETQ--AAE 693
PfAtg7          KKIFCEPFEKCMCCSERVILKYQEDKMDFIRNVIRDS-SILERITNMDQLKVEEN----D 1311
                      .:::* .** :*:  :     :*: :.:. .   ** ::.:  :: * :    :

ScAtg7          VFEWEDDESDEIA 630
HsATG7          IWDMSDDETI--- 703
PfAtg7          VIILE-------- 1316
```

2.3 Relation between Ubiquitin Proteasome System (UPS) and autophagy

Among two major pathways of degradation: UPS degrades 80-90% of proteins that includes many regulated, abnormal, short lived, denatured, or damaged proteins while autophagy degrades most long lived proteins, aggregated proteins as well as cellular organelles (mitochondria, peroxysomes, ribosomes, infectious organisms). UPS and autophagy keeps energetic homeostasis and proteostasis. Long lived proteins gets degraded by autophagy while short lived proteins by UPS. UPS is activated in cultured cells not subjected to stress condition but autophagy is activated during stress condition. The proteins that chooses either UPS or autophagy depends upon various parameters such as structure of the protein, the chaperone and co-chaperone molecules that constitute the primary detection device to the proteins that gets degraded.

The tagging system with ubiquitin or ubiquitin –like proteins determines the relation between the UPS and autophagy system for protein degradation. The type of the ubiquitination directs substrate to either degradation system such as K48- linked poly-ubiquitinated chains target UPS while K63-linked poly/mono-ubiquitinated chains target autophagy system. Some substrates are degraded by both the pathways such as α-synuclein and alpha1 anti¬trypsin in the endoplasmic reticulum(ER). It is seen that the proteasome inhibitors induces autophagy as compensatory response for a limited situation, where p62 protein is involved in both the process. The long term inhibition of autophagy slows the clearance of the short lived proteasome specific substrates like p53

because of the high levels of p62 due to sequestration of ubiquitinated short lived substrates. The endoplasmic reticulum (ER) coordinates the activity of the UPS and autophagy, where the post translation of the protein occurs. The accumulation of the premature or misfolded proteins in the cytosol or the lumen of ER causes ER stress, so UPS must degrade misfolded proteins exporting from the ER by the mechanism called unfolded protein response (UPR). UPR pathway can also activate autophagy by stimulating the expression of Atg5, Atg7, and LC3 genes.

The HDAC6 (histone deacetylase 6) plays role at the interface between the UPS and autophagy. The retrograde transport of autophagosomes and lysosomes to microtubule organizing center (MTOC) by HDAC6 where it facilitates the autophagic degradation of aggresomes. Therefore, HDAC6 favor autophagy upon the proteasome process.

The level of ATP constitute a link between the ubiquitin-proteasome and autophagy. The high level of ATP favours UPS working properly as this degradation process uses more energy. The lower ATP level slows down protein refolding as well as proteasomal degradation. The autophagy is activated in low ATP level in part due to the mechanistic increase of AMP that activates AMPK and finally inhibits the mTOR pathway.

The ubiquitin-proteasome system (UPS) and autophagy are the two synergistic degradation systems in eukaryotic cells that maintains energetic homeostasis and proteostasis. These two degradative pathways can be co-activated to degrade misfolded or damaged proteins and display also compensatory effects when one is dysfunctional. The overall understanding of the molecular mechanisms of communication between UPS and autophagy that response to different stimuli is important in manipulating the pathways needed for therapeutic goals and pharmaceutical development of novel treatment methods for a variety of diseases (Lilienbaum A. 2013).

2.4 Deubiquitination

The RPN 11 is one of the regulatory component of the proteasome. Ubiquitination is important for the protein degradation by the proteasome whereas the deubiquitinases (DUBs) catalyze the removal of ubiquitin from target proteins and also involves in maturation, recycling, and editing of ubiquitin. The ubiquitination process can be reversible. This process involves the coordination of many enzymes such as ubiquitin ligases which attach ubiquitin to proteins and allow their degradation. On the other hand, deubiquitylating (DUB) enzymes allow ubiquitin molecules to deconjugate from the target proteins and the proteasome mediated degradation is prevented. The RPN11 is a deubiquitylating enzyme and associates with the 19S regulatory particle lid of the proteasome. The RPN11 removes ubiquitin from target proteins and facilitates the

protein degradation by 20S proteasome core particle.RPN11 is an intrinsic subunit of the lid sub-complex of the 19S regulatory particle that contains a conserved predicted metalloprotease motif. The proteolysis occurs when the ubiquitinated substrate protein binds to the 19S regulatory particle and is followed by its unfolding and translocation into the lumen of the 20S core where it gets degraded by the action of the 20S peptidases. The ubiquitin targeting signal is detached from the substrate at some point in this process which then appeals the deubiquitination process that renders the targeting event irreversible and prevent unproductive turnover of ubiquitin and blockage of the 20S core entry portal of the ubiquitin chain. The lid sub-complex of the 19S regulatory particle plays necessary role for the ubiquitin-dependent degradation . The Rpn11 is an essential protein of the lid so the evaluation of the effect of the RPN11 mutation by plasmid shuffling was done. The metalloisopeptidase activity of RPN11 deubiquitinated the substrate . The substrate deubiquitination by RPN11 defines a new key step in protein degradation by 26S proteasome. The function of the lid is to serve as a specialized isopeptidase that tightly couples the deubiquitination and degradation of substrates (Verma et. al., 2002).

There are twenty deubiquitylating enzymes (DUBs) found in saccharomyces cerevisiae that catalyzes the hydrolysis of the isopeptide bonds that link ubiquitin to its targets . The DUBs are mostly thiol proteases but only RPN11 is a zinc metalloprotease. The DUBs recycle ubiquitin by recovering ubiquitin from ubiquitin–protein conjugates before the target protein gets degraded, and its defects give rise to reduced ubiquitin levels. The main DUBs that recovers ubiquitin from conjugates are Ubp6, RPN11and Doa4. The ubiquitin are rescued from degradation by the proteasome by Ubp6 and RPN11, whereas the ubiquitin are released by Doa4 from membrane proteins before being internalized within multivesicular bodies in route to the lysosome. The Ubp6 and RPN11 releases ubiquitin from proteasome substrates in the form of unanchored and are active only when associates with the proteasome (Finley et.al., 2012).The isopeptidase activity of the subunit RPN11 removes the polyubiquitin moieties. The RPN8-RPN11 heterodimer responds in removing polyubiquitin tags before substrate gets degraded in the proteasome (Pathare et. al., 2014).The polyubiquitination directs protein substrates to the 26S proteasome for degradation, whereas the polyubiquitin substrate gets removed by the deubiquitinase RPN11 during ATP-dependent substrate degradation (Worden et. al., 2017).

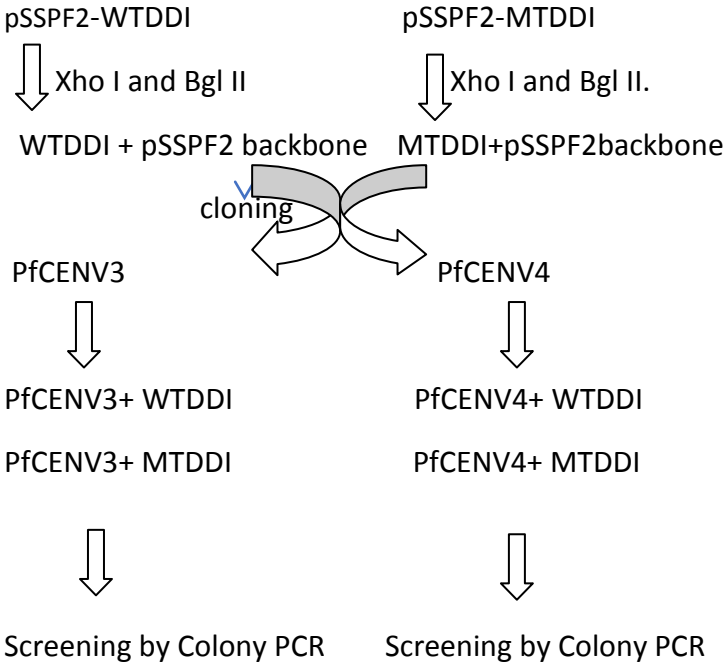
CHAPTER 3

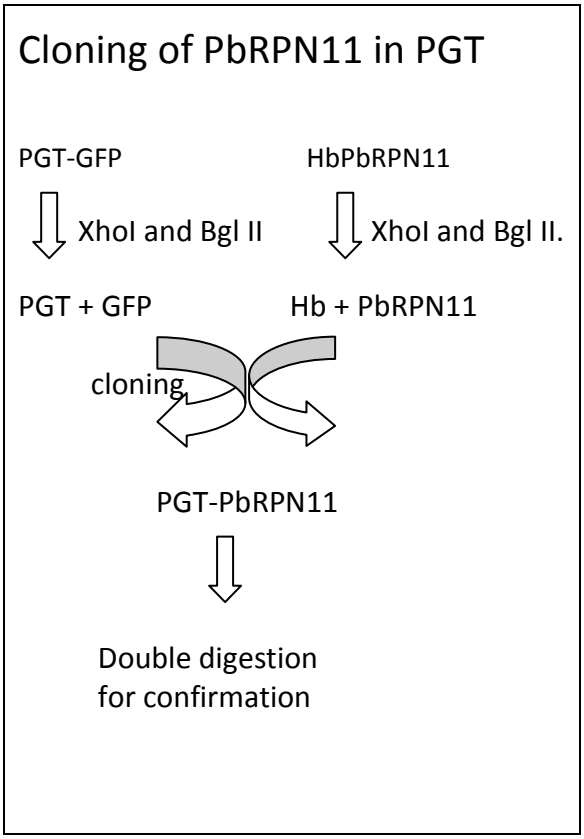
MATERIALS AND METHODS

All the biochemical were from Sigma, Serva or SRL Biosciences unless mentioned otherwise. The bacterial culture media Lauria Bertani (broth and agar) were from Thermoscientific. PCR reagent and restriction endonucleases were from New England Biolabs (NEB), ThermoScientific, Jonaki and Takara. PCR primers were purchased from Bioserve or Eurofins. The Gel extraction, PCR clean-up and Plasmid purification kits were from Machery-Nagel, Qiagen and Agilent.

Research strategy

Cloning of DDI in PfCENV3/V4





Production of recombinant Atg7 for generation of antibodies

pET32a-Atg7 plasmid expression in

Rosetta-gami cell

Primary culture

Secondary culture

Protein purification under denaturing condition

Protein refolding by Dialysis

Immunization in rats for antibody(Ab) production

ELISA method for titration of Ab

Western blotting for confirmation of Ab

3.1 For DDI gene

3.1.1 Bioinformatics Study of DDI, PfCENV3, PfCENV4

The plasmid PfCENV3 is used to clone sequences upstream (BglII-KpnI) and downstream (BamHI-XhoI of GFP); also, directly from the pSTCII and HB-based vectors. The wild type and mutated DDI gene (1.2Kbp) is cloned into downstream (BamHI-XhoI of GFP) region of PfCENV3.The mutation of DDI gene was seen in the 220 position was from aspartate to alanine. The work has already been carried out in Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India and we received the prepared plasmid vector and the

DDI gene. The cam5U region is present in PfCENV3 plasmid where as GAPDH5U is present in PfCENV4 instead of cam5U.

Construction of PfCENV3

The PfCENV3 was constructed in CCMB as mentioned below:

The GFPbsc region was amplified from pGT-GFPbsc plasmid with the help of GFPcen-F/GFPcen-R primers.

GFPcen-F: *attacctaggAGATCT***CAAA**ATG*GGTACC*

GFPcen-R: attaCTTAAGCTCGAGTTAGGATCCctg

It was digested with AvrII-AflII, and was then cloned into similarly digested PCENV2 plasmid, which generated PfCENV3 plasmid.

GAATTC*CCAATACTTATAATAATTTATGTATAATTAAATTAAATATTATAAACACACATATATATT*
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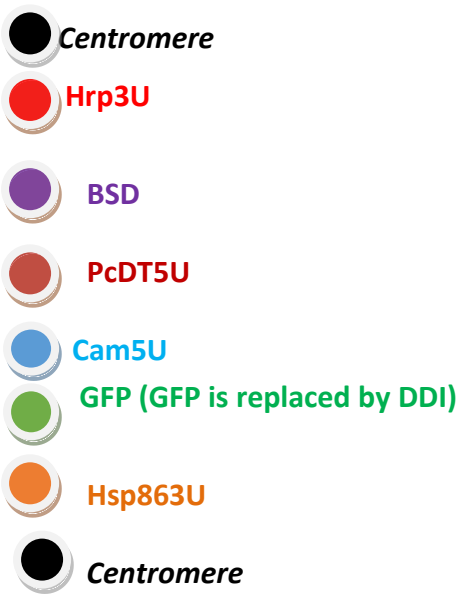

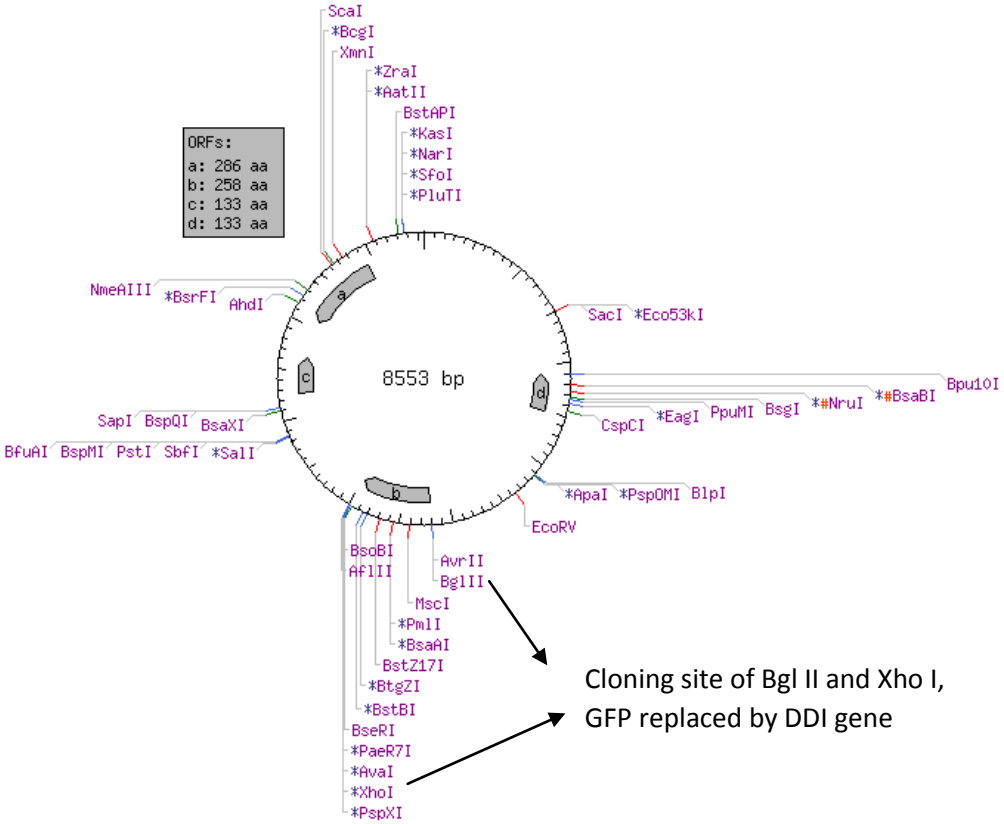

Figure: Circular plasmid Sequence of PfCENV3

(PfCENV3 has Cam5U instead of GAPDH5U)

Construction of PfCENV4

1. The BamHI-SalI sites was eliminated . The PfCENV2 was digested with BamHI-SalI and blunt end was ligated and was transform into DH5α cells. The absence of BamHI-SalI sites was checked. It was named as PfCENV2.1.
2. The amplification of hDHFR selection cassette (Pbef1-a5U/hDHFR/pbDT3U) from the PfCENV1 pDNA using the V1Sc-F/ V1Sc-F primers was done.
3. The PfCENV2.1 was digested with SacI and the blunt end was digested with ApaI and the pDNA backbone was purified.
4. The pDNA backbone was ligated with the PCR fragment to obtain PfCENV2.2.
5. The PfCENV2.2 was digested with AvrII-AflII and the backbone was purified.
6. The PfCENV3 was digested with AvrII-AflII to excise the GFP and it was then cloned into the PfCENV2.2 backbone to obtain PfCENV2.3.

7. The Cam5U of PfCENV2.3 was replaced with GAPDH-5U.The GAPDH-5U was amplified using GAPD5U-F/GAPD5U-R primers from PfgDNA.
8. The GAPDH 5U was digested with ApaI/BglII.
9. The PfCENV2.3 was digested with ApaI/BglII to excise Cam5U and the pDNA backbone was purified.
10. The GAPDH-5U was ligated with the pDNA backbone to construct PfCENV4.

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AGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCT
AGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGGCATTGCTACAGGCATCGTGG
TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAA
GTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCG
GCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTA
AAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGA
GATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCG
TTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG
AAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCA
TGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCC
CCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGC
GTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCA
GCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGG
CGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGT
ACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCAT
CAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTC
GCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGG
GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATA

Centromere

Hrp3U

BSD

PcDT5U

GAPDH5U

GFP (GFP is replaced by DDI)

Hsp863U

Centromere

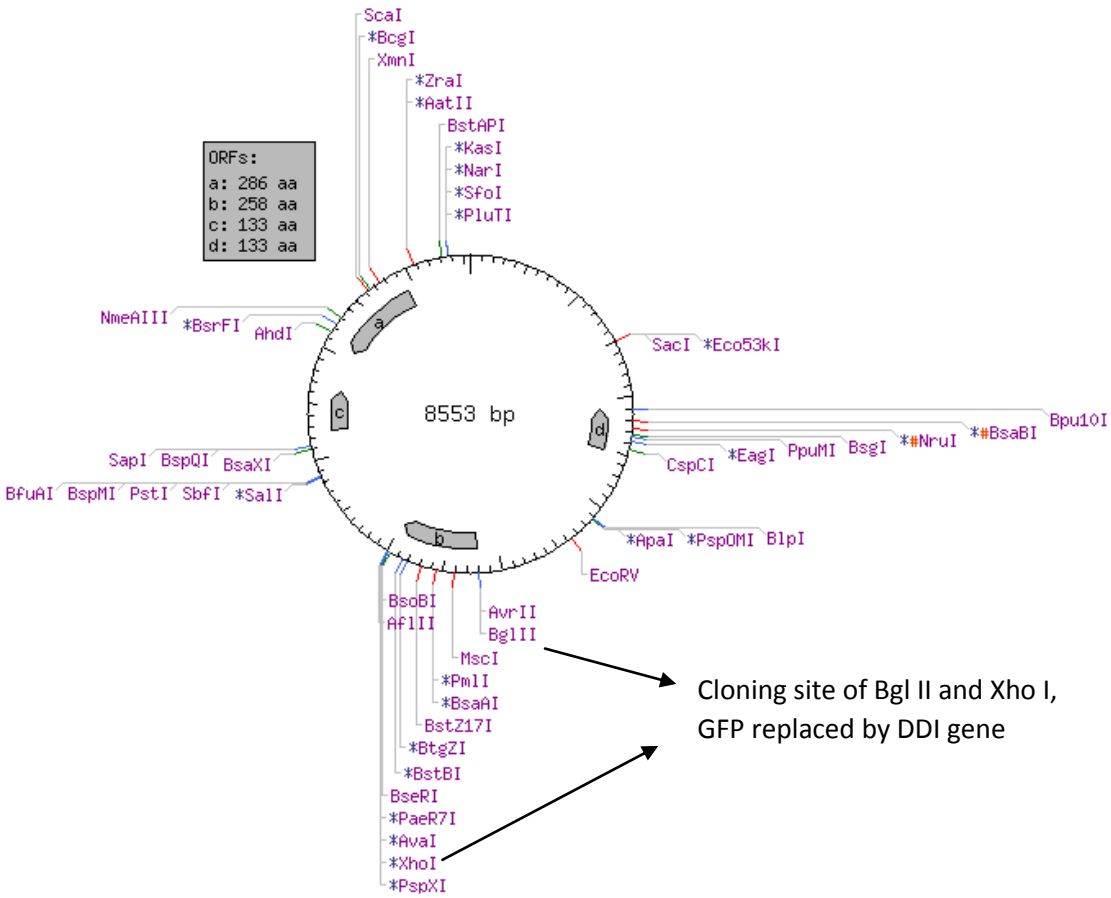


Figure: Circular plasmid Sequence of PfCENV4

(PfCENV4 has GAPDH5U instead of Cam5U)

I. Protein Sequence of *Plasmodium falciparum*- Pfddi1 (PF14_0090 / PF3D7 1409300)

MVFITISDDNNIITSLDVHEDTEIWTITNIIENDFSLNMNINELTYNGNAVDKFDTIKKL
NIKEGDLLFVRKKISADIMNDNVNNMSALNNILSTNNNVGNIGNIGNNLNNENVQNLLNN
PAFKTLLDQFKVYQENEYIKKESEILLEMKNDKSKMAVLKLQDEPLYNAIFSQNLEEIKK
IVKEKYETEKKEKEKEQQMYENALKNPLSEDSQKFIYENIYKNEINNNLALAQEHFPEAF
GVVFMLYIPVEINKNTVHAFVDSGAQSSIMSKKCAQKCNILRLMDKRFTGIAKGVGTKTI
LGKIHMIDIKIGNYFYAVSLTIIEDYDIDFIFGLDLLKRHQCLIDFKQNALIIEDNKIPF
LSEKDVISISTQSIDIDANNDL

Sequence Length: 382 aa

II. Predicted RNA/mRNA Sequence of Pfddi1 (introns spliced out)

ATGGTTTTTATTACAATATCAGACGATAATAATATAATAACTAGTCTTGATGTTCATGAG
GATACAGAAATTTGGACGATAACAAATATTATTGAAAATGATTTTTCTCTAAATATGAAT
ATAAATGAATTAACATATAATGGAAATGCTGTGGATAAATTTGATACGATAAAAAAATTA
AATATAAAAGAAGGAGACTTATTATTTGTACGTAAAAAGATTAGTGCTGATATAATGAAT
GATAATGTTAATAATATGTCAGCTTTAAATAATATTCTTTCCACAAATAATAATGTTGGA
AATATTGGAAATATTGGAAATAATTTAAATAACGAAAACGTACAAAATCTCTTAAATAAT
CCAGCTTTCAAAACATTATTAGATCAATTTAAAGTATATCAAGAAAATGAATATATTAAA
AAAGAATCTGAAATTTTATTGGAAATGAAAAATGATAAAAGCAAAATGGCAGTTTTGAAA
TTACAAGATGAACCTTTATATAATGCTATTTTTTCACAAAATTTAGAGGAGATAAAAAAA
ATTGTAAAAGAGAAATATGAAACTGAAAAAAAAGAAAAAGAAAAAGAACAACAAATGTAT
GAAAATGCTTTAAAAAATCCTTTATCAGAAGATTCACAGAAATTTATATATGAAAATATA
TATAAAAATGAAATTAATAATAATTTAGCTTTAGCTCAAGAACATTTCCCTGAAGCCTTT
GGTGTCGTTTTTATGTTATATATACCAGTAGAAATTAATAAAAACACAGTACATGCATTC
GTAGATTCTGGAGCACAATCAAGCATAATGTCAAAAAAATGTGCACAAAAATGTAATATT
CTAAGACTCATGGATAAAAGATTTACAGGAATAGCAAAGGGAGTAGGTACCAAAACTATT
CTAGGAAAAATACATATGATAGATATTAAAATTGGGAATTATTTTTATGCTGTATCCTTA
ACTATTATTGAAGATTATGATATTGATTTTATATTCGGATTAGATTTATTAAAAAGACAC
CAATGTTTAATAGATTTTAAGCAAAATGCTCTAATTATCGAAGACAATAAAATACCTTTC
TTATCAGAAAAAGATGTTATATCTATTTCAACACAAAGTATAGACATTGATGCAAACAAT
GATTTATAA

Sequence Length: 1149 bp

III. Gene sequence of mutant *Plasmodium falciparum* DDI (PfDDI) with myc-tag

ATGGTTTTTATTACAATATCAGACGATAATAATATAATAACTAGTCTTGATGTTCATGAG

GATACAGAAATTTGGACGATAACAAATATTATTGAAAATGATTTTTCTCTAAATATGAAT

ATAAATGAATTAACATATAATGGAAATGCTGTGGATAAATTTGATACGATAAAAAAATTA

AATATAAAAGAAGGAGACTTATTATTTGTACGTAAAAAGATTAGTGCTGATATAATGAAT

GATAATGTTAATAATATGTCAGCTTTAAATAATATTCTTTCCACAAATAATAATGTTGGA

AATATTGGAAATATTGGAAATAATTTAAATAACGAAAACGTACAAAATCTCTTAAATAAT

CCAGCTTTCAAAACATTATTAGATCAATTTAAAGTATATCAAGAAAATGAATATATTAAA

AAAGAATCTGAAATTTTATTGGAAATGAAAAATGATAAAAGCAAAATGGCAGTTTTGAAA

TTACAAGATGAACCTTTATATAATGCTATTTTTTCACAAAATTTAGAGGAGATAAAAAAA

ATTGTAAAAGAGAAATATGAAACTGAAAAAAAAGAAAAAGAAAAAGAACAACAAATGTAT

GAAAATGCTTTAAAAAATCCTTTATCAGAAGATTCACAGAAATTTATATATGAAAATATA

GCT (alanine) substituted GAT (aspartic acid) at 220 position

TATAAAAATGAAATTAATAATAATTTAGCTTTAGCTCAAGAACATTTCCCTGAAGCCTTT

GGTGTCGTTTTTATGTTATATATACCAGTAGAAATTAATAAAAACACAGTACATGCATTC

GTAGCTTCTGGAGCACAATCAAGCATAATGTCAAAAAAATGTGCACAAAAATGTAATATT

CTAAGACTCATGGATAAAAGATTTACAGGAATAGCAAAGGGAGTAGGTACCAAAACTATT

CTAGGAAAAATACATATGATAGATATTAAAATTGGGAATTATTTTTATGCTGTATCCTTA

ACTATTATTGAAGATTATGATATTGATTTTATATTCGGATTAGATTTATTAAAAAGACAC

CAATGTTTAATAGATTTTAAGCAAAATGCTCTAATTATCGAAGACAATAAAATACCTTTC

TTATCAGAAAAAGATGTTATATCTATTTCAACACAAAGTATAGACATTGATGCAAACAAT

GATTTAGAACAAAAGCTTATTTCTGAAGAAGA**C**CTG**TAA*CTCGAG***

Underlined segment is myc tag

3.1.2 Isolation of plasmid using Nucleospin kit

The bacterial colony was inoculated in 5mL LB media and grown at 37˚C. The pellet was collected after the centrifugation of sample at 4000rpm for 10 minutes. The pellet was suspended in 250µL A1 buffer and then treated with A2 buffer to liberate plasmid. The lysate was neutralized by treating with A3 buffer. It was then centrifuged at 13000 rpm for 15 minutes to clarify the lysate. The supernatant was transferred to spin column tube and centrifuged at 11,000g for 1 minute. The silica membrane was washed by adding 600 µL of wash buffer and dried by centrifuged it for 3 minutes. The bound plasmid DNA

was eluted by adding 20 µL of warm water and incubated at room temperature for 2 minutes and later centrifuged at 11,000g for 1 minute. The quality of the extracted plasmid was checked by agarose gel electrophoresis and nanodrop spectrometry.

3.1.3 Digestion of plasmid

The pSSPF2-WTDDI (90.87ng/µL), pSSPF2-MTDDI (122.5ng/µL) and PfCENV3 (402.61 ng/µL) plasmid was then digested with restriction endonuclease Xho I(10 units/µL) and Bgl II. The digestion process was carried out by adding reagents in a clean 1.5 mL microcentrifuge tube and incubating at 37˚C for 3 hours. The resultant mixture was then loaded in 0.8% agarose gel and the rest was stored at -20˚C for further use.

Table 1:- Components of reaction mixture for digestion

Component	Amount (µl)		
Plasmid	44 (pSSPF2-WT DDI)	38 (pSSPF22-MT DDI)	10 (PfCENV3)
10x NEB buffer 3.1	7	7	5
XhoI	0.7	0.7	0.7
Bgl II	0.7	0.7	0.7
MilliQ water	17.6	23.6	33.6
Total	70	70	50

3.1.4 Gel elution of extracted DNA

The desired band was cut from the agarose gel and the gel containing desired DNA was collected in centrifuge tube. The gel was melted by adding NT1 buffer (gel:buffer = 1:2). The mixture was transferred to a DNA binding column. It was washed twice with NT3 buffer and let it pass through. The column was dried by spinning for two minutes. The DNA was eluted in warm water.

3.1.5 Ligation

The ligation of desired fragment (insert DNA) was carried out in linear plasmid vector (PfCENV3) both wild and mutated type with T4 DNA ligase. The resultant volume was made up the volume to 20 µL with milliQ water and mixed gently. It was incubated at RT for 30 minutes and then stored at 20˚C.

Insert: vector – 3:1
Insert length:1200 bps

Vector length: 7200 bps
Insert mass: 25ng
Vector mass: 50ng

Table 2.Plasmid and insert concentrations for ligation.

Plasmid	Concentration
PfCENV3	35ng/µl
pSSPF2-WT DDI	6.3ng/µl
pSSPF2-MT DDI	9.4ng/µl

Table 3. Components of ligation reaction mixture WT DDI or MT DDI with PfCENV3

Components	Volume (µl)		
Insert DNA	4 (WT DDI)	2.6 (MT DDI)	0(control)
vector(pfCENV3)	1.42	1.42	1.42
10X Ligase buffer	2	2	2
T4 DNA ligase	1	1	1
AMQ water	11.58	12.98	15.58
Total	20	20	20

3.1.6 Transformation

The competent cells were thawed on ice for 5 minutes. DNA was added to the competent cells and incubated on ice for 30 minutes. The heat shock was given at 42˚C for 1 minutes. It was kept back on ice for 2 minutes after heat shock. 1mL of LB broth was added to the tube and incubated in a shaker incubator for 40 minutes at 37˚C. The sample was centrifuged at 4000 rpm for 10 minutes to pellet the cells. 800µL of supernatant was discarded, the cells were suspended in the remaining amount of supernatant and spread on LB-agar plates containing the desired antibiotics. The plates were incubated for overnight at 37˚C. The colonies were observed on the next day.

3.1.7 Colony PCR

In colony PCR, each colony acts as the template in which the desired genome is amplified only when the particular gene is present in the clone. Colony PCR is used to screen large number of colonies of true transformants in very short period of time.

Table 4. Composition of PCR reaction mixture

Reagents	Volume (µl)
NEB Taq. buffer (10x)	2
Template	Single clone
pfDdiexp-F(50pM)	0.2
pfDdiexp-R(50pM)	0.2
Mg^{2+}	0.2
NEB Taq.DNA polymerase	0.1
dNTPs (100mM)	0.2
AMQ water	17.1
Total	20

PfDdi-expF:ATGgatccGTTTTTATTACAATATCAGACGAT
PfDdi-expR: aattaaagcTTATAAATCATTGTTTGCATCAATGTC

3.2 Cloning of pGT-PbRPN11

Hb-PbRPN11 was inoculated in Luria-bertani broth and incubated at 37˚C for 12 hours. The plasmid isolation was done and the concentration was determined by Nano-drop spectrometer reading. The PbRPN11 was excised from Hb-PbRPN11 with Bgl II and Xho I restriction enzymes. The PbRPN11 fragment of 1.313 Kbp was purified. The PGT-GFP was digested with Bgl II and Xho I restriction enzymes which released PGT backbone (3Kbp) and GFP (800bp) was removed and the plasmid DNA backbone was purified. The PGT backbone and PbRPN11insert were ligated to obtain PGT-PbRPN11.

I.Protein sequence of *Plasmodium falciparum* RPN11: MAL13P1.343

MAGIPSSLRELFYSFSDGNGMNNETLADTSEQVYISPLALLKILKHGRAGVPMEVMGLMLGEIVDEY
TIRIVDVFAMPQSGNSVSVEAVDPVYQTNMLEELKKTGRHEMVVGWYHSHPGFGCWLSGTDVNT
QKSFEQLNPRTIGVVVDPIQSVKGKVVIDCFRLINPHILMLGQEPRQTTSNIGYLTKPTLTALVHGLNR
NYYSIVINYRKNELEKNMLLNLHKDMWTNPLKLNDFHEQKKSSDETLEDIKKLTTLYNKNLRNEMKK
TSEEILLENIGKIDAKKRIQNSVETLLNESILTCIGTMANTLFF

Sequence Length: 311 aa

II.Predicted PfRPN11 RNA/mRNA Sequences (introns spliced out)

ATGGCGGGTATTCCATCTTCATTACGTGAATTATTCTATTCCTTTTCTGATGGGAATGGAATGAAT
AATGAAACATTAGCAGATACCAGTGAACAAGTTTATATATCTCCTTTGGCTCTTCTAAAGATATT
AAAACATGGACGGGCTGGAGTACCTATGGAAGTTATGGGTTTAATGTTAGGCGAAATAGTTGA
TGAATACACCATAAGAATTGTTGATGTGTTCGCTATGCCTCAGTCAGGTAATAGTGTAAGTGTCG
AAGCAGTAGACCCAGTTTATCAAACGAATATGTTAGAGGAATTAAAAAAAACAGGCAGACATG
AAATGGTCGTTGGATGGTATCACTCGCATCCTGGTTTTGGTTGTTGGTTATCAGGTACTGATGTA
AATACTCAGAAAAGTTTTGAACAATTAAATCCAAGAACTATTGGTGTTGTAGTAGATCCTATACA
ATCTGTTAAAGGAAAAGTTGTTATTGATTGCTTTAGATTAATTAATCCACATATATTAATGTTAGG
ACAAGAACCAAGACAAACTACATCAAATATTGGTTATTTGACTAAACCAACCTTAACAGCTTTAG
TTCATGGATTAAATAGAAACTATTATTCAATTGTTATTAATTATAGAAAAAATGAATTAGAAAAA
AATATGTTATTAAATTTACATAAAGATATGTGGACCAACCCCTTAAAATTAAATGATTTCCATGA
ACAAAAAAAAAAGCTCAGATGAAACATTAGAAGATATAAAAAAATTAACTACCTTATATAATAAA
AATCTTCGTAATGAAATGAAAAAAACAAGTGAAGAAATACTTTTAGAAAATATTGGAAAAATTG
ATGCTAAAAAAAGAATACAAAATTCGGTTGAAACGTTGTTAAACGAATCAATTCTCACATGTATA
GGAACTATGGCAAATACTCTGTTCTTTTAA

Sequence Length: 936 bp

III.Genomic sequence of PfRPN11 (introns shown in lower case)

ATGGCGGGTATTCCATCTTCATTACGTGAATTATTCTATTCCTTTTCTGATGGGAATGGAATGAAT
AATGAAACATTAGCAGATACCAGTGAACAAGTTTATATATCTCCTTTGGCTCTTCTAAAGATATT
AAAACATGGACGGgtaaaacataaataaatttaaaattaaaataatgacatattataaatacctggaaatgtaacgttt
tgtataaacataatgtataaatttaatatttatttctacgttacactaacattatcacatatatatatatatatatatatttat
atatttatatatatatccaaatttatgaacaagtcaggtaatttttttttttttttttttttttttttttttaatttagGCTGGAGTA
CCTATGGAAGTTATGGGTTTAATGTTAGGCGAAATAGTTGATGAATACACCATAAGAATTGTTG
ATGTGTTCGCTATGCCTCAGTCAGGTAATAGTGTAAGTGTCGAAGCAGTAGACCCAGTTTATCA
AACGAATATGTTAGAGGAATTAAAAAAAACAGGCAGACATGAAATGGTCGTTGGATGGTATCA
CTCGCATCCTGGTTTTGGTTGTTGGTTATCAGGTACTGATGTAAATACTCAGAAAAGTTTTGAAC
AATTAAATCCAAGAACTATTGGTGTTGTAGTAGATCCTATACAATCTGTTAAAGGAAAAGTTGTT
ATTGATTGCTTTAGATTAATTAATCCACATATATTAATGTTAGGACAAGAACCAAGACAAACTAC
ATCAAATATTGGTTATTTGACTAAACCAACCTTAACAGCTTTAGTTCATGGATTAAATAGAAACT
ATTATTCAATTGTTATTAATTATAGAAAAAATGAATTAGAAAAAAATATGTTATTAAATTTACATA
AAGATATGTGGACCAACCCCTTAAAATTAAATGATTTCCATGAACAAAAAAAAAGCTCAGATGA
AACATTAGAAGATATAAAAAAATTAACTACCTTATATAATAAAAATCTTCGTAATGAAATGAAAA
AAACAAGTGAAGAAATACTTTTAGAAAATATTGGAAAAATTGATGCTAAAAAAAGAATACAAAA

TTCGGTTGAAACGTTGTTAAACGAATCAATTCTCACATGTATAGgtatattaaaaaaaaaatatatatata tatatatatatatatatgtgtgtgtgatgattatccttcggaacaaaaaaatatatatatatatatatatatatatatatata tataataatttatatttttttctttttagCAACTATGGCAAATACTCTGTTCTTTTAA

Sequence length:1313 bp

3.2.1 Isolation of plasmid using Nucleospin kit

The bacterial colony was inoculated in 5mL LB media and grown at 37˚C. The pellet was collected after the centrifugation of sample at 4000rpm for 10 minutes. The pellet was suspended in 250µL A1 buffer and then treated with A2 buffer to liberate plasmid. The lysate was neutralized by treating with A3 buffer. It was then centrifuged at 13000 rpm for 15 minutes to clarify the lysate. The supernatant was transferred to spin column tube and centrifuged at 11,000g for 1 minute. The silica membrane was washed by adding 600 µL of wash buffer and dried by centrifuged it for 3 minutes. The bound plasmid DNA was eluted by adding 20 µL of warm water and incubated at room temperature for 2 minutes and later centrifuged at 11,000g for 1 minute. The quality of the extracted plasmid was checked by agarose gel electrophoresis and nanodrop spectrometry.

3.2.2 Digestion of plasmid

The Hb-PbRPN11 (787.6ng/µL) and PGT-GFP (233.1 ng/µL) plasmid was then digested with restriction endonuclease Xho I(10 units/µL) and Bgl II. The digestion process was carried out by adding reagents in a clean 1.5 mL microcentrifuge tube and incubating at 37˚C for 3 hours. The resultant mixture was then loaded in 0.8% agarose gel and the rest was stored at -20˚C for further use.

Table:-5 Components of reaction mixture for digestion

Component	Amount (µl)	
Plasmid	6.5 (Hb-PbRPN11 -5µg)	13 (PGT-GFP -3µg)
10x NEB buffer 3.1	8	8
XhoI	1	1
Bgl II	1	1
NFW	63.5	57
Total	80	80

It was incubated for overnight at 37˚C.

3.2.3 Gel elution of extracted DNA

The desired band was cut from the agarose gel and the gel containing desired DNA was collected in centrifuge tube. The gel was melted by adding NT1 buffer (gel:buffer = 1:2). The mixture was transferred to a DNA binding column. It was washed twice with NT3 buffer and let it pass through. The column was dried by spinning for two minutes. The DNA was eluted in warm water.

3.2.4 Ligation

The ligation of desired fragment (insert Pb-RPN11 DNA) was carried out in linear plasmid vector (PGT backbone) with T4 DNA ligase. The resultant volume was made up the volume to 20 µL with nuclease free water (NFW) and mixed gently. It was incubated at RT for 30 minutes and then stored at 20˚C.

Insert: vector = 3:1
Insert length: 1313 bps
Vector length: 3000 bps
Insert mass: 65.65 ng
Vector mass: 50 ng

Table:-6 Plasmid and insert concentrations for ligation.

Plasmid	Concentration
Pb-RPN11 insert	19.2ng/µL
PGT backbone	31.5 ng/µL

Table :-7 Components of ligation reaction mixture of PGT backbone and Pb-RPN11 insert.

Component	Amount (µl)
10x DNA ligase buffer	2
T4 DNA ligase	1
PGT backbone (vector)	2
Pb RPN11 (insert)	4
NFW	11
Total	20

Calculation

PGT vector (µL) =50/31.5 =1.58 =2 µL

Pb-RPN11 insert (µL) = 65.65/19.2 =3.42 =4 µL

3.2.5 Transformation

The competent cells were thawed on ice for 5 minutes. DNA was added to the competent cells and incubated on ice for 30 minutes. The heat shock was given at 42˚C for 1 minutes. It was kept back on ice for 2 minutes after heat shock. 1mL of LB broth was added to the tube and incubated in a shaker incubator for 40 minutes at 37˚C. The sample was centrifuged at 4000 rpm for 10 minutes to pellet the cells. 800µL of supernatant was discarded, the cells were suspended in the remaining amount of supernatant and spread on LB-agar plates containing the desired antibiotics. The plates were incubated for overnight at 37˚C. The colonies were observed on the next day. After the transformation was done, it was screened by colony PCR.

3.2.6 Colony PCR

In colony PCR, each colony acts as the template in which the desired genome is amplified only when the particular gene is present in the clone. Colony PCR is used to screen large number of colonies of true transformants in very short period of time.

Table :-8 Composition of PCR reaction mixture

Reagents	Volume (µl)
NEB Taq. buffer (10x)	2
Template	Single clone
PfRpn11- epiF(50pM)	0.2
PfRpn11- epiR(50pM)	0.2
Mg^{2+}	0.2
NEB Taq.DNA polymerase	0.1
dNTPs (100mM)	0.2
AMQ water	17.1
Total	20

PfRpn11-epiF: TCCAAGATCTCAAAATGGCGGGTATTCCATCTT

PfRpn11-epiR: ATTAggtaccAAAGAACAGAGTATTTGCCATAGTTCCTA

3.3 For Atg7 gene

3.3.1 Transformation of pET32a-Atg7 into Rosetta-gami cells

Rosetta-gami cells are used for expression analysis. These cells are used for the expression of the desired protein. PET32a-Atg7 plasmid was isolated from BL21DE3 cells and transformed into Rosetta-gami cells. BL21DE3 cells are competent *E.coli* cells that are ideal for routine T7 expression system. The PET plasmids contain different antibiotic resistant genes that are not suitable for large expression in BL21DE3 cells. So for enhanced protein expression, Rosetta –gami cells are used. Rosetta–gami cells combine enhanced disulphide bond formation with enhanced protein expression. The native promoters drive rare and selected tRNA genes and are combatible for different antibiotic resistant plasmid such as chloramphenicol, kanamycin, tetracyclin and ampicillin resistant recommended for use with PET plasmids.

Rosetta-gami cells was thawed on ice for 5 minutes and DNA was added to the cells and incubated in ice for 30 minutes. The mixture was given heat shock at 42˚C for 1 minute and after 2 minutes of it, it was again kept back on ice. 1mL of LB broth was added to the tube and incubated in a shaker incubator for 40 minutes at 37˚C. It was centrifuged at 4000rpm for 10 minutes to yield pellet. 800 µL of supernatant was discarded and the cells are dispersed in remaining amount of supernatant which was eventually spread on LB-agar plates containing desired antibiotic. It was incubated at 37˚C overnight and the colonies was observed on next day. The colonies were then used to culture in large scale for protein expression.

3.3.2 Large scale protein expression and purification

The primary culture was first prepared by incubating 5mL culture overnight with respective antibiotics and which was later used to inoculate the secondary culture. The OD of the secondary culture was monitored to be in the range of 0.6-0.8 and the two sets of secondary cultures were prepared. One was labelled as induced which was supplemented with mM IPTG (1:1000 dilution) and the other was cultured without IPTG which was labelled as uninduced. The IPTG-induced cells were used for the protein purification under denaturing condition.

The induced cell pellets were suspended in urea buffer (5mL/g cell pellet weight) and incubated for 30 minutes. The suspension was subjected to sonication (22% amplitude for 9 second in on/off pulse) and then centrifuged at 15000 rpm for 30 minutes at room temperature. The supernatant was soluble fraction while the pellet was insoluble. The pellet was further used for purification.

The vector pET32a was added a 6X-His tag to the N-terminus of Atg7 and the resultant recombinant Atg7 was purified by Nickel-NTA affinity chromatography.

3.3.3 Purification

The Ni-NTA resin (0.125mL resin/gram initial weight of pellet) was loaded into protein purification column and was washed twice with AMQ and then twice with urea buffer (50mM NaH_2PO_4, 8M urea, 500ml NaCl, pH-8) containing 50mM imidazole, 0.5% TX and 5mM β-ME so as to equilibrate the beads. The protein pellets were dissolved in urea buffer (5mL buffer/gm of pellet) then incubated at room temperature for 30 minutes and sonicated for 4 minutes (22% amplitude for 9 second in on/off pulse). The suspension was centrifuged at 4˚C at 8000rpm for 30 minutes. The soluble fraction was then loaded in the column over the resin along with binding buffer (50mM Imidazole. 0.5% TX and 5mM β-ME) and left for binding for 30 minutes on the rotor at room temperature. After 30 minutes, they are allowed to pass out of column and flow through was collected. The protein in the soluble fractions bind to the Nickel-NTA resin. The undesirable proteins were separated by washing the resin with wash buffer (50mM imidazole, 0.5% TX and 5mM β-ME in urea buffer) such that the proteins that bound the resin with lesser affinity than 50mM imidazole were washed out. This will help in removal of proteins that has lesser affinity towards Nickel-NTA resin than His-tagged Atg7. It was repeated twice and the elute were named as wash 1 and wash 2. The third washing was carried out. The third washing was carried out with 50mM imidazole in urea buffer (50mL) and the eluted collected was named as wash 3. The protein was eluted with 250mM imidazole (in urea buffer up to 25mL). This high concentration imidazole replace the bound His-tagged Atg7, which was collected in 5 fractions each of 5mL after 5 minutes of incubation and labelled as E1,E2,E3,E4 and E5 respectively. All fractions (UN-IND-FT-W1-W2-W3-E1-E2-E3-E3-E4-E5 were run in 12% SDS PAGE gel.

3.3.4 Refolding of denatured Atg7 protein by dialysis

The protein in original conformation is essential for functional analysis. The cut off size of the dialysis membrane used was 10KDa.

The eluted protein was kept in a dialysis bag and diluted with urea buffer. The dialysis bag was then kept in 2 litre of refolding buffer and incubated with stirring at 4˚C for 14 hours. The buffer was changed and incubated for 3 hours again. After 3 hours, the sample in dialysis bag was centrifuged at 16000 rpm for 30 minutes to remove any precipitated proteins. The protein samples were loaded into the centricons, equilibrated

with refolding buffer and centrifuged at 4000rpm for 20 minutes to concentrate the protein.

The BCA assay was performed to estimate the protein concentration and titrated to determine the Atg7 concentration.

3.3.5 Immunization

The expression and purification of Atg7 was done for the generation of antibodies. The purified Atg7 was then injected into rat for antibody production

Pre-immune serum was collected prior to immunization and was used as a control in assays.

3.3.6 Immunization with recombinant Atg7

The refolded recombinant Atg7 was concentrated to 1mL using a centricon. It was then mixed with equal volume of Freunds complete adjuvant in order to increase the immunogenicity of the protein and emulsion was made. In later immunizations, the emulsion was made with Freunds incomplete adjuvant (1:1 ratio). The emulsion was injected intraperitoneally in the rats (50-100µg/rat).

1mL of blood was collected from each of the rats before the first immunization. Serum was separated by incubating blood at 37˚C for 2-3 hours. It was then centrifuged at 16000 rpm for 10 minutes. The supernatant was obtained and labelled as pre-immune serum which was then stored at -80˚C for further experiments.

Table:-9 The period of immunization in rat .

Days	**Immunization**	**Protein**
Day (23/1/16)	Pre-immune bleed (1mL)	Serum collected
Day 1 (23/03/16)	Priming	Atg7 (0.1mg/mL)+ CFA(1:1;600µL each)
Day 48 (10/05/16)	First booster	Atg7 (0.2mg/mL)+ IFA(1:1;500µL each)
Day 58	----------	Serum collected from 1mL blood

Day 62 (24/05/16)	Second booster	Atg7 (0.2mg/mL)+ IFA(1:1;500µL each)
Day 72 (03/06/16)	Third bleed	Serum collected from 1mL of blood
Day 76 (07/06/16)	Third booster	Atg7 (0.314mg/mL)+ IFA(1:1;300µL each)
Day 89 (20/06/16)	Fourth bleed	Serum collected from 1mL of blood
Day 102 (03/07/16)	Fourth booster	Atg7 (1.25mg/mL)+ IFA(1:1;900µL each)
Day 113 (14/07/16)	Fifth bleed(1mL)	Serum collected from 1mL blood

3.3.7 Immunization schedule

The serum from the blood was separated that contains antibodies against Atg7 protein. The antibodies were used for ELISA and western blotting.

3.3.8 ELISA

ELISA was used to test the presence and quantity of antibodies present in the serum. The wells were coated with Atg7 protein (10µg/mL). The serum was serially diluted two folds (1/10000 to 1/1280000) to titrate antibody present in the serum.

The 96 well plates were coated with 50µL of 10µg/mL antigen (Atg7 protein) by incubating overnight at 4˚C. The plate was inverted, shaken off and tapped on dry paper towel. 200µL of blocking solution was added per well and incubated for 2 hours at 37˚C. Five washes were done by flooding the wells with wash buffer at the interval of 5 minutes. 100µL of primary antibodies were added per well and incubated at 37˚C for 2 hours. Five washes were done by flooding the wells with wash buffer at the interval of 5 minutes. 100µL of secondary antibodies were added per well and incubated at 37˚C for 2 hours. Five washes were done by flooding the wells with wash buffer at the interval of 5 minutes. 100µL TMB/H_2O_2 per well was added and incubated for 15 minutes and 50µL 1N H_2SO_4 per well was added to stop the reaction. The OD reading was taken at 450nm and the result was analysed.

3.3.9 Western blotting

The serum was obtained from rat's blood immunized with PfAtg7 and titrated at dilutions of 1/500, 1/1000, 1/5000, 1/10000 (in 5% milk solution 1X TBS-T). Pre-immune serum was also diluted to 1/500 (in 5% milk solution 1X TBS-T) is used as control.

Recombinant Atg7 protein and 3D7 parasite lysate sample containing Atg7 were run on SDS-PAGE. PVDF membrane was first equilibrated with 100% methanol and then transfer buffer. The transfer was set in the order of Black side, 3Xspacer, blotting paper, SDS gel, PVDF membrane, Blotting paper, 3X spacer, Red side.

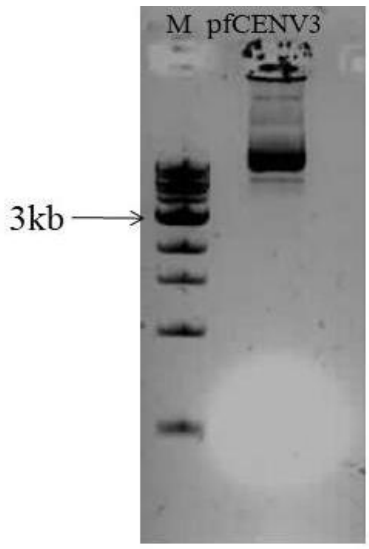
The transfer buffer was allowed to run at constant 200mA for 2 hours in cold room at 4˚C then the membrane was blocked in 5% milk solution in 1X TBS-T) for 2 hours in shaker at 35rpm. The serum dilutions were prepared in 5% milk solution and incubated for 2 hours at standardised dilution. The membrane was then washed four times with 1X TBS-T at the interval of 10 minutes. Anti-HRP antibody solution as secondary antibody (1/10000 dilution in 5% milk solution) was added and incubated for 1 hour at room temperature in shaker at 35rpm. The membrane was washed 4 times with 1X TBS-T at the interval of 10 minutes each. The blot was developed with Super Signal West Pico/Femto Chemiluminescent Substrate reagents on X-ray sheets.

CHAPTER 4

RESULTS

4.1 Cloning of DDI gene into PfCENV3

The pfCENV3, pSSPF2-WTDDI and pSSPF2-MTDDI plasmids were isolated and analyzed by agarose gel electrophoresis for quality and integrity (Fig. 4.1 and 4.2). The size of the plasmid backbone is around 8kb, and the size of the DDI gene is 1.2kbp . The DDI gene is cloned in BglII- XhoI restriction sites. The DDI genes were excised from the pSSPF2 vector and cloned into pfCENV3 vectors for expression in *Plasmodium falciparum.*



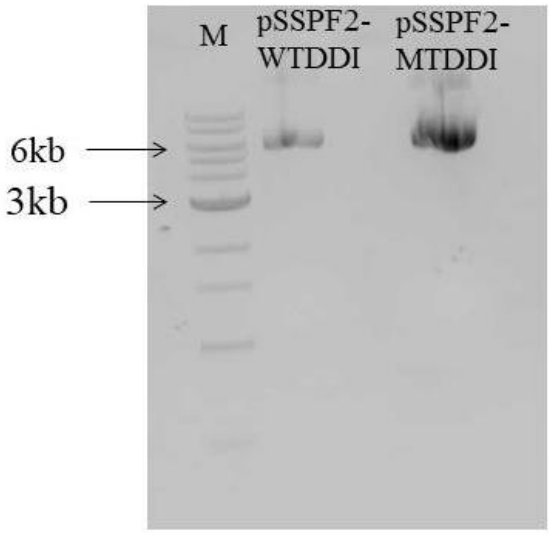


Fig 4.1 Agarose gel of the undigested PfCENV3 Fig 4.2 Agarose gel of pSSPF2-WTDDI and pSSPF2-MTDDI

Digestion of the pfCENV3 released the plasmid backbone of 7.2 kbp size and GFP coding gene of 800 bps (Fig 4.3). Digestion of the pSSPF2-WTDDI and pSSPF2-MTDDI plasmids released the DDI coding genes of 1.2 kbp and the pSSPF2 plasmid backbone. The DDI coding fragments were purified and ligated with the pfCENV3 backbone, transformed in the DH5α cells. Colony PCR was done to identify recombinant colonies, which were further confirmed by restriction enzyme digestion for confirming presence of the inserts (Fig 4.4). Plasmid DNA from the confirmed colonies were sequenced, sequences were analyzed, and glycerol stocks were made from the confirmed clones.

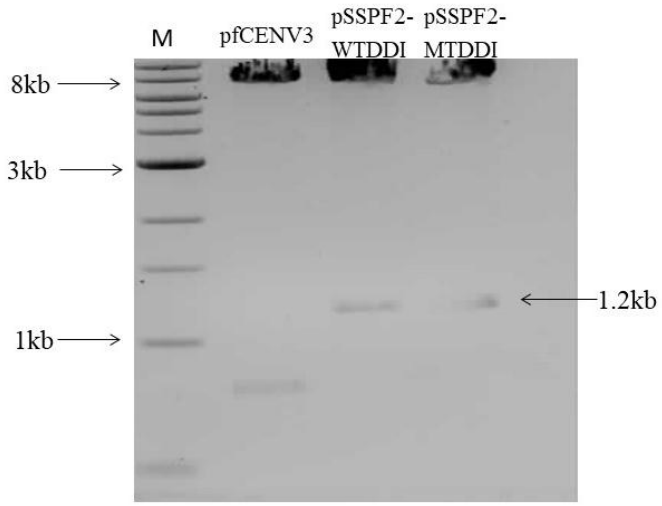


Fig 4.3 BglII and XhoI digestion of PfCENV3, pSSPF2-WT DDI and pSSPF2-MT DDI plasmids

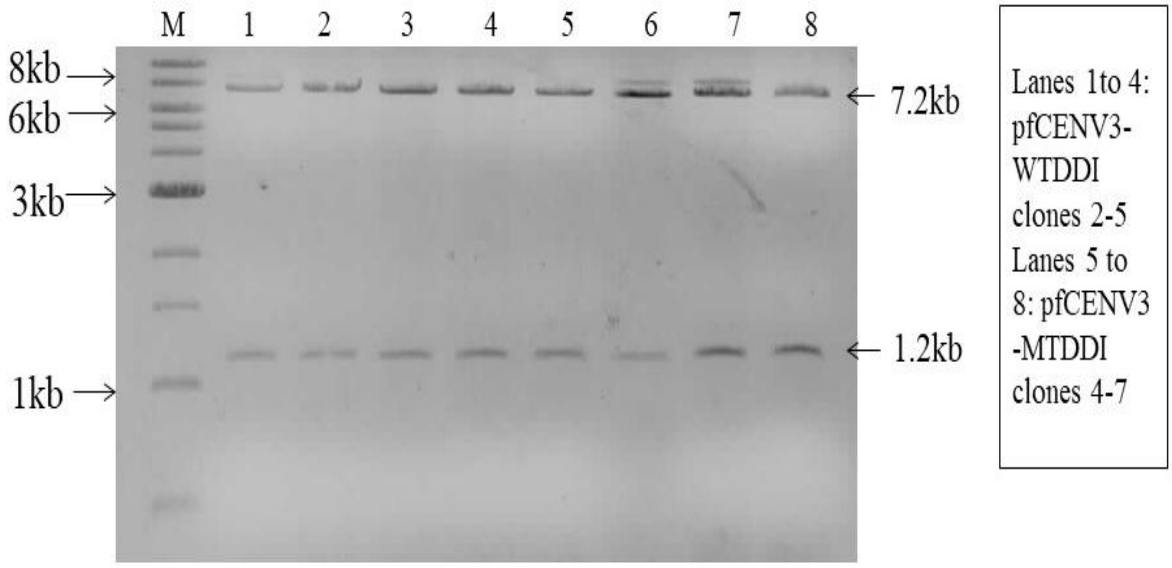


Fig 4.4. Colony PCR of PfCENV3 transformants showing presence of DDI insert. LANE M is 1kb ladder, L1-L4 are transformants with WT DDI gene insert and L5-L8 are transformants with mutated DDI gene insert obtained by BglII-XhoI digestion of PfCENV3-WT DDI and PfCENV3-MTDDI

4.2 Cloning of DDI gene into PfCENV4

The DDI gene (both wild and mutant type) from pSSPF2 was also cloned into pfCENV4 vector exactly as done in pfCENV3 vector. The plasmid of pfCENV4 was ran on 0.8% agarose gel. It was digested, backbone was purified, ligated with DDI inserts, and used to transformed DH5α cells (Figures 4.5 and 4.6).

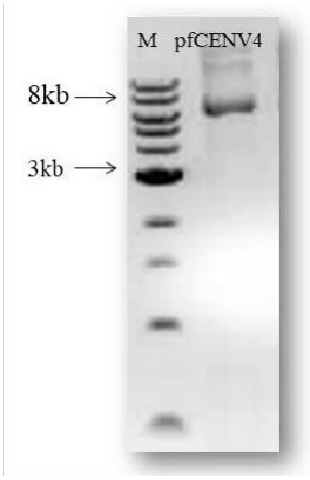


Fig 4.5 Agarose gel of undigested pfCENV4

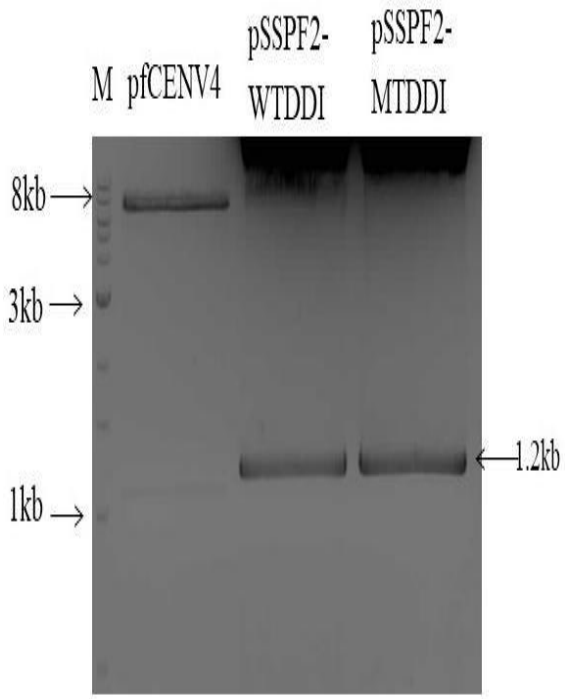


Fig 4.6 Double digested plasmid for gel elution.

Transformants were screened for the presence of insert by colony PCR, plasmid DNA was isolated from positive colonies, and digested with BglII-XhoI for insert release. Most of the clones showed release of expected size of insert (1.2 kb), suggesting the presence of DDI. Selected plasmid DNA of these clones were sequenced, and sequence confirmed clones were used for preparing glycerol stocks.

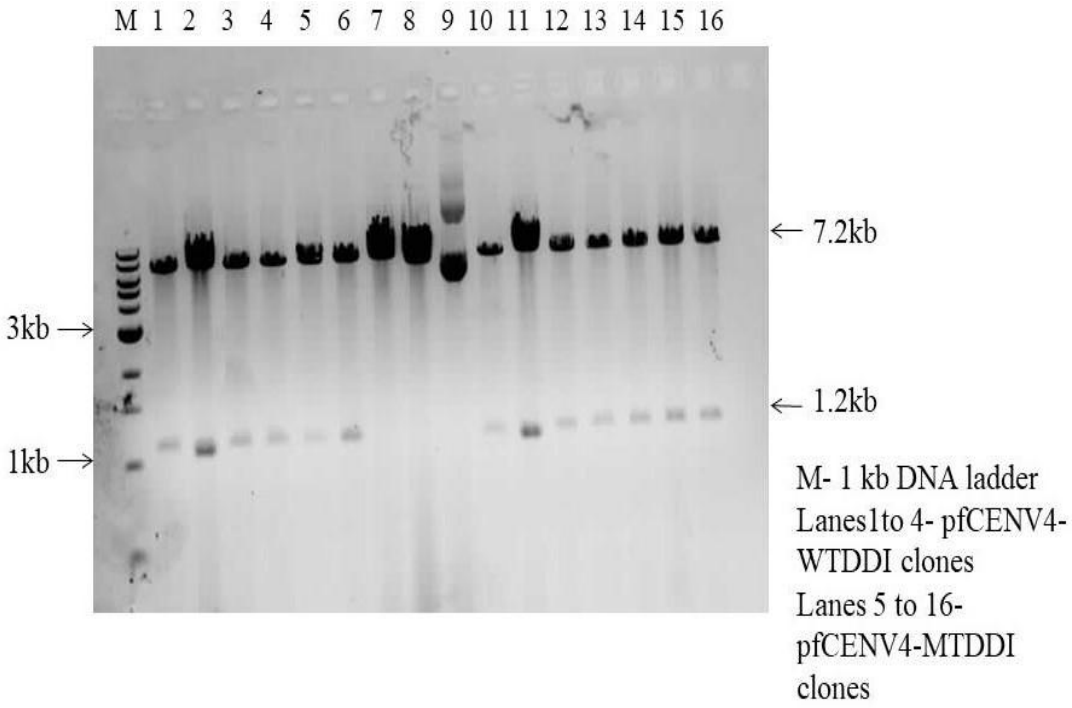


Fig 4.7 Colony PCR of pFCENV4 –DDI transformants showing presence of DDI insert. LANE M is 1kb ladder, L1-L4 are transformants with wtDDI gene insert and L5-L16 are transformants with mutated DDI gene insert obtained by BglII-XhoI digestion of pfCENV4-WTDDI and pfCENV4-MTDDI

4.3 Cloning of PGT PbRPN11

RPN11 is one of the components of the regulatory unit of proteasome. To clone PbRPN11, the PGT-GFP and Hb-PbRPN11 plasmids were digested with BglII-XhoI, which excised GFP coding sequence (800bp) from PGT-GFP and PbRPN11(1.3 kb) from Hb-PbRPN11 (Fig. 4.8). The PbRPN11 insert and PGT backbone were purified (Fig. 4.9) and ligated to obtain PGT-PbRPN11 construct, and transformed into DH5α cells.

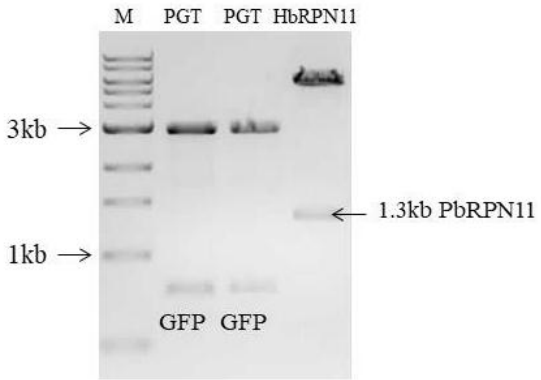


Fig 4.8 Digested PGT-GFP and Hb-PbRPN11 with BglII-XhoI.

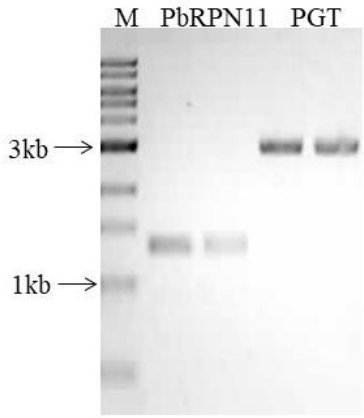


Fig 4.9 Gel eluted PbRPN11 and PGT

PGT-PbRPN11 transformants were digested with BglII-XhoI to check for the presence of insert (Fig.4.10). The insert of expected size was released, but in some cases the sizes of vector backbone were not of expected length, which might be due to incomplete digestion.

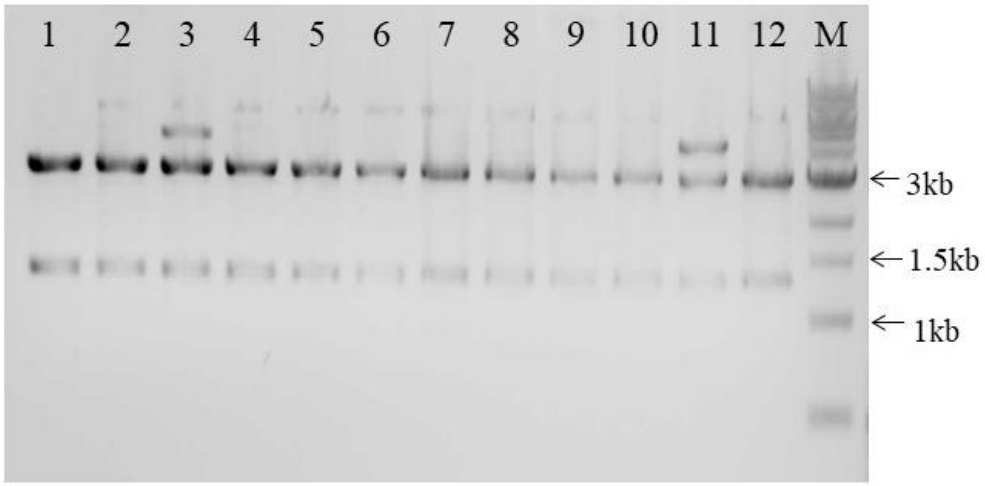


Fig4.10 Digested PGT-PbRPN11 transformants (1-12) and insert release

4.4 Atg7 protein expression and purification

The Atg7 protein was expressed and purified under denatured conditions. The expression of the protein was very low, and a band of expected size of His-Atg7 fusion protein was present in both uninduced and induced samples, which might be due to leaky expression (Fig 4.11). The protein was puried by passing through Ni-NTA column. The separation of protein was based on the affinity properties of the molecules. Higher concentration of imidazole (250 mM) in urea buffer was used as elution buffer which has higher affinity to Ni^{+2} ions that releases the bound protein and comes out as elute. The quality of the protein obtained was checked

by running it on the SDSPAGE, which indicated that it was purified to homogeneity. The protein was refolded into soluble form by dialysis.

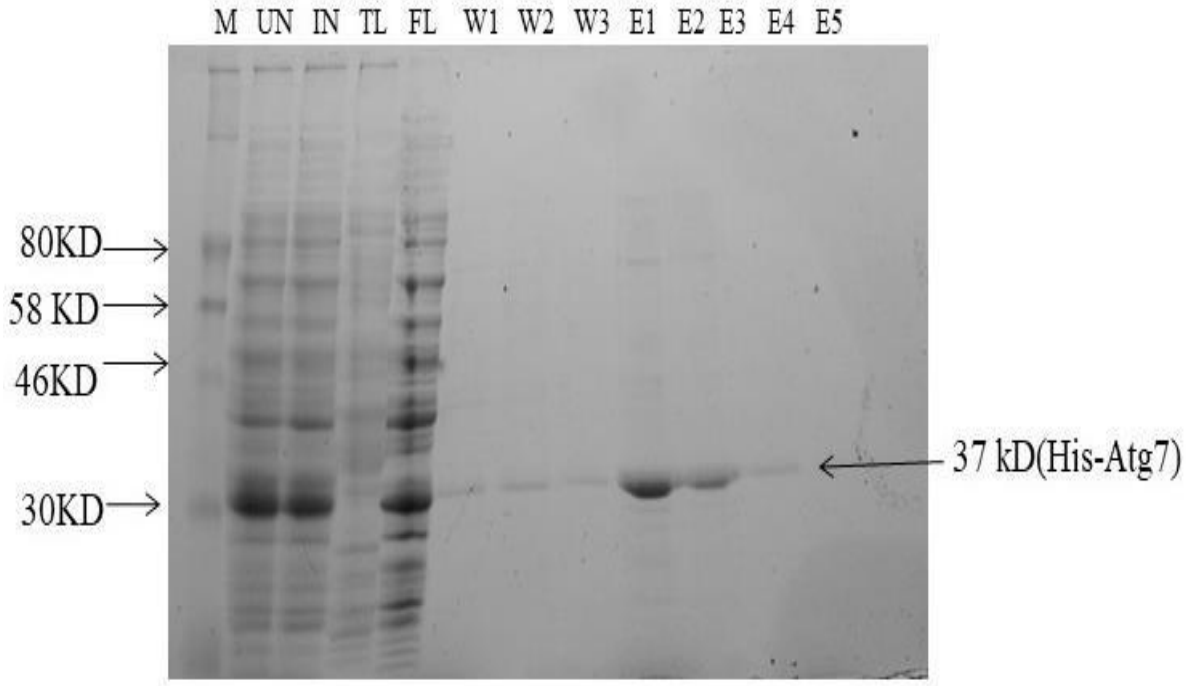


Fig4.11 SDS PAGE showing protein purification of recombinant Atg7 under denaturing condition.

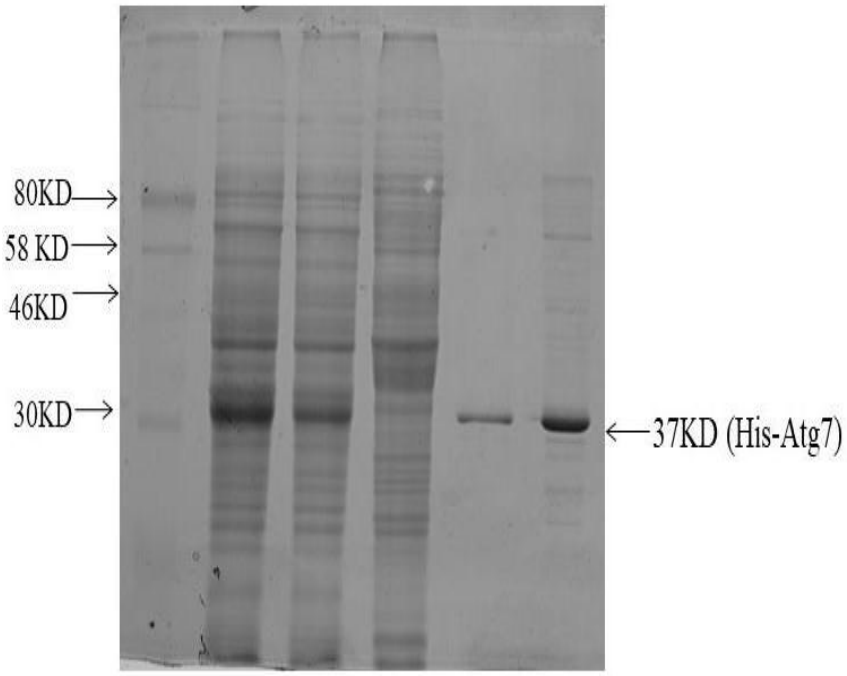


Fig 4.12.Protein purification under denaturing condition.

The dialyzed protein was concentrated and also assessed for homogeneity. Its concentration was estimated by BCA assay.

Exp.	Vol of BSA	Vol of water	OD at 562
BSA (µg/µl)	µl	µl	Avg
0	0	25	0.088
1.5	0.75	24.25	0.20775
3	1.5	23.5	0.2945
6	3	22	0.4475
9	4.5	20.5	0.61575
12	6	19	0.773
15	7.5	17.5	0.912
18	9	16	1.06325
A1	3	22	0.216
A2	6	19	0.32125
A3	9	16	0.427
A4	12	13	0.52975

Protein (µg)	Protein Conc(µg/µl)	Avg.(µg/µl)
1.781954887	0.593984962	0.624836832
3.760338346	0.626723058	
5.748120301	0.638680033	
7.679511278	0.639959273	

Calculation

Stock conc: Working Conc = 2X:X. Total protein Conc= 2* avg = 1.249674 mg/mL

The total protein concentration was found to be 1.249674 mg/mL

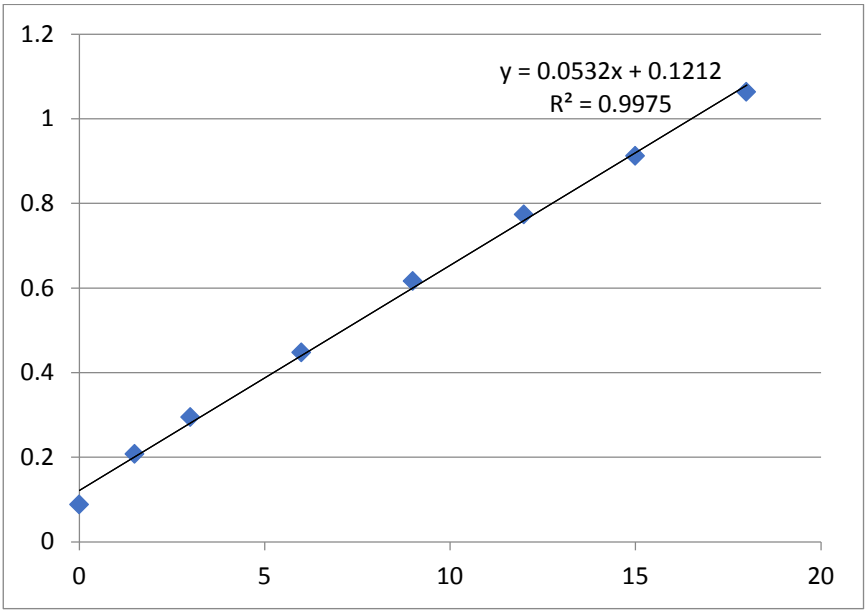


Figure.4.13 Graph showing the concentration of the dialyzed Atg7 protein.

The dialyzed proteins were then used for generation of antibodies by immunizing in rats with adjuvants (Freund's complete or incomplete adjuvants), and the antibodies generated were then quantified by using ELISA titration method.

4.5 Anti-Atg7 Antibodies titration by ELISA assay

Sera containing anti-Atg7 antibodies were titrated by ELISA for reactivity with the antigen. The pattern of rise in antibody levels should increase with each immunization, as the memory cells recognizes same antigen and produces higher amount of antibody. Emulsion made by mixing adjuvant with protein adds in the immunogenicity and increases in-vivo availability of the antigen for immune cells, which would activate immune cells for longer time.

The antibody titer increased with subsequent booster doses. The pattern of rise of antibody titer in the Rat showed increasing order. The third bleed showed higher antibody titer than second bleed. This shows that Atg7 protein is immunogenic. The sera were stored at -80 °C for future use.

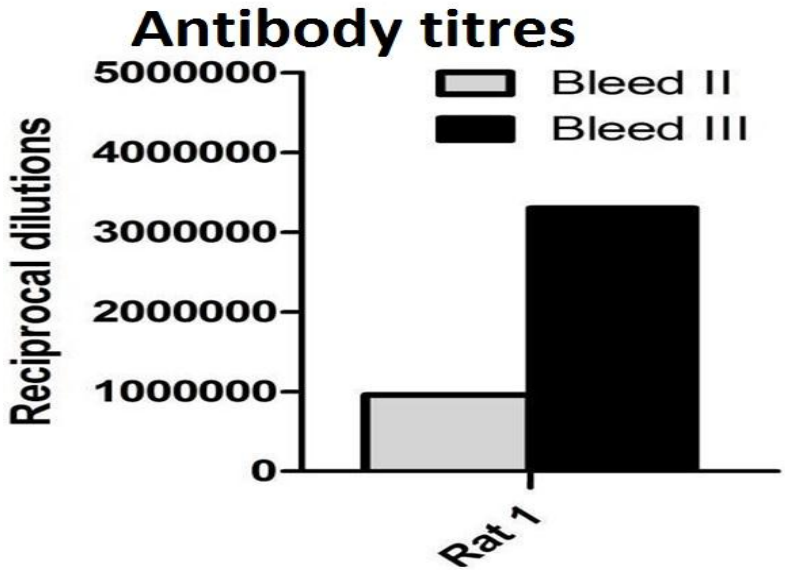


Fig 4.14. Anti-Atg7 antibodies titration by ELISA

4.6 Western blotting of recombinant Atg7 protein and native Atg7 in 3D7 parasite lysate

Western blotting of PfAtg7 protein with anti-Atg7 antibodies showed several bands, including the desired protein band of 37 kD (Fig. 4.15). The non-specific bands could be the presence of contaminants in the preparation of recombinant Atg7, hence, these antibodies would need to be purified.

Western blotting of 3D7 parasite lysate with Anti-Atg7 antibodies was done to assess the presence of native Atg7. The blot showed the desired protein band of 156.65 kD along with several non- specific bands. The predicted size of full-length Atg7 is 156.65 kD; a signal corresponding to this size is present in the blot, which was not detected by the pre-immune serum , suggesting that the antibodies produced also recognize Atg7 in the parasite lysate. As there are several nonspecific bands detected, the antibodies need to be purified.

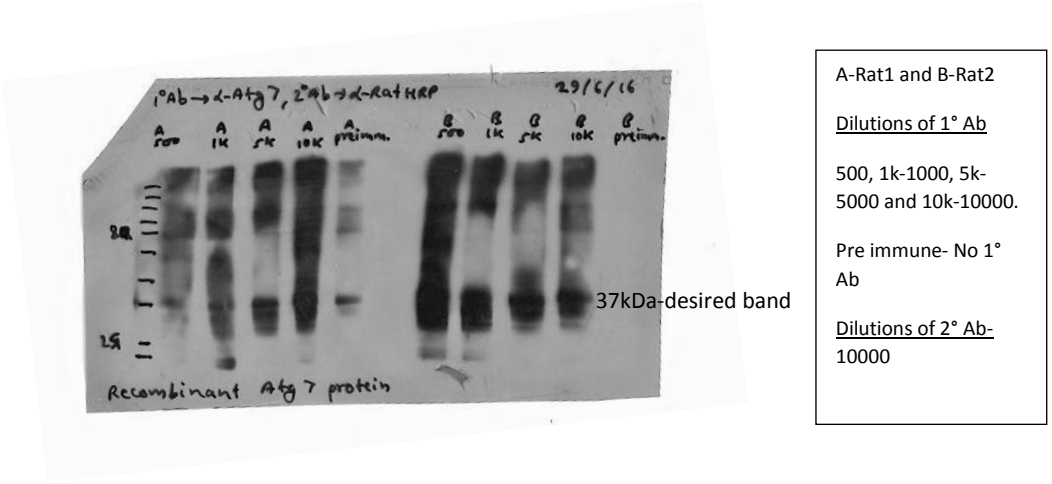


Fig 4.15 Western blot of recombinant Atg7 protein using anti-Atg7 antibodies

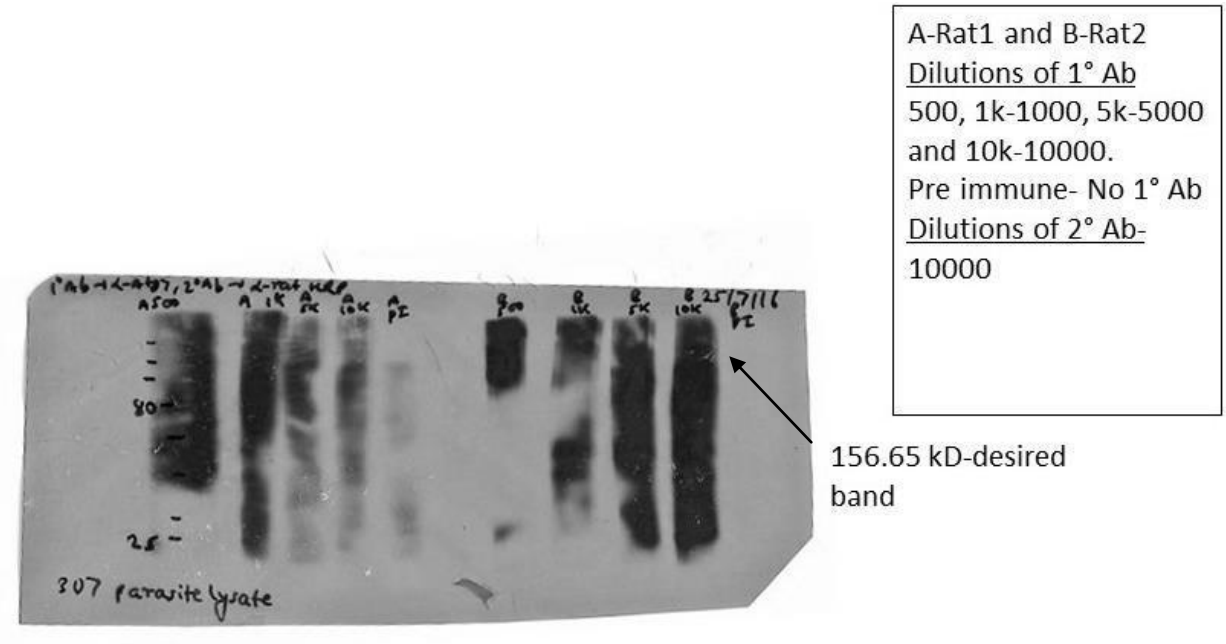


Fig4.16 Western blot of 3D7 parasite lysate using anti-Atg7 antibodies

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The antibodies generated will be used for various immunological tests such as agglutination, immunoprecipitation etc., which will be helpful in tracing the role of the PfAtg7 protein in autophagy pathway in *Plasmodium falciparum*.

CHAPTER 5

DISCUSSION

Cloning of DDI in PfCENV3 and PfCENV4

The cloning of the PfCENV3 and PfCENV4 transfection vectors had already been optimized in Centre for Cellular and Molecular Biology (CCMB) and the method of the cloning has been described. The PfCENV3 plasmid has cam5U region where as the PfCENV4 plasmid has GAPDH5U instead of cam5U. The wild type DDI (WTDDI) and mutant type DDI (MTDDI) were cloned in Xho I and Bgl II sites. The GFP was replaced by the DDI in PfCENV3 and PfCENV4 plasmid vectors. The aspartate amino acid residue of WTDDI in position 220 was substituted with alanine in MTDDI. The WTDDI has aspartic acid in the active site and is catalytic in nature where as MTDDI has alanine substituted which is non-catalytic in nature. The DDI (MT and WT type) were obtained from pSSPF2 plasmids and cloned into PfCENV3 and PFCENV4 plasmid vectors.

The cloning of the DDI in PfCENV3 and PfCENV4 vectors were done for effective transfection in *Plasmodium falciparum* because these vectors have conserved centromere region of the *Plasmodium falciparum* which is very effective than pSSPF2 vector. The DDI was already cloned in pSSPF2 which was not effective vector for transfection of *Plasmodium falciparum*.

The RVP domain of the DDI is the target site where HIV protease inhibitors show antimalarial activity. The plasmodial aspartic protease inhibition was possible because they are biochemically similar to the HIV protease (Achan et.al.,2012). The HIV and malaria geographically overlap and the HIV protease inhibitors, lopinavir –ritonavir (LPV-RTV) used were in combination with malaria treatment showed lower risk with 41% reduction of recurrent positive blood smear (BS) in HIV-infected children.

Cloning of PGT-PbRPN11

The PGT-PbRPN11 cloning was done by excising PbRPN11 from HB vector and ligating in PGT backbone where GFP was already excised out. The restriction digestion was done with Bgl II and Xho I enzymes. The insert release of expected size of the transformants on restriction digestion showed the cloning was done successfully. The RPN11 is an important component of the lid of the proteasome which plays an important role in deubiquitination process that recycles the ubiquitin molecules. The polyubiquitin substrates are removed by the deubiquitinase RPN11 during ATP-dependent substrate degradation (Worden et. al., 2017). So the cloning of the RPN11 in plasmid vector plays an effective role for the study of the regulatory mechanism of the *Plasmodium* parasite.

Expression of Atg7 protein in large quantity and production of anti-Atg7 antibodies

The Atg protein sets are partially conserved in *Plasmodium falciparum* (Kitamura et.al 2012). PfAtg7 has relatively low identity (14.7%) and similarity (32.2%) to ScAtg7 due to presence of long insertions and on excluding it, the identity is 38.0% and similarity is 70.8%. The PfAtg7 is conserved and is the activating enzyme of PfAtg8. The PfAtg7 is necessary for the normal parasite growth which was shown by the attenuation experiments that suggests PfAtg7 is a druggable target to treat malaria. The ScAtg7 is involved in the Atg5-Atg12 conjugation pathway, but the bioinformatic analysis confirmed the absence of a PfAtg5 in the *Plasmodium* genome which shows PfAtg7 may also be involved in other roles which needs to be fully elucidated (Walker et. al.,2013).

The PbAtg7 protein shares similarity with yeast (34%) and human (32%). The study shows that ScAtg7 binds to Atg8 via its C terminus and to Atg3 via the N terminus. The adenylation domain of ScAtg7 exhibits high similarity with the PbAtg7. The PbAtg3 has similarity of 50% in yeast, 48% in human and 54% in *Arabidopsis thaliana.* PbAtg3, PbAtg7, and PbAtg8 are constitutively transcribed throughout the parasite life cycle. In the mammalian host, PbAtg8 and PfAtg8 are found at the membranes of the apicoplast. PfAtg8 localization is found in the tubular structures throughout the lifecycle of *P. falciparum* (Jayabalasingham et. al, 2014).

The cloning of PET32a Atg7 in Rosetta-gami cells for protein expression was done from BL21DE3 cells because Rosetta-gami cells have high expression capacity than BL21DE3 cells for Atg7 protein. The recombinant Atg7 protein has small size of 37KDa in comparison to the PfAtg7 in 3D7 parasite lysate having size of 156.65 KDa. The small size of the recombinant Atg7 is due to the presence of selective DNA inserts by excision of the introns segments whereas 3D7 parasite has large size because of the presence of the long genomic DNA typically present in the parasite.

The non-specific binding of anti-Atg7 antibody is seen in Western blot. This is due to the presence of small amount of contaminants of tag proteins during purification by Ni-NTA chromatography. The protein obtained was checked in SDS PAGE which showed desired bands of appropriate size of 37KDa and was in pure form. The antibody was raised against this Atg7 protein and was in high titre of 1000000 and above 3000000 in bleed II and III respectively (bleed I is Pre-immune sera and contains no antibody), so the result of the ELISA is reliable. Though, the non-specific binding of anti-Atg7 antibody is seen in Western blot, it will not affect the result of the ELISA.

CHAPTER 6

CONCLUSION

The dissertation project was taken up to clone WTDDI and MTDDI genes in transfection vectors pfCENV3 and pfCENV4 and produce recombinant Atg7 antibodies. This would subsequently allow us to study the role of DDI genes and Atg7 genes in malarial pathogenesis that will be helpful in elucidation of UPS and autophagy pathway in malarial parasite, *Plasmodium falciparum*. The cloning of DDI gene was successful. The cloning of the RPN11 was successful in PGT vector which gives the way for the construction of the more effective form PfCENV3-PfRPN11 transfection vector which was not complete due to the time limit. The production of recombinant Atg7 antibodies were done successfully. The recombinant Atg7 protein was found to be immunogenic as indicated by the presence of high antibody titres. The antibodies recognized recombinant Atg7 as well native Atg7 in the parasite lysate. However, the antibodies need to be purified before using in various immunological assays.

LIMITATIONS

The DDI gene cloning was successful but the transfection in Plasmodium was not done to study the episomal expression of the genes. The cloning of the RPN11 in PGT vector was successful but the construction of the full PfCENV3-PfRPN11 transfection vector was underway. The recombinant Atg7 was purified but it was not ideal for further study and require further down streaming process which was not carried because of timeline and financial resources which will help in elucidating the role of Atg7 in autophagy in Plasmodium.

RECOMMENDATIONS/ FUTURE PERSPECTIVES

1. The cloned genes will be used in transfection for episomal expression of these genes in the parasite.

2. The future study on DDI may reveal if DDI is the target of HIV protease inhibitors or not.

3. The construction of PfCENV3-PfRPN11 transfection vector was underway which will be useful in switching the regulation control of the parasite pathogenesis/physiology.

4. Purified antibodies will likely help us to understand the role of Atg7 in autophagy in *Plasmodium*.

5. Recombinant antibodies may be useful as a therapeutic agent against malaria

CHAPTER 6

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APPENDICES

Preparation of buffers and reagents

1.1 Luria-Bertani (LB) Media

10 gram of tryptone, 10 gram of sodium chloride and 5 gram of yeast extract were dissolved in milliQ water and the volume was adjusted to 1000 milliliter. The medium was sterilized by autoclaving.

For **LB agar**, agar (1.5% w/v) was added to the LB broth. The volume was adjusted to required quantity and the medium was autoclaved.

1.2 **Agarose gel electrophoresis** (TBE) buffer (10X) was prepared by mixing 109 gram Tris base, 55 gram Boric acid and 40 ml 0.5 M EDTA (Ph 8.0) in milliQ water. The final volume was made up to 1000 milliliter, and the solution was sterilized by autoclaving.

1.3 **Ethidium bromide stock** (10 mg/ml in milli Q water)

1.4 **Urea buffer** (50 mM NaH_2PO_4, 8M urea, 500 mMNaCl, pH 8.0)

1.5 **Binding buffer** (20 mM imidazole, 0.5% Triton-X, 5 mMβ-ME; in urea buffer)

1.6 **Wash buffer 1 and 2** (30 mM Imidazole, 0.5% Triton-X, 5 mMβ-ME; in urea buffer)

1.7 **Wash buffer 3** (50 mM imidazole in in urea buffer)

1.8 **Elution buffer** (250 mM imidazole in in urea buffer]

1.9 **The SDS PAGE running buffer/TGS buffer (10X)** was prepared by mixing 30 gm of Tris base, 144 gm of Glycine and 10 ml of 10% SDS in milliQ water. The volume was adjusted to 1000 ml.

1.10 **SDS-PAGE Sample Buffer (2X)** was prepared by mixing 1.25 ml of 1MTris-HCl (pH 6.8), 2.5 gm β- merceptoethanol, 0.49 gm SDS, 2.5 mg Bromophenol blue and 2 ml of Glycerol in milliQ water and the volume was adjusted to 10 ml.

1.11 **The SDS-PAGE staining solution** was prepared by mixing 500 ml methanol, 100 ml Glacial Acetic acid, 2.5 gm Coomassie brilliant blue and milliQ water. The volume was adjusted to 1000ml.

1.12 **De-staining solution** contained all the components of the staining solution except coomassie brilliant blue.

1.13 **Refolding buffer**: 20 mMTris buffer (pH 7.5), 1 mM EDTA(pH 8) ,0.5 mM DTT, 50 mMNaCl,10% Glycerol and Chilled AMQ

1.14 **Change buffer**: 20 mMTris buffer(pH 7.5), 50 mMNaCl, Chilled AMQ.

1.15 **The ELISA coating buffer** was prepared by mixing 60.6 mg Na_2CO_3, 120 mg $NaHCO_3$ and water. The final volume was adjusted to 20 ml (pH 9.6).

1.16 **The PBS (10X) buffer** was prepared by mixing 2 gm of KCl, 14.4gm of Na_2HPO_4, 80 gm of NaCl, 2.4 gm of KH_2PO_4 in water. The pH was adjusted to 7.4 in final volume of 1000ml.

1.17. **5% BSA in 1X PBS**

1.18 **Wash buffer**: 0.05% Tween 20 in 1X PBS

1.19 **Primary antibody** (Ab against Atg7) in 1% BSA (in 1X PBS)

1.20 **Secondary antibody** (anti-rat Ab) in 1% BSA (in 1X PBS)

1.21 **The Transfer buffer** was prepared by mixing 3.3 gm of Tris, 14.68 gm of Glycine, 200 ml of Methanol in AMQ water and the final volume was adjusted to 1000 m. It was Kept at 4°C.

1.22 **The TBST (1X)** was prepared by mixing 10 ml of 1 M Tris buffer (pH7.5), 3 ml of 5 M NaCl, 1 ml of Tween 20 in AMQ water and the volume was adjusted to 1000 ml.

1.23 **5% milk in 1X TBST**

1.24 **Primary antibody** (Ab against Atg7) in 5% milk (in 1X TBST)

1.25 **Secondary antibody** (anti-rat Ab) in 5% milk (in 1X TBST)

I. *Plasmodium vivax* Atg7 (PvAtg7) (PVX_091922)

MDHTLGPKRDPSLREPPSGEKNTKKGEPNSEAAEGVDVLKHCHNEFKMDISFFTKLYEQK
IEIYKHRCEYINLLSSSSVHRMNVTVNRPYRVDSSEPVVFEHPYVSCSVVEIDRNSFAVV
EGEIGQAAVGSMTSEEMAQKGNVTLEDIIPSGADDKAEEPPLTMGTPKSADVRSADVRSA
DVRGAQGKDTKEKRRNTFSKKYRGILLNFNTLEEFLTMNSCTHINYTMSHLRSYLKRASQ
EKNAPGDGTKKDSIYEDSFWVYNPNELTYIEKINAYLILTFFDLQNFVCYYSLANPVVNP
PVHFKLVEPSERLYFLIHPEVMYLDSRRNHINVGDLLYLSHQIDTLFEGEKPFVDSGAFL
LLKFDAEEFSLHNEHLYVETYQSSLHRLGVAEELRERKEERAFASLDSIKGVCDYLSGIR
EKHSQGSNPCMCACADDSKEQITISGAFHCKCHLDDETTKCDCPYRILRSYAGLTVLPLN
CLGELKEELQNMKEKKLKYVKGDFFHLYICLVDPNCVFTSLGWDFRNLLYCLSLKYELHD
FEIELLVFRDFSLLSEELICKAFSTHLLWRYPHVGIPTGALPGMCHAYMPPVKSAQSTMV
CKMVARDYGGTQVDAAQVGGERGWSGKREESHVGGEASHQLGEPNAGATDLSARNPQTVK

YLLNSSVFKIRVPGRGDLEGATTGEAVRGKTATEAAPTPRTASPIRCAPGWKRRAGAKGD
SAIHRVSLRNFVNRDVVQRIALEQHVKLIKWKLLKDLKEEKIASLKVLILGMGTVGCNVA
RTCVTWGIKNLTVVDNSVVKHSNLGRQSLYTTADVEDECKLPLHKVVAAKRRLLEIAPDL
KIQAKEIDIPMPGHSIYVSDCRVSKTREAITQLRSLIATHDAVFLATDSKESRYFPSLLI
AERQYSAMRELQKGQRCGGQTLPSLGRTVPAGDYHGDYHGDYHSDCHSDCHSDHHSDLYM
HFSKDSLTEESIRERQTFYNNILTNVSQLAKMPPLGISVALGFDSLHIIRHPYLYFKGGC
FFCNDLHSPQNSSFGQPLDEKCTVSRGGLSSIGGGLSVELLVTLTQHPLGFFAPHTNRDQ
YVHSRQVHTSQVYAAERDASKEQTGVEQTGVKNTTVEATTVEATTVGKKGAAANSLVSCL
GATPHIVSLNLAEFTMRKMYCGAFDRCLCCSEKVILSYQACPEAFVENVIRDSSVLEKIT
GIDELRAEESDVIMLD

Sequence Length: 1216 aa

II. *Plasmodium bergei* Atg7 (PbAtg7) (PBANKA_092220)

MNINKCEAYSEELEKVQTCGQEKGQILKHCYNEFKIDISFFLKLHEQKINIYKLKSDYVN
LISETYVKKIKVEYKYKKINNLDIIEFYYPYKNTSFIEINKNSFETDKICIEFRKNEEKK
FQKINNYNKKYQGLLLNFNTLEEFLNANKTKHVNNSMNDIKMYVVCENNEKKKKKCIYDN
SFWEYKQDELDFFEKINKYLILSYFDLKKCICYYSISNPVIKPLGNFKEILPSQRLHIYI
NTENVYLNKEIKEINVIDTIYLSQKIDDCFNSHKMFVKSRVFLLLRFKNDDINDGNYYDE
FMQNSYEILSNIQSEENNEFYKINSFKKLYEYIFYDDKYKENSMNDNQQNEYILHCSILR
KNLNLVVLPINCLSELKEEINMSKDKVLKNIKKKYFDIYICMIDHNLIHNSLNWDARNLL
YFLTIKYELYNFEISLLAFRDIGLLGDQIICLFNKNNELIFKCPIFSKDHQTSSNDNSQI
VFKAVSEILGISYDLVEVIDYSEKKKKFNFELIKQYNNSSNNANKLDNKNLNEIKIDLSD
KKNKLVKFFLNSSIFNIKMMGISDFFKIDKNMNSLKYEIIPGWKKYIEKKNNKIKENIIY
VINLNNFLNKNTIQRISLELNIKLIKWRILKNFKFEK**IHDLKILIIGLGTLGC**SVARTCV
AWGIKNFTFIDNSRVSFSNVSRQSLFNLENAESYNNIGEYKSIAAKNNLLKISPDLNIIS
KIMDIPMPGHLNYLKNENLYNTIEELDKIIDNHDVVFLLTDSKESRYFPSLLIAEKHYNC
LKKYKNFQNNNIYDTSKLDEWKNTTIQYTENITYKNYMYVLNDGIENININPKLISNKKN
ELNNHRNIFYSNILSTITQINKMPPLGISVALGFDSFQVIRHSYLYFKGGCYFCNDMNSP
TDSMSYRTIDEK**C**TVTRPGISSISSSIATELLISLTQHPLQFSAPHVENDQYICFDSECD
NLKNKNIETSNSFASCLGATPHIITFNLSNLSIRKIYSDAFDRCVCCSEPVILKYQENKS
EFVKKVISESLVLEDITNMNVLKQADEKDVIIFE

Sequence Length: 1054 aa