Molecular characterization of membrane associated Inner membrane complex proteins in *Plasmodium falciparum* (Welch, 1897)

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Abstract

The Alveolates (dinoflagellates and ciliates and apicomplexans) have a similar architecture of the cortical pellicle. The alveolate pellicle consists of the plasma membrane, the inner membrane complex (IMC- flattened membranous sacs, a defining feature of this superphylum), membrane skeleton (that supports the IMC vesicles) and a network of microtubules. IMC provides the required rigidity to the protists cells. The apicomplexans however evolved further to make use of the IMC in critical processes like host cell invasion, transmigration and daughter cell formation. A family of proteins called the alveolins, associated with the cytoplasmic face of the IMC, is ubiquitous among the members of the superphylum and forms a key structural unit of the pellicle. These alveolins are identifiable by the presence of iterations of repeatitive stretches of certain conserved amino acid residues, which are implicated in proper targetting of the protein to the IMC.

In the malaria parasite, *Plasmodium spp.* only a handful of IMC/Alveolins have been identified and deletions of some of the alveolins have resulted in drastically reduced mechanical strength and motility of the ookinetes and sporozoites. In this study, we used an algorithm (XSTREAM- Seed extension) previously used to identify some classical alveolins (Gould *et al.,* 2010) and reduced the stringency of the parameters to trace out more members of the alveolin family in the *Plasmodium falciparum* genome. This approach enabled us to identify PF13_0226 and PF08_0033 as putative alveolin-like candidates. The localization studies of the two above mentioned putative alveolin-like proteins, revealed that the former is not and the latter is an IMC protein. We investigated the role of the crucial and conserved amino acid stretches called the alveolin-repeats in the identified alveolin –like protein, PF08_0033 by generating mutants devoid of the alveolin-like domains. Of the three such alveolin-like motifs identified in the protein, the third repeat or motif proved to be crucial for the protein to localize properly in the IMC. The acylation sites in the flanking regions were mutated to neutral amino acid residues. The mutation of posttranslational modification sites did not hamper localization of the mutant protein. This study reconfirmed the previous findings, that the well-conserved alveolin motifs help in membrane attachment of the protein. Furthermore, the resistance to detergent solubilitzation of the concerned alveolin-like protein, PF08_0033 was in compliance that it is a part of the rigid pellicle much like the classical alveolins.

Additionally we tried to assess the essestiality of the *Plasmodium* specific IMC-gene, MAL13P1.228 (non-alveolin) by truncating the gene. The full-length protein is implicated in sealing the adjoining IMC vesicles during the presexual and sexual stages of the parasite's life cycle and owing to this reason, the protein displays a stripy pattern of distribution during the sexual stages. During the asexual erythrocytic stage however, it displays a localization comparable with that of the classical alveolins. The truncated protein, on the contrary had a cytosolic distribution during the late asexual stage and in the presexual forms. The cell-line with truncated MAL13P1.228, did not have detectable changes in the parasite growth rate and morphology. The apparent truncation did not have an impact on the IMC biogenesis, the ongoing cytokinesis and the sexual differentiation leading to the formation of the presexual forms. The function of MAL13P1.228 might not be essential for the parasite development *in vitro*.

Moreover, in order to gain a deeper insight of the functionality of the IMC proteins, an alveolin (PFE1285w) and non-alveolin (MAL13P1.228) were tagged with destabilization domain (DD, a ligand based regulatable system) and expressed episomally. As a consequence of dominant negative effect, the entire complex of the native and the tagged protein was expected to get degraded in the absence of the appropriate ligand (Shld-1) of the destabilization domain. However, this approach failed to serve the purpose and degrade the tagged proteins primarily because the proteasomes could not access the tagged proteins. The two IMC proteins are entrapped in a meshwork of other interacting proteins as made apparent by the solubility assays, performed in this study and this made their degradation unattainable.

Dedication

I dedicate this piece of work to Ira and Arti for instilling the faith in my ownself.

Acknowledgements

I would like to sincerely thank my supervisor Dr. Tim. W. Gilberger. It was because of his support and help I could procure the coveted fellowship to fund my research and studies in Germany in the prestigious Bernhard-Nocht-Institute for tropical medicine (BNI). He has been a tremendous source of encouragement and energy that always rejuvenated my enthusiasm for the project. The immense support from him right from my first day in the organization, is highly appreciated by me. Despite his busy work schedule, he was available and responsive to my circumstances throughout my tenure, for which I shall always be grateful to him. The countless fruitful discussions with him and suggestions from him improved my understanding of science and the methodologies. Last but not the least, the degree of patience shown by him with me, has made me grow as an individual that always gave me a second chance to correct my mistakes.

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I highly appreciate the patience shown by my parents, sister (Arpita), my brother-in-law (Easwer) and my lovely nieces (Ishita and Elina) for which I am short of words to thank them. I am indebted to my uncles (specially Bibhas), aunts and my dear cousins (Sarba, Kuntu, Sumitro, Deepanjan, Meenakshi, Dibyajyoti, Siddhartha, Sayan and Sarthak) for their support and love that always restored the belief in me.

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I would like to end this chapter with the wise words of Rick Springfield, which I might have used several number of times in my life:

‘I am thankful for the serendipitous moments in my life, where things could have gone the other way. ’

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ABBREVIATIONS

%	Percent
α	Alpha, Anti
β	Beta
A	Alanine, Adenine
AA	Amino acid
AMA-1	Apical Membrane Antigen-1
AMP	Ampicillin
APS	Ammoniumpersulfate
ATG	Start Codon (Methionine)
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BPB	Bromphenolblue
BSA	Bovine Serum Albumin
°C	Grade Celsius
C	Cytosine
$CaCl_2$	Calciumchloride
cam	Promotor of Calmodulin Gene
cDNA	Complementary DNA
cm	Centimeter
CO_2	Carbon dioxide
crt	Promotor from Chloroquine Resistance Transporter
D	Asparagine amino acid
DAPI	4',6-Diamidino-2-phenylindol
dH_2O	Distilled Water
DHFR	Dihydrofolatreductase
dNTP	Desoxyribonucleoside-5´-triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleotide
DTT	4,4'- (2,2,2- trichloroethane- 1,1-diyl) bis (chlorobenzene)
E	Glutamine amino acid

E. coli	*Escherichia coli*
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ER	Endoplasmic Reticulum
et al.	*et alteri* (Latin: and others)
EtBr	Ethidiumbromide
EtOH	Ethanol
F	Phenylalanine amino acid
G	Guanine
GAP	Glideosome associated protein
gDNA	genomic DNA
GFP	Green Fluorescent Protein
Gln	Glutamin
Glu	Glutaminsäure
GTP	Guanosine- 5`- triphosphate
hr	Hours
H	Histidine
HCl	Hydrochloric acid
hdhfr	Human Dihydrofolatreductase
HRP	Horseradish peroxidase
IFA	Immunofluorescence - Assay
IMC	Inner membrane complex
ISP	IMC Subcompartment Proteins
i. e	Latin *id est* (That is)
in vitro	Latin: *in glass*
in vivo	Latin: within the living
K	Lysine
kDa	KiloDalton
K_2HPO4	Di- Potassium hydrogen phosphate
KH_2PO4	Potassium dihydrogen phosphate
kb	Kilo bases
KCl	Potassium chloride
l	Liter

L	Leucine
LB	Luria Bertani
M molar	Methionine; (Mol per Liter)
m	Meter
MetOH	Methanol
mg	Milligramm
ml	Milliliter
mM	Millimolar
µg	Microgramm
µm	Micrometer
MCS	Multiple Cloning Site
MG	Molecular weight
$MgCl_2$	Magnesium chloride
min	Minute
MnCl2	Manganese chloride
MSP 1	Merozoite Surface Protein 1
N	Asparagine
N_2	Nitrogen
Na	Sodium
NaAc	Sodium acetate
NaCl	Sodium chloride
Na_2HPO4	Di- Sodium hydrogen phosphate
NaH_2PO4	Sodium dihydrogen phosphate
NaH_2CO_3	Sodium dihydrogen carbonate
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
ng	Nanogram
nm	Nanometer
O_2	Oxygen
OD	Optical density
O/N	Overnight
ori	Start point of DNA- Replication
*Pb*DT	*Plasmodium berghei* dhfr-thymidylate

	synthase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
pH	pH - Word (Latin: *potentia hydroxii*)
PlasmoDB	*Plasmodium*- Data bank
Pro	Proline
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	Rotation per Minute
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
S	Serine
SDS	Sodium dodecyl Sulfate
SDS-PAGE SDS	Polyacrylamide gel electrophoresis
sec	Second
Ser	Serine
SP	Signalpeptide
SPM	Subpellicular Microtubuli
SPN	Subpellicular Network
ssp.	Latin: *Subspecies*
T	Thymine
Tg	*Toxoplasma gondii*
Tab.	Table
TAE	Tris- Acetate
Taq	*Thermus aquaticus*
TE	Tris- EDTA Puffer
TEMED	N,N,N,N´- Tetramethylethylendiamine
Temp.	Temperature
TMD	Trans membrane domain
Tris	Trishydroxymethylaminomethane
Trp	Tryptophan

U	Enzymatic Unit
UTR	Untranslated region
UV	Ultraviolet
V	Volt
(v/v)	Volume per volume
Vol.	Volume
W	Tryptophan amino acid
(w/v)	Weight per volume
WHO	World Health Organisation
XSTREAM	Variable Sequence tandem repeats extraction and architecture modelling

2. Introduction

2. 1 Malaria – a threat to public health

The deadly disease malaria is caused by the protozoan parasite *Plasmodium sp*. transmitted from one human host to another by *Anopheles* mosquitoes during its blood meal. Each year about 200 million-malaria cases and 660,000 deaths are reported, mostly of the children under the age of five (WHO 2012 report) (Fig. 2. 1).

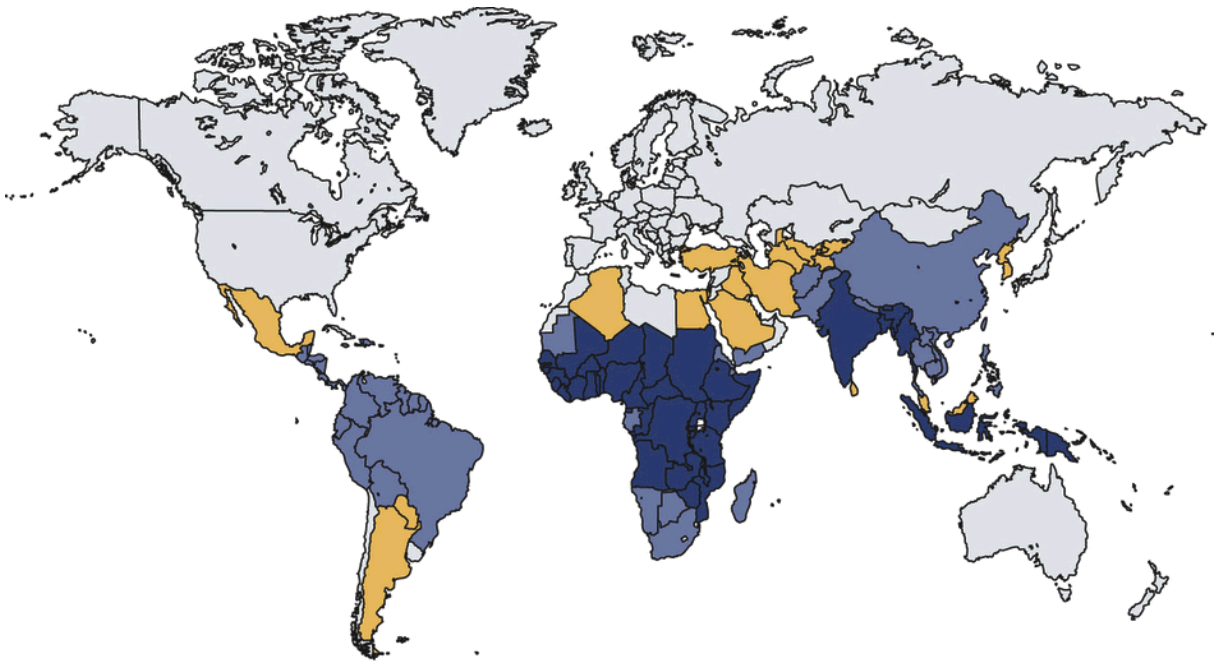


Fig 2. 1: Malaria endemic areas across the globe: The tropical and sub-tropical regions marked in light and dark purple show the areas worst affected by the malaria parasite (Courtsey: WHO 2010).

Plasmodium falciparum causes the maximum mortality and morbidity of the five known species of the *Plasmodium* family, *P.vivax*, *P.ovale, P. malariae, P. knowlesi* and *P. falciparum* that infect humans. Most of the cases of the malaria infection generate mild symptoms like that of influenza infection e.g. headache and body ache. The clinical symptoms in cases of children with severe form of malaria include cerebral malaria, severe anaemia, severe respiratory distress, renal failure, hypoglycaemia and pulmonary oedema.

2. 2 Life Cycle of the Malaria Parasite

The malaria parasite *Plasmodium* has a complex life cycle, which includes a sexual and an asexual phase. The infection in humans starts with the bite of a female *Anopheles* mosquito (Fig. 2. 2). Along with the insects saliva the sporozoites of the parasite are injected into the subcutaneous tissues of the human. These injected sporozoites migrate into the blood vessels in order to get transported to the liver with the flow of the blood (Amino *et al.,* 2006). The sporozoites transmigrate through several hepatocytes and Kupffer cells to infect a single hepatocyte and resides within a parasitophorous vacuole (PV) (Mota *et al.,* 2002). The parasite then differentiates into merozoites from the hepatic schizonts. These merozoites travel to the liver sinusoids in vesicles after being ejected from the infected hepatocytes, termed merosomes (Sturm *et al.,* 2006). A poorly understood phenomenon is involved in the release of the parasites from the merosomes, however once released into the blood stream, the merozoites invade the red blood cells (Sturm and Heussler 2007). The *P.falciparum* parasite multiplies asexually through schizogony within 48 hours. Other parasites like *P.berghei* need 72 hours for one cycle of multiplication. At the end of schizogony, the infected erythrocyte bursts open, releasing up to 32 merozoites that can readily infect novel erythrocytes. It is this repeated cycles of release of parasites into the blood stream that causes the acute symptoms of a malaria infection.

Some of the merozoites take a different course upon invasion into the erythrocyte and differentiate into presexual forms called gametocytes. These presexual forms are the transmissive stages of the parasite life cycle. Approximately in 7-10 days time after the commencement of the erythrocytic stage, some of the parasites differentiate into the mature sexual forms, which is much longer than the time needed for asexual development (Garnham *et al.,* 1966; Gautret *et al.,* 1999, Keuhn *et al.,* 2009).

After successfully gaining access into the female mosquitoes body during its blood meal from the infected human, the parasite develops into female macrogametocytes and male microgametocytes. In the stomach of the insect the male and female forms of the parasite gametes fuse to give rise to the motile diploid zygote called the ookinete. The ookinete then penetrates the mid-gut wall and differentiates into the oocyst. After maturation the oocyst

forms a large number of sporozoites that migrate into the salivary glands (Frischknecht *et al.,* 2004), ready to be injected into a human host during a blood meal.

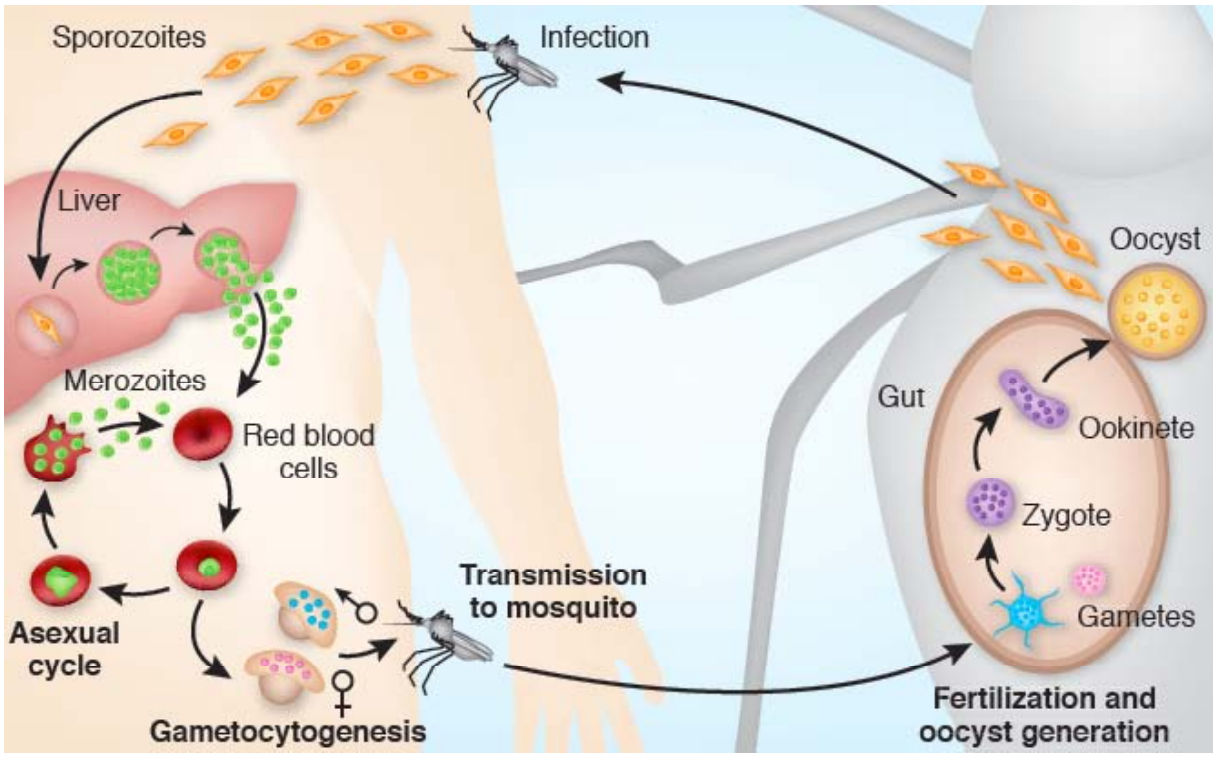


Figure 2. 2: The life cycle of malaria parasite: *Plasmodium falciparum*: The complex life cycle of the malaria parasite comprises two hosts. The primary host is humans where it completes its asexual stages and differenriates into sexual forms prior to the retransmission into other humans via the secondary host, a female *Anopheles* mosquito during the blood meal. In the mosquito the sexual forms differentiate further to reach the salivary glands (Pasvol *et al.,* 2010).

2. 2. 1 Blood stages of the malaria parasite

The obligate intracellular parasite, *Plasmodium sp.* needs to invade the erythrocytes soon after the completion of the hepatocytic stage or after being released from infected erythrocytes. The invasive stages of the *Plasmodium* parasites namely the merozoites, sporozoites and ookinetes bear a highly specialized set of organelles at their anterior end, called the apical tip. In merozoites, the apical tip carries a pair of membrane bound rhoptries, several micronemes and a large number of vesicular dense granules, positioned apically (Counihan *et al.,* 2013). The apical tip with the help of these secretory organelles plays a crucial role in merozoites egress and their reinvasion into the erythrocytes (Preiser *et al.,* 2000).

2. 2. 2 Invasion of the erythrocyte

A single cycle of asexual intracellular development and proliferation takes 48 hours for the *Plasmodium falciparum* to complete. Nascent merozoites are released into the blood stream as the parasites burst open the infected erythrocyte to invade the fresh ones. This invasion of the parasites into the erythrocytes is rather a rapid and complex event involving an extremely co-ordinated cascade of interactions between the merozoite and the RBC (Cowman and Crabb, 2006).

The merozoite attaches to the erythrocyte surface, which is brought about by the Merozoite Surface Proteins (MSPs) covering the entire outer membrane of the merozoite in the form of a dense coat (Gaur *et al.,* 2004). Then it re-orientates in order to make the apical tip form an initial junction with the host cell, which later modifies to a tight-junction. This tight-junction (TJ) eventually transforms into a moving-junction (MJ) and engulfs the invading merozoite as it drives its way into the host cell (Riglar *et al.,* 2011) (Fig. 2. 3).

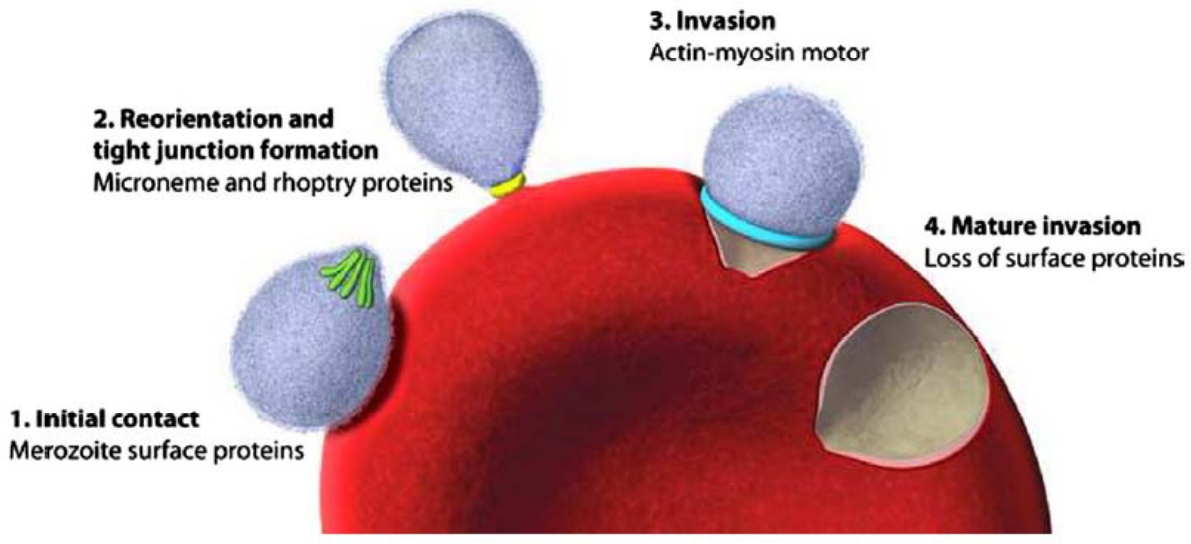


Fig 2. 3: The events involved in the invasion of the erythrocyte: The entire process can be divided into 4 major events namely (i) initial contact (apical organelles like rhoptries marked with green), (ii) re-orientation and formation of Tight-junction (TJ- marked in yellow), (iii) formation of Moving-junction (MJ-marked in blue)-invasion and (iv) complete invasion (formation of parasitophorous vacuole (PV) (Boyle *et al.,* 2012).

This set of entire events is driven by the parasite's translocation machinery, which involves transmembrane proteins forming a part of the actinomyosin motor system (Baum *et al.,* 2008). The actinomyosin system is seated between the parasite's plasma membrane and the outer layer of the inner membrane complex (IMC_O). The myosin is coupled to the IMC where as the actin filament is connected via an array of transmembrane adhesins (Erythrocyte binding like family-EBL and Thrombospondin-related anonymous protein - TRAP) to a junction (tight and moving junctions) formed by the parasite's plasma membrane (PPM) and the host cell-surface membrane (Baker *et al.,* 2006). The immobile myosin impels the filamentous actin towards the rear end of the parasite as a result of which the adhesins get pulled through. Since the adhesins are connected to receptors on the host cell surface, the net outcome is a forward motion, which in turn enables the parasite to drill into the host cell (Baum *et al*., 2008). The motor complex includes myosin A (type XIV myosin), myosin tail-domain interacting protein (MTIP) and Glideosome Associated Proteins (GAPs) like GAP45 and GAP50 (Farrow *et al.,* 2011) (Fig. 2.4). This highly evolved actinomyosin motor complex not only helps in the invasion of the erythrocytes but also in gliding motility, transmigration and egress as well.

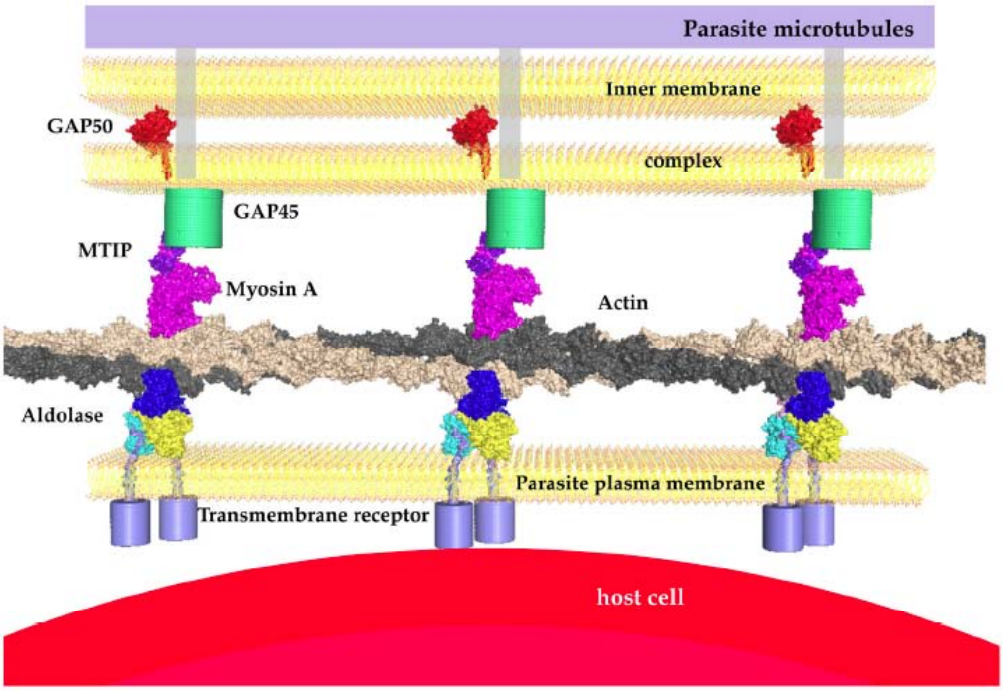


Fig 2. 4: The Actino-myosin motor complex anchored in the IMC: Motor complexes comprising the GAPs, MyoA, MTIP and GAPMs that power the gliding motility and invasion process are tethered into the IMC (Bosch *et al.,* 2012).

2. 2. 3 Presexual forms of the parasite

Certain parasites in the blood stage are predestined to differentiate into the sexual forms. The early stages (stages I and II a) of the presexual forms remain indistinguishable from the late trophozoite stage (asexual form), differing just in the size occupied within the infected erythrocyte and have single nucleus with pigment granules dispersed in the cytoplasm (Sinden *et al.,* 1998; Duval *et al.,* 2009). The mid staged gametocytes (II a, b) remain more or less spherical and are single-nucleated. The stage III gametocytes display a D-shaped form as the parasite elongates and the length to width ratio becomes roughly 2:1. By stage IV, the gametocytes elongate further and the length to width ratio changes to 4:1 and bear pointed tapering ends. The host cell merely becomes a thin coat around the parasite body. Stage V marks the change in pointed ends of the gametocytes to more rounded structure and the length to width ratio declines to 3:1 and the total volume of the parasite is about 50% of the uninfected erythrocyte (Hanssen *et al.,* 2012; Aingaran *et al.,* 2012) (Fig. 2. 5). The drastic change in the parasite morphology during the gametocytogenesis is driven by the modifications in the inner membrane complex and the microtubules lying underneath (Sinden *et al.,* 1982, 1983).

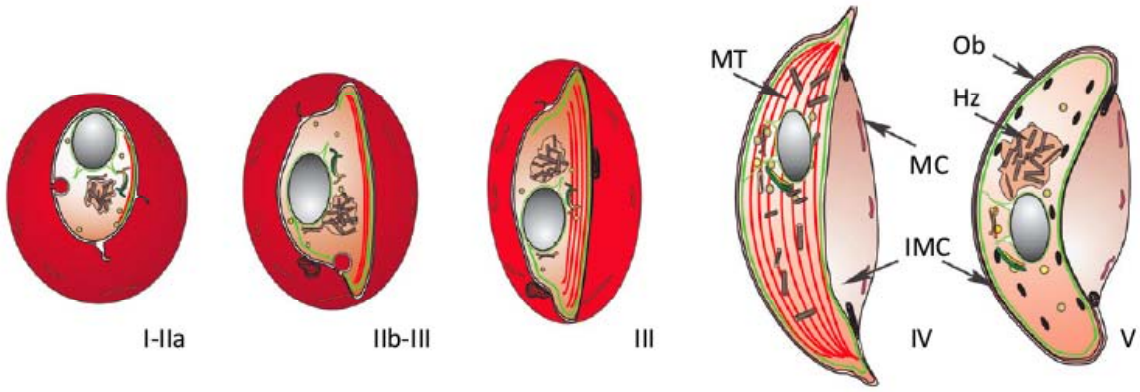


Fig 2. 5: Successive stages in gametocyte development of the malaria parasite: The parasite undergoes profound morphological changes during the gametocytogenesis and on complete maturation they are ready to be picked up by the mosquito during its blood meal (Dixon *et al.,* 2012).

2. 3 A unique membranous structure- the Inner Membrane Complex (IMC)

The malaria parasite *Plasmodium spp*. belongs to a phylogenetic group of organisms that is termed *'Alveolata'* (Cavalier-Smith 1993) (Fig. 2. 6). The Alveolates comprises three phyla: *Ciliates, Dinoflagellates and Apicomplexa* (Fig. 2. 7). While the ciliates and dinoflagellates are found exclusively in the aquatic environment the apicomplexans have a parasitic mode of survival. *Plasmodium,* as well as *Toxoplasma* and *Babesia* belong to the phylum *Apicomplexa*. These obligate intracellular parasites cause malaria, toxoplasmosis and babesiosis respectively in their vertebrate hosts. *Plasmodium* as the cause for malaria is the most dangerous and prominent one. Perhaps the only connecting feature of the members of this super phylum is the presence of the flattened membranous sacs and the recent advances achieved in the data from sequences of 18 rRNA that point to their common descent (Cavalier-Smith *et al.,* 1993).

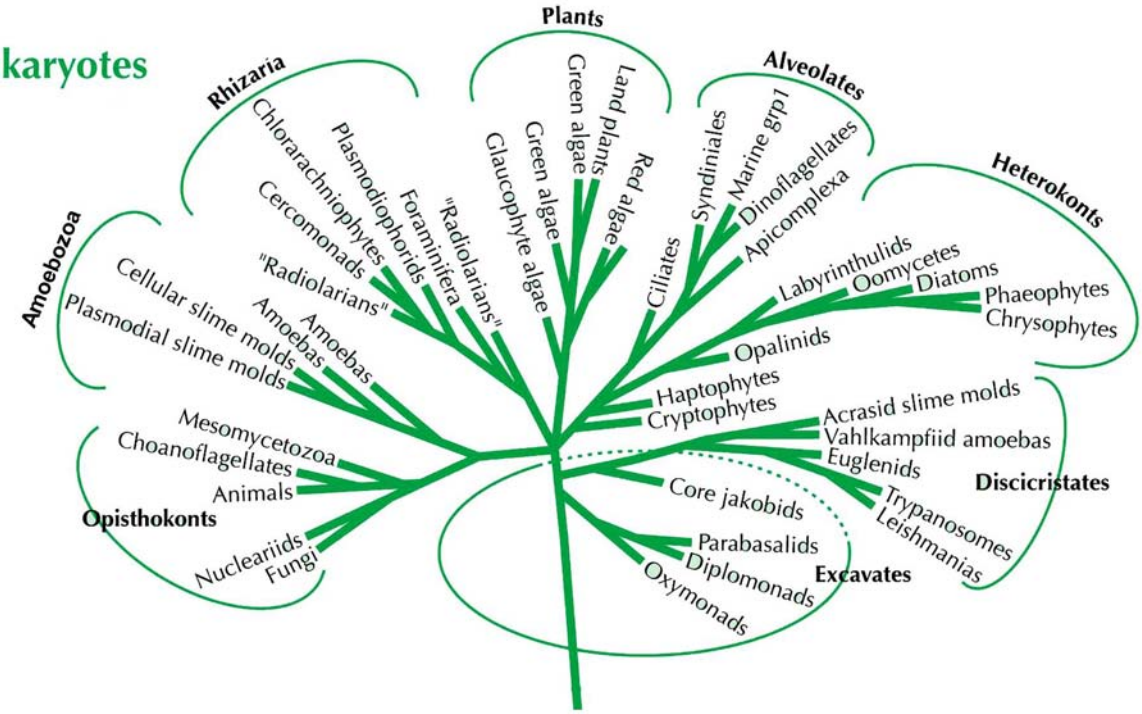


Figure 2. 6: The tree of life: The apicomplexans, dinoflagellates and ciliates which comprise the *Alveolata* super group share common ancestry, a glimpse given by the tree of life (Courtesy: Department of Biology, UNC Charlotte).

These flattened membranous sacs found exclusively underneath the plasma membrane are called 'Alveoli' in ciliates, 'Amphiesmal vesicles' in dinoflagellates and 'Inner Membrane

Complex (IMC) in the apicomplexans (Morrill *et al.,* 1983; Hausmann *et al.,* 2010; Hausmann *et al.,* 2010).

In ciliates, which include species like *Paramecium* and *Tetrahymena,* the alveolar vesicles or sacs are connected to plasma membrane via short stubble-like entities towards the exterior side. A network of filamentous proteins known as the epiplasm underlines the cytoplasmic side of these alveolar vesicles (Hausmann *et al.,* 1979). There are modifications in the alveoli suited for the needs of the individual species of the *Alveolata* cluster. These alveolar vesicles or sacs run longitudinally in *Tetrahymena sp*. and in *Paramecium* the alveoli are in shape of tiny pillow. The alveoli in *Paramecium* serve the purpose of storing calcium, act as scaffold during cytokinesis and support the ejectile structures and ciliary motion for cell for mobility (Stelly *et al.,* 1991; Plattner *et al.,* 2001). On the other hand the amphiesmal vesicles in *Dinoflagellates* are usually made up of polymers of glucose linked by glycosidic bonds, that give rise to plate-like structures (Nevo *et al.,* 1969; Dodge *et al.,* 1969). These plate shaped vesicles support the maintenance of the cell shape and architecture (Hausmann *et al.,* 2010).

The inner membrane complex (IMC) of the apicomplexans, which has been more extensively studied in the *Toxoplasma sp.* suggests that in the 'zoites' (motile stages) of the coccidians there is a single IMC vesicle in the shape of a cone towards the apical tip seated above numerous IMC cisternae (Russell *et al.,* 1984; Morrissette *et al.,* 1997). The IMC plates are interwoven with one another forming a single rigid structure. The IMC in *Plasmodium spp.* has different architecture during the successive stages of its life cycle. The non-motile presexual and sexual gametocytes have several IMC vesicles, which are equivalent to the ones in *Toxoplasma.* The patchwork of the IMC plates is riveted together by connecting sutures. The motile stages of *Plasmodium* merozoite, ookinete, the motile zygote and the sporozoites have only a single vesicle even though the cell length varies considerably (Meszoely *et al.,* 1987; Dearnley *et al.,* 2012; Kono *et al.,* 2012; Bannister *et al.,* 1995; Hu *et al.,* 2010; Meszoely *et al.,* 1982) (Fig. 2. 8).

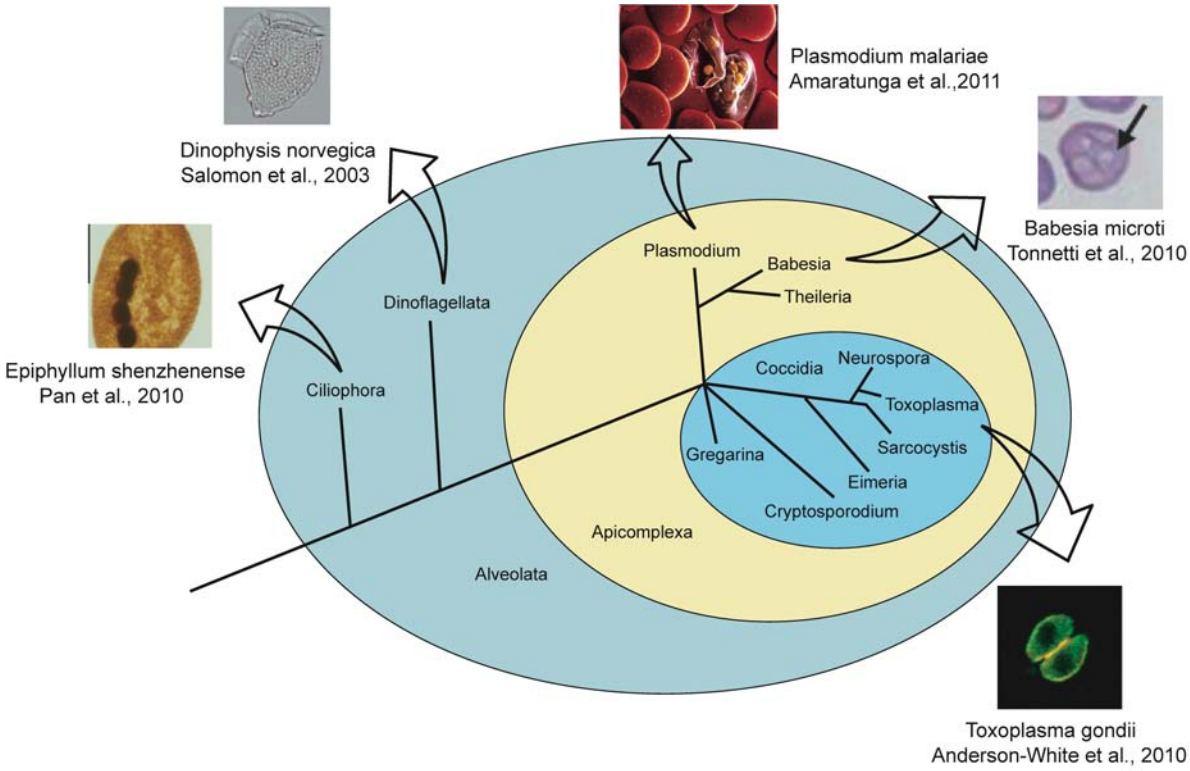


Figure 2. 7: The Alveolates: The varied species of organisms comprising this super group include the ciliates e.g.: *Paramecium spp.,* Dinoflagellates e.g.: *Dinophysis sp*., and the apicomplexans which have medicinal importance. The organisms categorized under this group are *Toxoplasma sp*., *Babesia sp.* and the *Plasmodium sp.* organisms.

The cell-shape determining 'pellicle' of the apicomplexans comprises the plasma membrane, the inner membrane complex (IMC), subpellicular microtubules (SM) and the subpellicular network (SPN) (Mann *et al*., 2001). Freeze fracture studies of the zoites show that inter membranous particles (IMP) act as connections between IMC and the microtubule associated proteins (MAPs) of SM (Morissette *et al.,* 1997). The SPN forms a membrane basket that encloses the entire parasite. Studies performed on the subpellicular network (SPN) of pellicle from *Toxoplasma sp.* show that it is highly agile and is very sensitive to detergents during the formation of the daughter cells as the developing cells still undergo changes in the shape and length. This sensitivity towards the detergent is however lost when the daughter cells have attained complete maturity. This is indicative of the fact that IMC and the SPN play a crucial role in the determination of the cellular integrity and shape opposed to the previous notion that the tubulin-based cytoskeletal SM only does the same (Mann *et al.,* 2002; Lemgruber *et al.,* 2009). But in case of merozoites or the gametocytes of the malaria parasite, *Plasmodium* ultra structure studies do not indicate the presence of the SPN. The recent discoveries however show that the merozoites carry 2-3 SMs referred to as f-MAST, seated exactly underneath the IMC (Fowler *et al.,* 1998). Whereas the gametocyte

of the parasite has IMC cisternae ranging from 10-15 in number and underlying them there is set of SMs (Meszoely *et al.,* 1987). Connecting sutures that are transversal in nature hold the IMC vesicles together (Meszoely *et al.,* 1987; Kono *et al.,* 2012).

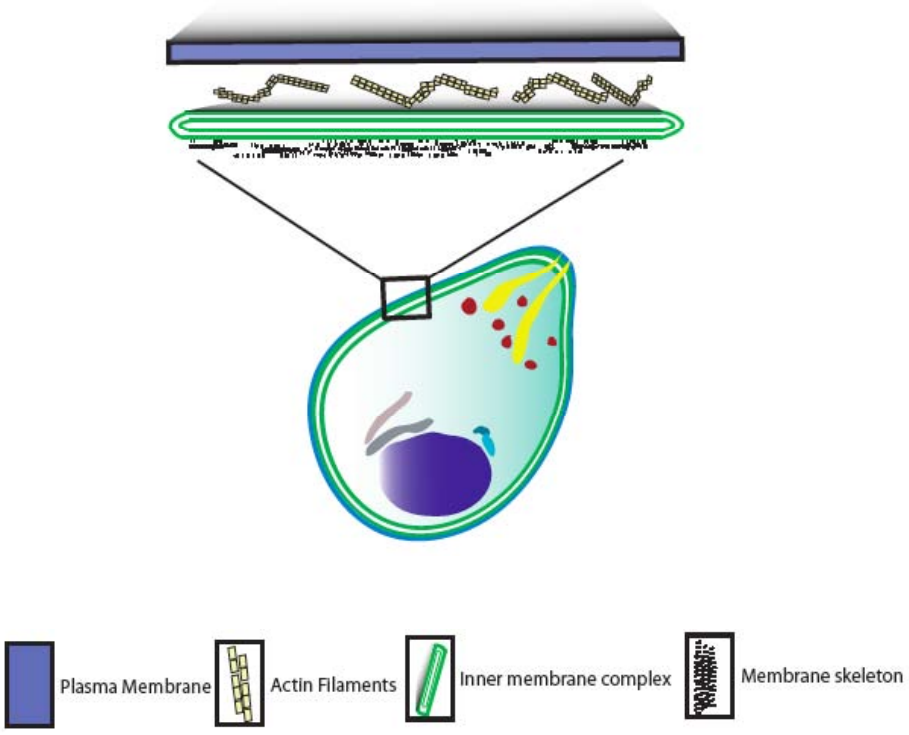


Figure 2. 8: The inner membrane complex in merozoite of *Plasmodium sp*.: The bi-layered IMC marked in green, lying underneath the plasma membrane (purple), completely surrounds the nascent merozoite.

2. 3. 1 Structural and phylogenetic diverse proteins characterize the IMC in *Apicomplexans*

The varied role of the IMC in *Alveolata* superfamily possibly makes its composition heterogeneous. A glimpse of which we get to see in the ever-growing number of the diverse IMC proteins present in the organisms of different genera constituting the cluster, *Alveolata*. During the early stages of evolution of the phylum *Alveolata* certain proteins of eukaryotic origin from progenitor species were recruited to the then developing IMC (Kono *et al.,* 2012). Along with these comparatively older eukaryotic IMC targeted proteins, certain highly alveolata-specific proteins evolved which conferred the ability to the members of this super phyla to adapt in diverse niches (Gould *et al.,* 2008). To date only 17 IMC proteins in

Plasmodium falciparum have been characterized. With the knowledge that about 40% of the genes in *Plasmodium* are genus specific and remain largely uncharacterized, the probability for large pool of proteins to be identified and annotated as forming a part of the IMC remain open (Wasmuth *et al.,* 2009).

IMC proteins can be grouped according to their structural features into multi-transmembrane proteins, alveolins and non-alveolins. An example for multi-transmembrane proteins is the glideosome-associated protein with multiple-membrane spans (GAPMs), which have 6-transmembrane domains. These transmembrane proteins weave in and out through the bilayer of the IMC from both the sides and are possibly implicated in keeping the IMC vesicles flattened (Sanders *et al.,* 2007; Bullen *et al.,* 2009). The GAPMs and PF14_0578 show a completely different distribution from the alveolins during their biogenesis in the erythrocytic stage of the parasite development (Kono *et al.,* 2012). GAP50, which is embedded in the IMC, is in close interaction with the complex formed by GAP45, MyoA and MTIP. The entire conglomerate of these proteins is in close conjuction with the transmembrane GAPMs (Johnson *et al.,* 2007; Frenal *et al.,* 2010; Sanders *et al.,* 2007). The best characterized IMC protein groups are the non-alveolins, more precisely those non-alveolins that are building up the glideosme (GAP45, GAP50) and act as markers for the IMC biogenesis and compartmentalisation as they are associated with the membrane all through schizogony (Kono *et al.,* 2013). Additionally the GAPs show distribution restricted to specific compartments within the IMC, a phenomenon first reported in matured tachyzoites of *Toxoplasma sp.* in case of IMC Subcompartment Proteins (ISPs) (Beck *et al.,* 2010). Based on this pattern of the localization, the IMC is known to have apical, central and basal sub-compartments in the nascent merozoites of *Plasmodium spp*. (Yeoman *et al.,* 2011).

The first group of alveolins was initially identified and characterized in *Toxoplasma sp.* and reported to form an integral part of the SPN (*Tg*IMC1 and *Tg*IMC2) (Mann *et al.,* 2001). The alveolins possess characteristic repetitive sequence motifs that and were established as the first molecular nexus uniting the members of *Alveolata* (Fig. 2. 9) (Gould *et al.,* 2008, 2011). The core repeats of the alveolins mainly comprise valine and proline rich sub domains: EKIIEVPQ, EKIIEVPK, EKIVEVPH, DKIVEVPQ, EKLIHIPK, ERIKKCSK, ERIIPVPK, EKIVEIPQ, EKVQEIPE and EKIVDRNV. These repeatitive amino acid stretches are the key determinants for the proteins to get targeted to the IMC (Gould *et al.,*

2010; Fung *et al.,* 2012). Studies of alveolins in *Plasmodium berghei* show that they are of absolute necessity for normal motility of the zoites and cell shape maintenance through out the life cycle of the parasite (Khater *et al.,* 2004; Tremp *et al.,* 2011; Volkmann *et al.,* 2012).

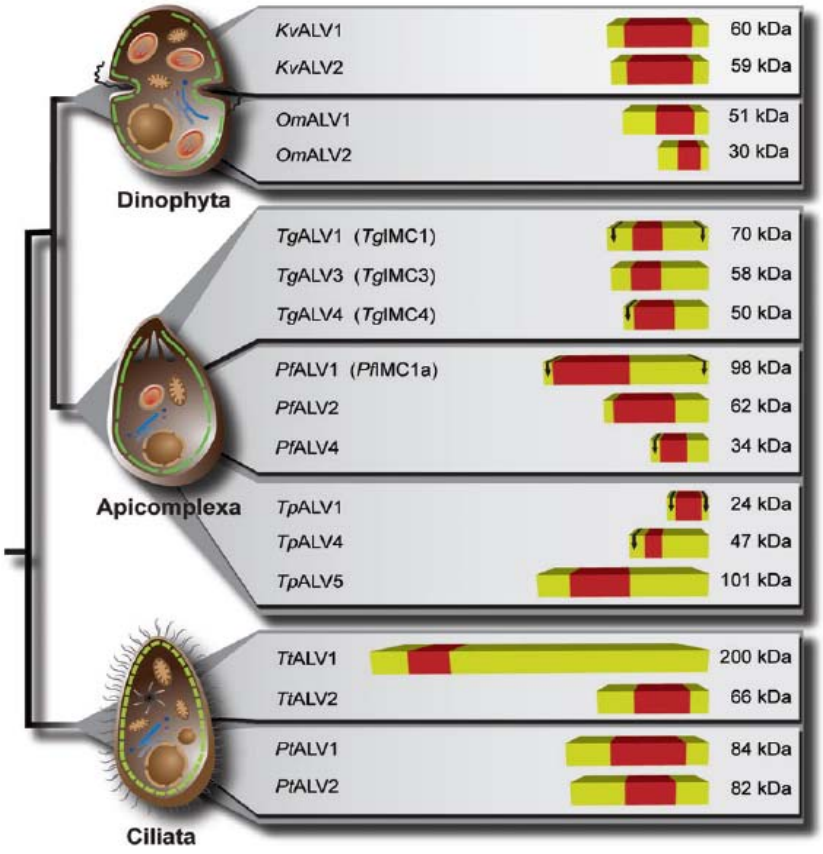


Figure 2. 9: Highly conserved alveolin domains: The alveolin repeats marked with red boxes in the alveolin proteins remain conserved all across the members of the Alveolata family- Ciliates, Dinoflagellates and Apicomplexans (Gould *et al.,* 2008).

Certain IMC proteins can neither be categorized as alveolins nor they form a part of the glideosome machinery. One such IMC protein is the Membrane occupation and nexus protein 1 (MORN1), which acts as bridge between the IMC and the cytoskeleton (Gubbels *et al.,* 2006; Lorestani *et al.,* 2010). The ISPs (*Toxoplasma sp.*) fall under this category of IMC proteins which bear a high content of charged amino acid residues and N-terminal myristoylation and palmitoylation motifs (Beck *et al.,* 2010; Fung *et al.,* 2012).

The emerging genomic and proteomic data from various species within the Alveolata not only exemplifies the complex and adaptive molecular composition of the pellicle and the IMC but also supports its monophylogenetic origin (Gould *et al.,* 2011).

2. 3. 2 IMC – biogenesis and development during endodyogeny and schizogony

During the cell division, the *Toxoplasma sp.* exhibit unique phenomenon of formation of daughter cell within the mother cell, by internal budding in a highly synchronous and symmetrical manner and is referred to as 'endodyogeny' (Sheffield and Melton, 1968; Gubbels *et al*., 2006; Nishi *et al*., 2008). Shortly after karyokinesis, the nascent inner membrane complex along with the conoid (spirally arranged apical microtubules) and subpellicular microtubules form the scaffold for the cytokinesis of the developing daughter cells (Nichols and Chiappino, 1987; Tilney and Tilney, 1996; Morrissette *et al.,* 1997; Hu *et al*., 2002; Nishi *et al*., 2008). The biogenesis of IMC during the cell division remains obscure but initial studies show that nascent IMC is supposedly derived from the golgi apparatus (Bannister *et al.,* 2000). The plasma membrane, which is inherited from the mother cell, then forms a sheath around the fully matured buds.

The biogenesis of IMC in *Plasmodium sp*. during the erythrocytic stage starts with the onset of schizogony. Based on the spatial distribution, the IMC member proteins can be broadly classified into two groups (Kono *et al.,* 2012). The group-A proteins (includes transmembrane proteins, glideosome associated proteins e.g. GAP45 & GAP50 and some specific non-alveolins) display a cramp-like structure, embedding the two centrosomes during the early schizogony with ratio of 2:1 with the nuclei. The cramps merge to form rings with a diameter of about 600 ± 100 nm. Towards the end of the schizogony the rings expand and extend towards the posterior end of the nascent merozoites and encompass them (Kono *et al.,* 2012) (Fig. 2. 10).

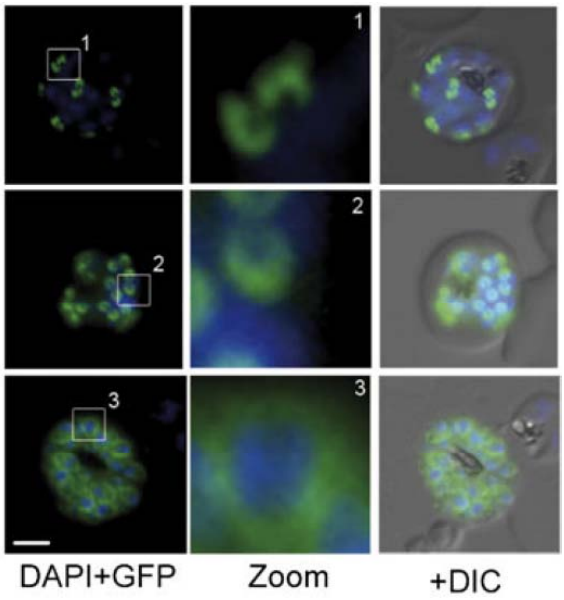


Figure 2. 10: Group-A IMC proteins spatial distribution during schizogony: The transmembrane IMC proteins and non-alveolins display cramp-like structures with the onset of their biogenesis (1) and the cramps fuse to form rings (2) and eventually encompass the nascent merozoites formed (3) (Kono *et al.,* 2012).

The other group of IMC member proteins which includes alveolins and the *Plasmodium sp.* specific MAL13P1.228, termed as group-B mark their biogenesis with the formation of thin ring like structures during the mid schizont stages with an avarage diameter of 900 ± 100 nm (T1). These rings further expand with the maturation of the schizonts (T2) and gradually enlarge and expand towards the posterior end of merozoites in formation and eventually encapsulate the nascent merozoites (Kono *et al.,* 2012) (Fig. 2. 11).

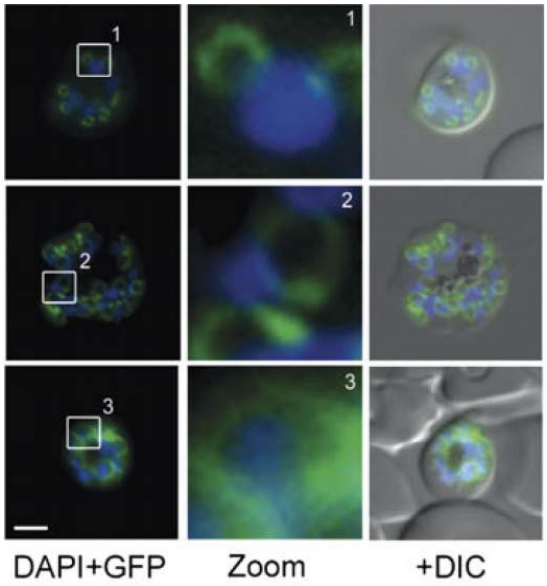


Figure 2. 11: Group-B IMC proteins display a different dynamics during schizogony: The alveolins and MAL13P1.228 display large ring-like structures with the onset of their biogenesis (1) and the rings expand in diameter with the ongoing cytokinesis (2) and with the complete maturation of the schizonts they display the same subpellicular distribution as the group-A IMC candidates do (3) (Kono *et al.,* 2012).

Prior to the cytokinesis, the *Plasmodium* parasites go through several rounds of division and a multinucleated syncytium containing about 30 nuclei is formed. The pre-merozoite forms lie towards the periphery of the cell and are in close conjuction with the plasmalemma. The penultimate step before the fully mature nascent merozoites burst open the erythrocyte is the formation of the plasma membrane sheath around them. The plasma membrane folds towards the inner side of the apical areas and gradually surrounds the nascent merozoites but the precise mechanism of this event needs further investigation.

2. 3. 3 Organization and composition of IMC of the gametocytes

The mature non-motile presexual forms of the *Plasmodium sp.* are much distinct from the asexual forms. The mature stages of the gametocytes have much elongated cell shape. This elongation takes place along with the establishment of the trilaminar membrane system in the parasite. This trilaminar system comprises parasitophorous vacuole membrane (PVM), parasite plasma membrane (PPM) and the IMC, originally referred to as subpellicular membrane complex, which is associated with subpellicular microtubules (Olivieri *et al.,* 2009). The protein composition and the ultra structure of this subpellicular membrane complex later established that it is analogous to the inner membrane complex (IMC) as it

bears some key components of IMC from asexual stages, GAPs (Dearnley *et al.,* 2011). The inner membrane complex during the presexual and sexual stages serves to maintain the structural integrity of the parasite cell. Depending on the stage of the life cycle of the parasite the architecture and composition of the IMC seems to vary. While the IMC of the merozoite seems to have one single vesicle, the presexual and ookinetes possess patchwork of IMC plates of approximately 400 nm in width and connecting bands of 100 nm in width (Fig. 2. 12) (Dearnley *et al.,* 2011).

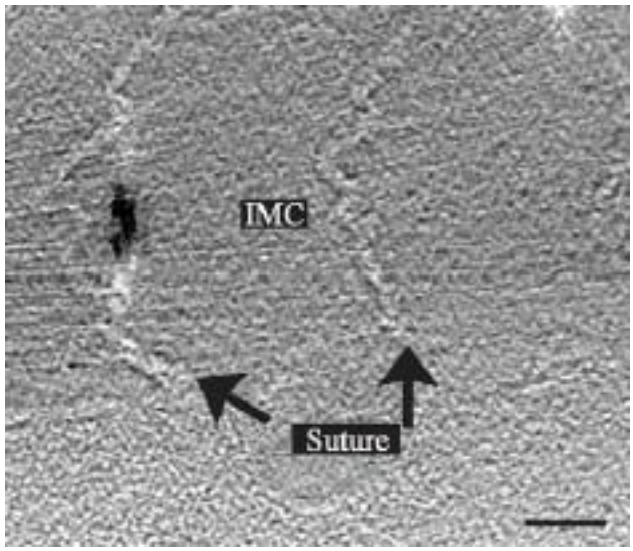


Figure 2. 12: Presence of multiple IMC vesicles during sexual stages: Cryo-electron microscopy of the gametocytes from *Plasmodium sp*. show the IMC plates that sealed end to end by sutures formed by some specific IMC proteins (Dearnley *et al.,* 2011).

The establishment of cytoskeleton of microtubules underneath the IMC drives the elongation of the gametocytes. Electron microscopic studies reveal the close association of the IMC and microtubules, which are found at regular intervals of 10 nm (Sinden *et al.,* 1979; Bannister *et al.,* 2000).

The transmembrane IMC proteins e.g. PFD1110w, qualify as markers of IMC in every stage. During the early stages (I and II) of the gametocytogenesis, in the nascent IMC being formed, PFD1110w shows spine-like pattern with transversal polarity to one side of the parasite cell. In the later stages (III, IV and V) the distribution is uniform and reaches out to the inner face of the parasite. The gametocytes expressing *Plasmodium* specific MAL13P1.228-GFP display a completely distinct pattern of distribution, which arises from

same structure, marked by PFD1110w but forms discreet lines traversing across the cell. These lines expand with the maturation of the gametocytes and encapsulate the parasite in a highly uniform meshwork. These studies are suggestive of the fact that MAL13P1.228 forms a component of sutures that seal the IMC vesicles (Fig. 2. 13) (Kono *et al.,* 2012).

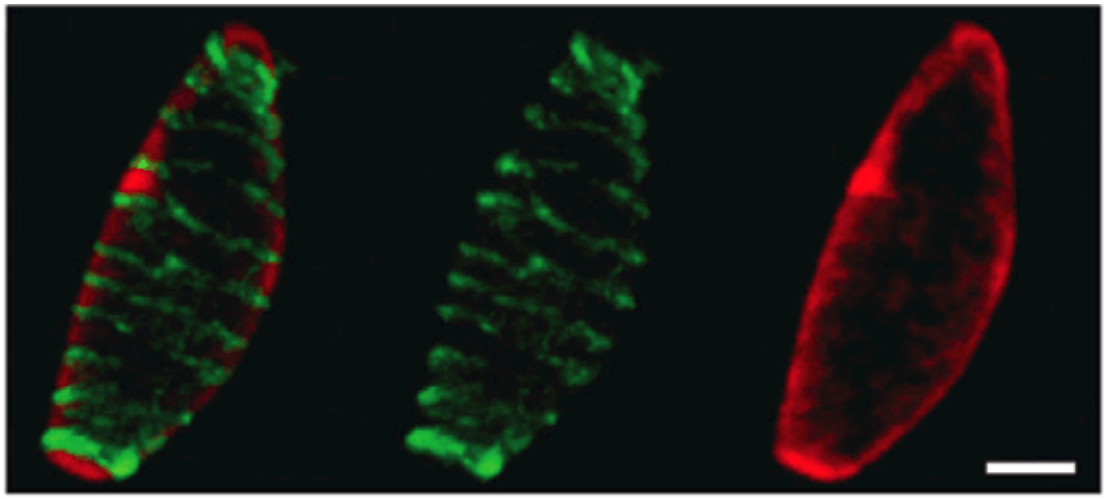


Figure 2. 13: Co localization of PFD1110w (red) and MAL13P1.228 (green) in stage IV gametocytes: Transmembrane protein, PFD1110w and MAL13P1.228, restricted to only *Plasmodium sp.,* mark the IMC distinctly (Dixon *et al.,* 2012).

2. 3. 4 The IMC in Ookinetes and Sporozoites

The IMC found in the ookinetes, the motile sexual forms in the midgut of the female mosquito and in the sporozoites, which are injected into the human host with the bite of the mosquito during the blood meal, exists as a single vesicle (Meszoely *et al.,* 1982; Raibaud *et al.,* 2001).

A dense layer analogous to the SPN in *Toxoplasma sp*. has been reported in the sporozoites of the *Plasmodium berghei* as disclosed by cryo-electron tomography studies (Kudryashev *et al.,* 2010). Thorough investigations revealed that 'Linker molecules', analogous to *Toxoplasma* IMP, about 27 nm in length connect the IMC and the SM. These linkers are found at regular periodicities of 32 nm on the cytoplasmic side of the IMC and are believed to provide the much needed stability and agility to the parasite cell. Localization studies performed in ookinetes (Fig. 2. 14 A) and sporozoites (Fig. 2. 14 B) using the homologous of IMC protein from *P.falciparum* in *P.berghei* (PB000207.03.0) showed its distribution in

the periphery of the parasite, possibly in the pellicle (Kono *et al.,* 2012). The investigations pertaining to the IMC clearly indicate its presence throughout the life cycle of the parasite with immense changes in its architecture and function, which is brought about by the varying composition of the constituent proteins in different stages.

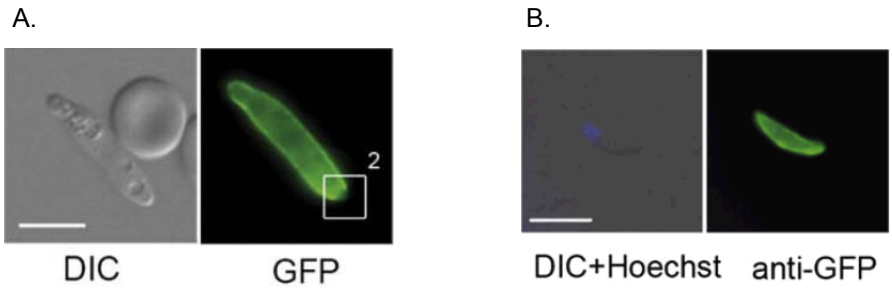


Figure 2. 14: Localization of the IMC proteins in ookinete and sporozoites of *P.berghei:* The peripheral distribution of the tagged IMC protein is suggestive of the association of the same with the pellicle and the presence of IMC in both the stages (Kono *et al.,* 2012).

2. 4 Membrane Proteins and their association with the membrane

Though all membrane proteins are associated with the membrane, their structure, function and mode of attachment with the membrane vary immensely. The group or subset of proteins associated with the membrane differs depending upon the cell-type and the sub-cellular location. On the basis of membrane-protein interaction and their localization with the membrane, the proteins can be grouped into two broad categories- integral (intrinsic) and peripheral (extrinsic).

The integral membrane proteins have certain segments embedded in the phospholipid bilayer of the membrane. These segments have amino acid stretches that have hydrophobic side chains, which in turn interact with the fatty acyl group of the membrane phospholipids. The transmembrane proteins best exemplify this class of proteins, which have membrane-spanning domains made up of α helices or multiple β strands. However, another class of integral membrane proteins are acylated and the lipid moiety gets incorporated in the lipid bilayer of the membrane, with the polypeptide chain dangling outside towards the cytoplasmic side. On the other hand the extrinsic or peripheral membrane proteins adhere temporarily to the biological membrane, either to the lipid bilayer or integral proteins. However the peripheral membrane proteins are transiently attached to the membrane and

these proteins can be disassociated from the membrane using elevated pH and high salt concentrations (Singer and Nicolson, 1972). These proteins are located in the peripheral regions of the lipid bilayer of the membrane and attach to it with the help of hydrophobic, electrostatic and other non-covalent interactions or could be a combination of them all.

2. 4. 1 Covalently attached hydrocarbons anchor some proteins to the IMC membrane

Some peripheral membrane proteins attach to the cytoplasmic side of the membrane by a hydrocarbon moiety commonly prenyl and farnesyl lipid-groups that are covalently fused to the cysteine residues. The anchor moiety forms a thioester bond with the thiol group of the cysteine residue. The protein then undergoes proteolysis and methylation, which enables it to attach to the membrane. In some cases palmitate, a lipid moiety forms the thioester bond with the cysteine residue of the protein to anchor it to the membrane. In addition to this, another fatty-acyl moiety, myristate forms an amide bond with the glycine residues and in turn anchors the protein to the membrane (Jones *et al.,* 2012).

2. 4. 2 Protein-lipid electrostatic interactions

The electrostatic interactions promote attachment of proteins on to the cytoplasmic leaflet of the membrane. The anionic components of the bilayered membrane attract the polycationic proteins or cationic domains in some proteins. As a result of this the proteins bind to the membrane causing a significant reduction in the net surface charge and a decrease in the electrical double layer and in turns brings stabilization (Denisov *et al.,* 1998). This stabilized domain grows by recruiting more cationic peptides and anionic lipids involving proteins like Phosphatidylinositol (4,5) biphosphate (PIP_2), which help in signal transduction.

2. 4. 3 Proteins binding specific lipids

The initial membrane adsorption of the proteins can be based on the non-specific electrostatic interaction but it might not anchor the protein for a sustained period of time (Murray *et al.,* 2002). Many protein-folding domains recognize and bind to very specific lipid moieties after the initial attachment to the membrane. Different proteins get recruited to the membrane on the basis of highly specific interaction between the protein domains and the lipids in the membrane (Cho *et al.,* 2005). Proteins involved in signal transduction

usually bind very particularly to phosphatidylinositol and its different forms. This interaction is more specific than electrostatic interaction, involving van der Waals interaction, hydrophobic interactions and formation of hydrogen bonds.

2. 4. 4 Non-specific hydrophobic interactions

Amphipathic proteins, which have hydrophobic and hydrophilic amino acid side chains distributed at the opposite ends of the alpha helices or beta-sheet structures, interact with the hydrophobic face of the target membrane by exposing their hydrophobic loop or chain. This interaction is much stronger than the non-specific electrostatic interaction. This hydrophobic interaction results in lowering of free energy of the entire complex thereby providing stability. The best-studied peptide is from the bee venom, melittin, which is lytic peptide and forms 80% alpha helical conformation when it binds to a phospholipids membrane (Sato *el al.,* 2006).

2. 4. 5 Membrane targeting of the IMC proteins

Protein recruitment to the IMC is a complex process and various means are implicated in this process. These IMC proteins undergo many modifications at the co- and post-translational levels. Studies show that for IMC protein GAP50 in *Toxoplasma*, it is an absolute necessity to get glycosylated at the N-terminus for its transport from endoplasmic reticulum to the Golgi apparatus and eventually to the IMC (Gaskins *et al.,* 2004; Fauquenoy *et al.,* 2011). In *Plasmodium falciparum*, the IMC protein PF14_0578 is known to get palmitoylated and myristoylated in order to attach to the membrane (Linder and Deschenes *et al.,* 2007; Cabrera *et al.,* 2012). IMC-specific palmitoyl acyl transferase (PAT) is known to bring about the posttranslational modification in proteins like ISPs and GAP45 in *Toxoplasma* and *Plasmodium* (Beck *et al.,* 2010; Frenal *et al.,* 2010; Fung *et al.,* 2012; Cabrera *et al.,* 2012). Some IMC proteins like GAP45 and MAL13P1.228 undergo phosphorylation, a posttranslational modification but the exact correlation between the addition of the phosphate group to the IMC protein and its attachment to the membrane is not very clear (Nebl *et al.,* 2011; Jones *et al.,* 2009; Ridzuan *et al.,* 2012). The signal-peptide bearing transmembrane spanning protein PFE1130w and multi-transmembrane domain possessing proteins like MAL13P1.130 and PFD1110w, possibly get incorporated into the IMC membrane via vesicle-mediated transport system (Raibaud *et al.,* 2001;

Yeoman *et al.,* 2011; Kono *et al.,* 2012). The trafficking of *Plasmodium* specific MAL13P1.228 to the IMC membrane is not clearly understood but it is believed that due to particular protein-protein interactions the protein gets localized in the IMC (Kono *et al.,* 2012). The alveolin domains are known to play a crucial role in the membrane association for the alveolins, as revealed by studies in *Toxoplasma sp.* (IMC3 and IMC8) wherein despite the deletion of the acylation motifs, the proteins displayed localization pattern like that of the wild-type (Anderson-white *et al.,* 2011; Fung *et al.,* 2012).

2. 5 Gene manipulations in *Plasmodium spp.*

Over the last decade substantial progress has been made in the area of localization studies of different proteins in *Plasmodium* using various different fluorescent tags. However the tools and approaches to effectively target essential genes by using double crossover are limited in this haploid parasite (Crabb *et al.,* 2004). The gene disruption strategies, which use homologous recombination via a single cross over do not delete the gene and the Open Reading Frame (ORF) is just disrupted (Maier *et al.,* 2006). In both the cases the drug-pressure has to be applied for long periods of time to obtain a pure population of the parasites. The RNA interference (RNAi) tool to carry out functional analysis was reported previously but the classical RNAi machinery is not present in the parasite's genome, making the RNAi approach unusable (Ullu *et al.,* 2004).

To overcome these limitations, a handful of regulatable expression systems have been recently developed and reported.

2. 5. 1 Tetracycline repressible transactivator system

Initially reported as a handy tool to effectively regulate the gene expression in *Toxoplasma*, this system is based on transactivator that comprises TetRep, which in turn is fused to nonendogenous activating domain (TATi1) (Meissner *et al.,* 2002). The transcription is activated when the transactivator binds to the tet operator (TetO) with the help of tet repressor (TetRep). In the presence of tetracycline, the TetRep doesn't bind to the TetO and the transcription is turned off (Bujard, 1999; Meissner *et al.,* 2001) (Fig. 2. 15). To mimic the similar phenomenon in *Plasmodium spp.*, transcription activation domains within apicomplexans AP2 (Api AP2) was identified and a conditional expression system was

reported to be successful for some genes of *Plasmodium berghei* (Profilin-involved in parasite invasion and N-myristoyltransferase) (Pino *et al.,* 2012).

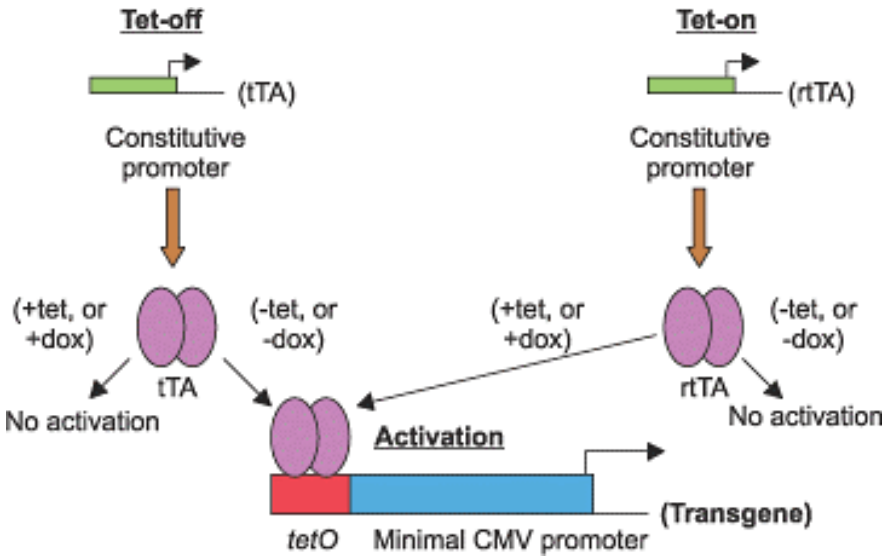


Figure 2. 15: A generalized illustration of the tetracycline repressible transactivator system: The transcription is very effectively turned off in the presence of tetracycline, as the TetRep doesn't bind to the TetO (Romano G, 2004).

The usage of this technique is limited as in many reported cases of studies of genes of malaria parasites, the tetracycline-sensitive transactivator, which is a fusion between TetR and the *Herpes simplex* virus VP16 protein, fails to activate the minimal promoters (Meissner *et al.,* 2001). The introduction of functional transactivation domains from apicomplexan, AP2 domain added to the efficacy and specificity of the approach but it is dependent on the transcriptional profile of the minimal promoter that mimics the gene of interest's transcriptional activity.

2. 5. 2 Destabilization domain based protein modulation system

In recent advances made in approaches to regulate the protein levels, a destabilization domain (dd) based technique has found immense importance. The engineered version of human FKBP12 (F36V, L106P) when fused to the protein of interest brings about the degradation of the entire complex. This however, is mitigated in presence of the ligand of the ddFKBP, Shld-1 (Armstrong and Goldberg 2007) (2. 16). In case of essential gene like

Calpain, cysteine proteases in *Plasmodium*, the degradation domain based protein modulation system proved to be very effective when conventional techniques failed to give an insight (Russo *et al.,* 2009).

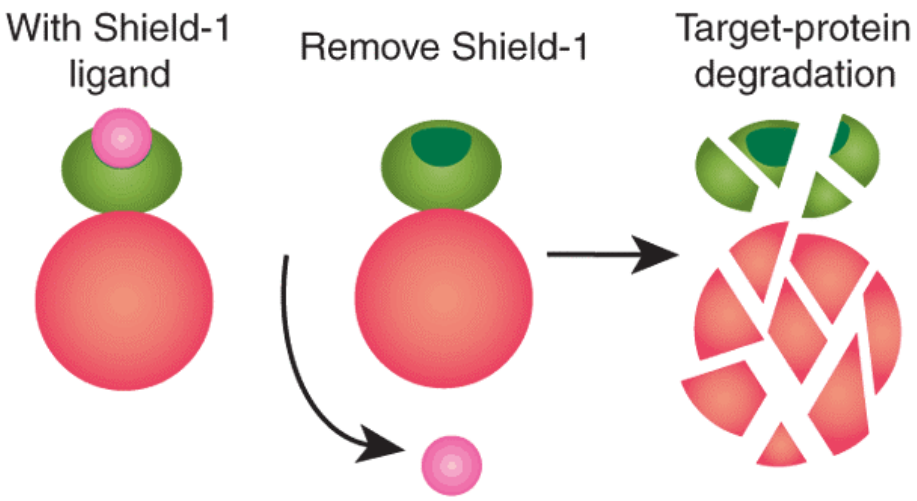


Figure 2. 16: Ligand-dependant protein level regulation: The ligand (Shield-1) of the degradation domain (in green) clearly salvages the tagged protein complex (in pink) when it is docked in the pocket of the degradation domain. The proteasomes help downregulate the tagged protein levels by degrading the entire protein conglomerate (Source: Nature Methods- Daniel Evanko).

The ligand of the destabilization domain, Shld-1 is expensive and in higher dozes can prove to be toxic for the parasite. A further breakthrough was achieved and *Escherichia coli.* Dihydrofolate reductase (DHFR) was engineered and used as degradation domain. The ligand that stabilizes the DHFR Destabilization Domain (DDD), Trimethoprim (TMP) is relatively inexpensive and equally effective (Iwamoto *et al.,* 2010). The adaptability of the DDD system in malaria parasite was remarkable and role of the asparagine repeats in a proteasome, Rpn6 lid subunit 6 was shown to be essential with the help of this technique (Muralidharan *et al.,* 2011).

This system however has a few drawbacks. The DD or DDD tag should not interfere with the normal functioning of the tagged protein when the entire complex is reversibly stabilized by the ligand of DD or DDD. Since the degradation of the protein complex is carried out by proteasome machinery, the approach has limited usage and is not effective for secretory proteins.

2. 5. 3 Cre and FLP recombinase based site-specific recombination

Manipulating the genome of the parasite in a highly specific manner is a task, which still needs more precision. The most convinient way to achieve this is to make use of recombination of the DNA in a desired way and this is carried out by set of enzymes called Recombinases. Site-specific recombinases like Cre, a tyrosine recombinase from P1 bacteriophage and Flippase recombination enzyme (FLP) from *S.cerevisiae* have been widely used for this purpose (Kilby *et al.,* 1993). The cre-based recombination has efficiently worked in case of *Toxoplasma spp.* by mediating recombination between 34 bp sites called *lox*P (Brecht *et al.,* 1999). The conventional methods of homologous recombination in the malaria parasite takes a long period of time and takes several rounds of erythrocytic cycle but when the recombination is mediated by the recombinases it is much quicker and can be achieved in single erythrocytic growth cycle with upto 100% effieciency (Collins *et al.,* 2013). A study in *Plasmodium falciparum*, showed that Cre based recombination could effectively remove the targeted gene, human dihydrofolate reductase (hDHFR) while FLP based recombination failed to do so (O'Neill *et al.,* 2011).

2. 6 Aims

The IMC is an essential structure for the parasite. A phylogenetic and functional mosaic of proteins is mediating the multiple and different function of this structure during the life cycle. In this thesis work I tried to delve deep to identify and characterize additional alveolins in *P. falciparum* using novel *in silico* tools. Since the *Plasmodium* genus specific MAL13P1.228 plays a critical role especially during the sexual stages, an attempt to functionally characterize it was made using a gene truncation approach. As most of the conventional techniques do not guarantee precise targeting of the essential genes, an approach to generate a conditional knock-out system for the IMC/Alveolins using a ddFKBP-GFP tag was also contemplated and improvised.

3. Materials

Table 3.1: Consumables

Consumables	Manufacturer
Coverslips, 13mm	Engelbrecht, Furth
Disposable cannulas	Braun, Melsungen
Disposable syringes	Braun, Melsungen
Eppendorf tubes (1.5 and 2 ml)	Eppendorf, Hamburg
Falcon centrifuge tubes (15 and 50ml)	Sarstedt, Nürnbrecht-Rommelsdorf
Microscopy glass slide	Roth, Karlsruhe
Gloves	Kimberly Clark, Koblenz- Rheinhafen
Cryotubes	Nunc, Langenselbold
Plastic pipettes (10 and 25 ml)	Sarstedt, Nürnbrecht-Rommelsdorf
Nitrocellulose transfer membrane	Protran® Schleicher & Schuell, Dassel
Pasteur pipettes	Brand, Wertheim
PCR tubes	Sarstedt, Nürnbrecht-Rommelsdorf
Petri dishes (5 and 10 ml)	Sarstedt, Nürnbrecht-Rommelsdorf
Pipette tips (20, 200, 1000µl)	Sarstedt, Nürnbrecht-Rommelsdorf
Pipette tips (10 µl)	Greiner, Solingen-Wald
Pipette tips packed (20, 200, 1000 µl)	Sarstedt, Nürnbrecht-Rommelsdorf
Scalpel	Braun, Tuttlingen
Sterile filter, 0.22 micron, medium filtration	Sarstedt, Nürnbrecht-Rommelsdorf
Whatmann filter paper	Schleicher & Schuell, Dassel

Table 3.2: Devices

Equipment	Description	Manufacturer
Acyl amide gel chamber	Protean	Bio-Rad, Munich
Agarose gel chamber	192 Cell	Bio-Rad, Munich
Analytical balance	SBA 32	Scaltech, Göttingen
Systec autoclave	V120	Wettenberg
Incubator	CB53	Binder, Tuttlingen
Incubator	B6200	Heraeus, Hannover

Flow pump	VP 03	Laboport, Lübeck
Flow pump	N86KN18	Hassa, Lübeck
Electroporator	X-Cell	Bio-Rad, Munich
Ice machine	AF-10	Vernon Hills, USA
Developer	Curix 60	AGFA- Gevaert, Mortsel, Belgium
Fine Scale	SBA32	Saltec, Göttingen
Fluorescence microscope	Axioskop 2 plus with Hamamatsu digital camera C4742 -95	Zeiss, Jena and Hamamatsu Photo tonics KK, Hamamatsu, Japan
Gel Documentation	Gel Doc XR	Bio-Rad, Munich
Laboratory Balance	MC1 Laboratory LC 2200P	Sartorius, Göttingen
Magnetic stirrer	19 R3001	Heidolph, Schwabach
Microwave	Micromaxx MM41568	Medion, Mülheim
Millipore Milli-Q	Millipore analgesia	Bedfort, USA
PCR Mastercycler	Epgradient	Eppendorf, Hamburg
pH meter	211 Microprocessor	Hanna Instruments, Kehl
Photometer	Eppendorf BioPhotometer plus	Eppendorf, Hamburg
Pipette 20µl, 100µl, 1000µl	Gilson	Middleton / USA
Power Supply	Power PAC 300	Bio-Rad, Munich
Shaker	GFL 1083	Eppendorf, Hamburg
Laminar bench	Terile Guard III	Advance Baker Company, USA
Eppendorf table-top centrifuge	5415 D	Eppendorf, Hamburg
Thermoblock Thermomixer	5436	Eppendorf, Hamburg
UV table	PHEROlum289	Biotec Fischer, Reiskirchen
Ultracentrifuge	J2-MI	Beckman, München
Ultracentrifuge	Sorvall Evolution, SS34-Rotor	Du Pont Instruments, Bad Hamburg

Wet electroblotting system	MiniTrans-Blot Cell	Bio-Rad, Munich

Table 3.3 Chemicals and reagents

Reagents	Manufacturer
Acrylamide	Roth, Karlsruhe
Agar	Becton Dickinson, Heidelberg
Agarose	Invitrogen, Karlsruhe
Albumax™ II	Gibco, Auckland, New Zealand
BSA	Biomol, Hamburg
APS	Merck, Darmstadt
Ampicillin	Roche, Mannheim
Bacto™ Yeast Extract	Becton Dickinson, Heidelberg
Blasticidin	Invitrogen, Karlsruhe
Sorbitol	Sigma, Steinheim
Bromphenolblue	Merck, Darmstadt
Calcium chloride	Sigma, Steinheim
Colchicine	Sigma, Steinheim
Coomassie Brilliant Blue G-250	Merck, Darmstadt
dNTPs	Fermentas, St. Leon-Rot
DAPI	Roche, Mannheim
DPBS	PAN Biotech, Aidenbach
1,4 DTT	Biomol, Hamburg
Acetic acid	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Ethidiumbromide	Sigma, Steinheim
EDTA	Biomol, Hamburg
Formaldehyde (10%)	Polysciences, Inc., Warrington PA, USA
Gentamycin	Rathiopharm, Ulm
Giemsa- solution	Merck, Darmstadt
Glucose	Merck, Darmstadt
Glutaraldehyde (25%)	Roth, Karlsruhe
Glycerine	Merck, Darmstadt

Yeast extract	Becton Dickinson, Heidelberg
HEPES	Roche, Mannheim
Hypoxanthine	Biomol, Hamburg
Isopropanol	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
Calcium dihydrogenphosphate	Roth, Karlsruhe
Lysozyme	Sigma, Steinheim
Magnesium chloride	Merck, Darmstadt
ß-Mercaptoethanol	Merck, Darmstadt
Methanol	Sigma, Steinheim
Milk powder	Merck, Darmstadt
MOPS	Merck, Darmstadt
TEMED	Merck, Darmstadt
SDS	Serva, Heidelberg
Sodium dihydrogenphosphate	Merck, Darmstadt
Peptone	Merck, Darmstadt
Phenol/Chloroform/Isoamylalcohol (25/24/1)	Sigma, Steinheim
Ponceau	Sigma, Steinheim
RNase	Merck, Darmstadt
RPMI	Applichem, Karlsruhe
Hydrochloric acid	Merck, Darmstadt
Saponin	Sigma, Steinheim
Sigma, Steinheim	Merck, Darmstadt
Triton X-100	Biomol, Hamburg
Tween 20	Sigma, Steinheim
WR99210	Jacobus Pharmaceuticals, Washington, USA

Table 3.4: Kits and reagents

Description	**Manufacturer**
NucleoSpin® Plasmid-Kit	Macherey-Nagel, Düren
NucleoSpin® Extract-Kit	Macherey-Nagel, Düren

PureLink™ HiPure Plasmid Midiprep Kit	Invitrogen, Karlsruhe
Western Blot Detection Kit	Pierce, Rockford, USA
1kb DNA- Ladder GeneRuler™	Fermentas, St. Leon-Rot
Protein- Ladder PageRuler ™	Fermentas, St. Leon-Rot
PageRuler™ Prestained	Fermentas, St. Leon-Rot

Table 3.5 List of Restriction endonucleases used

Name	Manufacturer
Kpn I	NEB/Fermentas
Not I	NEB/Fermentas
Avr II	NEB
BamH I	NEB/Fermentas
Xho I	NEB/Fermentas

Table 3.6: Software

Name	Description	Maker
Adobe® Photoshop®	CS3Version 10.0.1	Adobe
Adobe® Illustrator®	Illustrator 10	Adobe
AxioVision	40 V 4.7.0.0	Zeiss

Table 3.7: Databank and programs used

Data bank	URL/ Address	Specifications
PlasmoDB	http://www.plasmodb.org	Plasmodium-Data bank
GeneDB	http://www.genedb.org	Plasmodium-Data bank
IBIVUServer/ Praline MSA	http://www.ibi.vu.nl/programs/pralinewww/	Homologous region search tool
XSTREAM v[1.7]	http://jimcooperlab.mcdb.ucsb.edu/xstream/	To identify tandem repeat motifs in protein/nucleotide sequence.

EXPASY Proteomic Tools	www.expasy.ch/tools/#proteome	For posttranslational modification searches

3. 1 Buffers, Solutions and Medium

3. 1. 1 *P. falciparum* cell culture

RPMI-Complete medium

15.87g RMPI 1640,
1g NaH_2CO_3,
2g Glucose,
0.5% AlbumaxII,
0.0272g Hypoxanthine,
0.28g Gentamycin, pH 7.2
Adjust the total volume to 1l with dH_2O, sterilisation by filtration

10x PBS

5.7g Na_2HPO_4,
1.25g NaH_2PO_4,
15.2g NaCl
Adjust the volume to 1l with dH_2O, pH 7.4

Synchronisation Solution

5% Sorbitol in dH_2O; sterilisation by filtration

Freezing Solution

4.2% D-Sorbitol,
0.9% NaCl,
28% Glycerol
in dH_2O; sterilisation by filtration

Thawing Solution

3.5% NaCl in dH_2O; sterilisation by filtration

Cytomix (Transfection Buffer)

120mM KCl,
0.15mM $CaCl_2$,
2mM EGTA,
5mM $MgCl_2$,
10mM K_2HPO_4,
10mM KH_2PO_2
25mM HEPES pH 7.6; sterilisation by filtration

Lysis Buffer

0.03% Saponin in 1xPBS

Fixative Solution

4% Formaldehyde,
0.0075% Glutaraldehyde,
500µl 10x PBS
Adjust the total volume to 5.0 ml with dH_2O

Hypotonic Solution

5 mM Tris pH 8.0

Carbonate Buffer

0.1 M Na_2CO_3

Permeabilization buffer

0.1% TritonX- 100 in 1x PBS

Blocking Solution

3% BSA in 1x PBS

Medium Supplements

Human erythrocyte concentrate O^+ (Blood bank UKE, Hamburg)
Selection marker drug: WR99210
Stock solution: 20mM WR in DMSO,
Working solution: 20µM in Medium,
Sterilisation by filtration

Selection marker drug: Blasticidin S HCl 2µg/ml working solution in Medium,
Sterilisation by filtration

3. 1. 2 General microbiology reagents

For *E. coli*-Culture

LB-Medium

1% Yeast extract,
0.5% Peptone,
1% NaCl in dH_2O, autoclaved

LB-Agar

LB-Medium,
1.5% Agar

Ampicillin

100mg/ml Ampicillin in 70% Ethanol

Freezing solution for bacterial cells

50% (w/v) Glycerine in dH_2O

Competent Cells

TFBI

30mM Sodium acetate,

50mM $MnCl_2$,

100mM NaCl,

10mM $CaCl_2$,

15% Glycerine, sterilisation by filtration

TFBII

10mM MOPS,

75mM$CaCl_2$,

10mM NaCl,

15% Glycerine, sterilisation by filtration

3. 1. 3 Biochemical and molecular biological reagents

For separation of DNA

50x TAE-Buffer

242 g Tris-Base,
57.1 ml glacial acetic acid, 100ml

0.5 M EDTA,

Final volume adjusted to 1l with dH_2O

6x DNA-Loading dye

0.25% Bromphenolblue,

0.25% Xylencyanol,

40% (w/v) Glycerine

For separation of proteins (SDS-PAGE)

Electrophoresis running buffer

0.1% SDS,

192 mM Glycine,

25 mM Tris-HCl

Stacking buffer

1 M Tris, pH 6.8

Separation buffer

1.5 M Tris, pH 8.8

Ammoniumpersulfate (APS)

10% (w/v) in dH_2O

Stacking Gel (5%)

1 ml Stacking buffer,
2.3 ml dH_2O,
667 µl acrylamide (40%),
5 µl TEMED,
40 µl SDS (10%),
20 µl APS (10%)

Separating Gel (10%)

1.5 ml Separating buffer,
2.5 ml H_2O,
2 ml acrylamide (40%),
5 µl TEMED,
60 µl SDS (10%),
25 µl APS (10%)

5x SDS Loading dye

12% SDS,
300 mM Tris pH 6.8,
60% Glycerine,
0.05% BPB,
600 mM DTT
1x non-reducing sample buffer

Western Blot buffer (for transfer of proteins)

10x Tris-Glycine transfer buffer (Stock)

30.0 g Tris base,

144 g glycine

10.0 g SDS; final volume adjusted to 1l with dH_2O

1x Tris-Glycine Buffer (Working solution)

100 ml 10X Tris-Glycine transfer buffer
700 ml dH_2O
200 ml Methanol

Ponceau S staining solution

0.2% Ponceau S,
1% Glacial acetic acid

Blocking Solution

5% Milk powder in 1x PBS

Wash Buffer

1x PBS,
0.05% Tween

3. 1. 4 Bacterial and *Plasmodium* strains

E.coli XL-10 Gold

Tetr *(mcrA)183 (mcrCB-hsdSMRmrr) 173*
endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte
[F' *proAB lacIqZ M15 Tn10* (Tetr) Amy Camr]

3D7, *Plasmodium* Culture strain

MR4, Manasses/ USA, Origin: Africa

3. 1. 5 Antibodies

Primary antibodies

Table 3.8

Antigen / Organism	Dilution	Source
GFP / Mouse	1:1000 for western blot & 1:500 for IFA	Roche, Mannheim
PFD1110w/ Mouse	1:1000 for IFA	Gilberger Laboratory, Hamburg, Germany
GAPDH / Rabbit	1:3000 for western blot	Spielmann Laboratory, Hamburg, Germany
Aldolase / Rabbit	1:3000 for western blot	Spielmann Laboratory, Hamburg, Germany

Secondary antibodies

Table 3.9

Antigen	Conjugate	Dilution	Source
Mouse	HRP	1:3000 for Western blot	Dianova, Hamburg, Sigma, Steinheim
Rabbit	HRP	1:3000 for Western blot	Dianova, Hamburg, Sigma, Steinheim
Mouse	ALEXA 488	1:3000 for IFA	Molecular Probes, Leiden, Netherland
Rabbit	ALEXA 594	1:3000 for IFA	Molecular Probes, Leiden, Netherland

3. 2 Vectors

3. 2. 1 pARL1a- (Crabb *et al*., 2004a)

The plasmid pARL1a (-) is called a shuttle vector as well. It can be transformed into *E.Coli*, where the beta-lactamase resistance gene allows the selection of transformed cells using antibiotic, ampicillin. This vector can be used for transfecting *Plasmodium sp.* cells as well. This vector carries human dihydrofolate reductase gene (hDHFR) with the help of which the

transgenic parasites gain resistance towards the antifolate WR99210. The desired gene of interest can be cloned into the multiple cloning site (MCS) of the vector by using the restriction endonucleases KpnI and AvrII to generate sticky ends and seal them with ligases. In this case, the gene of interest is expressed under the control of 'Apical membrane antigen1' (*ama-1*) promoter, which marks the expression of the gene during schizogony.

3. 2. 2 pBcam (Flueck *et al.*, 2009)

The pBcam vector has a blasticidin S deaminase gene (BSD) selection cassette, which enables the selection of the transgenic parasites with the antibiotic blasticidin-S. In this study the calmodulin promoter, which allows a constitutive expression, is replaced with the ama-1 promoter (*ama-1*) to restrict the expression of the cloned gene to schizont stage in order to mimic the expression profile of the previously characterized IMC proteins. The fluorophore 'mCherry' is used as a marker at the C-terminus of the cloned gene. Ligation of the gene of interest is carried out after the restriction cleavage of the sites at the MCS by enzymes BamHI / NotI.

3. 3 Oligonucleotides (Primers)

All the primers used in this research study were synthesized in Sigma Aldrich, Germany. The list of primers is given below, in 5'-3' direction.

1. PF08_0033 (Sense)- ATGCAAGGTGAAACAATTAATGTA
2. PF08_0033 (Anti-Sense)- CATATTACCACAGCATTTATTAAT
3. PF13_0226 (Sense)- ATGGACGAAAAATCAGAAATTAAC
4. PF13_0226 (Anti-Sense)- TGAATTTATATATAGTTGTTGTTTTCC
5. PF08_0033 (Anti-Sense: Alveolin repeats 1, 2 deletion)- CATCTACATATTTATTTACATATTTAATTTGGCGTATTGGT TTAAGAATTTTAC
6. PF08_0033 (Sense: Alveolin repeats 1, 2 deletion)- GTAAAATTCTTAAACCAATACGCCAAATTAAATATGTAAATAAA GATG
7. PF08_0033 (Anti-Sense: Alveolin repeat 3 deletion)- CTACAATTTTGTTTCTGTATTCTATCTTTTCTACTACTTTTGGCT GATAC
8. PF08_0033 (Sense: Alveolin repeat 3 deletion)- GTATCAGCCAAAAGTAGTAGAAAAGATAGAATACAGAAACAAAAT TGTAG
9. PF08_0033 (Anti-Sense: C492A)- CATATTACCACAGCATTTATTAATAAAATTGGCCAATGAAGC
10. PF08_0033 (Anti-Sense: C498A)- CATATTACCACAGGCTTTATTAATAAAATTGCACAATGAAGC
11. PF08_0033 (Anti-Sense: C499A)- CATATTACCAGCGCATTTATTAATAAAATTGCACAATGAAGC
12. PF08_0033 (Anti-Sense: C492, 498, 499A)- CATATTACCAGCGGCTTTATTAATAAAATTGGCCAATGAAGC
13. PF08_0033 (3' tagging- Sense)- CAAACATTTGAAGAGTAAAATTC
14. MAL13P1.228 Truncation (Sense)- TAAATTTTTTTCTATTTAAGAAC
15. MAL13P1.228 Truncation (Anti-Sense)- ATTTAATGAATTTGACTTTTTATTAGCC
16. GFP (Sense)- ATGAGTAAAGGAGGAGAACTTTTC
17. GFP (Sense- overlap)- ATGAGTAAAGGAGAAGAACTTTTC

18. GFP (Anti-Sense- overlap)-
    GGAGATGGTTTCCACCTGCACTCCCTGCAGTTTGTATAGTT
    CATCCATGCC
19. DD (Sense- overlap)-
    GGCATGGATGAACTATACAAACTGCAGGGAGTGCAGGTGGAA
    ACCATCTCC
20. DD (Anti-Sense- overlap)- TTAATGCATGGCCATGGCCAGGTCCTC

4. Methods

4. 1 Bioinformatic tools used for identification of alveolins:

4. 1. 1 BLASTP – a protein-to-protein search (Altschul *et al.*, 1990)

Basic **L**ocal **A**lignment **S**earch **T**ool **P**rotein, or **BLASTP**, is an algorithm for comparing primary protein sequence information. The sequences of 14 alveolins from *Toxoplasma gondii* (Anderson-White *et al.,* 2011, please refer to table 5.1) were used for a BLASTP searched (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Homologues with an E-value above 4e-27 were selected for further assessment that included 7 putative alveolins of *Plasmodium falciparum* (Please refer to table 5.1 of result section).

4. 1. 2 XSTREAM: A algorithm for identification and architecture modeling of tandem repeats in protein sequences (Newman A. M. and Cooper J. B., 2007)

XSTREAM is a tool for easy detection of tandem repeats in proteins and nucleotide sequences. It is a precision means for working at multiple genome scale and determining tandem repeat domains with different architecture and extreme degeneracy. The programme identifies a consensus sequence of the amino acid or nucleotide stretch, which is preset at as minimum 'consensus value'. This consensus sequence bears a predetermined set or number of characters referred to as 'minimum period'. This handy tool was previously used in identification of alveolins in different species of Alveolata superfamily, making it an extremely successful tool for this purpose (Gould *et al.,* 2010). In this study here the preset values for the 'minimum consensus match' and the 'minimum period' were relaxed than previously used by Gould *et al.,* 2010.
The settings used by Gould *et al.* (2010):
Min Consensus Match = 0.5, Min Period = 10
Setting of the parameters of XSTREAM: Min consensus match = 0.45, Min period = 4,

XSTREAM was used to identify the short exact substring repeats or seeds which had 45% of consensus in their repeats and were least 4 residues long. The algorithm sought for initial

seeds and then expanded word with adjoining sequences while keeping the 45% of the word-match accurate. The proteins were further analyzed and the tandem repeats of those that maintained the E >= 6%, K >= 6%, Q >= 3%, and the sum of L, V, and I >= 10% were selected. Repeats with any single amino acid component > 30% was removed as low a complexity sequence. This approach identified putative alveolin-like proteins in the *Plasmodium* genome (please refer to table 5.2 of result section).

4. 1. 3 Phylogenetic profiling of the alveolin/IMC proteins

Orthologs of the 17 initially IMC proteins identified, were sought in the set of 120 complete eukaryotic genome. The multiple sequence alignments (MSAs) of these proteins were carried out using MUSCLE $^{V3.6}$ (Edgar, 2004). These MSAs were used to generate Hidden Markov Model (HMM) with the help of HMMER3 (Eddy, 2009). Remote homologs of these 17 IMC/alveolins were traced in four independent databases, using the HMM profiles of these proteins and HMM search algorithm (Kono *et al.,* 2012). These databases are:

a. PhyloPro (Xiong *et al.,* 2011) – the set of 120 genomes taken into consideration.
b. Ensembl (Flicek *et al.,* 2011)
c. PartiGeneDB (Peregrin-Alvarez *et al.,* 2005) – about 700 peptide data sets constructed with the compilation of expressed sequence tags (ESTs)
d. The non-redundant NCBI protein data base

4. 2 Molecular biology methods

4. 2. 1 Generation of competent bacterial cells

Competent bacterial cells can freely uptake foriegn DNA from the surroudings. A 2 ml *E.coli* culture was grown overnight (O/N) and then added to 200 ml of fresh LB culture medium (1:1000 dilution) and put on shaker at 37°C 180 rpm. The OD_{600} was measured and once it was in the range of 0.5 to 0.7 the culture was spun down and the supernatant was discarded. The pellet was resuspended in 30 ml of pre-cooled TFB I solution and incubated on ice for 10 minutes. Subsequently the cells were spun down at 4°C, 2600 rpm for 20 minutes. The pellet was then resuspened in 10 ml of TFBII solution and 100 µl aliquotes were made and stored at - 80 ° C.

4. 2. 2 Transformation of the competent cells (Dower *et al.,* 1988; Taketo 1988)

The phenomenon with which a foreign DNA is introduced in a prokaryotic cell is called transformation. The chemically treated competent cells were first thawed on ice and then mixed with the desired plasmid DNA. The mixture was incubated on ice for 10 minutes then subjected to heat shock treatment (42°C for 90 seconds) and then placed back on ice for 10 minutes. Then 1 ml of LB medium was added to mixture and incubated at 37°C for 40 minutes. This incubation helps the cells expressing the selection marker cassette to grow. Subsequently the culture mixture was plated on LB agar plate with appropriate amount of antibiotic in it and put for O/N incubation at 37°C.

4. 2. 3 Overnight Cultures

To isolate plasmid DNA from the specific bactrial colonies from plates or from frozen stocks, tiny amounts of inoculum were added to sterile LB medum with specific antibiotic in appropriate amounts. It was then incubated at 37°C for 16-17 hours.

4. 2. 4 Glycerol stocks of the transformed bacterial cells

In order to make glycerol stocks of specific transforned bacterial cells carrying the desired plasmid, 600 µl from an over night culture was mixed with 600 ul of 40% autoclaved sterile glycerol in a 1.5 ml eppendorf tube and immediately stored at -20° C.

4. 2. 5 Isolation of plasmid DNA

The plasmid DNA (mini or midi preparation) from overnight cultures was isolated using plasmid DNA isolation kits as per the instructions provided along with them. For mini preparations of plasmid DNA, NucleoSpin ® Plasmid kit (Macherey -Nagel) kit and for midi preparations Qiagen ® midi preparation kit were used. Isolation of DNA using the kits is based on alkaline lysis of the cells and then binding of the DNA to a silica membrane. The overnight culture (2 ml for mini preparation and 100 ml for midi preparation) was spun down and the pellet was resuspended in a buffer containing RNase and then lysed using a lysis buffer. A neutralization buffer was added to it so as to restore the normal physiological pH. The mixture was then centrifuged to separate the cellular debries from the plasmid

DNA, which is present in the supernatant. The DNA is bound on silica membrane and then eluted using TE buffer (50 µl for mini preparation and 100 ul for midi preparation). The DNA so isolated was used for restriction digestion analysis or for sequencing (mini preparations) or for transfection into the malaria parasite for which 100 µg DNA is required ideally (midi preparation).

4. 2. 6 Agarose gel electrophoresis (Garoff and Ansorge, 1981)

In electrophoresis the DNA is separated on the basis of the net charge it bears, its conformation, the concentration of the agarose in the gel and the field strength of electricity applied. The sugar-phosphate backbone gives a net negative charge to the macromolecule henceforth it migrates towards the positive electrode. 1% agarose gel is appropriate for separating DNA ranging from 0.5 kbp to 8 kbp. The migration of the DNA is inversely proportional to the natural logarithmic value of the molecular weight of the DNA molecule. The DNA is detected using an interaclating agent (ethidum bromide), which fluoresces when it binds to DNA, and exposed to UV light.

4. 2. 7 Purification of PCR products

PCR products were purified according to instructions provided with the NucleoSpin ® Extraction II Kit (Macherey-Nagel). In this method the DNA is reversibly bound to a silica membrane at high salt concentration and eluted with an elution buffer (5 mM Tris / HCl, pH 8.5) or ddH_2O eluted. The purification of the PCR product helps in removing the polymerases, oligonucleotides and salt that can hamper the other applications such as restriction digestions of the DNA.

4. 2. 8 Restriction digestions

Restriction endonucleases are a class of bacterial enzymes that hydrolyze the phosphodiester bond of the DNA backbone in a highly specific manner (Summers *et al.,* 1975). The recognition sequences generally consist of four to eight nucleotides in a double-stranded DNA in a palindromic arrangement. The nucleases generate blunt ends or single-cohesive DNA overhangs (sticky ends). The endonucleases that generate these sticky ends in the DNA are vastly used for the cloning purposes as the desired foreign DNA with the same overhanging sequences can be easily ligated to an appropriate vector, which have been cut

by the same restriction enzymes. The reaction mixture (either a PCR product or plasmid from mini preparation) was set up with enzyme concentration of 1.5 U/ µg of DNA and incubated at 37°C for 2 hours.

4. 2. 9 Ligation of DNA fragments

The DNA ligases catalyze the formation of phosphodiester bond between the 5' phosphate and 3' hydroxyl group of the two DNA molecules with compatible ends. This linkage of two DNA molecules with the help of ligase enzyme is called ligation. This tool called ligation is extensively used in fusing the insert DNA molecule into a vector/plasmid. The insert DNA molecule and preferably the plasmid after being digested with the same restriction endonucleases have compatible sticky ends. The two are mixed in a molar ratio of 3:1 respectively with 2 U of ligase and appropriate volume of ligase buffer in a reaction volume of 20 µl and incubated at RT for 1 hour or overnight at 16°C.

4. 2. 10 Precipitation of DNA

The precipitation of a solution of DNA is done by addition of 0.1 volume of 3 M Sodium acetate pH 5.6 and 2.5 volumes of cold, absolute ethanol. The mixture is first inverted and then centrifuged for 15 min at 12.000xg. The supernatant is carefully removed and the DNA sediment is washed with ice-cold 70% ethanol. After a further centrifugation for 5 minutes at 13.000xg, the supernatant is discarded and the DNA sediment is dried at room temperature. Subsequently, the DNA can be dissolved in a desired volume of dd H_2O or TE buffer.

4. 2. 11 Determination of DNA concentrations

The quantification of nucleic acids like DNA or RNA is usually performed to determine its net concentration. In a spectrophotometer, the nucleic acid is exposed to ultra violet light and the concentration is estimated. The nucleic acids have absorption maximum at 260 nm and the polypeptides at 280 nm. The DNA is diluted in dd H_2O or TE in a ratio of 1:1000. The diluent is used to set the 'blank' at 260 nm and 280 nm. It is common for nucleic acid samples to have contaminants like proteins and other organic molecules. The actual purity of the sample can be determined by taking the ratio of absorbance at 260 to 280 nm. If the ratio

is greater than 1.8 the absorption is due to nucleic acids. High quality preparation should have a ratio of 2.0 to 1.8. If the ratio is below 1.6 there may be proteins or other organic contaminants and the DNA can be extracted for a final clean up.

4. 2. 12 DNA amplification by Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987)

The polymerase chain reaction is used for selective amplification of DNA fragments. Using a set of specific primers that bind with the DNA at the flanking regions, a thermostable DNA polymerase, dNTPs and corresponding buffer, the amplification of a DNA fragment is achieved. Taq polymerase, isolated from the eubacterium *Thermus aquaticus* has an optimum functional temperature of 72°C, but is still stable up to 92°C. Enzyme has a high rate of synthesis (1000bp/min), but the enzyme lacks proof reading activity as a result of which there is an accumulation of significant number of mutations (1.1 x 104, Tindal and Kunkel, 1988). To amplify a mutation- free gene fragment or gene a ‘proof -reading polymerase’ is used that detects the error and corrects them. The ‘Deep Vent polymerase’ (from *Pyrococcus sp.*) is known for its correction capability and with only a mutation rate of 5.7 x 105 (Mattila *et al.,* 1991), but a lower synthesis rate. To ensure a high synthesis rate and a low mutation rate in the PCR product, a mixture of Taq and Deep Vent polymerase or the ‘Phusion - Proof Reading’ is used which has high synthesis rate with lower mutation rate (4 x 107 according to the manufacturer). In a consecutive series of different temperature steps (1) The separation of the two DNA strands (92°C), (2) the binding of the oligonucleotides to the DNA (42 - 60°C, depending on GC content of the primers) and (3) the subsequent amplification of the fragments (elongation at 72°C) the product of PCR, an amplicon is generated. By adjusting the salt concentration and the time intervals for annealing and elongation (depending on each primer set), the reaction can be optimized. The elongation depends on the length of the fragment to be amplified and the speed of the selected polymerase. The genome of *P.falciparum* has a high AT content, which is why the temperature conditions are kept relatively low (Gardner *et al.,* 2002a and 2002b). The annealing temperature is 42-48°C, the elongation temperature of 60-64°C (1- 3min).

Table 4.1: Standard set up for a PCR using Phusion polymerase (in a volume of 50 µl)

Reagents	Volume
5x Phusion HF Reaction buffer	10 µl
dNTPs (10mM)	5 µl
Sense-Primer (10µM)	1 µl
Antisense-Primer (10µM)	1 µl
Template-DNA (1-200ng/µl)	0,5 µl
Phusion-Polymerase	0,5 µl
dH_2O	32 µl

Table 4.2 Standard PCR programme

PCR steps	Temperature	Time	Cycles
Initial denaturation	94°C	2 minutes	
denaturation	94°C	15 seconds	30 X
Annealing	42°C	30 seconds	
Elongation	62°C	1-3 minutes	
Final elongation	64°C	4 minutes	
Programme end	4°C	∞	

4. 2. 13 PCR screening for identification of *E. coli* colonies with the required plasmid

The PCR screening is used for the rapid identification of *E. coli* colonies harbouring plasmids with the required DNA fragment. Bacterial colonies to be examined are directly used as a template and identified as positive or negative by using insert specific and vector specific primer set. The bacterial cells rupture in the denaturing temperatures of the PCR releasing the DNA, which in turn serves as the template for the diagnostic primers. Based on the size of the amplicon on the agarose gel, the positive colonies can be identified. The bacterial colonies are selectively picked using a sterile pipette tip from LB plate and added to the PCR reaction mixture. The PCR screening is carried out with a reduced number of cycles, approximately 25.

Table 4.3: Standard set up for a PCR for screening

Reagents	Volume
10xFirePol® Reaction buffer	2 µl
dNTPs (10mM)	2 µl
MgCl2	2 µl
Sense-Primer (10µM)	0,5 µl
Antisense-Primer (10µM)	0,5 µl
FirePol-Polymerase	0,2 µl
dH_2O	12.8 µl

Table 4.4: Standard PCR programme for colony screening

PCR steps	Temperature	Time	Cycles
Initial denaturation	94°C	2 minutes	
denaturation	94°C	15 seconds	25 X
Annealing	42°C	30 seconds	
Elongation	62°C	1-3 minutes	
Final elongation	64°C	4 minutes	
Programme end	4°C	∞	

4. 3 Biochemical Methods

4. 3. 1 Separation of proteins by SDS -PAGE (polyacrylamide gel electrophoresis) (Laemmli, 1970)

Proteins can be separated from a mixture of proteins or cell lysate according to their molecular weight by SDS-PAGE. Proteins have an amphoteric character, thus their net charge is dependant on the surrounding buffer. Sodium dodecyl sulphate (SDS) is an anionic detergent, which binds to the proteins with almost a uniform ratio (1.4 g SDS / 1 g Protein) and linearizes the proteins and imparts an uniform negative charge to the denatured molecules which results in a fractionation by approximate size during electrophoresis. The addition of dithiothreitol (DTT), which cleaves the disulphide bonds further, denatures the proteins. The determination of size of the proteins is done using a marker. The gel used is divided into an upper "stacking" gel of low percentage (with large pore size) and low pH

(6.8), where the protein bands get squeezed down as a thin layer migrating towards the anode and a resolving gel (pH 8.8) with smaller pores. The Cl^- is the only mobile anion present in both gels. When electrophoresis begins, glycine present in the electrophoresis buffer enters the stacking gel, where the equilibrium favors zwitterionic form with zero net charge. The glycine front moves through the stacking gel slowly, lagging behind the strongly charged, Cl^- ions. Since these two current carrying species separate, a region of low conductivity, with high voltage drop, is formed between them. This zone sweeps the proteins through the large pores of the stacking gel, and depositing it at the top of the resolving gel as a narrow band. The separating/resolving gel is more basic (pH 8.8), and has a higher polyacrylamide content, which causes the gel to have narrower channels or pores. As a protein, concentrated into sharp bands by the stacking gel, travels through the separating gel, the narrower pores have a sieving effect, allowing smaller proteins to travel more easily, and hence rapidly, than larger proteins.

4. 3. 2 Western Blotting (Towbin *et al.*, 1979)

Western blotting is a powerful technique for identifying specific proteins. The proteins are first separated by electrophoresis, then transferred from the polyacrylamide gel to nitrocellulose or PVDF membrane, and probed with specific antibodies. This technique can give us information about the size of the protein (with comparison to a size marker or ladder in kDa), and also about the level of expression of the protein (in comparison to a control). This transfer of the proteins from the gel to membrane is accomplished by using specific transfer buffers and Tris-Glycine transfer buffer being one of them. This buffer works excellently for the wet transfer of the proteins wherein the entire process is carried out in a tank filled with the transfer buffer (Towbin *et al.,* 1979). The gel and the membrane are tightly clamped together, padded with whatmann filter papers and foams, using a provided apparatus and then immersed into the tank filled with the tris-glycine buffer, with 20% methanol (to immobilize the proteins onto the membrane). An electric pressure of 120 V is applied for 90 minutes as a result of which the negatively charged protein molecules migrate towards direction of positive electrode and in turn get immobilized on the membrane. This generates a singnificant amount of heat, so the buffer is cooled to 4°C and ice pack is included in the buffer tank and the entire process is carried out in cold room.

4. 3. 3 Ponceau Staining

The proper transfer proteins to the nitrocellulose/PVDF membrane can be checked by means of ponceau staining. The Ponceau dye reversibly binds the basic side chains of the amino acids of the transferred proteins and can thus also provide insight into the separation type of proteins during SDS-PAGE. It can be washed off from the nitrocellulose/PVDF membrane with water or 1x PBS.

4. 3. 4 Immunological detection of proteins

The proteins immobilized on the membrane after the western blotting, can be detected using specific antibodies. The free binding sites were blocked at first by incubating the membrane with 5% milk powder in TBS-Tween (1 x TBS, 0.05% Tween). Subsequently, the membrane was then incubated with the primary antibody in the appropriate dilution either for about 3 hours at RT or at 4°C and rotated overnight. After the membrane was washed 3 times with TBS-Tween, it was then incubated with the HRP-conjugated secondary antibodies for 2 hours. Before the development of the blot, it was again washed five times with TBS-Tween. ECL working solution is added to it followed by the development by X-ray film.

4. 3. 5 Solubility Assay

In order to determine the degree to which proteins are associated with the different membranes in the cell, solubility assay was performed which allowed the extraction of the cellular proteins in different fractions. The parasite lysate was lysed in about 100 µl of hypotonic buffer and frozen at -80°C overnight. Then it was put on ice and thawed and mechanically disrupted by passing it through a narrow gauge syringe repeatedly. It was frozen again at -80°C for 2 hours. It was then again thawed on ice and centrifuged at 14000 rpm for 5 minutes. The supernatant was collected as hypotonic fraction containing the cytosolic pool of proteins. The pellet was subsequently washed 3 times with 1x PBS, after which it was resuspended in 100 µl of carbonate buffer. It was kept on ice of 30 minutes. Following which it was spun at 14000 rpm for 5 minutes. The supernatant was kept and it contained the peripheral membrane proteins. The pellet was again washed 3 times with 1x PBS and then was resuspended and incubated in 100 µl of 1% Triton-X solution for 30

minutes. After centrifugation at 14000 rpm for 5 minutes the supernatant was collected which contained the integral proteins. The remaining pellet was washed 3 times in 1x PBS and then resuspened in 100 µl of 1x PBS. Equal volumes from each fraction was loaded in the polyacrylamide gel and processed for immunoblotting.

4. 4 Microscopy

4. 4. 1 Light Microscopy

A light microscope uses a lens close to the object being viewed to collect light, which focuses a real image of the object inside the microscope. A second lens or set of lenses (eye piece) then magnifies this image. This in turn gives the viewer an enlarged inverted virtual image of the object. Giemsa-stained *Plasmodia* specimens are visualized using 100x objective lens of the microscope with immersion oil.

4. 4. 2 Fluorescence microscopy with live specimens

The fluorescence microscopic analysis of parasite material is used to validate the localization of certain proteins. The proteins of interest in this work are fused with the fluorescent protein GFP (Green Fluorescent Protein) of the marine organism *Aequoria victoria* (Chalfie *et al.,* 1994) or mCherry (Shaner *et al.,* 2004). GFP is composed of 238 amino acids and has a molecular weight of 26.9 kDa. The intrinsic fluorescence of the protein is due to a unique covalently attached chromophore, which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-67, Ser-Tyr-Gly (Cubitt *et al.,* 1995). The GFP from *A. victoria* has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum. MCherry is a red fluorescent protein isolated from *Discosoma spp.* This protein has an excitation maximum at 587 nm and an emission maximum at 610 nm. The transgenic cells of *Plasmodium* expressing the GFP or mCherry tagged proteins were examined by fluorescence microscopy using appropriate filters. Approximately 10 µl of a *Plasmodium* culture was mounted on a microscope slide and covered with a cover slip and then visualized under the fluorescent microscope. The documentation of the images was carried out using the program ‘Axiovision 4.4’ of the microscope manufacturer Zeiss and the high-resolution Hamamatsu Orca -ER digital

camera.

4. 4. 3 Indirect immunofluorescence microscopy

Fixed parasites (Section 4.5.8) were used for indirect immunofluorescence microscopy. The cells were blocked in 3% BSA (in 1x PBS) for 1 hour. The primary antibody was diluted appropriately in 3% BSA and added to the fixed cells after which the cells were incubated for 3 hours on ice. The primary antibodies specifically bind to the desired protein or to the epitope of fused tag. Following which the cells were washed 3 times for 10 minutes with 1x PBS. The secondary antibodies were diluted in a required dilution in 3% BSA and added to the cells. The secondary antibodies, which are conjugated with appropriate fluorophores (Alexa-488 or Alexa-594) bind to the primary antibodies. The cells were washed thoroughly 3 x 10 mins to get rid of the unbound antibodies. The localization of the secondary antibodies in the specimen was then determined by fluorescence microscopy using the suitable filters.

4. 5 Cell-biology Methods

4. 5. 1 *In vitro* cultivation of *P. falciparum* (Trager and Jensen, 1976)

The blood stages of *P. falciparum* (3D7) can be cultured continuously in human erythrocytes. The cultivation takes place in petri-dish with 10 ml RPMI complete medium and O^+ human erythrocytes (5% hematocrit) in a low oxygen gas mix ambiance (5% CO_2, 1% O_2, 94% N_2) at 37°C in an incubator. The asexual intraerythrocytic development cycle lasts 48 hours. After visualizing the Giemsa stained blood smears (see 4.5.2) under a light microscope, the parasitemia (percentage of infected erythrocytes) of the culture was estimated. Based on the parasitemia, the culture was adjusted every 48 hours to approximately to 1%, replenishing it with new fresh medium and appropriate amount of the hematocrit.

4. 5. 2 Giemsa staining the blood smears (Giemsa, 1904)

For the preparation of a blood smears about1.5 ul of *Plasmodium* culture was transferred on a glass slide. This drop of infected blood was spread evenly by using second clean slide. The

slide with the blood smear was dried in the air for 20sec and fixed in methanol. Then the slide was immersed in 10% Giemsa stain (Azur-eosin methylene blue) for 5-10 min. Giemsa is specific for the phosphate groups of DNA and attaches itself to regions of DNA where there are high amounts of adenine-thymine bonding. The DNA of the parasite appears deep blue under the light microscope whereas the cytoplasm of erythrocytes light bluish.

4 .5. 3 Cryo-preservation of the parasites

The parasites can be stored permanently in liquid nitrogen. Ring staged parasite culture with a high parasitemia (> 5%) was spun down and mixed with cryo-protectant solution (freezing solution) and added into a 1ml cryo vial. It was then stored in liquid nitrogen at -196 ° C.

4. 5. 4 Thawing of the frozen cultures

In order to thaw and recultivate *P. falciparum*, a vial was quickly thawed at 37°C and transferred to a 15 ml Falcon tube. It was then centrifuged at 1500 rpm for 5 min. The supernatant was carefully discarded and to the pellet, pre-warmed (37°C) 1 ml of thawing solution (NaCl solution) was added and mixed gently. After a quick centrifugation, the supernatant was removed and the pellet was subsequently washed with 10 ml of culture medium. The pellet was resuspended and added to a 10 ml petri plate with culture medium containing 5% hematocrit.

4. 5. 5 Synchronization of *Plasmodium* culture with sorbitol (Lambros and Vanderberg, 1979)

Synchronization was performed on cultures containing mainly young ring staged parasites. The culture was centrifuged at 1500 rpm for 5 min at room temperature and the supernatant was discarded. The cell pellet was resuspended in 5 ml of sterile 5 % sorbitol in H_2O and the suspension was incubated for 10 min at 37°C. Thereafter, the cells were centrifuged at 1500 rpm for 5 min at room temperature and the supernatant was removed. The cells were washed once with RPMI-1640 complete medium and were put back for cultivation.

4. 5. 6 Transfection of *P. falciparum* (Fidock and Wellems, 1997)

In contrast to transformation, transfection is the introduction of foreign DNA in eukaryotic cells. This can be achieved using a variety of techniques and in numerous sorts of eukaryotic cells. In case of *P. falciparum* it proves to be a tedious process as the foreign DNA has to cross a set of multiple membranes (erythrocyte membrane, parasitophorous vacuolar membrane (PVM), parasite plasma membrane (PPM) and nuclear membrane) owing to which, the success rate of transfection is very poor. Introduction of the foreign DNA into the parasite is obtained by means of electroporation. The ring staged *Plasmodium* culture with high parasitemia was mixed with 100µg plasmid DNA. The DNA was precipitated with ethanol, washed and resuspended in 15µl TE buffer. To this 385 µl of cytomix (electroporation buffer) was added so that the final volume was 400 µl. The electroporation is the strong application of electric field and it results in the formation of numerous pores in the membrane of the cells and through these minute pores the foreign DNA sneak into the cell. Electroporation was performed in a 20 mm cuvette at 310 V and 950 µF. Once electroporated the cells were put back into the culture by adjusting the volume of medium and hematocrit. After 5 hours the spent medium was replaced with fresh one and selection marker drug, an antifolate WR99210 (final concentration 2 nM) or Blasticidin (final concentration 30 nM) was added to select the transgenic parasites. For the selection of double transgenic parasites both the selection markers drugs were added to the culture in the needed amounts. The medium was initially changed consecutively for the first ten days then every alternate day. This was continued for the next 20-35 days post-transfection till the first transgenic parasite was seen in the thin blood smear after staining it with giemsa.

4. 5. 7 Isolation of parasites by Saponin lysis (Umlas and Fallon, 1971)

To isolate parasite from the erythrocytes of the culture, a very mild saponin treatment is given to the erythrocyte membrane. This breaks open the red blood cell membrane without harming the parasite's membrane. The detergent saponin (Latin sapo = soap) has hemolytic properties that lyse the plasma membrane of the erythrocytes and the PVM. The resulting parasite material can be used for DNA or protein analysis. For this, after the parasite culture was spun down at 1500 rpm for 5 min, the supernatant was removed and an equal volume of 0.03% saponin/PBS was added to the pellet. It was then incubated on ice for 10-15 minutes. The lysate was then centrifuged at 15000 rpm for 5 min and the pellet was washed 3-5 times

with 1x PBS.

4. 5. 8 Fixation of parasite material for immunofluorescence analysis of transgenic parasites

Formaldehyde/Glutaraldehyde- fixation

Formaldehyde fixes tissues by cross-linking the proteins, primarily the residues of the basic amino acid lysine. However, adding excess water can reverse its effect. On the other hand glutaraldehyde functions in a similar way by deforming the alpha-helix structures in proteins. Glutaraldehyde is a larger molecule, and so its rate of diffusion across membranes is slower than formaldehyde. A combination of formaldehyde and glutaraldehyde operates in an optimum manner as their respective strengths complement one another.

A parasite culture was centrifuged for 3 min at 1500 rpm, the supernatant was removed and the pellet was resuspended in 1 ml of fixing solution followed by a 30-minute incubation at room temperature. Subsequently, the parasites were centrifuged at 3400 rpm for 2 min and washed 3 times with PBS. Then a 10 minute incubation was carried out with 0.1% Triton-X to permeabilize the membrane. The pellet was then washed again 3 times with PBS and incubated for 1 h with 3% BSA buffer for blocking. An hour of incubation with the primary antibodies was done. The parasite material was then re- washed 3 times with PBS, following which the fixed cells were subjected to an incubation with the secondary antibodies, conjugated some appropriate fluorophore. Along with this, DAPI (1: 2000) was also added to stain the nuclei. The cells were thoroughly washed for 3 x 10 min with PBS. Post washing, the cells were resuspended in desired volume of PBS. From this, about 10 µl of the cell suspension was mounted on a slide and covered with a cover slip and visualized under the fluorescent microscope. All the steps were carried out at RT, however, the processed cells were stored at 4°C for later use.

4. 5. 9 Isolation of genomic DNA from *P. falciparum*

A culture rich in the late stages of the parasite is ideal for the genomic DNA isolation. The culture was centrifuged at 1500 rpm for 3 min and the supernatant was removed. Buffer-A (5 times pellet volume) was added and the pellet was resuspended. Then for the lysis of the

cells 18% SDS, in a proportion of 1 part of the volume of the pellet was added and incubated for 3 min at RT. Then 2.5 times the volume of the pellet, phenol/chloroform/isoamyl alcohol was added and mixed vigorously. The mixture was centrifuged at 3300 rpm for 10 min, which separated the DNA from proteins, the DNA accumulated in the aqueous phase whereas the protein in the interphase region of the supernatant and the pellet, containing the debries. The phenol/chloroform/isoamyl alcohol step was repeated twice to free the DNA from any impurity. The final extraction of DNA with chloroform cleared the phenolic residues completely. The DNA was finally precipitated overnight using salt and absolute alcohol at -20°C. The precipitate is subsequently washed with 70% ethanol and tried at RT. It was then resuspended in 50 µl of TE buffer, quantified and stored at 4°C in the refrigerator.

4. 5. 10 Cultivation of gametocytes (Fivelman *et al.,* 2007)

It is by and large a notion that starvation of the parasites during the asexual erythrocytic stages can induce gametocytogenesis. To stimulate differentiation into presexual and sexual forms, the parasites were synchronized with 5% sorbitol and the parasitemia was adjusted to 1% and put back into culture. Two days later, when the parasitemia was about 6-8%, only 1/3 of the medium was replaced with fresh one. Then on the third day, the hematocrit was reset to 5% and medium was changed. The fourth day usually marks the beginning of the sexual stage and the medium was changed on a daily basis hence on. The sexual forms were harvested during different stages and analyzed in a desired manner.

5. Results

5. 1 Alveolins- a conserved protein family

The alveolins are IMC localized proteins that are characterized by the presence of conserved stretches of amino acid residues referred to as 'alveolin repeats' (Gould *et al.,* 2008). To date 29 IMC proteins have been discovered of which 16 are alveolins (Kono *et al.,* 2013). In comparison in *Toxoplasma* only 14 alveolins are described so far (Anderson-White *et al.,* 2011).

5. 1. 1 Identification of putative alveolin-like genes

In order to identify additional genes coding for putative alveolins in the genome of *P. falciparum*, three complementary approaches were used: i) Identification of homologues of known *T. gondii* alveolins, ii) blast search of alveolin like proteins using a less stringent conservation of alveolin repeats amino acid motif and iii) phylogenetic profiling.

First, through the homology search of all known alveolins in *T. gondii* (Anderson-White *et al.,* 2011, Gould *et al.,* 2008) we were able to retrieve 7 proteins in the *P. falciparum* genome that are listed in Table 5. 1.

Table 5. 1: Putative alveolins/IMC candidates list identified from homology search of known alveolins present in *T. gondii.*

Toxoplasma **Gene ID**	**Trivial names**	***P.falciparum*** **Gene ID**	**Trivial names**
TGME49_031640	*Tg*ALV1 / *Tg*IMC1	PFC0180c	*Pf*ALV1 / IMC1a
		PFC0185w	*Pf*ALV2
TGME49_031600	*Tg*ALV3 / *Tg*IMC3	PFL1030w	*Pf*ALV3
TGME49_031630	*Tg*ALV4 / *Tg*IMC4	PFE1285w	*Pf*ALV4
TGME49_075670	*Tg*ALV5 / *Tg*IMC15	PF10_0039	*Pf*ALV5
TGME49_026220	*Tg*ALV6 / *Tg*IMC9	MAL13P1.260	*Pf*ALV6
TGME49_039770	*Tg*ALV7 / *Tg*IMC11	PFF1035w	*Pf*ALV7 / *Pf*77
TGME49_053470	*Tg*ALV8 / *Tg*IMC13		

TGME49_060540	*Tg*ALV9 / *Tg*IMC14		
TGME49_024520	*Tg*ALV10 / *Tg*IMC8		
TGME49_024530	*Tg*ALV11 / IMC5		
TGME49_030210	*Tg*ALV12/ *Tg*IMC10		

Second, a genome-wide BLAST search was performed using a reduced stringency of the XSTREAM algorithm to detect alveolin-like repeats with the following parameter (Gould *et al.,* 2010)

E >= 6%, K >= 6%, Q >= 3% the sum of I, L, and V >= 10%

(E- Glutamic acid, K- Lysine, Q- Glutamine, I- Isoleucine, L- Leucine and V- Valine)

The approach uses a seed extension algorithm and seeks the above-mentioned amino acid residues within the preset threshold limit (Newman and Cooper, 2007). This enables the algorithm to identify tandem repeats in the query sequence within the preset 'period number' of 4 and a size of 'copy number' of 3. On finding a putative repeat the algorithm expands the repeat size till the consensus limit of 45% is reached (0.45).

This approach helped in the identification of 17 putative alveolin like proteins listed in table 5. 2:

Table 5. 2: Putative alveolin-like proteins retrieved using the XSTREAM algorithm:

***P.falciparum* / Gene ID**	**Trivial names**
PFE1285w	*Pf*ALV4
PF10_0039	*Pf*ALV5
PFL1030w	*Pf*ALV3
PFC0185w	*Pf*ALV2
MAL13P1.260	*Pf*ALV6
PFF1035w	*Pf*ALV7 / *Pf*77
PFC0180c	*Pf*ALV1 / IMC1a
PF13_0226	
PF14_0290	
PF14_0287	
PF08_0033	
PF14_0168	

PF14_0614	
PF07_0031	
PF11_0431	*Pf*IMC1b
PF10_0283	

Finally, using phylogenetic profiling additional three putative alveolins PF14_0168, PF13_0226 and PF14_0164 could be retrieved.

5. 1. 2 Characterization of two, novel alveolin like proteins: PF13_0226 and PF08_0033

The putative alveolins PF13_0226 and PF08_0033 were both identified by either blast search using a "relaxed" alveolin motif search or by phylogenetic profiling (Fig 5. 1). They have either two (Fig. 5. 4 A) or three (Fig. 5. 5 A) alveolin-like repeats respectively. Further, PF08_0033 has three predicted palmitoylation sites at the C-terminus that might play a role in membrane attachment or protein complex formation (Linder and Deschenes *et al.,* 2007). Previously published expression profiling of both genes does not indicate a strong up-regulation of transcription in late blood stages like other alveolins (www.plasmodb.org).

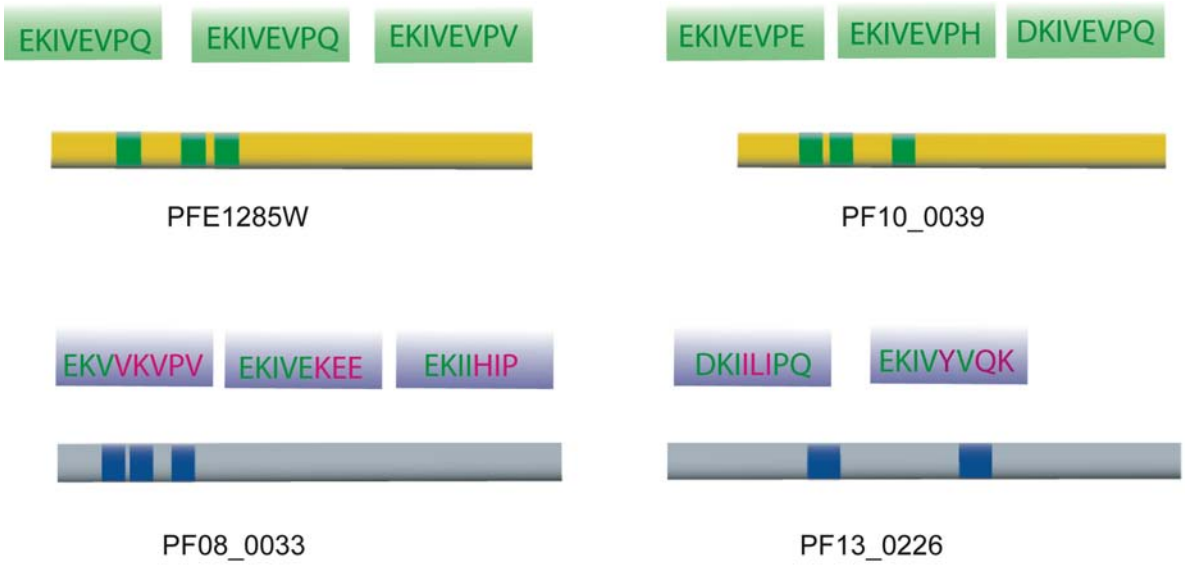


Figure 5. 1: The comparison of the alveolin repeats of the alveolins and alveolin-like: PFE1285w and PF10_0039 have conserved alveolin repeats marked within the green boxes whereas the PF08_0033 and PF13_0226 have alveolin-like repeats (violet boxes), which are comparatively less conserved (unconserved amino acid residues marked with pink).

To initiate the characterization of these two proteins, both were over expressed in *P. falciparum* as GFP fusion proteins. To achieve this, the corresponding genes were PCR amplified, cloned into the transfection vector pARL-GFP (Fig. 5. 2), sequenced to exclude mutations and transfected into 3D7 parasites (Fiddock and Wellems, 1997).

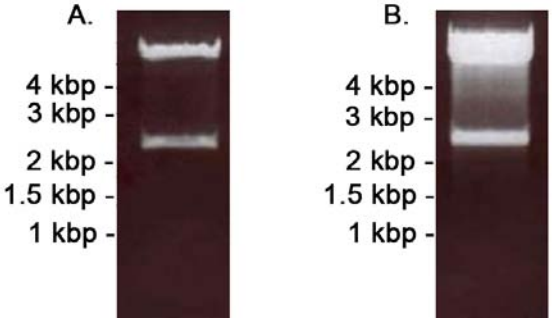


Figure 5. 2: Restriction digestions: The vectors (pARL) with chimeric alveolins (GFP tagged) digested with KpnI/XhoI to release the same. PF13_0226 (1389 bp), (A) and PF08_0033 (1509 bp), (B) fused to GFP (714 bp). 1% agarose gel was used to separate and visualise the restriction-digested product. Ladder depicted in kilo base pairs (kbp).

5. 1. 3 Localization studies of the putative alveolin-like proteins

Expression of both fusion proteins in *P. falciparum* was confirmed using western blot analysis (Fig. 5. 3).

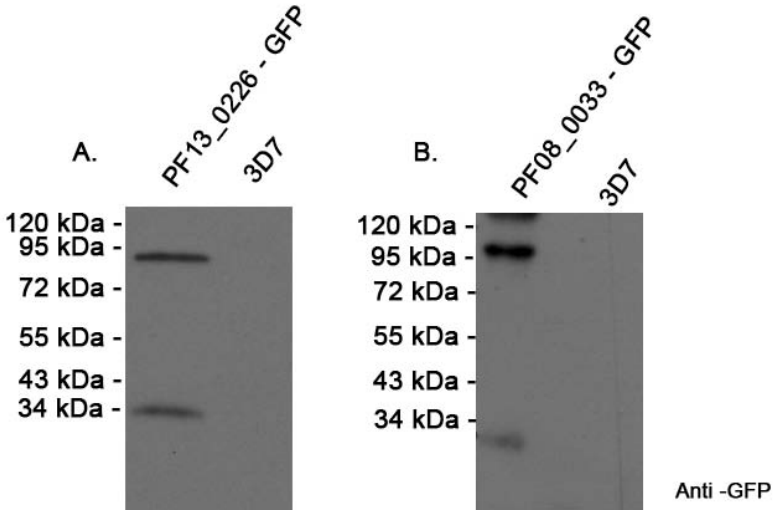


Figure 5. 3: Western blot analysis: Late schizont staged parasites material from either PF13_0226-GFP (A) or PF08_0033-GFP (B) were harvested and subjected to western blot analysis and probed with anti-GFP antibodies which generate bands of 76 kDa and 110 kDa respectively. PF08_033 migrates at a higher molecular weight due to the presence of excessive positively charged amino acid residues.

The localization of the protein in the parasites was assessed by live-cell microscopy during late schizont stages. This revealed a cytosolic distribution of PF13_0226-GFP (Fig. 5. 4 B) and indicated that this protein might not represent an additional alveolin in the parasite. No further studies were done pertaining to this protein.

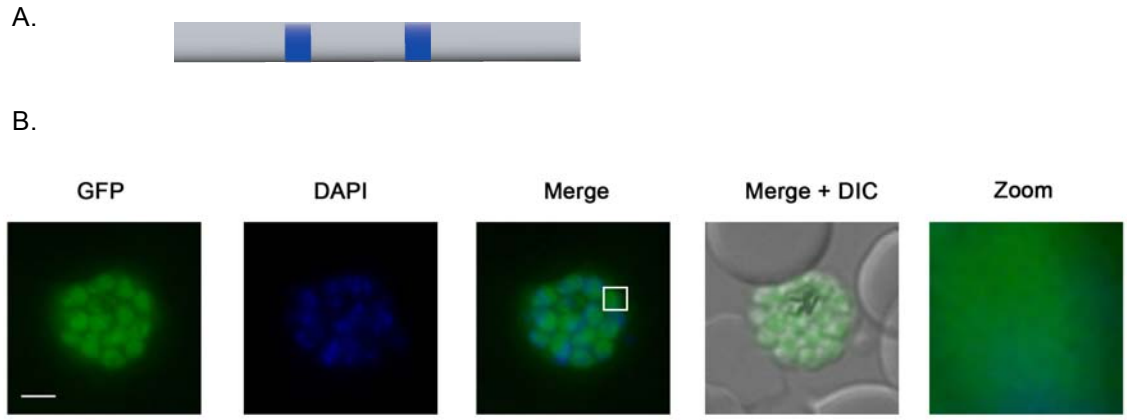


Figure 5. 4: Putative Alveolin-like, PF13_0226: A. The gene has 1389 base pairs in size i.e. 462 amino acids. It has two 'alveolin-like' repeats, marked with blue bars. B. Live cell microscopy of the GFP tagged protein, during schizogony, shows that despite the presence of two-alveolin-like repeats the protein fails to get targeted to the IMC. The white-bordered box marks the area zoomed in. Scale bars, 2µm.

In contrast, PF08_0033-GFP appears membrane attached in late schizont parasites. Unlike other alveolins, PF08_0033 is pronounced towards the basal pole of the nascent merozoites (Fig. 5. 5 B).

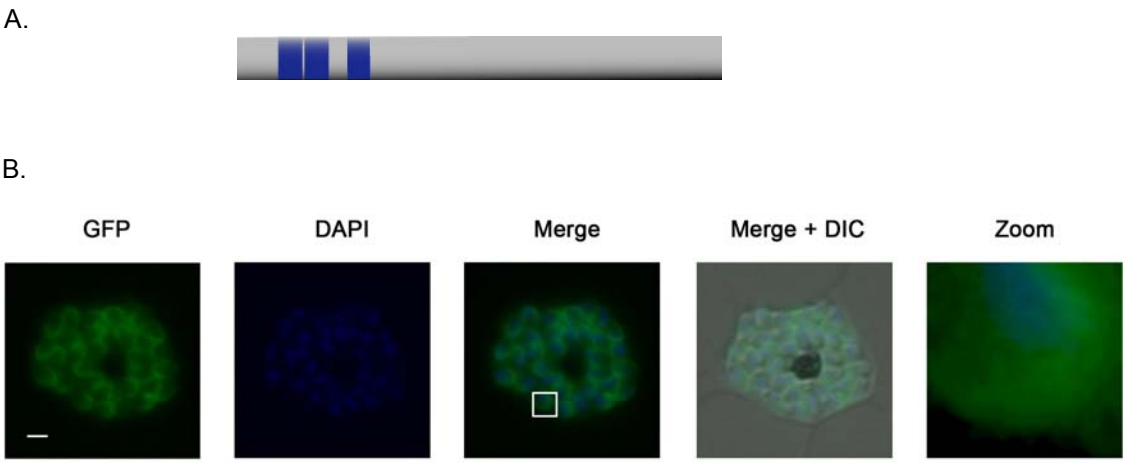


Figure 5. 5: Putative alveolin –like PF08_0033: A. The gene has 1509 base pairs in size i.e. 502 amino acids. It has three 'alveolin-like' repeats, marked with blue bars. B. The protein gets sub- compartmentalized within the IMC at the basal end of the newly formed merozoites in the live cell microscopy after staining the nuclei with DAPI. The white-bordered box indicates zoomed area. Scale bars, 2µm.

5. 1. 4 Co-localization of alveolin PF10_0039 and alveolin-like PF08_0033

To provide evidence of IMC association of the novel alveolin like protein it was co-localized with a previously established alveolin, PF10_0039 (Kono *et al.,* 2012). To achieve this a double transgenic cell-line was generated expressing PF10_0039 as a mchery fusion and PF08_0033-GFP.

Co-localization of the two proteins in late schizont staged parasites revealed some co-localization with PF08_0033 –GFP more prominent at the basal pole of the merozoites formed (Fig. 5. 6).

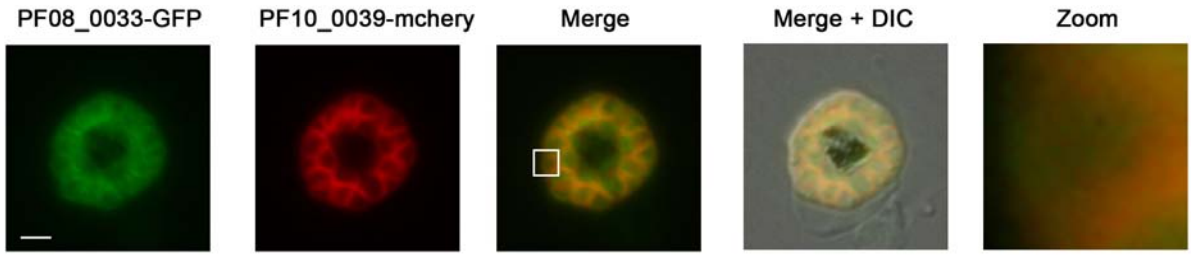


Figure 5. 6: Live Cell-microscopy of PF08_0033-GFP and PF10_0039-mchery: Both PF08_0033 and PF10_0039 show a localization pattern more profound at the basal end of the merozoites. Area zoomed in is indicated by the white-bordered box. Scale bar 2µm.

5. 2 Sequence requirements for membrane attachment of PF08_0033

In order to assess the membrane attachment of PF08_0033 two functional motifs were targeted: the alveolin like repeats and the C-terminal palmitoylation motifs (summarized in Fig. 5. 7). Appropriate mutations were introduced in PF08_0033 and expressed as GFP fusion in the parasite (Fig. 5. 8).

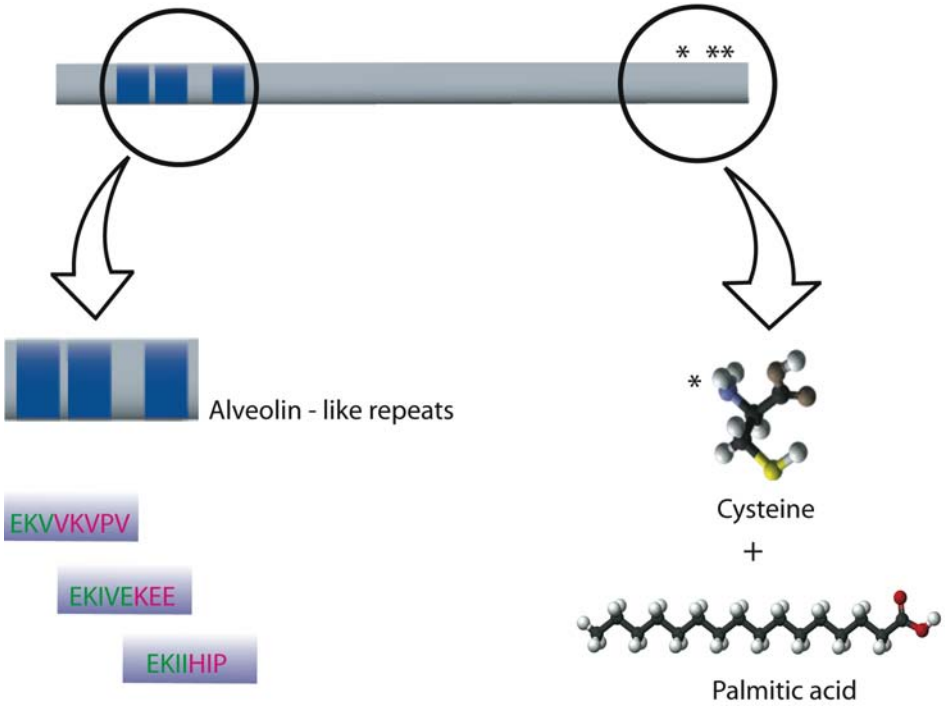


Figure 5. 7: The alveolin-like repeats and putative palmitoylation sites in PF08_0033: The alveolin-like repeats are present towards the N-terminus and the palmitoylation sites (*) at the very C-terminus.

Mutants of the protein, devoid of the conserved amino acid stretches were generated to assess the crucial role of the alveolin repeats (Fig. 5. 8 A, B, C) and the palmitoylation sites mutated to neutral amino acid residue alanine (Fig. 5. 8 D, E, F, G). To do the same the conserved stretches of amino acid residues were deleted and fused to GFP and then transfected into the parasite.

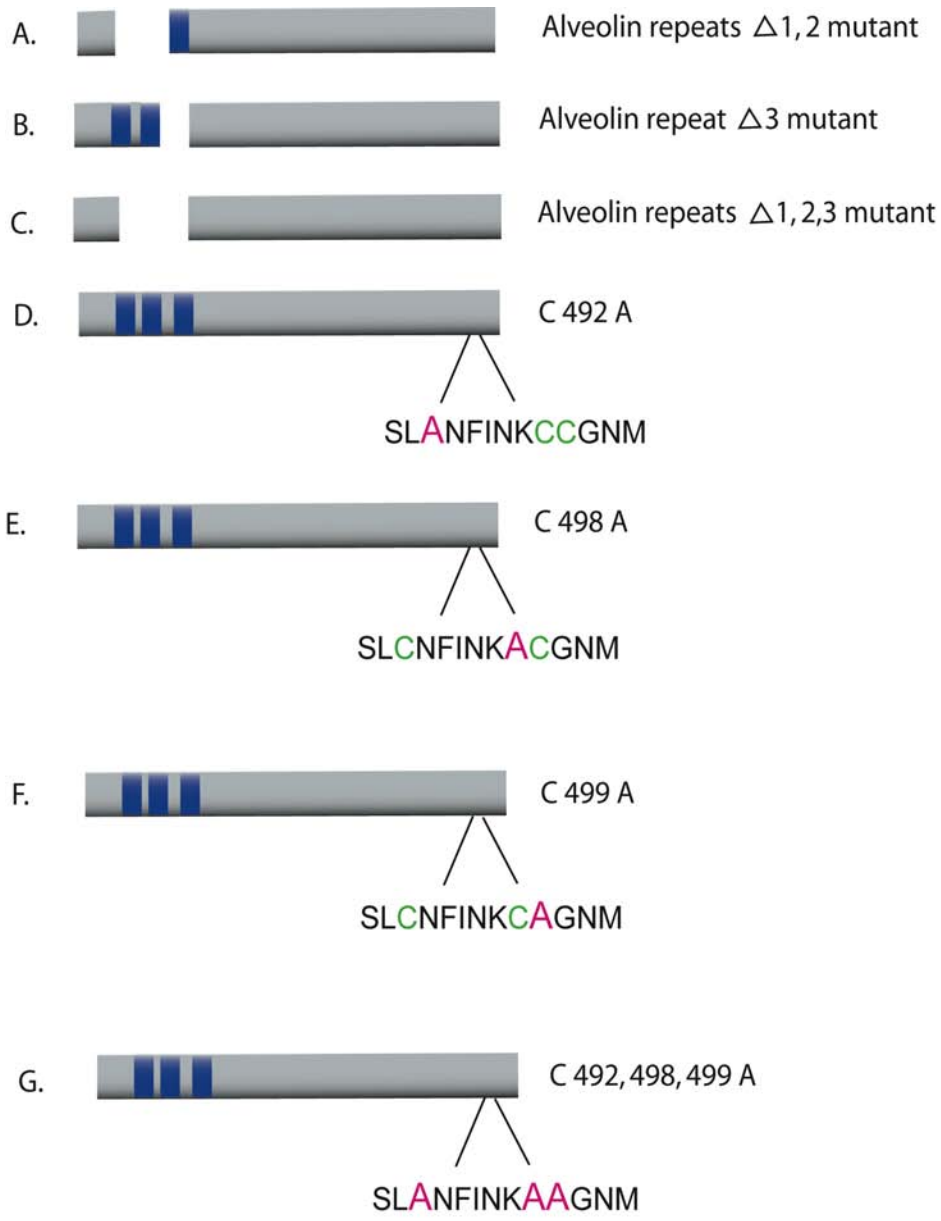


Figure 5. 8: Mutational analysis of putative membrane attachment motifs: Schematic representation of the generated mutants. A: Δ1,2 represent a mutant without alveolin-like repeats 1 and 2, B: Δ3 represent a mutant without repeat 3, C: Δ1,2,3 represent a mutant without any of the alveolin-like repeats, D-G: schematics showing the 'palmitoylation sites' sites towards the C-terminus, cysteine residues mutated to alanine at the positions 492, 498 and 499.

All mutant proteins (Fig. 5. 8) were expressed and localized (Fig. 5. 9) in parasites. The alveolin repeats 1 and 2 deletion mutant and the 'palmitoylation sites' point mutants (C492A, C498A, C499A and C492, 498, 499A) show a similar distribution pattern compared to the wild type protein and appear to get targeted to the IMC pronounced towards the basal pole (Fig. 5. 9 A, B, E, F, G and H). Interestingly the deletion of alveolin-like repeat 3 rendered the protein to a cytosolic distribution (Fig. 5. 9 B). This finding is in line

with the cytosolic distribution of the mutant devoid of all the alveolin-like repeats (Fig. 5. 9 C).

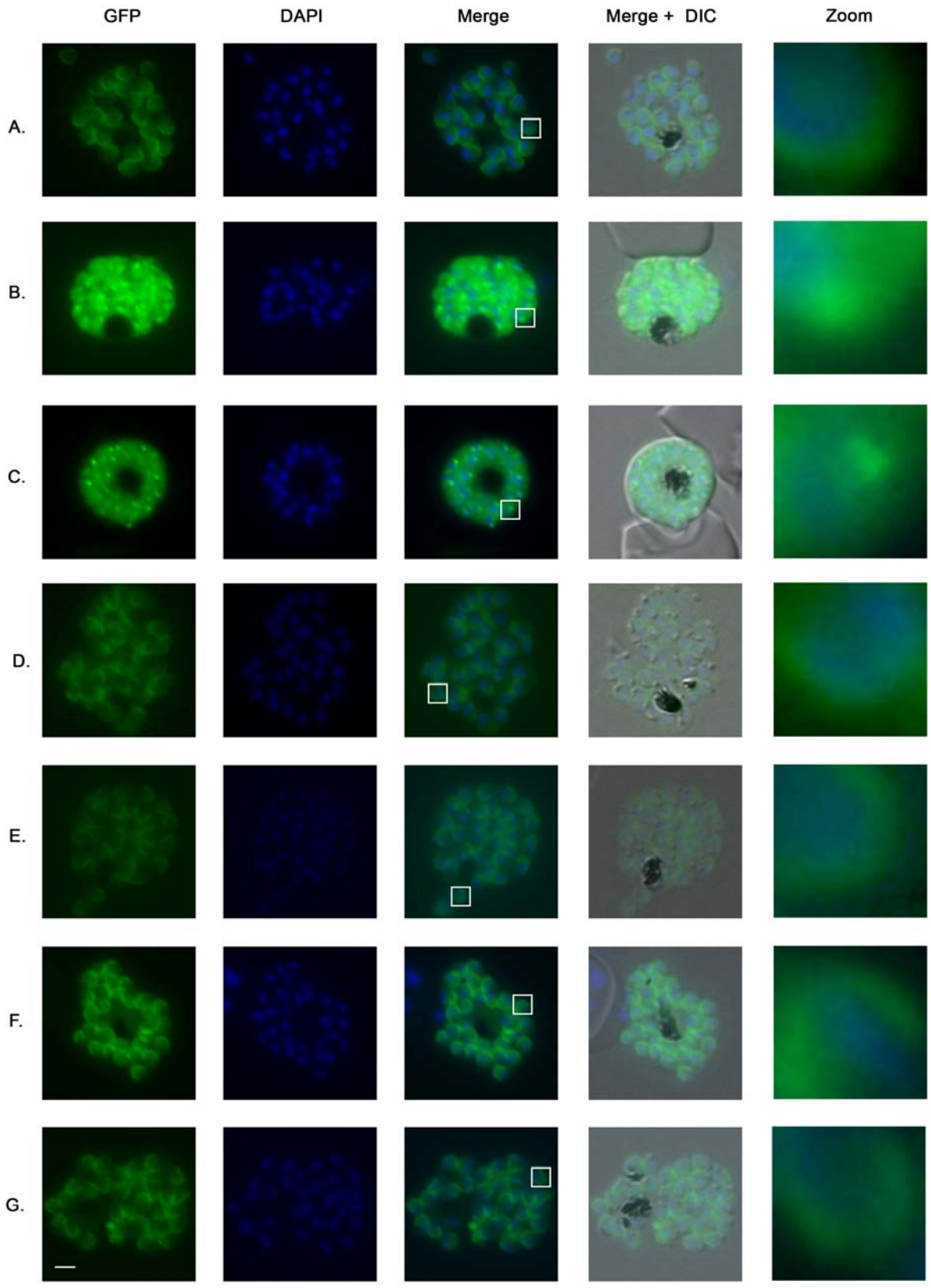


Figure 5. 9: Live-cell microscopy of PF08_0033-GFP mutants: The alveolin repeats 1 and 2 deletion mutant and the 'palmitoylation site' point mutants (C492A, C498A, C499A and C492, 498, 499 A) A, D E, F and G exhibit the same localization pattern like the wild type. The alveolin repeat Δ 3 (B) and alveolin repeats Δ1, 2, 3 (C) mutants do not display localization in the IMC. White-bordered boxes indicate zoomed area. Scale bars, 2µm.

This drastic change in membrane affinity of PF08_0033 was confirmed in solubility assays. While the mutant protein (PF08_0033 alveolin-like repeats 1, 2 and 3 deletion mutant) could only be detected in the hypotonic fraction (Fig. 5. 10 A), PF08-0033 (Fig. 5. 10 B) is like other alveolins (PFE1285w-GFP and PF10_0039-GFP) exclusively present in the insoluble pellet fraction (Fig. 5. 10 C and D).

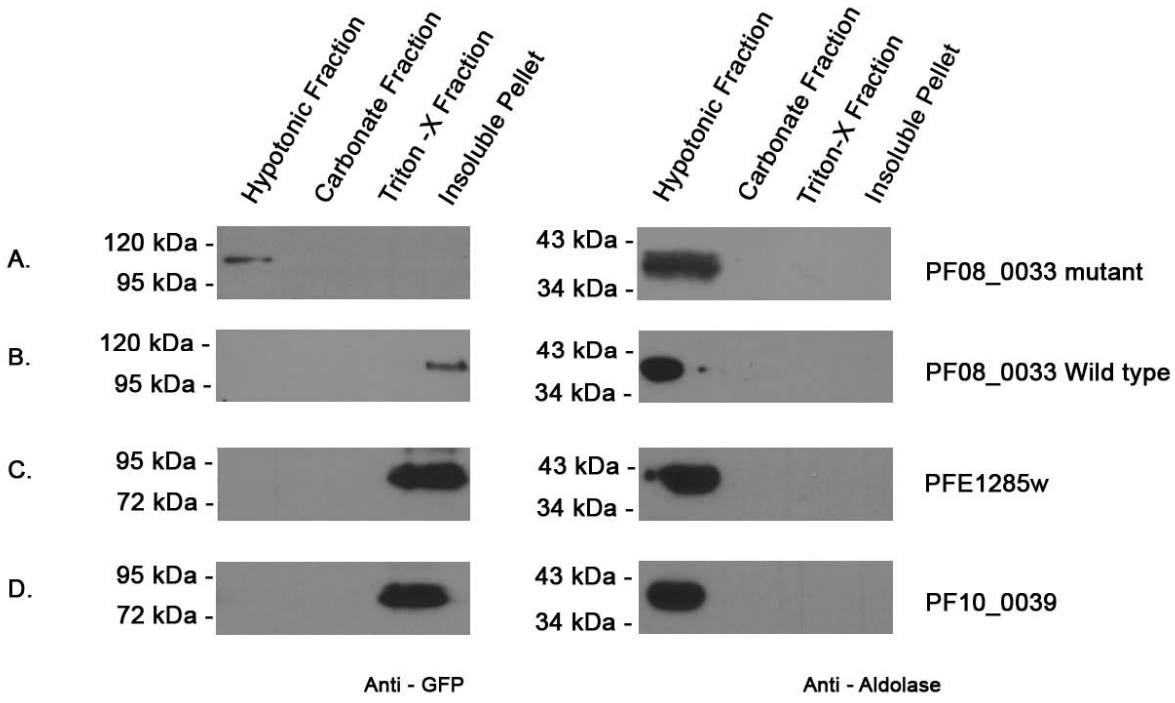


Figure 5. 10: Solubility assays confirmed the role of an individual alveolin repeat for membrane attachment in PF08_0033: The solubility assays were performed using anti- GFP antibodies for protein detection in the individual fractions. The PF08_0033 Δ 1,2,3 was detected in the hypotonic fraction (A) (left side panel) as opposed to the PF08_0033-GFP wild type (B), PFE 1285w-GFP (C) and PF10_0039-GFP (D) respectively (left side panel) which could be detected in the insoluble pellet fraction only. α Aldolase anti-bodies were used as a control for the hypotonic solubilitzation of the cytosolic protein aldolase (right – hand side panel).

The insolubility of all alveolins was additionally confirmed with NP40 detergent (Fig. 5. 11). NP40 is a tergitol-type, non-ionic surfactant water-soluble detergent and is known to solubilize cyotosolic and peripherally attached IMC proteins (Beck *et al.,* 2010). Again, the alveolins and alveolin-like proteins remained insoluble suggesting that they are trapped in the meshwork of proteins that bind strongly to the membrane forming components of a rigid pellicle.

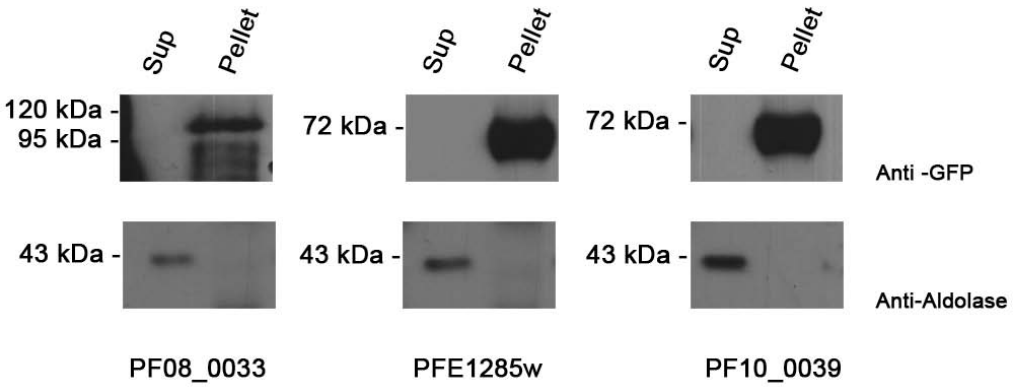


Figure 5. 11: NP40 resistance of PF08_0033 and other alveolins: Parasites expressing PF08_0033-GFP, PFE1285w-GFP and PFE10_0039-GFP were harvested and treated with detergent NP40. None of the GFP fusion proteins got solubilized in NP40. The GFP tagged proteins were detectable in the pellet fraction using α GFP anti-bodies (upper panel). Anti-bodies against the soluble aldolase were used as control for the supernatant (Sup) (lower panel).

5. 3 Tagging Green fluorescent protein to the endogenous locus of PF08_0033

To verify expression of PF08_0033 in blood stages and its localization to the IMC the endogenous locus of PF08_0033 was tagged with GFP. To achieve this a plasmid was constructed that allows the 3' replacement in the endogenous locus by homologous recombination (Fig. 5. 12A). After transfection and selection integration was confirmed by a diagnostic PCR (Fig. 5. 12 B).

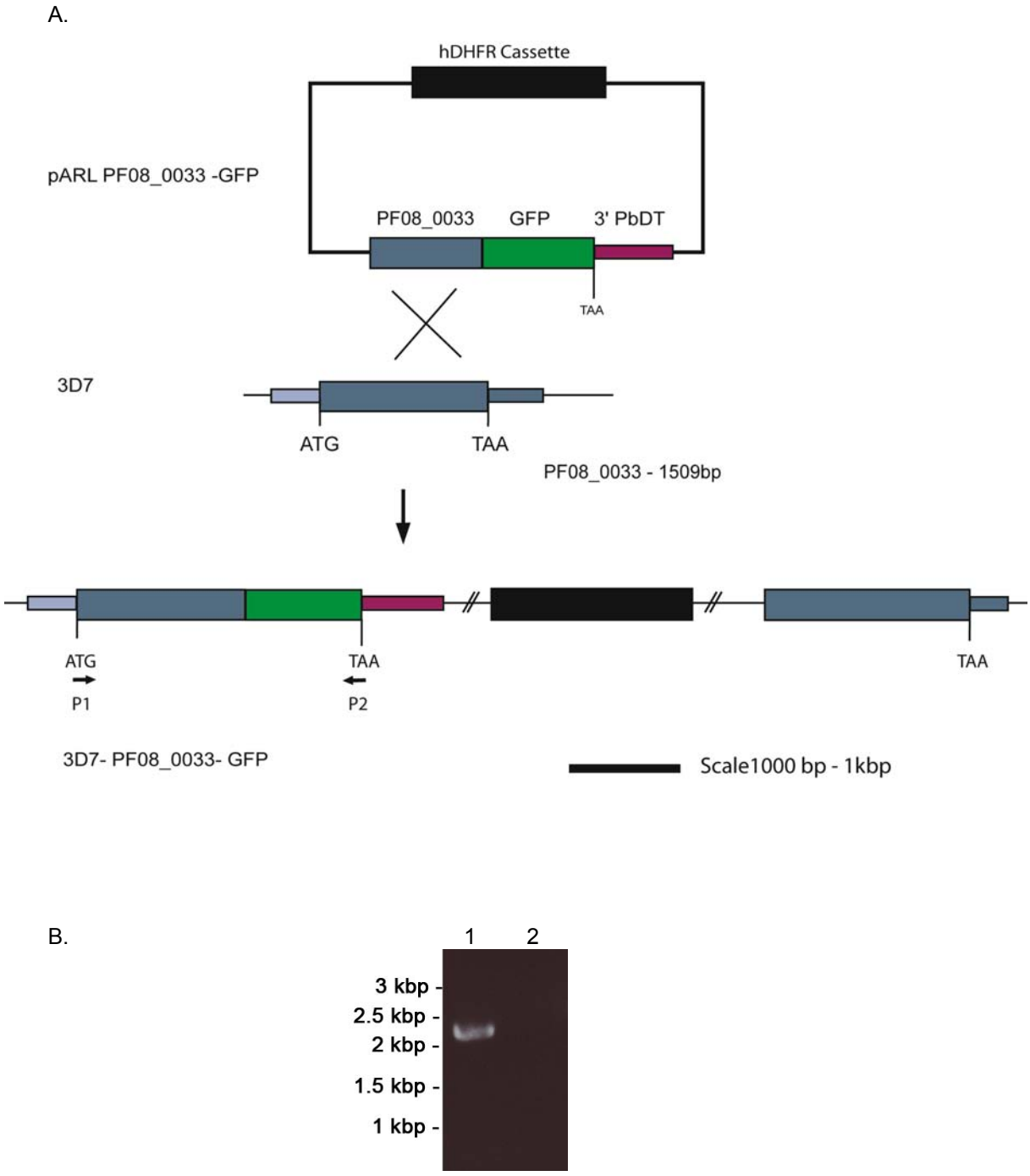


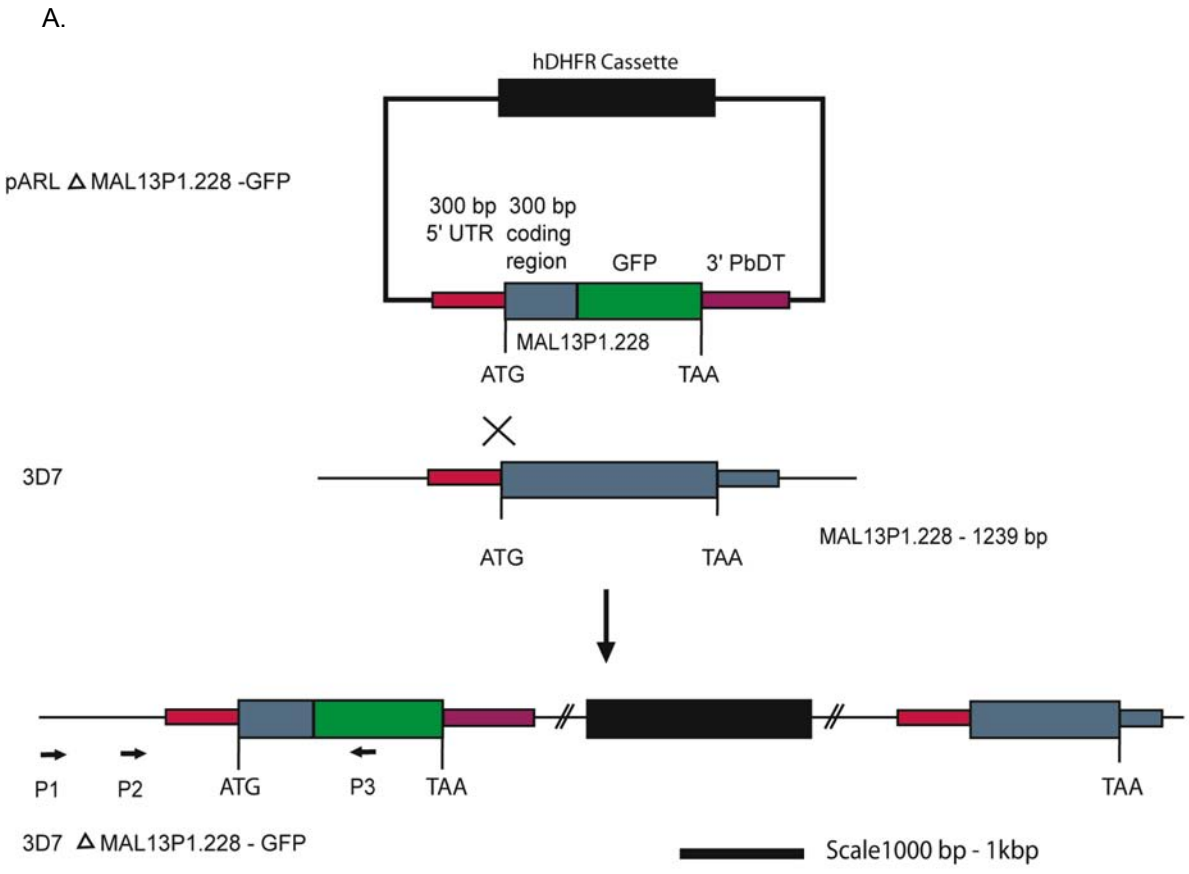
Figure 5. 12: Tagging GFP to the 3' of PF08_0033: A. Schematic representation of 3' replacement in the endogenous locus. 1Kb fragment of the PF08_0033 (grey blue) was fused with GFP (green) and cloned into the expression vector pARL derivate that allows selection with the WR99220 (black box). The open reading frame constituted by the fusion of the 3' end of PF08_0033 and GFP is flanked by the 3'UTR of the of *P. berghei* (pink) Homologous recombination is depicted with a X. Positions of the sense primer (P1) and anti-sense primer (P2) used in diagnostic PCR is indicated. B. Using gDNA derived either from or 3D7-PF08_0033-GFP (1) or 3D7 (2) the primer combination as mentioned in the schematic, a pcr product of 2200 bp was generated in the transgenic only but not in the parental cell line indicating the integration of the GFP into the endogenous PF08_0033 locus.

Although the PF08_0033 locus was successfully tagged with GFP, expression of the PF08_033-GFP protein could not be detected in western blot analysis, live microscopy or IFA. This is consistent with the available expression data and might indicate that PF08_0033 plays a specialized role exclusively in the stages other than the erythrocytic- stage of the parasite's life cycle e.g. during the sexual stages in the mosquito or in sporozoite stage.

Although the PF08_0033 locus was successfully tagged with GFP, expression of the PF08_033-GFP protein could not be detected in western blot analysis, live microscopy or IFA in asexual and presexual blood stages (data not shown).

5. 4 Gene deletion of MAL13P1.228 in *P. falciparum*

The alveolins represent the molecular nexus of the super group '*Alveolata*' and are highly conserved but MAL13P1.228 is so far the only IMC protein in *Plasmodium sp.* that has no homologues in any other apicomplexan or in fact any other organism (Kono *et al.,* 2012). Additionally, as summarized in the introduction it has a unique restricted distribution with in the IMC of gametocytes: It highlights transversal structures that are thought to represent the sutures of individual IMC vesicles in gametocytes (Kono *et al.,* 2012). In contrast, in asexual blood stages it shows the same localization like alveolins or other group B IMC proteins (Kono *et al.,* 2012). Therefore it was hypothesized that MAL13P1.228 plays a pivotal role in gametocytogenesis but might be dispensable for asexual proliferation. To test this, a plasmid vector was generated that mediates through homologous recombination a 900 bp deletion of the endogenous MAL13P1.228 gene and the tagging of resulting cryptic gene with GFP (Fig. 5. 13 A).



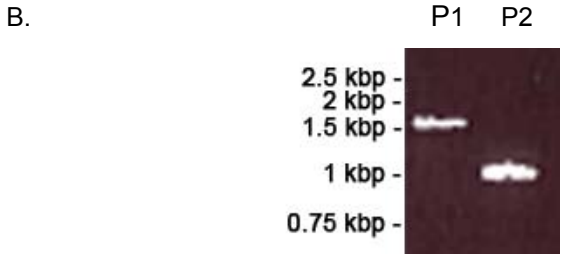


Figure 5. 13: Gene targeting of MAL13P1.228: A. Schematic representation of the gene deletion approach: 600 bp of the MAL13P1.228 gene **(**300 bp from the 5' UTR (red) and 300 b from coding region, grey blue) was fused with GFP (green) and cloned into a pARL derivate that allows selection with the WR99220 (black box). The open reading frame constituted by the fusion of the MAL13P1.228 and GFP is flanked by the 3'UTR of the of *P. berghei* (pink) Homologous recombination is depicted with a X. B. gDNA from 3D7 Δ MAL13P1.228 –GFP was used to perform PCR to confirm proper tagging. (P1) A diagnostic forward primer, P1 (binding at 1000 bp 5' UTR) and reverse primer, P3 binding at 272 bp of GFP generated a product of 1600 bp. (P2) Another diagnostic forward primer, P2 (binding at 400 bp 5' UTR) and a reverse primer, P3 binding at 272 bp of GFP yielded a product of 1000 bp.

After integration of the plasmid was verified by PCR (Fig. 5. 13B), expression of the 3D7 Δ MAL13P1.228 was analyzed by western blot. (Fig. 5. 14).

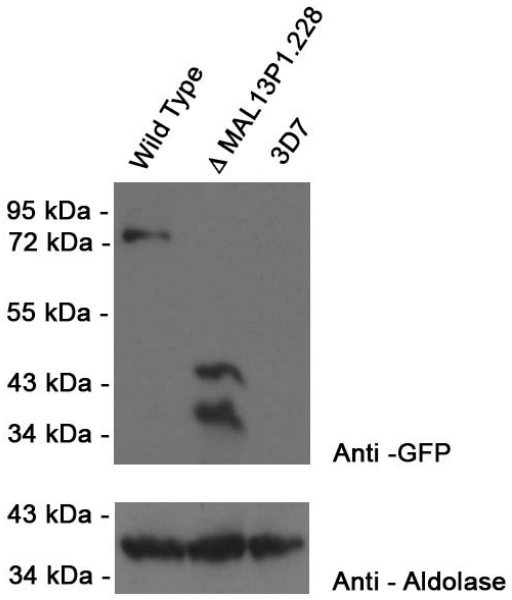
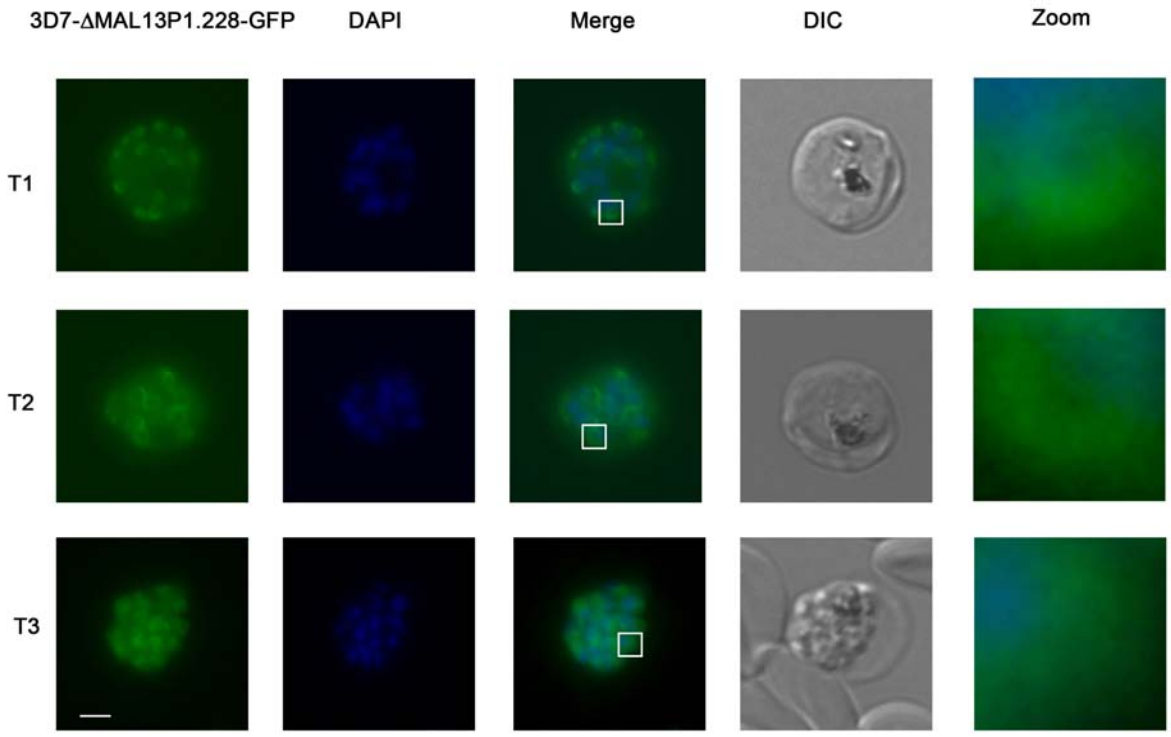


Figure 5. 14: Expression of 3D7-MAL13P1.228-GFP and 3D7-ΔMAL13P1.228-GFP: The western blot analysis of the two cell-lines, MAL13P1.228 (wild type), Δ MAL13P1.228 –GFP. The theoretical MW for the WT-GFP is 82 kDa for the MAL13P1.228-GFP. Using anti-GFP anti-bodies (upper panel) a 82 kDa protein was detected in 3D7-MAL13P1.228-GFP but two proteins of 45 kDa and 38 kDa protein were detectable in 3D7-ΔMAL13P1.228-GFP. As a loading control α aldolase anti-bodies were used (lower panel) which generate bands of 36 kDa of equal intensity (lower panel).

5. 4. 1 Localization and phenotypic consequences of cryptic MAL13P1.228 in asexual stages

Live-cell microscopy was performed with 3D7-Δ MAL13P1.228-GFP (Fig. 5. 15 A) and compared with the full-length MAL13P1.228-GFP expression (Fig. 5. 15 B). In the early phase of IMC biogenesis (T1 and T2), Δ MAL13P1.228-GFP shows a very similar distribution compared with the full-length protein commencing with the characteristic ring like structures that gradually enlarge (T2), although with a much higher cytosolic background. Interestingly, in the late stages the Δ MAL13P1.228 becomes completely cytosolic.

A.



B.

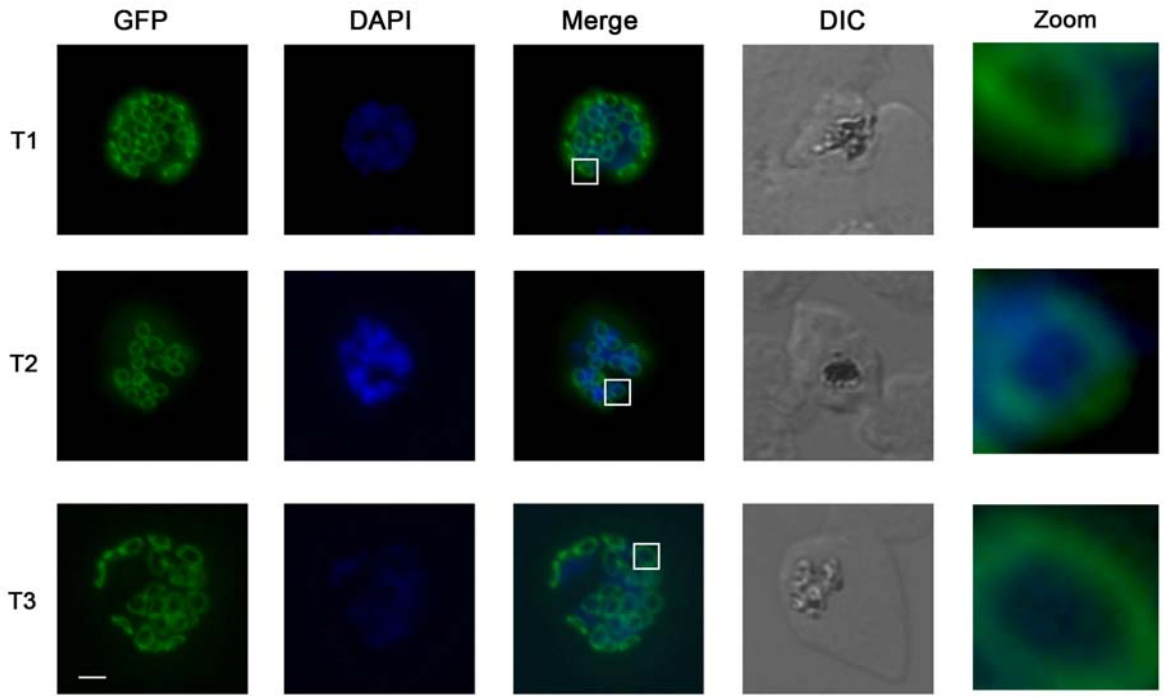


Figure 5. 15: Live cell microscopy of ΔMAL13P1.228-GFP (A) and wild type MAL13P1.228 (B): The distribution of both proteins during the early phase of schizogony (T1 and T2) is identical, although the Δ MAL13P1.228-GFP is accompanied by a cytosolic pool. In later stages (T3) the truncated protein is exclusively cytosolic (T3, Panel A) in contrast to the wild type protein that is localized with the IMC in the periphery of the nascent merozoite (T3, Panel B). White-bordered boxes indicate zoomed area. Scale bars, 2µm.

These striking differences in membrane association during the late stages can be also shown in solubility assays (Fig. 5. 16). While the full-length protein remains during schizogony in the insoluble fraction the truncated protein is already in the early stage partially in the hypotonic fraction and in late stages nearly exclusively.

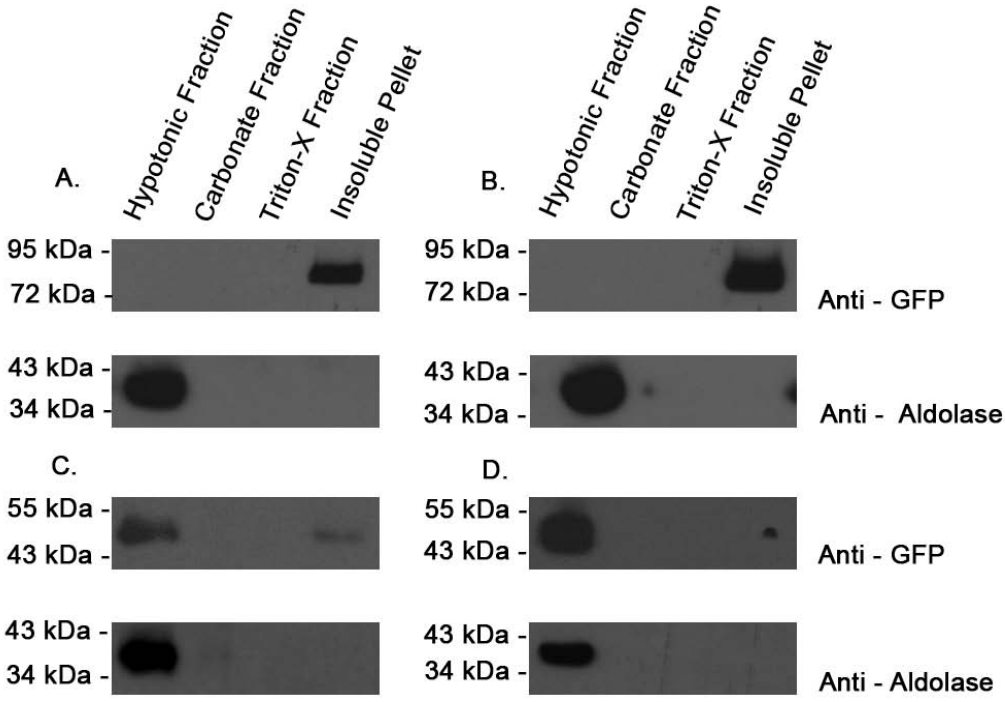


Figure 5. 16: Solubility assays of full length and Δ MAL13P1.228-GFP: Immunoblots from the solubility assay performed with the wild type MAL13P1.228-GFP (A and B) cell line during two time points, early (left side) and late schizont (right side) using anti-GFP anti-bodies. While MAL13P1.228 is always in the insoluble pellet fraction, the Δ MAL13P1.228-GFP on the other hand could be detected in hypotonic and pellet fractions during early schizont stages(C) and loses the attachment to membrane completely during the late schizont stages (D). Anti-aldolase anti-bodies were used as a control for the hypotonic fractions.

5. 4. 2 Localization and characterization in asexual stages

To further probe in the aberrant localization of truncated MAL13P1.228 and to visualize any putative impact of this truncation on IMC biogenesis, co-localization studies were performed with an independent IMC marker protein PFD1110w. This protein was previously described as a glideosome associated 6-transmembrane protein (Bullen *et al.,* 2009) and is classified as a group A protein (Kono *et al.,* 2012). As shown in Fig. 5. 17, the truncation of MAL13P1.228 does not lead to a visible perturbation of the IMC biogenesis and as shown in live microscopy membrane attachment of the truncated protein is completely lost in late stages.

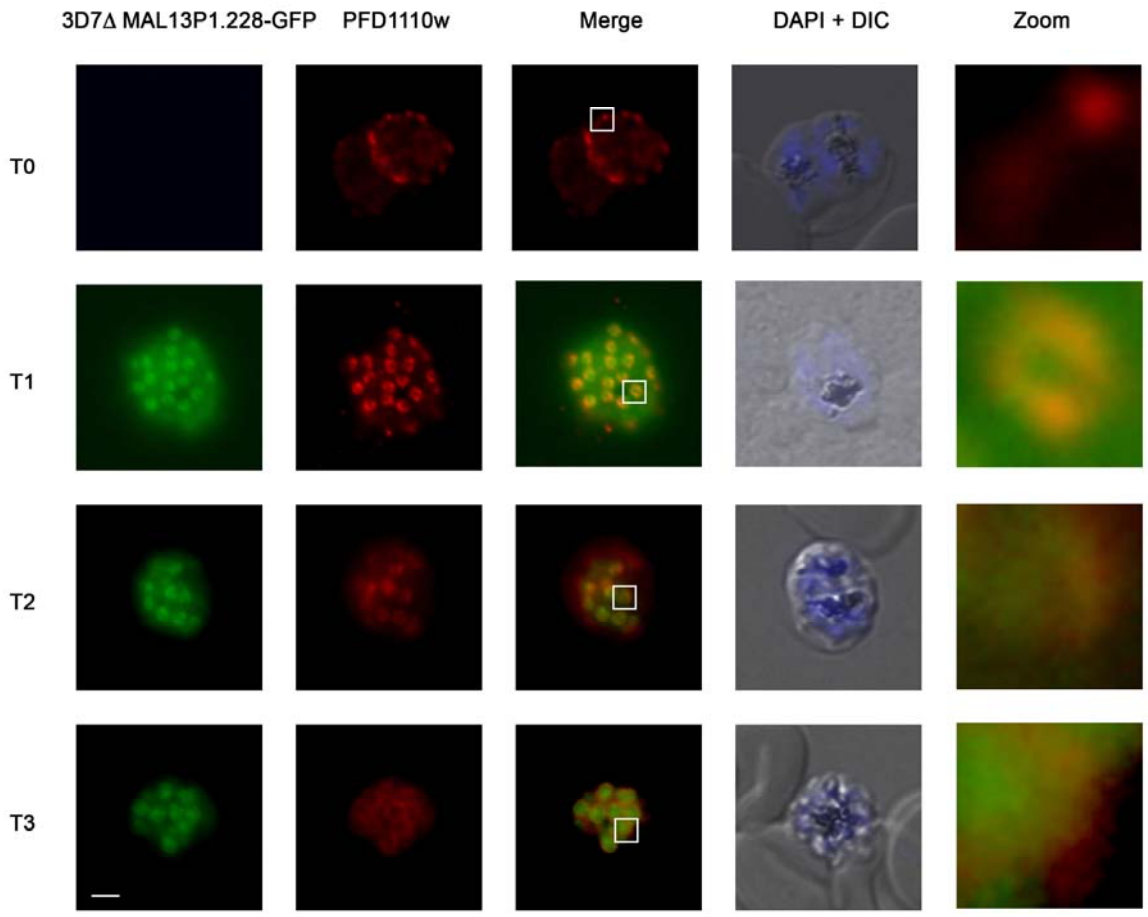


Figure 5. 17: Co-localization of 3D7-ΔMAL13P1.228-GFP with the IMC marker PFD1110w: The localization of 3D7-ΔMAL13P1.228-GFP shows the formation of rings during the T2 and T3 stages accompanied by a cytosolic pool. During the T4 stage the truncated protein is completely cytosolic. The antibodies against PFD1110w stain the unperturbed IMC, from the onset of its biogenesis to complete maturation. Scale bars, 2µm.

5. 4. 3 Proliferation of 3D7-ΔMAL13P1.228-GFP during asexual development

3D7-ΔMAL13P1.228-GFP has no obvious proliferation defect compared with 3D7-MAL13P1.228-GFP expressing parasites during its continuous cultivation. In order to probe into this notion a growth competition assay was performed where both transgenic parasites lines were co-cultured for 20 cycles. The parasitemia of both the cell-lines was initially adjusted to 1% and before they were mixed. After 20 cycles of schizogony, the parasites were harvested and a western blot analysis was performed. Both the proteins, full length and cryptic MAL13P1.228, appear to be expressed in the same quantity (Fig. 5. 18).

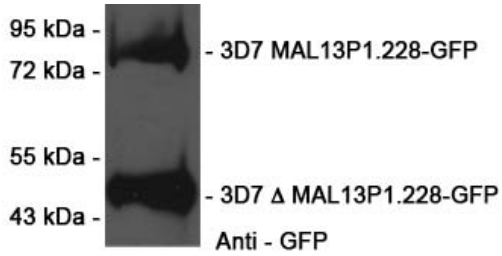


Figure 5. 18: Growth competition assay of 3D7-MAL13P1.228-GFP and 3D7-ΔMAL13P1.228-GFP in co-culture: Parasite material was harvested and subjected Western blot analysis. Two proteins could be detected by anti-GFP anti-bodies: A 82 kDa (full length 3D7-MAL13P1.228-GFP) and a 45 kDa (3D7-ΔMAL13P1.228-GFP), respectively.

5. 4. 4 Localization of truncated MAL13P1.228 during gametocytogenesis

To assess whether the truncated MAL13P1.228-GFP cell-line could undergo gametocytogenesis presexual differentiation was induced by starvation (please refer to section 4.4.10 in Material and Methods). Although the cryptic MAL13P1.228-GFP is cytosolic during all presexual differentiation stage this process proceeds in the 3D7-ΔMAL13P1.228-GFP-parasite line identical compared with 3D7-MAL13P1.228-GFP-cell line (Fig. 5. 19A and B).

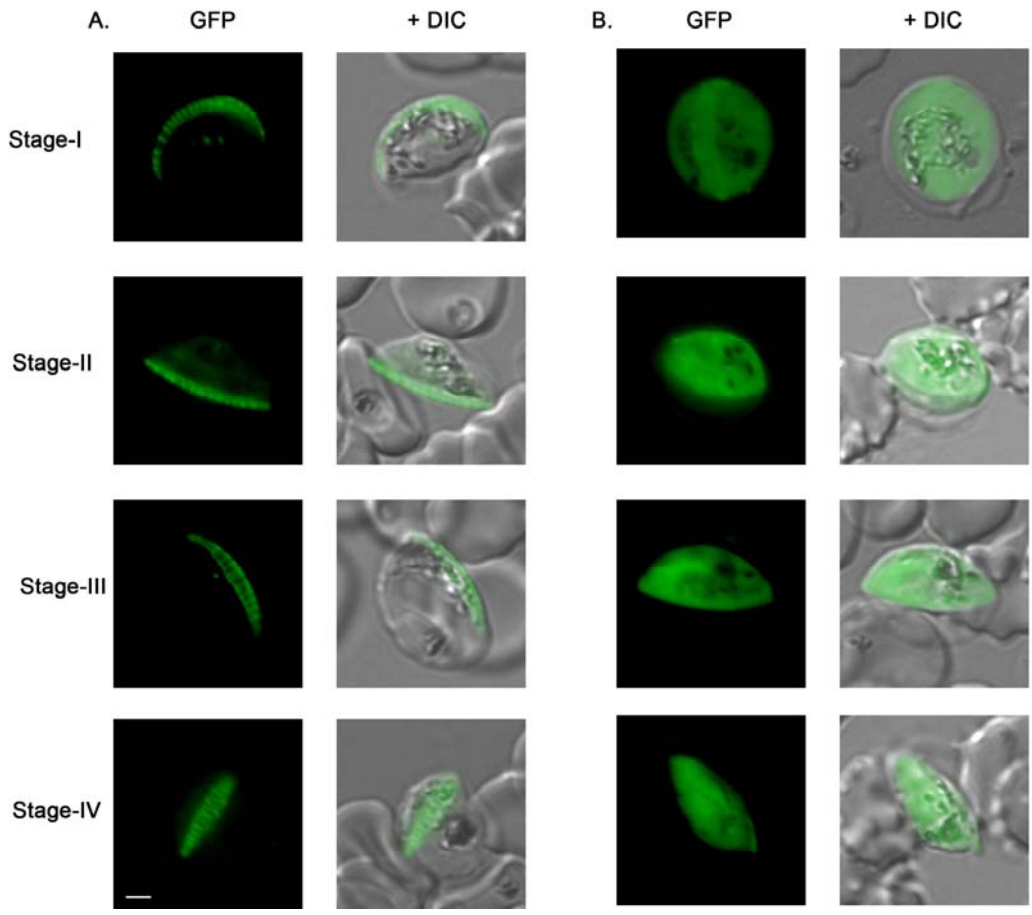


Figure 5. 19: Gametocytogenesis in 3D7-MAL13P1.228-GFP and 3D7-ΔMAL13P1.228-GFP parasite lines: Gametocytes of both transgenic parasite lines where harvested at different time points. A. The wild type of the protein displays a characteristic transversal structure. B. The 3D7-Δ MAL13P1.228 –GFP predominantly remains cytosolic in all stages of gametocyte. Scale bars, 2µm.

5. 4. 5 Localization and impact of MAL13P1.228 truncation during gametocytogenesis

Although truncation of MAL13P1.228 shows no recognizable morphological effect on gametocytogenesis a putative defect in IMC biogenesis was analyzed in the 3D7-ΔMAL13P1.228-GFP-cell line. To achieve this all gametocytes stages were harvested subjected to immunofluorescent assay (IFA) with an independent IMC marker protein (PFD1110w). As previously described IMC proteins except MAL13P1.228 tested so far revealed a restricted localization in early stages of gametocytogenesis (Kono *et al.,* 2012), forming a spine-like structure with longitudinal orientation (Fig. 5. 20). This defined flank of the spherical gametocyte is converting into the straight axis of the parasite as it transforms into the characteristic D-shaped stage II from. From here the defined structure expands congruent with the longitudinal growth of the cell (stage III) and spreads along the inner face of the parasite. Importantly, the IMC biogenesis visualized by anti-bodies directed

against PFD1110w is not impaired in the 3D7-ΔMAL13P1.228-GFP-cell line (Fig. 5. 21). This data might indicate, that MAL13P1.228 has a redundant function not only during asexual proliferation, but also during presexual differentiation.

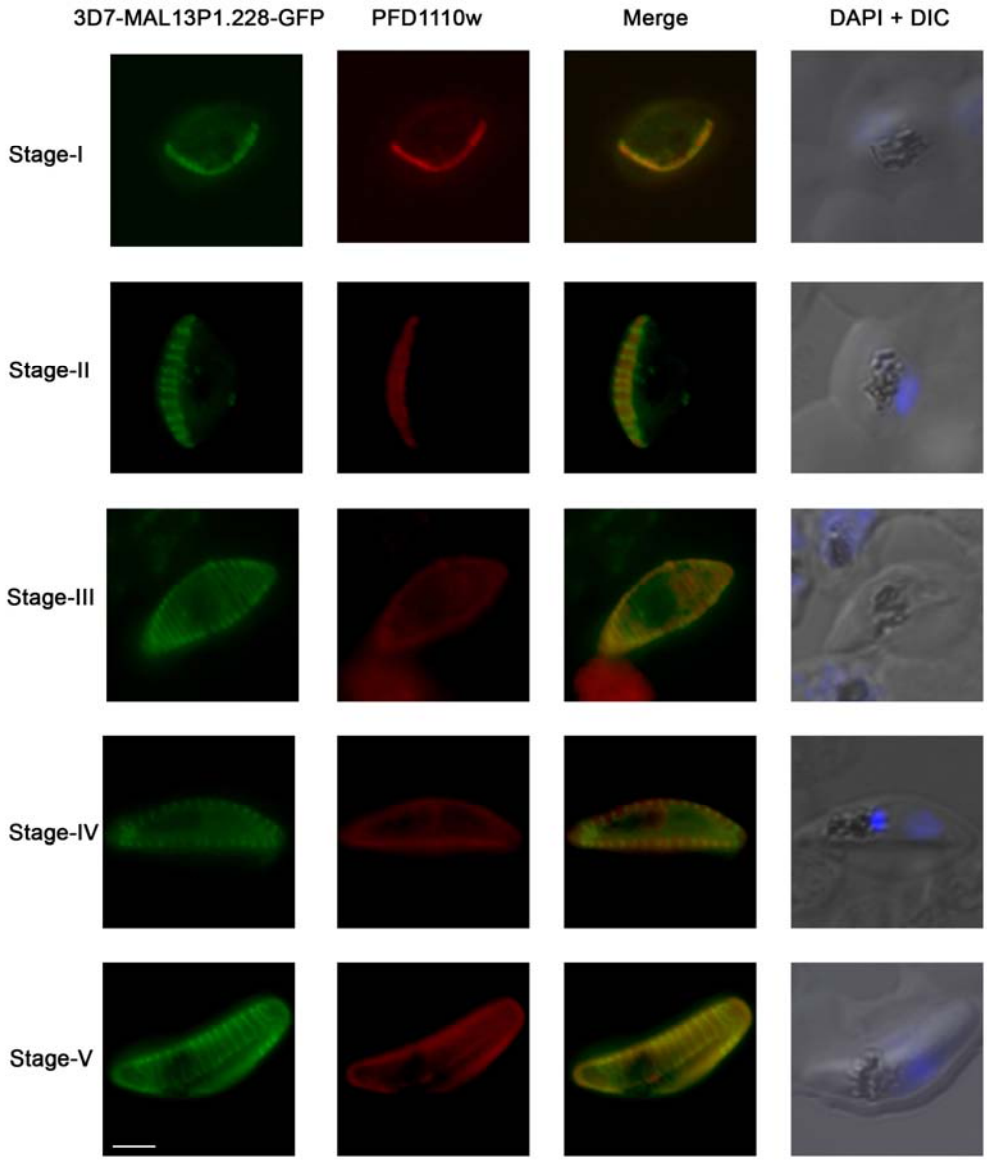


Figure 5. 20: Co-localization of full length MAL13P1.228 with the IMC marker PFD1110w: Immunofluorescent assays using gametocytes of 3D7MAL13P1.228-GFP were performed in conjunction with α PFD1110w antibodies (red). 3D7-MAL13P1.228-GFP protein highlights sutures that are expanding from a restricted transversal structure in early stage (stage I-II). These symmetric sutures are expanding with ongoing maturation of the gametes (stage III-IV). Co-localization of MAL13P1.228 with the TMD protein PFD1110w (red) shows their spatial relation in stage II and stage IV gametocytes. Scale bars, 2µm.

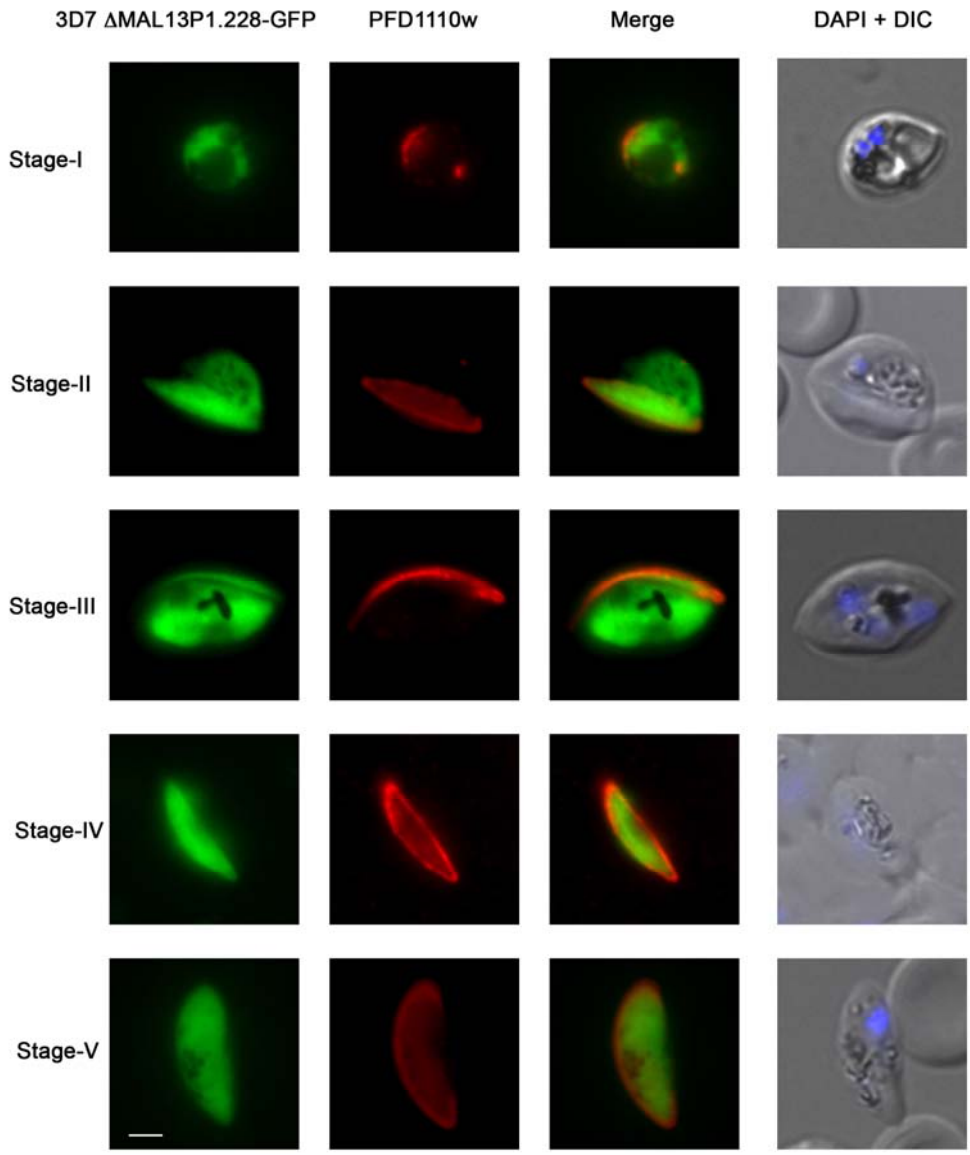


Figure 5. 21: Co-localization of 3D7-ΔMAL13P1.228-GFP with the IMC marker PFD1110w: Immunofluorescent assays using gametocytes of 3D7-Δ MAL13P1.228-GFP were performed in conjunction with α PFD1110w antibodies (red). While the IMC visualized by anti-PFD1110w antibodies is unimpaired, 3D7-Δ MAL13P1.228-GFP is only cytosolic. Scale bars, 2µm.

5. 5 Studies of inducible protein degradation as a tool for the functional analysis of IMC proteins.

FKBP-based inducible protein degradation has bee proven to be a usable instrument in the functional characterization of proteins (Armstrong *et al.,* 2007, Dvorin *et al.,* 2010; Russo *et al.,* 2008). In order to establish this system for the functional analysis of IMC proteins, GFP was fused with the DD domain and expressed under the control of *crt*-promoter (Fig. 5. 22).

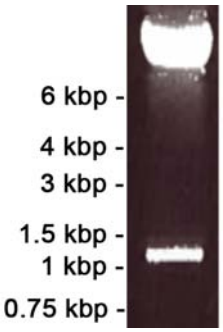
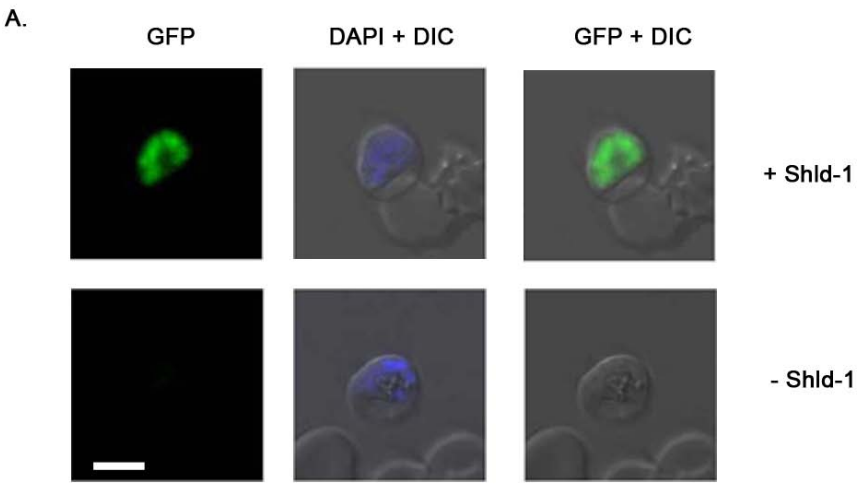


Figure 5. 22: Restriction digestion- The vector (pARL) with GFP-DD expressed under crt-promoter, digested with NotI/XhoI to release the same.1% agarose gel was used to separate and visualise the restriction-digested product, GFP (714 bp) and Destabilization domain (300 bp).

The plasmid was transfected in parasites. Expression in presence or absence of Shld-1 was monitored by microscopy (Fig. 5. 23 A) and western blot analysis (Fig. 5. 23 B).



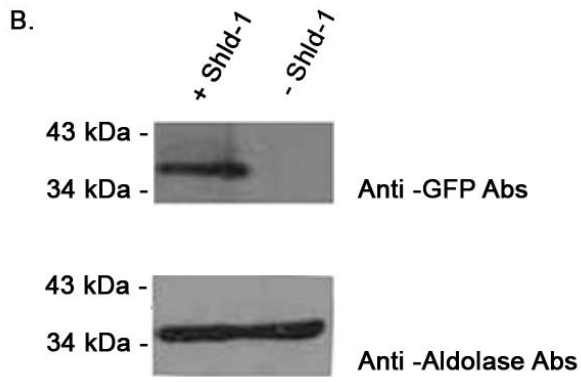


Figure 5. 23: Expression of cytosolic GFP-DD in presence or absence of Shield-1: (A) Live cell microscopy of expression of GFP-DD with (+ shld-1) and without (- shld-1) Shield-1 in trophozoite stage. Scale bars, 2µm. (B) Immunoblot for the same parasite material using anti-GFP anti-bodies. As a loading control anti-bodies against aldolase were used.

After establishing the system in the laboratory, two IMC proteins were attempted to be targeted by the over-expression and subsequently induced degradation of the tagged IMC proteins. Implying a dominant-negative effect this degradation might also lead to a degradation of the endogenous protein might get impaired. MAL13P1.228 and the alveolin PFE1285w were chosen because i) both proteins are peripheral membrane proteins and therefore accessible for the protein degradation machinery of the parasite and ii) allows the re-investigation of the apparent dispensability of MAL13P1.228 (please refer to 5. 4).

In order to achieve this the plasmid pARL-PFE1285w-GFP-DD and pARL-MAL13P1.228-GFP-DD were constructed and sequenced.

The plasmids were transfected into the parasite and expression of the GFP-DD tagged proteins were monitored by microscopy (Fig. 5. 24 A) and western blot. The expression levels of the both proteins were unimpaired by the presence or absence of Shld-1 (Fig. 5. 24 B).

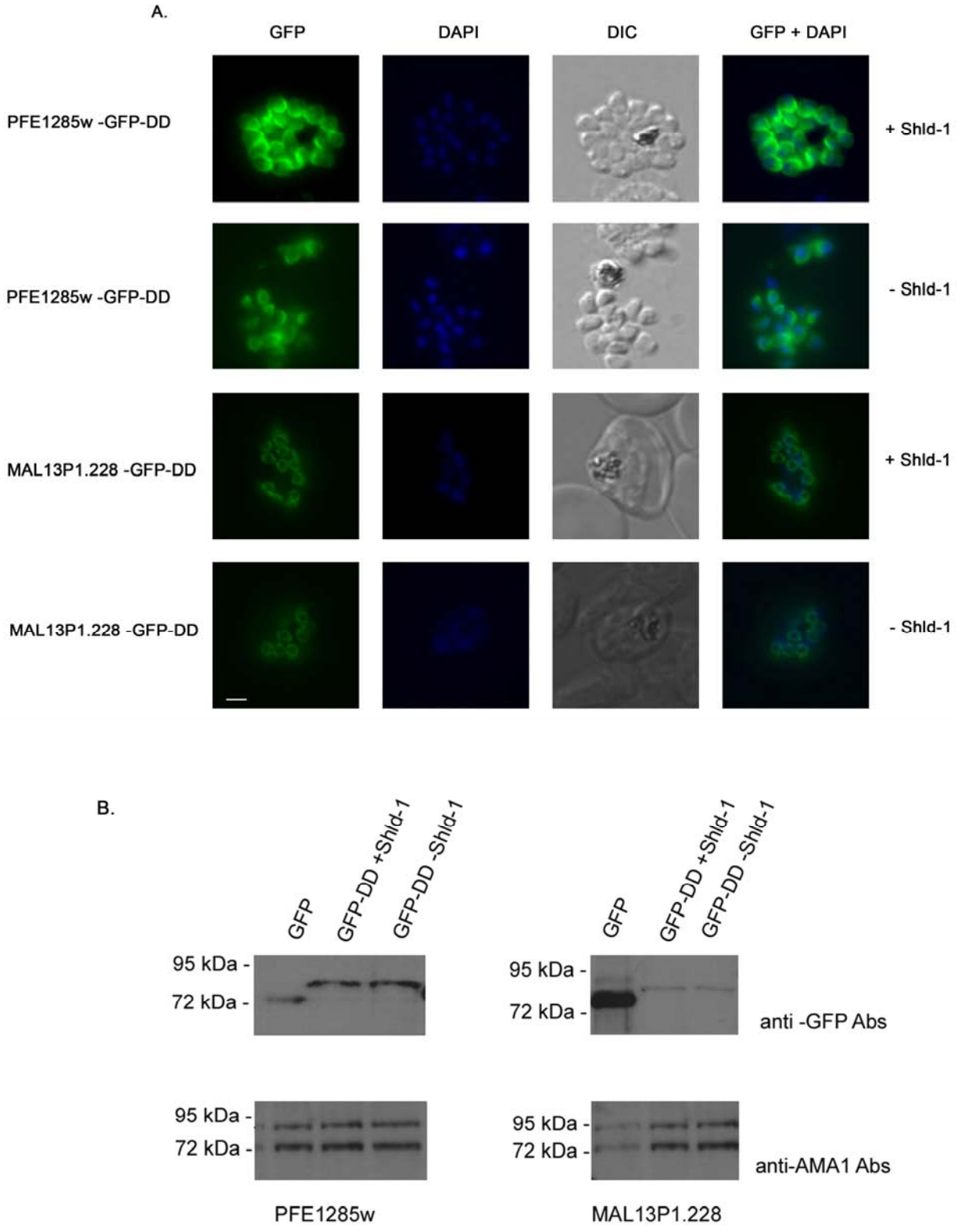


Figure 5. 24: Expression of GFP-DD tagged IMC proteins PFE1285w and MAL13P1.228 in the presence or absence of Shield-1: A. Live cell microscopy revealed that the GFP-DD tag does not interfere with the IMC localization of both proteins and is not impaired by the removal of Shld-1. Scale bars, 2µm. B. Western blot analysis of the two proteins with GFP-DD tag maintain a similar expression level with or without Shld-1 (upper panel), as shown by the bands generated when probed with anti-GFP anti-bodies. As loading control, anti-bodies against AMA-1 were used (lower panel).

6. Discussion:

The Inner Membrane Complex (IMC), the only unifying feature of the members of the *Alveolata* cluster, is a highly specialized flattened cisternal compartment, compressed against the plasma membrane and helps define the morphological feature of the cell (Allen 1971; Lee and Kugrens 1992; Morrissette and Sibley 2002). In Apicomplexans, it plays a critical role in host cell invasion and cytokinesis (Striepen *et al.,* 2007; Agop-Nersesian *et al.,* 2009). The composition of the IMC varies significantly among the individual members comprising the superfamily, suited for the adaptation according to the environment they dwell in (Kono *et al.,* 2012, 2013), but we are still far away from a comprehensive overview and functional understanding of the proteome associated or integrated with the IMC.

6. 1 Evolution of the IMC and its proteins contents

Phylogenetic analysis confirms the monophyletic origin of the members of the infrakingdom *Alveolata* (Baldauf *et al.,* 2000; Wolters 1991). Despite this common origin, the members of this superphylum have profound diversity in the manner of obtaining their nutrition e.g. photoautotropism, intracellular parasitism and predation. The majority of the species of the alveolata can be categorized and placed in the phylum of ciliates, dinoflagellates and apicomplexans. Some of the lineages like Colpodella, Chromera, Rastrimonas, Parvilucifera and Perkinsus cannot be broadly distinguished and classified into any of the three major phyla mentioned above. These organisms bear features that give a glimpse of the evolution of the alveolates (Leander and Keeling 2003; Cavalier-Smith and Chao 2004; Siddall *et al.,* 2001). The information generated from the gene sequences of rRNA of the small subunit from Colpodellids suggest they are very closely related to the apicomplexans and form a sister group (Kuvardina *et al.,* 2002). Perkinsids, in a similar manner constitute the sister lineage group of the dinoflagellates. It is henceforth believed that along with the apicomplexans and dinoflagellates, these two sub-phyla (colpodellids and perkinsids) bequeathed the common characteristic features from a common ancestor. (Kuvardina *et al.,* 2002; Leander and Keeling, 2003, 2004; Leander *et al.,* 2003; Saldarriaga *et al.,* 2003; Moore *et al.,* 2008). Together with this common ancestor, the present day apicomplexans, dinoflagellates, colpodellids and perkinsids form a clade referred to as ‘Myzozoa’ (Cavalier-Smith and Chao, 2004). This primogenitor was supposedly a myzocytotic predator, which bore two flagella, trichocysts, rhoptries, micronemes, a polar ring and a conoid (Kuvardina

et al., 2002). From this piece of information it appears that this predecessor organism was most likely characterized by the presence of sac-like alveoli as well that formed a continuous layer just under the plasma membrane and has been maintained in the present day alveolata members. This is upheld by the family of proteins called alveolins, which is not only associated with the alveoli but also is common among the alveolates (Gould *et al.,* 2008).

6. 2 Alveolins and related proteins

The alveolins belong to a unique family of proteins that is ubiquitous throughout the diverse clade of alveolata (Gould *et al.,* 2010). These proteins are marked by the presence of highly conserved iterations of amino acid stretches commonly termed as alveolin repeats. Alveolins form structural units of the subpellicular network (SPN), which is attached to the cytoplasmic side of the IMC. The SPN is a network of intermediate filaments (IF) that forms a cytoskeletal basket (Volkmann *et al.,* 2012; Tremp *et al.,* 2013). The first alveolin reported and characterized in *Toxoplasma gondii* (*Tg*ALV1), is believed to form coiled-coil domains that give rise to a 10 nm filamentous network lying in close association with the cytosolic face of the IMC and is found between the subpellicular microtubules (Mann and Beckers, 2001). Mutational studies pertaining to *Tg*ALV1 elucidate that it is a key player in maintenance of the cell shape as the parasites devoid of this alveolin had reduced mechanical strength and displayed hampered motility (Mann *et al.,* 2002). Investigations indicate that the N-terminus of *Tg*ALV1 has sites for palmitoylation and myristoylation whereas the C-terminus possibly bears a motif for prenylation, which help this alveolin to attach to the IMC (Mann and Beckers, 2001). The other reported alveolins in *Toxoplasma*, *Tg*ALV3 and *Tg*ALV4 are also highly conserved. The flanking termini of the alveolins do not share any homology and interestingly these alveolins display a similar sub pellicular localization (Gubbels *et al.,* 2004).

The alveolin homologs traced in the *Plasmodium* genome have been identified to be essential for normal motility and morphogenesis (Tremp *et al.,* 2011; Volkmann *et al.,* 2012). The homolog of *Tg*ALV1 in *Plasmodium berghei* (*Pb*ALV1) further confirmed the role played by the alveolins in defining the cell shape and maintaining the tensile strength (Khater *et al.,* 2004). From a minimum of 2 alveolin-repeats in PFF1035w (*Pf*ALV7) to 13 such repeats in PFC0185w (*Pf*ALV2), the members of the alveolin family have a variable

number of repetitive domains and exhibit a huge difference in their sizes within the protein family and yet serve the same designated function (Gould *et al.,* 2008; Kono *et al.,* 2013).

Interestingly, not all *Apicomplexans* have orthologs of the classical alveolins that are found in most of the members of this supergroup. *Theileria spp.* lacks some of these key alveolins and possibly it has evolved its own repertoire of proteins that compensate for the alveolins (Kono *et al.,* 2012).

The classical alveolins are marked with repetitions of conserved amino residues of polar hydrophilic- E and K that form hydrogen bonds and aliphatic – I, V and P which are non-polar hydrophobic. Another group of proteins referred to as 'Articulins' characterized by the presence of conserved repetitive motifs (VPVPV) shares a striking similarity with the alveolin domains. This group of proteins is a key constituent of the fibrous epiplasm, seated underneath plasmalemmal structure, found in the euglenoids like *Euglena sp.* and ciliates like *Pseudomicrothorax sp.* (Marrs and Bouck 1992; Huttenlauch *et al.,* 1998). Studies show that antibodies raised against the repetitive domains of articulins cross-react with proteins in Dinoflagellates that localize in the cortical regions (Huttenlauch *et al.,* 1998). This likely indicates a common ancestry or a merger of the phyla in the course of evolution (Gould *et al.,* 2008).

A uniquely different group of ciliates, Euplotide (*Euplotes aediculatus*) has the cytoskeletal elements localized inside the lumen of the cortical alveoli, unlike the other members of the Ciliata phylum which have the cytoskeletal elements around the alveoli (Kloetzel *et al.,* 2003). One such group of cytoskeletal proteins reported in Euplotide is 'Plateins' which carries iterations of conserved amino acid residues (EVVPDV) that bears a comparable homology with the alveolin repeats found in alveolins (Kloetzel *et al.,* 2003). However in contrast to the classical alveolins, plateins carry a signal peptide and possibly get incoporated into the membrane of alveoli via vesicular mode of transport. These revelations could suggest that plateins are paralogs of alveolins. Interestingly some fungal members of the phylum Basidiomycete have been reported to possess two proteins that are similar to alveolins and carry similar repetitive domains (EKIVEVP). The functional assessment of these two proteins akin to the alveolins has not been established yet. However it is believed the presence of these proteins in the fungal cells is a result of horizontal transfer of genes (Gould *et al.,* 2008).

The presence of the families of proteins like articulins and plateins, which have a passing resemblance with the alveolins and contribute to more or less a similar function of maintaining the cellular shape and integrity, give a hint of the possibility of existence of more family of proteins related to the classical alveolins. In this study we have identified IMC proteins that bear iterations similar to those of alveolins but are significantly less conserved. This extends the family of alveolins to 'Alveolin-like' proteins, which also form a part of the pellicle in *Apicomplexans*. In the present study a seed extension algorithm, which forms the backbone of the XSTREAM software, was used to identify more putative members of the alveolin family (Gould *et al.,* 2008). The minimum preset value for the "consensus match" in the XSTREAM software $V^{1.7}$ is 80%. This consensus match value used to identify the tandem repeats in classical alveolins was lowered and set at 50% and the "minimum period" value was set to 10 (Gould *et al.,* 2010). Pertaining to this study both the values were further reduced to 45% and 4 respectively. This, however, detected putative alveolin-like proteins and at the same time opened the possibility of inclusion of false hits in the list of alveolin-like proteins due to the lowered stringency values of the algorithm. With relaxed settings of 'consensus value' and 'minimum period' to seek tandem repeats forming the alveolin-like domains, the algorithm generated a list of alveolin-like proteins which included PF13_0226 and PF08_0033, which have been analysed in this study. The classical alveolins were also picked up by the search analysis as they fulfilled the search conditions. However, the amino acid composition of the alveolin-like domains of these two putative alveolins display a certain level of disparity when scrutinized under the light of the classical alveolin domains.

6. 3 Evolution of the alveolins in *Plasmodium spp.*

The phylogenetic investigation of alveolins so far reported in *Plasmodium spp.* give an insight of their evolution at different time points in the clock of evolution. A well-conserved alveolin PFE1285 (*Pf*ALV4) has homologs present across the eukarya, pointing to its ancient ancestry and it subsequently evolved as an IMC protein (Kono *et al.,* 2012). The other alveolin PFF1035w (*Pf*ALV7) is found exclusively among the members of Apicomplexans suggesting its origin after the divergence of Apicomplexans from Ciliates and Dinoflagellates. In case of alveolins like PFC0180c (*Pf*ALV1), PFL1030w (*Pf*ALV3) and PF10_0039 (*Pf*ALV5, part of this study) no homologs are found other than in few insect

species, which possibly could have resulted due to the phenomenon of lateral transfer of genes between the two species (Kono *et al.,* 2012).

The phylogenetic metanetwork for the alveolins suggests that PF13_0226 is closely related to a *Cryptosporidium* protein. As a result of duplication event during the evolutionary bifurcation of phyla *Cryptosporidium* and *Coccidia*, PF13_0226, a conserved protein with alveolin-like repeats came into existence. PFF1035w (*Pf*ALV7) also evolved as an ortholog of *Cryptosporidium* alveolin lineage during this split of the two phyla and is an established alveolin in the *Plasmodium* family (Kono *et al.,* 2012). The homology search of PF13_0226 indicates the precise conservation of the novel protein across *Alveolata* family and the homologs possess the designated function of the alveolins. Previously characterized alveolins in *Toxoplasma sp.*, TGME49_020270 (IMC6), Alveolin1 in *Neospora caninum*, Alveolin2 in *Tetrahymena thermophila*, a Ciliate and Alveolin3 of *Dinoflagellates* (*Karlodinium veneficum*) represent homologs of PF13_0226 (Anderson-White *et al.,* 2011; Gould *et al.,* 2008; Reid *et al.,* 2012). Possibly during the divergence of the phylum *Plasmodium* from rest of the *Alveolata* members, PF13_0226 lost its function as an alveolin/IMC protein, which is justified by the cytosolic localization pattern of this protein as shown in this study. The amino acid residues that compose the alveolin-like repeats one and two of PF13_0226 are DKIILIPQ and EKIVYVQK. The alveolin repeats essentially help the proteins to get targeted to the IMC, which appear to be degenerate in case of PF13_0226 as a consequence of which the protein fails to get incorporated into the IMC.

The evolutionary studies of the alveolins support PFC0185w (*Pf*ALV2), PFL1030w (*Pf*ALV3) as well as PFE1285w (*Pf*ALV4) and PF08_0033 as paralogs of one another (Kono *et al.,* 2012). The lack of orthologs of PFE1285w and PF08_0033 in other species suggests that the two seemingly evolved in the Aconoidasida lineage before the divergence of the Haemosporidia from Piroplasmida (Kono *et al.,* 2012). PF08_0033 annotated as 'membrane skeletal protein IMC1-related' can be an intermediate between the classical alveolins and the articulins, which form a major portion of the eukaryotic peripheral membrane skeleton (Marrs and Bouck 1992). Homologs of PF08_0033 are restricted to the Apicomplexans only, namely the membrane skeletal protein-IMC1 in *Eimeria necatrix* and alveolin domain containing intermediate filament-IMC8 in *Toxoplasma spp.* which is an indicative of its evolution after the cluster of genus comprising *Plasmodium spp. Toxoplasma spp. Babesia spp.* and Coccidians had split from the Dinoflagellates and

Ciliates. Though a paralog of PFE1285w (*Pf*ALV4), PF08_0033 is a much newer addition to the IMC/Alveolin repertoire. The level of expressions, spatial and temporal dynamics displayed by the two during schizogony of erythrocytic development of the parasite are also very much different. Like any other classical alveolins, PFE125w (*Pf*ALV4) appears as ring-like structure in mid stages of schizogony and at the terminal stages, it accumulates uniformly at the periphery of the nascent merozoites formed (Kono *et al.,* 2012). On the other hand PF08_0033 seemingly remains untranscribed. However, the localization of PF08_0033, when expressed episomally under the control of an artificial promoter (*ama-1*), is aggregated at the basal end of the merozoites. This observation provides a glimpse of a possible functional divergence of the two paralogs.

6. 4 Differential expression of the IMC resident proteins

Previously characterized IMC proteins in *Plasmodium spp.* were mostly co-translationally expressed and predicted to be implicated in invasion process by PlasmoINT (Hu *et al.,* 2010). Therefore the IMC proteins so far studied were expressed under the control of the *ama-1* promotor in order to mimic their natural promoters (Kono *et al.,* 2012). However with the identification of new members of the IMC it was established that not all of these proteins get upregulated during schizogony. The *Plasmodium* IMC proteins can be classified based on their available transcriptional profiles generated from their microarray data (www.Plasmodb.org). Depending on the stage of the parasite life cycle the genes coding for IMC proteins display a significant variation in their expressions as depicted in the chart below (Fig. 6.1), primarily targeting the asexual erythrocytic stage and the sexual gametocytic stages.

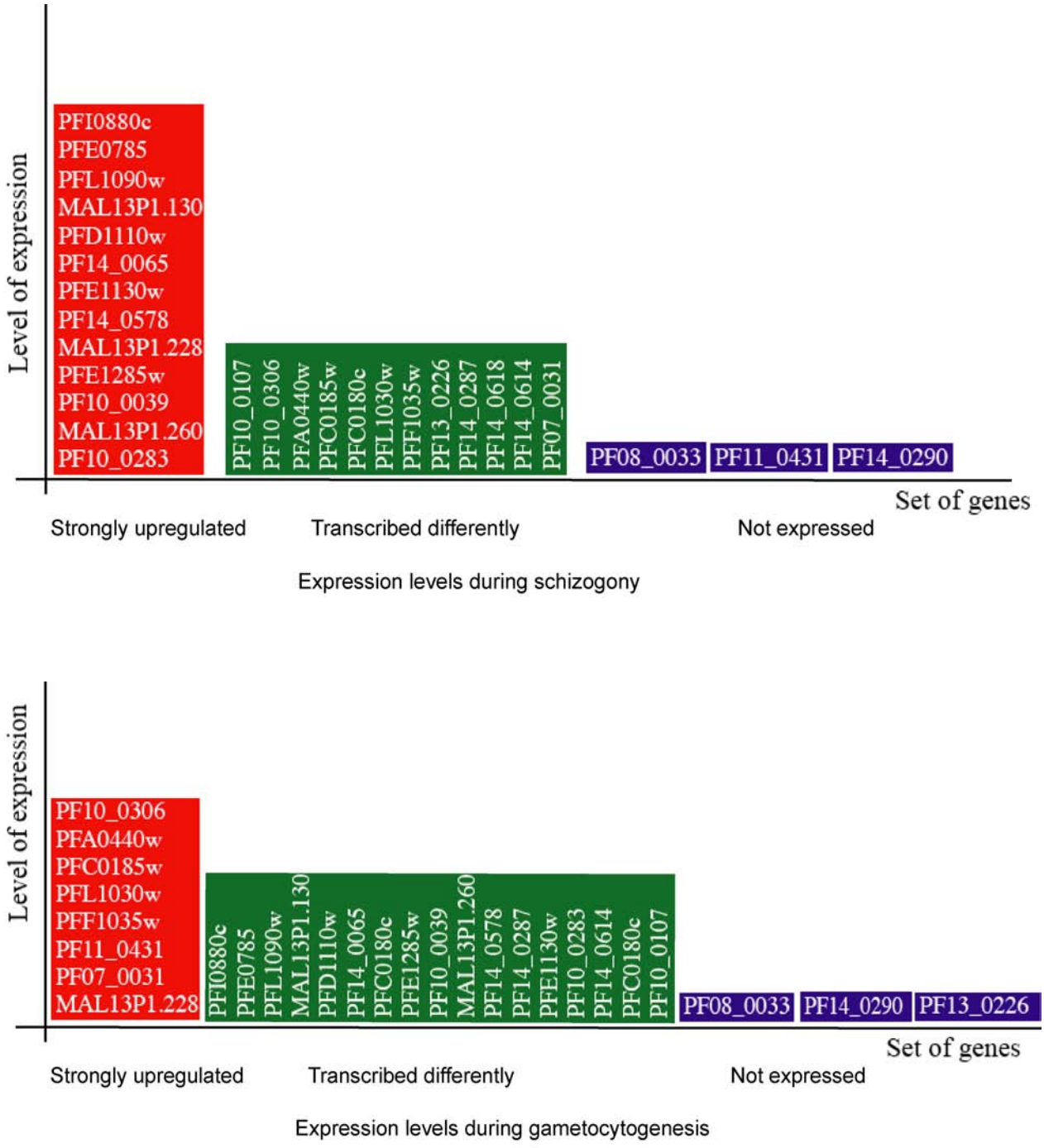


Figure 6. 1: Variation in the expression of different IMC proteins/Alveolins: The microarray profiles clearly indicate the disparity in the levels of expression of the various IMC proteins so far identified. The upper panel represents the expression profile during schizogony and the lower panel during gametocytogenesis. Red-bar marks the strongly upregulated set of genes, green bar signifies the genes that are transcribed differently, independent from one another and the genes denoted in the purple bar represent the ones that remain untranscribed.

This tabulation of the IMC/Alveolins indicates that most of the alveolins that get strongly upregulated during schizogony namely PFE1285, PF10_0039 and MAL13P1.260, do not get transcribed at the same levels during the sexual stages (Kono *et al.,* 2012). Whereas the alveolins that have a relatively inconspicuous expression during the intra-erythrocytic development stages, show a massive upregulation during the gametocytic stages. This group includes alveolins like PFC0185w, PFL1030w and PFF1035w. However, PFC0180c is the only alveolin, which is expressed in trace amounts during schizogony, and in sexual forms its expression is marginalized. The change in the expressions of the alveolins during different stages is indicative of their functional diversification within the sub group of the protein family. Most of the alveolin-like proteins maintain extremely low levels of expression through out the life cycle of the parasite. The same was proven with the 3' replacement of the gene, PF08_0033 with a GFP tag. The expression was not detected in any of the stages of the parasites life cycle. However, the localization of the protein when expressed under an artificial promotor (*ama-1*) was comparable to that of any other alveolin. During the evolution as a paralog of PFE1285w, PF08_0033 retained the alveolin repeats but possibly lost its functionality and owing to which the gene remains unexpressed. The only candidate from this family of proteins is PF10_0283, which is expressed notably in the asexual stage. During the sexual stages however, its expression drops notably and two other alveolin-like proteins, PF11_0431 and PF07_0031 mark their presence with strong levels of expression.

The alveolins are known to form an integral part of the pellicle and maintain the cell shape. The sexual forms of the parasite are much bigger than the asexual ones and the IMC in the former is a patchwork of these cortical vesicles. It is likely that the tensile strength required by the protozoan cell during the different stages varies significantly depending on the morphology it acquires. The disparity in the upregulation of the alveolins and alveolin-likes is possibly attributed to these profound changes in the cellular architecture of the parasite (Fig. 6. 2).

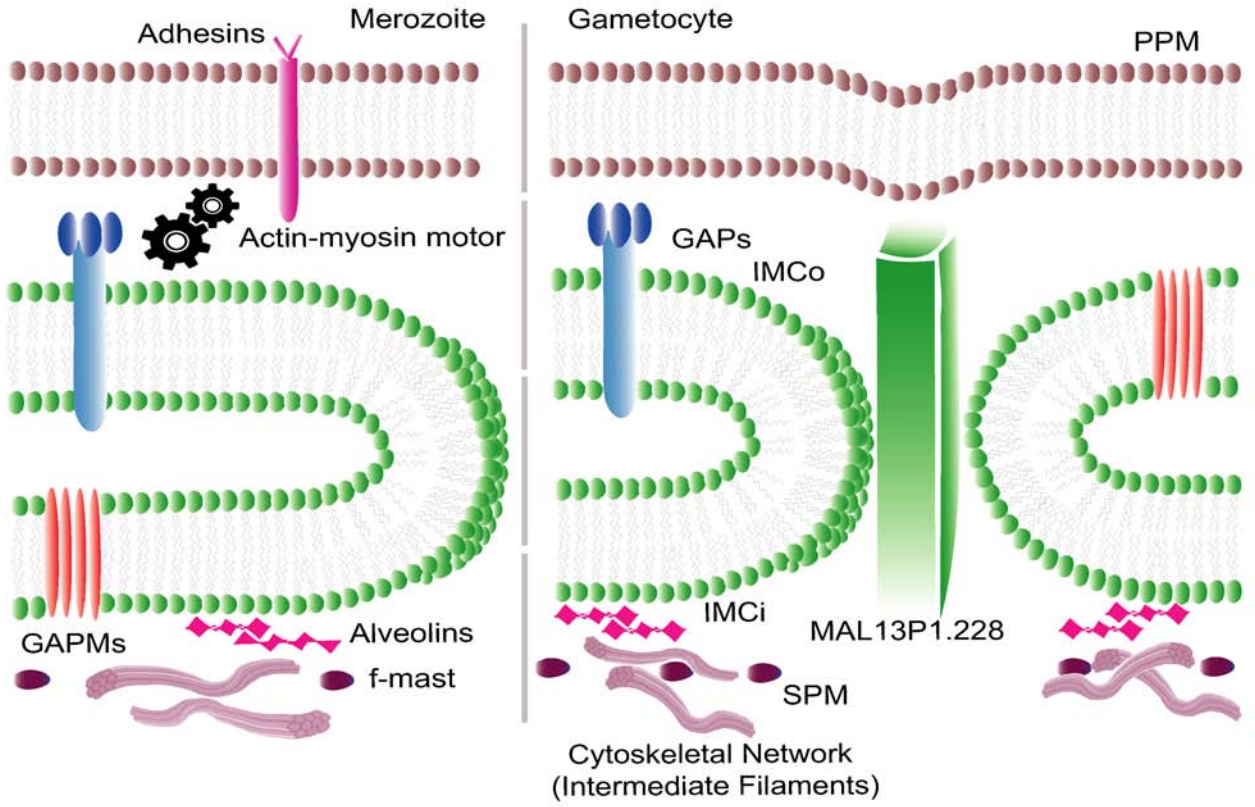


Figure 6. 2: Architecture of the IMC: The flattened IMC vesicles are seated underneath the parasite plasma membrane (PPM). On the cytoplasmic side of IMC (IMC i), the alveolins and alveolin-likes (marked with pink) form molecular connections between cytoskeletal network (Intermediate filaments) and the IMC vesicles.

6. 5 Membrane recruitment and attachment of the alveolins

The precise manner in which the alveolins get associated with the IMC membrane remains an enigma till date. However, what is known so far is that the alveolin domains confer the ability to the alveolins and alveolin-likes to localize in the IMC. The N- and C- termini of the alveolins bear posttranslational modification sites, mostly acylation sites. Mutational analysis of the alveolins in *Toxoplasma gondii* (IMC3 and IMC8) demonstrates that just the alveolin domains are potent enough to get targeted to the IMC. However, IMC8 alveolin repeats devoid of the flanking regions did not exactly localize like the wild type with pronounced basal end localization in the developing buds (Anderson-White *et al.,* 2011). A similar approach was undertaken in the present study of PF08_0033, and the palmitoylation sites at the C-teriminus were mutated and alveolin-like repeats were deleted. The mutation of the C-terminus flanking region of this alveolin-like protein did not alter the localization pattern of the protein, which is in agreement with the mutational studies of the previously characterized alveolins in *Toxoplasma*. Of the three alveolin-like repeats present in this

protein, the third domain is the crucial one to target the protein to the IMC. Upon the deletion of the first two alveolin-like domains, the proteins membrane attachment remained unhampered. The first two repeats could be the devolved forms of alveolin motifs that no longer serve the characteristic function or they are false alveolin-like repeats, which qualified as putative repeats.

The typical alveolin domain is a stretch of amides, polar uncharged amino acids like glutamic acid (E), aspartic acid (D) followed by a basic amino acid like lysine (K); both of these groups of amino acid residues are extremely hydrophilic and easily form hydrogen bonds. The next amino acid type in the stretch is aliphatic one like valine (V) and isoleucine (I), which are non-polar and highly hydrophobic in nature. Proline (P), the only cyclic amino acid is also a residue common to the alveolin domain and shares the physical and chemical properties with the aliphatic amino acids. The aliphatic amino acid residues can form a hydrophobic core or hydrophobic anchor structures like amphiphilic α–helixes or exposed non-polar loops, which can help the protein to attach with the membrane via non-specific hydrophobic association commonly exhibited by Histactophilin, a cytoskeletal protein in *Dictyostelium* cells, involved in osmoregulation (Hanakam *et al.,* 1996). The amphiphilic α–helixes act like act anchors for almost all monotopic proteins, many channel forming proteins and some membrane associated enzymes. In addition to this, it is reported in snake venom toxins, defensins and some microbial peptides possess a stretch of hydrophobic amino acid residues that can form a amphiphilic β–sheets, β- hairpins or β–turns which promotes interaction with the membrane leading to their anchorage. Investigations reveal that even unfolded polypeptides comprising few non-polar amino acid residues are potent enough to penetrate through the lipid head-group region of the membrane (Zhang *et al.,* 2003; Marcotte *et al.,* 2003; Ellena *et al.,* 2004). The polar uncharged and basic amino acids present in alveolin motifs can later confer additional anchorage to the docked protein by forming hydrogen bonds with the interacting molecules in the vicinity. However in case of alveolins it is likely that once they are bound to the membrane, they get trapped in a meshwork of interacting proteins that form a part of the SPN making them resistant to solubilitzation.

Apart from the alveolins, the IMC harbors a variety of different kinds of proteins that have designated functions and diverse mechanisms to associate with the IMC membrane. A unique family of IMC proteins gives a novel insight of the architecture of the IMC and the

posttranslational modifications implicated in its membrane association. The IMC of *Toxoplasma* tachyzoite is divided into sub-domains of apical, central and basal regions and are highlighted by the unique IMC-subcompartment proteins (ISPs) (Fung *et al.,* 2012). The ISPs studied extensively in *Toxoplasma spp.* reveal they do not carry any iterative motifs. Reports confirm the existence of similar compartments in the IMC of merozoites in *P.falciparum* (Yeoman *et al.,* 2011). The IMC in merozoites most likely comprises only a single vesicle and the ISPs ambiguously demark IMC of the zoite into apical and basal parts. These *Apicomplexan* specific proteins are myristoylated in the cytosol that allows their transient attachment to the membrane. In a coordinated manner these proteins are further palmitoylated by IMC-specific Palmitoyl acyl transferases (PATs) to anchor them to precise sub-domains of the IMC (Beck *et al.,* 2010; Fung *et al.,* 2012). However the current advances made in protein trafficking indicate that just palmitoylation is essential for the ISPs to localize in the IMC (Fung *et al.,* 2012). This posttranslational modification (palmitoylation) has proven to be extremely crucial for the *P.falciparum* Glideosome Associated Protein 45 (*Pf*GAP45- PFL1090w) as well. An IMC-specific PAT palmitoylates it and docks the protein firmly within the IMC (Rees-Channer *et al.,* 2006). The expression of certain PATs is strongly upregulated during schizogony along with the co-translational upregulation of GAPs and some of the other IMC proteins implicated in invasion (Bozdech *et al.,* 2003; Hu *et al.,* 2010). Not much is known about the PATs in apicomplexans, however investigations reveal that different organelles and compartments of the cells carry their specific PATs. This hints to the point that proteins first must reach to the destination via some other means or posttranslational modifications, most commonly acylation and then the site specific PATs do the needful. Recently a study on rhoptry localized PAT (*Tg*DHHC7) in *Toxoplasma* showed that these enzymes are extremely essential for the proper assembly of the rhoptries at the apical tip of the parasite cell (Beck *et al.,* 2012).

However palmitoylation, does not seem to play a role in the localization of the alveolin-like protein, PF08_0033 investigated in this study. This IMC protein does not get upregulated during schizogony and remains unexpressed in the presexual stages as well but displays a distribution in the IMC when expressed episomally using a powerful artificial promoter (*ama-1*). The third alveolin-like repeat is critical for it's trafficking to the IMC, where this protein is caught in a meshwork of other interacting proteins, like the alveolins and cannot be yielded upon solubilitzation. The comprehensive methodology used to identify this alveolin-like protein and the thorough investigation to ascertain it's attributes similar to the

classical alveolins, indicate to the possibility of existence of other related proteins which form a part of the SPN.

6. 6 MAL13P1.228 and IMC architecture in gametocytes

In an attempt to generate a broader picture of *Plasmodium* family proteins involved in invasion, a sub-network of proteins based on previously established invasion associated proteins that get upregulated during schizogony was created, which led to the identification of MAL13P1.228 as one such candidate, associated with the IMC (Hu *et al.,* 2010). Of the till date characterized IMC proteins in *Apicomplexans*, MAL13P1.228 is the only IMC protein that is restricted to the *Plasmodium spp.* indicating that it evolved only after the *Plasmodium* genus diverged from other members constituting the Apicomplexa phylum (Kono *et al.,* 2012). Interestingly, this IMC member protein displays similar spatio-temporal distribution like the alveolins during schizogony but unlike alveolins, it does not possess any recognizable repetitive motifs or structural domains that can mediate hydrophobic interactions with the membrane or any known posttranslational modification sites to help the protein attach with the IMC. It is likely that MAL13P1.228 is escorted to the membrane by some carrier protein or by highly specific protein-protein interactions. Similar to this, PhIL1 (photosentsitized 5- [125I] iodonaphthalene-1-azidelabeledprotein 1), a cytoskeletal protein of the pellicle, well characterized in *Toxoplasma spp.*, bears no transmembrane domains or sites predicted for posttranslational modifications (Gilk *et al.,* 2006). This *Apicomplexan* specific protein shows peripheral localization, more aggregated at the apical ends (Gilk *et al.,* 2006). MAL13P1.228 could follow the same suit for getting trafficked to the IMC (Kono et al., 2012).

Recent studies reveal that the merozoite IMC arises due to the redistribution of certain sub compartments of the endoplasmic reticulum to the developing apical caps (Yeoman *et al.,* 2011). However as the parasite differentiates from the asexual to the presexual form, the shape of the cell undergoes a remarkable remodeling. This change in the morphology is driven by the modifications in the dynamics of the microtubules underneath the IMC, which in turn alters the architecture of the IMC seated under the PPM (Kono *et al.,* 2012; Dearnley *et al.,* 2012). The fundamental protein composition of the IMC anyhow, in the asexual and sexual forms is very much similar as marked by the presence of the characteristic proteins like GAP50, GAP45, MyoA and MTIP (Dearnley *et al.,* 2012). Due to the unavailability of

a comparative ultrastructural investigation of merozoites or gametocytes in *Plasmodium*, the presence or the exact arrangement of SPN is not clear. Nonetheless, it is confirmed that the cytoplasmic face of IMC of merozoites and the gametocytes is associated with SMs (sub pellicular microtubules), which vary only in their numbers in the two above-mentioned distinct stages (Fowler *et al.,* 1998; Meszoely *et al.,* 1987). The cryo-electron microscopy and freeze-fracture microscopy shed light on the basic difference in the IMC architecture in merozoites and gametocytes. In a method unknown so far the IMC, which exists in the form of a single vesicle in asexual stage, divides and forms a patchwork of rectangular IMC cisternae sealed together at the peripheries during the presexual and sexual stages (Kono *et al.,* 2013). This ultra microscopy information also unmasks the details of the intervening regions of the IMC plates in the gametocytes and reveals the existence of proteinaceous sutures holding the IMC plates together in place (Meszoely *et al.,* 1987; Dearnley *et al.,* 2012). These sutures could also be implicated in pinning the plasma membrane to the outer membrane of the IMC. The localization displayed by MAL13P1.228 in the late-staged gametocytes has a stripy- pattern that forms a symmetric meshwork enclosing the parasite body. It is henceforth assumed to be a component of these sutures (Meszoely *et al.,* 1987; Kono *et al.,* 2012) (Fig. 6.3). The architecture of the IMC in gametocytes of the *Plasmodium spp.* and the tachyzoites from *Toxoplasma* are comparable and exist in multiple vesicular forms. However, MAL13P1.228 is just restricted to *Plasmodium* family that indicates towards the possibility of existence of a functional homolog in *Toxoplasma spp.* These sutures might confer extra agility to the gametocytes, which is needed as the cell matures from stage-IV to stage-V and the two ends of the cell rounds up. Usually the early forms of gametocytes adhere to the endothelial cells of the blood vessels, bone marrow or spleen where they can get sequestered. The terminal stage of presexual forms marks the loss of adhesins, which results in their release from the adhesion sites and the parasite wriggles through the sinusoidal slits to re-enter the circulation (Dixon *et al.,* 2012). MAL13P1.228, a *Plasmodium* genus specific innovation could have evolved to quench this need during sexual stages, which not only cements the IMC vesicles but most likely acts as a pivot which gives a certain degree of flexure to the adjoining IMC plates.

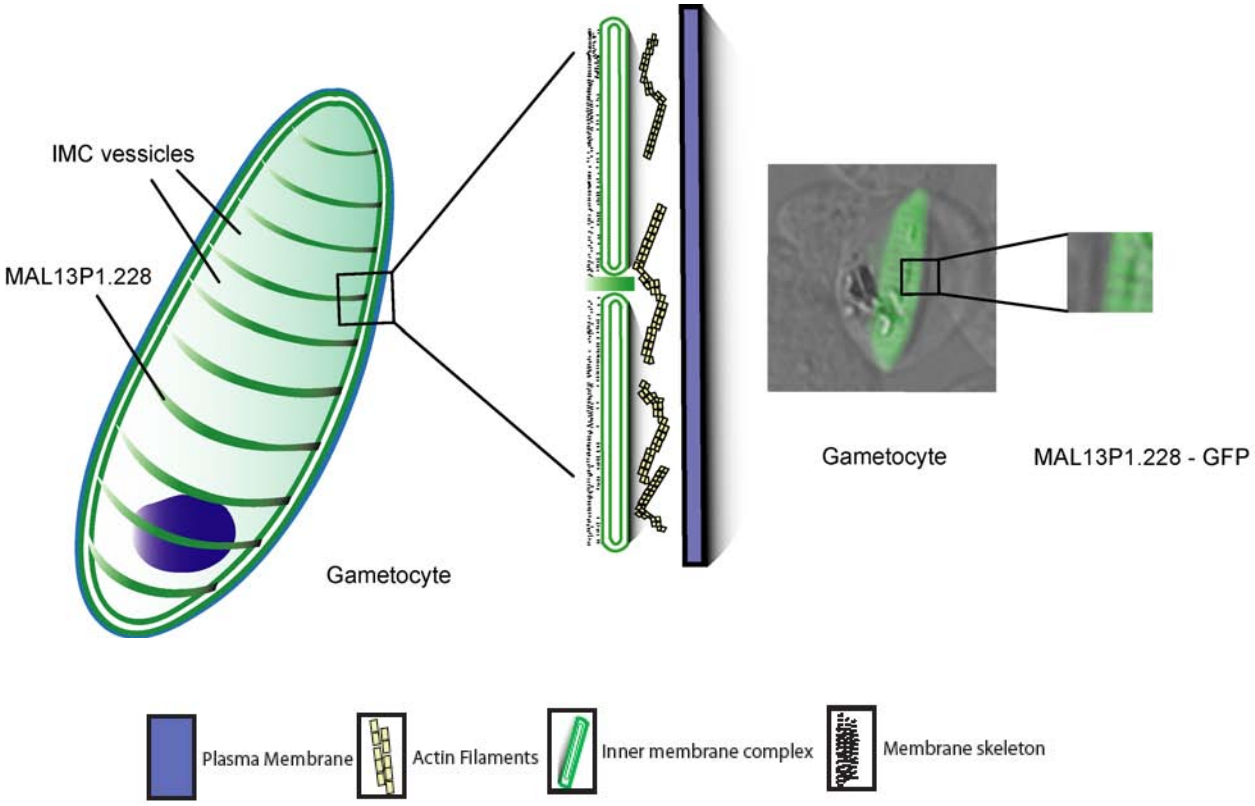


Figure 6. 3: IMC vesicles are sealed by sutures: MAL13P1.228 forms a key component of the molecular sutures that hold the IMC patches together in gametocytes. The GFP tagged *Plasmodium* specific IMC protein; MAL13P1.228 clearly highlights sutures (on the right side).

In this work, we report on the apparent truncation of MAL13P1.228. Diagnostic PCR with the gDNA from 3D7-Δ MAL13P1.228-GFP cell-line confirmed the tagging of the vector at the proper locus and accomplishment of the disruption of the gene. The A-T richness of the *Plasmodium* genome makes reliable targeting of UTRs a tough task (Weber, 1986). To ensure homologous recombination at the precise locus, along with the preceding 300 bp of the gene (from 5' UTR), the first 300 bp from the coding region of the gene was also included. The truncation of MAL13P1.228 in this study sheds light on this unique gene and makes it seem more cryptic. A transcript of initial 300 bp remained expressed as a consequence of which the ORF was just disrupted. The western blot analysis using total cell-lysate from the full-length and truncated cell-lines clearly showed the massive difference in the sizes of the two versions of the protein. Using antibodies directed against GFP, in case of the truncated cell-line, two bands were generated at 45 kDa and approximately at 38 kDa. This could possibly be attributed to the reason that truncated protein gets processed differently and that could lead to the generation of a yet smaller fragment of 38 kDa in size. However, degradation of the protein can also contribute to the same and as a consequence of which a smaller fragment of the protein is noticed.

The spatial distribution of the truncated protein resembles that of the full-length to a certain extent. During mid schizogony, the truncated protein appears as rings at the apical tip of the developing merozoites, accompanied by a cytosolic pool of the same. It is likely the reminiscent of its wild type counterpart. However, there is a drastic change in localization towards completion of the cytokinesis as the nascent merozoites are formed, the truncated protein becomes purely cytosolic. This distribution pattern is continued to be seen in the presexual forms as well. With this token of information it appears that this cryptic gene could be redundant in the parasite's genome as an outcome of gene duplication event. The presence of the truncated protein in the cytosol of the gametocytes could also hint at the functional salvage of the gene by some other related or unrelated gene. A microarray analysis with the RNA from the truncated cell-line can point out the gene or a set of genes, which gets upregulated upon of truncation of MAL13P1.228 and functionally complement the loss of the gene.

However, from the given set of functions associated with the full-length protein, it appears to be crucial for the parasites development especially during the presexual stages. The success associated with the reliable targeting of such kind of genes is extremely low. The UTR region included in this approach for a recombination increases the chances of a possible heterologous recombination at an undesired locus owing to the high content of A-T in *Plasmodium*'s genome, which in the intronic and intergenic regions rises upto 90% (Weber, 1986; Gardner *et al.,* 2002). To address this problem, the UTR region can be omitted and a homologous recombination mediated by a tiny stretch from the coding region only be used to guarantee precision. In order to obtain a pure cell line with the truncated form of this gene, long period of time had to be dedicated to the passaging of the cells for several rounds of erythrocytic developmental stage. However, a recent development in this recombination technique has been achieved, wherein Cre, a tyrosine recombinase from P1 bacteriophage-I is used that mediates an extremely quick recombination with a great accuracy (Kilby *et al.,* 1993; Collins *et al.,* 2013). This Cre- based recombination could be used in the study pertaining to this cryptic gene to curb the problems associated with the fidelity of the site of recombination and the time spent in achieving the same. The other limitation with this strategy however remains that the gene locus is not entirely disrupted and the ORF is still intact. A complete knock out of the gene mediated by a double crossover using the flanking regions of the gene can be used to overcome this hurdle which in turn can provide a much clearer insight of the role of this gene (Maier *et al.,* 2006).

6. 7 Towards a comprehensive analysis of IMC proteins.

The conventional methods of gene manipulation in *Plasmodium spp.* usually fail to provide a better understanding of the parasite biology. A technique to regulate the levels of concerned protein within the cell of the parasite can give better a perception of its functions. To get a better idea of the IMC/Alveolins functioning, a conditional knockout system was undertaken. Dominant negative phenomenon to selectively abolish the protein of interest using a destabilization domain (DD) tag was used. The level of DD or the protein to which it is fused can be regulated in a ligand dependant manner. The alveolin, PFE1285w (*Pf*ALV4) and *Plasmodium* specific, non-alveolin IMC protein MAL13P1.228 fused with the GFP-DD tag were expressed episomally in the parasite. However, the approach could not help provide much insight, as the levels of the proteins did not get downregulated. The degradation of the tagged proteins remained unachieveable possibly because the proteasome machinery was not accessible to them. Both the proteins had displayed strong conjuction with the membrane in their respective solubility assays (please refer to sections 5.2 and 5.4.1), indicating that they are entrapped in mesh of interacting proteins. The degradation domain works most efficiently for the cytosolic proteins as made apparent by GFP, which was tagged with DD and expressed in the cytosol of the parasite, in the present study. The tagged protein complex (GFP-DD) was effectively stabilized when the ligand of the degradation domain, Shld-1 was added to the culture. To circumvent the issues associated with the dominant negative approach, the 3' of the genes can also be replaced by GFP-DD tag and generate a conditional knockdown *in situ*. This approach can however function if the tagged proteins get degraded co-translationally before they reach their destination and escape from the proteasome machinery. The major flaw with this ligand dependant protein regulation method is that the level of down-regulation of the tagged protein might not be sufficient to generate a phenotype. This makes the system quite leaky. Nonetheless, a tetracycline regulatable expression system can be simultaneously used to tightly control the protein amounts (Meissner *et al.,* 2002).

6. 8 Outlook

A recent development in the techniques to identify putative interacting partners of any particular protein, BirA fusion protein is used to figure out the interactome of the same. BirA also known as AviTag is a promiscuous *Escherichia coli* biotin ligase that site-

specifically biotinylates a lysine side chain within a 15-amino acid acceptor peptide or in its close proximity. In order to achieve this, protein of interest tagged with BirA is stably expressed. The tagged protein interacts with specific proteins or the proximal proteins. Upon addition of biotin, the BirA of the chimeric protein, biotinylates the entire set of interacting proteins. This mixture of proteins can be affinity purified and subjected to mass spectrometry or immunoblot analysis using specific antibodies (Roux *et al.,* 2012). A similar approach was used to identify three different cytoplasmic adhesin tail peptides from the family of thrombospondin-related anonymous proteins (TRAPs) as interacting partners of *Plasmodium falciparum* aldolase (*Pf*Aldolase) tagged with AviTag (Boucher *et al.,* 2013). This technique can be brought in use to trace out the interacting partners of MAL13P1.228 and gain an insight into the possible protein-protein interaction that possibly targets it to the membrane.

The IMC remains a conserved subcompartment among the extremely varied and divergent organisms comprising the alveolata infrakingdom over the period of long evolution. This holds proof of its multifaceted utility. However the protein composition of this vesicular structure makes it appear as a conundrum and after the identification of every newer member in its protein repertoire its complexity deepens. Along with the identification of a novel alveolin-like protein in this study, an attempt to get a glance of the functional importance of the non-alveolin, *Plasmodium* specific protein MAL13P1.228 was made. Though many other techniques can be improvised to overcome the limitations or the difficulties faced in the study here, this approach however exemplifies the cryptic role played by the protein during the different stages. The identification and subsequent characterization of other putative IMC proteins can provide a better insight of this enigmatic and ever evolving subcellular structure.

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