Deciphering the role of putative ORC proteins in DNA replication and non-replicative function in *Plasmodium falciparum*

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DOCTOR OF PHILOSOPHY

BHUMIKA SHARMA

SPECIAL CENTRE FOR MOLECULAR MEDICINE
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI

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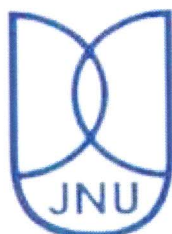
Special Centre for Molecular Medicine
Jawaharlal Nehru University
New Delhi-110067, India



CERTIFICATE

The research work embodied in this thesis entitled **"Deciphering the role of putative ORC proteins in DNA replication and non-replicative function in *Plasmodium falciparum*"** has been carried out by Ms. Bhumika Sharma under my guidance at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any university/Institution elsewhere.

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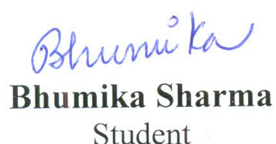

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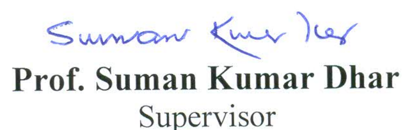
Student



Prof. Suman Kumar Dhar
Supervisor

Prof. Gobardhan Das
Chairperson

dedicado a mi madre

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"Success is not final; failure is not fatal:
It is the courage to continue that counts."

-- Winston S. Churchill

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ABBREVIATIONS

APS	:	Ammonium Per Sulfate
Amp	:	Ampicillin
ATP	:	Adenosine 5’-triphophate
bp	:	Base pair
BFA	:	Brefeldin A
BSA	:	Bovine Serum Albumin
DTT	:	Di thiothreitol
EDTA	:	Ethylene DiamineTetraacetic acid
hpi	:	hours post invasion
IPTG	:	Isoproryl-ß-D-thiogalactopyranoside
kb	:	kilo base
kDa	:	kilo Dalton
ORC	:	Origin recognition complex
PAGE	:	Poly Acrylamide Gel Electrophoresis
PBS	:	Phosphate Buffer Saline
PDI	:	Protein disulphide isomerase
PMSF	:	Phenyl Methyl Suphonyl Flouride
PMV	:	Plasmepsin V
PCR	:	Polymerase Chain PCR
PEG	:	Polyethylene glycol
rpm	:	Revolution Per Minute
SDS	:	Sodium-Dodecyl Sulfate
SPP	:	Signal peptide petidase
ssDNA	:	Single Stranded DNA
TARE	:	Telomere associated repeat elements
TEMED	:	N, N, N’, N’,Tetramethylethylenediamine
Tris	:	Tris (hydroxymethyl) amino ethane
βME	:	β-Mercaptoethanol
µl	:	Micro litre

Review of Literature

I. Introduction

Malaria is caused by a protozoan parasite, *Plasmodium*, which is transmitted through bite of infected *Anopheles* mosquito. The disease is a global health problem majorly in the tropics and subtropics. The Centers for Disease Control and Prevention estimates that there are around 200 million cases of malaria each year, and more than 1 million people succumb to death due to the disease. Symptoms may include six to twelve hours period of nausea, vomiting, cold and shivering alternating with fever and headaches and then a stage of sweating and tiredness. Very severe form of malaria would exhibit anaemia, kidney failure, convulsions (cerebral malaria) and cardiovascular collapse besides other clinical signs.

Multitudes of measure have been taken to control and prevent the occurrence of malaria. These include insecticide sprays in area where mosquito breeds, use of insecticide repellent mosquito nets, prophylactic anti-malarial tablets, etc. Early diagnosis of malarial fever also improves prognosis. The current regime of antimalarial drugs falls under three categories based on their mode of action. These are aryl amino-alcohol compounds (quinine based), antifolate compounds (trimethoprim) and artemisinin compounds. It is common for pathogens to develop resistance against drugs, and anti-malarial drugs are not spared as well. An ever-increasing number of cases of antimalarial drug resistance have been reported, especially in the African region, responsible for malarial deaths.

Although, humans have known to suffer from malarial fever since a long time, there is no commercially available vaccine available till date. The most advanced vaccine candidate is RTS,S/AS01; which is currently in Phase II of human trial (Organization 2018). The need of the hour is to explore other molecules and pathways which can be targeted for devising new and more efficient drugs. For this purpose, we need to decipher the basic biology of malarial parasite and identify the processes indispensable for its survival. Proteins involved in DNA replication, surface proteins accountable for antigenicity, proteins in haem crystallization are few of the examples of essential malarial proteins, which can be a good target for antimalarials.

After a brief discussion of malaria, its causative agents, DNA replication with emphasis on replication initiation, we propose to study the role of two putative Origin recognition

complex subunits (Orc2 and Orc4) in *Plasmodium falciparum*. This biochemical and functional study of these proteins will help in understanding the replication biology of *P. falciparum* in the intraerythrocytic stages.

1. History

The first description of symptoms of malaria dates back to 2700 BC in the Chinese medical records and to 1550 BC by Egyptians wherein it was related with fever and wet ground (Cox 1992). Greek physician Hippocrates observed the connection between high fever and propinquity of patients to stagnant water bodies. The root word "*mal aria*" alias 'bad air' was coined by Italians who also co-related the occurrence of rising body temperature to marshy wetlands and standing water in early 16th century. Closer to the second century, *Qinghao* plant was used as a treatment for high fever in China. The active component of *Qinghao* was later isolated and identified as artemisinin by Chinese researchers in 1971.

In 1880, the presence of malarial parasites was discovered by Charles Louis Alphonse Laveran in the blood smears of infected patients and was awarded the Nobel Prize for this discovery in 1907. Laveran believed in the existence of only one species of this parasite and named it *Oscillaria malariae*. However, other species of malarial parasites were isolated from human subjects and named *Plasmodium vivax* and *P. malariae* respectively in 1890s. In 1897, William H. Welch coined the term *P. falciparum* for the causative agent of tertian fever in humans. In 1902, Ronald Ross was presented with the Nobel Prize for demonstrating the transmission of malarial parasite from human to mosquito and from bird to bird respectively. A group of Italian researchers headed by Giovanni Batista Grassi deciphered the sporogonic life cycle of *Plasmodium vivax* and *P. falciparum* in mosquito 1898.

2. Taxonomy and diversity of *Plasmodium*

Malaria is the oldest disease ever known to mankind and is known to infect humans, rodents, aves, reptiles and amphibian. *Plasmodium* has been placed in the phylum Apicomplexa which is named after the presence of an microtubular apical complex within the cell (Morrison 2009). Amongst the apicomplexans, *Plasmodium* belongs to the order Haemosporida that include blood parasites. More than 200 species belong to

the genus *Plasmodium* affecting different vertebrate hosts and invertebrate vectors (Garnham 1966; Martinsen and Perkins 2013). These species are classified according to their morphology, structure, life cycle & selection of their hosts and have been depicted in Fig. 1 below. *P. falciparum, P. malariae, P. vivax,* and *P. ovale* are the four species of *Plasmodium* which primarily cause disease in humans [reviewed in (Singh and Daneshvar 2013)]. *P. knowlesi* is the fifth species known to infect humans besides macaques (Kantele and Jokiranta 2010).

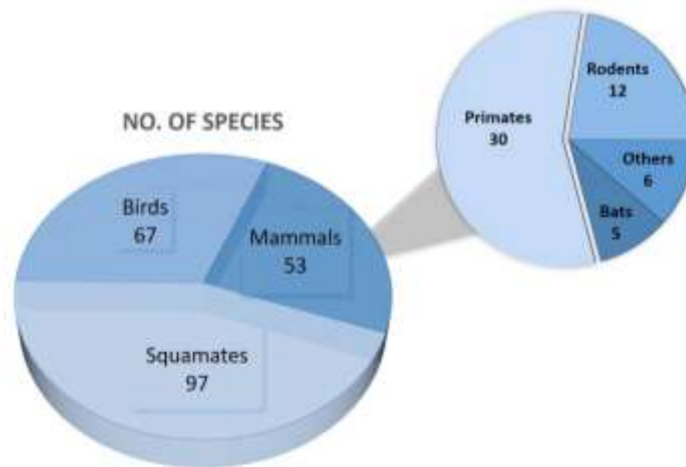

Fig. 1 Numerical distribution of various *Plasmodium* species according to their primary vertebrate hosts [Modified and adapted from (Martinsen and Perkins 2013)].

3. Transmission & epidemiology and of malaria

Malaria is a vector-borne disease transmitted through bite of infected female *Anopheles* mosquito. Other less common means of transmission are through infected needles, blood transfusion, organ transplantation and rarely through congenital route (Owusu-Ofori, Betson et al. 2013; Mace, Arguin et al. 2018).

More than half of the world population is at the risk of malaria infection. Neonates, children under the age of 5 years, immunocompromised individuals and pregnant women are more vulnerable to contracting malaria. Malaria is a preventable and curable disease; yet, approximately 216 million cases of malaria were reported from 91 countries in the year 2016 (Organization 2018). Statistically, sub-Saharan Africa region contributes to ninety percent of the disease burden globally. As evident from the map below (Fig. 2), transmission of malaria is highest in Africa. *P. falciparum* is the major pathogen behind

99 % of the malaria incidences reported in Africa. The incidences of disease and death are more for *P. falciparum* that causes the most severe form of malaria. The weather conditions prevailing in African sub-continent aid in better transmission of parasite via highly efficient mosquito vector *Anopheles gambiae.* Further, an estimated 64% cases of malaria in WHO region of Americas and 30% in South-East Asia respectively were a result of transmission of *P. vivax* (Organization 2018).

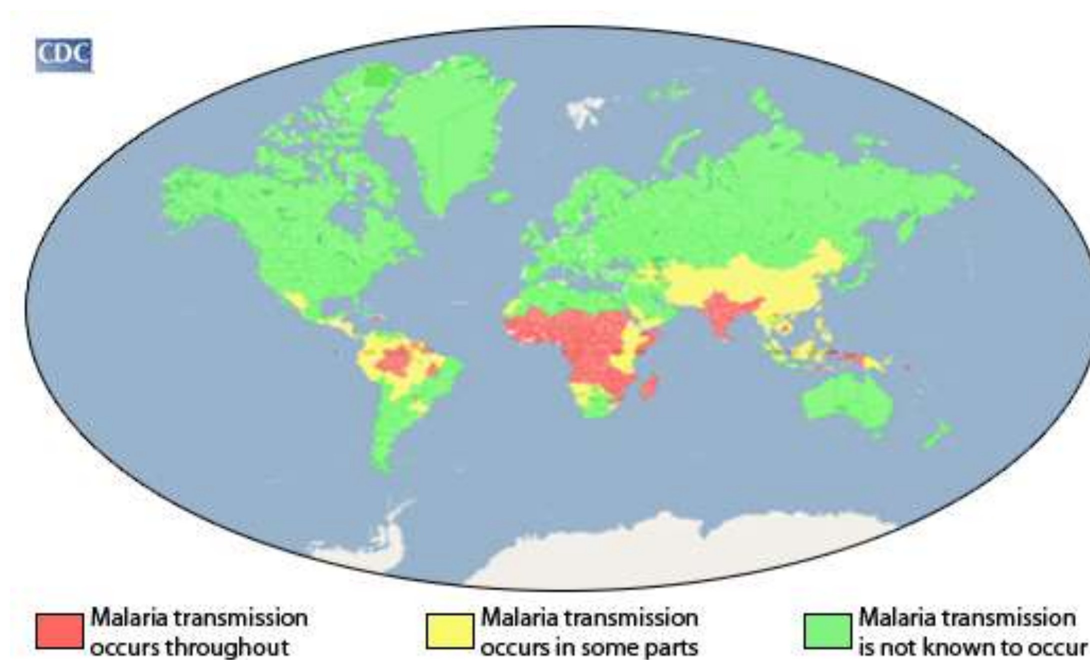

Fig. 2 Burden of malaria transmission across the globe. Africa and India both come under the regions of high transmission of malaria. However, the causative agents of these cases are *P. falciparum* and *P. vivax* respectively. (Adapted from https://www.cdc. gov/malaria/malaria_ worldwide/impact.html)

The world malaria report 2017 states that India was accountable for half the cases of *P. vivax* infection and six percent of all the malaria cases reported worldwide in 2016. Out of the 0.94-1.83 million cases reported, 23,990 cases proved to be fatal (Organization 2018). Thus, in spite of best of the efforts from government and healthcare workers to check the occurrence of malaria and improve prognosis, a large percentage of population still suffers from malaria infection. This is because the type of *Plasmodium* sp, species of vector, local environment prevalent in the region, preventive measures and socio-economic condition of people all influence the manifestation of malaria

4. Life cycle of *Plasmodium*

The malarial parasites alter between vertebrate human hosts and mosquito *Anopheles* during their life cycle. The life cycle is starts with the release of diploid sporozoites into human blood stream when the mosquito takes a blood meal. These sporozoites migrate to and replicate in the hepatocytes in liver within one hour of inoculation producing multinucleated schizonts (Fig. 3).

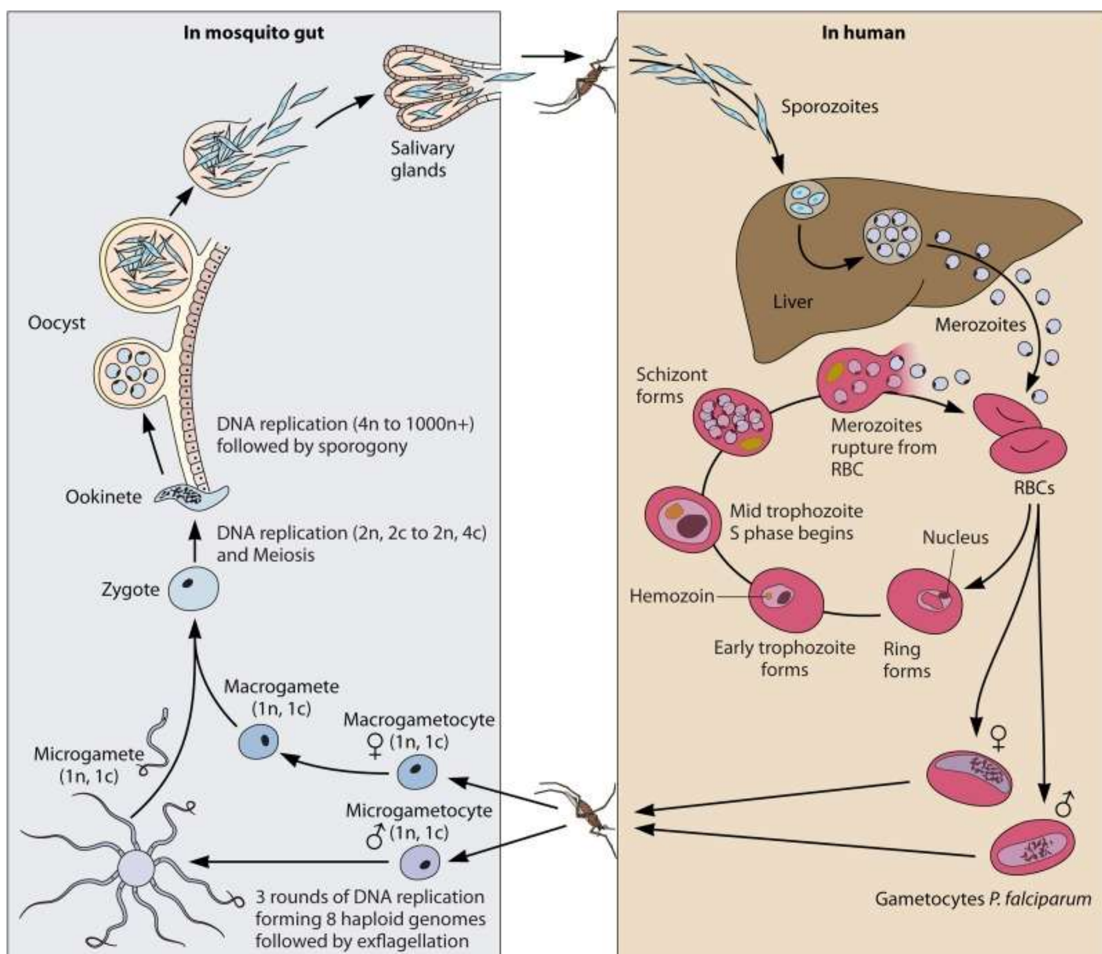

Fig 3 Life cycle of *Plasmodium falciparum. P. falciparum* undergoes asexual reproductive cycle in humans. Mosquito bite ejects numerous haploid sporozoites into the host bloodstream. The parasites undergo multiple rounds of division in the hepatocytes where multinucleated schizonts are formed. The schizonts burst out releasing merozoites into peripheral circulation of host, from where the intraerythrocytic cycle begins. Inside the RBC, the ring-shaped parasite grows and replicates its DNA in the trophozoite stage. Several rounds of DNA replication and nuclear division without cytokinesis result in multinucleated schizont stage. Rupture of schizonts culminates in release of merozoites which invade other erythrocytes. A fraction of the merozoites form mature gametocytes that are transferred to the mosquito in the next blood meal. *P. falciparum* parasites undergo mitotic and meiotic divisions inside the mosquito to again form sporozoites as described in the text [The figure is adapted from (Lee, Symington et al. 2014)].

The intra-hepatocytic cycle for continues for 5-7 days before the schizonts rupture to release thousands of haploid merozoites. The latter invade erythrocytes (RBCs) where the parasite cycles between ring, trophozoite and multinucleated schizont stages. The duration of asexual cycle is 48 hours in case of *P. falciparum*, *P. ovale* and *P. vivax* as compared to 72 hours for *P. malariae* (Garnhan 1988). A few of the merozoites exit from the erythrocytic cycle and develop into sexual erythrocytic stages i.e. male and female gametocytes (Fig. 3). These gametocytes are taken up by the mosquito in the next blood meal where they fuse together to form a diploid zygote in mosquito's mid gut (Bannister and Mitchell 2003). Encystment of motile zygotic form called ookinete occurs in the midgut wall leading to the formation of an oocyst. Multiple round of nuclear division without cytokinesis takes place inside the oocyst resulting in formation of elongated sporozoites. The egressed sporozoites traverse the mosquito hemolymph to invade the salivary glands. The sporozoites remain in the mosquito salivary gland until their release into human host during next blood meal (Bannister and Mitchell 2003).

5. Symptoms of malaria

The initial phase termed as the prepatent period (time between mosquito bite and appearance of parasite in blood stream) is 6-9 days for *P. falciparum*, 8-12days for *P. vivax*, 10-14 days for *P. ovale* and 15-18 days for *P. malariae* infection respectively. The incubation period required for onset of symptoms varies from 7-14 days in case of *P. falciparum* to upto 40 days in case of *P. malariae*. The common symptoms include fever, cough, chills, headache, weakness, dysentery and anorexia (Fig. 4). These are usually associated with the intraerythrocytic schizogony of the parasites rather than the hepatic stages. Classical cases of malarial fever are characterized by febrile paroxysms (Karunaweera, Wijesekera et al. 2003). The patient experiences a cold stage exhibited by chills, high fever and shivering, followed by hot stage of elevated body temperature and severe headache. Eventually, the fever starts to decline in the sweating stage (Crutcher and Hoffman 1996). The paroxysms occur at distinct intervals at the onset of each schizogony (release of merozoites into blood stream) and leave the patient weak and exhausted. Moreover, *P. falciparum* infection has been associated with complexities of nervous system (cerebral malaria), splenomegaly, renal failure, and severe anemia (Trampuz, Jereb et al. 2003).

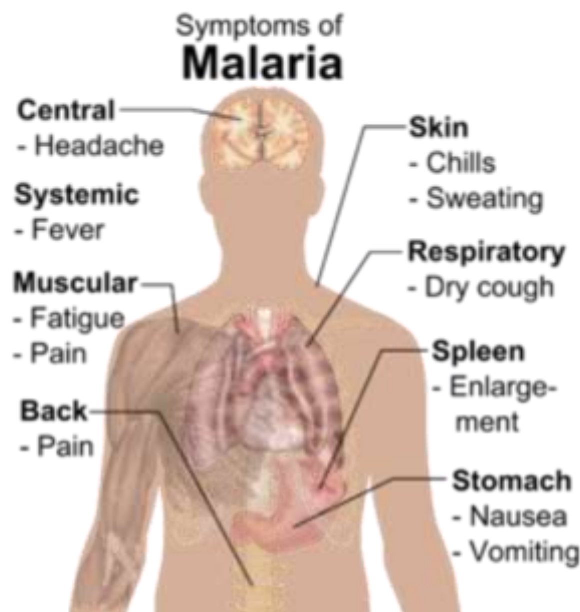

Fig. 3 Illustration of common symptoms of *Plasmodium* infection causing malaria in humans (Adapted from http://learn.e-limu.org/topic/view/?c=2&t=13).

6. *Plasmodium falciparum*

6.1 Genomic organization

As mentioned earlier, *P. falciparum* accounts for the maximum morbidity arising out of all episodes of malaria. It is believed that *P. falciparum* (Pf) evolved from the species *P. laverania*, parasite of gorillas in Africa (Liu, Li et al. 2010). In 1996, the malarial genome project consortium was set up with the aim to decipher the genomic structure of Pf (Hoffman, Bancroft et al. 1997). The complete genome sequencing and its analysis revealed the presence of fourteen chromosomes adding up to a 22.8 Mb genome (Gardner, Hall et al. 2002). Pf genome is the most AT-rich genome sequenced till date and codes for 5268 proteins. A significant proportion of proteins are involved in evading host-immune response and interacting with the host, undoubtedly making *P. falciparum* a highly successful parasite ever encountered by humans. Interestingly, nearly 60% of the proteins do not show sequence similarity with any functional proteins from other organisms and hence are termed hypothetical proteins. About 31% of the proteins are predicted to have at least one transmembrane domain and ~17% have signal peptide sequences (Gardner, Hall et al. 2002).

6.2 Structural organization

P. falciparum is a multi-staged unicellular protozoan protist. It continuously remodels itself with the progression of its life cycle. Each of the parasitic stages (ring, trophozoite and schizont) is distinct from one another in terms of morphology as well as the protein expression. Ring and trophozoite parasites harbor a nucleus, ribosomes, endoplasmic reticulum, Golgi, vesicles, mitochondria, and an apicoplast. Parasites feed on the hemoglobin present in the RBC cytoplasm and convert the biproducts of haem catabolism into haemozoin crystals (Francis, Sullivan et al. 1997; Coronado, Nadovich et al. 2014). The brown crystals are confined in the food vacuole of the parasite whose size increases with the transition into late stages. The schizont stage is exhibited by 16-32 nuclei in a single cell which releases individual merozoites upon rupture. Merozoites are distinguished with the presence of apical organelles required for invasion (dense granules, rhoptries & micronemes), numerous ribosomes, a basal nucleus and a clump of basal filaments (Bannister, Hopkins et al. 2000). The 3D representation of the ultrastructure obtained by electron microscopy is depicted in Fig 5.

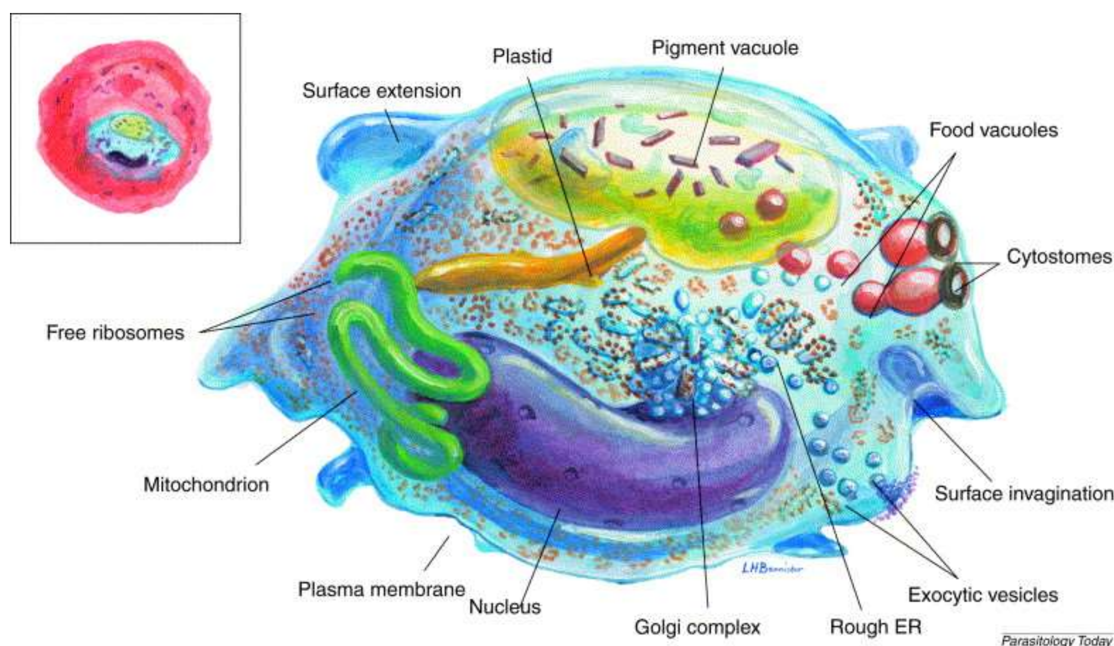

Fig. 5 Illustration of a mid-trophozoite stage *P. falciparum* parasite inside an erythrocyte. The actual proportion of area taken up by trophozoite in the RBC as seen by light microscopy is represented in the inset [Figure is adapted from (Bannister, Hopkins et al. 2000)].

7. DNA replication

Complete and faithful replication of DNA is important for maintaining genomic integrity for all organisms. The core sequence of events taking place during replication remains same in most eukaryotes. Briefly, DNA replication commences at replicator sequences, called origins of replication (*ori* sites). A pre-replication complex of several proteins is assembled at the *ori* sites, followed by employment of regulatory proteins which signal the commencement of DNA replication. Several DNA polymerases are employed to synthesize the daughter strands in a semi-conservative manner.

There is an ordered recruitment of proteins at the *ori* sites which initiate and regulate formation of bidirectional replication forks only once per cell cycle at each *ori.* Initially, origins were defined as the sequences capable of rendering ability for autonomous replication to plasmids (Stinchcomb, Struhl et al. 1979; Huberman, Spotila et al. 1987). These sequences were identified in yeast and named as Autonomously Replicating Sequences (ARS). (Stinchcomb, Struhl et al. 1979). The ARS is constituted by several essential motifs, one of them being the highly conserved A-element, which consists of 11 bp ARS consensus sequence (ACS). It is the principle site where the binding of initiator protein such as Origin Recognition Complex (ORC) takes place (Bell and Stillman 1992). However, not all ACS sites assist in recruitment of ORC, likewise not all ORC binding sites are potential origins. Additionally, less conserved B elements (B1, B2 and B3) are also present within the ARS that help in recruitment of DNA unwinding element at the origin (Bell 1995).

Binding of hexameric ORC at *ori* sites is the foremost step of replication initiation. Binding of ScORC to both A-element & B1 element extending upto 30 bp region has been demonstrated in yeast *S. cerevisiae* (Rao and Stillman 1995). The mechanism of ORC binding is dissimilar in different organisms. It was found that though ScOrc1-5 are required for identification of origins, only four subunits, ScOrc1-2 and ScOrc4-5 directly bind to the DNA (Lee and Bell 1997). On the contrary, DmOrc6 in conjunction with other ORC subunits in *Drosophila* is absolutely essential for recognition and binding to origin (Chesnokov, Remus et al. 2001). Not all ORC proteins have well defined origin DNA binding motifs, however, *S. pombe* has distinct AT-hooks at N-terminus of SpOrc4 which are responsible for DNA binding (Chuang and Kelly 1999). Further, direct interaction of ORC with Cdc6 allows the recruitment of Cdc6 onto the

chromatin (Liang, Weinreich et al. 1995). Primary sequence of Cdc6 divulges the presence of an ATP-binding site as well as a site of ATP-hydrolysis having close homology to that of Orc1 (Bell, Mitchell et al. 1995). The next step in licensing of eukaryotic replication is the loading of MCM helicases with the help of C-terminus region of Cdt1 protein (Takara and Bell 2011). Two hexamers of Mcm2-7 are loaded in head-to-head orientation one after the other. Both the events are dependent on association and dissociation of Cdc6 and Cdt1 (Remus, Beuron et al. 2009). Together, ORC1-6, Cdc6, Cdt1 and Mcm2-7 constitute a functional pre-RC.

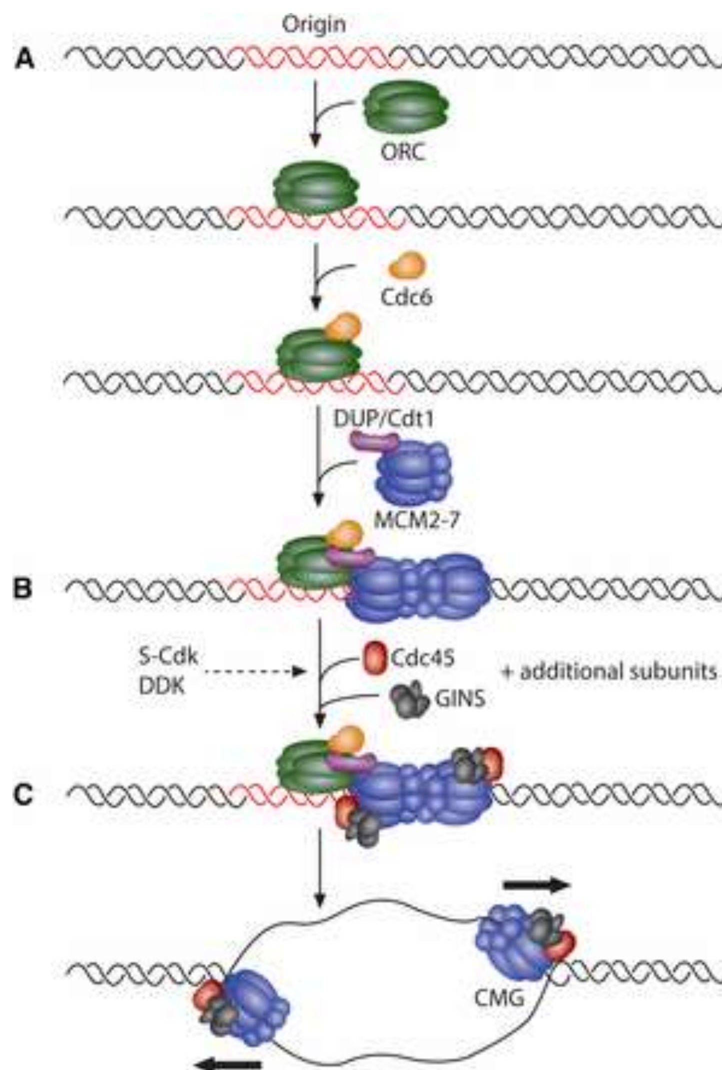

Fig. 6 The figure depicts sequential assembly of components of pre-replication and initiation complex. A. ORC binds to cis-acting origin sequences and serves to recruit other replication factors Cdc6 and Cdt1 along with hexameric Mcm2-7 complex. B. Mcm hexamers associate with Cdc45 and GINS to form CMG complex. C. Bidirection CMG complex is reponsible for helicase activity required for unwinding of DNA before proceeding for S-phase of replication (Figure is adopted from (Hua and Orr-Weaver 2017).

For accomplishing unwinding of double stranded DNA, the double hexamers get separated and traverse in opposite directions (Yardimci, Loveland et al. 2010). There is simultaneous recruitment of Cdc45 and GINS (Go, Ichi, Nii, San) proteins at the site of dissolution of double hexamer Mcm2-7, forming a CMG complex (Gambus, Jones et al. 2006). A strong helicase activity of CMG complex, constituted by Mcm2-7 hexamer, Cdc45 and heterotetramer of GINS helps to establsih replisome progression complexes in opposite directions (Moyer, Lewis et al. 2006). DNA polymerase can not initiate addition of nucleotides *de novo*; therefore, short ~10 nucleotide RNA primers are synthesized by Pol alpha (Polα) primase (Perera, Torella et al. 2013). It is universally accepted that synthesis of DNA is discontinous and semi-conservative. Synthesis of leading strand is brought about by Pol epsilon (Polε) and that of lagging strand by Pol delta (Polδ).

8. Origin recognition complex proteins with emphasis on *Plasmodium* homologs

As mentioned above, ORC is a central component of DNA replication machinery responsible for binding to replication origins. A plethora of work has been documented with regard to ORC subunits. A brief discription of individual ORC proteins and their status in *P. falciparum* is discussed below.

8.1 Orc1

It is the largest subunit of ORC and plays an important role in replication initiation as well as its regulation. It is known to directly bind to DNA coupled with ATP hydrolysis. Expression of Orc1 is regulated through E2F/Rb pathway, therefore the same pathway indirectly controls ORC activity at the origin (Ohtani, DeGregori et al. 1996). Orc1 is known to exhibit high sequence similarity with Cdc6 protein. Besides replication initiation, Orc1 has been assigned a role in maintaining the copy number of centriole and centrosome (Hemerly, Prasanth et al. 2009). In *Plasmodium falciparum*, PfOrc1 has been annotated as PF3D7_1203000. It has a conserved C-terminus, however the N-terminal region exhibits less sequence conservation. PfOrc1 and PfSirc2 have been demonstrated to be enriched at the regions of telomeric, sub-telomeric repeats and promoters of *var* genes, thus suggesting a role of these proteins in *var* gene silencing (Mancio-Silva, Rojas-Meza et al. 2008; Deshmukh, Srivastava et al. 2012). Various post translational modifications of PfOrc1 like phosphorylation, ubiquitination and sumoylation dictate its stability as well as the sub-cellular localisation.

8.2 Orc2

Orc2 forms part of the core replication complex (Orc2-5). Its human homolog, HsOrc2 has been shown to interact with other ORC subunits- Orc3, Orc4 and Orc6 respectively using immunoprecipitation-based assays (Dhar, Delmolino et al. 2001; Vashee, Simancek et al. 2001). In *Drosophila*, DmOrc2 has been shown to interact with HP1 which is implicated in gene silencing and heterochromatinization (Pak, Pflumm et al. 1997). The same phenomenon has been reported in humans, where disruption of HsOrc2 resulted in altered localization of HP1. Orc2 has been shown to employ a histone demethylase protein, KDM5A in order to maintain the permissive methylated state of centromeric DNA and inhibit reduplication of satellite DNA, thus maintaining integrity of the genome (Huang, Cheng et al. 2016). In *P. falciparum*, Orc2 has been identified as PF3D7_0705300 encoded as 825 amino acids long protein. Its role in DNA replication has been suggested by yeast complementation assays wherein a chimera consisting of N-terminal of yeast Orc2 and C-terminal of PfOrc2 was able to complement Ts-yeast *orc2* knock-out strain (Sharma, Sharma et al. 2018). Initial characterization using antibodies against its C-terminal region has been carried out earlier (Sharma, Sharma et al. 2018).

8.3 Orc3

Orc3 is the third largest subunit of ORC and forms part of the core complex required for replication initiation. Orc3 is not well-studied as compared to other ORC homologs. However, N-terminus of HsOrc3 has been shown to interact with C-terminus of HsOrc2 in a heterologous system (Dhar, Delmolino et al. 2001). Besides replication, HsOrc3 has been reported to suppress Rho signaling pathway and helps in growth and maturation of neuronal cells (Cappuccio, Colapicchioni et al. 2010). In *Plasmodium*, Orc3 (PF3D7_1029900) has been annotated as a conserved protein of unknown function in PlasmoDB. No experimental work on PfOrc3 has been reported yet.

8.4 Orc4

Orc4 protein belongs to a family of AAA+ ATPases which are known to associate with diverse functions. Orc4 homologs are capable of directly binding to chromatin and have a preference for triple-stranded DNA structure (Kusic, Tomic et al. 2010). Interestingly,

N-terminus of Orc4 homolog in *S. pombe* consists of 9 AT-hook structures which aid in DNA binding (Chuang and Kelly 1999). In mice, oligomers of Orc4 have been demonstrated to form a cage-like structure around the polar bodies, resulting in their exclusion during meiosis (Nguyen, James et al. 2017). Further, genetic mutations in *orc4* gene have direct links with appearance of dwarf phenotypic disease called Meier-Gorlin syndrome (Guernsey, Matsuoka et al. 2011).

8.5 Orc5

Orc5 is another ORC homolog that belongs to the AAA+ family of ATP-hydrolyzing proteins. The DNA binding ability of Orc5 is ATP-driven too, similar to Orc1. Orc5 was found to be immunoprecipitated with Orc2 and Orc4 proteins, suggesting its participation in formation of hexameric ORC (Quintana, Thome et al. 1998). An acetylation dependent role of Orc5 in opening up of chromatin has been reported. It has been shown to interact with GCN5 protein and increase acetylation at origins (Giri, Chakraborty et al. 2016). Orc5 homolog in *P. falciparum* has been well-characterized. Chimeric PfOrc5 has been shown to functionally complement yeast ScOrc5 in yeast *orc5* knock-out strain (Gupta, Mehra et al. 2008). PfOrc5 displayed marked co-localization with replication foci marker PfPCNA1, which indicated its role in replication (Gupta, Mehra et al. 2008).

8.6 Orc6

Orc6 is the smallest ORC subunit and is known to interact with Cdc6 protein in addition to rest of the ORC proteins (Thomae, Baltin et al. 2011). It has been experimentally shown that Orc6 mediates the interaction between the ORC complex and a chaperonin HMGA1 protein that guides ORC onto origin sites (Thomae, Baltin et al. 2011). In *Drosophila*, Orc6 is involved in cytokinesis by aiding in formation of septin filaments (Akhmetova, Balasov et al. 2015). Similar to *orc4* gene, *orc6* gene mutations have been implicated in manifestation of Meier-Gorlin syndrome (Balasov, Akhmetova et al. 2015).

9. Pathogenicity of *Plasmodium falciparum*

As mentioned earlier, pathogenicity of *P. falciparum* is attributed to a well-defined mechanism of antigenic variation, owing to which it can evade host immunity and thrive successfully in the host. *P. falciparum* achieves this antigenic variation by expressing an

adhesive membrane protein on the surface of erythrocyte. There are 60 representative genes belonging to *var* gene family, and these are expressed in a mutually exclusive manner (Su, Heatwole et al. 1995; Gardner, Hall et al. 2002). Regulated expression of the chosen *var* gene occurs only in early ring stage and not in the late stages. Each of the fourteen chromosomes present in the parasite consists of a few *var* genes located next to a repertoire of TAREs (telomere-associated repeat elements) besides other multigene families such as *rif* and *stevor* in different orientations. Since the orientation of var genes is different, four distinct classes of 5' upstream promotors have been formed. These are UpsA, UpsB, UpsC, and UpsE respectively (Scherf, Lopez-Rubio et al. 2008).

Various epigenetic marks are associated with the activation or suppression. For example, H3K4 dimethylation & trimethylation or H3K9 acetylation are markers of var gene activation, whereas H3K9 trimethylation is a signature of silencing (Scherf, Lopez-Rubio et al. 2008). Chromatin suppressers like histone deacetylases are recruited at the telomeres which spread around 50 kb into the regions covering *var* genes. One such example is PfSir2 which brings about repression of 5' upstream regions (UpsB and UpsE). Sir2 mutants exhibit derepression of certain UpsA and UpsE regions but not UpsB. This suggests that these promoters are under another level of repression (Duraisingh, Voss et al. 2005). Further, it has been shown that heterochromatin protein (HP1) specifically associates with the regions marked with H3K9Me3 (Flueck, Bartfai et al. 2009).

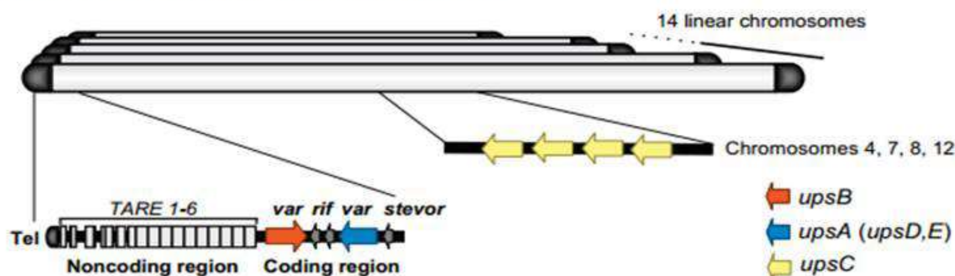

Fig. 7 Structural organization of *var* genes located on all the 14 chromosomes of *P. falciparum.* The var genes are positioned at the telomeric ends of the all chromosomes, with addition to central location in chromosome 4, 7, 8 and 12 too. The figure also illustrates the arrangement of genes at sub-telomeric region. *Var* genes are present adjacent to telomeric repeats (TAREs) at extreme end and with other multigenic family genes *rif* and *stevor* on the other end. *Var* genes are present in different orientations resulting in different orientations of their 5' promoter regions (Scherf, Lopez-Rubio et al. 2008).

10. The components of secretory system

10.1 Outline of classical secretory pathway

Every organism (prokaryote or eukaryote) has established a mechanism for trafficking of proteins intracellularly or for secretion of proteins outside the cell. The main players of this system are endoplasmic reticulum and Golgi apparatus and their intermediary vesicles. These organelles not only transport proteins but are also a reservoir of several enzymes responsible for post-translational modification, proteolysis and proper folding. In general, the proteins entering the secretory system are on the ribosomes of rough ER (Nyathi, Wilkinson et al. 2013). Entry into the ER is facilitated by a short signal peptide usually present at the N-terminus of protein. This process of co-translational translocation is made possible by signal recognition particle (SRP) which recognizes and binds to the signal peptide of nascent polypeptide chain being synthesized from ribosome. SRP binds to its receptor (SR) present in the ER membrane, thereby bringing the nascent peptide along with the ribosome closer to the Sec61 translocon embedded in the ER membrane (Nyathi, Wilkinson et al. 2013).

The newly synthesized proteins are encompassed into small vesicles which fuse with the *cis*-Golgi apparatus. These vesicles bringing cargo towards Golgi are COPII coated. Vesicles move across the cisternal network of Golgi membrane and reach the *trans* Golgi phase. Golgi is the site of active protein modifications such as glycosylation or sulphation. From here, the cargo are released in clathrin coated endosomes that fuse with the lysosomal organelles or the plasma membrane (Day, Staehelin et al. 2013).

Certain ER-resident proteins that mistakenly escape out of the ER into the *cis*- Golgi network are sent back to the ER relying on the ER retention sequence present at the C-terminus. ER resident proteins carrying KDEL sequence are recognized by KDEL receptors present in *cis* Golgi (Lewis and Pelham 1992). Binding of KDEL receptor to KDEL sequence in ER resident protein brings about conformational change in the receptor which prompts its loading in COPI vesicle and transported back to the ER (Majoul, Straub et al. 2001).

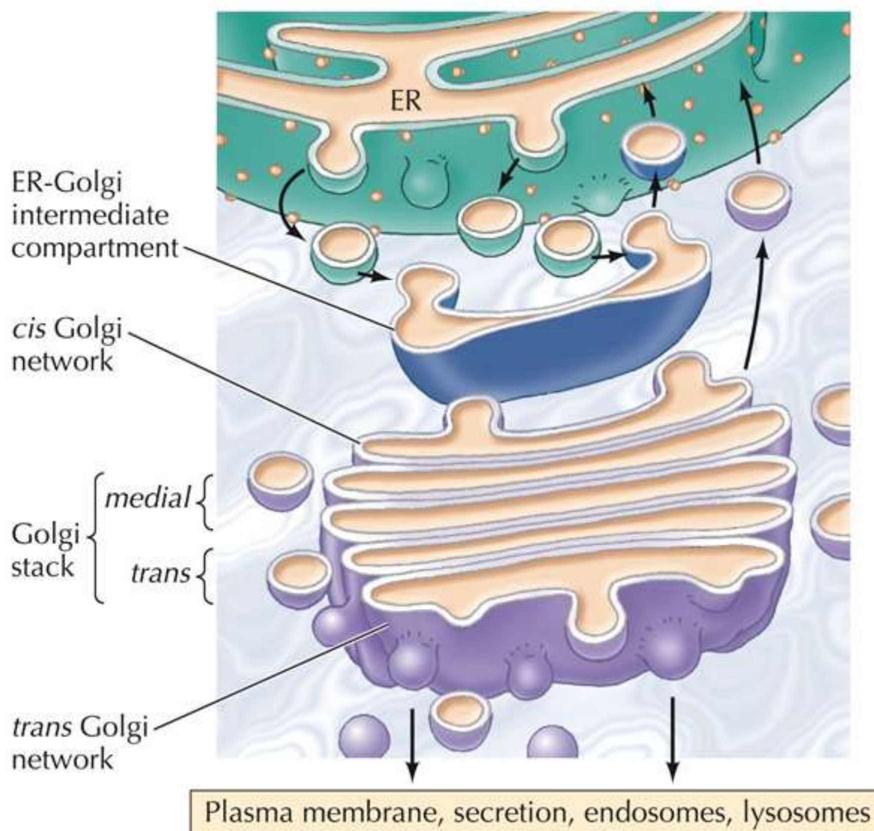

Fig. 8 Illustration of budding and fusion of vesicles signifying the anterograde (towards Golgi) and retrograde (towards ER) transport between ER and Golgi (Adapted from the book "Molecular biology of the cell, 4th edition, 2006).

10.2 Secretory system in *P. falciparum*

The malarial parasite has a very elaborate secretory system which transports proteins within itself as well as into host cytoplasm and cell surface. Initial studies described the presence of a rudimentary ER in the form of loose network of vesicles (Langreth, Jensen et al. 1978). Similarly, Golgi was identified as a small coated vesicle structure located adjoining the ER (Elmendorf and Haldar 1993; Van Wye, Ghori et al. 1996). Protein trafficking in P. falciparum is a highly ordered and regulated as the parasite is surrounded by a parasitophorous vacuole membrane present inside an erythrocyte. Secretory pathway is absolutely crucial for the pathogenicity of parasite. It transports its virulence proteins onto the erythrocyte surface via this route. Examples of such proteins are PfEMP and knob associated histidine rich protein, KAHRP (Howard, Lyon et al. 1987; Taylor, Parra et al. 1987). These proteins form part of the knob-like structure on RBC membrane that aid in endothelial cytoadherence.

The trafficking of intracellular proteins follows the conventional secretory pathway followed by proteins in other eukaryotes. The set of proteins which require translocation to the parasitophorous vacuole are delivered through fusion of vesicle membrane with the parasite plasma membrane (Taraschi, Trelka et al. 2001). It has been suggested that the proteins moving beyond the PV into the host cytosol or RBC membrane are also first released into the PV in specialized compartments (Mattei, Ward et al. 1999).

Majority of the secretory proteins belonging to multigene family such as RIFIN, STEVOR and RESA consist of a short PEXEL (Plasmodium export element) motif at the N-terminus, at a conserved distance from the ER signal peptide. This sequence is cleaved and N-acetylated in the endoplasmic reticulum (Chang, Falick et al. 2008). It has been shown that this proteolytic cleavage of PEXEL is catalyzed by an ER membrane protease Plasmepsin V and the N-terminally processed proteins are recognized prior to export out of the PV (Chang, Falick et al. 2008; Russo, Babbitt et al. 2010). However, there are a number of secretory proteins that lack any obvious PEXEL motif; they either have a recessed element or a transmembrane domain anchored in the ER membrane. These proteins are exported with the help of chaperonins and loaded onto cargo receptors which export the protein into parasite plasma membrane. From here, the protein associates with another chaperon Hsp101, which channels it into erythrocyte (Gruring, Heiber et al. 2012).

10.3 Exception to classical secretory pathway

Since biology is a science of exceptions, there are numerous examples where a conserved pathway followed for protein homeostasis is altered to suit the need of an organism. One such example is a cholesterol biosynthesis related protein SREBP (sterol regulatory element binding proteins). These are transcription factors which regulate few of the genes implicated in maintaining cholesterol homeostasis like HMG CoA reductase and HMG CoA synthase (Brown and Goldstein 1997). Under normal conditions when cholesterol is present in adequate amount in the cell membrane, the full-length SREBPs are present in the ER. In condition of depleted cholesterol levels, SREBP preproteins are translocated from ER to Golgi in association with SREBP-cleavage-activating protein (SCAP) through COPII coated vesicles. Once, the vesicle is unloaded on the Golgi membrane, two consecutive proteolytic events take place at the

N-terminal of SREBP. The enzymes responsible for this cleavage are intramembrane proteases- Site-1 protease and Site-2 protease respectively (Duncan, Brown et al. 1997). The released SREBP translocates to the nucleus in an importin β (karyopherin) dependent manner, where it binds through basic H-L-H-leucine zipper domain to promoters of Sterol regulatory genes. SREBP also promotes transcription of its own gene by a positive feedback loop (Nagoshi, Imamoto et al. 1999). This is one of the very few examples of deviation from the classical secretory pathway.

11. Scope of the current work

As evident from the literature, ORC subunits play a vital role in the sustenance of the malarial parasite. The individual ORC subunits have been worked upon, but how they interact with each other and other replication factors to form Pre-RC still needs to be elucidated. Only PfOrc1 and PfOrc5 subunits have been studied thoroughly so far. It is crucial to study and characterize all the ORC subunits in the parasite to decrypt the molecular mechanism of its DNA replication. Complete understanding of the *Plasmodium* ORC is a preliminary step in apprehending the replicative and non-replicating function of ORC, and how it influences pathogenicity. In this direction, work in the present study revolves around PfOrc2 and PfOrc4 with the following objectives:

I. **Biochemical characterization of origin recognition complex subunit 2 of *Plasmodium falciparum***

II. **Role of endoplasmic reticulum in trafficking of origin recognition complex subunit 2 of *Plasmodium falciparum***

III. **Biochemical and functional characterization of origin recognition complex subunit 4 of *Plasmodium falciparum***

II. References

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Objectives

Objectives of the study are:

I. **Biochemical characterization of origin recognition complex subunit 2 of *Plasmodium falciparum***

II. **Role of endoplasmic reticulum in trafficking of origin recognition complex subunit 2 of *Plasmodium falciparum***

III. **Biochemical and functional characterization of origin recognition complex subunit 4 of *Plasmodium falciparum***

Materials and Methods

I. Materials

1. Chemicals and Media

All the enzymes employed in cloning-restriction enzymes, Taq polymerase, Phusion, etc. were procured from New England Biolabs (NEB). Gel elution and PCR purification kit was obtained from Real Genomics and Plasmid mini-preparation kits were purchased from MDI. All the routine chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA). Roche Protease inhibitor cocktail was used in all the protein purification and cell lysis-based experiments. IPTG was purchased from G-biosciences. Ni2+-NTA Agarose and GST Sepharose beads were purchased from Qiagen and Amersham Biosciences respectively. Complete and Incomplete Freund's Adjuvant used for raising antibodies was bought from Sigma Aldrich, USA. For *Plasmodium* culture, RPMI 1640 media and Albumax II were procured from Invitrogen. All the other components of culture media were obtained from Sigma. Brefeldin A and Z-$(LL)_2$-ketone were obtained from Sigma and Calbiochem respectively. Fluorescence labelled secondary antibodies and nuclear stain, DAPI (4, 6 Diamidino-2-Phenylindole, Dihydrochloride) used for IFA were purchased from Invitrogen.

2. Bacterial and yeast strains

Two common laboratory strains of bacteria were used for this study for cloning and protein expression. Their genotype is mentioned below in addition to the yeast swapper strain used for carrying out complementation studies.

Table 1 List of strains used in the present study.

***E. coli* strain**	**Genotype**
DH10β	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 galU galK nupG rpsL λ-*
BL21CodonPlus (DE3)-RIL	*F– ompT hsdS (rB– mB –) dcm+ Tetr gal λ (DE3) endA Hte[argU ileY leuW Camr]*
Yeast *orc4* knock-out swapper strain	*W303-1A or B (ade2-1 ura3-1 his3-11 trp1-1 leu2-3 112 can1-100); orc4::Trp [pSPB46] orc4 pRS416 Amp URA3*

3. Plasmids and vectors used for cloning and expression

Table 2 List of vectors employed in this study

Plasmid	Relevant characteristic
pGEX6P2	*lacIq*, *tac*, N-GST tag, Ampicillin resistance
pET28a	T7 promoter, N- and C- terminal His_6 tag
pRS416	Gal1, URA3, CEN6, Ampicillin resistance
pRS423	HIS3, CEN6, LacZ, Ampicillin resistance

4. Oligonucleotides

Following is the list of primers used for cloning various regions of *PfOrc2*, *PfOrc4* and *ScOrc4*.

Table 3 List of oligonucleotides used for cloning

S.No.	Name of the Primer	Oligonucleotides sequence (5' to 3')
1	ORC2_Fwd_KpnI	CGGGGTACCATGCTGAAAAACTTTGAAG
2	ORC2_90bp_pARL_REV_AvrII	TGGACCTAGGTAAATACTCTTCTTTGTCTTC
3	ORC2_150bp_piggy_Avr_FW	TGGACCTAGGATGCTGAAAAACTTTGAAG
4	ORC2N150-CGFP Rev_NheI	CTAGCTAGCTGCTGCTGCATCACCATTACTAGAGC
5	pARL.GFP_SEEL.PstI Rev	AACTGCAGGTCTGGACATTTATAGCTCCTCTGATT TGTATAGTTCATCCATGC
6	ScOrc4.Fw_BamHI	CGGGATCCATGACTATAAGCGAAGCTCGTCTATC
7	ScOrc4.Rv_HindIII	CCCAAGCTTTCACAGTTGTGTCCAGGAGTAGTACA
8	PfOrc4_Fw_BamH1	CGGGATCCATGAAAGAAACCCTGAATAATTTGAAT
9	PfOrc4_Rv_EcoRI	CGGAATTCTTATTGCATAACTTCTGTATTATGC
10	Ch1ScOrc4.Rv750bp	ATCATCATTGCAGCCAAAAATGCAAACAG
11	Ch1PfOrc4.Fw1251bp	TTTGGCTGCAATGATGATGAGAACTTAGATTTTAAT
12	PfOrc4_Fw_BamHI	CGGGATCCATGAAAGAAACCCTGAATAATTTG
13	PfOrc4Rv1161bp_XhoI	CCGCTCGAGTTTTCTTAAAGGTGATAAGTCG

Table 4 List of oligonucleotides used for ChIP-PCR

S.No.	Primer Name	Oligonucleotide sequence (5' to 3')
1	ARS1_Fwd	CAAATTTTTATAATTTAAATGACATAA
2	ARS1_Rev	GGATATATAAATCTTTATTTAATTAAAA
3	ARS3_Fwd	CGGGGTACCCCTTATATTTAAATTGTCCAATTGG
4	ARS3_Rev	TTATTATACCTATTTTTCTTTTATTTC
5	AT-rich_Fwd	AAAAATGTTAAAGTAGAAACCCTATTT
6	AT-rich_Rev	CATTTTTATCAAATAAATTTATAA
7	TARE1_Fwd	GTGTTGATGTTGTTAGTTGG
8	TARE1_Rev	AAGACTAAGTAGATGATAATAGG

5. Recombinant Plasmid Constructs

The details of the plasmids used in this study is given below:

Table 5 List of recombinant plasmids constructs

S.No.	Name of the construct	Vector	Restriction site	Source
1	PfOrc2N-His, $_{1\text{-}996\ bp}$	pET-21a(+)	BamH1, EcoRI	In-house
2	Orc2N$_{1\text{-}30}$GFP, $_{1\text{-}90\ bp}$	pARL	KpnI, AvrII	This study
2	Orc2N$_{1\text{-}150}$GFP, $_{1\text{-}150bp}$	PiggyBac	AvrII, NheI	This study
3	Orc2N$_{1\text{-}30}$SEEL, $_{1\text{-}90\ bp}$	pARL	KpnI, PstI	This study
4	Orc2N$_{1\text{-}150}$SEEL,$_{1\text{-}150bp}$	pARL	KpnI, PstI	This study
5	ScOrc4.pRS416, $_{1\text{-}1590\ bp}$	pRS416	BamHI, HindIII	This study
6	ScOrc4.pRS423, $_{1\text{-}1590\ bp}$	pRS423	SacI, XhoI	This study
7	PfOrc4.pRS416, $_{1\text{-}2952\ bp}$	pRS416	BamHI, EcoRI	This study
8	PfOrc4.pRS423, $_{1\text{-}2952\ bp}$	pRS423	SacI, XhoI	This study
9	ScO4N$_{1\text{-}750}$.PfO4C.pRS416$_{1251\text{-}2952}$	pRS416	BamHI, EcoRI	This study
10	ScO4N$_{1\text{-}750}$PfO4C.pRS423$_{1251\text{-}2952}$	pRS423	SacI, XhoI	This study
11	PfOrc4N, $_{1\text{-}1161\ bp}$	pGEX-6P2	BamHI, XhoI	This study

6. Antibodies

Various antibodies used in this study along with their animal source is listed below:

Table 6 List of antibodies used

S.No.	Protein	Animal used	Dilution used		Antibody profile in WB	Source
			WB	IFA		
1	PfOrc2N	Mice	1:2000	1:500	~100 kDa	This study
2	PfOrc2C	Rabbit	1:500	1:100	~100 kDa, ~45 kDa	Abexome Biosciences
3	PfActin	Mice	1:3000	1:500	~43 kDa	In-house
4	PfBip	Rabbit	1:15000	-	~72 kDa	Prof. John H Adams (USF),
5	Histone H3	Mice/Rabbit	1:3000	-	~15 kDa	Abcam
6	PfAldolase	Rabbit	1:3000	-	~43 kDa	In-house
7	6X-His	Mice	1:5000	-	-	Abcam
8	GFP	Rabbit	1:5000	1:500	~26K kDa	Sigma/ Abcam
9	GST	Rabbit	1:1000	-	~26K kDa	Santa Cruz
10	PfPDI	Rabbit	-	1:500	-	Prof. Philippe Grellier (CNRS)
11	PfPMV	Rabbit	-	1:50	-	Prof. Daniel E Goldberg
12	PfHP1	Mice	-	1:500	-	In-house
13	PfOrc4M (aff. pur.)	Rabbit	1:2000	1:100	~100 kDa, ~55 kDa, ~35 kDa	This study
14	PfOrc4N	Mice	1:2000	-	~100 kDa	This study
15	PfMcm6	Mice	-	1:500	-	In-house

II. Methods

1. DNA manipulations

For cloning of PfOrc2N_{1-30}GFP, specific primers (P1 & P2) were used to amplify 1-90 bp of *PfOrc2* (PF3D7_0705300) from *P. falciparum* gDNA and cloned in pARL vector between the sites KpnI and AvrII. Similarly, region from 1-450 bp of *PfOrc2* was PCR amplified using specific primer (P3 & P4) and cloned in PiggyBac vector between the sites AvrII and NheI to generate PfOrc2N_{1-150}GFP. For generating GFP-SEEL constructs, nucleotides coding for SEEL were added downstream to GFP coding sequence in the reverse primer (P5). Likewise, PfOrc4 N-terminal region (1-1161 bp) was amplified from Pf genomic DNA (using primers P12 & P13) and cloned between BamHI and HindIII sites in pGEX-6P2 vector. Sequence of the primer sets mentioned above is listed in Table 3.

2. Expression and purification of His-fusion proteins

Standard protocol as recommended by the manufacturer (Qiagen) was employed to purify His_6-tagged recombinant protein. Firstly, PfOrc2N-pET28a plasmid DNA construct was used to transform *E. coli* BL21 CodonPlus (DE3) cells and spread on LA agar plate under Kanamycin (50 µg/ml) and Chloramphenicol (25µg/ml) selection. Single colony was picked and used to inoculate primary culture. Next, two liters LB broth was inoculated with 1% primary inoculums and incubated at 37°C. After the OD_{600} reached 0.6, the culture was induced with 0.5 mM IPTG and allowed to grow overnight at 22°C. Next day, the cells were pelleted down and resuspended in lysis buffer containing 20mM Tris-Cl (pH 8), 300mM NaCl, 10mM Imidazole, 10mg/ml lysozyme and 1 mM PMSF. The cell suspension was sonicated and incubated with Triton X-100 for thirty minutes. Further, the lysate was centrifuged at 16,000 rpm for thirty minutes. After separating the supernatant, the pellet was resuspended in buffer containing 20mM Tris-Cl (pH 8), 300mM NaCl, 10mM Imidazole, 1% N-lauryl sarcosine and 1 mM PMSF and incubated on ice for two hours. The suspension obtained was again centrifuged at 16,000 rpm for thirty minutes. The supernatant obtained was allowed to bind to equilibrated Ni-NTA resin for one hour. Post binding, the unbound protein fraction was separated from Ni-NTA beads by centrifugation at

2,000 rpm for 3 minutes. The protein-bound beads were then washed with buffer containing 30-50 mM Imidazole. Lastly, elution buffer containing 500mM imidazole was used to elute the bound protein from beads. All the steps were carried out at 4 ºC.

3. Expression and purification of GST-fusion proteins

PfOrc4N-GST fusion protein was purified using Glutathione Sepharose beads. PfOrc4N-GST plasmid DNA construct was used to transform *E. coli* BL21 CodonPlus (DE3) cells and spread on LA agar plate under Ampicillin (100 µg/ml) and Chloramphenicol (25µg/ml) selection. Single colony was picked and used to inoculate primary culture. Next, three liters LB broth was inoculated with 1% primary inoculums and incubated at 37°C. After the OD_{600} reached 0.6, the culture was induced with 0.5 mM IPTG allowed to grow overnight at 22°C. Next day, the cells were pelleted down and resuspended in lysis buffer containing 1XPBS, 10 mM DTT, 2 mM EDTA and 1 mM PMSF. The cell suspension was sonicated and incubated with Triton X-100 for sixty minutes. Further, the lysate was centrifuged at 16,000 rpm for thirty minutes. After separating the supernatant, the pellet was resuspended in buffer containing 1X PBS, 10 mM DTT, lauryl sarcosine and 1 mM PMSF and incubated on ice for two hours. The sample was again centrifuged at 16,000 rpm for thirty minutes. The supernatant obtained was incubated with GST-beads for 4-5 hours followed by centrifugation to separate the unbound protein sample. Protein bound GST beads were washed with buffer containing 1X PBS, 10 mM DTT, 1M NaCl and 1 mM PMSF. Finally, elution buffer containing 50 mM Tris.Cl, pH 8.0, 10 mM DTT, 20 mM Glutathione, 0.1% NP-40, 10% Glycerol and 100 mM NaCl was used to elute the bound protein from beads.

4. Antibody generation

Purified protein (~100µg) was resolved by SDS-PAGE and the band of interest was excised. The gel piece was finely crushed and mixed with Complete Freund's adjuvant for the 1st dose and incomplete Freund's adjuvant for booster dose after 7 days. The mixture was administered into mice sub-cutaneously and the bleed was collected after 7 days of 1st booster. Pre-immune sera were collected before immunizing the mice**.** Mice were administered several booster doses and sera were collected every 7th day of the injection.

5. *Plasmodium falciparum* culture

P. falciparum 3D7 parasite were cultured in RPMI 1640 (Sigma) medium supplemented with 0.5% Albumax (Invitrogen), 5% NaHCO3 (Sigma), 50 µg/mL gentamicin (Sigma) and 50 µg/mL ampicillin with human erythrocytes maintained at 3-5% hematocrit. Culture was grown in 5% CO_2 at 37˚C. Ring stage parasites were synchronized with 5% sorbitol (Sigma) wherever required.

6. Parasite lysate preparation and western blotting

Infected RBCs were lysed with 0.015% saponin and washed in 1X PBS until clear black pellet was obtained. Western blotting was performed using standard procedure. The antibodies used were: PfOrc2N, 1:2000; PfOrc2C, 1:500, PfActin, 1:3000; Bip, 1:15000; Aldolase, 1:3000; histone H3, 1:3000; His, 1:5000; GFP,1:5000.

7. Immunoprecipitation of PfOrc2C from Pf lysate

Saponin lysed trophozoite stage parasite pellet was resuspended in IP Lysis buffer (10mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% NP40, 10% Glycerol) and incubated for 2 hours on a rocker at 4ºC. Sample was then centrifuged at 13,000 rpm for 30 minutes and the supernatant was collected. After separating the input sample, the supernatant was diluted with a buffer containing Tris7.5, EDTA and Glycerol and then equally divided into two microfuge tubes. Pre-immune and immune sera were added to the respective tubes and allowed to bind overnight at 4ºC. Protein A Sepharose beads were added to the samples and incubated for 1 hour. Samples were then centrifuged at 2,500 rpm and the supernatant was removed. The beads were washed twice with wash buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% Glycerol). Washed beads were finally resuspended in SDS-loading dye and boiled for 5 minutes at 95ºC. The pre-immune, immune and input samples were resolved by 12% SDS PAGE and probed by respective antibodies post transfer onto PVDF membrane.

8. Preparation of sample for MS analysis

For mass-spectroscopic analysis, *P. falciparum* 3D7 parasite pellet (~40 hpi) was first lysed using 8 M urea. The lysate was resolved by 10 % SDS-PAGE followed by staining the proteins with Coomassie dye. Portions of gel were excised from three

regions (~ 100 kDa, 40-55 kDa and 25-35 kDa) and subsequently treated with trypsin to obtain short peptides. The samples were further extracted and desalted before proceeding with LC/MS.

9. Immunofluorescence assay

P. falciparum infected RBC culture was used to draw smears on a glass slide. The cells were fixed using methanol for a few seconds. Fixed slides were air dried and incubated in blocking solution (3 % BSA, 0.01 % saponin in 1X PBS). After a brief wash with 1X PBST, the slides were incubated in desired dilution of primary antibody (or pre-immune sera) for ~12-16 hours at 4ºC. The slides were then washed with 1X PBST thrice prior to incubation with fluorescently labeled secondary antibodies (Anti rabbit/mice AF 488, anti-rabbit AF 594 or anti-mice AF 568 respectively) along with a nuclear stain (4', 6-diamidino-2phenylindole; DAPI) for one hour at RT. Further, the slides were washed with 1X PBST thrice and finally mounted in the presence of antifade solution. Images were analyzed and processed with Zeiss Imager.Z1 (Apotome) using Axiovision 4.6.3 SP1 software. The dilution of various immune sera used were: PfOrc2N, 1:500; PfOrc2C, 1:100; GFP,1:500; PfPDI, 1:500; PfPMV, 1:50; PfOrc4 affinity purified, 1:100; PfMcm, 1:500 PfActin, 1:1000 PfHP1, 1:500)

10. Immunoelectron microscopy

Pf3D7 parasites were fixed in a solution of 4% paraformaldehyde and 0.1% glutaraldehyde for ~12-16 hours at 4 °C. The sample was washed thrice in 100 mM 1X PBS for 30 mins at 4 °C followed by serial dehydration steps using increasing alcohol concentrations (30%, 50%, 70%, 80% and 90%) for 30 mins each at 4 °C. The sample was again dehydrated using absolute ethanol for 1 h at 4 °C twice. Equal parts of LR white resin and absolute alcohol were used to infiltrate the pellet for 1 hour at 4°C twice. The sample was again treated with LR white overnight at 4 °C and then at RT for 4 hours respectively. Subsequently, pellet was embedded in 4-5 volumes of LR white and kept at 55 °C for 48-72 hours. The constructed block was used to make ultra-thin slices using ultramicrotome. The grids were incubated with antibodies against PfOrc2C and labeled using gold conjugated secondary antibodies. Imaging was done using Transmission Electron Microscope (JEOL2100F).

11. Sub-cellular fractionation of proteins

Synchronized Pf3D7 iRBCs were lysed by saponin and washed with 1X PBS. Lysed parasite pellet was resuspended in five volumes of buffer A (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.65% NP-40) in addition to 1X protease inhibitor cocktail (Complete, Roche) and 1mM PMSF. After 5 minutes incubation on ice, the suspension was centrifuged at 3,500 rpm for 4 minutes and the supernatant was separated as the cytoplasmic fraction. The remaining pellet was washed with buffer A and centrifuged at 3,500 rpm for 4 mins. The remnant was collected as the nuclear fraction. Equal concentrations of cytoplasmic and nuclear fractions were analyzed by SDS-PAGE and Western blotting using specific antibodies.

12. Protein fractionation

Saponin lysed parasites were freeze thawed twice in 5mM Tris pH8.0 and centrifuged at 13,000 rpm for 30minutes. The resulting supernatant was separated as the soluble protein fraction. Remaining pellet was incubated with 0.1M sodium carbonate, pH 11.5 for thirty minutes on iceand centrifuged at 13,000rpm to obtain the peripheral membrane proteins. Further, the remaining pellet was treated with 1% Triton X for 30 minutes and centrifuged to separate the integral membrane proteins from the insoluble proteins. Protein samples were prepared by adding SDS loading dye to the different fractions obtained. Equal concentrations of respective fractions were resolved by SDS-PAGE followed by Western blotting using specific antibodies.

13. Transfection of *Plasmodium falciparum* 3D7

Fresh RBCs (500μl, compact) were washed in RPMI media in a falcon. Traces of media were removed, and cells were washed with cytomix twice (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, pH 7.6). 100 μg of DNA (20μL) was introduced into tube containing compact RBCs along with 30 μL cytomix and mixed well. The mixture was transferred to electroporation cuvette and a pulse was given according to standard protocol. The cuvette was immediately transferred on ice after electroporation. The content of the cuvette was transferred into a falcon and washed with 10 mL incomplete RPMI twice. 8-10% parasitemia schizont stage parasites were diluted in such a way that ~10 μL parasite can be added per

transfected RBCs. The mixture was then transferred into T25 flask and allowed to grow in 10 mL complete RPMI in 5% CO_2 at 37˚C. Parasites were selected under 2.5 nM WR99210 drug after 48 hours and 60 µL compact blood was added on alternate days.

14. Live-cell imaging using ER-tracker

Parasites transfected with PfOrc2N$_{1-30}$GFP and PfOrc2N$_{1-150}$GFP respectively were incubated with 200 nM ER tracker red (Invitrogen) for 20minutes and 0.1 µg/µl DAPI was added for another 10 minutes. Cells were harvested and washed twice with 1X PBS and imaged using Carl ZEISS Apotome microscope.

15. Protease protection assay

Saponin-lysed parasites were suspended in chilled hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl) and incubated on ice for 10 mins. The cells were homogenized using a Dounce homogenizer and the suspension was centrifuged at 10,000 rpm for 15 minutes. The pellet fraction was immediately resuspended in 2X SDS loading dye while the supernatant was equally divided into two tubes. One part was treated with Trypsin (Promega) in the ratio of 1:50 and both samples were incubated at 37℃ for 10 mins. 1 mM PMSF and 6X SDS loading dye were added to both the tubes to stop trypsin digestion. The prepared samples were further analyzed by SDS-PAGE and immunoblotting with respective antibodies.

16. Yeast complementation

ScOrc4 gene was amplified by PCR using specific primers from the gDNA from *S. cerevisiae* and cloned in pRS416 expression vector between BamHI and HindIII sites. Similarly, *PfOrc4* gene was amplified from *P. falciparum* gDNA and cloned in pRS416 between BamH1 and EcoRI sites. A chimera of N-terminal ScOrc4 and C-terminal PfOrc4 was created by employing a PCR-based SOE technique (gene Splicing by Overlap Extension). We designed a set of overlapping primers by adding few nucleotides of 3’ *ScOrc4* reverse primer to 5’ *PfOrc4C* forward primer without the need of any additional restriction site. Different domains and the secondary structure of ScOrc4 and PfOrc4 were taken into consideration while generating chimera. The chimera was also PCR amplified and cloned into pRS416 vector between BamH1 and

EcoRI sites. For all the three constructs, the cassette containing the gene of interest and *Gal* promoter was excised and ligated in another vector, pRS423 having *His3* as a selection marker. All the three constructs in pRS423 vector were used to transform yeast *orc4* knock-out strain as described below.

17. Yeast transformation

Yeast o*rc4* swapper strain was grown in 10 ml YEPD medium overnight at 30ºC with shaking at 200 rpm. 5×10^6 cells from primary culture were inoculated into 50 ml YEPD media and allowed to grow at 30ºC till cell number reaches 2×10^7 cells. Culture was harvested in a sterile 50 mL centrifuge tube. Cells were re-suspended in 25 mL sterile water and centrifuged again. 1 mL 100 mM LiAc was added to the pellet and the suspension was transferred to a 1.5 ml microfuge tube. Cells were then centrifuged at 13,000 rpm to remove LiAc. Cells were resuspended to a final volume of 500 µL of 100 mM LiAc. 50 µL of the mix was added to the microfuge tubes. Cells were centrifuged and LiAc was removed. 240 µL of PEG (50%), 36 µl lM LiAc, 25 µL ss-DNA (2 mg/ml), 50 µL water and DNA construct was added to the pellet. Each tube was vortexed to resuspend the cell pellet. Mixture was incubated at 30ºC for 30 min. Heat shock was given at 42ºC for 20 minutes followed by centrifugation to remove the supernatant. Pellet was resuspended in 100 µL of sterile water and plated on the selective media-agar plate.

18. Chromatin immunoprecipitation assay (ChIP) assay

ChIP assay was performed as per the manufacturer’s protocol. Briefly, trophozoite stage parasites were crosslinked using 1% formaldehyde and incubated for six minutes at 37ºC. The sample was centrifuged at 2,000 rpm for 4 minutes and supernatant was discarded. The pellet was washed with 1X PBS twice in the present of 1 mM PMSF at 4ºC followed by saponin lysis. The parasite pellet obtained was resuspended in SDS lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCL pH 8.1, 1mM PMSF and protease inhibitor cocktail, followed by incubation on ice for 10 minutes. The lysate was sonicated for 1 minute with 6 cycles (10’’ on, 10’’ off) to shear the DNA into shorter DNA fragments of ~200 to ~500 bp. A clear lysate was obtained after centrifugation at 13,000 rpm for thirty minutes at 4ºC. 5% input was isolated from the

supernatant and remaining sample was diluted in ChIP dilution buffer, followed by pre-clearing the lysate using Protein A Sepharose beads. The pre-cleared sample was divided into two equal parts and incubated with immune and pre-immune sera overnight at 4ºC. The immunoprecipitated DNA samples were screened by semi quantitative PCR for enrichment of PfOrc4 protein on specific sites.

Chapter I

Biochemical characterization of origin recognition complex subunit 2 of Plasmodium falciparum

I. Introduction

DNA replication is a vital process and forms the basis of inheritance in all living forms. Thus, making a precise copy of the parent DNA is fundamental to every living organism. DNA replication is brought about by an initiator which binds the replicator DNA sequences called origin of replication (*ori*), within the genome (Jacob, Brenner et al. 1963). These *ori* sites are present in both prokaryotes and eukaryotes, difference being the existence of complex and multiple origins in eukaryotes. In general, Origin recognition complex (ORC) is the conserved initiator which binds to origins of replication. It provides a platform for other proteins to be recruited at the Ori, thereby forming a pre-replication complex. This complex consists of Origin Recognition Complex (Orc1-6) protein subunits (Bell and Stillman 1992), Cdc6 & Cdt1 proteins (Hofmann and Beach 1994; Cocker, Piatti et al. 1996) and a hexamer of Mcm2-7 helicase proteins (Aparicio, Weinstein et al. 1997).

The function of ORC is not limited to DNA initiation only. Role of ORC subunits in transcriptional silencing has been well documented (Foss, McNally et al. 1993). Besides these, ORC subunits also have a defined role in chromatid separation (Shimada and Gasser 2007), cytokinesis (Prasanth, Prasanth et al. 2002), and regulation of dendritic spine development in postmitotic neurons (Huang, Zang et al. 2005).

Replication of DNA in *P. falciparum* is well orchestrated and timed in a manner that multiple rounds of replication take place in a short span of time (White and Kilbey 1996). This could be made possible by presence of multiple replicator sequences along with a highly efficient replication machinery to recognize and initiate replication at those sites. Thus, DNA replication plays an important role in *Plasmodium* biology. However, different steps of DNA replication, particularly the replication initiation are poorly understood. Among the ORC subunits, PfOrc1 and PfOrc5 have been characterized thoroughly. Role of PfOrc1 has been established in DNA replication as well as Sir-2 dependent *var* gene silencing (Mehra, Biswas et al. 2005; Gupta, Mehra et al. 2009; Deshmukh, Srivastava et al. 2012). Furthermore, PfOrc5 has been shown to co-localize and interact with replication foci marker PfPCNA during trophozoite stage

(Gupta, Mehra et al. 2008). In addition, putative homologs of PfOrc2 [PF3D7_0705300], PfOrc3 [PF3D7_1029900] and PfOrc4 [PF3D7_1334100] have been identified and annotated in the *Plasmodium* genome database (PlasmoDB). The presence of Orc6 homolog in *P. falciparum* has not been reported yet.

Origin recognition complex 2 (Orc2) plays a central role in formation of ORC core complex, consisting of Orc2-5. It has been reported that the C-terminal region of human Orc2 directly interacts with the N-terminus of HsOrc3 (Dhar, Delmolino et al. 2001). *In vitro* immunoprecipitation experiments revealed that HsOrc2 interacts with HsOrc4 and HsOrc6 as well (Vashee, Simancek et al. 2001). Orc2 has been shown to bind to DNA through ChIP-based experiments, where it remains bound to the chromatin throughout the G1 to S-phase (Ladenburger, Keller et al. 2002). ORC has also been implicated in the heterochromatin formation in *Drosophila.* DmOr2 was shown to co-localize with HP1, a protein involved in gene silencing and heterochromatinization (Pak, Pflumm et al. 1997). Interaction between Hp1 and human Orc2 has also been reported, where depletion of Orc2 using anti-sense is correlated with mis-localization of Hp1 (Prasanth, Prasanth et al. 2004). Thus, function of Orc2 is not limited to DNA replication initiation only. In *Plasmodium falciparum,* putative Orc2 had been identified in our lab and established as a homolog of yeast Orc2 (studied by Gupta A and Dhar SK). In this chapter, we are focusing on PfOrc2 protein with unique characteristics as reported earlier by our laboratory.

II. Results

1. *In-silico* analysis of PfOrc2 sequence

P. falciparum Orc2 (PfOrc2) is an 825 amino acids long protein encoded by 2,478 bp long *orc2* gene (PF3D7_0705300). The complete amino acid sequence of PfOrc2 is given below (Fig.1.1A). The various domains identified in the protein sequence have been illustrated in the schematic below (Fig.1.1B). It consists of an Orc2 super family domain (495-818 aa), two putative nuclear localizing signal sequences (NLS; 262-276 aa & 281-295 aa), and an ER-retention sequence at the extreme C-terminus (822-825 aa).

A. PF3D7_0705300; origin recognition complex subunit 2, putative (ORC2)

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MLKNFEVNSPKKLIRKIEEETYEEDKEEYLITNLTENKKNMPSVIIRIPRDLNKFLSNEE
TTIKYNENIKSSNNKMNKEQKLTSFKMDNKYDMNSEEKISAMDNIVLDSTYQSDSTHESI
SFFNKIKGQNYTIDNINNENENNNSSSNGDSDDDSNDDSDNNSYDNSDYYTDDDKPIDES
LLKSFKNKYVDVYESNVSNNEYITFIKNHTKNELEHFLQRRKLHHTNEMKVNDIINIDYD
KLNEKNIIPYFLTNVFDMEQKKKREKMKLELEKKRKIMEEKRKTKTKVDDKAKTKNDTHI
NEKKKKKRNENNKVDTKKSVENFTKAANTQKIVKEGKMKTEDNDNSYDEYEDDNSYENKN
RRNKKNKTNSNENQKINEDNEESYSDSYNDEFDKEDIDINIYNDPTLYGIEGSSDYSEDM
KSDADNHTQEDDTGKEQITNNNSKTPKKKKGKKNIDEKNSEEEEKLDDIVDACTQRLISY
DYYSSLNIKEIVKPNNKIKSLSSYIPIQENLDNLDHIQKLQYLIKNLPYTHIKEKRSLYH
YNIKQFIKWKVYLLNNINICLYGIGSKFHLLNLFTNICLNDGNKCIILGFEDEINFEEIL
VRILEYHYKYKSSKTLKSFDLLYELIQRVNDSNVPLYFIIHNLDNTKLYPYYEYFSFLSQ
YENIYFVCSIDDVSFELNMNFKNISSINFFYIKCHTWLDYRHEILRQWNKFLPEWVFNKK
CEEIDIKKNIETILNALSINHKRLFKIIASIQLENLDKGIYGVEKESLLQDKRIFTVGAS
SIRINSLLVEFVSHNVITETRLKEGNTFLKINADKEELKRISEEL
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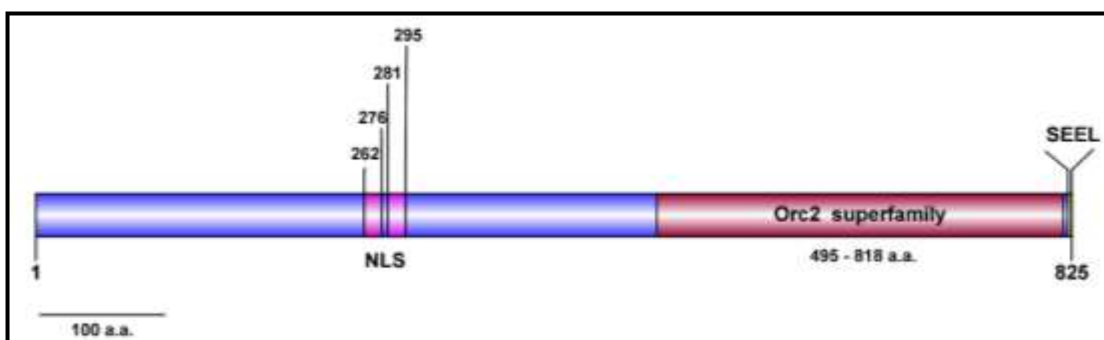

Fig. 1.1 (**A**) Primary sequence of Origin Recognition complex 2 subunit of *Plasmodium falciparum* 3D7. The gene is annotated as putative PfOrc2 in PlasmoDB database. (**B**) Schematic representation of various domains present in PfOrc2. Two putative NLSs have been identified by SMART software (https://prosite.expasy.org) besides an Orc2 superfamily domain and a putative ER-retention sequence; SEEL.

2. Generation of polyclonal antibodies against PfOrc2

Antibodies have proved to be a great tool to study any protein. Earlier, antibodies against the C-terminus of PfOrc2 (PfOrc2C) have been raised in rabbit in our laboratory. These were polyclonal antibodies raised against two peptides from the C-terminal region of PfOrc2 (604-617 aa and 756-771 aa respectively). In order to fully characterize PfOrc2, we decided to generate antibodies against the extreme N-terminus of PfOrc2 as well. The cloning of Pforc2N (1-996 bp) in expression vector pET-21a(+) was performed by Dr Ashish Gupta, Dhar lab. We used this construct to transform *E. coli* BL21 CodonPlus (DE3) cells and checked the expression of PfOrc2N. The N-terminal domain is constituted by 332 amino acids resulting in 36.5 kDa protein

fragment. *E. coli* BL21 cells were transformed with the recombinant clone as described above. The protein was expressed and purified using Ni-NTA affinity purification as described in the materials and methods. Different protein samples were subjected to SDS-PAGE followed by staining the gel with Coomassie blue (Fig.1.2). The purified protein obtained at ~40 kDa was confirmed as PfOrc2 (PF3D7_0705300) after MALDI analysis prior to antibody generation.

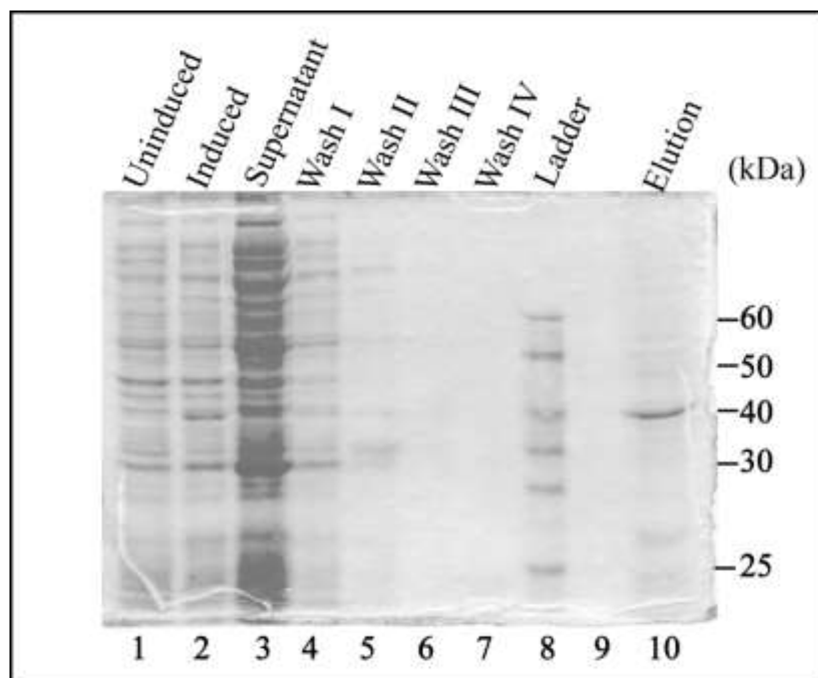

Fig. 1.2 Purification profile of His$_6$-PfOrc2N protein from *E. coli* BL21 CodonPlus (DE3) lysate. Different protein samples (Lane 1: Uninduced *E. coli* lysate; Lane 2: Induced *E. coli* lysate; Lane 3: supernatant after sarcosine treatment; Lane 4: Wash I, 10 mM Imidazole buffer; Lane 5-7: Washes II-IV, 35 mM Imidazole buffer; Lane 8: protein ladder; Lane 10: Elution, 250mM imidazole buffer.) A band corresponding to PfOrc2N was obtained in the eluate at ~40 kDa after Ni-NTA chromatography purification. The sizes of molecular weight standards (protein ladder marker) are shown on the right.

For the purpose of antibody generation, ~100 µg of purified PfOrc2N protein was injected into mice at stipulated time interval according to the method explained in materials and methods. Pre-immune sera were collected prior to injecting the protein into mouse. Both the immune sera and pre-immune sera were used to immunoblot uninduced and induced bacterial lysates along with the purified protein to check the authenticity of the antibodies (Fig.1.3). The antibodies against PfOrc2N and PfOrc2C were employed in subsequent experiments to further characterize the endogenous expression of PfOrc2.

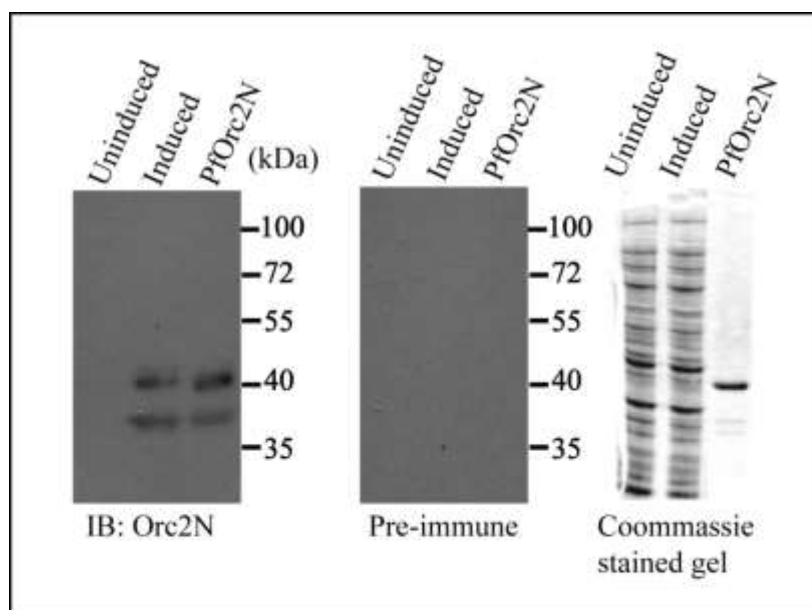

Fig. 1.3 Uninduced and IPTG-induced bacterial lysates were resolved by SDS-PAGE along with the purified PfOrc2N protein followed by Western blot analysis using antibodies against PfOrc2N and pre-immune sera. Specific bands of PfOrc2N were recognized in the induced and purified lanes, pre-immune sera did not recognize any such band. (In some experimental replicates, purification of recombinant PfOrc2N yielded shorter bands along with ~40 kDa band which could be a result of protein degradation).

3. Intra-erythrocytic protein expression of PfOrc2

3.1 PfOrc2 is expressed as a full-length protein endogenously

As discussed earlier, *P. falciparum* parasites cycle among ring, trophozoite and schizont stages during their intra-erythrocytic life cycle inside the human host. It is essential to study the endogenous expression of any protein in these stages to fully understand its function. A stage specific expression profile of PfOrc2 using the antibodies against C-terminus has been studied earlier (Sharma R & Dhar SK). We decided to check the expression of PfOrc2 using antibodies against PfOrc2N. *P. falciparum* infected erythrocytes pellet and only RBCs were subjected to saponin lysis and resolved by SDS-PAGE followed by immunoblotting by using antibodies against PfOrc2N. A full-length band was obtained around the expected size of ~ 100 kDa (molecular wt. 98 kDa) which was corroborated by using antibodies against C-terminal region of PfOrc2. It is interesting to note that the antibodies against PfOrc2C always pick up a smaller and more intense band at ~45 kDa along with the full-length band (Fig.1.4B). These results clearly show that PfOrc2 is expressed as a full-length protein of ~100 kDa as two

antibodies raised against different regions of the same protein recognize a common band at the expected size of ~100 kDa.

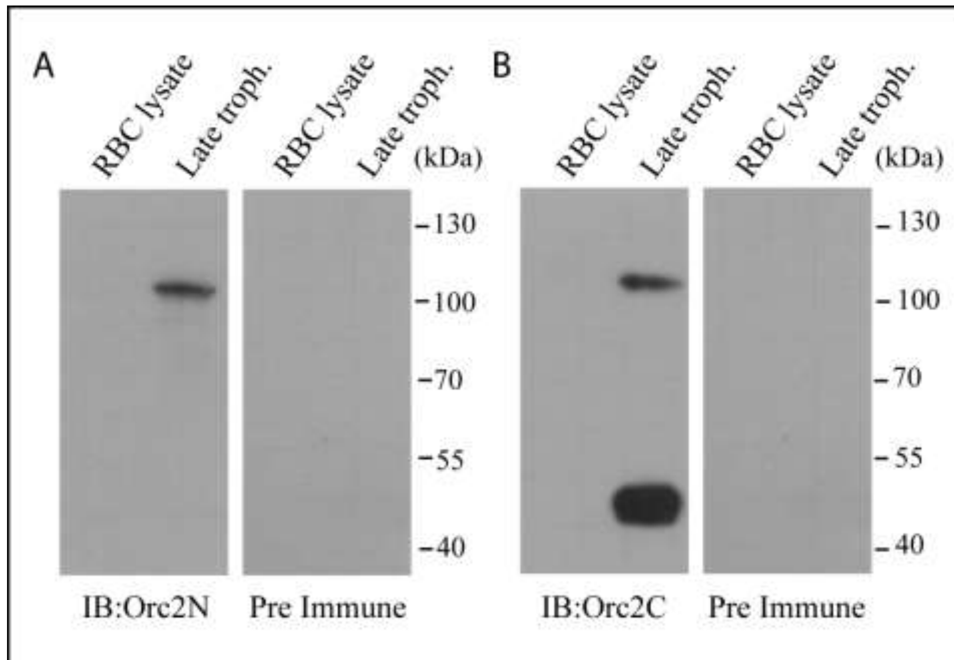

Fig. 1.4 Expression of PfOrc2 was evaluated in the late trophozoite stage using antibodies against PfOrc2N and PfOrc2C. **(A)** Antibodies against PfOrc2N picked up one single band around ~100 kDa, which is the expected size of the full-length PfOrc2. **(B)** Western blotting of same trophozoite lysate using antibodies against PfOrc2C resulted in two distinct bands at ~100 kDa and ~45kDa.

3.2 Immunoprecipitation and Mass Spectrometric analysis of parasite lysate to confirm the identity of PfOrc2C

Whenever two or more discrete bands are observed after immunoblotting with specific primary antibodies, it is presumed to be either post-translational modification or an alternate gene product besides the possibility of a site-specific protein cleavage. PfOrc2 sequence does not contain any intron or internal ribosome entry site (IRES); thus, ruling out the possibility of any isoform. Various bioinformatic tools indicate several PTMs like phosphorylation, glycosylation and myristylation of PfOrc2. However, these modifications might not account for difference of ~55 kDa between the full-length protein and the shorter ~45 kDa fragment. To ensure that the lower band obtained in Western blotting experiments is indeed a part of full-length protein, we performed immunoprecipitation assay using antibodies against PfOrc2C. Immunoprecipitated

protein samples were resolved by SDS-PAGE and immunoblotted using antibodies against PfOrc2C. Band corresponding to ~45 kDa fragment of PfOrc2C was obtained in the input lane and immune sample lane but not in pre-immune lane (Fig. 1.5). Although the immunoprecipitation was not very efficient as compared to input, this experiment gave us confidence to claim that the band acquired at ~45 kDa is a processed form of PfOrc2 and not a cross-reacting band, since it would not have been immunoprecipitated by specific antibodies.

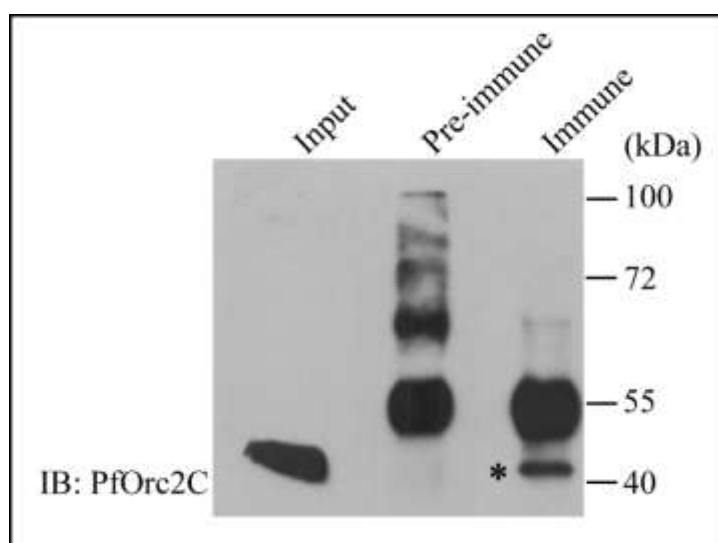

Fig. 1.5 Immunoprecipitaion experiment of Pf3D7 lysate was performed using pre-immune sera and antibodies against PfOrc2C. The samples obtained were resolved by SDS-PAGE followed by Western blotting analysis using antibodies against PfOrc2C. Specific band of PfOrc2C fragment was obtained at ~45 kDa in the immune and input lanes, which was absent in the lane loaded with pre-immune sample.

Additionally, to corroborate the above result, we used 8M urea to lyse the *P. falciparum* 3D7 parasites and the prepared lysate was resolved through SDS-PAGE followed by Coomassie staining. Gel slices were excised from regions pertaining to ~100 kDa (region I), ~40-55 kDa (region II) and ~25-35 kDa (region III) and subjected to mass-spectrometric analysis as described in methods section. As illustrated in Fig.1.6, PfOrc2C peptides were identified from both regions I and II, whereas PfOrc2N peptide was found in the sample of region I only. We also took trypsinized sample from region III as a control, from where no peptides of PfOrc2 were retrieved. These results gave us confidence that the lower band of ~45 kDa was possibly a part of PfOrc2 which is being released after processing and not a non-specific band.

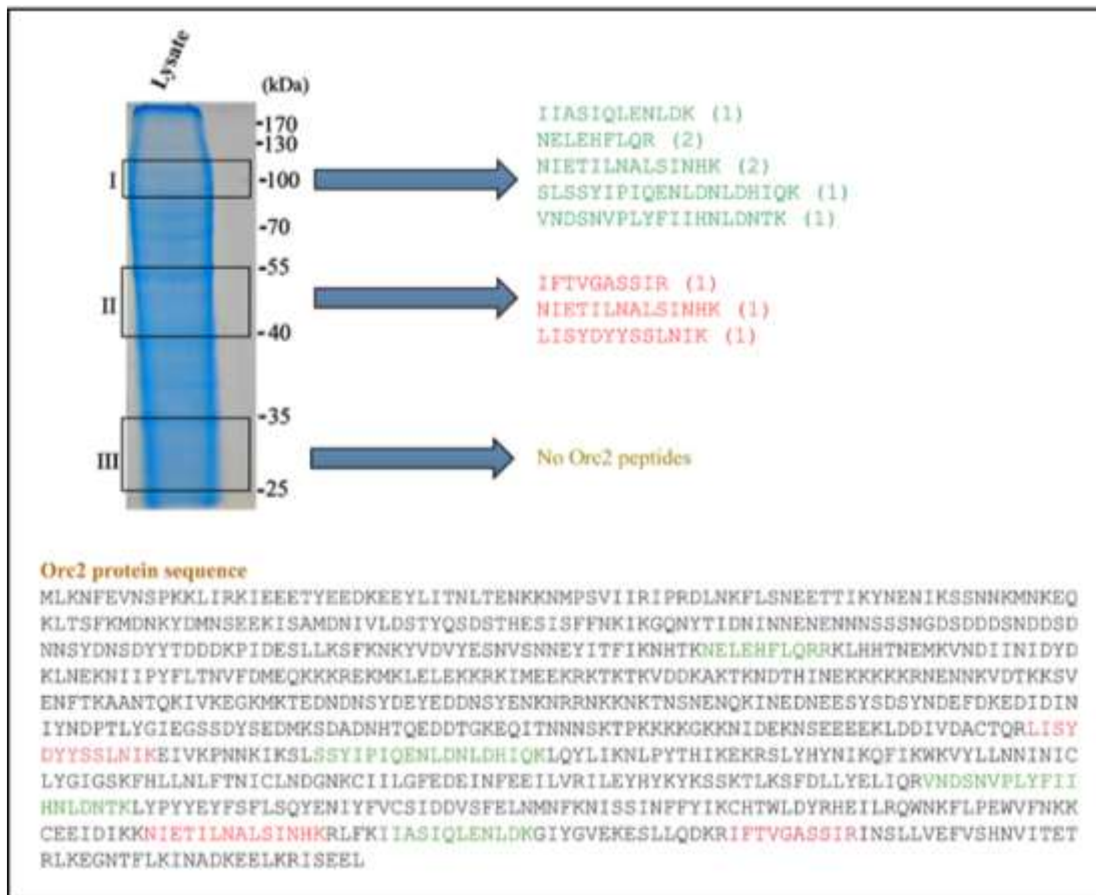

Fig. 1.6 Mass-spectrometric analysis of *P. falciparum* lysate. Parasites were treated with 8M Urea and whole cell lysate was subjected to SDS-PAGE. Three different regions corresponding to (I) ~100 kDa, (II) ~40-55 kDa and (III) ~25-35 kDa were selected and excised from the coommassie-stained gel. The three samples were trypsinised and the peptides were analysed mass-spectrometerically. The peptide sequences retreived from MS analysis are highlighted in the complete PfOrc2 protein sequence. Green and red colors are used to distinguish between the sequences obtained from region I and II respectively.

3.3 Stage specific expression of PfOrc2

Further, we wanted to explore the expression pattern of PfOrc2 in different parasitic stages of *P. falciparum*. Tightly synchronized parasites were harvested at different stages viz. ring (18 ± 4hpi), trophozoite (32 ± 4 hpi) and schizonts (40 ± 4hpi) and lysed using saponin. The samples were resolved through SDS-PAGE followed by immunoblotting using antibodies against PfOrc2N. The intensity of the band corresponding to full-length PfOrc2 was found to be maximum in trophozoite stage as compared to ring and schizont stages (Fig.1.7A). The stage specific expression of PfOrc2 protein had been studied earlier by using the antibodies against PfOrc2C.

Intensity of band corresponding to PfOrc2C fragment was found to increase as stage progressed from ring to trophozoite stage and decreased gradually as schizont stage was reached (Sharma, Sharma et al. 2018). The stage specific expression obtained for full-length protein (probed by antibodies against PfOrc2N) corroborates the pattern of stage specific expression exhibited by PfOrc2C fragment reported earlier. Thus, the overall protein expression of PfOrc2 was found to be maximum in the trophozoite stage, which is the time of active DNA replication. Also, the amount of full-length protein is negligible in ring stage. The *in-silico* transcript level of PfOrc2 as described in PlasmoDB also resembles with the protein levels in different parasitic stages (Fig.1.7B).

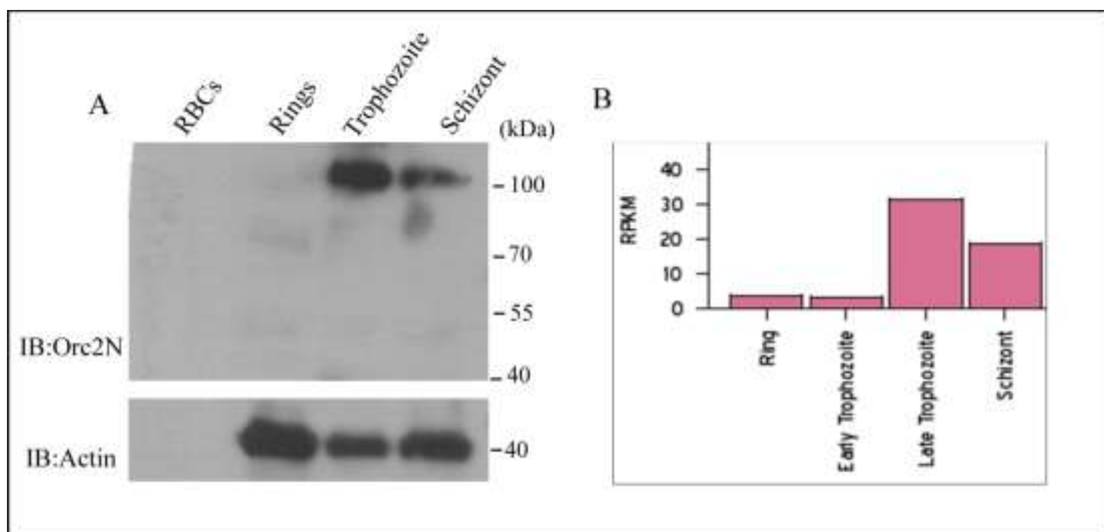

Fig. 1.7 (A) Endogenous expression of PfOrc2 protein was checked in different erythrocytic developmental stages of *P. falciparum*. Western blotting experiment using antibodies against PfOrc2N identified the full-length band in trophozoite (32 ± 4 hpi) and schizont stages (40 ± 4 hpi). Expression of PfOrc2 was found to be maximum in the trophozoite stage. (**B**) Transcriptome data of PfOrc2 available on PlasmoDB (http://plasmodb.org/plasmo/) suggest that indeed more transcripts are present in late trophozoite stage, possibly accounting for higher protein level.

4. Intracellular localization of PfOrc2

4.1 Immunofluorescence assay using antibodies against PfOrc2N and PfOrc2C

We performed immunofluorescence assay (IFA) using the antibodies against PfOrc2N and found a diffused signal around the nuclear stain DAPI in all the parasitic stages (Fig. 1.8A). Parasites from late ring (20 ± 4 hpi), mid-trophozoite (30 ± 4 hpi) and schizont stages (40 ± 4 hpi) have been shown for representation. Immunostaining using

antibodies against PfOrc2C gave intense punctate localization over nuclear DAPI in all stages viz. rings (18 ± 4 hpi), trophozoite (25 ± 4 hpi) and schizont (42 ± 4 hpi) as shown in Fig.1.8B. Even though the full-length protein is localized around the nucleus (as shown by IFA results using antibodies against PfOrc2N that predominantly recognize the full-length protein), the perinuclear signal obtained by IFA of PfOrc2C strengthens the fact that PfOrc2C is indeed a nuclear protein.

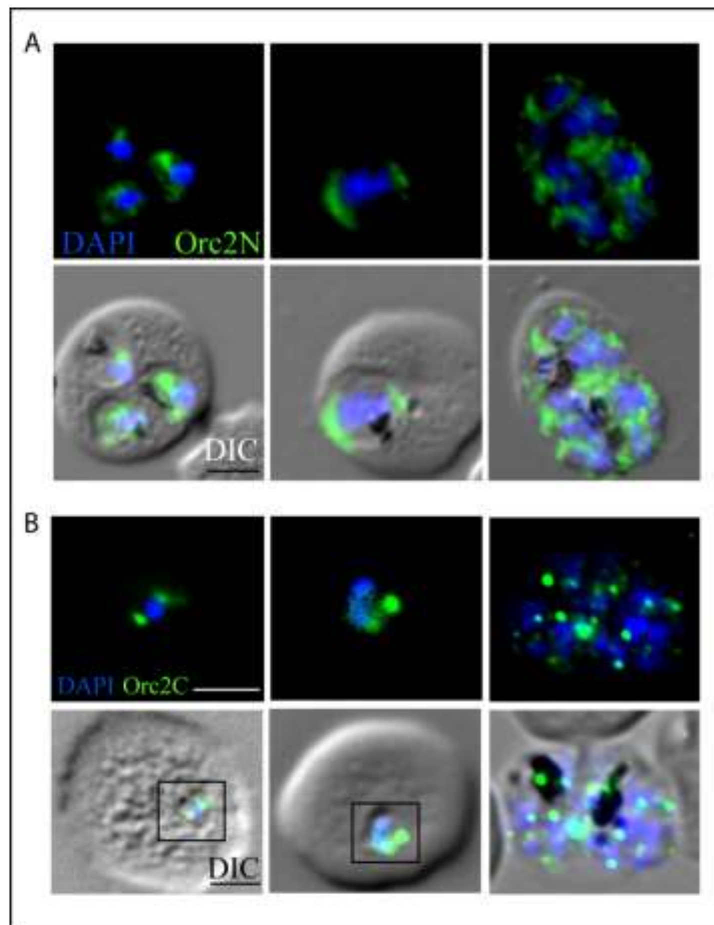

Fig. 1.8 Immunofluorescence assay (IFA) was performed to study the localisation of PfOrc2 in different parasitic stages. **A.** IFA using PfOrc2N antibodies presented a diffused extra-nuclear signal in all the stages. **B**. Punctate nuclear staining was exhibited by PfOrc2C antibodies in rings (18 ± 4 hpi), trophozoite (25 ± 4 hpi) and schizont (42 ± 4 hpi) parasitic stages (scale represents 2 μm in all images; DAPI is used to stain nucleus).

4.2 PfOrc2C localizes in the nucleus

To confirm the nuclear localization of PfOrc2 by another method, we tried to find the immunolocalization of PfOrc2C using gold labeled secondary antibodies. The protocol for preparing blocks for microtomy and labelling of samples has been described in

methods section. After scanning several IEM images, we noted that gold particles predominantly accumulated in the nucleus. As a representative image, Fig. 1.9 clearly demonstrates the presence of PfOrc2C in the nucleus. We also observed that no gold particles were present in the cytoplasm when an equal area was taken for comparison.

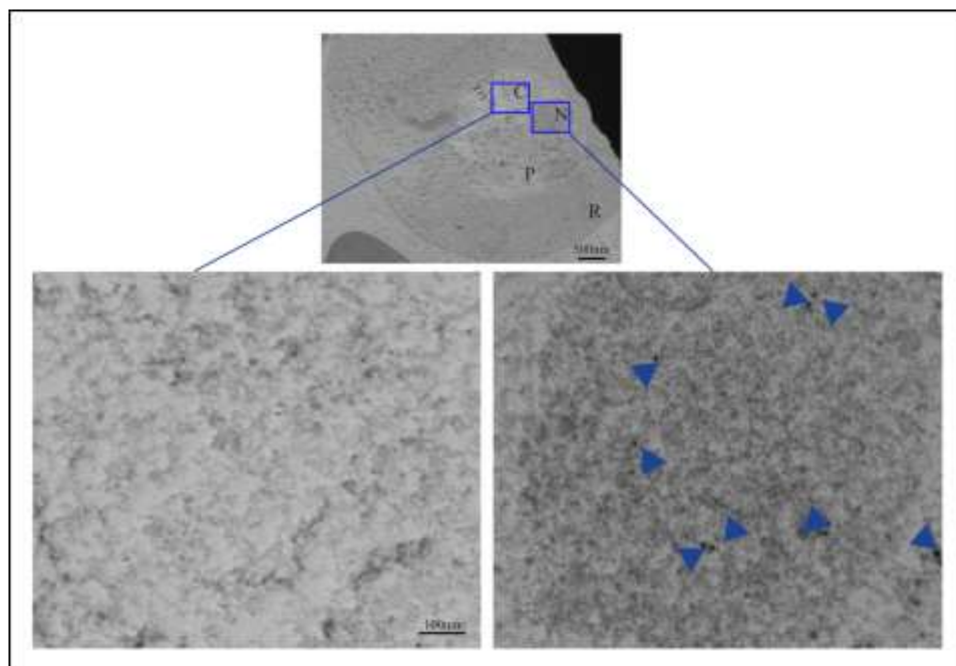

Fig. 1.9 Immuno-electron microscopy of *P. falciparum* trophozoite stage parasite depicting the enrichment of gold particles in the nucleus. Gold-labelled secondary antibodies were used to probe the region where PfOrc2C primary antibodies were bound. No gold particles were present in the cytoplasm (C and N denote cytoplasm and nucleus of the parasite; P represents the parasite inside the RBC; R. Scale bar is as indicated).

4.3 Sub-cellular fractionation of PfOrc2

Since, PfOrc2 full-length protein and PfOrc2C fragment showed different localization pattern by immunofluorescence assay, we deemed it necessary to check their sub-cellular fractionation profile. Following the NP40-detergent based fractionation protocol, which separated the cytoplasmic proteins from the nuclear proteins, we obtained the full-length PfOrc2 (probed with N-terminal antibodies) in the nuclear pool. In the same experiment, PfOrc2C protein fragment also remained in the nuclear fraction. In this experiment, we also checked the fractionation status of PfBip, which is an ER resident chaperone. Although ER proteins should be separated in the cytoplasmic fraction, we also obtained a faint band of Bip in the nuclear fraction. This indicates that

a non-nuclear protein may be recovered from nuclear pool due to close proximity to the nucleus. We used aldolase and histone as controls for cytoplasmic and nuclear protein fractions respectively (Fig. 1.10).

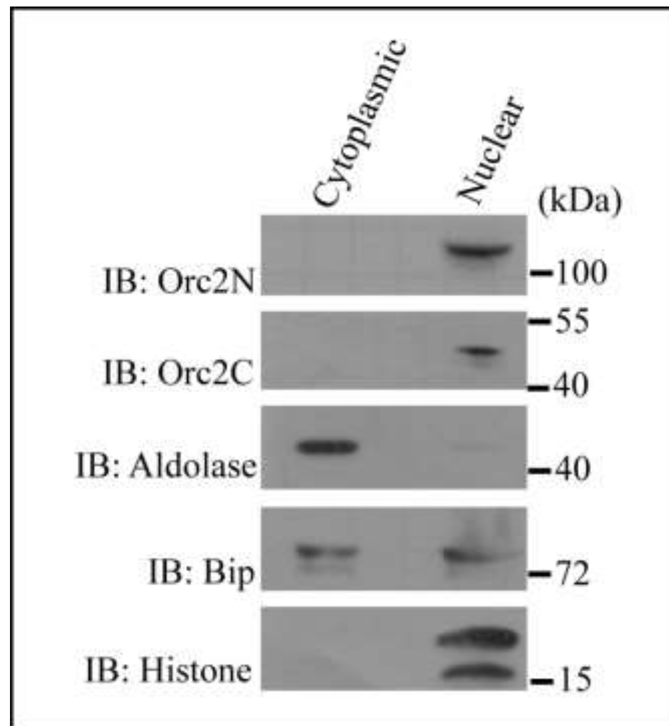

Fig. 1.10 Sub-cellular fractionation of *P. falciparum* trophozoite stage parasites was carried out using NP-40 detergent, as described in the materials and methods section. The cytoplasmic and nuclear fractions were subjected to SDS-PAGE followed by Western blot analysis using antibodies against PfOrc2N, PfOrc2C, PfAldolase (cytoplasmic protein marker), PfBip (ER protein marker) and histone (nuclear protein marker) respectively. Both the full-length PfOrc2 and PfOrc2C fragment were found to be present in the nuclear pool.

4.4 Full-length PfOrc2 is peripherally associated with membrane

The presence of full-length PfOrc2 protein in the nuclear pool was contrasting to the diffused non-nuclear IFA pattern obtained by using PfOrc2N antibodies (Fig. 1.8 A). We further decided to extract the membrane bound proteins using sodium carbonate (Na_2CO_3). This treatment discriminates between the peripheral and integral membrane proteins. Firstly, parasites were freeze-thawed to break open the cells and collect the soluble proteins. The basic pH solubilizes the peripheral proteins leaving the integral proteins intact in the lipid bilayer. Triton X-100 was employed to separate the integral membrane proteins from the insoluble chromatin bound proteins. Full length PfOrc2 was obtained in the soluble and peripheral protein fraction. PfOrc2C could withstand

both alkaline pH and Triton X-100 treatment and thus, remained in the insoluble protein fraction. Aldolase was used as a cytoplasmic control and Bip, an ER resident protein was used a control for proteins present near the nuclear periphery (Fig.1.11) These two fractionation experiments clearly indicate that PfOrc2 full-length is an extra-nuclear peripheral membrane protein, whereas the shorter PfOrc2C fragment is tightly bound to the chromatin and present in the nucleus.

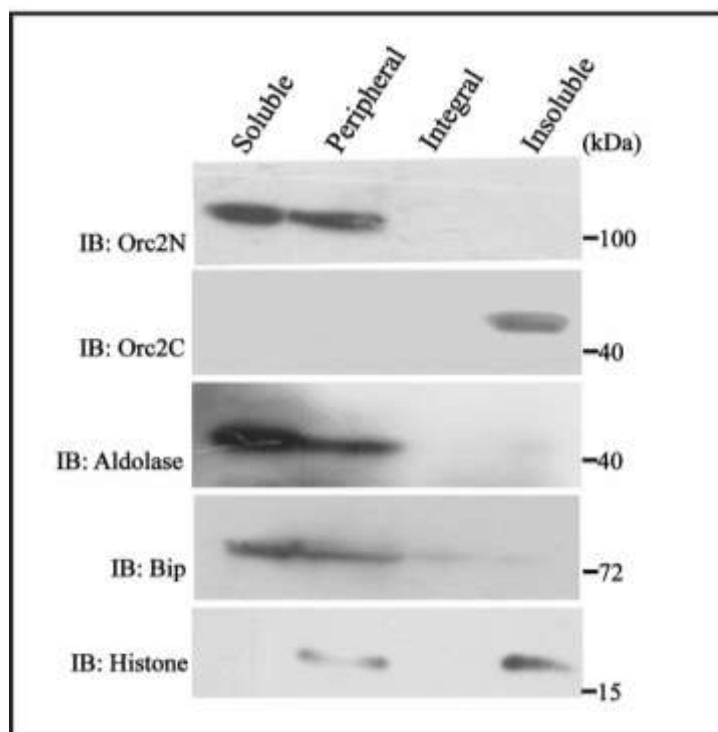

Fig. 1.11 Protein fractionation was performed in order to distinguish between soluble, membrane bound and chromatin bound proteins as described in materials and methods. Full length PfOrc2 was recovered from the soluble and loosely bound peripheral membrane protein fractions (Na_2CO_3 extracted). PfOrc2C stayed in the insoluble fraction (neither Na_2CO_3 nor Triton X-100 extractable). Aldolase was majorly present in the soluble fraction. PfBip appeared in both soluble and peripheral membrane fraction.

III. Discussion

As mentioned earlier, DNA replication is a vital and fundamental phenomenon occurring in all the organisms. Owing to its importance, it is one of the most studied fields of elemental biology. Replication initiation is the primary step in the process of DNA replication, hence aberrations in the genes coding for initiation proteins deleteriously affect the growth and multiplication of the organism. It has been reported that mutations

in genes coding for ORC subunits inhibit the loading of accessory replication factors required for forming Pre-RC and thus leads to defects in DNA replication (Romanowski, Madine et al. 1996; Chesnokov 2007). As cited earlier, ORC proteins have been implicated in transcriptional silencing, chromatid separation and cytokinesis as well. It can be inferred from the above examples that ORC as a complex has been conserved during evolution, but the different ORC subunits play diverse roles inside the cell.

Five of the six ORC subunits have been identified in *P. falciparum*. The individual ORC subunits have been worked upon, but how they interact with each other and other replication factors to form Pre-RC still needs to be elucidated. It is crucial to study and characterize all the ORC subunits in the parasite so as to decipher the molecular mechanism of its DNA replication. Complete understanding of the *Plasmodium* ORC is a preliminary step in apprehending the replicative and non-replicating function of ORC, and whether and how it influences pathogenicity. In this direction, work in the present study revolves around PfOrc2 and PfOrc4. The latter has also been identified and partially characterized (Sharma R and Dhar SK, unpublished data).

PfOrc2 has 22% identity with yeast *Saccharomyces cerevisiae* homolog ScOrc2. PfOrc2 has a conserved Orc2 superfamily domain which might be responsible for performing replicative function. A chimera of N-terminal ScOrc2 and C-terminus of PfOrc2 is able to complement the function of ScOrc2 in temperature sensitive *orc2* knock out yeast strain suggesting its essential role in DNA replication (studied by Dr Ashish Gupta, Dhar lab). Although the primary sequence suggests it to be alike any other ORC homolog consisting of Orc2 domain, but the protein expression profile obtained after using two different antibodies suggests otherwise. PfOrc2 has an SEEL sequence at the extreme C-terminus. Most of the bonafide ER-resident proteins have -KDEL or -SDEL amino acid sequence at the C-terminus which binds to the KDEL receptors and aids in retrieval of the protein back into the ER. ‘SEEL’ is not a common ER-retention signal, nonetheless, it is present in another *Plasmodium* ER protein, PfPDI (Protein disulphide isomerase). Since, the final destination of PfOrc2 (PfOrc2C) is nucleus, we assume that the putative ER-retention signal in Pforc2 is redundant or there is a possible protease cleavage which removes this sequence, thereby not allowing the protein to be retained in the ER.

Western blotting analysis of *Plasmodium falciparum* lysate in the presence of antibodies against PfOrc2N and PfOrc2C recognized the full-length band at ~100 kDa corresponding to endogenous PfOrc2 respectively, along with a stronger band at ~45 kDa with antibodies against PfOrc2C. As mentioned earlier, *Plasmodium orc2* does not contain any intron, or any internal ribosomal entry site, therefore, possibility of any isoform is remote. The predominant presence of shorter C-terminus band in all the stages indicates a possible processing event of full-length PfOrc2 which yields PfOrc2C fragment. We have also shown that peptides of PfOrc2C region are present in the region corresponding to ~ 40-55 kDa, which confirms the identity of shorter fragment. Several proteases have been accounted for site-specific cleavage of proteins. We will study the processing of PfOrc2 in the subsequent chapter.

PfOrc2 has a cytoplasmic pool in the form of full-length and a nuclear pool of C-terminal fragment. The full-length PfOrc2 is found to be present around the nucleus as seen in Fig. 1.8A, and extractable as a peripherally bound membrane protein (Fig. 1.11). Further, the presence of a putative ER-retention sequence in a conserved nuclear protein is thought-provoking. How and why the protein associates with the ER will be detailed in the following chapter. In this chapter, we have tried to biochemically characterize PfOrc2 by giving more attention to the expression and localization of the protein in the parasite. These results are groundwork for the detailed study of PfOrc2 in connection with Endoplasmic reticulum.

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Chapter II

Role of endoplasmic reticulum in trafficking of origin recognition complex subunit 2 of Plasmodium falciparum

I. Introduction

The secretory system is an essential player in the day-to-day maintenance and growth of organisms. It allows efficient regulation of the delivery of newly synthesized proteins, amino acids and carbohydrate to intracellular organelles as well as outside the cell, thus supporting the cellular growth and homeostasis (Lippincott-Schwartz, Roberts et al. 2000). This trafficking of molecules is more complex in an apicomplexan parasite such as *Plasmodium falciparum* since it needs to transport proteins to multiple destinations like parasite organelles, parasite membrane, parasitophorous vacuole, host erythrocyte cytoplasm and the erythrocyte membrane (Cooke, Lingelbach et al. 2004). The mechanism and route of protein trafficking in the parasite is being actively deciphered by various research groups. Questions arise when a nuclear protein is found to be trafficked through the secretory pathway, as is the case with PfOrc2 protein.

We have seen that PfOrc2 is a part of origin recognition complex, and possibly involved in replication related function as a chimera of yeast and *Plasmodium orc2* can complement yeast *orc2* mutant strain. Initial characterization of PfOrc2 using antibodies against N-terminus and C-terminus revealed unique protein expression profile and localization pattern. In this chapter, we will try to delve deeper into the trafficking of PfOrc2 to nucleus through its association of with the ER.

II. Results

1. Tracking the possible link of PfOrc2 with endoplasmic reticulum (ER)

1.1 Bioinformatic analysis of PfOrc2 amino acid sequence

Thorough *in silico* analysis of amino acid sequence of PfOrc2 revealed the presence of putative ER-retention signal in PfOrc2 in the form of SEEL at the extreme C-terminus. ‘SEEL’ is a non-contemporary ER-retention signal. We were surprised to find an ER-retention signal in a nuclear protein and hence examined whether this sequence is conserved among Orc2 protein of other apicomplexans and higher eukaryotes. Following sequence analysis of extreme C-terminus of different Orc2 homologs, we observed that no such ER retention sequence was present in other organisms but PfOrc2 (Fig. 2.1 A). We also generated an evolutionary tree using Clustal omega alignment and noticed that PfOrc2 and PbOrc2 were present in the same branch, which indicates

sequence similarity (Fig. 2.1B). However, absence of ER retention sequence in other Orc2 homologs may imply *Plasmodium falciparum* specific function of PfOrc2.

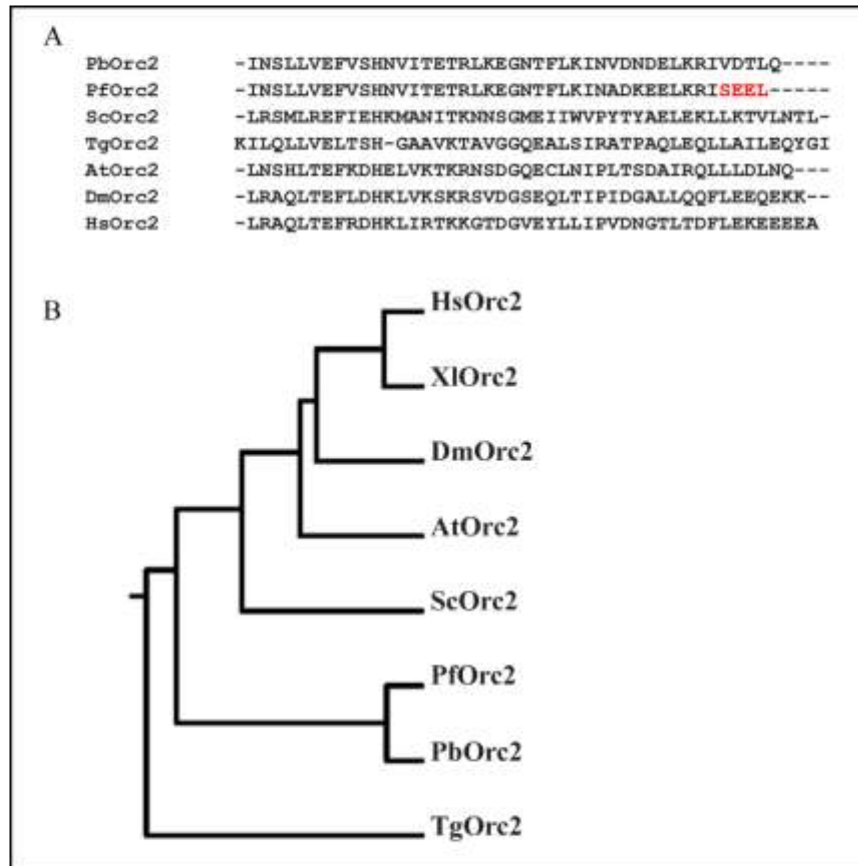

Fig 2.1 A. Sequence comparison of extreme C-terminal region of Orc2 homologs from different organisms using Clustal Omega. The putative ER retention signal is present exclusively in the *Plasmodium falciparum* Orc2, and not in other eukaryotes. **B.** Phylogenetic tree depicting the evolutionary relationship amongst the Orc2 homologs. [*Plasmodium berghei* (CDS46085.1), *Plasmodium falciparum* (XP_001348986.1), *Saccharomyces cerevisiae* (CAA85003.1), *Toxoplasma gondii* (KFG47135.1), *Arabidopsis thaliana* (NP_181292.1), *Drosophila melanogaster* (AAF99606.1) and *Homo sapiens* (NP_006181.1).

1.2 PfOrc2 is sensitive to Brefeldin A

The presence of a putative ER retention signal in a bona fide nuclear protein prompted us to investigate whether PfOrc2 enters the secretory pathway through ER. The proteins which are destined to localize in organelles, cell membrane or are secreted outside the cell are trafficked through the endoplasmic reticulum (Rapoport 1991). To test this hypothesis, we employed Brefeldin A (BFA), a fungal metabolite that inhibits the anterograde transport between ER and Golgi. BFA binds to the complex of Guanine nucleotide exchange factor called, GBF1 and GDP bound Arf1p proteins, thereby not allowing the

binding of GTP (Niu, Pfeifer et al. 2005. Consequently, uncoated vesicles in Golgi accumulate and fuse up, adversely affecting the dynamics between ER and Golgi (Niu, Pfeifer et al. 2005). 5 µg/mL BFA was introduced into the parasite culture in early trophozoite stage (20 ± 3 hpi) and parasites were harvested after 18 hours. Western blot analysis of vehicle only (ethanol) and BFA-treated parasite lysates displayed a moderate rise in the full-length protein when probed with antibodies against PfOrc2N (Fig. 2.2). Huge accumulation of shorter PfOrc2C fragment was also detected in BFA treated samples. Accrual of PfOrc2 (full-length as well as PfOrc2C) suggests possible link of ER-Golgi for PfOrc2 trafficking (Fig. 2.2). It is possible that the full-length protein gets processed in the ER and the PfOrc2C fragment moves ahead to the nucleus. We used antibodies against an apicoplast resident protein, single stranded binding protein (PfSSB) to know the status of proteins being trafficked from ER to apicoplast. PfSSB is an example of nuclear encoded, rough ER translated protein which is translocated to the apicoplast through ER (Foth, Ralph et al. 2003; Prusty, Dar et al. 2010). BFA treatment inhibited the processing of PfSSB leading to accumulation of its unprocessed form (Fig. 2.2).

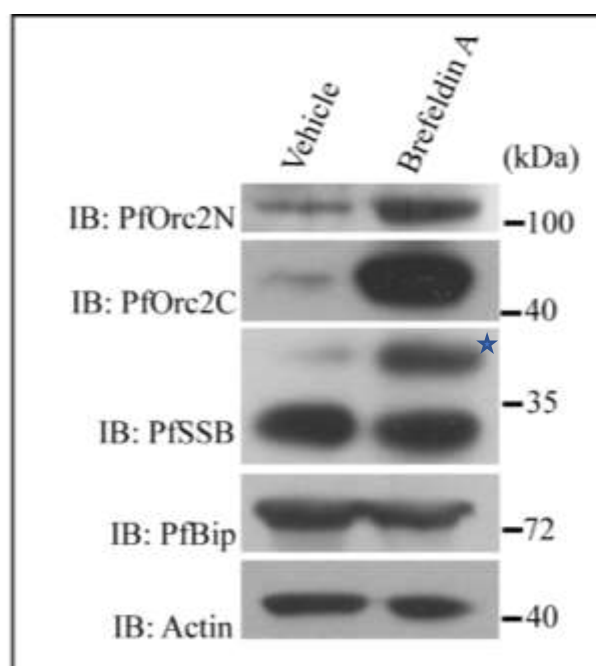

Fig. 2.2 PfOrc2 gets trafficked through the classical secretory pathway. 5 µg/mL Brefeldin A was added to the early trophozoite stage parasites for 18 hours and status of PfOrc2 was examined using antibodies against PfOrc2N and PfOrc2C respectively. Western blot analysis of vehicle and BFA treated samples exhibited increase in the protein level of full-length PfOrc2 in the BFA treated lane. PfOrc2C protein was found to accumulate as a result of BFA treatment. PfSSB was used a positive control to verify the effect of BFA on ER-translocated apicoplast protein. * denotes the unprocessed for of PfSSB. PfBip showed no change in protein level in the similar condition. PfActin was used as a loading control.

On suppressing the traffic from ER to Golgi, the unprocessed form of protein tends to accumulate too, which seems to be happening with both full-length PfOrc2 and PfSSB. Further, there was no effect on the overall protein level of ER-resident protein PfBip reaffirming that only the proteins moving ahead of the ER get accumulated in the presence of BFA. This experiment strongly indicates the potential role of ER in processing and trafficking of PfOrc2.

2. Identification of a putative ER-targeting sequence in PfOrc2

2.1 Prediction of putative ER targeting signal

Generally, the proteins entering the endoplasmic reticulum have a canonical ER-targeting signal at the N-terminus (Martoglio and Dobberstein 1998; Paetzel, Karla et al. 2002). This signal peptide is usually 10-30 amino acids long, constituted by a stretch of N-terminal hydrophobic residues (mainly form alpha helix), and then a cleavage site. The signal peptide is recognized and cleaved by signal peptidase enzyme (SP) at the time of entry into the ER (Paetzel, Karla et al. 2002). We looked for the presence of any such signal peptide in PfOrc2 using the conventional signal sequence prediction tools like SignalP, PrediSi or SignalBlast. However, we could not identify any such signal sequence (data not shown). However, failure of finding ER targeting signal by bioinformatics tools does not rule out the possibility of the presence of putative ER targeting signal at the N-terminus of PfOrc2. It may be noted that certain proteins entering the secretory pathway have a recessed signal sequence and may not be identified by the commonly used signal peptide prediction software (van Ooij, Tamez et al. 2008). Sequence analysis using another signal peptide prediction tool, Phobius, identified a putative ER-targeting signal at the N-terminus of PfOrc2. This signal peptide consists of a hydrophobic region between 5-10 aa (sequence, FEVNSP) and a cleavage site between 15/16 aa) (Fig. 2.3).

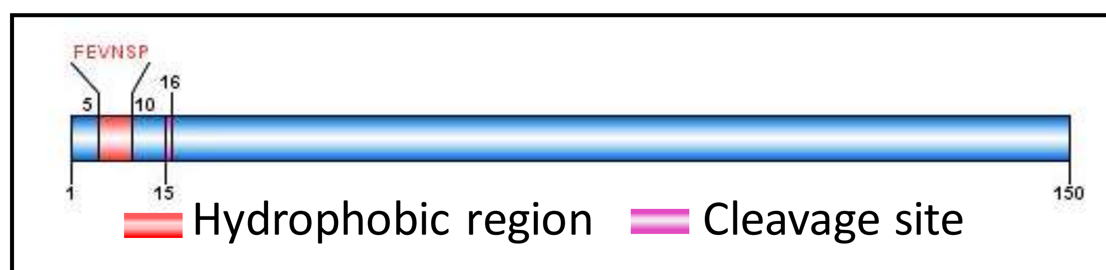

Fig. 2.3 Schematic representation of extreme N-terminus of PfOrc2. Signal peptide prediction software Phobius identified a hydrophobic region between 5-10 amino acid region. A cleavage site for signal peptidase was identified at 15th /16th amino acid residues.

2.2 Generation of parasite lines expressing PfOrc2N-GFP

In order to test whether extreme N-terminus of PfOrc2 contain putative ER-targeting signal, we cloned the first 30 amino acids from the N terminus of Orc2 into pARL vector (with GFP at C-terminus) and used this construct to transfect Pf3D7 parasites. Cell lines expressing only GFP were also generated by transfecting Pf3D7 parasites with pARL vector alone. We also cloned the first 150 amino acids from N-terminal region of PfOrc2 into a transposon mediated vector, PiggyBac which expressed GFP as a fusion protein. The protein expression following transfection in each case was confirmed by Western blotting using the antibodies against GFP. Bands of expected sizes ~26 kDa and ~30 kDa were obtained for GFP alone and PfOrc2N$_{1-30}$GFP respectively (Fig. 2.4 A). Surprisingly, parasites expressing PfOrc2N$_{1-150}$GFP gave multiple bands at ~50 kDa (marked **) and two bands below 40 kDa in molecular weight (Fig. 2.4 B). The expected molecular weight of PfOrc2N$_{1-150}$GFP is ~43 kDa. This points towards a possible cleavage taking place at the N-terminal of PfOrc2. The exact site and nature of cleavage still remains to be studied in detail. We also checked the expression of another GFP tagged cell line (Sir2 GFP) as a positive control for authenticity of antibodies against GFP.

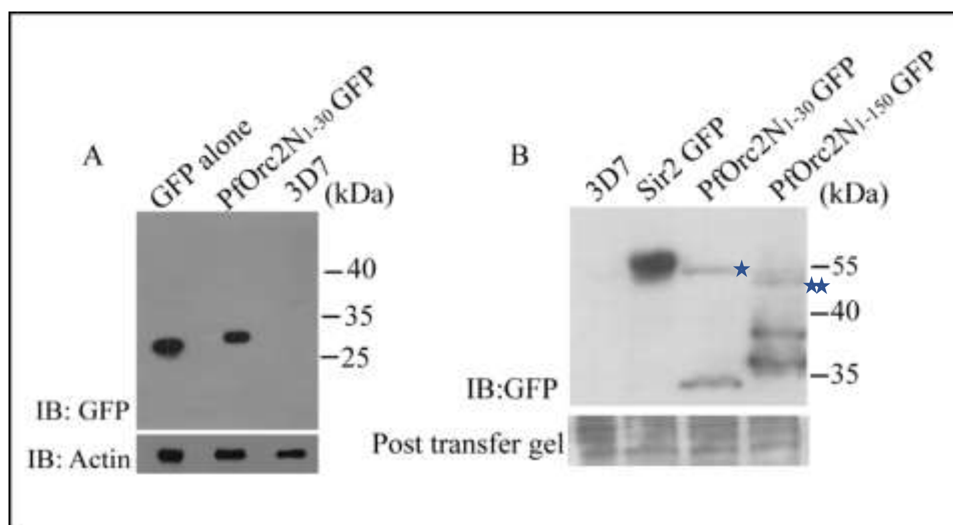

Fig. 2.4 Expression of GFP tagged proteins in different parasite lines. Different parasite lysates as labelled in the figure were subjected to SDS-PAGE and immunoblotted using antibodies against GFP. **A.** Bands corresponding to GFP and PfOrc2N$_{1-30}$GFP were obtained at expected molecular weight of ~26 kDa and ~30 kDa respectively. No bands were obtained in the untransfected Pf3D7 lane. **B.** A band of expected size ~45 kDa (marked **) along with two bands of <40 kDa were obtained in the PfOrc2N$_{1-150}$GFP lysate lane. Sir2 GFP lysate was loaded as a positive control for authenticity of GFP antibodies. (* indicates a non-specific band at ~55 kDa). Post-transfer gel is shown as loading control.

2.3 Live cell imaging of PfOrc2N-GFP proteins

To find out whether the first N-terminal 30 aa or 150 aa are sufficient to drive GFP into ER, we tracked localization of GFP along with ER tracker red dye, which binds to sulphonylurea receptors of ATP-sensitive K+ channels that are prominent on ER. The fusion proteins gave a crescent-shaped green signal throughout the cell. We observed partial co-localization of both the constructs in the live cell imaging experiment (Fig. 2.5 and 2.6). However, GFP signal was also obtained around the nuclear stain DAPI. To exclude the possibility of GFP being mis-localized in the nucleus, we performed sub-cellular fractionation of PfOrc2N$_{1-30}$GFP and PfOrc2N$_{1-150}$GFP parasite lysate using NP 40 detergent, which confirmed that the fusion proteins are not present in nucleus (Fig. 2.7).

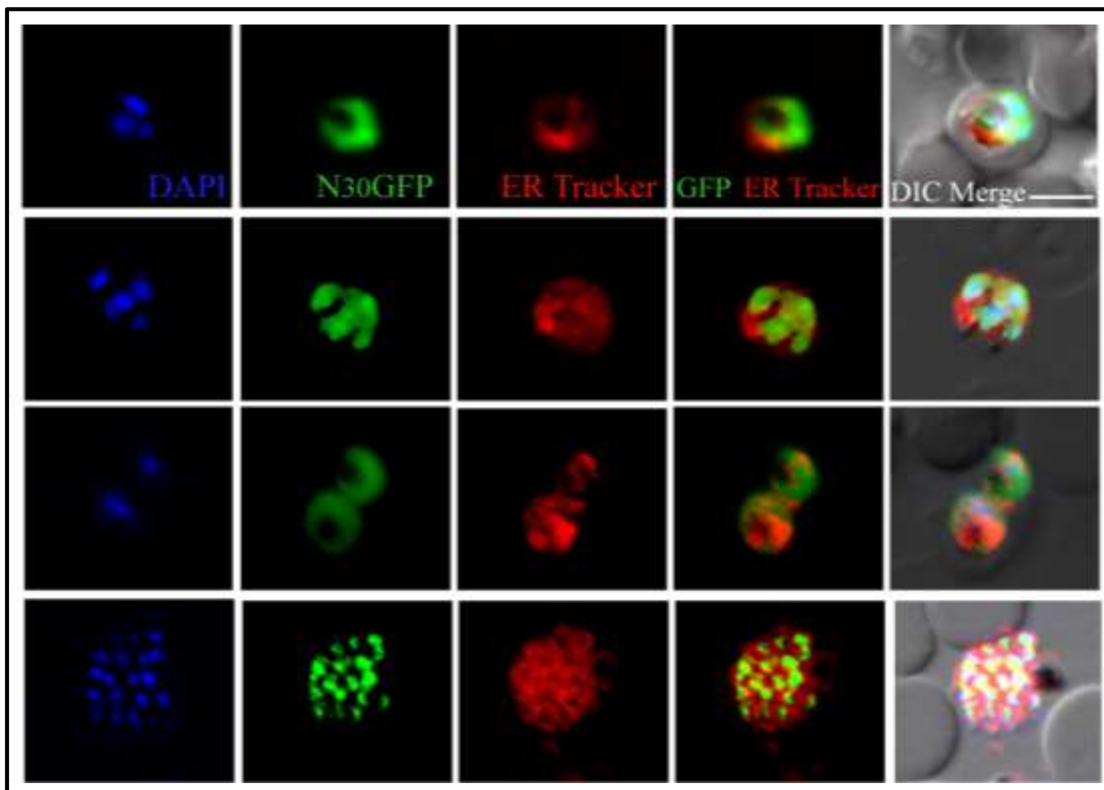

Fig. 2.5 Live cell imaging of GFP and ER tracker red in PfOrc2N$_{1-30}$GFP parasites. Nominal co-localization was observed between GFP and the ER marker in different parasite stages. DAPI was used to stain the nucleus (scale bar is 2 µm).

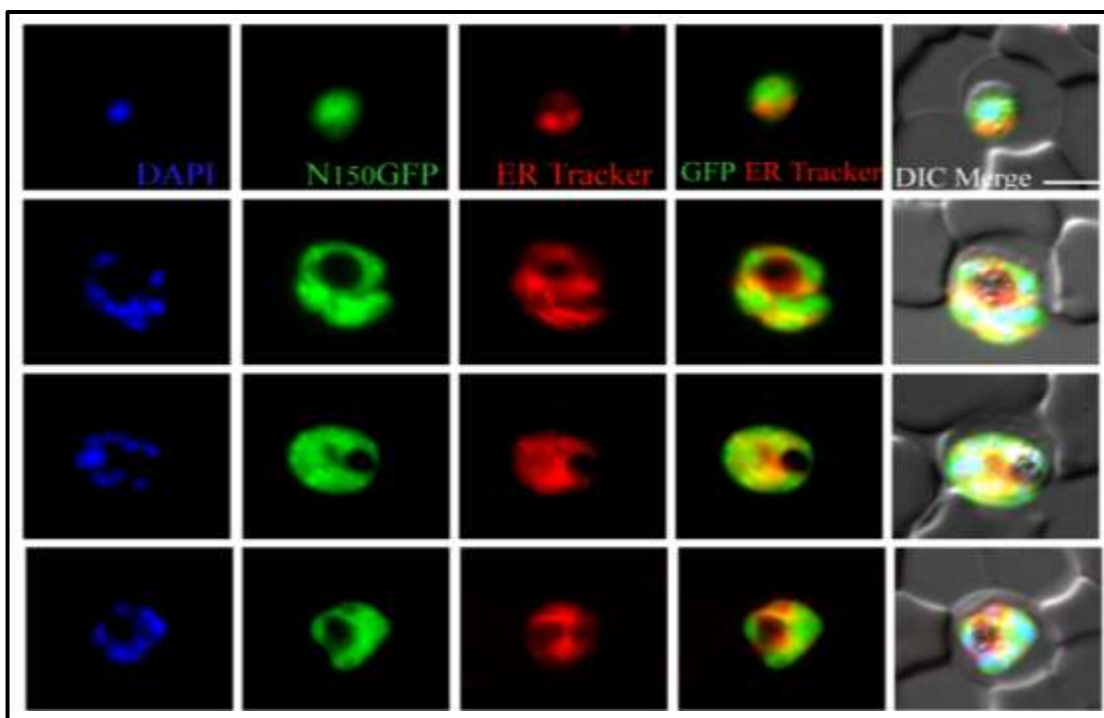

Fig. 2.6 Live cell imaging of GFP and ER tracker red in PfOrc2N$_{1-150}$GFP parasites. Partial co-localization was observed between GFP and the ER marker. DAPI was used to stain the nucleus (scale bar is 2 µm).

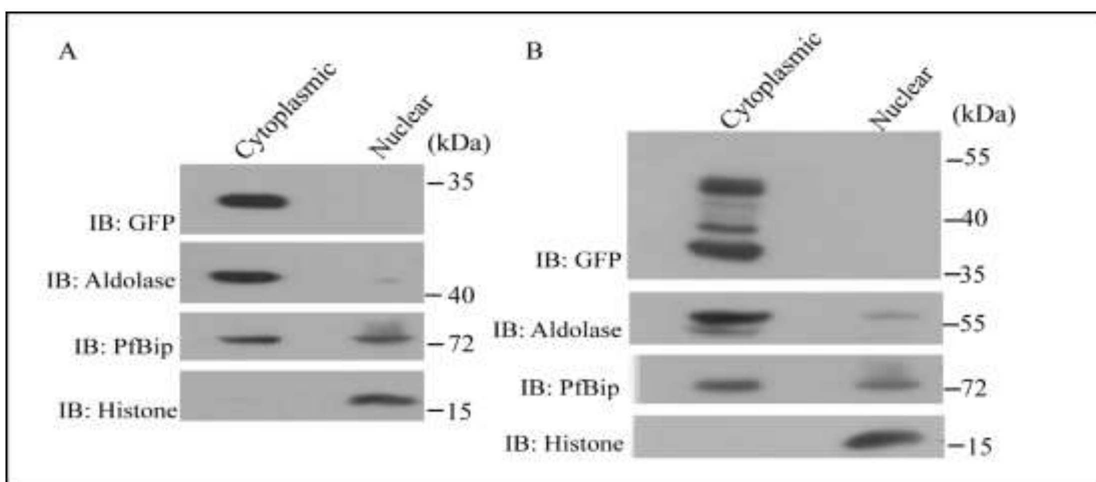

Fig. 2.7 Sub-cellular fractionation of PfOrc2N$_{1-30}$GFP and PfOrc2N$_{1-150}$GFP parasite cell lines. **A**. Parasites expressing PfOrc2N$_{1-30}$GFP were fractionated using the protocol described in materials and methods and the cytoplasmic and nuclear protein fractions were subjected to SDS-PAGE. Western blotting experiments using antibodies against GFP resulted in a band corresponding to PfOrc2N$_{1-30}$GFP protein (at ~35kDa) in the cytoplasmic fraction. **B**. Similar fractionation experiments were performed on parasites expressing PfOrc2N$_{1-150}$GFP. Here, antibodies against GFP recognized the band at expected size of ~45 kDa, along with the lower molecular weight bands around ~40 kDa in the cytoplasmic fraction. Aldolase was used as a cytoplasmic marker in both the experiments. We also checked the status of PfBip, which was majorly present in the cytoplasmic fraction, but gave a faint band in nuclear fraction. This could be due to close proximity of ER and nucleus. Histone was used as a control for nuclear protein.

2.4 Co-localization of PfOrc2N-GFP proteins with ER

Since, GFP is expressed under a very strong CRT (Chloroquine resistance transporter) and Calmodulin promoter (in PfOrc2N$_{1-30}$GFP and PfOrc2N$_{1-150}$GFP respectively), during live cell microscopy the whole parasite becomes saturated with green signal even under low exposure. It even masks the nuclear DAPI stain. To retain only the actual signal, we proceeded with Immunofluorescence assay (IFA) which allows leeching out the excess GFP signal at the time of fixation and adequate washing in subsequent steps. IFA experiments were performed using antibodies against GFP and PDI, an ER resident protein. PDI is a thiol-metabolizing enzyme and required for proper folding of proteins in ER (Mouray, Moutiez et al. 2007). We again obtained partial colocalization between PfOrc2N$_{1-30}$GFP and PfPDI in IFA experiment (Fig. 2.8). Further, fairly moderate colocalization of GFP and PfPDI was witnessed in IFA experiment in parasites expressing PfOrc2N$_{1-150}$GFP (Fig. 2.9).

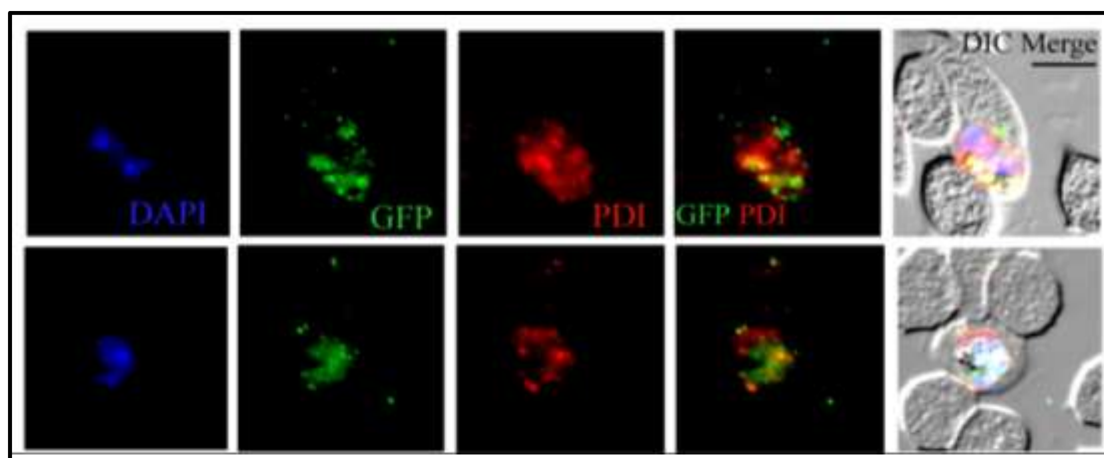

Fig. 2.8 IFA experiment using antibodies against GFP and an ER marker PfPDI in PfOrc2N$_{1-30}$GFP parasites. Partial colocalization was obtained between PfOrc2N$_{1-30}$GFP and PfPDI. DAPI was used to stain the nucleus (scale bar is 2 µm).

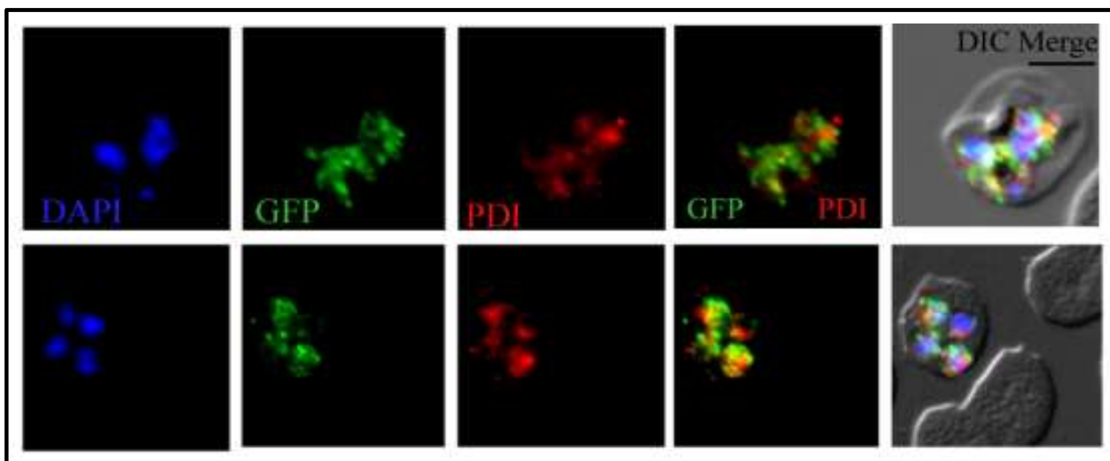

Fig. 2.9 IFA experiment using antibodies against GFP and PfPDI in parasites expressing PfOrc2N_{1-150}GFP. Average co-localization was obtained between PfOrc2N_{1-150}GFP and the ER marker. DAPI was used to stain the nucleus (scale bar is 2 µm).

We observed only partial co-localization between PfOrc2 N-terminal GFP constructs (PfOrc2N_{1-30}GFP and PfOrc2N_{1-150}GFP) and ER proteins. We reasoned that the short N-terminal constructs lacked any signal for ER-retention or for transport to nucleus. It is possible that the fusion proteins come out into the cytoplasm, responsible for only a partial colocalization with ER marker. We tried to resolve this issue by adding an SEEL sequence at the C-terminus of PfOrc2N_{1-30}GFP and PfOrc2N_{1-150}GFP respectively using appropriate oligonucleotides as mentioned in material and methods section. Further, we performed double labelled IFA between PfOrc2N_{1-30}SEEL and PfPDI using antibodies against GFP and PDI respectively (Fig. 2.10). The pattern exhibited by PfOrc2N_{1-30}GFP was no longer crescent shaped, and overlapped with ER protein, PDI. We also investigated the co-localization between PfOrc2N_{1-150}GFP and another ER membrane protein, Plasmepsin V (Fig. 2.11). PMV is an aspartyl protease and responsible for cleaving the PEXEL motif present in *Plasmodium* secretory proteins (Boddey, Hodder et al. 2010). We obtained an average co-localization between PfOrc2N_{1-150}GFP and PMV. Furthermore, we also performed immunofluorescence assay between GFP alone and PfPDI (Fig. 2.12). GFP alone presented a cytoplasmic staining pattern, distinct from that observed by different PFOrc2N GFP constructs.

In essence, all the results presented above together support our assertion that a possible ER-targeting sequence is present at the N-terminus region of Orc2 which can direct GFP into ER.

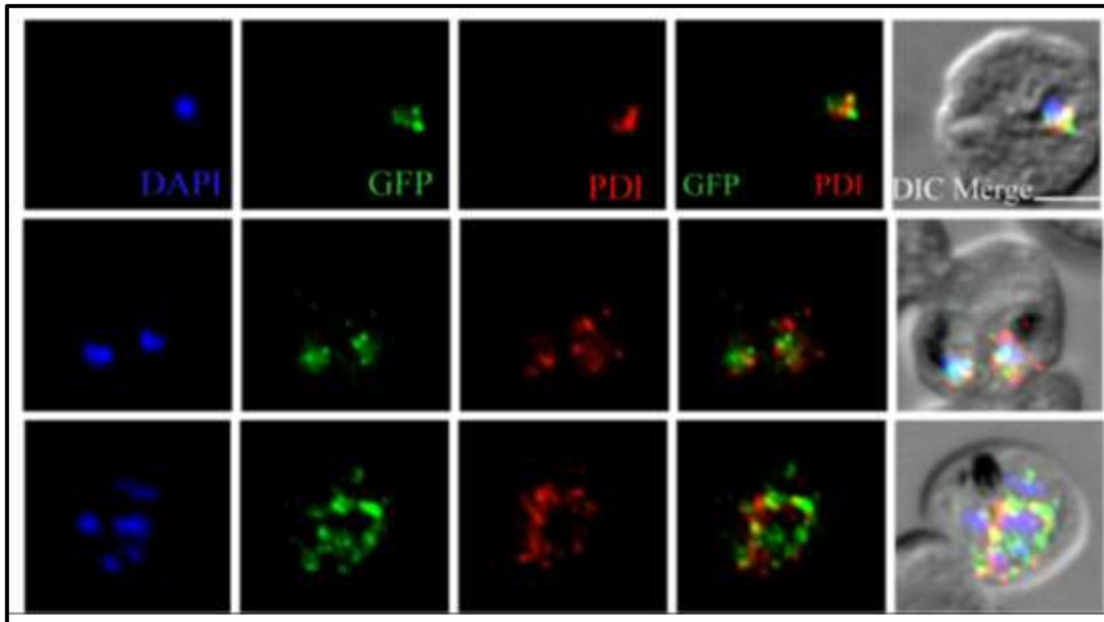

Fig. 2.10 IFA study of parasites expressing PfOrc2N$_{1\text{-}30}$SEEL as a GFP fusion protein. Moderate co-localization was observed between PfOrc2N$_{1\text{-}30}$SEEL and PfPDI in all the stages. DAPI was used to stain the nucleus (scale bar is 2 µm).

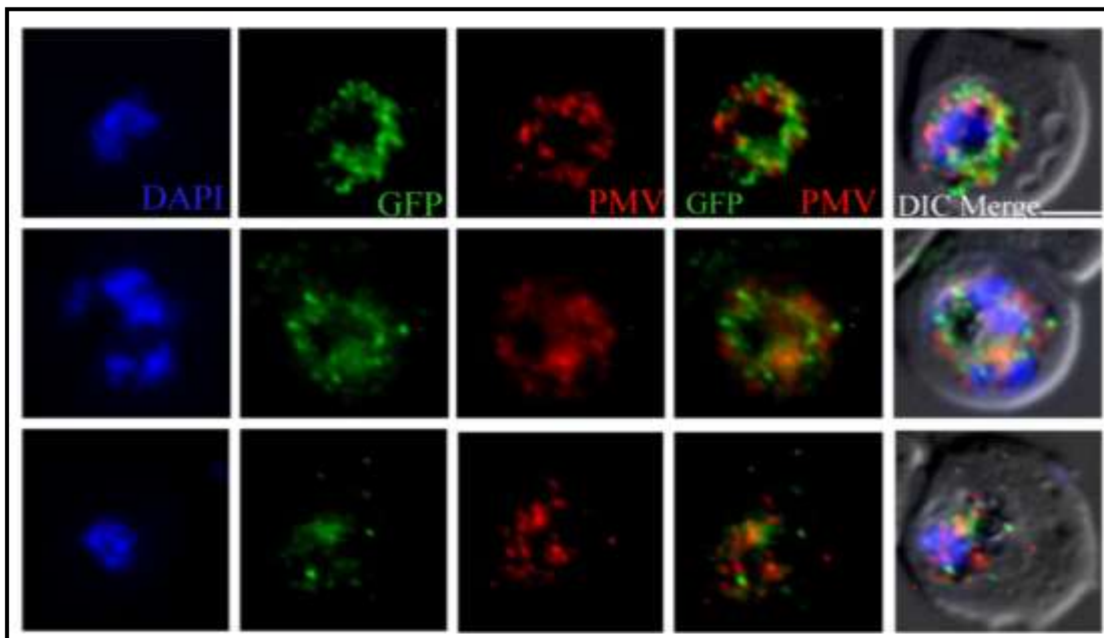

Fig. 2.11 IFA study of parasites expressing PfOrc2N$_{1\text{-}150}$GFP as a GFP fusion protein. Partial co-localization was observed between PfOrc2N$_{1\text{-}150}$GFP and PfPDI. DAPI was used to stain the nucleus (scale bar is 2 µm).

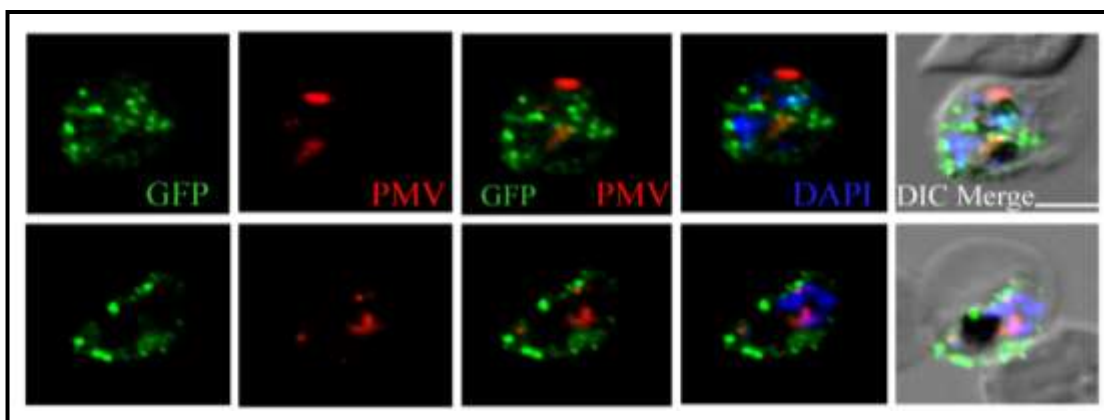

Fig. 2.12 IFA of parasites expressing only GFP. These parasites transfected with GFP alone did not show colocalization of GFP and ER protein, Plasmepsin V. DAPI was used to stain the nucleus (scale bar is 2 μm).

3. PfOrc2 peripherally associates with the endoplasmic reticulum

We have seen that Brefeldin A leads to accumulation of PfOrc2 in the ER and affects migration of PfOrc2C to the nucleus. Also, we obtained the full-length PfOrc2 protein in the sodium carbonate extractable fraction. Further, we have demonstrated the presence of a possible ER-targeting signal in the N-terminus of PfOrc2. Together, these results point towards an ER-association of PfOrc2 as peripheral membrane protein. To further validate the above points, we performed a protease-protection assay. As described in methods section, hypotonic lysis buffer was used to resuspend *P. falciparum* parasites, and subsequently the soluble cytosolic proteins were separated from the nuclear proteins by centrifugation. The cytosolic fraction containing the proteins of ER and other lighter organelles was treated with Trypsin and the samples were subjected SDS-PAGE and Western blotting. Full-length PfOrc2 protein was obtained in the cytosolic protein fraction and was susceptible to trypsin digestion (Fig. 2.13). ER resident protein, PfBip which was also present in the cytosolic fraction was protected from enzymatic activity in the same experimental setup. As expected, PfOrc2C was present in the nuclear pellet fraction. Aldolase was used as a control for cytoplasmic proteins and was completely digested by trypsin. These results clearly suggest that PfOrc2 full-length is peripherally associated with ER, though it does not enter the ER lumen.

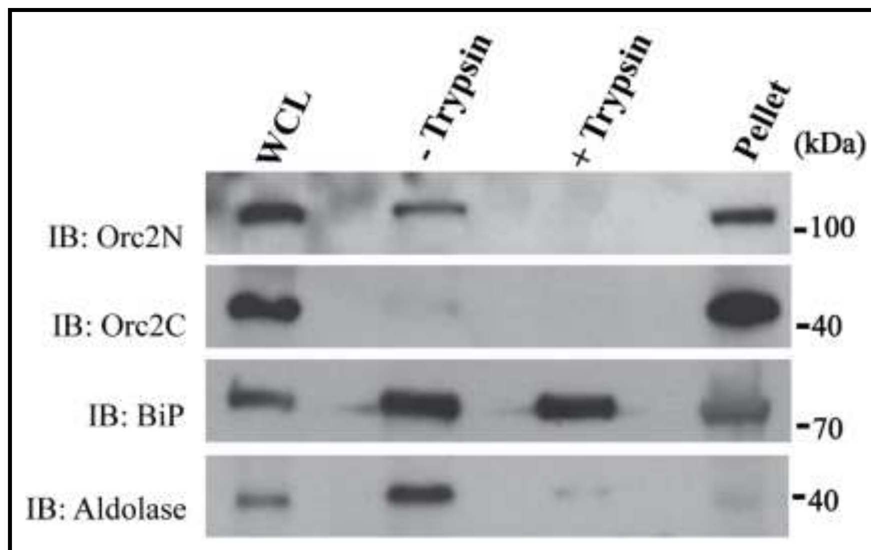

Fig. 2.13 Protease protection assay was carried out using trophozoite stage *P. falciparum* parasites. Cell lysate was prepared by homogenizing the parasite in hypotonic buffer and repeated freeze-thaw. The cytoplasmic protein sample obtained after centrifugation contained the full-length PfOrc2 protein. Full-length PfOrc2 was completely digested in the presence of trypsin, however ER lumen protein, PfBip was protected against trypsin digestion. PfOrc2C, which resides in the nucleus was obtained in the pellet fraction. Aldolase was used as a marker for cytoplasmic proteins.

4. Processing of PfOrc2 takes place in endoplasmic reticulum

It was evident that PfOrc2 associates with the ER through its N-terminus, possibly for its processing. ER is a reservoir of number of chaperones, enzymes and proteases that help in proper protein folding, any post-translational modification or processing of a pre-protein. In a quest to find the enzyme responsible for processing of PfOrc2, we treated mid-trophozoite stage parasites (30 ± 5 hpi) with $(Z\text{-}LL)_2$-ketone, an inhibitor of signal peptide peptidase, SPP (Weihofen, Lemberg et al. 2000). As mentioned earlier, any protein entering the ER usually consists of a signal peptide which gets cleaved by a signal peptidase present in the ER membrane. Signal peptides of several proteins get subsequently cleaved by Signal peptide peptidase (SPP) which is also present in the ER membrane. SPP treated parasite lysates were subjected to SDS-PAGE and subsequent Western blot analysis. The intensity of the band corresponding to PfOrc2 full-length protein was found to increase when probed with antibodies against PfOrc2N (Fig. 2.14). There was a reduction in the intensity of PfOrc2C protein as well as ER resident protein PfBip. These results suggested that the full-length protein fails to get processed when SPP-like activity is inhibited. As a result, the level of mature form of the protein

declines. Aldolase was used as a control for cytoplasmic protein, whose level did not get affected by $(Z\text{-}LL)_2$-ketone.

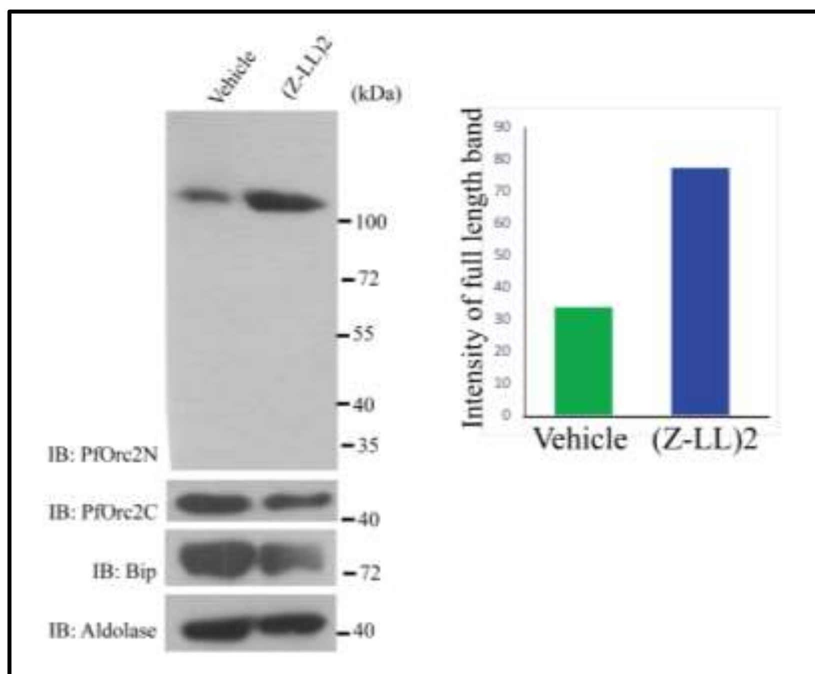

Fig. 2.14 PfOrc2 gets processed in the ER by Signal peptide peptidase like enzyme. Mid-trophozoite stage parasites were treated with 10 μM of $(Z\text{-}LL)_2$-ketone, a known inhibitor of SPP enzyme for 10 hours. Full length unprocessed PfOrc2 was accumulated as a result of inhibition of SPP-like enzyme in contrast to vehicle (DMSO) only lane. As a result, the processed form PfOrc2C showed considerable reduction in amount. PfBip and Aldolase were used as a positive control and loading control respectively. Statistical representation of increase in the full length Orc2 normalized to Aldolase.

5. Possible function of PfOrc2 in the parasites

In the preceding sections, we have detailed how a nuclear protein enters the secretory pathway to get processed and eventually travels to the nucleus. Orc2 is a known replication protein and has been implicated in diverse functions. What could be the possible function of Orc2 in *P. falciparum*? Complementation of chimera of *ScOrc2* & *PfOrc2* in yeast is the only evidence that indicates a possible role of PfOrc2 in replication. However, in *P. falciparum* it may also be involved in non-replicative functions. PfOrc2 shows partial co-localization with another ORC subunit, PfOrc1 (Sharma, Sharma et al. 2018). The latter has been shown to bind to regions responsible for *var* gene silencing in ring stage (Deshmukh, Srivastava et al. 2012) and to replication origin sequences

identified in *P. falciparum* in trophozoite stage (Agarwal, Bhowmick et al. 2017). We reasoned that the partial co-localization observed between PfOrc2 and PfOrc1 might be attributed to the fact that they may come together only at the sites of replication and may have different individual functions as well. Moreover, inefficient immunoprecipitation of PfOrc2 did not allow us to search for possible interacting proteins which could validate the exact function of PfOrc2 in *Plasmodium.* Lately, it has been reported that Actin I protein mediates physical rearrangement of *var* gene from transcriptionally repressed sites to the site of active gene expression within the nucleus by binding to *var* introns (Zhang, Huang et al. 2011). In a quest to look for involvement of PfOrc2 in *var* gene regulation, we tried to immunolocalize PfOrc2C and Actin I in early ring stage and observed substantial co-localization between the two (Fig. 2.15).

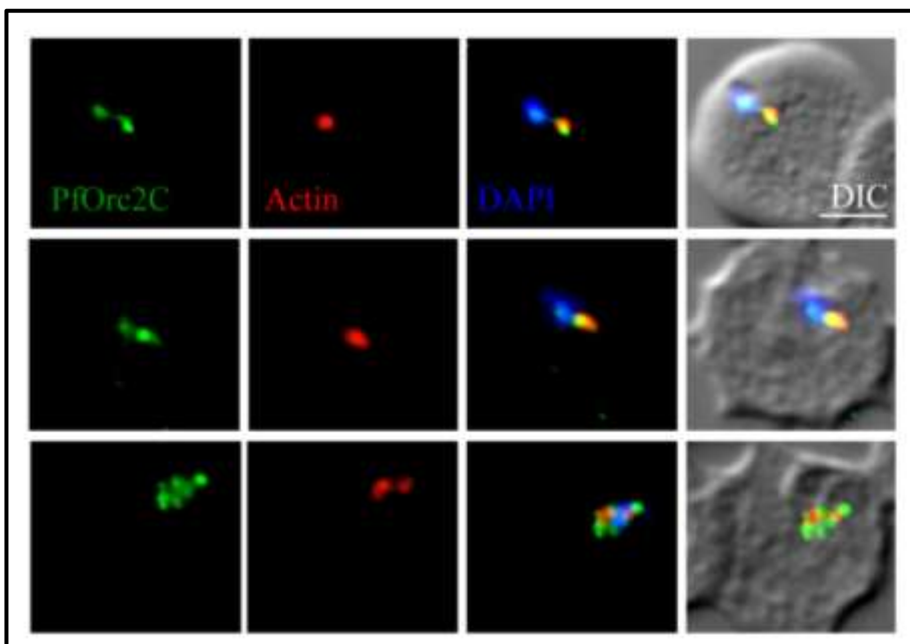

Fig. 2.15 Immunofluorescence assay using antibodies against PfOrc2C and PfActin revealed excellent co-localization between the two proteins in early ring stage. DAPI was used to stain the nucleus (scale bar is 2 µm).

Further, it has been reported that Orc2 binds to heterochromatin in interphase and mitotic stages in *Drosophila (*Pak, Pflumm et al. 1997). This phenomenon is not restricted to only *Drosophila*, since the interaction between Orc2 and centromeric DNA has also been shown in humans. HsOrc2 demonstrates preferential binding to heterochromatin

mediated by Heterochromatin protein (HP1), and they mutually need each other for loading onto transcriptionally silenced regions (Prasanth, Prasanth et al. 2004; Prasanth, Shen et al. 2010). HP1 homolog in *P. falciparum* (PfHP1) is also involved in heterochromatin formation and most importantly in the regulation of singular *var* gene expression (Flueck, Bartfai et al. 2009). We were interested in studying whether the interaction between HP1 and Orc2 is conserved in *P. falciparum* as well. We performed an IFA using antibodies against PfOrc2C and PfHP1 and observed an interesting pattern. The two proteins co-localized significantly in the schizont stage. However, the ring and trophozoite stage parasites did not show significant co-localization (Fig. 2.16).

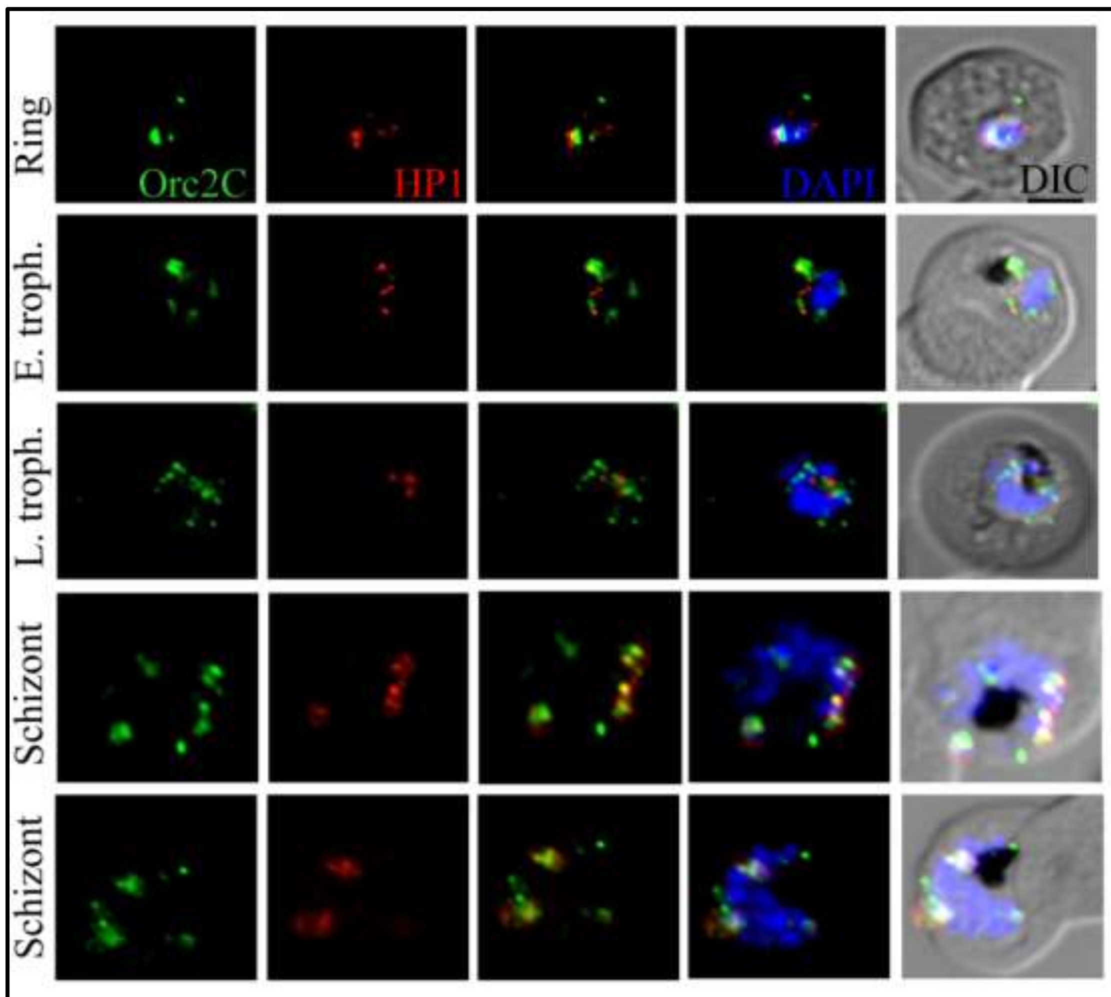

Fig. 2.16 IFA experiment in different stages of *P. falciparum*. PfOrc2C and PfHP1 did not show co-localization in ring stage. Fairly less co-localization of PfOrc2 and PfHP1 was observed in trophozoite stage in contrast to moderately good co-localization in schizont stage. DAPI was used to stain the nucleus (scale bar is 2 µm).

III. Discussion

Plasmodium falciparum resides in the human erythrocytes, which provide enough room for its survival but lesser cellular components to support its growth and pathogenesis. *P. falciparum* has evolved an intricate secretory system within itself as well as in the host cells for its survival. The proteins that are destined to be translocated into either its own organelles and membrane or host's cytoplasm or surface need to enter the classical secretory pathway that begins with the endoplasmic reticulum and is mediated by budding and fusion of small vesicles.

In the present study, we have unraveled the unusual behavior of a nuclear protein, PfOrc2 which is fed into the secretory system so as to translocate to the nucleus. The very first clue that directed our attention to the association of PfOrc2 with the ER is the presence of ER-retention signal. This sequence is present only in the resident proteins of ER. For instance, PfPDI (PF3D7_0827900) has an -SEEL motif at the C-terminus which acts as a bonafide ER-retention sequence (Mouray, Moutiez et al. 2007). PfPDI is a well-established ER-resident protein. However, we propose that 'SEEL' in PfOrc2 is either redundant or it is cleaved, thereby not allowing the protein to be retained in the ER.

Further, we have shown that PfOrc2 has a putative ER-targeting signal at the N-terminal region as identified by Phobius. With the help of GFP constructs consisting of first 30 and 150 amino acids of PfOrc2, we have tried to investigate the presence of a signal peptide by virtue of which the protein can enter into ER. Partial to moderate co-localization of GFP fusion proteins was obtained with the ER (Fig. 2.8 - 2.11). Addition of 'SEEL' amino acids at the C-terminus of PfOrc2N_{1-30}GFP and PfOrc2N_{1-150}GFP improved the co-localization signal of PfOrc2N and ER-resident proteins (PDI/PMV). However, further point mutation studies are required to narrow down to exact signal peptide present in PfOrc2 responsible for its ER association.

We have proposed a hypothetical model based on our observations as shown in Fig 2.16. The full-length protein enters the ER with the help of a signal peptide. Usually, proteins undergo co-translational translocation at the ER membrane through translocon, Sec61. Effect of a translocon inhibitor, Eeyarestatin I (ESI) on PfOrc2 has been

demonstrated earlier, wherein the level of overall PfOrc2 protein reduced on increasing the ES I concentration (Sharma R and Dhar SK, unpublished data). The presence of putative ER targeting signal at the N-terminus of PfOrc2 may direct the protein to the ER. Consequently, the full-length PfOrc2 protein peripherally associates with the ER and is later cleaved by an SPP- like enzyme resulting in a mature PfOrc2C fragment. At present, we have no knowledge on the status of N-terminus of PfOrc2; perhaps, it might be degraded as antibodies against N-terminal region of PfOrc2 do not recognize any smaller fragment besides the full-length protein. The released PfOrc2C is ultimately trafficked to the nucleus. The exact mechanism how PfOrc2C fragment reaches the nucleus from ER is not understood. PfOrc2 trafficking is sensitive to Brefeldin A, which implies that PfOrc2 moves beyond the ER. Whether it leaves in a vesicle bound form via Golgi or as a cytoplasmic fragment needs to be researched further.

The exact function of PfOrc2 inside the nucleus is still speculated. It may be involved in non-replicative function as suggested by co-localization data of PfOrc2C with Actin I in early ring stage and with PfHP1 in later stages respectively. We have presented preliminary data with respect to the role of these proteins in *var* gene regulation. We hypothesize that PfOrc2 may be involved in activation of *var* gene based on Immunofluorescence assay and enrichment of PfOrc2 and Actin on *var* intron regions by ChIP PCR (Sharma R and Dhar SK, unpublished data). Nevertheless, the moderate co-localization observed between PfOrc2C and PfHP1 cannot be disregarded. It may be possible that in early ring stage, PfOrc2 associates with Actin and not with HP1 protein. Co-localization of PfOrc2 and PfHP1 directs our attention towards a possible role of the former in heterochromatin formation during the later stages. However, further direct experiments such as co-IP and ChIP are required to conclusively propose the interaction of these proteins. Alternatively, a role of PfHP1 (gamma form) has also been implicated in euchromatin in Drosophila (Piacentini, Fanti et al. 2003). However, whether PfHP1 is also involved in gene expression in euchromatin is not clear yet. The above two results are strikingly opposite but shed light on the complex regulatory mechanisms adopted by *P. falciparum.*

Our work gives a mechanistic view on the unusual trafficking of a nuclear protein through endoplasmic reticulum. *P. falciparum* exhibits several deviations in the

trafficking of secretory proteins, however such processing and translocation of a replication protein in the *Plasmodium* parasite has not been reported earlier. The findings presented in this chapter reflect upon the evolutionary changes the malaria parasite has gone through. It would be interesting to find out whether *P. falciparum* has devised this novel intracellular trafficking pathway specifically for PfOrc2 or others nuclear proteins also follow such route.

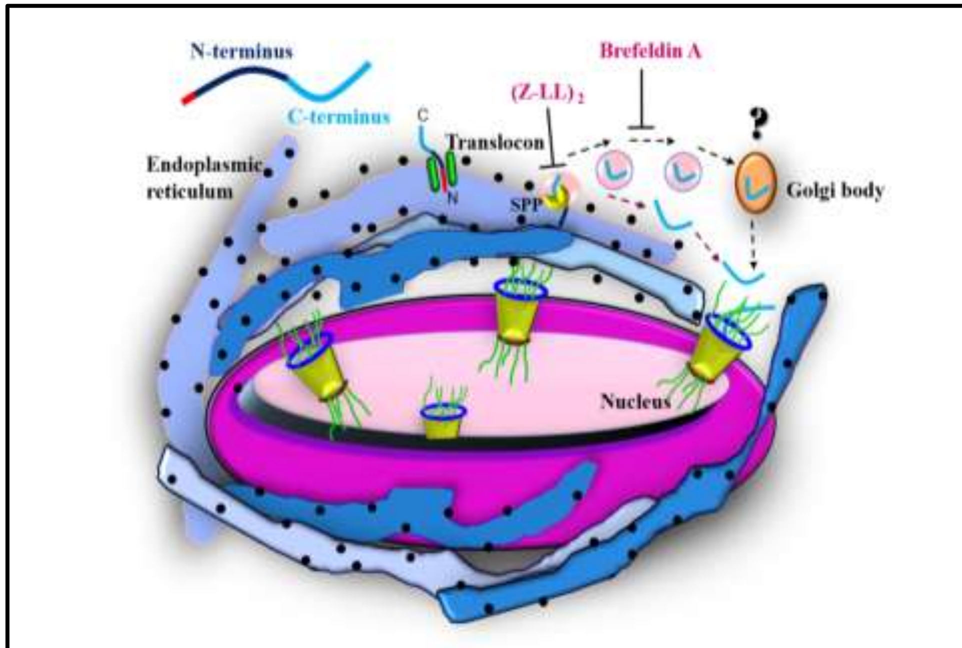

Fig. 2.16 A model depicting the various observations made in the present study. PfOrc2 may gain entry into ER by virtue of a putative ER-targeting signal. Next, PfOrc2 is processed on the ER membrane by an SPP-like enzyme which releases the C-terminal fragment. Whether PfOrc2C travels to the nucleus as a cytoplasmic fragment or inside a vesicle is not understood yet.

IV. References

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Chapter III

Biochemical and functional characterization of origin recognition complex subunit 4 of *Plasmodium falciparum*

I. Introduction

DNA replication is a tightly regulated process ensuring replication of DNA only once per cell cycle. This phenomenon is warranted by a complex of initiation proteins that assemble at the origin of replication. These proteins primarily include the ORC proteins, CDT1, Cdc6 and MCM helicases. The mechanism of DNA replication initiation is not completely understood in eukaryotes because of multiple origins and interplay of protein complexes at *ori* sites. Besides DNA replication, the individual ORC proteins have been implicated in non-replication related but essential function for maintaining cellular homeostasis too.

A subunit of a hexameric ORC, Origin recognition complex 4 (Orc4) is a part of AAA+ superfamily of adenosine triphosphate ATPase and requires ATP to bring about conformational change in itself and its interacting partners (Divac, Tomic et al. 2010). In humans, a report suggests that Orc4 protein can restructure DNA into non-canonical forms (Stefanovic, Kusic et al. 2008). Intriguingly, *S. pombe* Orc4 has nine AT-hook regions at the N-terminus which are required for DNA binding, whereas the C-terminal region is required for interacting and binding with other ORC subunits (Chuang and Kelly 1999; Kong and DePamphilis 2001). *In vitro* studies using human Orc4 protein have revealed that the structural architecture of DNA governs the preferential binding of Orc4 to triple-stranded DNA (Kusic, Tomic et al. 2010). Role of Orc4 has also been described during oogenesis in mice (Nguyen, James et al. 2017). A cage consisting of oligomers of Orc4 protein is responsible for physical extrusion of polar bodies during meiosis (Nguyen, James et al. 2017). In humans, missense mutations in *orc4* gene have been associated with Meier-Gorlin syndrome, a form of dwarfism (Guernsey, Matsuoka et al. 2011). Hence, Orc4 plays crucial roles in DNA replication and its regulation in all eukaryotes.

Replication initiation is poorly understood in *P. falciparum* which maintains tight synchrony in replicating its genome multiple times such that multiple nuclei are present in a single cell before the merozoites are released for re-infection. As mentioned before, homologs of Orc1, Orc2 and Orc5 only have been well-studied in *P. falcifarum*. The identity of homolog of Orc4 and Orc3 in *P. falcifarum* has not been validated yet. The

putative PfOrc4 protein has been identified and partially characterized (Sharma R and Dhar SK, unpublished data). Whether this putative Orc4 homolog is a true component of *Plasmodium* ORC and its possible role in DNA replication are still not known clearly. Some of these aspects will be explored in this chapter.

II. Results

1. *In-silico* analysis of PfOrc4 amino acid sequence

P. falciparum Orc4 (PfOrc4) is constituted by 983 amino acids that are encoded by a 2,952 bp long gene (PF3D7_1334100). It is annotated as a conserved *Plasmodium* protein in PlasmoDB. The putative PfOrc4 consists of three ATPase domains (133-179 aa, 347-385 aa & 429-570 aa, labeled as AAA+) and a P-loop region responsible for binding to ATP (Fig. 3.1A). Hydrolysis of ATP is required for assembly of ORC at the site of replication initiation. PfOrc4 shows ~22% homology with ScOrc4, its homolog present in yeast *Saccharomyces cerevisiae* (Fig. 3.1A). PfOrc4 also consists of asparagine-rich regions present at (78-393) a.a. and (780-842) a.a. Stretches of glutamine/asparagine repeats in proteins have been implicated in protein-protein interactions and antigenic variation in *P. falciparum* (Hughes 2004).

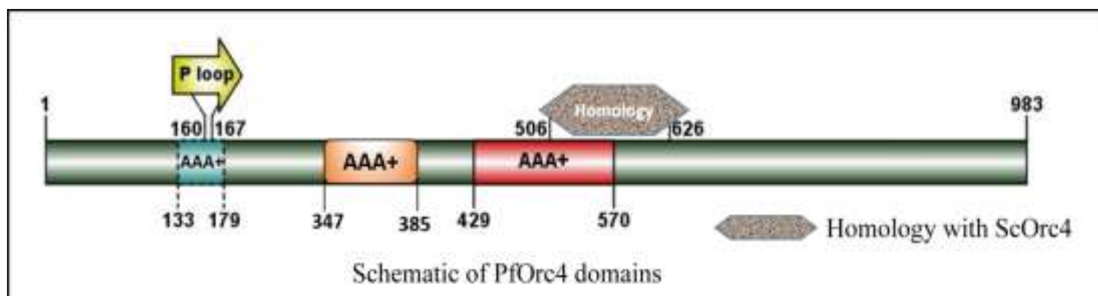

Fig. 3.1 Schematic depiction of various domains present in putative PfOrc4 protein. It is 983 amino acid long protein and consists of three AAA+ domains for ATP hydrolysis. A P-loop containing site for binding to ATP was identified between 160-167 a.a. The homologous region of PfOrc4 and ScOrc4 is present from 506-626 a.a. and shares ~22% homology with each other.

Although, we could not identify the exact sequence of Orc4 superfamily domain in PfOrc4, we aligned the complete amino acid sequences of different Orc4 homologs from other apicomplexans and higher eukaryotes using ClustalW and looked for the presence of conserved sequences, if any (Fig. 3.2). The sequence highlighted in upper

panel signifies the NTP-binding motif (p-loop or Walker A) present in ORC proteins (Fig. 3.2A). Further, another sequence 'EKRV/IKSRFS/T' that was first identified in yeast and human Orc4 (Quintana, Hou et al. 1997) was also found to be conserved in different Orc4 homologs (555-563 a.a. in Pf; Fig. 3.2B). The exact function of the second sequence is not yet understood, but its presence in all the Orc4 homologs indicates a conserved role. Hence, sequence analysis using bioinformatic tools indicates that the conserved protein annotated as PF3D7_1334100 could be a putative Orc4 protein in *P. falciparum*.

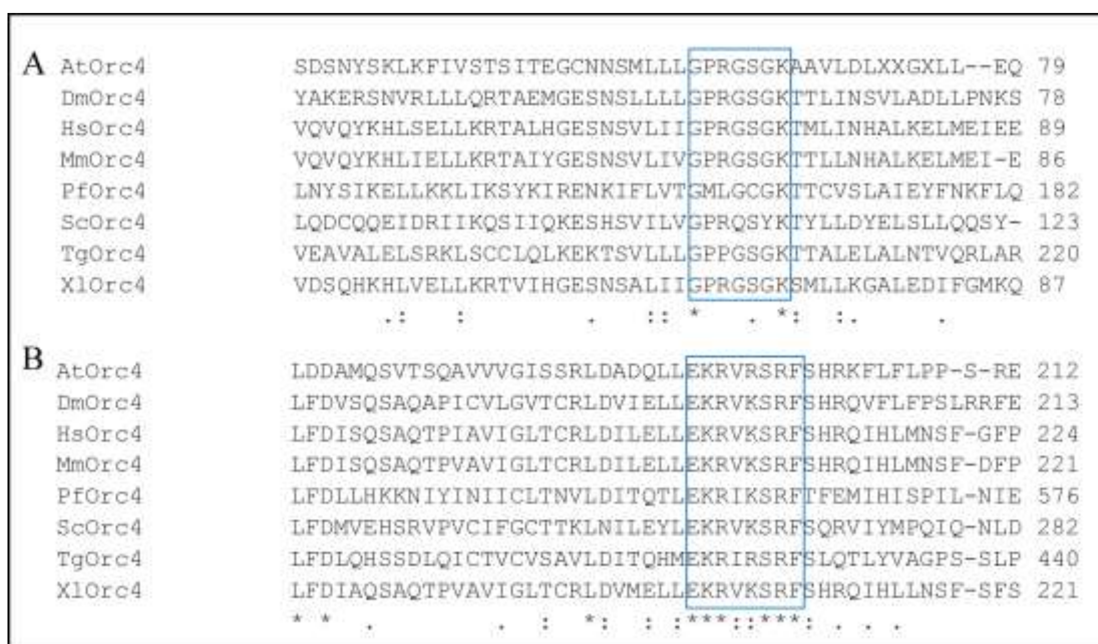

Fig. 3.2 Multiple sequence alignment of Orc4 homologs from different organisms. (**A**). The P-loop containing the nucleotide binding site motif (Walker A) was found to be semi-conserved in all Orc4 homologs. (**B**). Another stretch of sequence found in PfOrc4 (EKRIKSRFT) was found to be conserved across eukaryotic phyla. [*Arabidopsis thaliana*, AEC05404.1; *Drosophila melanogaster*, NP_477320.1; *Homo sapiens*, NP_001177808.1; *Mus musculus*, NP_036088.3; *Plasmodium falciparum* 3D7, XP_001350072.1; *Saccharomyces cerevisiae*, KZV07676.1; *Toxoplasma gondii*, CEL72616.1 and *Xenopus laevis*, AAI06364.1].

2. Complementation of *orc4* in yeast

The next question was whether the putative PfOrc4 protein is indeed an Orc4 homolog which is involved in replication. For this purpose, we tried to genetically complement yeast *orc4* (as a positive control), *Pforc4* and a chimera consisting of N-terminus of *Scorc4* and C-terminus of *Pforc4* in a yeast swapper strain whose genomic copy of *orc4*

is knocked-out, but an episomal copy is maintained under Ura selection. Different domains and the secondary structure of ScOrc4 and PfOrc4 (Fig. 3.3) were taken into consideration while generating chimera of *Scorc4* (1-750 bp) & *Pforc4* (1251-2952 bp) by SOE (gene Splicing by Overlap Extension) method. The chimera contained one AAA+ domain from ScOrc4 and one AAA+ domain from PfOrc4.

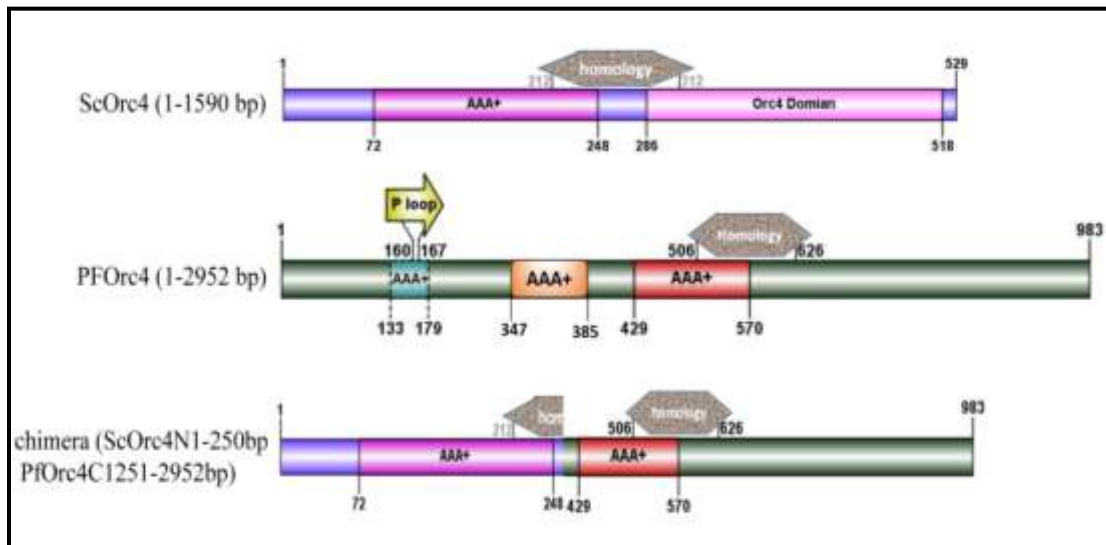

Fig. 3.3 Schematic representation of Orc4 homologs of yeast (Scorc4) and *P. falciparum* (PfOrc4). ScOrc4 is a 529 amino acid long protein and consists of an AAA+ domain (72-248 a.a.) in the N-terminal region. It also contains an Orc4 superfamily domain in the C-terminal region (286-518 a.a.). Full-length PfOrc4 contains three ATPase domains (133-179 a.a., 347-385 a.a. and 429-570 a.a.). The homology region between ScOrc4 and PfOrc4 lies at 212-312 a.a. in case of yeast and 506-626 a.a. in case of *P. falciparum.* The chimeric construct is comprised of complete homology domain from PfOrc4, apart from two AAA+ domains from ScOrc4 and PfOrc4 respectively. The amino acid coordinates are also shown in the figure.

All the three constructs (ScOrc4, PfOrc4 and ScO4NPfO4C) were first cloned in pRS416 vector (Ura selection) which has a Galactose-inducible promoter. Cells transformed with pRS416 are routinely selected on SD Ura media. However, the yeast *orc4* knock-out strain to be used for complementation inhabited wild-type *Scorc4* gene in an episomal vector under Ura selection. Therefore, the insert along with the Gal cassette was sub-cloned into another plasmid, pRS423 which can be selected in SD His media (Fig. 3.4). This construct was used to transform yeast *orc4* knock-out strain cells, wherein SD His medium plates were used to select the transformed cells and URA3/FOA screening method to select cells that functionally complement *orc4* gene.

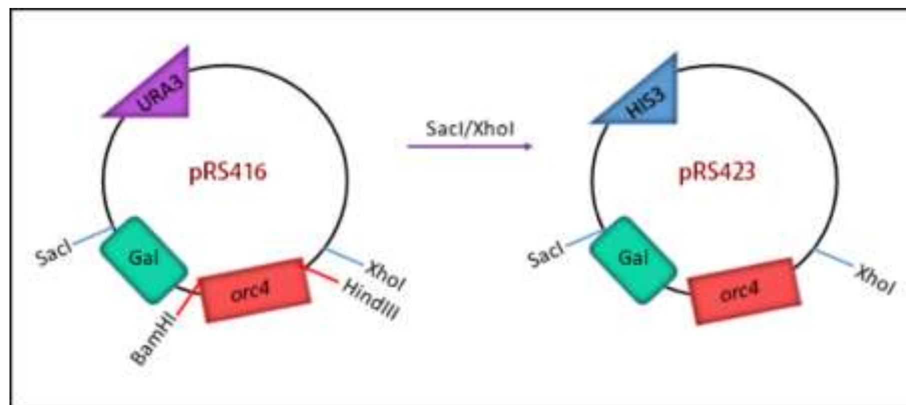

Fig. 3.4 Strategy for yeast complementation. *Orc4* gene from yeast or chimeric orc4 were cloned in pRS416 vector in sites BamHI and HindIII. The insert and its upstream Gal cassette were together sub-cloned in pRS423 vector between SacI and XhoI sites. This construct was used for transforming yeast cells.

Yeast orc4 swapper strain was transformed with all the three constructs along with vector alone and grown on agar plates containing selective dropout (SD) His media for 3-4 days at 30ºC. Only transformed yeast cells were able to grow under His selection (Fig. 3.5, upper panel). We did not obtain any colony for yeast cells transformed with PfOrc4 under His selection (data not shown). Colonies obtained on the SD His plates were subsequently streaked on SD His and SD His+FOA (5-Fluoroorotic acid) plates. The yeast swapper strain is URA3+ which codes for Orotidine 5'-phosphate decarboxylase, an enzyme involved in synthesis of pyrimidine ribonucleotides. This enzyme converts 5-FOA into a toxic compound, fluorouracil which kills the yeast cell (Boeke, Trueheart et al. 1987). However, only those cells in which *orc4* is complemented would lose the episomal URA3 plasmid and would be able grow on His+FOA selection media. Wild type ScOrc4 was able to survive His+FOA selection (Fig 3.5, lower panel). Also, ChimeraOrc4 (ScO4NPfO4C) transformants were able to grow under FOA selection but to a lesser extent, which indicate that it could partially complement yeast *orc4* function (Fig. 3.5). Neither pRS423 nor the yeast *orc4* knock-out strain could grow under the same experimental condition.

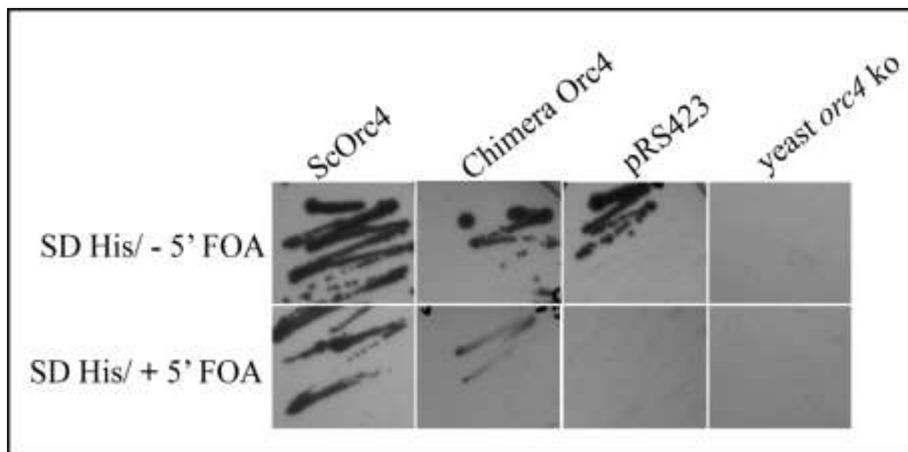

Fig. 3.5 Yeast complementation of PfOrc4. Yeast *orc4* swapper strain was transformed with plasmids containing full-length *ScOrc4*, chimera *Orc4* or only pRS423 vector. The three transformants were able to grow on SD His media due to the presence of *His3* in pRS423 vector. Further selection of transformants in the presence of 5' FOA allowed growth of *ScOrc4* and Chimera *Orc4*, but to a lesser extent. Only pRS423 transformants could not grow under 5'FOA selection. As expected, untransformed yeast *orc4* knock-out cells did not grow in SD His media.

3. Endogenous expression of PfOrc4

As mentioned earlier, putative PfOrc4 is constituted by 983 amino acids with the expected molecular weight of ~117 kDa. Antibodies against the middle domain of PfOrc4 (417-749 a.a.) region were raised in rabbit (Sharma R and Dhar SK, unpublished data). However, these antibodies picked up several non-specific bands in the Pf3D7 parasite lysate. To resolve this issue, we affinity-purified the crude antibodies using the protocol described in methods section. Briefly, the purified PfOrc4M protein was resolved by SDS-PAGE and transferred onto nitro-cellulose membrane. Following Ponceau S staining, the membrane was excised from the region corresponding to recombinant PfOrc4M and allowed to bind to crude sera containing antibodies against PfOrc4M. The specific antibodies bound to recombinant PfOrc4M were eluted twice using Glycine pH 3.0 and subsequently neutralized with Tris.Cl pH 9.5 (Elution I and II respectively). Pf3D7 parasite lysate was resolved by SDS-PAGE and different strips were immunoblotted using antibodies against PfOrc4M. Western blot analysis using the crude antibodies and affinity purified antibodies is shown in Fig. 3.6. All the antibodies recognized a band above ~100 kDa corresponding to full-length PfOrc4 (marked by *). PfOrc4M crude antibodies picked up quite a few bands, of which two bands were more

discrete and evident in the elution I of affinity purified antibodies. These were obtained at ~55 kDa and ~35 kDa respectively. The elution II of affinity purified antibodies recognized only two bands at ~100 kDa and ~55 kDa. The pre-immune sera did not detect any band (Fig. 3.6).

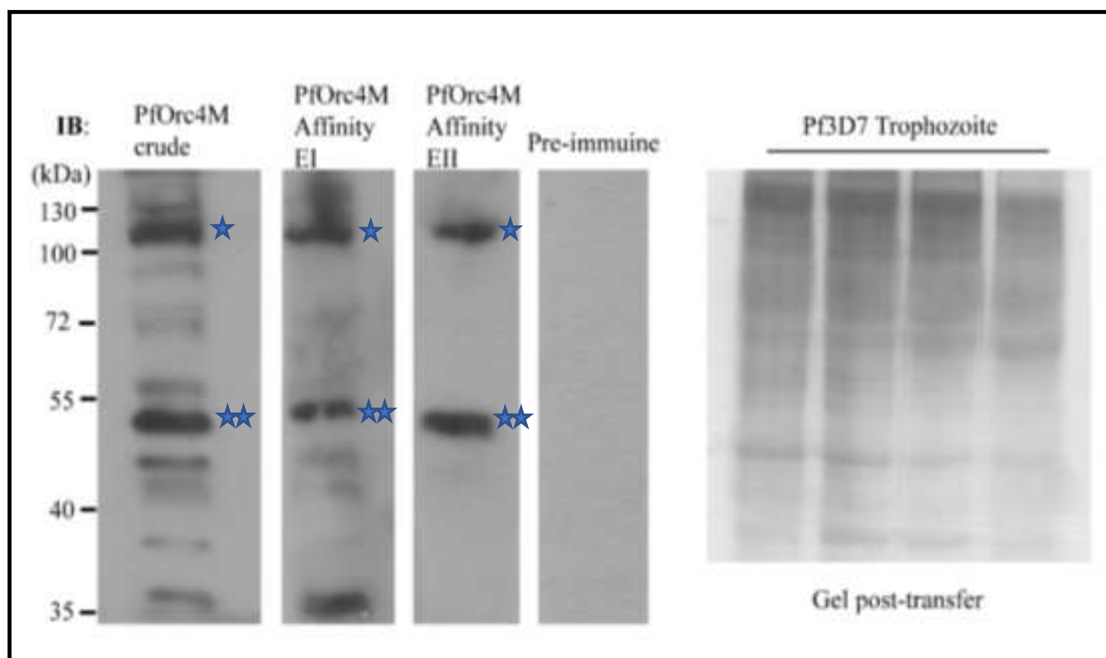

Fig. 3.6 Endogenous expression of PfOrc4 in intra-erythrocytic stage. Pf3D7 trophozoite stage parasite lysate was subjected to SDS-PAGE and Western blotting analysis using antibodies (pre-immune sera, crude anti-sera or affinity-purified antibodies) against PfOrc4M. The crude antibodies picked up intense bands around ~100 kDa and ~55 kDa besides several other bands. The affinity purified antibodies picked up majorly three bands at ~100 kDa, ~55 kDa and ~35 kDa. The Elution II after affinity purification recognized only two bands at ~100 kDa and ~55 kDa. Thus, the full-length band of PfOrc2 was obtained by both crude and affinity-purified antibodies, indicating the efficiency of affinity purification (* denotes the ~100 kDa full-length band and ** denotes the ~55 kDa band of PfOrc4).

PfOrc4 is a large protein of ~117 kDa and gave more than one band in Western blotting experiments. We were interested to know the status of PfOrc4 when probed with antibodies against its N-terminal region. For this purpose, we cloned the first 1161bp region (1-387 a.a.) from the N-terminus of PfOrc4 into pGEX-6P2 vector and used this clone to transform *E. coli* BL21 CodonPlus cells. We expressed recombinant PfOrc4N-GST protein by inducing with IPTG and different protein samples, as illustrated in Fig. 3.7 A, B were resolved by SDS-PAGE in duplicate followed by Coomassie staining and western blotting respectively. Band corresponding to PfOrc4N-GST fusion protein was

obtained at expected size of ~70 kDa in the induced lane (Fig. 3.7A). However, a more prominent band was also observed ~60 kDa, which was also recognized by antibodies against GST (Fig. 3.7B). Further, we resuspended the induced pellet in lysis buffer and centrifuged the sample after sonication to obtain supernatant and pellet fractions. The two bands corresponding to PfOrc4N-GST were obtained in the supernatant as well as pellet fraction (Fig. 3.7B). The lower band was most-probably a degradation of the GST-fusion protein. For the purpose of generating antibodies against PfOrc4N, we purified PfOrc4N-GST using GST-affinity chromatography and resolved the collected protein samples by SDS-PAGE (Fig. 3.7C). We used both the protein fragments (marked with * in fig 3.7 C) to raise antibodies in mice according to the protocol described in methods section.

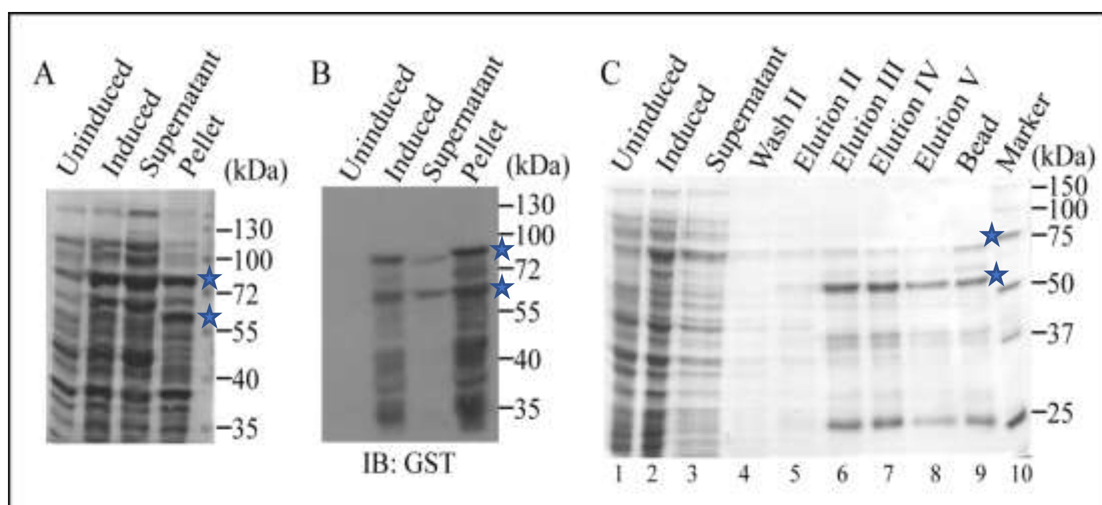

Fig. 3.7 Expression and purification of PfOrc4N from *E. coli* BL21 CodonPlus (DE3) cells. (**A**) Coomassie-stained gel showing the induction profile of PfOrc4N. Bands corresponding to PfOrc4N-GST were observed in lanes loaded with induced *E. coli* lysate, cell supernatant and cell pellet samples. (**B**) Western blotting of same samples using antibodies against GST identified the bands corresponding to PfOrc4N-GST at ~70 kDa and another band at ~60 kDa. (**C**) Affinity purification of PfOrc4N using GST-sepharose beads yielded the same bands at ~ 70 kDa and ~60 kDa in Coomassie-stained gel. The protein samples are loaded in the following sequence: Lane 1: Uninduced *E. coli* lysate, Lane 2: induce *E. coli* lysate, Lane 3: supernatant obtained after sarcosine treatment, Lane 4: Wash II (with buffer containing 1M NaCl), Lanes 5-8: Elutions using buffer containing reduced glutathione, Lane 9: remaining beads after elution, lane 10: unstained protein marker. The lower bands obtained in (C) could be degradation products of PfOrc4N since they are being picked up by antibodies against GST as shown in (B).

We determined the authenticity of the antibodies raised against PfOrc4N by analyzing the *E. coli* uninduced and induced protein lysate along with the purified PfOrc4N-GST

protein by Western blotting. PfOrc4N antibodies recognized the protein in the induced and recombinant protein lane at ~70 kDa and ~ 60 kDa (Fig. 8A, left panel). Western blotting of the same protein samples using GST antibodies corroborated the same result (Fig. 8A, right panel). Further, we examined the endogenous expression of PfOrc4 by resolving RBC and Pf3D7 trophozoite stage parasite lysate by SDS-PAGE and subsequent Western blotting in the presence of antibodies against PfOrc4N. A band corresponding to full-length PfOrc4 was obtained ~100 kDa. Pre-immune sera were screened for any non-specific band (Fig. 3.8 B). Recognition of ~100 kDa band by antibodies from two different regions of the same protein authenticates the identity of full-length PfOrc4.

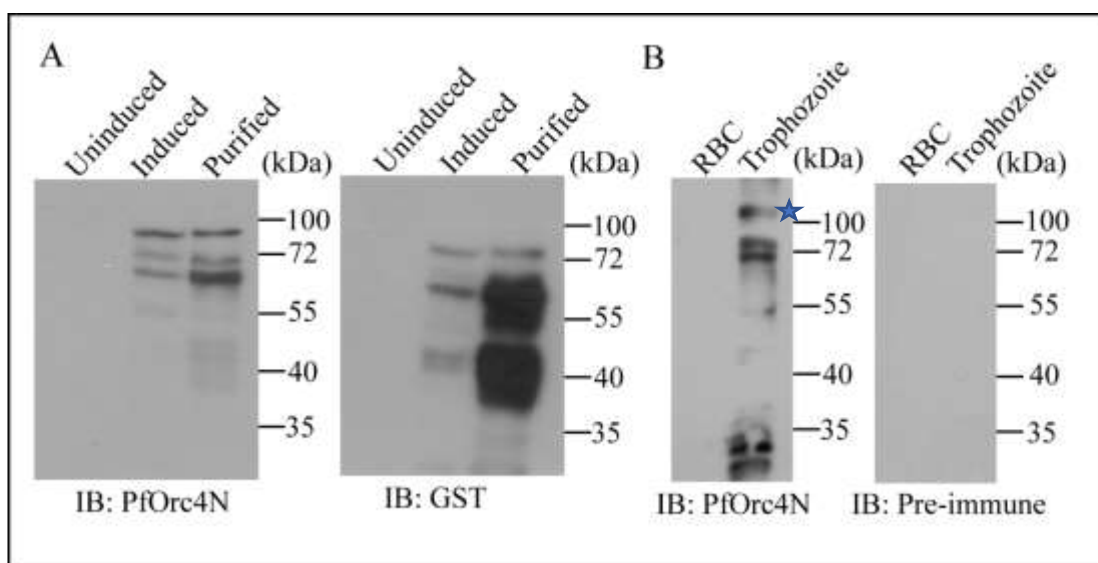

Fig. 3.8 Determining the specificity of PfOrc4N antibodies. (**A**). *E. coli* BL21 CodonPlus uninduced & induced cell lysate and purified PfOrc4N protein were resolved by SDS-PAGE in duplicate. Antibodies against PfOrc4N (left panel) and GST (right panel) identified the bands corresponding to PfOrc4N in the induced and purified protein lanes respectively. (**B**). Full-length band (marked with *) corresponding to PfOrc4 was observed in Western blotting experiment of saponin-lysed RBC and Pf3D7 trophozoite lysate using antibodies against PfOrc4N (left panel). No band was detected by Western blotting in the presence of pre-immune sera (right panel). (Non-specific bands ~70 kDa in panel (B) are obtained in lane loaded with Pf lysate. These bands could be cross-reactive bands due to generation of antibodies against GST which was a part of recombinant PfOrc4N-GST fusion protein).

4. Intracellular localization of PfOrc4

We were interested in determining the intracellular localization of PfOrc4. Since, antibodies against both PfOrc4N and PfOrc4M recognized the endogenous full-length

PfOrc4, we had employed affinity-purified antibodies against PfOrc4M in subsequent experiments. Before proceeding with the Immunofluorescence assay (IFA) using antibodies against PfOrc4M, we screened the rabbit pre-immune sera where no signal was detected during IFA (Fig. 3.9). IFA of PfOrc4 divulged an interesting pattern. Peri-nuclear punctate staining was obtained in case of early parasitic stage as shown in Fig. 3.10. However, the intensity of signal increased as the stage progressed towards trophozoite and schizont. PfOrc4 exhibited ring-shaped staining pattern around the nuclear DAPI in late stages (Fig. 3.10).

The foremost step leading to initiation of replication is binding of ORC to *ori* sites, which then recruits other accessory proteins like licensing factors CDT1 and hexamer of MCM helicase proteins. Mini chromosome maintenance complex proteins play an essential role in DNA replication initiation and help in unwinding of DNA through their ATPase activity. In *P. falciparum*, three of the six subunits (Mcm2, Mcm6 and Mcm7) have been characterized (Patterson, Robert et al. 2006). Mcm6 was found to be associated with the chromatin throughout the parasite's life cycle. We wanted to ascertain whether PfOrc4 colocalizes with any other protein of the pre-replication complex. We performed double-labeled IFA using antibodies against PfOrc4M and PfMcm6 and observed moderate colocalization between the two proteins (Fig. 3.11). Co-localization with a bonafide replication protein points towards a possible involvement of PfOrc4 in replication.

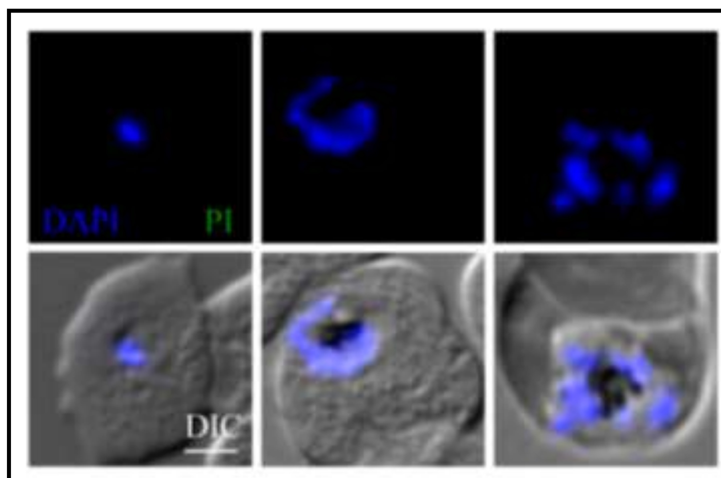

Fig. 3.9 IFA using pre-immune sera did not yield any signal in any of the stages. Ring, trophozoite and schizont stages have been shown for representation (DAPI stains the nucleus; scale bar represents 2 μm).

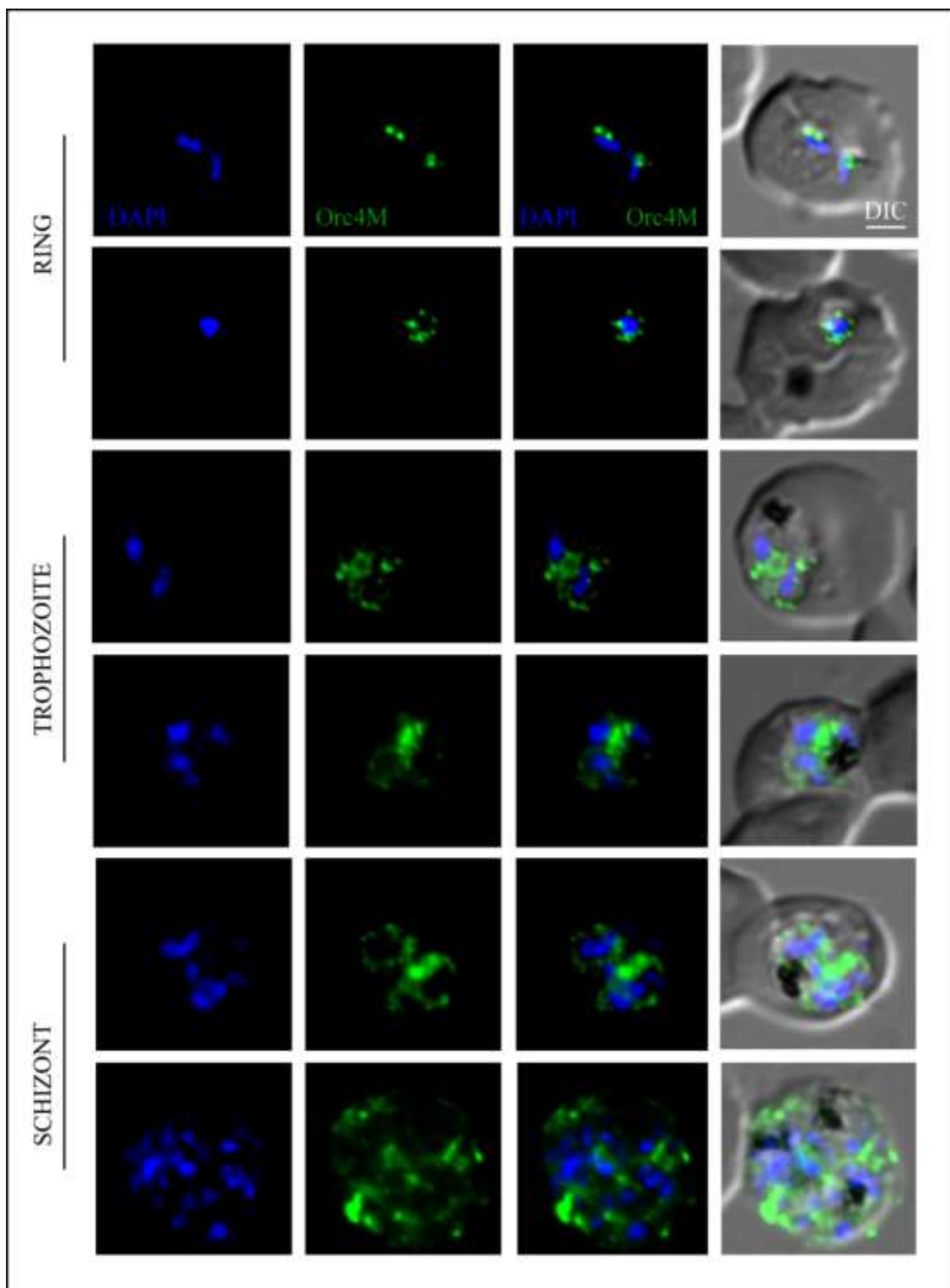

Fig. 3.10 Immunofluorescence assay for studying the localization of PfOrc4 in *P. falciparum*. **(A).** Immunostaining of ring stage parasites (18 ± 4 hpi) using antibodies against PfOrc4M presented with a punctate pattern in and around the nuclear DAPI. The punctate pattern changed to ring-shaped perinuclear pattern as the parasites advanced towards later stage. In the trophozoite and schizont stages, a diffused signal around the nucleus was observed. (Scale bar represents 2 μm.

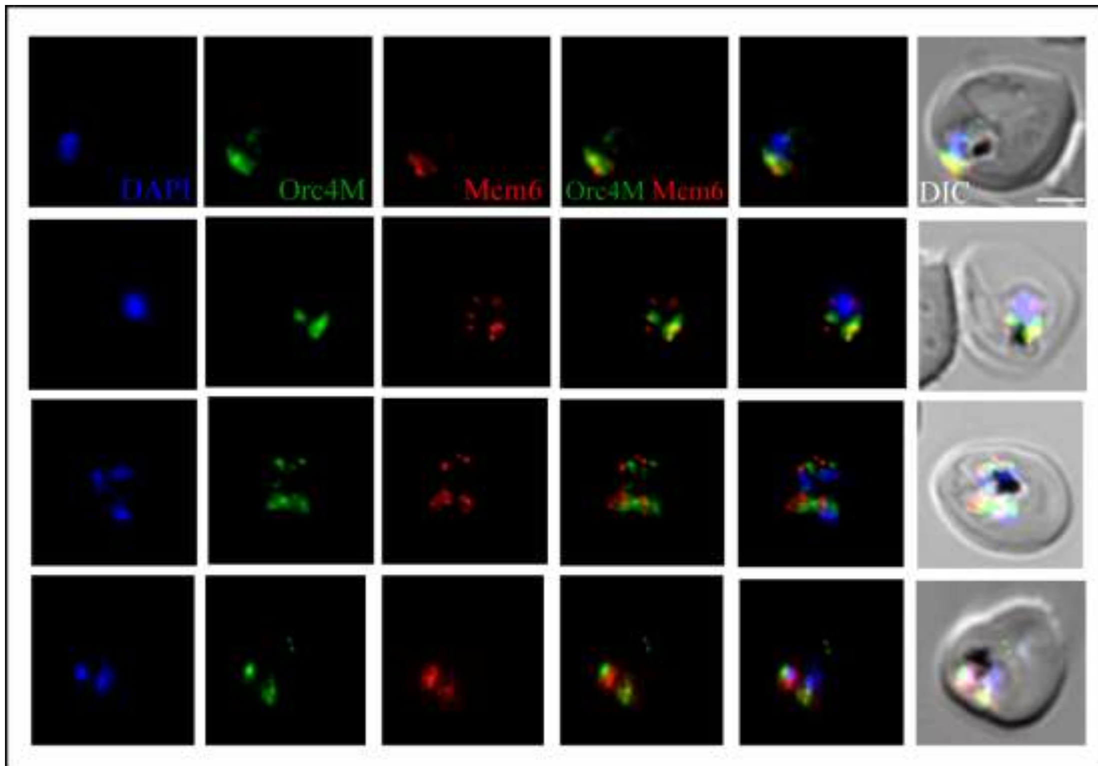

Fig. 3.11 Immunofluorescence assay illustrating the co-localization of PfOrc4 and Mcm6 protein in *P. falciparum* using the respective antibodies (DAPI stains the nucleus; scale bar represents 2 µm).

5. Possible function of PfOrc4

Origin recognition complex is required for identifying and binding to origin of replication sites in all eukaryotes. In yeast, these well-established replicator sites are called autonomously replicating sequences (ARS) and are sub-divided into ARS consensus sequences (ACS/ 'A element') and stretches of 15-20 bps 'B elements' that are required for origin function (Broach, Li et al. 1983; Theis and Newlon 1997). Recently, multiple ARSs have been characterized in *P. falciparum* (Agarwal, Bhowmick et al. 2017). We studied the occupancy of PfOrc4 on the putative origin sites ARS1 and ARS3, present on chromosome 10 by chromatin immunoprecipitation in trophozoite stage parasites. We found that PfOrc4 was enriched at both the ARS sequences (Fig. 3.12). We employed an AT-rich non-ARS region as a control wherein negligible enrichment of PfOrc4 was obtained as compared to pre-immune sample. Further, we could not observe any enrichment of PfOrc4 at TARE 1 (telomere associated repetitive element) which is associated with regulation of *var* gene silencing.

TARE1 was taken as negative control since regulatory proteins control *var* gene silencing by binding to TARE regions only in ring stage. This ChIP-based experiment provides preliminary information that PfOrc4 may be involved in binding to *ori* sites in *P. falciparum.*

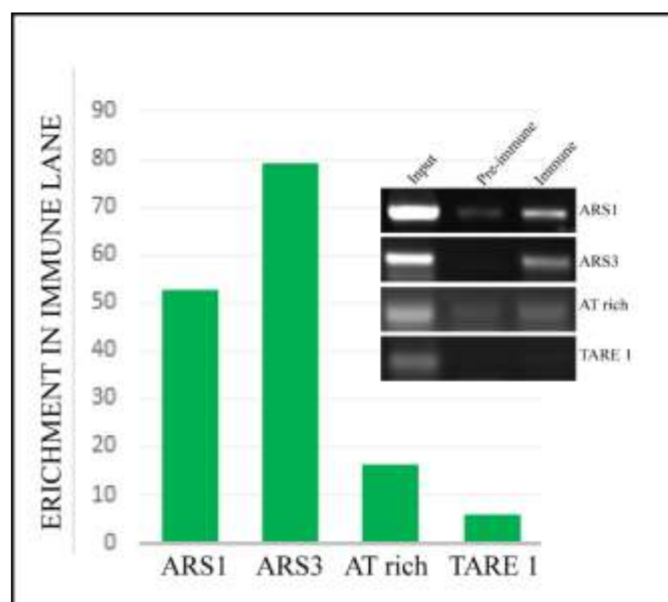

Fig. 3.12 Chromatin immunoprecipitation experiment followed by semi quantitative PCR demonstrates the enrichment of PfOrc4 at *P. falciparum* putative origin sequences ARS1 and ARS3 as compared to a random AT-rich sequence or TARE sequence from Pf genome.

III. Discussion

Plasmodium falciparum replicates multiple times during its life cycle in mosquito and human hosts. DNA replication is a tightly regulated process and commences with binding of ORC proteins at the origin. Pf is known to have an AT-rich genome and several *ori* sites are presumed to be present in the parasite. It is necessary to study and characterize the ORC subunits in order to fully understand the replication biology of *Plasmodium*. In this regard, we have partially characterized the putative Orc4 homolog of *P. falciparum* (PfOrc4).

Unlike its other eukaryotic homologs, PfOrc4 does not contain an obvious ORC4 superfamily domain. Apparently, it has the presence of three ATPase domains. It is unusual for any protein belonging to AAA+ protein family to possess more than one ATPase domain. It will be interesting to know why PfOrc4 has three ATPase domains

and whether all of them are functionally active. We had cloned and expressed the N-terminal region of PfOrc4 with the aim of studying whether it has any ATPase activity. However, the protein being induced at ~70 kDa got degraded to yield a shorter fragment at ~60 kDa. Even after repeated efforts to purify PfOrc4N-GST, we could not purify the intact protein without any degradation, which could be attributed to presence of multiple asparagine repeats (78-393 aa, 780-842 aa) at the N-terminus. Codon bias could also hinder efficient expression of PfOrc4N in heterologous bacterial system.

Further, we have tried functional complementation of *PfOrc4* in yeast system. The chimera consisting of ScOrc4N and PfOrc4C could withstand URA3/FOA selection but not to an extent as compared to positive control wild type ScOrc4. This could be a result of poor expression of chimeric protein due to codon bias. Nevertheless, it gives us confidence that putative PfOrc4 indeed belongs to Orc4 family of protein and may be involved in replication.

PfOrc4 is expressed as a ~117 kDa full-length protein endogenously. We confirmed this by using antibodies against different domains of PfOrc4 (N-terminal and middle region respectively). However, western blotting of Pf3D7 parasite lysate in the presence of PfOrc4M antibodies yielded two additional bands at ~55 kDa and ~ 35kDa. The exact nature and amino acid composition of these shorter protein fragments are not understood. These might be processed form of full-length PfOrc4 protein. Furthermore, PfOrc4 localizes in the nucleus in early stages as demonstrated by IFA studies (Fig. 3.9). Yet, it seems to move towards the perinuclear compartment in the later stages. Since, PfOrc4M recognizes three major bands in Western blotting experiments, we speculate that it could be one of the processed forms which is responsible for ring-shaped immunostaining in IFA.

Our observations suggest that the putative PfOrc4 protein is involved in formation of initiation complex since it colocalizes with one of the helicase protein PfMcm6 which have been shown to be crucial for forming initiation complex and moving from Pre-replication to replicative stage. Further, there have been reports that the ATPase activity of PfOrc4 is required for binding of ORC to DNA sequences in yeast (Lee and Bell 1997). Unlike *S. pombe*, PfOrc4 lacks any AT hook region at N-terminus which are

responsible for binding to replicator sequences. Yet, the preferential binding of PfOrc4 to replication origin sequences (PfARS1 and PfARS3) indicates its direct role in binding to DNA sequences.

The observations and data reported in this chapter are our efforts to characterize one of the subunits of *Plasmodium* ORC by generating reagents and carrying out preliminary in-silico and biochemical analysis. Since, pathogenicity of a parasite is associated with efficient DNA replication, further detailed study of a replication protein will prove to be beneficial for understanding the biology of the parasite.

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Summary

Humans serve as hosts for millions of microorganisms, some of which are beneficial commensals and others that cause diseases. There are numerous examples of disease causing agents encountered by humans. These belong to different classes of bacteria, viruses, fungi, protozoan parasites or insects. While there is an intrinsic resistance offered by the host to combat the infectious agents, these disease-causing organisms have evolved various mechanisms to evade the host immunity. The twenty first century has witnessed pioneering research in the diverse fields of infectious diseases; still we have failed to control and eradicate a handful of pathogens responsible for high rate of mortality. These include *Mycobacterium tuberculosis*, Human immunodeficiency virus (HIV) and *Plasmodium falciparum* among several others.

Malaria is one of the oldest diseases acknowledged by humans and documented in medical literature centuries ago. Recent WHO report states that half the population of the world is at the risk of contracting malaria. The mortality rate due to malaria is such that a child below five years is lost every two minutes. Malaria is a vector-borne infectious disease caused by a protozoan of genus *Plasmodium*. More than 200 species of *Plasmodium* have been identified that specifically infect mammals, birds and reptile. Of these, five species have been known to cause malaria in humans. These are *P. malariae, P. vivax, P. ovale, P. knowlesi* and the deadliest of them all, *P. falciparum.* Despite of malaria being a preventable and curable disease, an alarming 4,45,000 numbers of deaths were reported in the year 2016. Majority of fatal incidences arise due to *P. falciparum* infection which is predominant in African region. On the contrary, *P. vivax* is the prevalent strain causing malaria in Indian population.

The success of any pathogen is measured by the efficiency with which it can multiply itself while parallelly escaping the host immune response. During evolution, pathogens have carefully chosen their hosts according to their requirements. Some pathogens do not infect the reservoir host but only humans. Similar is the case with *P. falciparum* which is dependent on two hosts to complete its life cycle. The asexual sporogonic cycle takes place in the vertebrate host; first inside hepatocytes succeeded by the erythrocytes. The haploid protozoan gametocytes circulating in human blood are taken up by *Anopheles* mosquito where rest of the maturation from gamete to oocyst takes place (described in the review of literature). The oocyst releases sporozoites which are

subsequently stored in the salivary gland till the next blood meal of mosquito. During its complete life cycle, *P. falciparum* replicates its DNA at least four times; in human hepatocytes & RBCs and in mosquito mid gut. This ubiquitous and indispensable phenomenon of DNA replication is the foundation of the present study. Exponential multiplication in the number of parasites is largely dependent on the precise duplication of its genome in stipulated time frame.

In eukaryotes, replication of genetic material follows an organized and regulated recruitment of protein complexes. The first set of proteins to be recruited at the site of replication initiation are collectively termed as the Origin recognition complex (ORC). ORC is composed of six subunits that recognize and bind to the origin sites on the chromatin. This hexamer in turns recruits other proteins such as Cdc6, Cdt1 and Mcm2-7 to form the pre-replication complex. Establishment of a number of pre-RC dictates the number of sites where origin is fired, which is important for duplication of large and complex eukaryotic genomes. As discussed earlier, *P. falciparum* has a highly AT-rich genome and various regulatory mechanisms are in operation to control the timing and position of replication initiation. For instance, *P. falciparum* replicates its ~ 23 Mb genome multiple times within 48 hours during intra-erythrocytic cycle drawing attention towards a possibility of numerous origins. With this background, it is critical to study the factors involved in parasite DNA replication, with emphasis on replication initiation proteins.

In addition to proficient DNA replication mechanism, *P. falciparum* cleverly avoids the host immune response by switching the epitopes of its antigenic protein. This protein is a surface antigen called PfEMP1 (erythrocyte membrane protein) encoded by one of the sixty *var* genes. EMP emerges on the RBC surface around early trophozoite stage and facilitates interaction with host endothelial proteins. This also prevents passage through spleen where the infected RBCs may have been destroyed. To this effect, *P. falciparum* has evolved an intricate secretory pathway as well as the ability to maneuver the host system according to its requirements. In a conventional secretory pathway, proteins translocated into endoplasmic reticulum (ER) are trafficked to the Golgi apparatus from where they are sorted to cellular organelles like lysosomes, food vacuole, mitochondria, plastid, and plasma membrane or secreted outside the cell. The mechanism of protein

trafficking in malarial parasite is carefully crafted because the secretory proteins need to traverse its own plasma membrane, followed by parasitophorous vacuole membrane, host cytoplasm and finally the host's cell membrane.

In accordance with the importance of DNA replication in *P. falciparum*, it is imperative to study the biology of molecules involved in this universal process. In the present study, we have biochemically and functionally characterized two putative ORC homologs in *P. falciparum* (PfOrc2 and PfOrc4). We have also tried to gather details on the pathway followed by PfOrc2 protein in order to reach the parasite nucleus. The results obtained have been distributed into three chapters as summarized below.

In the **first chapter**, we have attempted to biochemically characterize PfOrc2 (PF3D7_0705300) using various Molecular Biology techniques. First, we have generated antibodies against the N-terminal region of PfOrc2 (PfORc2N) and used these to examine the protein expression *in vivo*. We obtained a band at ~100 kDa corresponding to full-length PfOrc2 which was corroborated by antibodies against the C-terminal region of Orc2 (PfOrc2C). The antibodies against PfOrc2C were found to recognize two bands, one at ~100 kDa and a shorter but more intense band at ~ 45kDa. We confirmed the identity of the lower band by immunoprecipitation experiment using Pforc2C antibodies which specifically pulled out a band ~ 45 kDa. Further, mass spectrometric analysis of *P. falciparum* 3D7 lysate resolved by SDS-PAGE revealed the presence of PfOrc2 C-terminal peptides in the region corresponding to 40-55 kDa. Additionally, Western blotting of different parasitic stage lysate in the presence of PfOrc2N antibodies divulged that maximum amount of full-length protein exists in the trophozoite stage which gradually reduces in the schizont stage as compared to negligible level in ring stage.

Further, we ascertained the sub-cellular localization of PfOrc2 using various means. Immunofluorescence assays depicted an extra-nuclear localization of the full-length PrfOrc2 in contrast to punctate nuclear-presence of PfOrc2C in all the parasitic stages. We confirmed the nuclear enrichment of PfOrc2C through immunoelectron microscopy, wherein gold particles recognizing PfOrc2C were confined to the nuclear region. We also performed protein fractionation experiments in which full-length

PfOrc2 was obtained as a peripheral membrane protein and PfOrc2C as an insoluble nuclear protein.

Bioinformatic analysis of PfOrc2 protein sequence indicated the presence of an ER-retention signal at its C-terminus. Experiments reported in **chapter two** were performed to trace the possible link between ER and a bonafide nuclear protein. Our data suggest that PfOrc2 enters the secretory pathway through ER to get processed by a membrane protease present on ER membrane and the cleaved fragment (PfOrc2C) is then translocated to the nucleus. Besides, we have generated parasite lines expressing short extreme N-terminal region of PfOrc2 fused to GFP to demonstrate the presence of putative ER-targeting signal in the protein.

We have proposed this unusual pathway for PfOrc2 based on a series of specific inhibitors that include Brefeldin A (blocks ER-Golgi traffic) and Z-$(LL)_2$-ketone (signal peptide peptidase inhibitor) used in this study and Ivermectin (blocks nuclear import) as reported earlier. Nuclear proteins are generally translated on the cytoplasmic ribosomes and translocated into the nucleus by virtue of a nuclear localization signal or in association with other nuclear proteins. However, reports of nuclear trafficking of a protein through secretory pathway are rare. One such example is sterol regulatory element binding protein (SREBP) that translocates from Golgi to the nucleus during reduced cholesterol level. Another example of nuclear translocation has been reported in mouse mammary tumor virus (MMTV) protein called Rem whose N-terminal signal peptide is cleaved and translocated to the nucleoli and the C-terminus remains in the ER.

The obvious unanswered questions were pertaining to the possible function of PfOrc2, whether PfOrc2 associates with the ER only for its processing or there is some non-nuclear function of PfOrc2. It is difficult to comment on the ER-specific function of PfOrc2 in the absence of any data on its interacting partners. However, we obtained good co-localization of PfOrc2C with PfActin, which is a marker for *var* gene activation in the ring stage. This was an interesting observation, because Orc2 homologs have been usually associated with HP1 related heterochromatinization of chromosome across many taxa. We examined the status of PfOrc2C and PfHP1 proteins and found partial co-localization between the two. The above two results are

strikingly opposite but shed light on the complex regulatory mechanisms adopted by *P. falciparum.* It is possible that Orc2 is involved in actin-mediated activation of *var* gene in the very early stage. HP1 protein is largely associated with genomic loci that are transcriptionally silenced, but there are a few reports which establish its presence in the euchromatin as well. Co-localization of PfHP1 and PfOrc2 in late stages may be indicative of a non-replicative function of Orc2. However, further experiments revealing the exact genomic location of these proteins in stage-specific manner are required to come to any conclusion.

Finally, work in the **third chapter** revolves around another ORC subunit, putative PfOrc4 (PF3D7_1334100) where we have performed complementation studies in yeast *orc4* knock-out strain to confirm the identify of this putative protein. Next, we have tried to biochemically characterize PfOrc4 using antibodies against its middle region (PfOrc4M) which yielded a band corresponding to full-length PfOrc4 at ~100 kDa. The presence of full-length band was confirmed by using antibodies against the N-terminal region of PfOrc4. Interestingly, besides the full-length band, antibodies against PfOrc4 recognized two shorter bands at ~55 kDa and ~35 kDa whose identity and function are still elusive. Immunostaining signal of PfOrc4 got altered from punctate to that of ring-shaped diffused perinuclear pattern as the parasites progressed from early to late stages. We also obtained partial co-localization between PfOrc4 and another replication protein PfMcm6 which suggests a replication related function for putative PfOrc4. Furthermore, PfOrc4 was found to be enriched at the sites of putative replication origins ARS1 and ARS3 in a ChIP-based PCR experiment. These ARS like elements have been identified recently as putative origins in *Plasmodium falciparum*.

In a nutshell, we have tried to characterize two replication homologues of *P. falciparum* namely PfOrc2 and PfOrc4 and decipher their potential roles in the malarial parasite biology. Our observations with respect to ER association and processing of PfOrc2 define a novel role of secretory system in *Plasmodium*. Based on our data, we speculate that PfOrc2 is involved in replication as well as non-replicative functions whereas the role of PfOrc4 is restricted to DNA replication.

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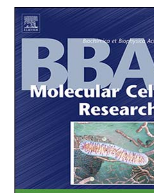

Identification of a novel trafficking pathway exporting a replication protein, Orc2 to nucleus via classical secretory pathway in *Plasmodium falciparum*

Rahul Sharma[a,1,2], Bhumika Sharma[a,1], Ashish Gupta[b], Suman Kumar Dhar[a,*]

[a] Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067, India
[b] Department of Life Sciences, School of Natural Sciences, Shiv Nadar University, Greater Noida 201314, India

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ABSTRACT

Malaria parasites use an extensive secretory pathway to traffic a number of proteins within itself and beyond. In higher eukaryotes, Endoplasmic Reticulum (ER) membrane bound transcription factors such as SREBP are reported to get processed *en route* and migrate to nucleus under the influence of specific cues. However, a protein constitutively trafficked to the nucleus via classical secretory pathway has not been reported. Herein, we report the presence of a novel trafficking pathway in an apicomplexan, *Plasmodium falciparum* where a homologue of an Origin Recognition Complex 2 (Orc2) goes to the nucleus following its association with the ER. Our work highlights the unconventional role of ER in protein trafficking and reports for the first time an ORC homologue getting trafficked through such a pathway to the nucleus where it may be involved in DNA replication and other ancillary functions. Such trafficking pathways may have a profound impact on the cell biology of a malaria parasite and have significant implications in strategizing new antimalarials.

1. Introduction

Malaria continues to be a major threat to the developing world claiming a large number of lives worldwide annually [1]. Crucial factors contributing to its severity include unavailability of an effective vaccine and new antimalarials. The malarial parasite, *P. falciparum* has evolved extremely ingenious ways to exhaustively remodel the host cell by trafficking proteins across its boundary and in particular on the surface of red blood cell for immune evasion [2]. This remarkable feat is achieved by a well-developed endomembrane system which plays a crucial role in trafficking proteins in and out of the cell. Protein trafficking is highly enigmatic in the malarial parasites because there are many unique features/exceptions or organelles which are not found in other eukaryotic cells such as parasitophorous vacuole (PV), rhopteries, micronemes, apicoplast etc. [2,3]. Under usual circumstances, the majority of proteins going through the secretory pathway either get secreted outside the cell or intracellularly transported to different organelles (other than nucleus) through vesicular transport and these fundamental processes of protein trafficking have been reported in *P. falciparum* too [4,5]. In a typical secretory pathway, endoplasmic reticulum is the organelle where proteins begin their journey and Golgi serves to sort them to different destinations. Other than this established itinerary, there are a few unconventional routes taken by different proteins depending on their specific roles within a cell e.g. Cystic fibrosis transmembrane conductance regulator (CFTR) is reported to be transported to the plasma membrane by bypassing the Golgi complex [6]. However, a pathway where a protein can be constitutively trafficked to the nucleus via secretory pathway has not been reported in any life form including the apicomplexans. Although some ER membrane bound transcription factors such as SREBP, ATF6 and an ER localized endonuclease DNAS1L3 are reported to migrate to the nucleus under the influence of specific signals [7–9], no protein so far has been reported to be constitutively trafficked to the nucleus by the classical secretory pathway.

In this work, we have identified a homologue of an Origin Recognition Complex (Orc2) which is a well-established nuclear protein and is reported to be a part of a multi subunit complex that plays important role in the initiation of DNA replication and cell cycle regulation in various systems [10]. Orc2 is an essential component of the ORC complex in several eukaryotes and plays a crucial role in initiating DNA replication. The regulation and function of Orc2 is diverse across various systems. The depletion of Orc2 has been reported to delay the progression through mitosis in *S. cerevisiae* [11]. It has also been found to interact with other replication initiation proteins Mcm10 and CDC45

* Corresponding author at: Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi 110067, India.
E-mail address: skdhar2002@yahoo.co.in (S.K. Dhar).
[1] These authors contributed equally to this work.
[2] Present address- Memorial Sloan Kettering Cancer Center, NY 10065.

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to initiate DNA replication [12,13]. Other than nucleus, Orc2 has also been reported to be present on the centrosome, centromeres and heterochromatin in tight association with HP1 in human cells [14]. These reports highlight the importance as well as the diversity of Orc2 functions. Herein, we report for the first time, the presence of a novel protein trafficking pathway in an apicomplexan parasite where Orc2 gets trafficked to the nucleus following its association with ER where the full-length protein gets processed. Subsequently, the C terminal fragment (PfOrc2C) migrates to the nucleus constitutively. In the nucleus, PfOrc2C localizes at the nuclear periphery raising the possibility of a novel role of an ORC protein in a pathogen.

In brief, we report a new protein trafficking pathway, where a protein may be trafficked to the nucleus by ER association thereby establishing a novel role of ER in an apicomplexan parasite. Our findings highlight an unconventional way of protein trafficking in an apicomplexan parasite and provide the first evidence of ER-nucleus traffic which helps us to understand its basic cell biology that may lead to strategizing the development of new antimalarials based on such pathways.

2. Materials and methods

2.1. Yeast complementation assay

Yeast cells were transformed with Lithium Acetate method [15]. For this purpose, yeast *Orc2* strain was grown in YEPD media overnight at 30 °C to set up the primary culture. $\sim 5 \times 10^6$ cells from the primary culture were inoculated again and grown until the number reached 2×10^7. Cells were washed with sterile water and then treated with 1 mL 100 mM LiAc. After washing, the pellet was treated with an aqueous solution containing: PEG (50%), 1 M LiAc, ss-DNA (2 mg/mL) and the desired plasmid DNA. The whole mixture was incubated at 30 °C for 30 mins. Heat shock was given at 42 °C for 20 mins followed by removal of the supernatant after centrifugation. The pellet was resuspended in 100 μl of sterile water and plated on the selective media-agar plate. To check successful transformation a single colony was picked and streaked onto SD-trp plates supplemented with 2%raffinose, 2% galactose. These plates were incubated at 25 °C for 3–4 days. Single colonies were streaked on *–Trp* plates separately and kept at 25 °C and 37 °C for incubation to check their viability.

Protein expression in yeast cells was checked by preparing yeast cell lysates as described by Kushnirov VV [16]. Briefly, yeast cells were resuspended in 0.2 M NaOH for 5 min at room temperature. Pellet obtained after centrifugation was resuspended in SDS loading buffer and boiled for 5 min. Supernatant containing the yeast protein lysate was resolved by SDS-PAGE. Expression of Orc2 protein was confirmed by using antibodies against PfOrc2C.

2.2. DNA manipulations

Different regions of Orc2N (1–332 amino acid residues, nucleotide positions 1–1450 bp) and Orc2C (561–825 amino acid residues, nucleotide positions 1624–2478 bp) were amplified from 3D7 genomic DNA by PCR using primers (Orc2N-P1/P2, Orc2C-P3/P4) listed in Table S1. These were cloned under *BamH*I restriction sites for Orc2N and *BamH*I-*Not*I for Orc2C in pET28a vector. All the clones were subsequently sequenced to rule out the possibility of any mutation.

Plasmid constructs used for yeast complementation studies were made in pRS416 yeast expression vector having *ura* gene as a selection marker and galactose inducible promoter and terminator. Five different DNA fragments were cloned in the MCS of pRS416 vector that included ScOrc2, PfOrc2 full length, Chimera Orc2 (having N terminus of *S. cerevisiae* and C terminus of *P. falciparum* Orc2), ScOrc2N and PfOrc2C. These DNA fragments were amplified by PCR using specific primers (*Sc*Orc2-P5/P6, *Pf*Orc2 full length- P7/P8, Chimera Orc2- P9/P10, ScOrc2N- P11/P12, *Pf*Orc2C- P13/P14) from *S. cerevisiae*/*P. falciparum* genomic DNA as per the requirement. The whole cassette containing inserts from pRS416 were sub-cloned along with galactose inducible promoter and terminator sequences in pRS314 vector between *Sac*I-*Kpn*I restriction sites having *trp* selection. These constructs were then used for transformation and complementation studies. Temperature sensitive yeast *Orc2* strain was used for complementation studies which allowed the yeast cells to survive only at temperature (25 °C).

2.3. Protein purification

Orc2 N-terminus (1–332 amino acids) and C-terminus (561–825 amino acids) recombinant proteins were expressed by transforming *E. coli* BL21 cells with pET28a-Orc2N and Orc2C constructs and induced with 0.5 mM IPTG at 22 °C overnight. The recombinant proteins were purified by batch purification method using Ni-NTA beads from Qiagen.

2.4. Polyclonal antibody generation

Polyclonal antibodies against Orc2N terminus (1–332 aa) were raised by injecting 15-25 μg of purified protein in mice followed by collection of immune sera after 10 days. Pre-immune sera were collected before immunization. Subsequent booster doses were given followed by collection of the immune sera after 7–10 days. PfOrc2C-terminal (561–825 aa) peptide antibody was raised in rabbit by Abexome Biosciences, Bangalore (India). The sequences of the peptides are: EYHYKYKSSKTLK and DKGIYGVEKESLLQD.

2.5. P. falciparum culture

The growth and maintenance of parasite culture has been followed according to the method described earlier [17]. In brief, *P. falciparum* 3D7 strain was grown at 4–10% parasitemia in RPMI 1640 medium supplemented with 0.5% Albumax (Invitrogen), 5% $NaHCO_3$, 50 μg/mL gentamycin and 50 μg/mL ampicillin at 37 °C. Wherever required, the culture was synchronized by incubating ring-staged parasites for 5 min with 5% sorbitol followed by 2 washes with RPMI only.

2.6. Reagents

Following antibodies were obtained as gift: Plasmepsin V (a kind gift from Prof. Daniel E Goldberg, St. Louis), PfBiP (kind gift from Prof. John Adams, University of Florida), PfPDI (kind gift from Prof. Philippe Grellier, CNRS, France). Dilutions of different antibodies used for Western Blotting (WB) and Immunofluorescence assays (IFA) are as follows: Orc2C (1:500 for WB, 1:100 for IFA), Orc2N (1:2000 for WB, 1:100 for IFA), Plasmepsin V (1:50 for IFA), PDI (1:500 for IFA), BiP (1:10,000 for WB), Orc1 (1:500 for IFA), SSB (1:3000 for WB and 1:500 for IFA), Aldolase (1:5000 for WB). Undiluted PfOrc2C antibodies were used for Immunoelectron microscopy. Yeast $Orc2T_s$strain was a kind gift from Prof Bruce Stillman, CSHL. Inhibitors used in this study: Brefeldin A (Sigma), Ivermectin (Sigma), $(\text{Z-LL})_{2-}$ketone (Calbiochem) and Cytochalasin D (Sigma).

2.7. Western blot analysis

The parasitized RBCs were saponin lysed using 0.1% saponin. The parasites released from the RBC after saponin lysis were washed with Phosphate Buffer Saline (PBS) twice and then mixed with an equal amount of 2× Laemmli SDS dye and boiled at 94 °C for 3–4 min. The lysate obtained was subjected to Bradford assay for determination of protein concentration. Depending on the experiment, ~60 μg to ~100 μg of total protein was loaded into each lane and separated by SDS-PAGE. The proteins were transferred to PVDF membranes and treated with primary and secondary antibodies as applicable. The blots were developed by ECL made of Luminol, Coumaric acid and Hydrogen Peroxide. Intensity quantification of the bands obtained after

developing was done by Image*J* software (NIH). Secondary rabbit/mouse HRP was procured from Santa Cruz (1:5000 dilution).

2.8. Immunofluorescence assay

A thin smear having 3–5% parasitemia was prepared on glass slides. These slides were fixed by normal methanol for 10 s and then air dried. Air dried slides were rehydrated with PBS for 10 mins. Permeabilization as well as blocking of slides containing parasitized RBCs was done using a solution containing 3% Bovine Serum Albumin (BSA) and 0.02% saponin. Subsequently, primary and secondary antibodies were added as applicable. All the washing was done in PBST (Phosphate Buffered saline with 0.1% v/v Tween 20). Secondary antibodies like Alexa Flour 488/568/594 rabbit/mice and DAPI were procured from Molecular Probes- Life Technologies (1:500 dilutions). Imaging was mostly done on an Olympus confocal microscope having a High Sensitivity Detector (HSD). However, imaging using Plasmepsin V, PDI and SSB antibodies was done using Carl Zeiss AXIO Imager Z1 fluorescence microscope. The software used for image capturing was AxioVision Rel. 4.8. Z-stacking was done on Carl Zeiss AXIO Imager Z1 and 3D image was reconstructed by AxioVision Rel. 4.8 software.

2.9. Immunoelectron microscopy

Parasites were fixed overnight in the fixative (4% paraformaldehyde + 0.1% glutaraldehyde) at 4 °C. The pellet was washed 3 times in 0.1 M PBS for 30 mins at 4 °C. The pellet was then dehydrated with graded alcohol concentrations (30%, 50%, 70%, 80% and 90%) for 30 mins each at 4 °C. Final dehydration step was done by treatment with absolute alcohol for 1 h at 4 °C twice. Infiltration of the parasite pellet was done by taking equal volume (1:1) of LR white (resin) and absolute alcohol as the infiltration solution. Pellet was treated with infiltration solution for 1 h at 4 °C twice. Subsequently, it was treated with LR white alone and incubated overnight at 4 °C. It was again treated with LR white for 4 h at room temperature. The final step of embedding was performed by adding 5 volumes of LR white over the pellet in a 1.5 μl microcentrifuge tube. The sample was further kept at 55 °C for 48 h. Orc2 peptide antibodies (Conc. 4 μg/mL) were used as the primary antibodies. 10 nm gold particle conjugated secondary antibodies were used for labelling. Imaging was done at AIRF-JNU on Transmission Electron Microscope (JEOL2100F).

2.10. Sub-cellular fractionation and Na_2CO_3 extraction method for membrane proteins

Protocol for sub-cellular fractionation of *P. falciparum* parasites was adapted from Flueck et al. [18]. Briefly, infected RBCs were lysed by saponin to release the parasites followed by washing with PBS. Further, parasites were lysed in a buffer containing 20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.65% NP-40, 1 mM DTT, protease inhibitor cocktail for 5 min on ice. Sample was centrifugated at 3000 rpm to separate the cytoplasmic fraction. The pellet was again washed with above buffer and pelleted at 3000 rpm to obtain the nuclear pool. Both the cytoplasmic fraction and nuclear pellet were solubilised in 2× SDS loading dye. Equal amounts of protein samples from both samples were resolved by SDS_PAGE.

Protocol for analysing protein solubility was adapted from Kulangara et al. [19]. Saponin lysed parasites were freeze thawed thrice in 5 mM Tris.Cl pH 8.0 and centrifuged (12,100*g*) to obtain soluble protein fraction. Peripheral membrane proteins were extracted by 0.1 M sodium carbonate pH 11.0. 1% Triton X was used for separating the integral membrane proteins from remaining insoluble protein pool. Respective protein fractions were solubilised in 2× SDS loading dye accordingly and separated by SDS-PAGE.

2.11. Protease protection assay

Infected erythrocytes were lysed using saponin and a fraction was removed that can be used for whole cell lysate (WCL) subsequently. Parasites were then resuspended in chilled hypotonic buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl) and kept on ice for 10 mins. Parasites were centrifuged at 10,000 × g for 15 min after gentle homogenization using Dounce homogenizer. 2× SDS loading dye was added to the pellet fraction and supernatant was equally divided into two parts. Trypsin (Promega) was added to one part in the ratio of 1:50 and both samples were incubated at 37 °C for 10 mins. Reaction was stopped by adding 1 mM PMSF and 6× SDS loading dye. All the samples were analysed by immunoblotting with respective antibodies.

2.12. Mass spectrometric analysis of peptides

Mass spectrometric analysis of peptides was performed essentially following the protocol as described elsewhere [20,21]. Parasite pellet (~40 hpi) was lysed using urea (8 M) followed by resolving the whole cell lysate in 10% SDS-Polyacrylamide gel. The gel was stained with Coomassie 250 based stain and the gel slices were excised from three different regions as shown in the figure (Fig. S6). The gel slices were further trypsinized and the peptides were extracted and desalted. The peptides were further subjected to LC-MS/MS analysis using a nano ACQUITY UPLC system (Water) which was coupled to the Q Exactive plus mass spectrometer (Thermo Scientific). The MS data were acquired and subsequently processed for protein identification using MaxQuant software.

2.13. Statistical calculations

Wherever applicable, the Student's *t*-test was performed on Microsoft Excel 2007.

3. Results and discussion

3.1. Functional characterization of a putative origin recognition complex 2 protein in Plasmodium falciparum

We have identified a putative homologue of the Orc2 protein in *P. falciparum* from PlasmoDB.org (PF3D7_0705300). The expected molecular mass of the protein is ~98 kDa containing 825 amino acid residues. The putative PfOrc2 shows overall ~22% identity with ScOrc2 and the homology is primarily restricted to the C-terminus (Fig. S1 A and B). An *in-silico* analysis suggests that the C terminus is a part of the Orc2 superfamily (Fig. S1A). Thus, in order to investigate whether it really acts as an Orc2 subunit, we attempted functional complementation of a temperature sensitive mutant strain of yeast *orc2* using C-terminus of PfOrc2. We used different constructs to complement the yeast strain based on homology (Fig. 1A) and found that the yeast cells expressing the chimeric Orc2 having N-terminus of ScOrc2 and C-terminus of putative PfOrc2 were viable at the restrictive temperature (37 °C) (Fig. 1B). Neither full length PfOrc2 nor the individual subfragment of the chimeric construct was able to complement the T_s strain. The expression of the chimeric Orc2 protein in yeast was confirmed by Western blot experiment using yeast protein extracts in the presence of antibodies against PfOrc2C (Fig. S2). These results suggest that the C terminus of PfOrc2 is capable of complementing ScOrc2 function in vivo and that PfOrc2 is indeed an Orc2 like protein with possible role in parasite DNA replication as previously reported in other systems [10].

3.2. Dynamics of Orc2 expression and its subcellular localization during the intra-erythrocytic developmental (IED) stages

For studying the protein expression profile in intra-erythrocytic

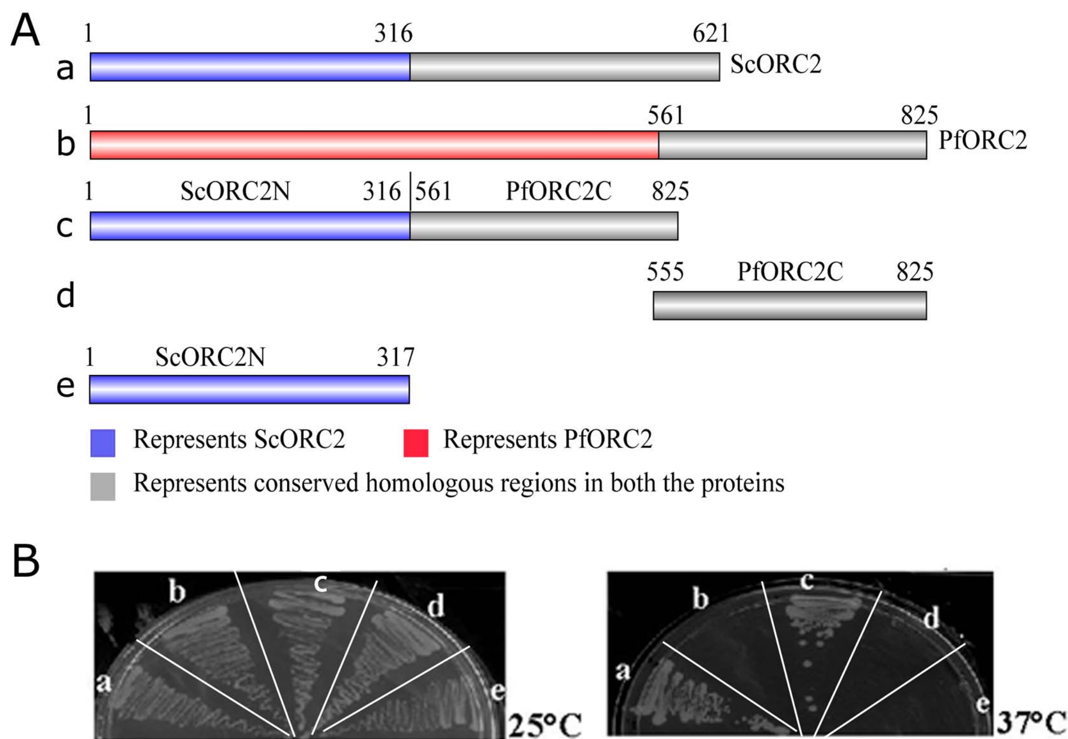

Fig. 1. Functional complementation of *Sc*Orc2 with the C-terminus of putative PfOrc2 in ScOrc2 temperature sensitive strain. (A) Different constructs (a–e) were made in yeast expression vector pRS314 having a *trp* gene selection marker and galactose inducible promoter and terminator. (B) Functional complementation showing the surviving yeast cells in restrictive conditions (37 °C) having the chimera Orc2 (c. ScOrc2N + PfOrc2C) along with the wild type *Sc*Orc2. All the other constructs failed to rescue the yeast strain. All the cells were grown at permissive temperature (25 °C) on –Trp SD medium plates with raffinose and galactose.

asexual cycle, we used polyclonal antibodies raised against the N-terminus and C-terminus of PfOrc2 as detailed in the materials and methods section. The region of PfOrc2 protein used for raising antibodies against PfOrc2N and the peptides used for raising antibodies against PfOrc2C are shown in Fig. S3. The antibodies specifically recognized the purified PfOrc2N and PfOrc2C in the Western blot experiments respectively and the pre-immune sera didn't recognize any such band (Fig. S4). The transcriptome data available in PlasmoDB suggests that the protein is highly expressed in the late trophozoite stages (Fig. S5). Immunoblotting of late trophozoite stage parasites using antibodies against PfOrc2N revealed a band at the expected size of ~100 kDa (Fig. 2A, left panel). Western blotting analysis of the same lysate samples with antibodies against PfOrc2C gave the same band at ~100 kDa but there was an additional band at about half the size (~45 kDa) of the full-length protein which was stronger in intensity than the upper band (Fig. 2A, right panel). Pre-immune sera for both the antibodies did not recognize any such band suggesting that the bands were specific for the protein of interest (Fig. 2A). Since the antibodies against PfOrc2C were generated against peptides from the C-terminus of PfOrc2, it is assumed that the ~45 kDa band is a part of the C-terminus of the full-length protein. Presence of a single copy gene for *orc2* and absence of intron(s) in the coding sequence precluded the possibility of an isoform or an alternate gene product. To further validate that the lower band (~45 kDa) is indeed a part of the C-terminus of PfOrc2, we resolved urea-lysed parasites by SDS-PAGE and excised three regions corresponding to ~100 kDa, ~40–55 kDa and ~25–35 kDa (Fig. S6). Gel slices were digested by trypsin and subjected to mass-spectrometric analysis as described in the methods section. ~40–55 kDa region provided three peptides exclusively from the C-terminal sequence of PfOrc2 (Table S2). Unsurprisingly, peptides from both N-terminal and C-terminal region pertaining to full-length PfOrc2 were obtained from the ~100 kDa region. No peptides of PfOrc2 were present in the ~25–35 kDa region, which was taken as a control (Table S2). Based on these results, we concluded that the lower band was possibly a processed product of the full-length protein and was less likely a non-specific band.

A stage specific Western blotting of the parasite lysate using PfOrc2C antibodies revealed that the lower band (~45 kDa) was more predominant in all the stages with the upper band being visible only in the late trophozoite stage (Fig. 2B). This might be due to the overall increase in the total protein content (as suggested by the transcriptome data from different stages; Fig. S5) which makes the unprocessed full-length protein visible in the late trophozoite stage.

Indirect Immunofluorescence assay (IFA) using antibodies raised against PfOrc2N showed a diffused pattern around the nuclear DAPI stain in all parasite stages (Fig. 2C, S7A). IFA using PfOrc2C antibodies showed punctate perinuclear foci of PfOrc2 around the nucleus in early stage (Fig. 2D). In the later stages, the foci became more intense which was consistent with the higher expression of the protein in the later stages (Fig. S7B). Being a DNA replication protein, PfOrc2 is expected to be present in the nucleus, but antibodies against two different regions of the same protein showed varied localization.

The nuclear localization of the PfOrc2C protein was confirmed by sub-cellular fractionation using NP-40 detergent as described in methods section (Fig. 3A), where the protein was exclusively present in the chromatin enriched fraction, similar to histone. We selected another ORC subunit PfOrc1 which has been established as a nuclear protein to check for possible co-localization. Apart from its function in DNA replication initiation, it has also been reported to bind to sub-telomeric sites and promoters of *var* genes which are localized at the nuclear periphery during the early trophozoite stages [22,23]. Indirect double labelled IFA with PfOrc1 showed partial co-localization in the early stages which supported *bonafide* nuclear localization of PfOrc2C (Fig. 3B). Since both Orc1 and Orc2 are homologues of replication proteins that form a multi protein complex in other systems, we had expected a significant co-localization unlike the partial co-localization observed here. This observation raised the possibility that besides replication initiation, these proteins might have some individual

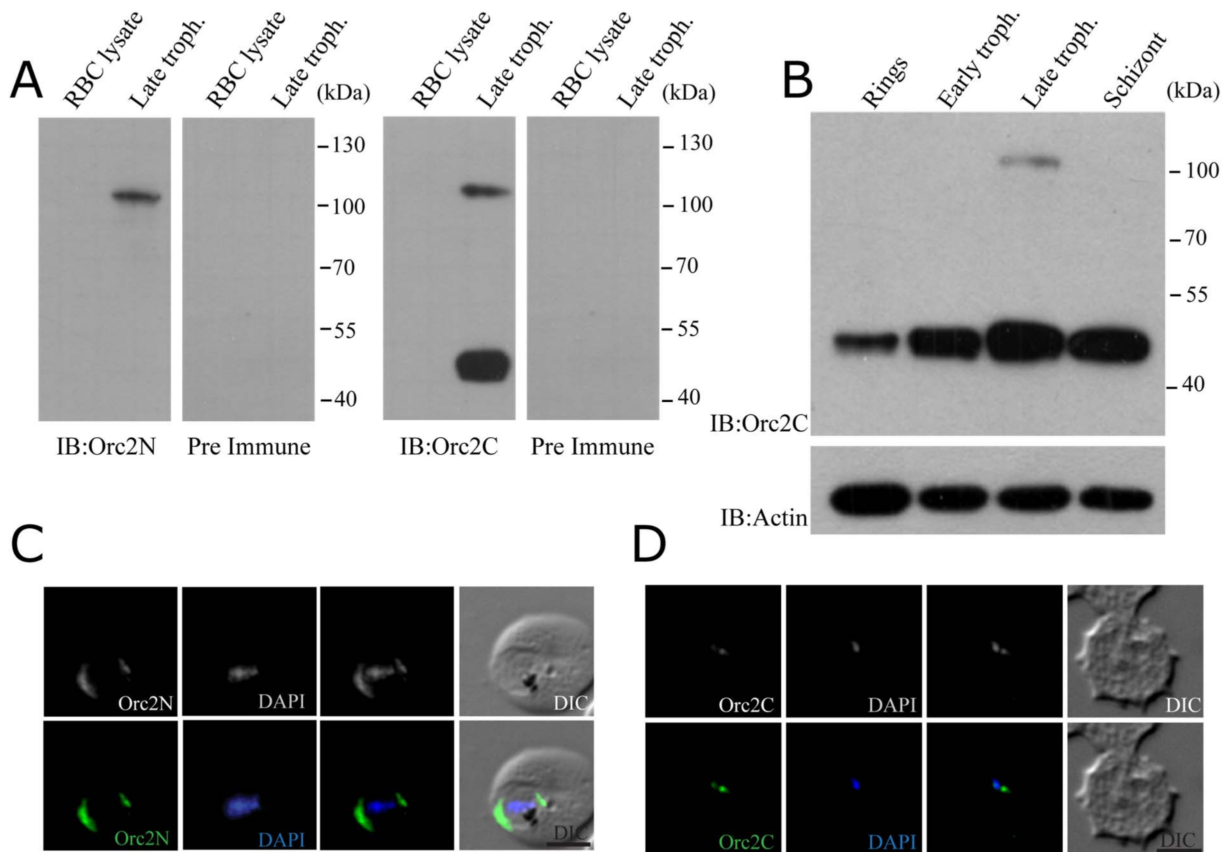

Fig. 2. Dynamics of Orc2 expression and its localization during the intra-erythrocytic developmental (IED) stages. (A) Western blot experiments using late trophozoite stage (35 ± 5 hpi) parasite lysate show the presence of full length Orc2 (~100 kDa) using antibodies against Orc2N and Orc2C respectively. An extra band at ~45 kDa can be seen only in the presence of antibodies against PfOrc2C. Pre-immune sera were clean. (B) Western blot experiment using antibodies against PfOrc2C during different stages of the intra-erythrocytic (IE) cycle (Ring 18–20 ± 4 hpi, Early troph 25 ± 4 hpi, Late Troph 35 ± 4 hpi, Schizont 44 ± 4 hpi) of the IE cycle. The results show the predominant presence of the 45 kDa band in all the stages while the full length Orc2 is visible only in the late trophozoite stage. (C) IFA using antibodies against Orc2N shows a diffused extra-nuclear pattern. (D) IFA showing the punctate localization of PfOrc2C. (DAPI was used to stain nucleus; early parasitic stages have been shown for representation in C, D; Scale bar represents 2 μm in each case). The top panels are shown in B/W and the bottom panels are shown in color.

functions like *var* gene silencing in case of PfOrc1.

What is the fate of the N-terminal region of PfOrc2? We used the same method as mentioned above to fractionate parasite lysate and obtained the full-length protein in nuclear fraction (data not shown). This observation was contrary to the diffused IFA pattern we had obtained for PfOrc2N. Subsequently, we employed another method to fractionate soluble and peripheral membrane proteins using sodium carbonate, followed by Triton X-100 to extract integral membrane protein. The remaining pellet contained the insoluble fraction. Upon sub-cellular fractionation by this method, we found the presence of majority of the full length PfOrc2 protein (using N terminus antibodies) in the soluble and peripheral protein fraction (Fig. 3C). Aldolase, a cytoplasmic soluble protein was majorly present in soluble fraction. We used PfBip, an ER resident luminal protein as a control for proteins present near the nuclear periphery. However, PfOrc2C (~45 kDa band) was exclusively found in the nuclear fraction, similar to histone under the same experimental conditions (Fig. 3C).

Since, PfOrc2 is believed to be a nuclear protein, and our results of IFA and fractionation experiments suggested nuclear localization of shorter fragment of PfOrc2 (Orc2C), we further confirmed its nuclear presence by Immunoelectron (IE) microscopy. PfOrc2C clearly showed nuclear localization in late trophozoite stage as the gold particles are predominantly present in the nucleus but not in the cytoplasm when similar areas of nucleus and cytoplasm were compared (Fig. 4).

Taken together, we observed an interesting pattern of protein expression in the asexual stage where we got a predominant protein band at about half the size (along with the full length) and the IFA (along with IE microscopy) as well as sub-cellular fractionation confirmed its nuclear localization, which is consistent with the previous reports on Orc2 [10].

3.3. Brefeldin A affects nuclear trafficking of PfOrc2

To investigate the origin of the lower molecular mass band, we analysed the amino acid sequence of PfOrc2 and carefully looked for specific signal sequences or motifs which might be responsible for its appearance. Upon in silico analysis of the sequence, we found Orc2 superfamily domain (496–818 amino acid residues) as well as an ER retention sequence (-SEEL; 821–825 amino acids) (Fig. 5A). Proteins targeted to the ER generally contain an N-terminal hydrophobic signal sequence [24]. Usually, proteins destined to go to the nucleus are translated on free ribosomes in the cytoplasm and those entering the secretory pathway are translated on the ribosomes attached to the surface of Rough ER. ER retention sequences are present in resident ER proteins and are retrieved from the Golgi once they move ahead of the ER [25]. We were surprised to find a putative ER retention sequence (at the extreme C terminus) in a nuclear protein. So far, a protein called GRP58 has been reported to contain an ER retention signal as well as Nuclear Localization Signal (NLS) but their function as well as mechanism are not clearly understood [26]. Such ER retention sequence was not found in Orc2 homologue of any other apicomplexan, common model systems as well as different species of *Plasmodium* (data not shown). Phylogenetic analysis of Orc2 sequences from different species showed the presence of PfOrc2 and PbOrc2 in the same branch (Fig.

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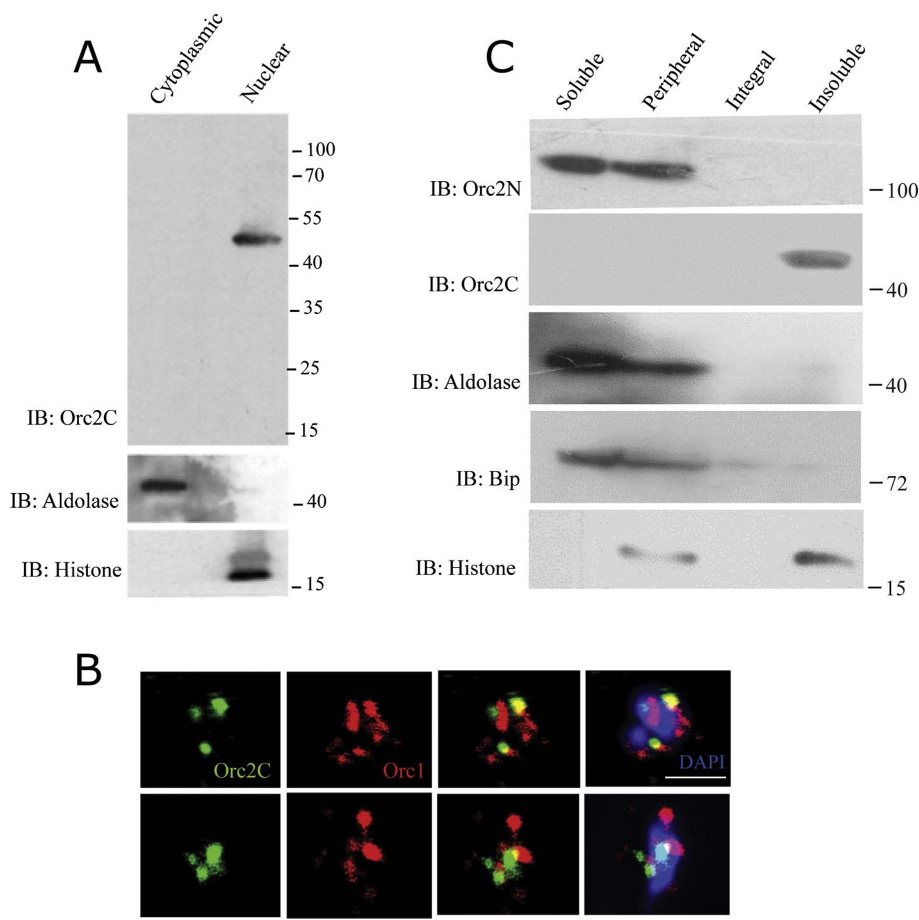


Fig. 3. Subcellular fractionation of Orc2. (A) NP-40 detergent based subcellular fractionation followed by Western blot experiment shows the nuclear localization of PfOrc2C. Aldolase was used as a cytoplasmic fraction marker and Histone H3 was used as a nuclear/chromatin fraction marker. (B) Co-IFA showing the localization of PfOrc2C with another ORC protein PfOrc1. PfOrc2 partially co-localizes with PfOrc1. (C) Trophozoite stage parasites (30 ± 4 hpi) were subjected to protein extraction using Na_2CO_3 (peripheral) and Triton X-100 (integral) as described in materials and methods. Full length PfOrc2 protein was obtained in the soluble and peripheral membrane protein fraction. Even after such harsh treatment, PfOrc2C fragment was present in the remaining insoluble protein fraction. Aldolase, Bip and Histone H3 were used as cytoplasmic marker, an ER protein present close to nucleus and a chromatin marker respectively.

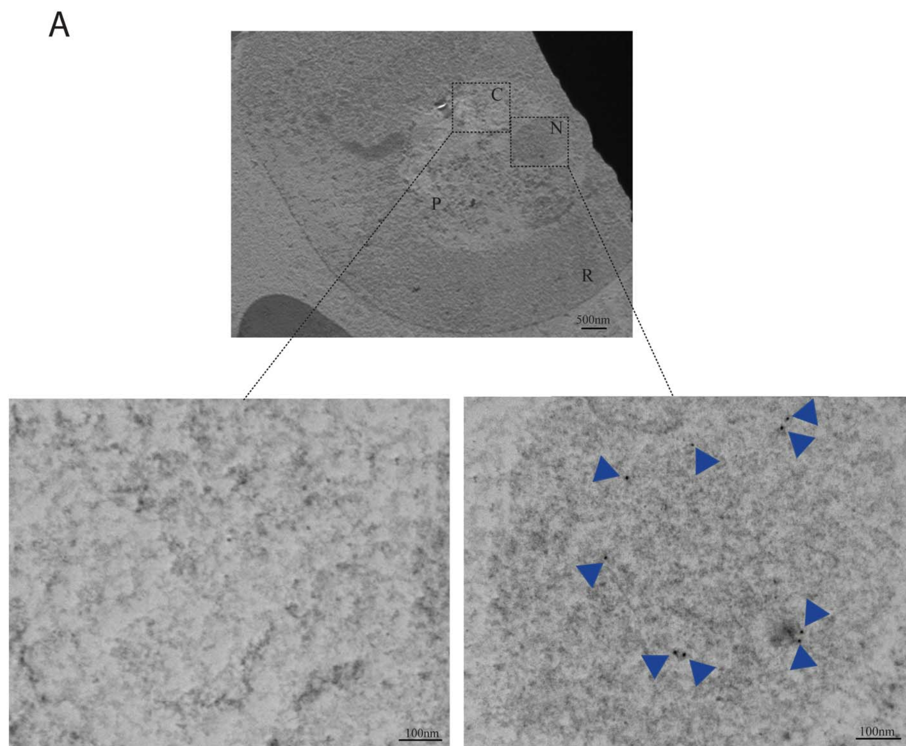


Fig. 4. Immunoelectron microscopy. IEM showing predominant nuclear localization of PfOrc2C. (Gold particles visible as uniform dark black dots have been marked with blue arrowheads, scale bar is indicated; C-cytoplasm, N-nucleus, P-parasite, R-RBC).

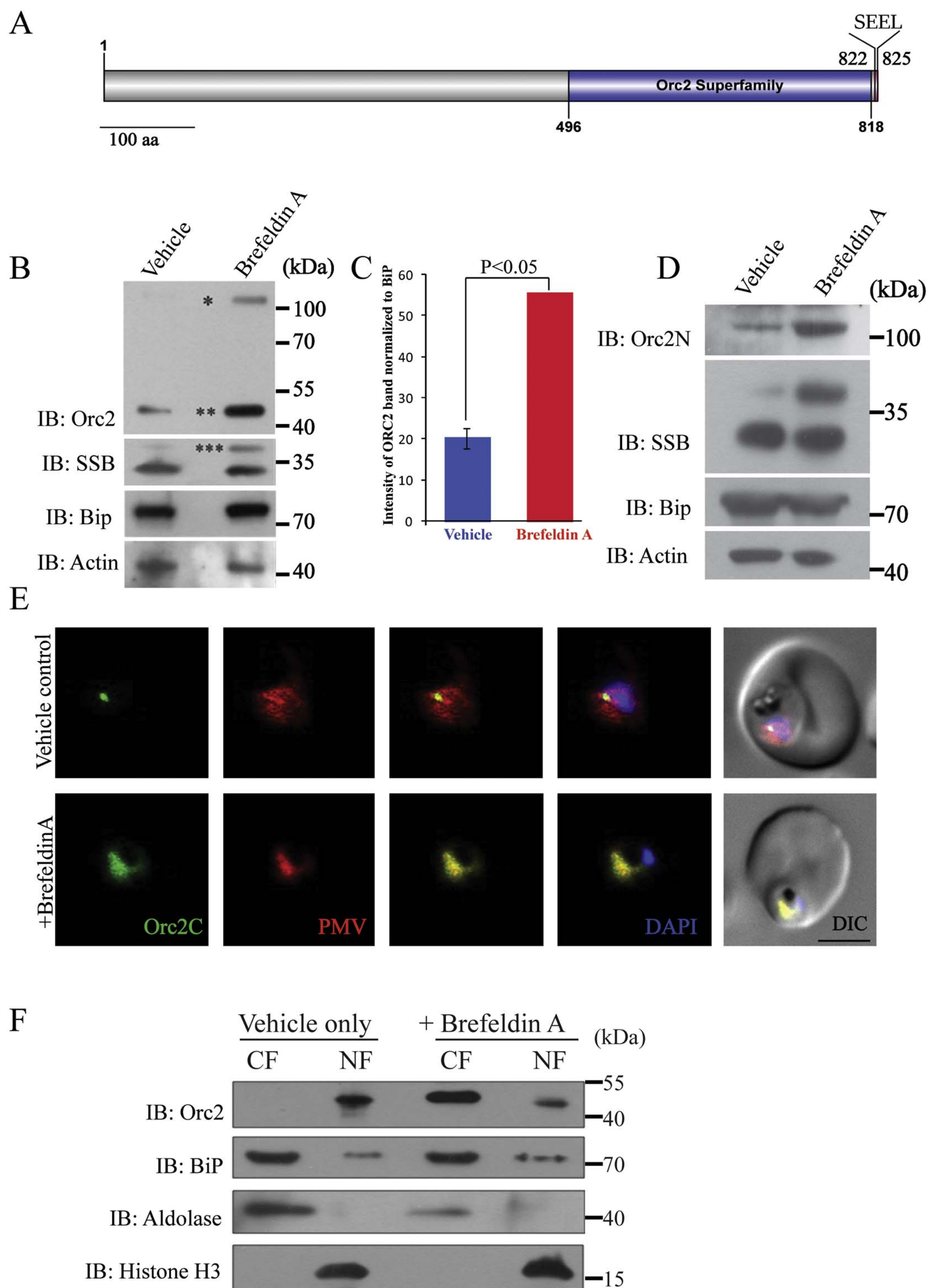


Fig. 5. Orc2 gets trafficked to the nucleus through the classical secretory pathway (A) Schematic representation of the key sequences present in PfOrc2 i.e. Orc2 superfamily domain and ER retention sequence. (B) Brefeldin A (BFA) treatment resulted in an increase in the intensity of the lower band (~45 kDa). Early trophozoite (20 ± 3 hpi) parasites were incubated with 5 μg/mL of BFA and ethanol (vehicle control) for 18 h. BiP and SSB were used as different controls mentioned in the text. Actin was used as a loading control. * Represents full length band which was present in a few experimental replicates. ** represents ~45 kDa band. *** represents the unprocessed form of SSB which appears upon BFA treatment. (C) Graph showing the accumulation of the lower band of PfOrc2 upon BFA treatment. The band intensity has been normalized to BiP. Error bar represents SEM. $*P < 0.05$ has been calculated using unpaired 2 tailed Student *t*-test. ($n = 4$). (D) Brefeldin A treatment yielded similar increase in full length PfOrc2 when probed by PfOrc2N antibodies. (E) IFA of PfOrc2C with Plasmepsin V (an ER marker) showed co-localization with PfOrc2C post BFA treatment. (F) Subcellular fractionation showing the presence of 45 kDa band of PfOrc2 in the cytoplasmic fraction post BFA treatment. Early trophozoites (20 ± 4 hpi) were incubated with 5 μg/mL of BFA along with the vehicle control for 10 h and then subjected to fractionation protocol. BiP was used as an ER marker. Aldolase and Histone H3 were used as cytoplasmic and nuclear marker respectively.

S8). However, the absence of putative ER retention signal in PbOrc2 may suggest *Plasmodium falciparum* specific function of this protein.

The presence of a putative ER retention sequence in PfOrc2C indicates its possible connection with the ER. However, we have demonstrated above that PfOrc2C is present in the nucleus. To test whether PfOrc2 is getting trafficked through the secretory pathway, we

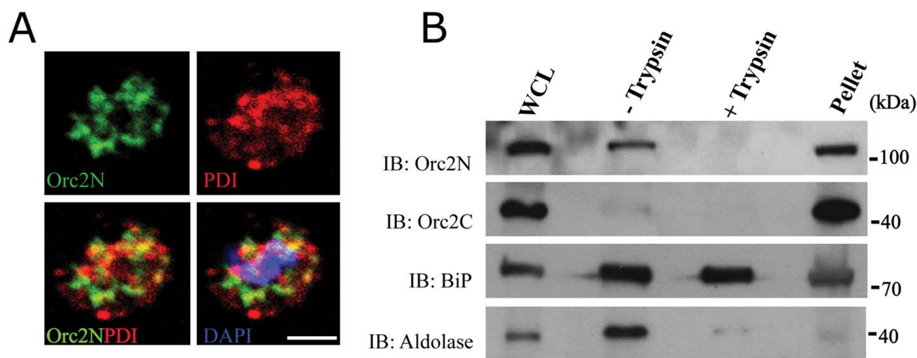


Fig. 6. Full length PfOrc2 protein is associated with the ER. (A) IFA showing the co-localization of PfOrc2N and an ER marker PDI. This indicates that the full-length PfOrc2 is associated with the ER. (B) Gentle homogenization and centrifugation of saponin lysed parasites renders the full-length PfOrc2 accessible to trypsin in the protease protection experiment. Full length PfOrc2 band was present in the cytoplasmic fraction without trypsin and absent after trypsin digestion. PfOrc2C band was obtained mainly in the pellet. Bip, being an ER protein was obtained in the cytoplasmic supernatant too and was protected against trypsin digestion by virtue of being a luminal protein. As expected for a cytoplasmic protein, Aldolase was digested by trypsin.

treated the parasites with Brefeldin A for 18 h. (BFA; which inhibits protein transport from ER to Golgi). Upon BFA treatment, we found that the intensity of the lower band increased significantly ($P < 0.05$, Student's *t*-test, unpaired, two-tailed) as compared to the vehicle only lane. These results suggested that the lower band was the processed or cleaved protein which was getting accumulated within the ER, in the presence of BFA (Fig. 5BC). The significant increase of the smaller protein fragment also suggested that possibly the full-length protein (as seen earlier) was getting processed in the ER and the C terminal fragment generated after the processing was moving ahead. We observed an increase in the full-length band which might be a result of incomplete processing due to BFA-induced ER stress (Fig. 5B). However, the level of BiP (ER resident protein) remained same following BFA treatment supporting the claim that PfOrc2C actually moves ahead of the ER and it gets accumulated upon disrupting the ER traffic (Fig. 5B). Apicoplast (a relict plastid found in apicomplexans) targeted proteins have been found to be sensitive to BFA as they are also targeted via secretory pathway [27,28]. Single Stranded DNA Binding protein (SSB) is a resident apicoplast protein [29] that was used as a control to show that the unprocessed form of SSB starts appearing in the presence of BFA (Fig. 5B). This confirmed that the increase in PfOrc2 level was due to BFA treatment and not a chance factor. Actin was used as a loading control. Further, we reaffirmed the effect of Brefeldin A on Orc2 by probing with antibodies against PfOrc2N. The full length PfOrc2 protein showed considerable accumulation post BFA treatment as it was shown for PfOrc2C (Fig. 5D).

To rule out the possibility of off-target effects of BFA due to long hours of incubation, we repeated the experiment with a shorter incubation time (~3 h). On incubation for 3 h, we found accumulation of PfOrc2 as previously seen for ~18 h of incubation although with a much lesser intensity, because of the short incubation time being proportional to a small amount of protein accumulation in the ER (Fig. S9). Similar accumulation of the protein at a shorter drug incubation time confirmed our hypothesis that the protein is indeed getting accumulated in the ER upon BFA treatment and is not an off-target effect due to long hours of drug treatment. Plasmepsin V (PMV) is a membrane bound aspartyl protease which is an ER resident protein [30–32]. We used PMV as an ER marker in double labelled IFA to investigate whether PfOrc2 colocalizes with it after BFA treatment. As expected, PfOrc2C colocalized with PMV after BFA treatment whereas it showed punctate foci different from that of PMV in untreated parasites (Fig. 5E). These results indeed suggest that PfOrc2 gets accumulated within the ER after BFA treatment and that PfOrc2C is continuously being trafficked from the ER to the nucleus.

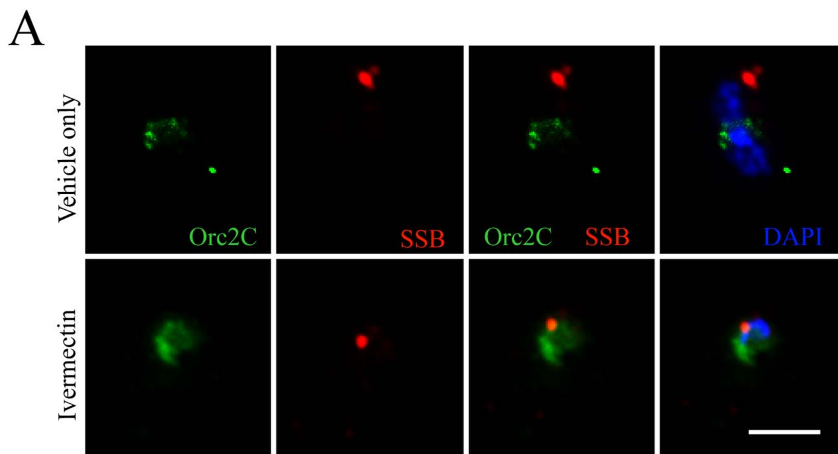
Proteins associated with the ER are majorly present in the cytoplasmic fraction (CF). Thus, upon BFA treatment proteins getting accumulated in the ER would appear in the CF which are otherwise not present in the CF. Upon sub-cellular fractionation after BFA treatment, PfOrc2C (~45 kDa band) started appearing in the CF whereas it was absent in the CF of control parasites supporting our claim that it is indeed getting accumulated within the ER (Fig. 5F). BiP was present mainly in the CF in the untreated parasites with a minor amount present in the nuclear fraction which may be due to the intimate contacts of the ER with the nuclear membrane. Another ER resident protein Plasmepsin V has been reported to be present in the nuclear proteome [33]. Thus, accumulation of PfOrc2 full length as well as the processed ~45 kDa fragment upon BFA treatment strengthened our claim that PfOrc2 indeed gets trafficked through the ER-Golgi pathway.

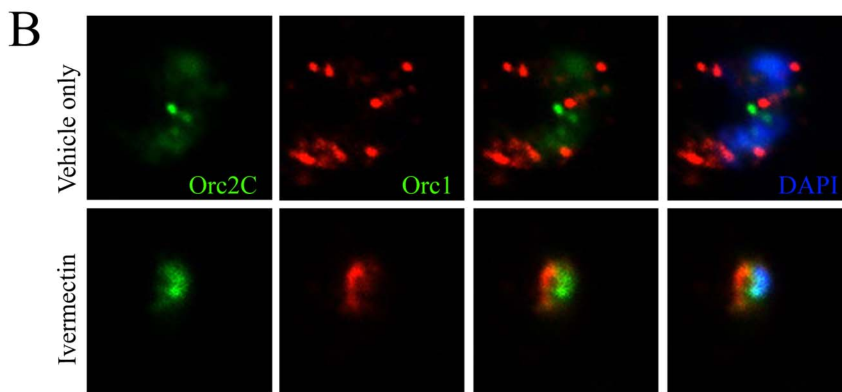
3.4. Orc2 associates with the ER for its processing

We have already shown that PfOrc2C does not co-localize with ER resident protein PMV (Fig. 5E, upper panel) suggesting redundancy of ER retention signal SEEL in PfOrc2. However, full length PfOrc2 may still show association with ER before its processing. For this purpose, we performed co-localization studies between Protein Disulphide Isomerase (PDI; PF3D7_0827900) and PfOrc2. PDI is another conventional ER resident protein with SEEL sequence at the C-terminus. It is a thiol metabolizing enzyme and helps in proper folding of proteins [34,35]. IFA using antibodies against PfOrc2N and PfPDI showed co-localization of both the proteins (Fig. 6A) suggesting the possibility of the association of the full-length protein with the ER.

The presence of full length PfOrc2 in the Na_2CO_3 extractable fraction (and not in the Triton-×100 extractable integral protein fraction, as shown in Fig. 3C) and its association with ER (Figs. 5, 6A) suggests that it is a peripheral membrane protein of the ER. In order to further validate this point, we performed a protease protection assay. Briefly, *P. falciparum* parasites were resuspended in hypotonic lysis buffer followed by separation of the cytosolic fraction containing ER and Golgi from the heavier organelle like nucleus as described in the materials and methods. Further, the cytosolic fraction was treated with trypsin and Western blot experiments were performed in the presence of antibodies against various proteins as indicated in the Fig. 6B. We found that full length PfOrc2 was present in the cytosolic fraction containing the cellular organelles like ER and it can be completely digested by trypsin like the cytosolic protein aldolase. Bip, an ER luminal protein was present in the cytosolic fraction too and it was completely protected by trypsin under the same experimental conditions while PfOrc2C was found mostly in the pellet fraction. These results clearly suggest that full length PfOrc2 is peripheral membrane protein associated with ER.

Further, we were interested to know whether the cleaved fragment (PfOrc2C) was a manifestation of the activity of any protease. For this purpose, we used E64 (cysteine protease inhibitor) which did not show any effect on the processing of PfOrc2 (data not shown). However, use of $(Z\text{-}LL)_2$-ketone, a signal peptide peptidase (SPP) inhibitor followed by Western blotting of $(Z\text{-}LL)_2$-ketone treated samples showed an increase in the intensity of the full-length band as compared to the control lane with concomitant decrease in the intensity of the processed PfOrc2C band (Fig. S10 A, B). This suggested that the full-length protein was not processed upon inhibition of an SPP like enzyme in the parasites. Similar increase in the intensity of the full-length band of PfOrc2





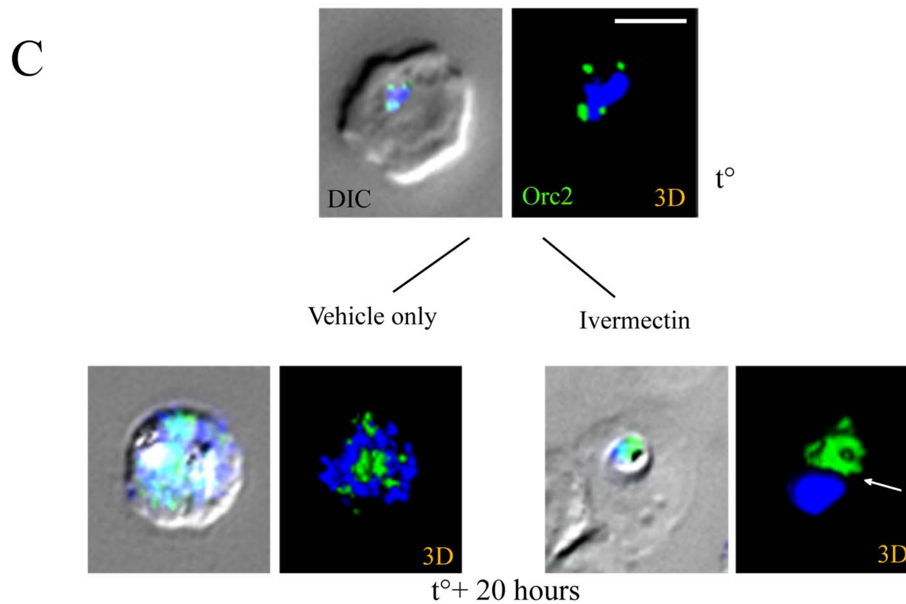


Fig. 7. Orc2 goes to the nucleus through the nuclear pore. (A) Orc2 showed a diffused pattern upon Ivermectin treatment. Ring (18 ± 4 hpi) parasites were incubated with 10 μM of Ivermectin along with DMSO as a vehicle control for 20 h. SSB was used as a non-nuclear negative control. (B) Orc1 as a positive control showed similar diffusion upon Ivermectin treatment which confirms that the diffusion of Orc2 is due to an inhibition of nuclear import (C) 3D reconstruction of Z stacked images showed the spatial localization of Orc2 with respect to the nucleus. Ivermectin treatment resulted in the loss of punctate foci as seen in the vehicle control lane. The white arrow shows the boundary of the nucleus seen due to the inability of Orc2 to enter the nucleus. ($n > 7$) ~300 cells were analysed in this study. DAPI was used to stain the nucleus.

was obtained when the effect of $(Z\text{-}LL)_2$ -ketone was investigated by antibodies against PfOrc2N (Fig. S10 C, D). BiP was used as a positive control as it is expected to be processed by SPP by virtue of being an ER resident protein. The level of BiP protein went down in the inhibitor treated lane suggesting a similar effect of the drug. Aldolase was used as a loading control (Fig. S10 A, C).

3.5. PfOrc2 enters the nucleus through the nuclear pore

From Golgi, proteins can be trafficked by vesicle mediated transport such as AMA-1 in *P. falciparum*, which is targeted to the micronemes and is involved in merozoite invasion [36]. Alternatively, proteins can be released as a cytoplasmic fragment (such as ER membrane bound

transcription factor SREBP in higher eukaryotes) which then enters through the nuclear pore [7]. Since there were no convincing reports on vesicle transport delivering cargo to the nuclear pore, we expected PfOrc2C to enter the nucleus through the nuclear pore. Recently, Ivermectin has been shown to block nuclear import by specifically inhibiting importin α/β in *P.falciparum* [37]. We used Ivermectin to evaluate its effect on the nuclear import of Orc2C. Treatment of the parasites with Ivermectin led to the growth arrest of the parasites in trophozoite stages (data not shown) as reported earlier [37]. IFA showed punctate foci of PfOrc2C in the untreated parasites (Fig. 7A). However, we observed a diffused pattern of PfOrc2C all around the cytoplasm in Ivermectin treated parasites (Fig. 7A). Diffused pattern of PfOrc2C in drug treated parasites suggested that it was getting inside the nucleus through the nuclear pore and upon inhibiting the nuclear import, the protein failed to enter the nucleus. As a control, we used a non-nuclear protein like apicoplast targeted SSB whose pattern and localization should not change with Ivermectin. IFA results showed no apparent change in the localization or pattern of SSB in the absence or presence of Ivermectin (Fig. 7A). We used another nuclear protein PfOrc1 as a positive control whose localization is likely to change upon inhibition of nuclear import. PfOrc1 showed similar disruption in its punctate, perinuclear foci and became diffused around the nucleus as seen for PfOrc2C (Fig. 7B). 3D reconstruction of Z stacked images clearly showed that PfOrc2C failed to enter the nucleus upon Ivermectin treatment and got accumulated in the cytoplasm (Fig. 7C). Both these controls support the hypothesis that PfOrc2C was entering the nucleus in an importin α/β dependent pathway and was therefore specifically inhibited by Ivermectin.

3.6. PfOrc2 occupies perinuclear sites

In a quest to understand the sub-nuclear localization of PfOrc2C, we tried co-localizing different nuclear proteins with it. Interestingly, we found PfOrc2C to co-localize with Actin I (Fig. 8A). Actin I has been reported to be present at the nuclear periphery in early ring stage parasites. Actin I is believed to be a part of a multi protein complex as previously reported [38,39]. To explore the possibility of PfOrc2C as a part of this complex, we tried depolymerising Actin I and then observe the subsequent effect on PfOrc2. Cytochalasin D (CD) has been a well-known inhibitor of Actin assembly and binds to F-Actin (polymeric form) and accelerates its depolymerisation to G-Actin (monomeric form) [40]. Upon CD treatment (10 μM) in ring stage parasites (18 ± 4 hpi), we found a diffused pattern of Actin I due to the loss of polymerization (Fig. 8B). Interestingly, we also found a considerable amount of diffusion for PfOrc2 signal. Intensity analysis of vehicle control and CD treated parasites clearly showed that the signal for PfOrc2/Actin I was less in intensity, continuous and distributed over a large area in CD treated parasites (Fig. 8C). On the other hand, the signals for control parasites were more intense, discontinuous and distributed over a smaller area. Diffusion of PfOrc2 upon CD treatment suggested its possible association with Actin I. Disruption of Actin I polymerization affected the association of PfOrc2 with multiprotein complex containing Actin I. However, careful observation of CD treated cells showed less intense foci of PfOrc2 compared to the untreated parasites suggesting the possibility of the presence of other PfOrc2 binding sites that are independent of Actin I assembly. Therefore, this evidence alludes to a possible perinuclear localization of Orc2 with some specific role that needs further investigation.

4. Discussion

The known paradigm for protein trafficking through the secretory pathway is that the protein travels through the ER and Golgi and then gets transported outside of the cell or to different organelles other than the nucleus. Although there are several reports of deviation from the accepted paradigm and proteins have been reported to undertake unconventional routes [41], there is no report till date where a protein is constitutively trafficked to the nucleus en route the ER-Golgi. The malarial parasite itself has lot of exceptions with respect to the secretory pathway [3] but neither it nor any other apicomplexan has been reported to have such an unconventional trafficking pathway.

The entry of any protein into endoplasmic reticulum requires a canonical ER-targeting sequence. We could not find any such sequence in PfOrc2 by conventional signal sequence prediction tools like SignalP, PrediSi or SignalBlast. It is possible that there is a cryptic ER targeting signal at the N-terminus of PfOrc2 which needs to be characterized further. A schematic model explains the details of the pathway followed by Orc2 in *P. falciparum* (Fig. 9). The full-length protein appears to be associated with the ER (as shown in Figs. 3C, 6A) and is subsequently processed by an SPP like enzyme that yields a stable C-terminal protein (PfOrc2C). The N-terminus of PfOrc2 is probably degraded as we do not see any fragment corresponding to N-terminus using antibodies specific to this region. The released PfOrc2C is further trafficked to the nucleus either via Golgi (because PfOrc2 is Brefeldin-A sensitive) or as a cytoplasmic fragment.

The accumulation of the PfOrc2C in the presence of BFA and full length PfOrc2 in the presence of $(Z\text{-LL})_2$-ketone (Fig. 5 and Fig. S10 respectively) indicate that PfOrc2 associates with ER for proteolytic processing thereby generating a C terminal peptide. The above two experiments also strongly suggest that PfOrc2C is indeed a processed form of PfOrc2.

It is intriguing that $(Z\text{-LL})_2$-ketone inhibits the processing of PfOrc2. Typically, the proteins entering the classical secretory pathway have an N-terminus ER targeting sequence which is cleaved by a Signal Peptidase (SP) as soon as the protein enters the ER. This cleaved N terminus is further cleaved by a Signal Peptide Peptidase (SPP) [42]. SPP plays a crucial role in the intra-erythrocytic growth of *P. falciparum* [43,44]. In the absence of a bonafide signal peptide in PfOrc2, it is not clear at this point how $(Z\text{-LL})_2$-ketone inhibits the processing of PfOrc2. However, the accumulation of the full-length protein with concomitant decrease of the processed form in the presence of the above inhibitor clearly suggest the role of an SPP like enzyme in the processing of PfOrc2.

There are reports of membrane bound transcription factors/enzymes/proteins such as SREBP, IRE1, and ATF6 [7,36] which are maintained as membrane bound forms in the ER as inactive precursors and are processed and released from the ER only when specifically required. These active forms enter the nucleus through the nuclear pore and bind to their target gene response elements. Interestingly, such transcription factors (like IRE-1, ATF6) are absent in the *Plasmodium* genome [45,46] and it has a simplified ER associated degradation pathway (ERAD) which plays a crucial role in unfolded protein response (UPR) [47]. Recently ER stress has been reported to trigger gametocytogenesis in *P. falciparum* by inducing AP2 transcription factors [48] which indicate that ER can affect gene expression under specific circumstances.

Our work sheds important light on the function of ER in trafficking proteins to the nucleus in apicomplexans in general, where no such membrane bound transcription factor has been reported earlier. Transcription factors/enzymes/nuclear proteins have a temporal mode of functioning and play a very crucial role in the overall physiology of a cell e.g. DNAS1L3 is a Ca^{2+} dependent endonuclease which usually resides in the ER, but translocates into the nucleus upon apoptosis induction and degrades the chromatin [9]. However, PfOrc2 may be trafficked to the nucleus constitutively. Arguably, there may be a number of such proteins in these apicomplexans and in particular in *P. falciparum* which are required for a specific purpose and time and are residing in the ER as inactive forms. Since most of the apicomplexans are deadly pathogens and *P. falciparum* being the deadliest of them, it would be of paramount importance to study ER associated transcription factors or other effector molecules as well as this trafficking pathway in greater detail to understand the uniqueness involved in their function as

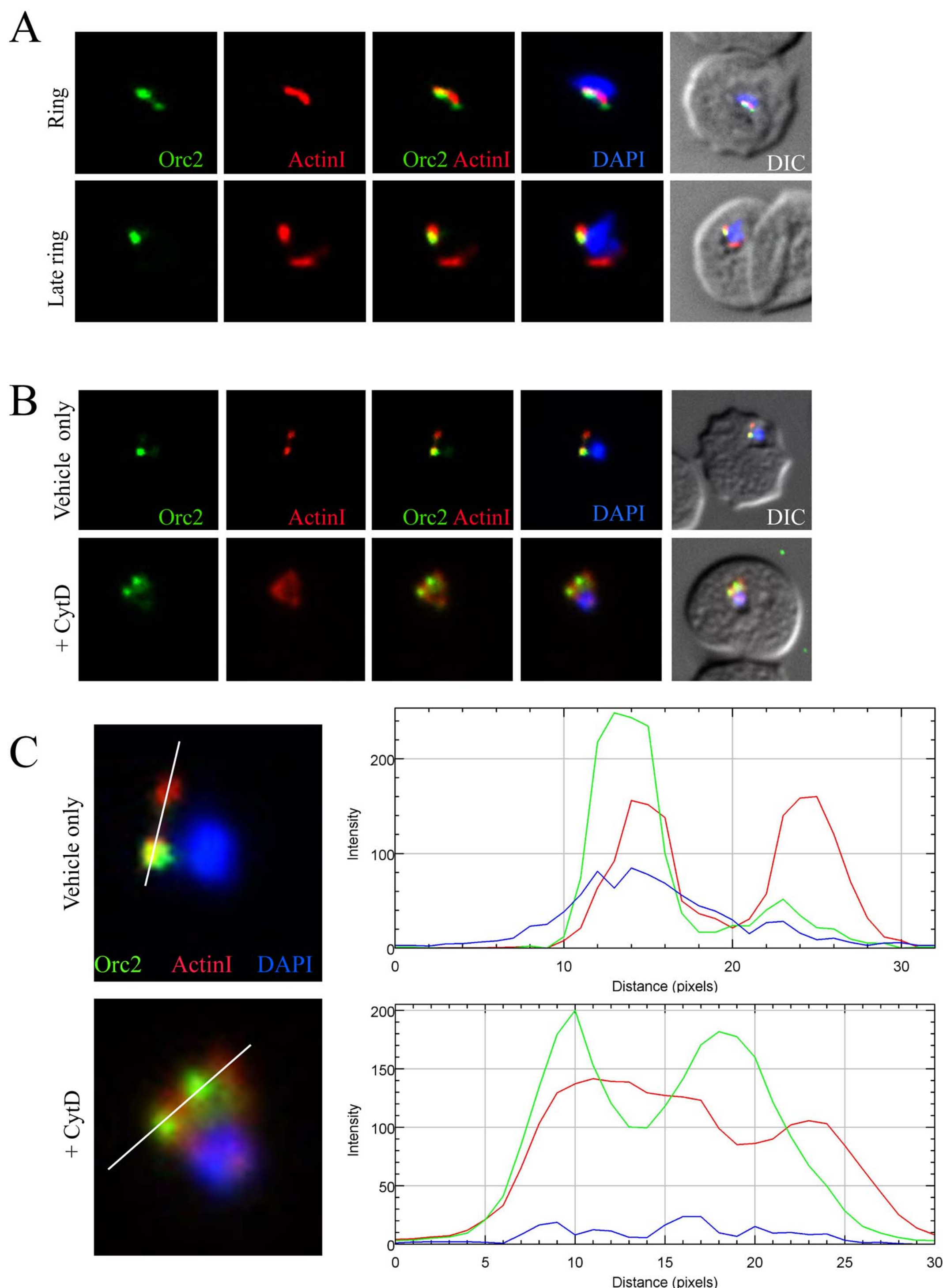


Fig. 8. Orc2 occupies perinuclear sites. (A) Co-IFA of Orc2 and Actin I shows a significant perinuclear colocalization of both the proteins. Actin I has been reported to be present at perinuclear sites. Ring stage parasites (18 ± 4 hpi) were taken for this IFA ($n > 4$). (B) Effect of CD (10 μM) on the polymerization of Actin I and subsequent effect on Orc2 localization. (C) Intensity analysis of CD treated and vehicle control IFA images using Image*J*.

well as their specific role in these deadly pathogens.

Orc2 is a part of an iconic multi subunit complex known as ORC (Origin Recognition Complex) which has been reported to play a role in DNA replication initiation in higher eukaryotes [10]. Orc2 in particular has also been shown to play a role in gene silencing [14,49]. Since it has not been reported in *P. falciparum* earlier (other than total proteome and phosphoproteome studies) [50,51], the function of the protein remains elusive in this pathogen. However, the yeast complementation assay gives us an insight on the possible role of PfOrc2 in DNA replication in the malarial parasite. Co-localization of PfOrc2C and Actin I is a unique finding with respect to an ORC protein. Since Actin I has been reported to be a marker of active *var* gene expression [39], the co-localization of Orc2 with Actin I may suggest some novel roles of Orc2 in *P. falciparum* that may include regulation of *var* gene expression.

To the best of our knowledge there are no reports of Orc2 being trafficked by such a pathway in any other biological system. It may serve as a good example of structural and functional evolution of an

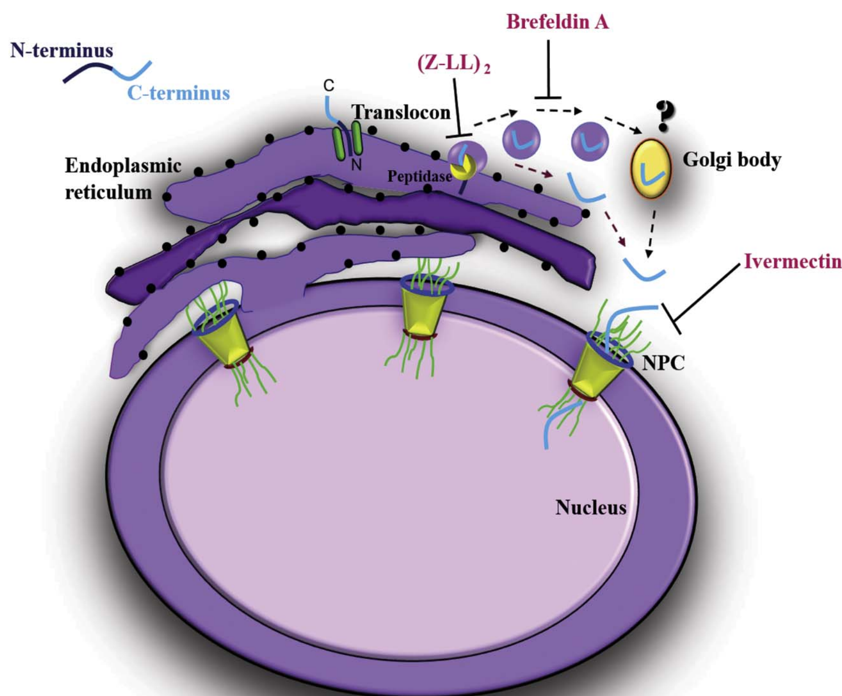


Fig. 9. Proposed model of Orc2 trafficking. The full-length protein gets cleaved by an SPP like peptidase present in the ER. The processed C-terminal fragment (PfOrc2C) might go to the Golgi by a vesicle mediated pathway which is inhibited by Brefeldin A. Alternatively, it may be released from the ER as a cytosolic fragment which then enters the nucleus through the nuclear pore by an importin dependent transport as it is Ivermectin sensitive. In the nucleus, PfOrc2C localizes to the nuclear periphery.

ORC subunit in the malaria parasite where PfOrc2 seems to have undertaken an alternative journey as compared to other ORC molecules in different systems. At this point, the exact mechanism by which the processed C-terminal fragment enters the nucleus is not known. However, several putative Nuclear Localization Signals (NLS) have been found in PfOrc2 (data not shown) which may help the transport of Orc2C into the nucleus mediated through importin α/β as mentioned in Fig. 7A. Alternatively, it may take the help of some other accessory nuclear proteins to enter the nucleus.

It is intriguing that unlike other *Plasmodium* sp., *P. falciparum* Orc2 contains putative ER retention signal although the protein is finally destined to the nucleus. This raises the issue whether the presence of ER retention signal and nuclear translocation of PfOrc2 through ER is unique for *P. falciparum*. *P. falciparum* is the most fatal form of malaria parasites which show various unique characteristics absent in other species. Therefore, it is possible that some specific function of Orc2 in *P. falciparum* has led to the incorporation of an ER retention sequence during the course of evolution.

Our work adds another dimension to the secretory pathway of the malaria parasite and this pathway resembles the trafficking of ER membrane bound transcription factors in higher eukaryotes suggesting that such a pathway might be conserved across taxa. Additionally, we show the first evidence of potential involvement of ER in some nuclear function. This unique and alternative pathway could be a new “hot spot” for investigating drug targets against malaria as specific proteins having a crucial function may be trafficked through it.

Author contributions

RS performed all the experiments except raising polyclonal antibodies against PfOrc2N and subsequent experiments (as shown in Figs. 2A, C, D, 3C, 4, 5D, 6B, S2, S3, S4A, S7A, S8 and S10 C, D performed by BS) and yeast complementation assay (Fig. 1; done by AG); and RS, SKD, BS and AG critically analysed the data. RS, BS and SKD wrote the manuscript.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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