BBA - Molecular Cell Research 1865 (2018) 817–829

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

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

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ARTICLE INFO

Keywords:
Protein trafficking
Origin recognition complex 2
Plasmodium falciparum
Classical secretory pathway
ER-nucleus traffic
Nuclear import

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

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https://doi.org/10.1016/j.bbamcr.2018.03.003
Received 15 September 2017; Received in revised form 15 February 2018; Accepted 6 March 2018
Available online 07 March 2018

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. ~5×10^6 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), $(Z\text{-}LL)_2$–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

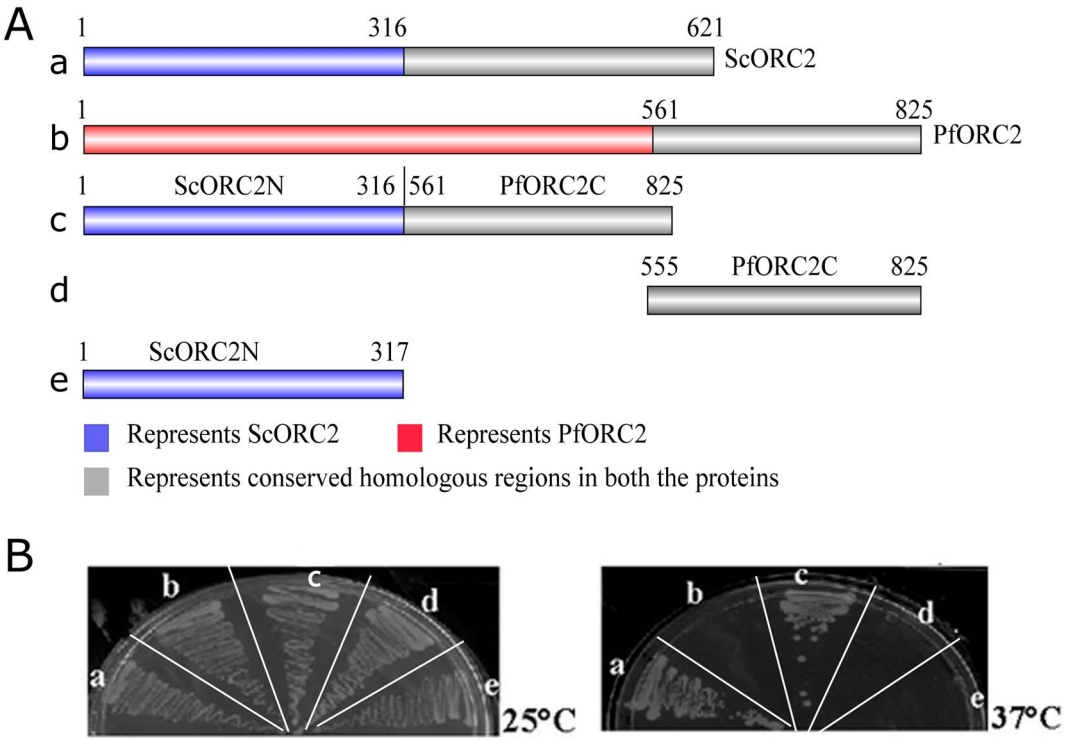


Fig. 1. Functional complementation of *Sc*Orc2 with the C-terminus of putative PfOrc2 in ScOrc2 temperature sensitive strain. (A) Different constructs (a–e) were made in yeast expression vector pRS314 having a *trp* gene selection marker and galactose inducible promoter and terminator. (B) Functional 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

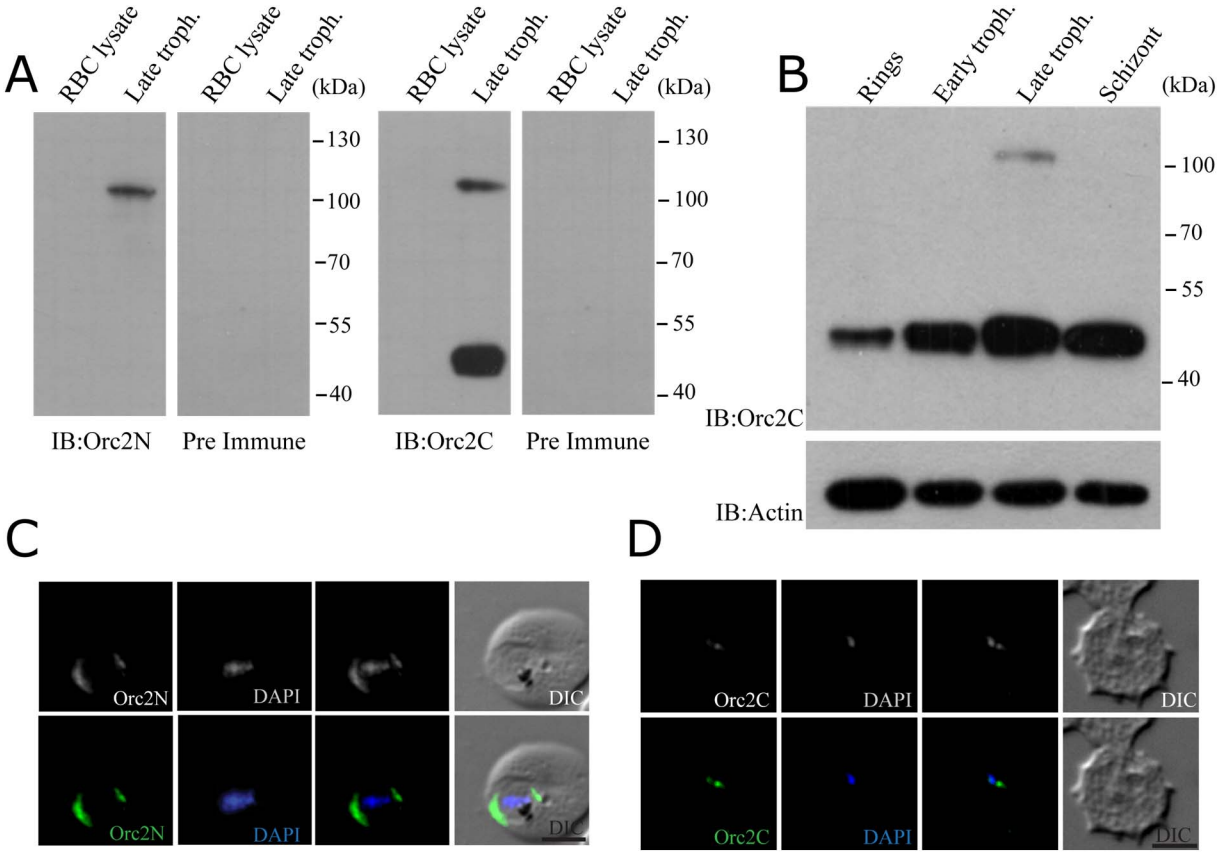


Fig. 2. Dynamics of Orc2 expression and its localization during the intra-erythrocytic developmental (IED) stages. (A) Western blot experiments using late trophozoite stage (35 ± 5 hpi) parasite lysate show the presence of full length Orc2 (~100 kDa) using antibodies against Orc2N and Orc2C respectively. 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.

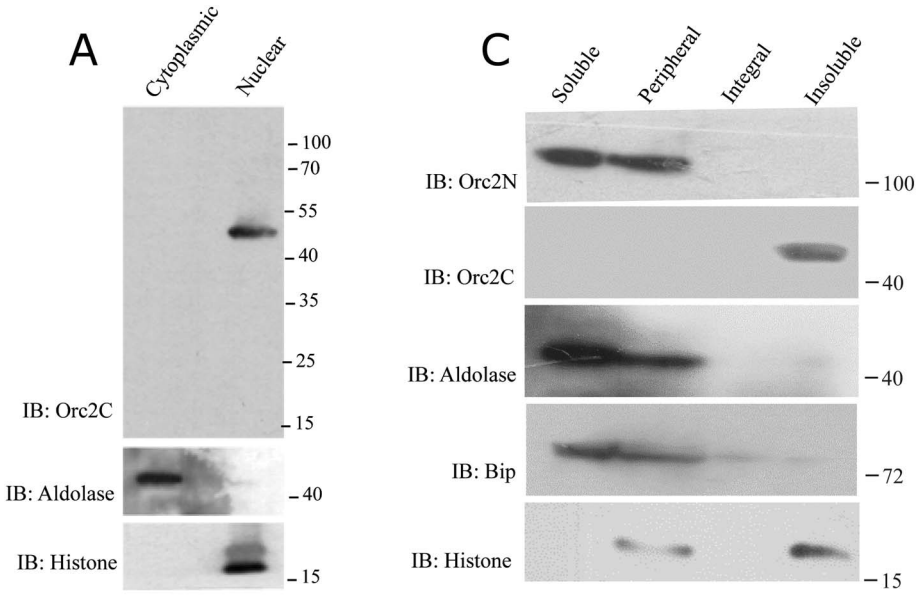
What is the fate of the N-terminal region of PfOrc2? We used the same method as mentioned above to fractionate parasite lysate and obtained the full-length protein in nuclear fraction (data not shown). This observation was contrary to the diffused IFA pattern we had obtained for PfOrc2N. Subsequently, 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.



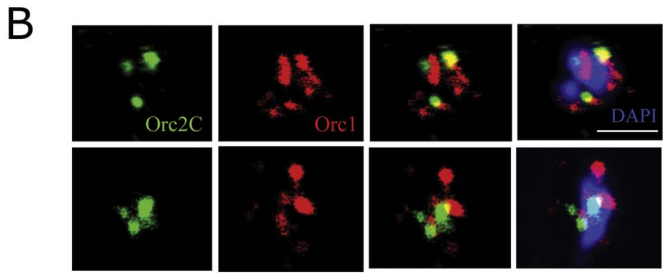


Fig. 3. Subcellular fractionation of Orc2. (A) NP-40 detergent based subcellular fractionation followed by Western blot experiment shows the nuclear localization of PfOrc2C. Aldolase was used as a cytoplasmic fraction marker and Histone H3 was used as a nuclear/chromatin fraction marker. (B) Co-IFA showing 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.

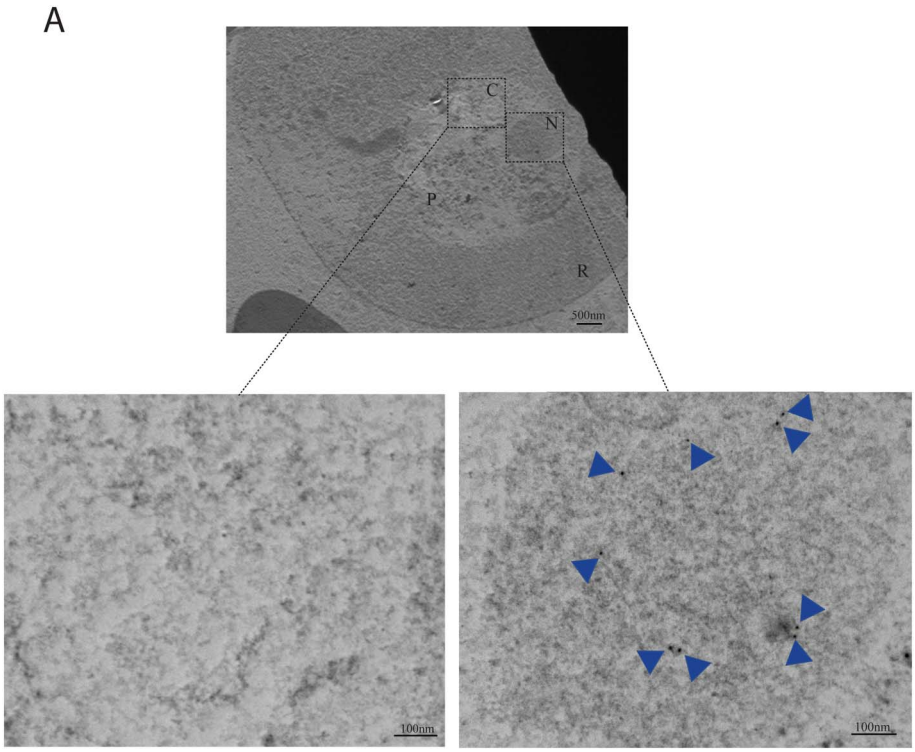


Fig. 4. Immunoelectron microscopy. IEM showing predominant nuclear localization of PfOrc2C. (Gold particles visible as uniform dark black dots have been marked with blue arrowheads, scale bar is indicated; C-cytoplasm, N-nucleus, P-parasite, R-RBC).

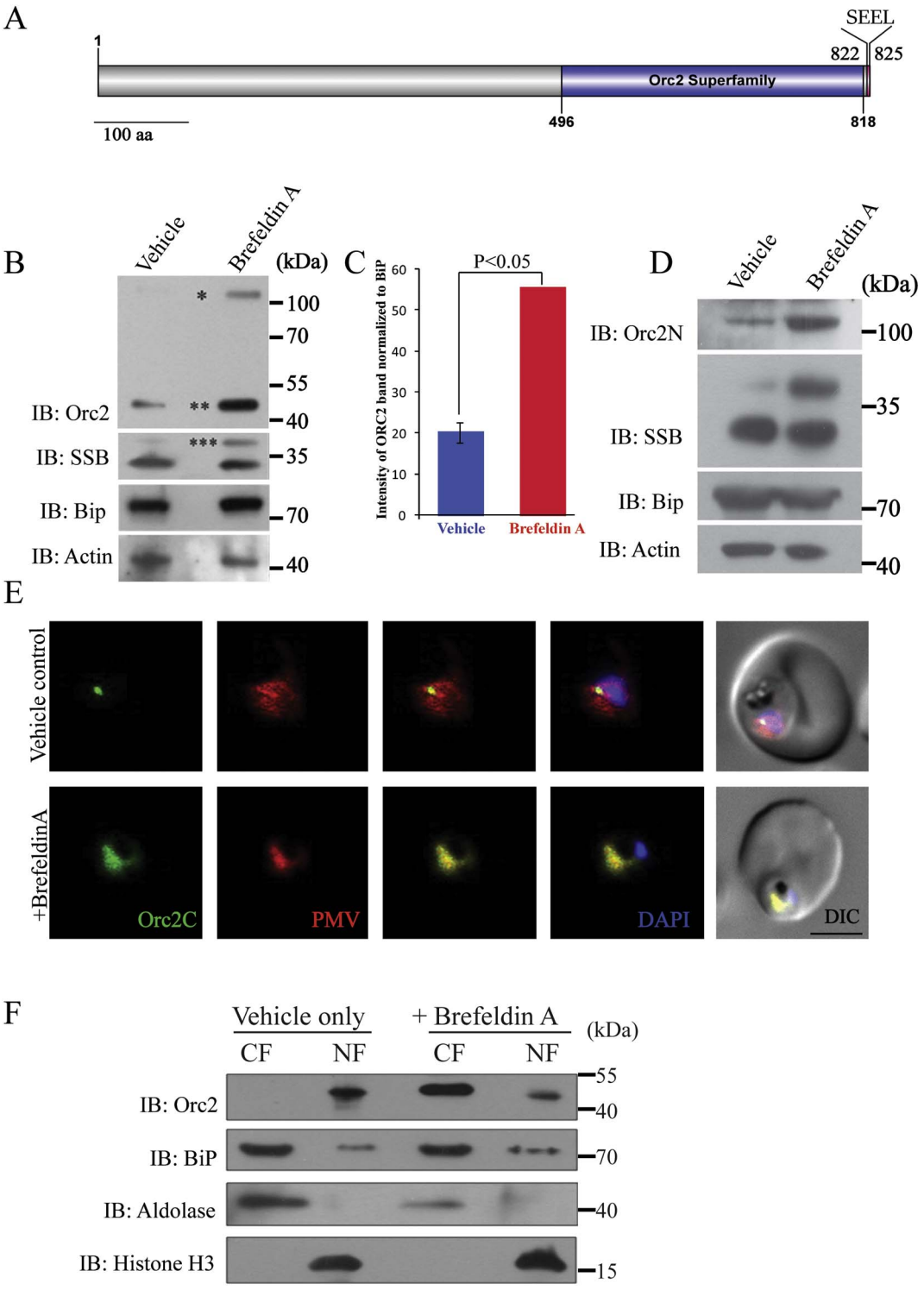


Fig. 5. Orc2 gets trafficked to the nucleus through the classical secretory pathway (A) Schematic representation of the key sequences present in PfOrc2 i.e. Orc2 superfamily domain and ER retention sequence. (B) Brefeldin A (BFA) treatment resulted in an increase in the intensity of the lower band (~45 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

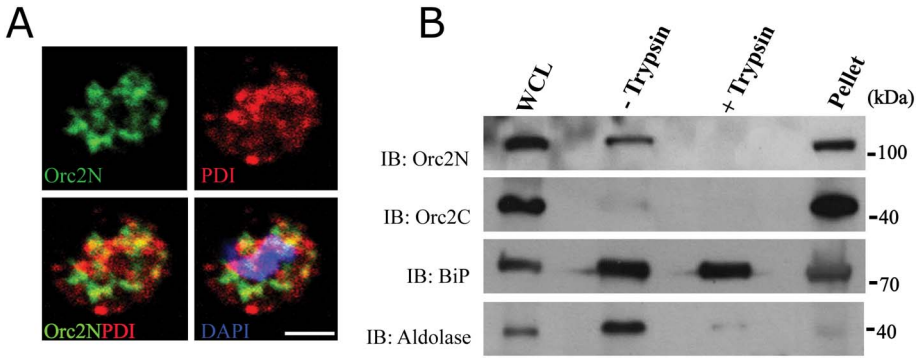


Fig. 6. Full length PfOrc2 protein is associated with the ER. (A) IFA showing the co-localization of PfOrc2N and an ER marker PDI. This indicates that the full-length PfOrc2 is associated with the ER. (B) Gentle homogenization and centrifugation of saponin lysed parasites renders the full-length PfOrc2 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

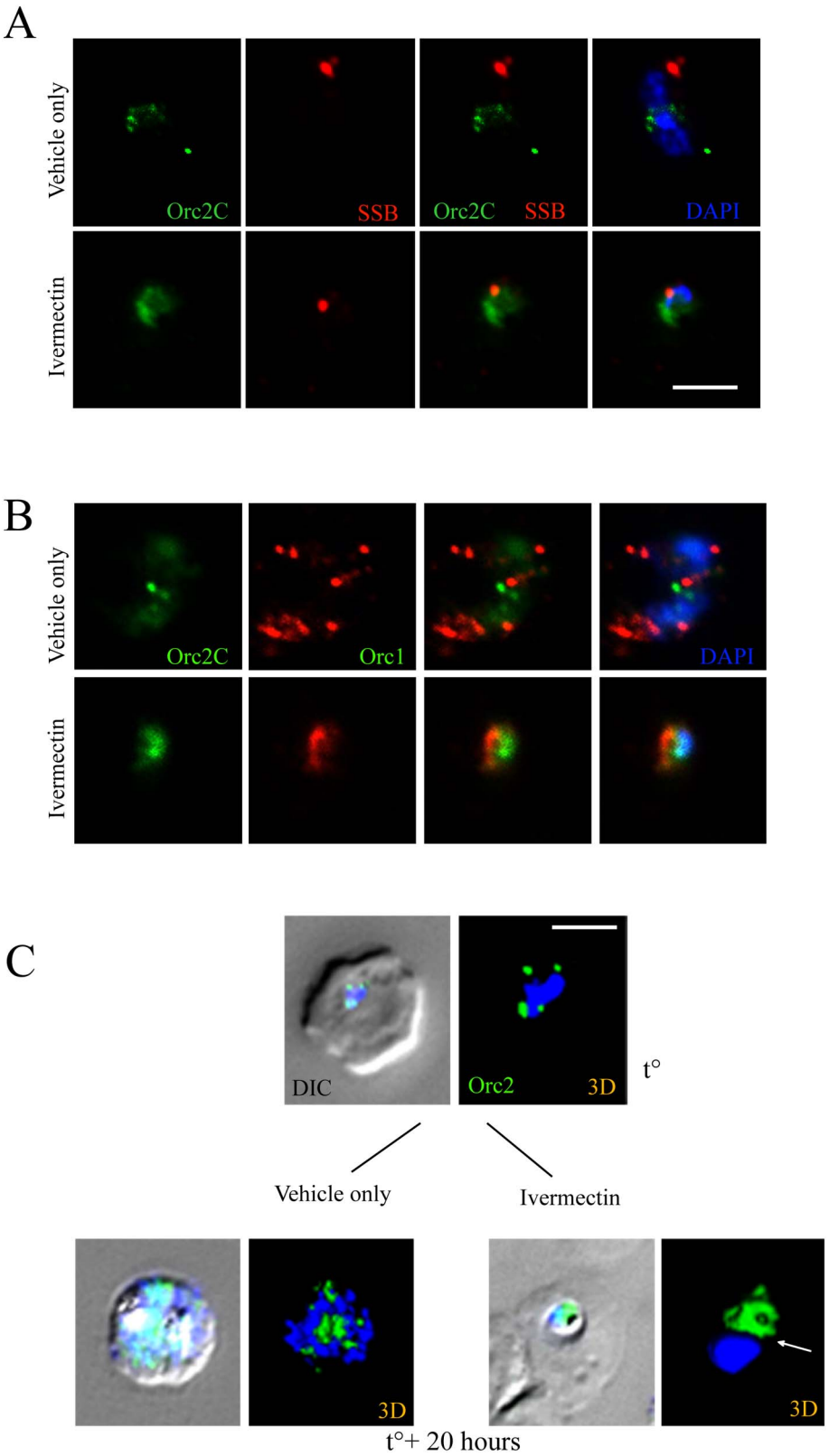


Fig. 7. Orc2 goes to the nucleus through the nuclear pore. (A) Orc2 showed a diffused pattern upon Ivermectin treatment. Ring (18 ± 4 hpi) parasites were incubated with 10 μM of Ivermectin along with DMSO as a vehicle control for 20 h. SSB was used as a non-nuclear negative control. (B) Orc1 as a positive 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

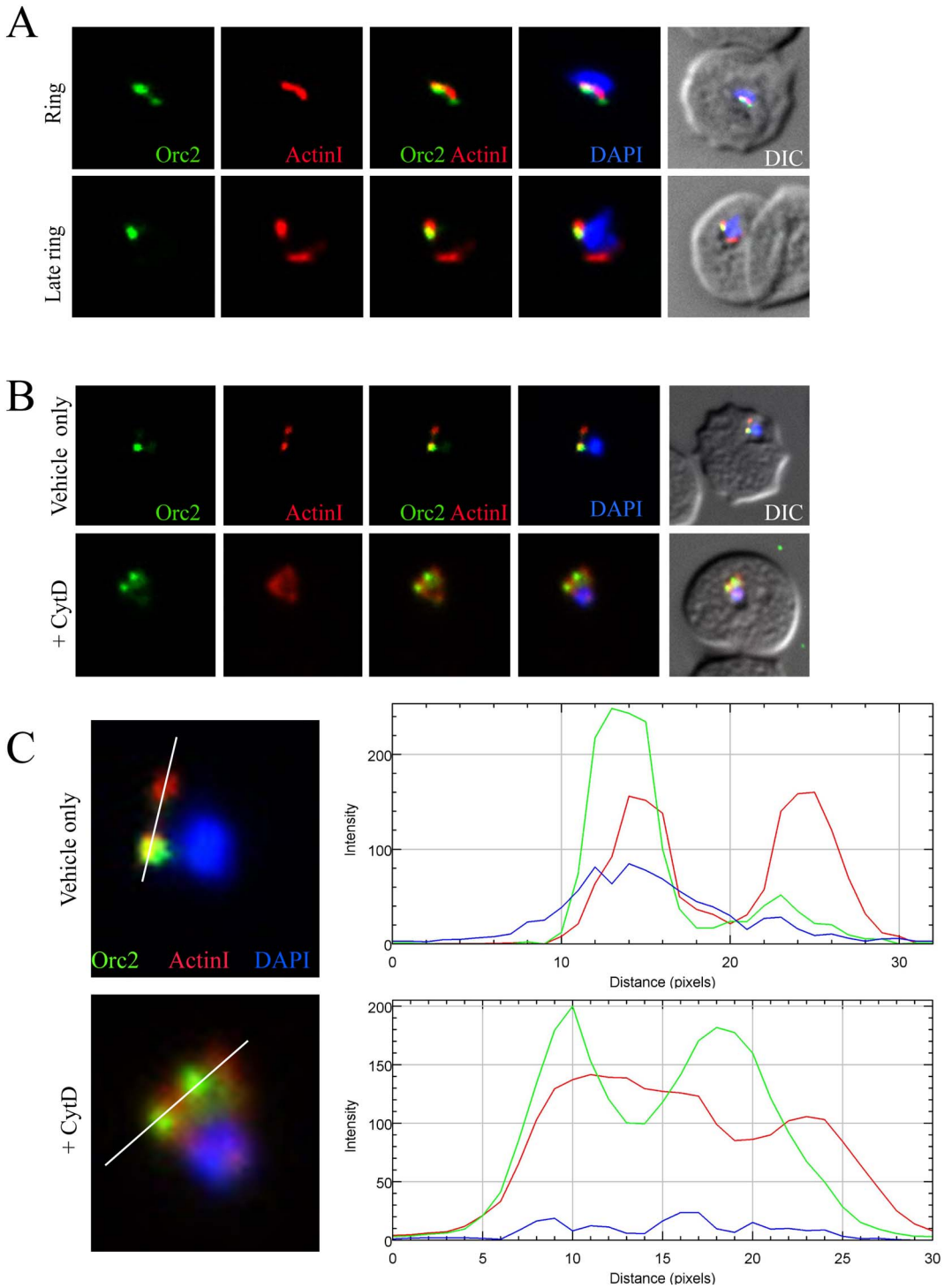


Fig. 8. Orc2 occupies perinuclear sites. (A) Co-IFA of Orc2 and Actin I shows a significant perinuclear colocalization of both the proteins. Actin I has been reported to be present at perinuclear sites. Ring stage parasites (18 ± 4 hpi) were taken for this IFA ($n > 4$). (B) Effect of CD (10 μM) on the 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

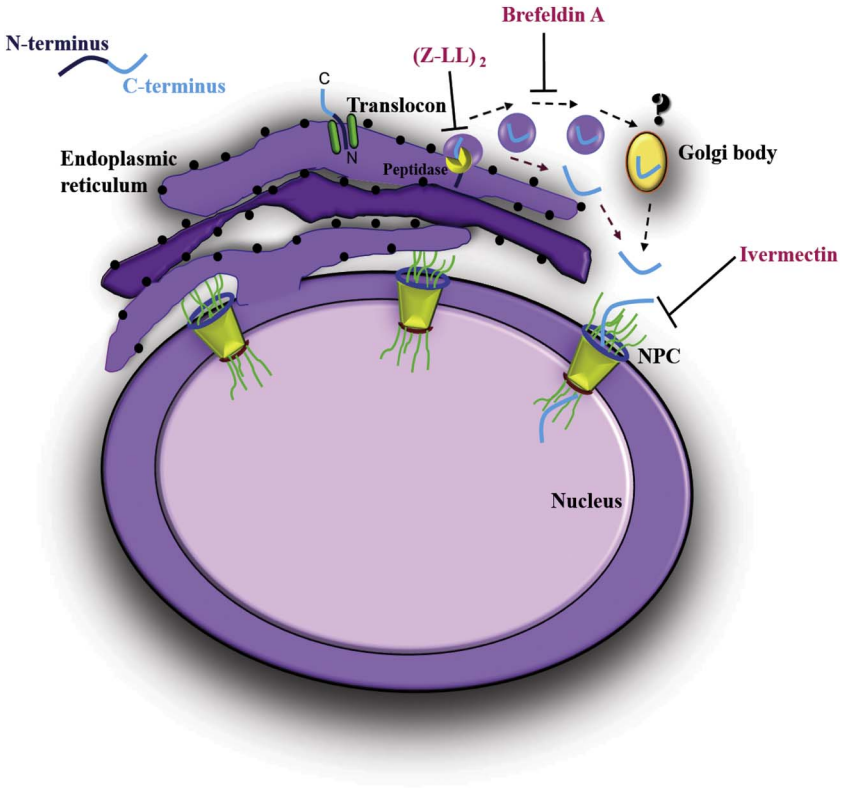


Fig. 9. Proposed model of Orc2 trafficking. The full-length protein gets cleaved by an SPP like peptidase present in the ER. The processed C-terminal fragment (PfOrc2C) might go to the Golgi by a vesicle mediated pathway which is inhibited by Brefeldin A. Alternatively, it may be released from the ER 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.

Acknowledgements

The authors sincerely thank Prof. Daniel E Goldberg (Washington University), Prof. John H Adams (USF), Prof. Philippe Grellier (CNRS, France) for Plasmepsin V, BiP and Protein Disulphide Isomerase (PDI) antibodies respectively. We also thank Dr. Meetu Agarwal for providing antibodies against PfOrc1 (mice). Electron-microscopy facility at AIIMS, New Delhi and Dr. Gajender Saini, AIRF, JNU are acknowledged or EM sample processing and imaging. For Mass Spectrometry based experiments, we sincerely acknowledge Dr. Rajesh Soni, Prof. Jayanta Chaudhuri, Dr. Ronald C. Hendrickson (Memorial Sloan Kettering Cancer Center, NY) and Dr. Inderjeet Kaur (ICGEB, New Delhi). We also thank Prof. Kirk W Deitsch (Weill Cornell Medical College) for providing synchronized schizont 3D7 pellets for mass spectrometry experiment. We also thank Dr. Souvik Bhattacharjee (JNU) for his help and suggestions. This work is supported by the Centre of Excellence (CoE) in Parasitology grant funded by Dept. of Biotechnology, Govt. of India (BT/01/CEIB/11/V/08). The authors acknowledge UGC-SAP, DST-PURSE, ICMR core funding (63/9/2010-BMS) and UPE-II funding (ID no. 28) awarded to JNU for financial support. RS acknowledges CSIR, India and BS acknowledges ICMR, India for fellowships.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2018.03.003.

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