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Regulation of *Plasmodium falciparum* Origin Recognition Complex subunit 1 (PfORC1) function through phosphorylation mediated by CDK-like kinase PK5

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Summary

***Plasmodium falciparum* Origin Recognition Complex subunit 1 (PfORC1) has been implicated in DNA replication and *var* gene regulation. While the C-terminus is involved in DNA replication, the specific role of N-terminus has been suggested in *var* gene regulation in a Sir2-dependent manner. PfORC1 is localized at the nuclear periphery, where the clustering of chromosomal ends at the early stage of parasite development may be crucial for the regulation of subtelomeric *var* gene expression. Upon disassembly of telomeric clusters at later stages of parasite development, ORC1 is distributed in the nucleus and parasite cytoplasm where it may be required for its other cellular functions including DNA replication. The level of ORC1 decreases dramatically at the late schizont stage. The mechanisms that mediate regulation of PfORC1 function are largely unknown. Here we show, by the use of recombinant proteins and of transgenic parasites expressing wild type or mutant forms of ORC1, that phosphorylation of the PfORC1-N terminal domain by the cyclin-dependent kinase (CDK) PfPK5 abolishes DNA-binding activity and leads to changes in subcellular localization and proteasome-mediated degradation of the protein in schizonts. These results reveal that PfORC1 phosphorylation by a CDK is central to the regulation of important biological functions like DNA replication and *var* gene silencing.**

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Introduction

The eukaryotic hetero-hexameric Origin Recognition Complex (ORC) binds to DNA replication origins to trigger the formation of the pre-replicative complex (pre-RC) and the initiation of DNA replication (Bell and Dutta, 2002). In each cell cycle, DNA replication occurs only once, and this is ensured by regulation of pre-RC components (Feng and Kipreos, 2003; Woo and Poon, 2003; DePamphilis, 2005). ORC subunits are regulated in a cell cycle dependent manner through phosphorylation and ubiquitination (Feng and Kipreos, 2003; Woo and Poon, 2003; DePamphilis, 2005). In metazoans, cycA/Cdk1 phosphorylates multiple components of the pre-RC, including ORC1. *Saccharomyces cerevisiae* ORC1, ORC2 and ORC6 subunits contain consensus sequences [(S/T)PX(K/R)] for phosphorylation by cyclin-dependent kinases (CDKs) (Findeisen *et al.*, 1999; Laman *et al.*, 2001; Remus *et al.*, 2005; Chen and Bell, 2011). Mutations in the CDK phosphorylation sites resulted in re-replication (Vas *et al.*, 2001), delayed S phase progression (Makise *et al.*, 2009) and impaired loading of Chromatin licensing and DNA replication factor 1 (CDT1) and minichromosome maintenance (MCM) proteins 2–7 onto the origin of replication (Chen and Bell, 2011). In mammalian cells, ORC1 is hyperphosphorylated by cycA/Cdk1 during the G_2 to M phase, which leads to a decrease in the stability of its association with chromatin during M phase. Subsequently, ORC1 is dephosphorylated during the M-to- G1 transition (Li *et al.*, 2004). In *Xenopus* egg extracts, ORC1 is underphosphorylated in interphase, but is phosphorylated by cycA/Cdk2 in S-phase, and is hyperphosphorylated in M phase that inhibits its binding to chromatin (Findeisen *et al.*, 1999).

CDKs are Ser/Thr kinases whose activation depends on heterodimerization with cognate cyclins (Morgan, 1997). Initially, cdc2 (now called Cdk1) was identified in complex with cyclin B as a regulatory factor. Subsequently, many more kinases and their cyclin partners were identified (Pines, 1993). In the human malaria parasite *Plasmodium*

falciparum, several cell cycle regulators, including cyclins and CDKs, have been described (Doerig *et al*., 2008).

During its 48 h intra-erythrocytic developmental cycle (IDC), *P. falciparum* matures through the ring, trophozoite and schizont stages. The ring stage corresponds to the G1 phase; S phase (DNA replication) is initiated at the trophozoite stage around 24 h post invasion and is followed by multiple rounds of asynchronous nuclear division in the absence of cytokinesis, resulting in a multinucleated schizont (Leete and Rubin, 1996). Neither distinct phases of the *P. falciparum* cell cycle nor the molecular mechanisms that underlie the regulation of this atypical cell cycle have been defined. Furthermore, the substrates of the CDKs, and their cognate cyclin partner(s), are unknown, and how CDKs regulate the malarial life cycle remains an open question.

In *P. falciparum*, six CDK-related kinases have been characterized so far: PfPK5 (Le Roch *et al*., 2000), PfPK6 (Bracchi-Ricard *et al*., 2000), Pfmrk (Li *et al*., 1996), PfCrk-1 (Halbert *et al*., 2010), PfCrk-3 (Halbert *et al*., 2010) and PfCrk-5 (Dorin-Semblat *et al*., 2013). Furthermore, four cyclin homologues, Pfcyc1, 2, 3 and 4, have been identified in the parasite genome (Le Roch *et al*., 2000; Merckx *et al*., 2003). PfPK5 is the only plasmodial kinase that clusters with mammalian cell cycle CDKs in phylogenetic trees, whereas PfCrk-1 and PfCrk-3 show similarity with the transcriptional CDKs (Ward *et al*., 2004; Halbert *et al*., 2010). Pfmrk is a putative homologue of mammalian CDK7, which activates other CDKs by phosphorylating their activation loop (Li *et al*., 1996). Cyclin-dependent kinase activity of PfPK5 and Pfmrk has been demonstrated (Le Roch *et al*., 2000; Jirage *et al*., 2010), and a role for PfPK5 in DNA replication has been proposed, initially based on the fact that it shows nuclear localization at the time of the onset of DNA synthesis (Ross-Macdonald *et al*., 1994; Graeser *et al*., 1996a). A possible role in S phase is further suggested by the observations that (i) parasites treated with the DNA synthesis inhibitor aphidicolin display elevated PfPK5 kinase activity, and (ii) treatment with the CDK inhibitors flavopiridol and olomucine results in an impairment of DNA synthesis (Graeser *et al*., 1996b). Pfmrk has also been implicated in DNA replication, as it colocalizes with DNA and phosphorylates Replication factor C subunit 5 (PfRFC-5) and PfMCM6, both of which are components of the DNA replication machinery (Jirage *et al*., 2010). Apart from the above-mentioned four classical-looking CDKs, the *P. falciparum* kinome contains two CDK-related genes that have no orthologue in the mammalian kinome. These are PfPK6 and PfCrk-5, which display cyclin-independent and cyclin-dependent kinase activity *in vitro* respectively (Bracchi-Ricard *et al*., 2000; Dorin-Semblat *et al*., 2013). PfCrk-5 deficient parasites display a slow parasitemia growth rate caused by a lower average number of daughter merozoites produced per schizont, exactly mimicking the phenotype of parasite lacking the atypical kinase PfPK7 (Dorin-Semblat *et al*., 2008; 2013).

We previously identified three structural domains in *P. falciparum* ORC1: a unique N terminal domain rich in low complexity regions; an ATP-binding domain of the AAA^+ type in the C-terminal half of the protein; and a potential winged-helix (WH) DNA binding domain at the extreme C-terminus (Mehra *et al*., 2005). The C-terminus of ORC1 is involved in DNA replication (Gupta *et al*., 2009), whereas the N-terminus is targeted to the nuclear periphery where chromosome ends cluster, suggesting a possible role in the regulation of *var* gene expression (Deshmukh *et al*., 2012).

Plasmodium falciparum chromosome ends form four to seven telomeric clusters at the nuclear periphery (Freitas-Junior *et al*., 2000). Telomere-associated proteins, such as PfORC1 (Mancio-Silva *et al*., 2008; Deshmukh *et al*., 2012), Sir2 (Mancio-Silva *et al*., 2008) and HP1 (Perez-Toledo *et al*., 2009), may have significant role in the regulation of subtelomeric *var* genes. During ring stage (G1 phase), Sir2 and PfORC1 bind to telomeric and subtelomeric repeat regions. Prior to DNA replication (trophozoite stages, S phase), telomere clusters disassemble with concomitant redistribution of Sir2 and PfORC1 in the nucleus and parasite cytoplasm (Mancio-Silva *et al*., 2008). Sir2 has been shown to be sumoylated (Issar *et al*., 2008), a modification that may play role in the redistribution of this protein. The redistribution of ORC1 during the transition from ring to trophozoite stage could possibly be associated with relocation to replication initiation sites that may trigger DNA replication. Following DNA replication, the level of ORC1 is drastically reduced due to its degradation by the proteasome, as suggested by the observation that treatment of the parasite with the proteasome inhibitor MG132 stabilizes ORC1 at the schizont stage (Gupta *et al*., 2008).

It is thus clear that the level and activity of ORC1 are highly regulated during erythrocytic developmental stages. Interestingly, two putative CDK phosphorylation sites (TPKK and SPTK, respectively) are present at the very N-terminus of PfORC1, suggesting phosphorylation may contribute to the regulation of ORC1 function.

We therefore hypothesized that the redistribution of ORC1 and modulation of its expression level during different stages of erythrocytic development requires post-translational modifications (PTMs) of the protein.

Here, we show that the N-terminus of PfORC1 (PfORC1N) can indeed be phosphorylated *in vitro* by human cycA/Cdk2 and cycE/Cdk2, as well as by PfPK5 in the presence of Ringo, a CDK activator previously shown to stimulate PfPK5 activity (Merckx *et al*., 2003). Phosphorylation takes place specifically at the putative CDK phosphorylation sites present at the N terminus, as deletion or

mutation of these sites completely abrogates phosphorylation *in vitro*. PfORC1N can also be phosphorylated by parasite lysate, and immunodepletion of PfPK5 from parasite lysate drastically affects *in vitro* phosphorylation, suggesting the possibility of PfPK5 being the cognate kinase for PfORC1. Phosphorylation of PfORC1 at its N-terminus was further confirmed by generating parasite lines expressing GFP tagged wild type or phosphorylation site mutant forms of PfORC1N. Phosphorylation of PfORC1N at the late schizont stage coincides with the change of subcellular localization of PfORC1, its degradation as well as its reduced occupancy at the telomere-associated repeat regions (TAREs) and *var* gene promoters compared with early trophozoite stage parasites. A reduced affinity of phosphorylated PfORC1 for DNA was further validated by gel shift assay *in vitro*.

Together, these results clearly suggest that the regulation of some important functions of PfORC1 is mediated by phosphorylation and establish PfPK5 as the kinase involved in this process.

Results

ORC1 relocates during IDC

Indirect immunofluorescence assays were performed to define the localization of PfORC1 in 3D7 parasites from ring, trophozoite and schizont stage. We found that PfORC1 underwent dynamic localization throughout the 48 h blood stage cycle. It localized predominantly to distinct perinuclear compartments during the late ring/early-to-mid trophozoite stage (~ 18–28 h post invasion), whereas it was distributed throughout the nucleus and cytoplasm during the early schizont stages (~ 38–40 h post invasion). In the very late schizont stages (~ 46 h post invasion), it was mainly present in the cytoplasm, with a drastic reduction in the overall PfORC1 signal, suggesting possible degradation of the protein (Fig. 1A). Overall, the localization pattern changed from well-defined perinuclear spots/foci during early stages of development to a rather more diffused pattern in schizonts. Western blot analysis using antibodies against PfORC1 (and antibodies against PfHSP70 as a loading control) followed by densitometric analysis showed increased expression of PfORC1 from ring to schizont stage (18–40 h post invasion) and a dramatic decrease in the late schizont stage (~ 46 h post invasion) (Fig. 1B and C). The stage-specific western blot experiment was reproduced a few times, and similar results were obtained (data not shown). The subcellular localization of ORC1 was further investigated by western blot analysis of cytoplasmic and nuclear fractions. Consistent with immunofluorescence data (Fig. 1A), PfORC1 was detected mostly in the nuclear fraction of trophozoites (~ 28 h post invasion) and in the cytoplasmic fraction of schizonts (~ 40 h post invasion) (Fig. 1D). The change in subcellular localization of ORC1 in the trophozoite–schizont transition and the drastic reduction of ORC1 levels at the late stages of the IDC suggest that PfORC1 may possibly be regulated through PTMs. We searched for different potential PTMs in the PfORC1 sequence manually and by using bioinformatics tools, and found two putative CDK phosphorylation sites (TPKK and SPTK) at the extreme N-terminus (positions 2–5 and 20–23) (Fig. S1). Moreover, two motifs shown to be important for protein degradation through the proteasome pathway, DEAD and KEN, were also found in the PfORC1 (Fig. S1). Thus, bioinformatics analyses predict that redistribution of PfORC1 during the IDC may take place through PTMs.

The ORC1 N-terminus (ORC1N) is phosphorylated on residues Thr2 and Ser20

Cdc2 phosphorylation sites [(S/T)PX(K/R)] (Chen and Bell, 2011) can be found in ORC1 homologues from fission yeast, budding yeast, *Drosophila melanogaster*, *Xenopus* and mammals. We therefore investigated whether two putative CDK phosphorylation sites at the PfORC1 amino-terminus were a target for CDKs *in vitro*. For that purpose, we generated the following GST-tagged fusion proteins: $ORC1N_{1–164}$, $ORC1N_{16–164}$ (the extreme CDK phosphorylation site deleted) and $ORC1N_{25–164}$ (both CDK phosphorylation sites deleted) (Fig. 2A). The activity of co-purified human cycA/Cdk2 and cycE/Cdk2 (Fig. 2B) was assessed using histone H1 as a substrate. Although both kinases could phosphorylate histone H1, hcycA/Cdk2 phosphorylated histone H1 more efficiently than hcycE/Cdk2 in our experimental conditions (Fig. 2C). *In vitro* kinase assays indicated that $ORC1_{1–164}$, and $ORC1_{16–164}$, but not $ORC1_{25–164}$, could be phosphorylated by hcycA/Cdk2 (Fig. 2D). Under similar experimental conditions, hcycE/Cdk2 phosphorylates $ORC1_{1–164}$ robustly but not the $ORC1_{25–164}$ (Fig. 2E). Moreover, $ORC1_{16–164}$ (with only one putative CDK phosphorylation site) showed reduced phosphorylation compared with $ORC1_{1–164}$ (with two CDK phosphorylation sites), indicating that ORC1N could be phosphorylated on both CDK phosphorylation sites (Fig. 2D). In short, $ORC1_{1–164}$ is clearly a substrate for both human S phase cyclin/Cdk complexes (cycA/Cdk2 and cycE/Cdk2).

The specificity of phosphorylation was verified by substituting T2 and S20 with alanine residues (T2A and S20A) in ORC1N. The substitution of the threonine and serine resulted in protein ($ORC1_{1-164T2AS20A}$) that did not undergo *in vitro* phosphorylation with hcycA/Cdk2 (Fig. S2A). Both substrate-dependent and kinase-dependent phosphorylation of $ORC1N_{1–164}$ further showed the specificity of kinase assays (Fig. S2B and C). Our results are corroborated by

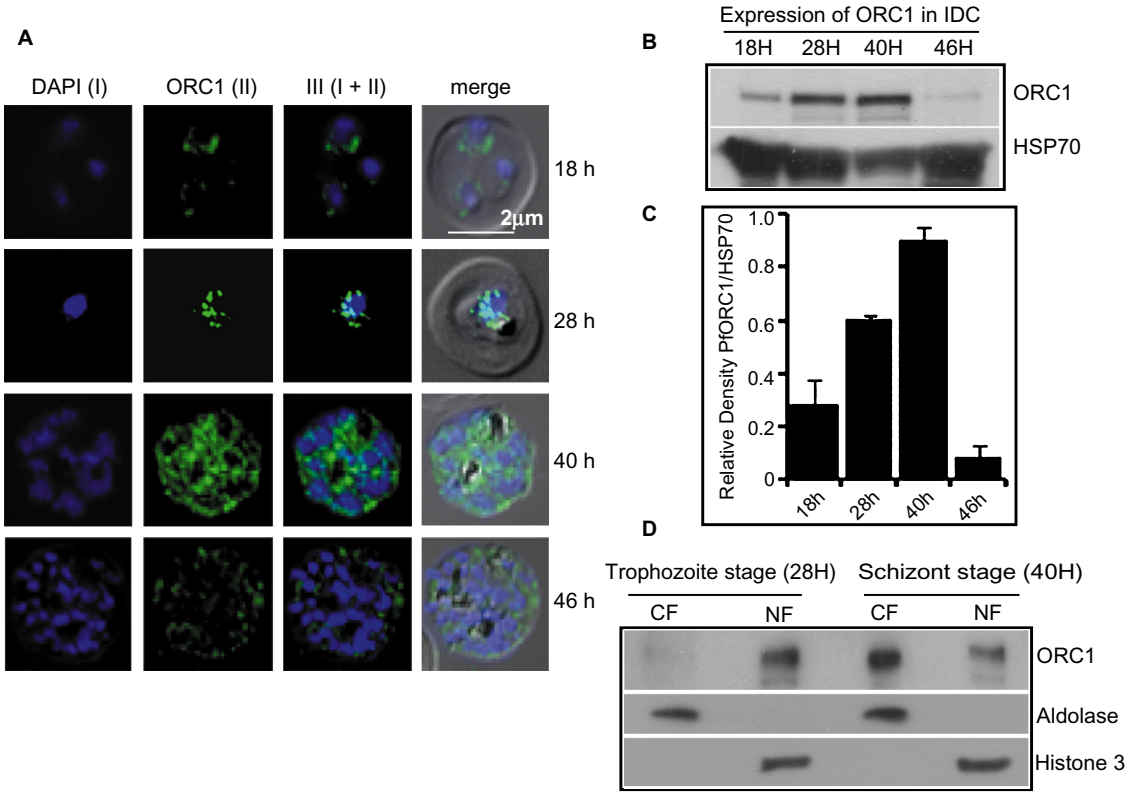


Fig. 1. Endogenous ORC1 is redistributed during blood-stage asexual cycle.
A. Immunofluorescence analysis using antibodies against PfORC1 reveals punctate pattern at the nuclear periphery in the ring stage (~ 18 h) and early-to-mid trophozoite stage (~ 28 h). At a later stage (~ 40 h), an apparent increase of protein level and diffused pattern in the cytoplasm is observed, whereas during very late schizont (~ 46 h), the PfORC1 signal decreases drastically.
B. Western blot analysis of ring, trophozoite and schizont lysates (the approximate age of the parasites have been indicated on the top) using antibodies against PfORC1. PfHSP70 was used as loading control.
C. Densitometric analysis of the PfORC1 and PfHSP70 signals as shown in (B). The band intensities of PfORC1 and PfHSP70 were calculated using ImageJ software from three exposures of an experiment and plotted accordingly using HSP70 as a control. Densitometry values represent the ratio of PfORC1/PfHSP70 band intensities for the respective stages of the parasite. Error bars represent the standard errors of the mean. These results indicate that the level of PfORC1 is enhanced during the trophozoite stage but falls drastically in late schizonts.
D. Subcellular localization of PfORC1 in trophozoites and schizonts. Parasite lysates were fractionated into cytoplasmic (CF) and nuclear (NF) samples and analyzed by western blot analysis using antibodies against PfORC1. PfORC1 was found mostly in the nuclear fraction in trophozoites, whereas the majority of PfORC1 is found in the cytoplasmic fraction in the schizont stage. Aldolase (a cytoplasmic protein) and Histone 3 (a nuclear protein) were used as markers for fractionation. These results indicate change in subcellular localization of ORC1 from nucleus to cytoplasm during parasite development.

available mass spectrometry data showing phosphorylation of PfORC1 at the S20 position in schizont (Treeck *et al*., 2011).

To test whether ORC1 is a substrate of *Plasmodium* kinase(s), we investigated ORC1N phosphorylation in the presence of two plasmodial CDKs, PfCrk-5 and PfPK5 respectively. The activity of GST-PfCrk-5 was tested in the presence of Pfcyc-1 and Pfcyc-4, the two plasmodial cyclins shown to be able to activate PfCrk-5 (Dorin-Semblat *et al*., 2013); and the activity of PfPK5 was mostly assayed in the presence of Ringo (a protein with no sequence homology to cyclins but that is nevertheless a strong activator of mammalian CDK1/2) as this protein was identified as the most potent activator of PfPK5 *in vitro* (Merckx *et al*., 2003). The purification profile of plasmodial cyclins, plasmodial CDKs and activator protein RINGO has been shown in the supplemental section (Fig. S3). After we verified that Pfcyc1/PfCrk-5, Pfcyc4/PfCrk-5 and PfPK5/RINGO were active against mammalian histone H1 as expected (Fig. 2F and G), these kinase complexes were further tested in *in vitro* kinase assays using ORC1N as a substrate. Activated hcycA/Cdk2 but not PfCrk-5 (in the presence of Pfcyc1 or Pfcyc4) was able to phosphorylate $ORC1_{1–164}$ (Fig. 2H). However, Ringo/PfPK5 was able to phosphorylate $ORC1_{1–164}$, but not $ORC1_{25–164}$ (Fig. 2I).

We have also used different cyclin combinations (Pfcyc1-4) for the activation of PfPK5 using histone and PfORC1N as substrates. Although we could see moderate

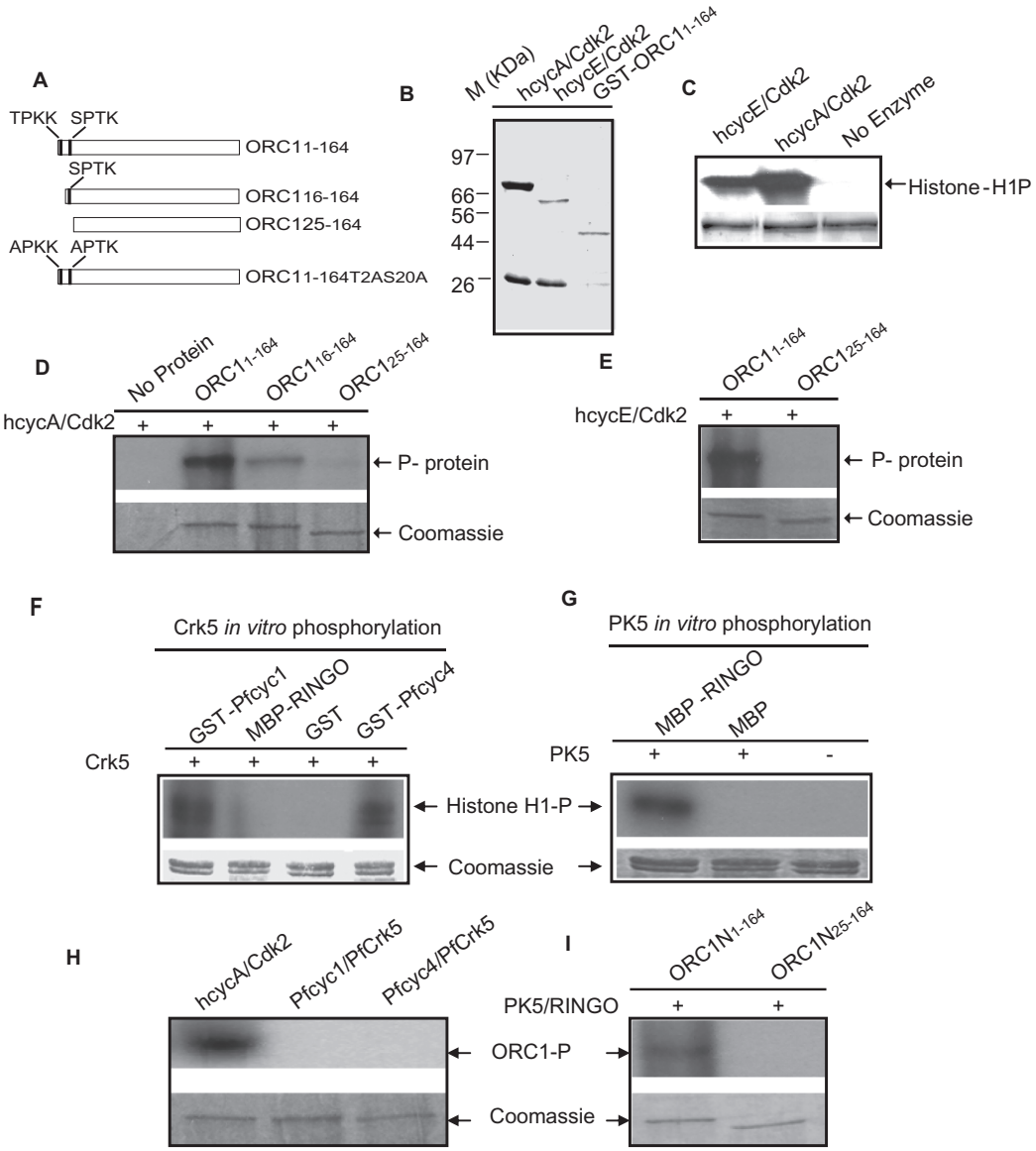


Fig. 2. *In vitro* phosphorylation of N terminus of ORC1 (ORC1N).
A. Schematic diagrams of various N-terminal domains of PfORC1 (wild type or mutant forms). The corresponding proteins were expressed and purified as GST fusion proteins.
B. Coomassie gel showing recombinant hcycA/Cdk2 (co-purified), hcycE/Cdk2 (co-purified) and GST-ORC1$_{1–164}$ proteins used in kinase assays.
C. Autoradiogram following kinase assay using histone H1 as a substrate. Both hcycE/Cdk2 and hcycA/Cdk2 phosphorylate histone H1. Coomassie stained gels (lower panel) show equal loading of histone H1. The third lane shows 'no enzyme' control.
D. Both putative CDK phosphorylation sites on the N-terminus of PfORC1 are phosphorylated. Two micrograms of ORC1N proteins as indicated on top (wild type, one CDK phosphorylation site deleted or both sites deleted) was incubated separately with hcycA/Cdk2 in kinase assay. Robust phosphorylation of ORC1$_{1–164}$ was observed, about half the level was observed with ORC1$_{16–164}$ (one phosphorylation site deleted), whereas no phosphorylation was detected in ORC1$_{25–164}$ (both phosphorylation site deleted).
E. Similarly, no phosphorylation was detected for ORC1N$_{25–164}$ (both phosphorylation sites deleted) with hcycE/Cdk2. Coomassie stained gel show equal loading of ORC1N wild type and mutant proteins.
F and G. Kinase assays were performed using recombinant GST-PfCrk-5 or His-PfPK5 in the presence of histone H1 as a substrate and recombinant cyclins GST-Pfcyc1, GST-Pfcyc4 (only GST as a control) or MBP-Ringo as an activator of kinase (only MBP as a control). Coomassie stained gels (bottom panels) show equal loading of histone H1. The third lane of panel G shows 'no enzyme' control.
H. Kinase activity of PfCrk-5 was tested in the presence of Pfcyc-1 and Pfcyc-4 using ORC1$_{1–164}$ as a substrate. PfCrk-5 did not phosphorylate ORC1$_{1–164}$, although hcycA/Cdk2 could phosphorylate under the same experimental conditions. Coomassie stained gel (bottom panel) showed equal loading of GST-ORC1$_{1–164}$.
I. Ringo/PfPK5 could phosphorylate ORC1$_{1–164}$ as a substrate but not the deletion mutant ORC1$_{25–164}$. Coomassie stained gel (bottom panel) shows equal loading of wild type and mutant proteins.

Fig. 3. PfPK5 is a likely kinase for PfORC1 *in vivo*.
A. Schematic diagrams of full-length PfORC1 with putative CDK phosphorylation sites and different deletion mutants. The corresponding proteins were expressed and purified as GST fusion protein.
B. Ringo/PfPK5 phosphorylates only $ORC1N_{1–238}$. None of the other ORC1 deletion mutant proteins as indicated could be phosphorylated *in vitro*. Five micrograms of each protein was used in a kinase assay followed by SDS-PAGE of the reaction mixture and autoradiography.
C. Kinase assay using mixed stage parasite lysates as a source of kinase. The ~ 5 μg each PfORC1 deletion mutant proteins were incubated with ~ 10 μg parasite lysate. Only $ORC1N_{1–238}$ could be phosphorylated.
D. $ORC1N_{1–238}$ is phosphorylated robustly in the presence of schizont stage parasite lysate. The ~ 10 μg parasite lysate from the ring or trophozoite or schizont stage (~ 20 h, ~ 30 h and ~ 40 h of post-invasion, respectively) were incubated in the presence of ~ 5 μg of GST-$ORC1N_{1–238}$ protein in *in vitro* kinase assay.
E. Stage-specific expression of PfPK5 was checked by western blot analysis using antibodies against PfPK5. Antibodies against PfActin were used as loading control. The parasite lysate were prepared from ring, trophozoite and schizont stage (~ 20 h, ~ 30 h and ~ 40 h of post-invasion respectively).
F. Densitometric analysis of the PfPK5 and PfActin signals as shown in (E). The band intensities of PfPK5 and PfActin were calculated using ImageJ software from three different exposures of an experiment to avoid the signal saturation and plotted accordingly using PfActin signal as control. Densitometry values represent the ratio of PfPK5/PfActin band intensities for the respective stages of the parasite. Error bars indicate the standard errors of the mean. Maximum expression of PfPK5 was observed during schizont stage corroborating highest *in vitro* phosphorylation during this stage as shown in 'D'. Experiments were repeated few times, and similar results were obtained.
G. Kinase assay in the presence of 0.5 mM and 1 mM of Olomucine. Olomucine inhibits phosphorylation of $ORC1N_{1–238}$. The top panel shows the autoradiogram, the bottom panel shows the Coomassie stained gel.
H. An immune-depleted parasite lysate using antibodies against PfPK5 was used in the kinase assay in the presence of GST$ORC1N_{1–238}$. Pre-immune sera against PfPK5 were used as control for immune-depletion. Coomassie stained gel shows equal loading of GST-$ORC1N_{1–238}$ protein.
I. *In vivo* phosphorylation of full length endogenous PfORC1. *P. falciparum* proteins were metabolically labeled with ^{32}[P] orthophosphate *in vivo* followed by extraction of the protein and immunoprecipitation of endogenous labeled ORC1 using antibodies against PfORC1. A Specific band corresponding to PfORC1 is seen only in the immune sera lane but not in the pre-immune sera lane (marked by asterisk).

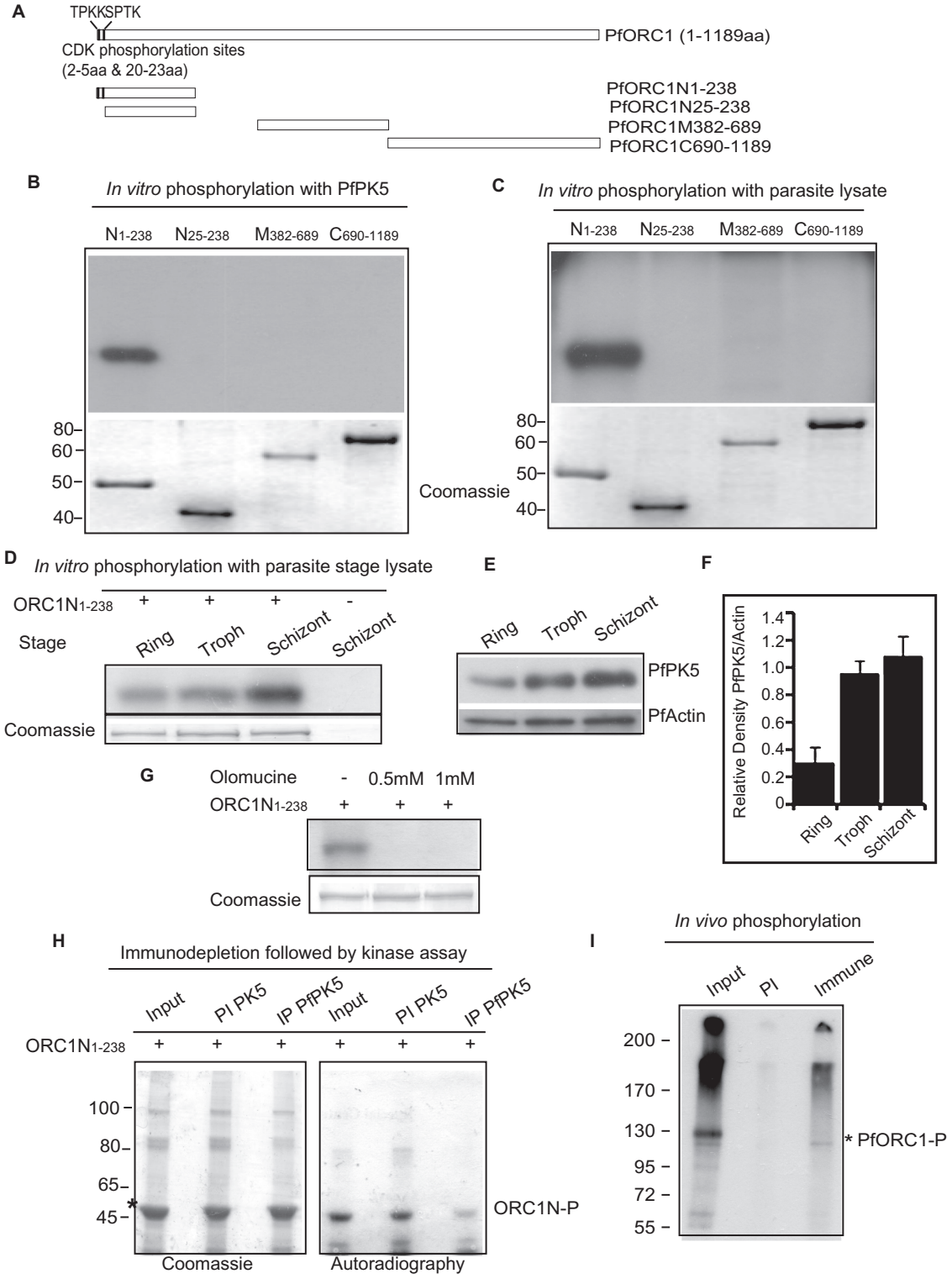
activation of PfPK5 in the presence of Pfcyc1 using histone as a substrate, none of the Pfcyclin combinations with PfPK5 could phosphorylate PfORC1N under our experimental conditions (data not shown).

Only the N terminus of ORC1 (ORC1N$_{1–238}$) is phosphorylated in a CDK dependent manner

PfORC1 is a large, 1189-residue protein. Could other regions of PfORC1 be phosphorylated by CDKs? To address this point, we purified GST tagged fusion proteins containing different domains of PfORC1 ($ORC1_{1–238}$, $ORC1_{25–238}$, $ORC1_{382–689}$ and $ORC_{690–1189}$, respectively) and performed *in vitro* kinase assay in the presence of activated PfPK5 and parasite lysate as a source of plasmodial kinase activity (Fig. 3A–C) (the region between residues 238–382 has many Ser/Thr residues but we were unsuccessful in expressing this region because of large low-complexity repeats). No domains could be phosphorylated apart from the N terminal region ($ORC1N_{1–238}$). The control N-terminal region without the putative CDK phosphorylation sites ($ORC1N_{25–238}$) was also not phosphorylated (Fig. 3B and C) and an uninfected RBC lysate did not cause any phosphorylation of PfORC1 (data not shown).

To investigate the developmental stage of the parasites that is a source of the kinase for PfORC1, we performed *in vitro* kinase assays in the presence of 10 μg of parasite lysates obtained from rings, trophozoites or schizonts stages (~ 20 h, ~ 30 h and ~ 40 h of post invasion respectively). The results showed highest phosphorylation of $ORC1N_{1–238}$ in the presence of schizont stage parasite lysate (Fig. 3D). Western blot analysis using antibodies against PfPK5 and PfActin as a loading control (Fig. 3E) followed by densitometric analysis (Fig. 3F) indeed detected a peak of PfPK5 expression level at the schizont stage. Interestingly, recombinant $ORC1N_{1–238}$ can also be phosphorylated in the presence of lysate obtained from ring and trophozoite stage parasites (Fig. 3D) that are correlated with the presence of PfPK5 in these lysate (Fig. 3E and F); however, the level of phosphorylation is not as high as seen in the presence of schizont stage lysate.

PfPK5 activity can be inhibited using the CDK inhibitor olomucine (Graeser *et al*., 1996a). To ascertain that a CDK is indeed responsible for PfORC1 phosphorylation, we performed kinase assays using a schizont lysate in the presence or absence of olomucine. Phosphorylation of $ORC1_{1–238}$ was abolished in the presence of 0.5 and 1.0 mM concentrations of olomucine, indicating that PfORC1 is phosphorylated by one or more CDK (Fig. 3G). To further check the specificity of PfPK5-mediated PfORC1 phosphorylation, we first performed immunoprecipitation of PfPK5 from a parasite lysate, and the PfPK5-depleted lysate was used in a kinase assay with $ORC1_{1–238}$ as a substrate. The PfPK5-depleted lysate was significantly less active in ORC1N phosphorylation than the native extract, whereas depletion using preimmune did not affect phosphorylation of ORC1N (Fig. 3H), further strengthening the candidature of PfPK5 as the PfORC1 kinase. Antibodies against PfPK5 are highly efficient to immunoprecipitate PfPK5 from the parasite extract (Graeser *et al*., 1996a). We have performed immunodepletion using a similar protocol as reported earlier (Graeser *et al*., 1996a) and efficient immunodepletion of PfPK5 from parasite



extract was achieved as well in our experimental conditions (Fig. S4). Using pre-immune sera in the immunodepletion protocol maintained the presence of a prominent PfPK5 band in the parasite lysate, whereas the lysate from the immune sera-depleted sample showed only residual amounts of PfPK5. The loading control actin showed that the immunodepletion was specific for PfPK5.

Finally, to examine the *in vivo* phosphorylation of endogenous PfORC1, we incubated 3D7 parasites with [^{32}P] orthophosphate followed by immunoprecipitation

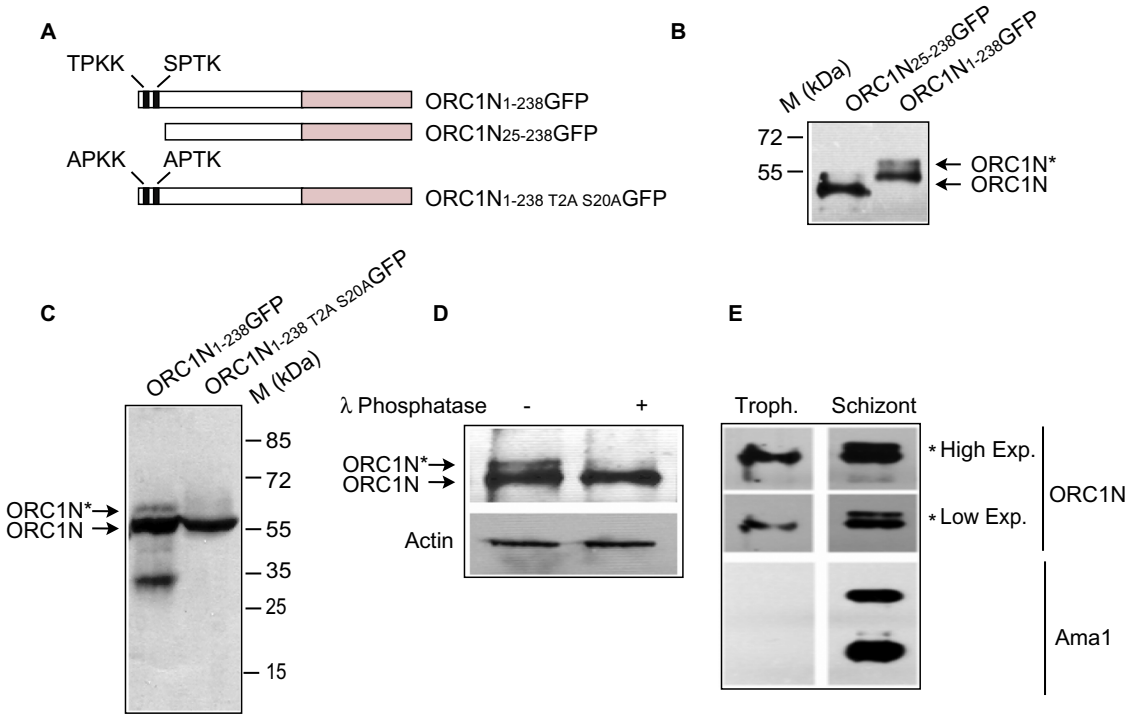


Fig. 4. *In vivo* phosphorylation of ORC1N$_{1–238}$.
A. Schematic diagram of wild type PfORC1 and different mutant version of ORC1N expressed as GFP fusion protein in *P. falciparum* parasites.
B. Western blot analysis of parasite lysate obtained from ORC1$_{1–238}$GFP and ORC1$_{25–238}$GFP expressing parasites using antibodies against GFP. ORC1N* likely represents the phosphorylated form of ORC1N (see text for details) that is clearly present in the ORC1$_{1–238}$GFP lane.
C. Western blot analysis of parasite lysate obtained from ORC1$_{1–238}$GFP and ORC1$_{1-238T2AS20A}$GFP using antibodies against GFP, confirming the top band as a phosphorylated form (ORC1N*) of ORC1N. Molecular mass markers are shown on the right.
D. Prior to western blot analysis the parasite lysate was incubated either with (+) or without (–) λ- phosphatase followed by western blot analysis using antibodies against GFP. Actin was used as loading control (bottom panel).
E. Western blot analysis using trophozoite and schizont lysates obtained from ORC1$_{1–238}$GFP expressing parasite reveals the presence of the slower migrating band primarily in the schizont stage but not in the trophozoite stage. The doublet can be visible clearly in the lower exposure. AMA1 was used as schizonts stage-specific marker (Narum and Thomas, 1994).

using antibodies against PfORC1. A specific radiolabeled band corresponding to PfORC1 could be seen, which was absent in the control pre-immune lane. This demonstrates that endogenous PfORC1 is phosphorylated *in vivo* (Fig. 3I), consistent with one of the phosphoproteomics data sets available on PlasmoDB (Treeck *et al.*, 2011). Interestingly, some bands could be seen in the immune lane only and not in the pre-immune lane above the PfORC1 band. It is tempting to speculate that these bands are possible phosphorylated interacting partners of PfORC1. However, further detailed experiments are required to resolve the identity of these proteins.

ORC1$_{1–238}$ is phosphorylated in vivo

We have shown above that the phosphorylation of ORC1N *in vitro* occurs on the amino-terminal consensus CDK phosphorylation sites. To investigate whether phosphorylation takes place at the same sites *in vivo*, we generated transgenic parasites expressing GFP-fusion proteins containing wild type ORC1$_{1–238}$, the deletion mutant, ORC1$_{25–238}$, or the phosphomutant, ORC1$_{1-238T2AS20A}$ (Fig. 4A). Expression of ORC1$_{1–238}$GFP and ORC1$_{25–238}$GFP was verified by western blot analysis using antibodies against GFP (Fig. 4B). Interestingly, a distinct slower migrating band was observed above the major band in ORC1N$_{1–238}$ lane, but the deletion mutant ORC1$_{25–238}$ lane failed to detect any such clear slower migrating band (Fig. 4B). The slower migrating band was also absent from the phosphomutant lane (ORC1$_{1-238T2AS20A)}$ (Fig. 4C), suggesting that the slower migrating band was the phosphorylated form of ORC1$_{1–238}$. Treatment of parasite lysate with λ- phosphatase followed by GFP western blot analysis led to the disappearance of the top band (Fig. 4D). These results confirmed the *in vivo* phosphorylation of PfORC1N. We further checked the presence of the phosphorylated form of PfORC1N in the trophozoite stage and schizont stage. Western blot analysis confirmed the presence of the phos-

phorylated form mostly in the schizont stage, as ascertained by AMA1 levels (AMA1 is known to be expressed exclusively in schizonts) (Narum and Thomas, 1994) (Fig. 4E).

The above results collectively show that PfORC1N is phosphorylated at the cdc2/Cdk phosphorylation sites *in vivo* and that this phosphorylation predominantly takes place in schizonts, corroborating our *in vitro* data presented in Fig. 3D and E, and consistent with published phosphoproteome data (Treeck *et al*., 2011).

Phosphorylation of ORC1N affects its DNA binding activity

We reported earlier that His-ORC1$_{1–238}$ protein binds directly to telomeric DNA (Deshmukh *et al*., 2012). Phosphorylation can increase or decrease the DNA binding activity of proteins (Lee *et al*., 2012). Generally, the effects of phosphorylation on DNA binding can be explained either by electrostatic repulsion or by conformational modification of the protein. In order to investigate the effect of phosphorylation on the DNA binding activity of PfORC1N, we performed electrophoretic mobility shift assay using a radiolabeled telomeric DNA probe in the presence of ORC$_{1–238}$ alone or hcycA/Cdk2 treated ORC1N$_{1–238}$ protein in the presence or absence of ATP. We found that kinase-treated ORC1N$_{1–238}$ in the presence of ATP lost its DNA-binding activity. Absence of ATP in the kinase reaction or ATP alone did not affect DNA binding activity of ORC1N$_{1–238}$ (Fig. 5A). These results clearly show that phosphorylation of ORC1N$_{1–238}$ abrogated its DNA-binding activity.

Endogenous and ORC1$_{1–238}$GFP bind to telomere-associated repetitive elements (TAREs) and promoters of *var* genes (*ups*) (Mancio-Silva *et al*., 2008; Deshmukh *et al*., 2012). The schizont specific phosphorylation and cytoplasmic localization of ORC1, as well as reduced DNA binding activity of phosphorylated His-ORC1$_{1–238}$, tempted us to investigate whether phosphorylation of endogenous and ORC1N$_{1–238}$ in schizont would lead to its dissociation from telomeric *var* promoters. We therefore performed chromatin immunoprecipitation (ChIP) followed by real time PCR (qPCR) in 3D7 and ORC1$_{1–238}$GFP transgenic parasites using antibodies against ORC1 and GFP (for endogenous and GFP-ORC1N respectively). We compared the occupancy of the above proteins at the subtelomeric regions and *var* gene promoter in rings (~ 20 h), trophozoites (~ 30 h) and schizonts (~ 40 h). This revealed a dramatic decrease in the occupancy of endogenous ORC1 and ORC1N$_{1–238}$ at TARE-1,-2,-3 and *ups*E promoter as the parasite progresses from ring stage to the trophozoite and schizont stages (Fig. 5B and C).

In order to validate the authenticity and validity of ChIP-qPCR experiments, various control experiments were performed as followed. ChIP experiments using antibodies against Histone H3 followed by qPCR analysis did not show drastic change in the occupancy of Histone H3 at the various TARE regions between the ring (~ 20 h) and schizont (~ 40 h) stage of 3D7 parasites (Fig. S6A). These results suggest the efficiency of ChIP experiments is similar at both stages. Furthermore, we compared the occupancy of ORC1 at the TARE-2 region (positive control) and HRP region (negative control) in rings and schizonts. PfORC1 showed much higher binding to the TARE-2 region compared with minimal binding at the HRP region during the ring stage. During the schizont stage, the binding of ORC1 at the TARE-2 region reduced drastically as compared with the ring stage. As observed in the ring stage, the binding of ORC1 at the HRP region was very minimal (Fig. S6B).

These results suggest phosphorylation-mediated displacement of ORC1 from the telomeric region and *var* gene promoters.

Mutation in CDK phosphorylation sites prevents change in subcellular localization of PfORC1N

We have shown that phosphorylation of PfORC1 leads to reduced DNA binding *in vitro* and to reduced occupancy of PfORC1N at the TAREs and *var* gene promoter in schizonts (where PfORC1N phosphorylation peaks) *in vivo*. In order to evaluate the direct impact of phosphorylation of PfORC1N on its subcellular localization and affinity towards TAREs and *var* gene promoter, we compared the subcellular localization of PfORC1N$_{1–238}$GFP (with intact phosphorylation sites) and PfORC1N$_{1\text{-}238T2AS20A}$GFP (with mutant phosphorylation sites) during trophozoites (~ 30 h) and schizonts (~ 40 h) stages. Western blot analysis using antibodies against GFP showed the presence of both proteins in the nuclear fractions of trophozoites (Fig. 6A). However, in schizonts, ORC1N$_{1–238}$ was mostly found in the cytoplasmic fraction, whereas PfORC1N$_{1\text{-}238T2AS20A}$ was still found in the nuclear fraction similar to trophozoite stage (Fig. 6B). Antibodies against *P. falciparum* aldolase and Histone H3 were used as control for cytoplasmic fraction and nuclear fraction respectively. Furthermore, we performed subcellular localization of PfORC1N$_{1\text{-}238T2AS20A}$ and endogenous ORC1 under the same experimental conditions during schizont stage (~ 40 h). PfORC1N$_{1\text{-}238T2AS20A}$ was found in the nuclear fraction, whereas the endogenous ORC1 was mostly found in the cytoplasmic fraction under these conditions (Fig. S7). These results suggest that mutations in the CDK phosphorylation sites may affect translocation of PfORC1N to the cytoplasm.

Do phosphosite mutations affect occupancy of ORC1N at the TAREs and *var* promoter? To test this hypothesis, we performed real-time PCR analysis of chromatin immunoprecipitated DNA (using antibodies against GFP)

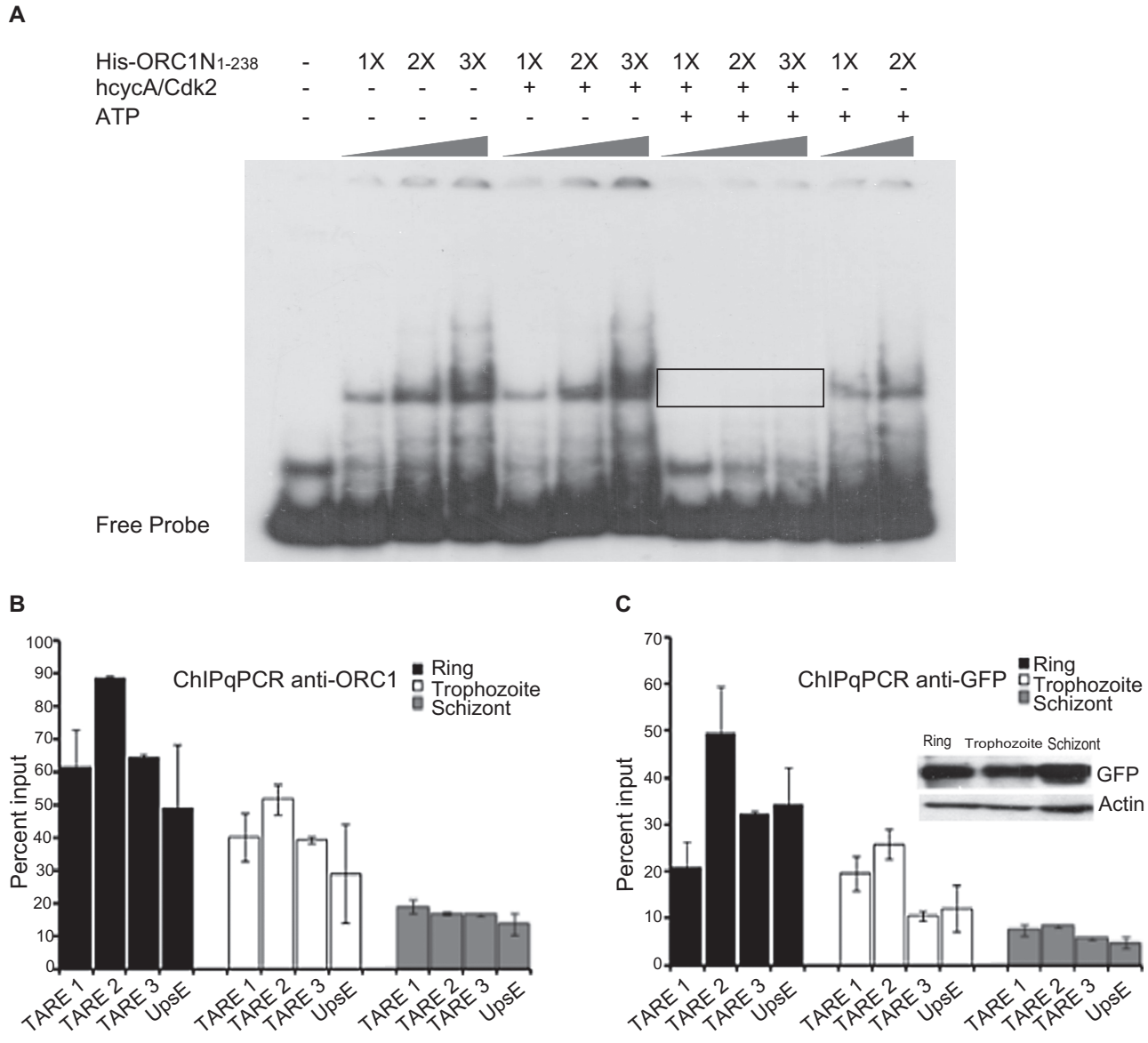


Fig. 5. Phosphorylation of PfORC1 causes its dissociation from DNA.
A. His-ORC1N$_{1–238}$ was phosphorylated *in vitro* using hcycA/Cdk2 and ATP. Mock kinase assay was performed without the addition of hcycA/Cdk2 or without adding ATP. His-ORC1N$_{1–238}$ proteins from different kinase assay were used in electrophoretic mobility shift assay (EMSA) using radiolabeled telomere probe. Phosphorylation of PfORC1N leads to loss of DNA binding activity as indicated by the absence of the bands in the inset rectangle.
B and C. Reduced association of ORC1 to TAREs and promoter of *var* gene as blood stage progresses. ChIP reactions were performed by immunoprecipitation of chromatin fraction obtained from different parasite stages (ring, ~ 20 h; trophozoite, ~ 30 h and schizont, ~ 40 h) using antibodies against PfORC1 (for 3D7 parasite) and GFP (for ORC1N$_{1–238}$-GFP expressing parasite) followed by real-time PCR using primer sets from the TARE and *var* promoter regions. Input of the respective stage was used for normalization of PCR. Enrichment was calculated as percent input (ChIP/Input) using the Ct value of the 'Input' and 'ChIP' after subtracting the corresponding Ct value of Pre-immune. Expression of GFP-ORC1N from different stages (as indicated on the top) is shown in the inset of (C). Each ChIP-qPCR reaction was reproduced three times, and qPCR reactions were set up in triplicates. The bars include mean and standard error of the mean.

obtained from rings, trophozoites and schizonts of the ORC1N$_{1\text{-}238T2AS20A}$GFP transgenic line. We have already shown that the occupancy of ORC1N$_{1–238}$GFP at the TAREs and *var* promoter reduces drastically from ring to schizont stage (Fig. 5C). Interestingly, in the case of parasites expressing ORC1N$_{1\text{-}238T2AS20A}$GFP, the occupancy of mutant protein at the above sites was comparable during ring and schizont stages (Fig. 6C). These results reconfirm that phosphorylation of PfORC1N is required for translocation of the protein from the nucleus to the cytoplasm (with concomitant decrease in the occupancy of the protein at the TAREs and *var* promoters).

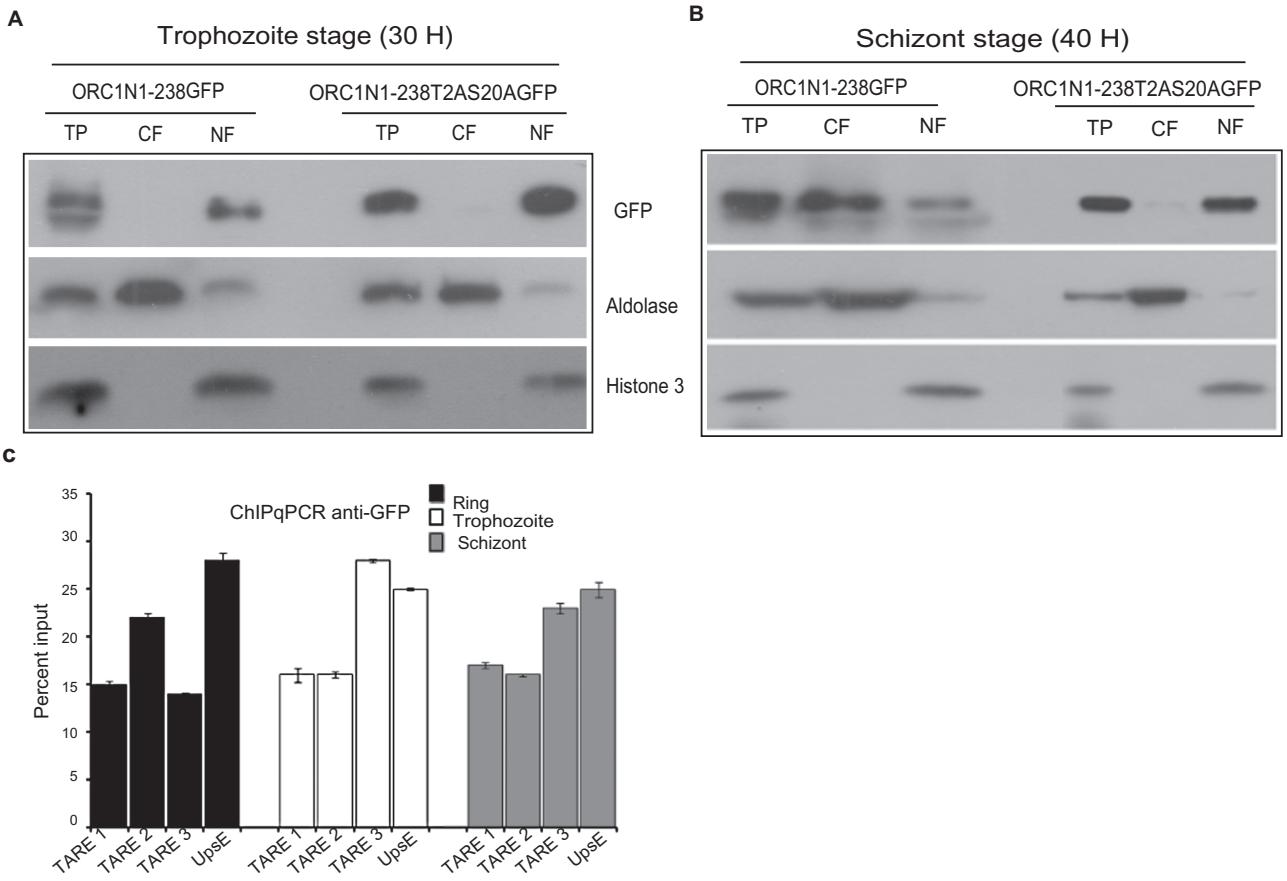


Fig. 6. Phosphorylation dependent change in subcellular localization of PfORC1N.
A. Western blot analysis of total protein (TP), cytoplasmic fraction (CF) or nuclear fraction (NF) from parasite lysates obtained from trophozoite stage (30 h) of ORC1N_{1-238}GFP and ORC1$_{1\text{-}238T2AS20A}$GFP-expressing parasites, using antibodies against GFP. Antibodies against aldolase and Histone 3 were used as marker for cytoplasmic fraction and nuclear fraction.
B. Western blot analysis using parasite lysate obtained from schizonts (40 h) under similar experimental conditions as above. Antibodies against aldolase and Histone 3 were used as markers for cytoplasmic fraction and nuclear fraction.
C. Chromatin Immunoprecipitation followed by quantitative PCR (ChIPqPCR). The chromatin fraction obtained from ORC1$_{1\text{-}238T2AS20A}$GFP expressing parasites from different stages (ring, ~ 20 h; trophozoite, ~ 30 h and schizont, ~ 40 h) was immunoprecipitated using antibodies against GFP, followed by qPCR analysis of ChIPed DNA using primer sets from TARE and *ups*E regions.

Proteasome-mediated degradation of ORC1 in late schizonts

We have shown that the amount of PfORC1 protein level is drastically reduced at the schizont stage (Fig. 1A and B). Phosphorylation of PfORC1 and its translocation to the cytoplasm also peaks in schizonts. It is therefore possible that phosphorylation leads not only to changes in subcellular localization (as detailed above) but also to degradation of the protein in a proteasome-mediated pathway (Gupta *et al*., 2008). Interestingly, transcriptome data (http://WWW.PlasmoDB.org) indicate that PfORC1 mRNA levels do not decrease at the schizont stage, even though we see a decrease in the protein level (Fig. 1B). Treatment of parasites using the proteasome inhibitor MG132 leads to the stabilization of PfORC1 in late schizonts, as shown by western blot analysis of parasite lysate obtained from MG132 treated and untreated parasites using antibodies against PfORC1 (Fig. 7). These results suggest that phosphorylation of endogenous ORC1 leads to cytoplasmic translocation and eventual degradation of protein.

Discussion

In this paper, we have shown that both endogenous PfORC1 and ORC1NGFP display dynamic localization and abundance during the *Plasmodium* blood stage cycle, and that this is mediated by PTMs of PfORC1, mainly phosphorylation at the very N-terminus, and proteosome-mediated degradation. Furthermore, we have demonstrated that PfPK5 phosphorylates PfORC1N in the amino terminal CDK phosphorylation sites *in vitro*. PfPK5 is the best candidate as the *in vivo* PfORC1 kinase in schizonts. Phosphorylation of PfORC1 leads to reduced affinity for DNA and concomitant translocation of PfORC1 to the

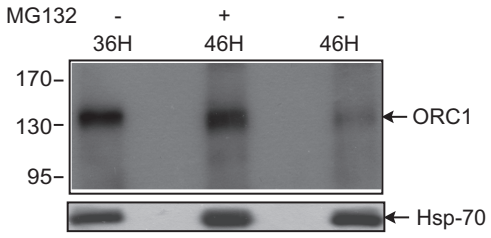


Fig. 7. PfORC1 is degraded at the late schizont stage. Western blot analysis using antibodies against PfORC1 showed increased signal of ORC1 at the late schizont stage in the presence of MG132 compared with the signal obtained in the absence of the proteosome inhibitor MG132. Antibodies against PfHSP70 were used as loading control.

cytoplasm. Finally, cytoplasmic translocation leads to the degradation of ORC1 through the proteasome pathway, as demonstrated by the effect of a proteasome inhibitor on the process.

The overall mechanism of DNA replication is well conserved among eukaryotes, although different regulatory mechanisms for different replication proteins are observed among eukaryotes. For example, in yeast, ScORC1 stably binds to origins throughout the entire cell cycle, whereas the interaction of *Xenopus* and mammalian ORC1 (XlORC1 and HsORC1, respectively) with chromatin appears to vary during different stages of the cell cycle (Findeisen *et al*., 1999; Mendez *et al*., 2002; DePamphilis, 2005). In *P. falciparum*, the association of PfORC1 with chromatin gradually decreases as the parasite develops from ring to schizont. In human cells, cycA/Cdk2 phosphorylates ORC1 during late S phase, which is followed by polyubiquitination and degradation of ORC1 before it reappears during M to G1 transition (Mendez *et al*., 2002). However, in hamster cells, ORC1 gets monoubiquitinated following phosphorylation, leading to the dissociation from the chromatin during M phase. Monoubiquitinated ORC1 is not degraded but possibly gets deubiquitinated to allow it to bind to origins during next G1 phase (DePamphilis, 2005). In *D. melanogaster*, N-terminus of ORC1 contains three types of degradation motifs (O box, KEN box, three D boxes, respectively), out of which the O box is responsible for Fzr-Cdh1 dependent polyubiquitination of ORC1 leading to the degradation of the protein during M phase (Araki *et al*., 2005). DmORC is a substrate of casein kinase 2 (CK2) *in vitro*, which modulates ATP-dependent DNA binding activity of DmORC (Remus *et al*., 2005). Our results demonstrate that PfORC1 is phosphorylated by the *Plasmodium* CDK PfPK5, leading to cytoplasmic translocation of PfORC1 from the nucleus and eventual degradation by the proteasome. At this point, it remains to be determined whether polyubiquitination of PfORC1 leads to its degradation. Like DmORC, *in vitro* phosphorylation of PfORC1 also modulates its DNA-binding activity, but the kinases involved are different (CK2 versus a CDK). Our results strongly suggest that ORC1, a crucial protein conserved in eukaryotes, is regulated in a similar fashion not only from yeast to mammals (which are both members of the Opisthokonta phylum), but also in the phylogenetically distant Apicomplexa taxon.

In the yeast *S. cerevisiae*, although ScORC1 binds to chromatin throughout the cell cycle, another important component, Cdc6, plays a crucial role. Phosphorylation of ScCdc6 by cdc28 in the presence of G1 cyclins Cln1/2 generates binding site for SCF^{CDC4} (E3 ubiquitin ligase), which leads to very rapid ubiquitin-mediated proteolysis of ScCdc6 during late G1 and S phase following the pre-RC formation (Perkins *et al*., 2001). Further interaction of Clb2/Cdc28 with the amino terminal region of Cdc6 during G2/M phase yields a stable complex preventing it from being loaded on DNA as well as preventing further loading of the MCM2-7 complex (Mimura *et al*., 2004). Interestingly, *P. falciparum* does not have a clear homologue for Cdc6; however, the C-terminus of PfORC1 shows homology with Cdc6 and thus may undergo similar regulation as cdc6 does in *S. cerevisiae*.

One fundamental difference between *P. falciparum* and other organisms is that multiple rounds of DNA replication take place in the absence of cytokinesis, contrary to the single round of DNA replication per cell division that occurs in the majority of higher eukaryotes. Therefore, the presence of ORC1 in the nucleus until the schizont stage ensures that PfORC1 is available to fulfill its function. Phosphorylation of PfORC1 affects its association with DNA *in vitro* and diminishes its association with chromatin *in vivo* in late schizonts. In this context, phosphorylation may regulate DNA replication as well as the *var* gene regulation function of PfORC1.

First, phosphorylation of PfORC1 leads to reduced occupancy at the telomeric/TARE regions in schizonts. This probably helps in reprogramming of *var* gene regulation/expression in the next cycle by the redistribution of PfORC1 at the TAREs and telomeric *var* gene promoters that may be important for the allelic exclusion process. Second, PfORC1 phosphorylation may also help to dissociate this protein from replication sites in late schizont when DNA replication is completed. Shuttling ORC1 from nucleus to cytoplasm ensures the termination of DNA replication. Cell-cycle dependent relocalization of replication proteins have also been reported from yeast to mammals (Petersen *et al*., 1999). The finding of putative origins in *Plasmodium* will be helpful to investigate their stage-specific occupancy by PfORC1.

Our findings identify PfORC1 as a possible substrate of PfPK5. Phosphorylation-mediated regulation of PfORC1 may influence both DNA replication and *var* gene regulation highlighting biological functions of PfPK5 that are important for both parasite multiplication and immune evasion. It is intriguing that Pfcyc1/PfCrk-5 and Pfcyc4/

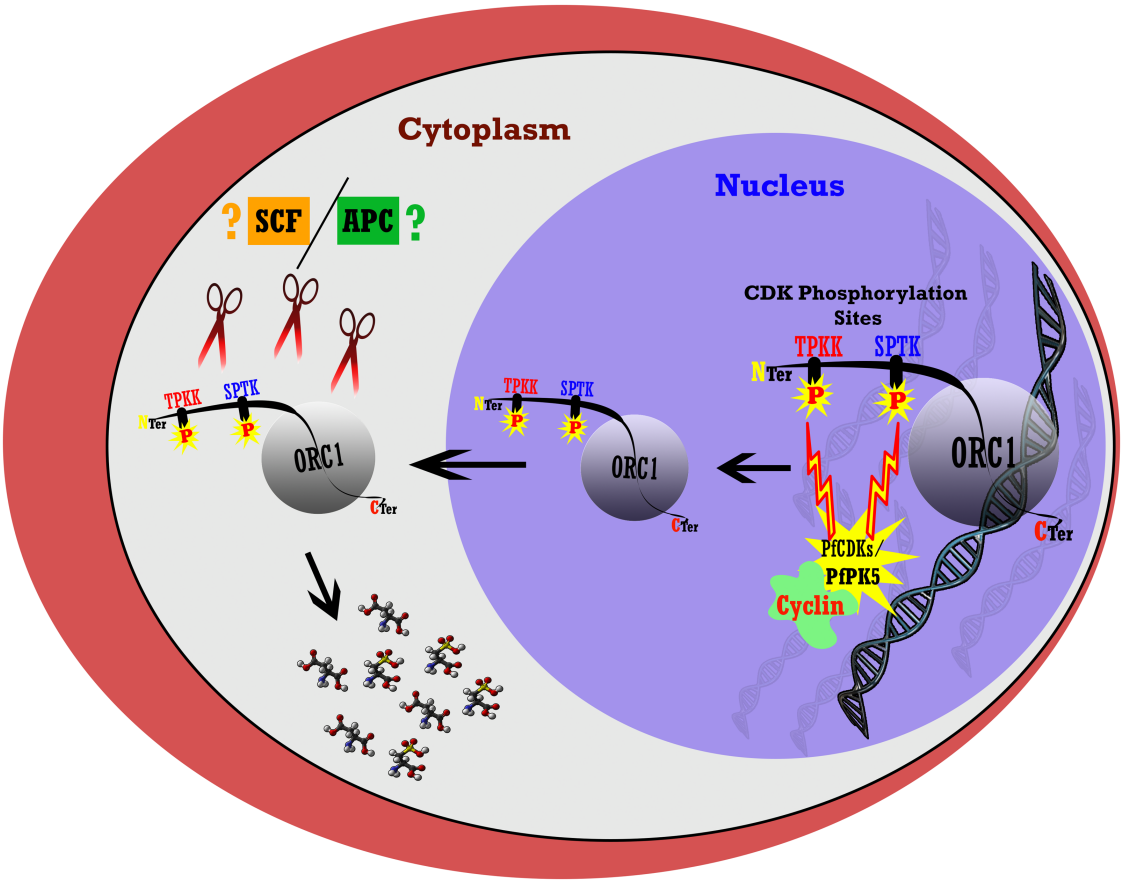


Fig. 8. Model for PfORC1 regulation through phosphorylation during blood-stage development. In the early stages of parasite development, PfORC1 remains into the nucleus in chromatin-bound form. During later stages, it becomes phosphorylated at its N terminus by PfPK5, dissociates from DNA and relocates to cytoplasm, where it is degraded by the proteasome following ubiquitination mediated by either the SCF or the APC protein complex.

PfCrk-5 could phosphorylate histone but not PfORC1N, whereas RINGO-activated PfPK5 could phosphorylate the latter substrate. This suggests possible substrate specificity of PfPK5 for PfORC1. The fact that RINGO is a non-natural activator of PfPK5 raises the question of the authenticity of PfORC1 as a substrate of PfPK5 (Fig. 2G). However, the kinase assay using immunodepleted parasite lysate (Fig. 3H) in the presence of antibodies against PfPK5 strengthens our claim that PfORC1 is a possible substrate of PfPK5. The inhibition of phosphorylation of PfORC1 in parasite extracts by the CDK inhibitor olomucine further confirms the involvement of a CDK activity in the process.

PfORC1 levels decrease drastically in late schizonts that may be explained either by low levels of transcripts or by specific degradation of the protein. Transcriptomic data (http://www.plasmodb.org) indicate that PfORC1 mRNA levels are essentially constant in trophozoite and schizont stages, whereas PfORC1 protein levels undergo a drastic decrease in late schizonts. As this decrease is prevented in the presence of a proteasome inhibitor (Fig. 7), it may be concluded that a significant fraction of PfORC1 is degraded by the proteasome-mediated pathway in the later stages of schizogony. In other eukaryotes, cell cycle-regulated proteolysis depends on either the Skp-/cullin-/F-box complex (SCF complex) or the anaphase promoting complex (APC). Both complexes catalyze ubiquitination of the target cell cycle regulatory proteins and direct them to proteasome degradation. To limit DNA replication to the S phase of the cell cycle, ORC1 levels are regulated by SCF complex in humans (Mendez *et al.*, 2002) and APC in *Drosophila* (Araki *et al.*, 2005). Interestingly sequence data mining suggests presence of all the aforementioned subunits of the SCF complex and several components of the APC in *P. falciparum*. With the presence of components of both regulatory pathways in the *P. falciparum*, it is now important to investigate which one of these pathways is responsible for ORC1 degradation.

Based on the above results, we propose the following model (Fig. 8). After invasion and until the trophozoite stage, PfORC1 primarily resides in the nucleus to fulfill its functions in *var* gene regulation and DNA replication.

However, in schizont stage, it is phosphorylated by PfPK5 that causes its dissociation from chromatin and translocation to the cytoplasm, where it is degraded by the proteasome. Therefore, PfPK5-mediated phosphorylation of ORC1 controls two fundamental aspects of parasite biology, *var* gene regulation and DNA replication that are central to malaria pathogenesis.

Experimental procedures

Parasite culture

Plasmodium falciparum 3D7 strain was cultured as described earlier (Trager and Jensen, 1976). Synchronization of the culture was achieved by using 5% sorbitol treatment of the parasites.

DNA manipulation

Cloning for expression in Escherichia coli. Sequences encoding various domains of ORC1; $ORC1_{1–164}$, $ORC1_{16–164}$, $ORC1_{25–164}$, $ORC1_{1\text{-}164T2AS20A}$, $ORC1_{1–238}$, $ORC1_{25–238}$, $ORC1_{382–689}$ and $ORC1_{690–1189}$ (subscript numbers denote amino acid co-ordinates) were amplified by PCR using *P. falciparum* 3D7 genomic DNA as template and cloned into pGEX-6P-2 vector at the *BamH*I and *Xho*I restriction sites. The coding regions of $ORC1N_{1–238}$ and PfPK5 were amplified by PCR using *P. falciparum* 3D7 genomic DNA and cDNA, respectively (as there are introns in the PfPK5 gene) as template and cloned into pET-28a vector using the *BamH*I and *Xho*I restriction sites and into pET-21c vector at the *Nhe1* and *Xho*I sites respectively. Primer sequences are mentioned in Table S1A.

Generation of GFP constructs. The DNA fragments corresponding to $ORC1N_{1–238}$ and $PfORC1N_{25–238}$ were PCR amplified from 3D7 genomic DNA using primers listed in Table S1B and cloned into pARL-GFP vector using the *Kpn*I and *Avr*II restriction sites (Crabb *et al.*, 2004). Phosphomutant of PfORC1N ($PfORC1N_{1\text{-}238T2AS20A}$; alanine replacement mutants of single and double amino acids at positions 2 and 20) were generated by site-directed mutagenesis primers listed in Table S1C. Fusion gene expression is driven by the *crt* (*P. falciparum* chloroquine resistance transporter) gene promoter.

Transfection

Transfection of the 3D7 parasites using the pARL fusion constructs were performed using the protocol described elsewhere (Fidock and Wellems, 1997). The transfected parasites were selected using the drug WR99210 (Jacobus Pharmaceuticals). Expression was verified by western blot analysis using transfected parasite lysate in the presence of anti-GFP antibodies and fluorescence microscopy.

Antibodies

Polyclonal antisera against PfORC1 were raised in rabbit (Deshmukh *et al.*, 2012). Rabbit polyclonal antibody to GFP was purchased from Abcam (ab290). The anti-PfPK5 antibody was raised in chicken against the peptide PfPK5-derived AKQALEHAYFKENN© coupled to rabbit albumin using 3-maleimidobenzoic acid N-hydroxysuccinimide ester.

Immunofluorescence microscopy

Localization of PfORC1 was observed using immunofluorescence assay with anti-PfORC1 antibodies as described elsewhere (Deshmukh *et al.*, 2012).

Expression and purification of recombinant proteins

Escherichia coli strain DH10β was used for cloning purposes. BL21 Codon plus (RIG) cells were used for the expression of the recombinant proteins. Protein purification was performed as described elsewhere and in the Supplementary method section (Deshmukh *et al.*, 2012).

Subcellular fractionation

Nuclear and cytoplasmic extracts were prepared using the protocol as previously described (Flueck *et al.*, 2009) with some modifications. Briefly, parasites from a synchronous culture of 3D7 *P. falciparum* strain were isolated from infected erythrocytes by saponin lysis. The parasite pellet was resuspended in 1 ml of lysis buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2% NP-40) and incubated for 5 min at 4°C. It was followed by centrifugation at 4000 *g* for 10 min. The supernatant was collected as cytoplasmic fraction. The Pellet containing the nuclei was resuspended in 1 ml of extraction buffer (20 mM Tris, pH 8.0, 0.3 M NaCl, 20 mM KCl, 15 mM $MgCl_2$, 10% glycerol, 0.6% NP-40, 1 mM DTT, 50 mM Na_3VO_4 and 50 mM β-glycerophosphate) and kept on ice for 20 min, after which the sample was sonicated for three times for 10 sec followed by incubation at 4°C for 20 min. The extract was further centrifuged, and the supernatant containing nuclear proteins was collected. All buffers used in this protocol contained protease inhibitors (Complete, Roche).

Kinase assay

In vitro phosphorylation with recombinant kinases

Kinase assays were performed as previously described (Leykauf *et al.*, 2010). Protein purification profile of various human cyclin-cdk complex (cyclin A/cdk2 and cyclin E/cdk2) has been shown in Figure 2B. Protein purification profiles of different *Plasmodium* cyclins (GST-Pfcyc1 and GST-Pfcyc4), kinases (PfPK5-His and GST-PfCrk-5), human cell cycle protein (MBP-Ringo) and deletion mutants of ORC1 (GST-$PfORC1_{1–164}$ and GST-$ORC1_{25–164}$) are shown in (Fig. S3). Histone H1 was purchased from Sigma.

Briefly, 5.0 µg of ORC1 proteins were assayed at 30°C for 30 min in a 30 µl kinase reaction buffer [50 mM Tris-HCl pH7.5, 10 mm $MgCl_2$, 0.1 mM EDTA, 200 µM ATP and 5 µCi (γ-^{32}P) ATP] supplemented with 3.0 µg of cycA/Cdk2 or cycE/Cdk2 or PfPk5 proteins. Following the incubation time, the

reaction was stopped by the addition of 4× SDS loading buffer and boiled. The reactions were resolved on a 10% SDS-PAGE gel, and the gel was subsequently dried. Imaging of ^{32}[P]-labeled protein bands was achieved by direct autoradiography of dry gels.

In vitro phosphorylation with parasite lysates

Synchronized parasites were first lysed with saponin. To prepare parasite lysates with kinase activity (Leykauf *et al.*, 2010), the parasite pellets were lysed in 10 volumes of ice cold buffer B (50 mM β -glycerophosphate pH 7.3, 1% Triton X-100, 1 mM DTT, Complete Protease Inhibitor Cocktail (Sigma) and phosphatase inhibitors, 50 mM Na_3VO_4 and 50 mM NaF) and incubated on a rotating wheel overnight at 4°C. Control extracts were made from similar amounts of whole uninfected red blood cells under identical conditions. Lysates were centrifuged at 15000 *g*, and the protein concentration of the supernatant was determined by Bradford assay (Bio-Rad). Kinase reaction were performed as above with 10 μg total protein lysate in the presence of 5 μg of the GST or GST-fused ORC1 proteins immobilized on glutathione-sepharose beads.

Immunodepletion

Hundred milliliters of 10% 3D7 schizont stage parasites were harvested and parasite were released from host cell using saponin, and washed with 1× cold PBS and lysed for 12 h at 4°C by placing them in a rotating platform (10 r.p.m.) in the presence of 10 volume of ice cold lysis buffer [50 mM Tris-HCL pH 7.5, 1% Triton X-100, 1 mM DTT, 50 mM Na_3VO_4, 50 mM NaF, 50 mM beta glycerol phosphate, phosphatase inhibitor cocktail (Sigma), complete protease inhibitor (Roche) and PMSF]. After separation of the membrane fraction by centrifugation, the supernatant was divided into two parts: one was mixed with antibodies against PfPK5, and the other part with pre-immune antibodies and incubated at a rotating platform at 4°C for 6 h. ~ 20 μl of protein L sepharose beads were added, and the mixture was further rotated for 4 h. The beads were then separated by centrifugation and supernatant were used for kinase assay.

Metabolic labeling of phosphorylated P. falciparum protein

Metabolic labeling was performed as described previously (Leykauf *et al.*, 2010). Metabolic labeling of parasite protein was achieved by incubating sorbitol-synchronized trophozoite stage 3D7 parasites with 100 mCi/ml of ^{32}[P]-monosodium phosphate in 50 ml phosphate-free RPMI medium supplemented with 25 mM HEPES (pH 7.2), 0.5% (w/v) Albumax, 0.4% (w/v) glucose, 0.2% (w/v) Na_2HCO_3 at 37°C overnight. Schizont stage parasite pellet was obtained by saponin lysis, and parasite pellets were extracted in 10 volumes of HNET buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) for 1 h at 4°C with intermittent vortexing. The detergent-soluble schizont stage extract was clarified by centrifugation at 10000 *g* for 30 min at 4°C. All procedures were carried out in the presence of protease and phosphatase inhibitors on ice, unless otherwise stated. Following metabolic labeling, immunoprecipitation was carried out with 2 μl each of polyclonal or pre-immune sera against PfORC1 in the ^{32}[P]-labeled parasite extracts.

Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of $ORC1N_{1–238}$ was investigated using 20 μl reaction mixture containing DNA binding buffer [10 mM Tris-Cl (pH 7.5), 100 mM KCl, 5 mM $Mgcl_2$, 2 mM dithiothreitol, 6% glycerol, 50 μg ml^{-1} bovine serum albumin] containing appropriate (γ-^{32}P) ATP labeled telomere DNA (Deshmukh *et al.*, 2012). Telomere DNA (175 bp) was amplified from a plasmid DNA using the primers listed in Table S1D (Mancio-Silva *et al.*, 2008). The ~ 2 ng radiolabeled telomere DNA probe was used per reaction. *In vitro* kinase assay was performed for His-$ORC1N_{1–238}$ using different concentrations of hcycA/Cdk2 and ATP prior to EMSA reaction.

Chromatin immunoprecipitation (ChIP) and real-time PCR (ChIPqPCR)

The ChIP assay was performed as described previously (Deshmukh *et al.*, 2012). Chromatin fragments obtained from ring trophozoite and early schizont stages were incubated overnight at 4°C with the following antibodies: 2 μl of rabbit anti-ORC1 and 1.0 μl of rabbit anti-GFP. The immunoprecipitated DNA was analyzed for real-time PCR amplification of TAREs (TARE-1, TARE-2 and TARE-3) and *upsE* promoter using primers listed in Table S1E. PCR conditions were optimized so that ~ 150 bps products were obtained (Fig. S5). Real-time PCR was performed by using an Applied Biosystem equipment and absolute SYBR Green Mix. All test and control reactions were performed in triplicate using the following cycle conditions: 95°C, 15 min followed by 40 cycles of 94°C, 30 s; 55°C, 40 s and 68°C, 50 s. Enrichment was calculated as (ChIP/Input) using the Ct value of the 'Input' and 'ChIP' after subtracting the corresponding Ct value of Pre-immune.

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