Identification and functional studies of *Plasmodium falciparum inhibitor 3 homolog*: a regulatory subunit of protein phosphatase type 1

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Summary

Growing evidence indicates that the protein regulators governing protein phosphatase 1 (PP1) activity have crucial functions since their deletion drastically affects cell growth and division. PP1 has been found to be essential in *Plasmodium falciparum*, but little is known about its regulators. In this study, we have identified a homolog of Inhibitor-3 of PP1, named PfI3. NMR analysis combined with GST-pull down assays demonstrated that PfI3 interacts with PfPP1 through the ^{41}KVVRW^{45} motif where the substitution of W45A abolished the binding to PfPP1. Reverse genetic approaches suggest an essential role of PfI3 in the growth and /or survival of blood-stage parasites since attempts to obtain knock out parasites were unsuccessful, although the locus of PfI3 is accessible. The use of a yeast model showed a lack of functional orthology as PfI3 was unable to rescue yeast deficient in I3 (Ypi1) expression. Functional assays *in vitro* revealed that PfI3, unlike yeast I3 and human I3, increased PfPP1 activity. Episomal expression of PfI3 as a GFP-tagged protein indicated a principal localisation of PfI3 in the nucleus of all blood-stage parasites, suggesting a potential regulatory role of PfI3 on the activity of nuclear PfPP1.

Introduction

Protein phosphatases are well known to play key roles in many biological functions by controlling essential nodes involved in cellular growth, differentiation and division. The elucidation of many events directed by these enzymes came initially from the discovery of diverse natural toxins that have been found to be potent and specific inhibitors of phosphatases (Berndt, 1999; Honkanen *et al.*, 1990; Ohta *et al.*, 1994; Sugiyama *et al.*, 1997). It is estimated that ~30% of cellular proteins are phosphorylated by kinases at a given time, implying that they are potentially submitted to a dephosphorylation process by phosphatases to control their activities. Protein Phosphatase type 1 (PP1) is considered as one of the major phosphatases involved in the control of numerous dephosphorylation steps. In this context, it has been reported that a decrease of PP1 activity by a reduction of its expression using antisense oligonucleotides resulted in a failure of cell division in a late stage of cytokinesis (Cheng *et al.*, 2000). Conversely, a hyperphosphorylation state of cellular proteins induced by an overexpression of some kinases blocked cell division (Sassoon *et al.*, 1999; Watanabe *et al.*, 1992; Wheatley *et al.*, 1997), indicating a fundamental role of the phosphorylation/dephosphorylation balance. Taken together, these observations point out that cell vitality and viability must be coordinated through multiple and tight regulations of both kinases and phosphatases. In eukaryotic cells a large number of endogenous proteins regulating PP1 have been identified, most of which have been found as 'permanent' ligands for this enzyme allowing the control of its localization, activity and/or its specificity (Bollen *et al.*, 2010). These regulators mainly comprise proteins with a degenerate sequence motif [K/R]-X0-1-[V/I]-{p}-[F/W], known as the RVXF binding motif to PP1 (Bollen *et al.*, 2010). Biochemical, interaction and genetic studies clearly indicated that PP1 regulators are as crucial as PP1 itself in the control of cell vitality and survival (Fardilha *et al.*, 2010). Hence, the multiple functions of PP1 seem to be organized and to operate according to the binding of distinct regulators. So far, more than 100 regulatory subunits of PP1 have been characterized, leading to a high number of holoenzymes which can explain the multiple and specific functions of this enzyme at different locations (Hendrickx *et al.*, 2009)

In *Plasmodium falciparum* (Pf), an apicomplexan parasite responsible for most of the morbidity and mortality attributable to human malaria, phosphatase activities and corresponding genes have been identified, including PP1 and PP2A (Bhattacharyya *et al.*, 2002; Dobson *et al.*, 1999; Dobson *et al.*, 2001; Li and Baker, 1997, 1998; Mamoun *et al.*,

1998). The use of natural toxins to phosphatases, such as okadaic acid (OA), indicated that blood stage parasites exhibited a high level of phosphatase activity associated with PP1 (Dobson *et al.*, 1999). In addition OA has been shown to inhibit parasite growth in vitro, mainly by blocking PP1-like activity (Yokoyama *et al.*, 1998). In this parasite, very little is known about the role of endogenous regulatory subunits of PP1, although we recently reported the first data on an inhibitory subunit of PfPP1, PfLRR1 (Daher *et al.*, 2006a). The gene product of PfLRR1 belongs to the Leucine Rich Repeat protein family, and is the ortholog of Sds22 described in yeast (Ohkura and Yanagida, 1991). We showed that PfLRR1 was able to interact physically with PfPP1 and to down-regulate its phosphatase activity. Our inability to obtain knock-out parasites for PfLRR1 (unpublished) and the fact that an overexpression of its ortholog in *Toxoplasma gondii* (Daher *et al.*, 2007) can impair parasite growth suggested an essential role of LRR1 in parasite survival.

In a continuing effort to characterize the regulators of PP1 in *P. falciparum*, a recent examination of its genome revealed the presence of a putative gene product encoded by PF10_0311 orf (designated in this study PfI3) which shared ~30% identity with inhibitor-3 (I3 in mammals, or Ypi1 in yeast) an essential regulator of PP1 expressed by different organisms (Takemiya *et al.*, 2009; Zhang *et al.*, 1998). In yeast, it has been shown that the deletion of Inhibitor 3 ortholog (Ypi1) is lethal for *Saccharomyces cerevisiae*, suggesting an essential function of the gene in the physiology of the yeast, and its depletion (conditional strain) affected the distribution of PP1 and provoked a blockage in anaphase with condensed chromosomes (Pedelini *et al.*, 2007). Indirect evidence supporting the idea that Inhibitor-3 inhibits PP1 is the fact that overexpression of this regulator reduces glycogen levels in the yeast because glycogen synthase requires dephosphorylation by PP1 to become active (Garcia-Gimeno *et al.*, 2003). Here, we report the identification and characterization of PfI3 using biochemical, structural and genetic approaches. Our results indicate that: 1) PfI3 is a partner of PfPP1 and, unlike other I3s, it acts *in vitro* as a positive regulator of PfPP1 towards a non-specific substrate; 2) NMR studies combined with GST pull-down experiments with wild type and mutated PfI3 proteins showed that the RVXF motif is the main binding site for PP1; 3) transfection experiments to obtain knock-out parasites strongly suggest its essentiality in blood parasite survival and 4) the generation of PfI3-GFP transgenic parasites revealed the PfI3 is mainly localized in the nucleus whatever the stage of blood parasite, suggesting the regulation of PfPP1 in this compartment.

Results

Molecular cloning and analysis of Pf Inhibitor-3.

BlastP analysis of PlasmoDB (http://www.plasmoDB.org) using known Inhibitor-3 sequences allowed the identification of PF10_0311 as a *P. falciparum* homolog. Amplification of the open reading frame using cDNA obtained from total RNA of erythrocytic stages and primers mentioned in Experimental Procedures showed a PCR product with the expected size. This confirmed the transcription of PF10_0311 in blood parasite stages and confirmed the microarray data available in PlasmoDB. The cDNA sequence, designated PfI3 in this work, confirmed the open reading frame (orf) predicted by PlasmoDB with only 1 different nucleotide at the position 263 (T → A), leading to a change of the amino acid sequence (L → Q) (Fig. 1.A). 3'-RACE combined with a walking approach on cDNA from the 5' side allowed the confirmation of the stop and start codons respectively. As *P. falciparum* proteomic data revealed the presence of a peptide covering the sequence PMHSSSTTTTTTYVQDTNTQNDTNENSSTIVR (a.a positions 3 to 33) (http://plasmodb.org/plasmo/showRecord.do?name=GeneRecordClasses.GeneRecordClass&source_id=PF10_0311&project_id=PlasmoDB; section protein features), it is very likely that the first methionine shown in Fig. 1.A corresponds to the correct start codon of PfI3.

The deduced amino acid sequence of the orf corresponds to a protein containing 116 aa with a predicted molecular mass of 13.1 kDa. The BLAST sequence analysis combined with visual inspection of PfI3 aa sequence of showed 31 % identity when compared to the human I3 (accession number CAC16920) and 28% with *S. cerevisiae* Ypi1 amino acid sequences (accession number NP_116658) respectively. However, the highest identity score (59% and 45% with the human and yeast counterparts respectively) was found in the middle of the sequence between aa 39 and 81 (Fig. 1.B). In this conserved sequence we identified the motif KVVRW which corresponds to the PP1-binding motif R/K(X_1)$_{0-1}$V/I(X_2)F/W where X_1 and X_2 can be any aa except proline for X_2. It is important to note that the PP1-binding motif mentioned above fits with 90% of the known PP1-binding proteins described so far (Wakula *et al.*, 2003)

Expression of the PfI3 gene product by *Plasmodium falciparum*.

To assess and to confirm the expression of the PfI3 gene product in *P. falciparum*, we raised polyclonal antibodies against a recombinant 6xHis-PfI3 fusion protein. These antibodies were able to recognize the recombinant protein in western blot analysis (Fig.2A). The recombinant protein shared a molecular weight of around 20 kDa, in agreement with the anomalous electrophoretic behaviour of I3 gene products of several species. However, immunoblot analysis using either soluble extracts from asynchronous erythrocytic parasites (20µg per lane) or whole parasites solubilized in loading buffer (10^6 parasites per lane) did not allow the detection of PfI3 with these antibodies. This could be due to the quality/low affinity of produced antibodies and/or to the low level of expression of PfI3 by *P. falciparum*. Based on the view that PfI3 could be a partner of PfPP1, we attempted to perform affinity purification of endogenous PfI3 from whole parasite extract using His-tagged PfPP1 retained on Ni-NTA beads. As shown in Fig. 2.B lane 2, antibodies against recombinant PfI3 reacted with one band at 20 kDa which corresponds to the SDS-PAGE migration of the recombinant PfI3. The presence of His-tagged PfPP1 in the eluted protein from the column was confirmed by the use of mAb anti-His antibody (Fig. 2.B lane 3).

Mapping of the PfPP1 interaction site on PfI3 using NMR

We used NMR (Nuclear Magnetic Resonance) spectroscopy to examine potential direct interaction(s) of PfI3 and PfPP1 and to additionally map the interaction site. The [^{1}H,^{15}N]-HSQC spectrum of free [^{15}N]-PfI3 shows the poor dispersion of the signals typical of globally disordered proteins (Fig. 3), as described for several interactors of human PP1 (Dancheck *et al.*, 2008). Most of the [^{1}H,^{15}N] signals were assigned to a specific amino acid residue in the protein sequence using classical 3D-spectra of a doubly labeled [^{15}N,^{13}C]-PfI3. The backbone assignment is not complete, however, due to weak signals in several regions of the protein ([58-71] and [91-106]). Nevertheless the assignment was spread over the entire length of the sequence, allowing us to probe the interaction with PfPP1 using HSQC spectra. Each [^{1}H,^{15}N] resonance in these spectra, corresponding to an amide group of an amino acid of PfI3 (except the proline residues), is sensitive to the chemical environment of the corresponding residue. Interaction translates into a modification of the chemical shift and/or a broadening of the signal of those residues located in the interaction region.

Comparison of [^{1}H,^{15}N]-HSQC spectra of [^{15}N]-PfI3, either free or in the presence of an equimolar amount of PfPP1, indeed showed perturbations of numerous signals, which are broadened (Fig. 3). This indicates a direct interaction between PfI3 and PfPP1. Comparison of

the intensities of the signal along the protein sequence, between the free or bound PfI3 protein, allowed the definition of a region of the protein [29-75] in which the corresponding resonances lose most of their intensity upon binding. This region corresponds to the sequence homologous to the PfPP1 binding site of yeast and human I3 (Fig. 1.B), including the RVXF binding motif. Two other segments [12-16] and [93-103], also show a decrease ratio of free versus bound resonance intensity, averaging 0.4. This suggests that these two regions could correspond to weak secondary binding sites of PfI3 to PfPP1.

Study of the interaction between PfI3 and PfPP1

From the above results, it seems that the region containing the KVVRW (RVXF motif) is the main binding site with PfPP1. Hence, GST-pull down experiments were carried out to explore the capacity of binding of recombinant PfI3 with PfPP1 and the contribution of the RVXF motif to this binding by replacing the W45 by A (PfI3W45A). Pull down experiments followed by western blot analysis (Fig. 4.A upper panel) showed that GST-PfI3 (lane 2) but not GST-alone (lane 1) was able to bind efficiently to the recombinant PfPP1protein. The pull down experiments carried out with mutated PfI3 (PfI3W45A) revealed only a very faint band corresponding to PfPP1 when the western blot was overexposed (Fig. 4.A, lane 3 upper panel), suggesting that the interaction between PfI3 and PfPP1 was mainly due to the KVVRW motif. Loading controls with GST and GST- PfI3 are shown in the lower panel of Fig. 4.A.

In order to further evaluate the binding capacity of PfI3 and PfI3W45A, the corresponding recombinant proteins coated to ELISA plates were used in binding experiments using labelled biotin-PfPP1. Results presented in Fig. 4.B evidenced the high capacity of PfPP1 to bind to PfI3 and the intensity of the signal was dependent on the amount of PfPP1 added. When PfI3W45A was coated, no significant binding to PfPP1 was observed. Based on the OD obtained and the quantity of PfPP1 added, it can be suggested that the KVVRW motif is necessary for PfI3 binding to PfPP1..

Genetic manipulations of Inhibitor-3 in *P. falciparum*

Previous observations revealed that the deletion of Inhibitor 3 (Ypi1) demonstrated an essential function of this gene in yeast physiology. In order to investigate the role of the I3 orthologue in the *Plasmodium* life cycle, we attempted to disrupt the *PfI3* gene using the pCAM vector system (Sidhu *et al.*, 2005). We transfected wild-type 3D7 parasites with a plasmid containing a 5' fragment derived from the genomic PfI3 sequence and the BSD gene,

conferring resistance to blasticidin (Fig. 5.A). We first attempted to detect the presence of the plasmid in transfected parasites by plasmid rescue. To this end, bacterial clones obtained after transformation with genomic DNA from blasticidin-resistant parasites were checked for the presence of the pCAM-BSD-I3 plasmid. As shown in Fig. 5.C lane 1, resistant parasites carried the correct construct. However, genotype analysis by specific PCR of stable transfectant parasites (3 independent transfection experiments) did not reveal the interruption of the gene (Fig. 5.D). The wild-type endogenous gene was still detectable in genomic DNA (Fig. 5.D, lane 1). The plasmid remained episomal even after prolonged culture (> 5 months of drug cycling, Fig. 5.C, lane 1 and Fig. 5.D, lane 2).

To check the accessibility for recombination of the genomic *PfI3* locus, we tried to modify the locus without causing loss-of-function of the gene product (Fig. 5.B). We transfected wild-type 3D7 parasites with a plasmid containing the 3'end of the PfI3 coding region fused to the haemagglutinin (HA) epitope. pCAM-I3-HA, blasticidin-resistant parasites showed the presence of the correct construct (Fig. 5.C, lane 2) and the integration of I3-HA into the *PfI3* locus (Fig. 5.E, lane 3). Further, western blot analysis of these resistant parasites using mAb anti-HA antibody revealed the presence of a specific band at about 20kDa (Fig. 5.F). Altogether these results indicate that the PfI3 locus is accessible to genetic manipulations. The unsuccessful attempts to recover I3 KO parasites suggest that I3 is required for the completion of the cycle in red blood cells *in vitro*.

Study of PfI3 function using the yeast model

The significant degree of similarity of yeast I3 (Ypi1) and PfI3 prompted us to examine whether expression of PfI3 might rescue Ypi1 depleted *S. cerevisiae*. First, we had to check whether PfI3 was able to interact with the yeast PP1 (also known as the *GLC7* gene product) by yeast two hybrid analysis. In these experiments, mammalian PP1α was also included. Unexpectedly and despite the sequence identity between all PP1s, (>85%) PfI3 did not show any interaction with mammalian PP1α as no ß-Gal activity was detectable (Table I). In contrast, the detection of β-Gal activity revealed an interaction of PfI3 with both PfPP1 and Glc7 (Table I and Fig. S1). Based on this latter result, we next carried out experiments aimed at complementing a yeast strain deficient for Ypi1 expression. To this end, we constructed an *ypi1Δ* conditional yeast strain mutant where Ypi1 was expressed at physiological levels from a centromeric vector under the control of the p*GAL* promoter In this way, cells were viable when growing in galactose containing media, but became unviable when cells were shifted to glucose containing media.. These cells were transformed either with empty vector (negative

control), pWS93-HA-PfI3 or pWS93-HA-Ypi1. Results presented in Fig. 6 show that the pWS93Ypi1 construct was able to restore the growth of the Ypi1 depleted cells in glucose media. However, the pWS93-HA-PfI3 construct, like the empty vector, failed to rescue the same depleted strain.

Western blots using extracts from transformed cells and mAb anti-HA antibody revealed that all strains tested expressed HA-PfI3 or HA-Ypi1 in similar amounts (Fig. 6.D). These results suggest the possibility that PfI3 is non-functional in yeast, even though it binds yeast PP1, or that it fulfils a role distinct from that of Ypi1.

Effect of PfI3 on phosphatase activity of PfPP1

Many observations in vitro have defined Inhibitor-3 as a regulatory subunit of PP1 in plants, mammals, and yeast that acts by decreasing the activity of the latter against different non-specific substrates including myelin basic protein, phosphorylase-a or p-nitrophenylphosphate (pNPP). Given that our results indicated that PfI3 was unable to complement the Ypi1 depleted yeast strain although it could interact with Glc7 (Table I), we investigated the effect of PfI3 on PfPP1 activity. No phosphatase activity could be detected with recombinant PfI3 alone when pNPP was used as substrate (not shown).

Unexpectedly, PfI3 strongly increased the dephosphorylation activity of PfPP1 in a concentration dependent manner (Fig. 7A, 7B). At all PfPP1 concentrations tested, the EC50 (effective concentration at which PfI3 confers 50% of maximal activation) was less than 500 nM of PfI3. The same experiments were repeated with PfI3W45A mutant but we did not observe any change in the PfPP1 activity (Fig. 7A, 7B).

To provide convincing evidence that PfI3 is an activator of PP1, it was important to investigate whether the activity of PfPP1 could be decreased by known inhibitors. To this end, we examined the effect of human I3 and yeast Ypi1 proteins on PfPP1 phosphatase activity. As shown in Fig. 7C, both proteins inhibit the phosphatase activity reaching almost 70 % inhibition (80 % for inhibitor 3) with the maximum amount assayed. Taking together all these data support the idea that the activation effect of PfI3 on PfPP1 phosphatase activity seems to be very specific and different from the mode of action of its mammalian and yeast homologs I3 and Ypi1.

Localization of PfI3

We next analyzed the localization of PfI3 protein in live 3D7 parasites transfected with pARL2 construct mediating the expression of full-length GFP-fused PfI3 (Fig 8A). It is

important to mention that the use of this vector by Kuhn *et al* showed that the trafficking was attributed to the sequence of the protein per se rather than to the promoter used (Kuhn *et al.*, 2010). The PfI3 GFP-tagged protein was successfully expressed and the integrity of the fused protein was maintained as observed by western blot analysis (Fig. 8.C). A single band with molecular mass of 48 kDa was observed, which is the expected molecular mass of the GFP tagged PfI3. Examination of the localization of PfI3 showed a distribution mainly in the nucleus of the parasite (Fig. 8.D) as it is demonstrated by the overlap of DNA staining with the fluorescence of PfI3-GFP. Examination of different parasite stages showed that the protein is imported into the nucleus of *P. falciparum* throughout the erythrocytic lifecycle. Even at the very early stage (young ring), the expression of PfI3 was observed in the nucleus (Fig. 8.D).

Discussion

Protein Phosphatase type 1 (PP1) belongs to the serine/threonine phosphatase family with representatives in animals (including helminth parasites), plants and in unicellular eukaryotes, including Apicomplexa that share a high level of identity of amino acid sequences (>80%) (Daher *et al.*, 2006a; Daher *et al.*, 2006b; Daher *et al.*, 2007). Several lines of evidence indicate that PP1 contributes to a wide range of physiological processes, including glycogen metabolism, smooth muscle contraction and sperm motility (Brady and Saltiel, 2001; Ceulemans and Bollen, 2004; Newgard *et al.*, 2000; Oliver and Shenolikar, 1998). Further observations pointed out that PP1 also exerts important functions within the nucleus. It has been shown that PP1 participates in the control of transcription by interacting with RNA polymerase II and in splicing process (Bennett, 2005; Hirano *et al.*, 1996; Moorhead *et al.*, 2007; Novoyatleva *et al.*, 2008). Moreover, it has been observed in many organisms that the impairment of PP1 activity leads to a mitotic arrest (Axton *et al.*, 1990; Baker *et al.*, 1997; Chen *et al.*, 2007; Ishii *et al.*, 1996; Thompson *et al.*, 1997). Further studies have shown that PP1 activity should be closely controlled to ensure correct centrosome separation and for the segregation/decondensation of chromosomes (Landsverk *et al.*, 2005; Lee *et al.*, 2010; Sassoon *et al.*, 1999).

In *Plasmodium falciparum*, although it has been shown that PP1 is essential for parasite survival and for the release of infectious merozoites (Bhattacharyya *et al.*, 2002; Blisnick *et al.*, 2006; Ward *et al.*, 1994; Yokoyama *et al.*, 1998), little is known about the expression of regulators of PP1 and on the nature of their exact functions. In this study, we have isolated a novel gene homolog to Inhibitor 3 (I3) of PP1, designated PfI3, which has significant sequence identity with I3 from a variety of organisms. Moreover, the inspection of its amino acid sequence revealed the presence of a KVVRW primary sequence that corresponds to the degenerate RVXF consensus sequence, identified as a binding motif to PP1 (Garcia-Gimeno *et al.*, 2003; Takemiya *et al.*, 2009; Zhang *et al.*, 1998). In order to assess whether PfI3 is a direct partner of PfPP1 and to map the PfI3 protein interactions regions, purified recombinant [^{15}N]-PfI3 and PfPP1 were used in NMR spectroscopy experiments. This approach confirmed that PfI3 is an unstructured protein and revealed the presence of a main binding site (Fig. 3, aa positions 29-75) containing the RVXF motif and two further weak zones of interaction at positions 12-16 and 93-103. The physical interaction between PfI3 and PfPP1 was confirmed by GST pull down assays and ELISA. Most importantly, mutation of the putative KVVRW motif present in PfI3 by substitution of W45 by A almost completely abolished the interaction between PfI3 and PfPP1. The results presented in this work suggest that the RVXF motif

functions as a primary anchor to PfPP1, subsequently promoting the interaction of secondary binding sites which can explain the observed NMR spectra. Our data concerning the implication of the RVXF motif are in agreement with previous binding data from both yeast and plant cells (Garcia-Gimeno *et al.*, 2003; Takemiya *et al.*, 2009) which revealed that this motif is critically required in PP1-binding. Regarding the region of PP1 involved in this interaction, structural studies of co-crystallized human PP1 and a peptide containing the RVXF motif demonstrated that the motif binds to a hydrophobic channel constituted by β-sheets within the Ct region of PP1 (Egloff *et al.*, 1997). Furthermore, the substitution of the phenylalanine residue of the binding motif by alanine abrogated the ability of the peptide to interact with PP1. Interestingly, the critical amino acids identified in the β-sheet conformation of mammalian PP1 are conserved in the sequence of PfPP1 (Bhattacharyya et al., 2002).

The detection of the expression of the PfI3 gene product by *P. falciparum,* attempted by direct western blot assays with total parasite extracts did not show any specific band using antisera raised against the recombinant protein. However, PfPP1 was able to pull-down endogenous PfI3 from parasite extract, clearly showing its expression by blood stage parasites. In order to evaluate PfI3 function(s) in *P. falciparum*, disruption of the corresponding gene was investigated. Although stable transfectants were obtained, no mutants could be selected with a disrupted *PfI3* locus. The absence of knock-out parasites was not due to the inaccessibility of its locus for genetic modifications as we were able to obtain knock-in parasites expressing HA tagged PfI3. These data suggest that PfI3 is essential for blood stage parasites and are in agreement with the *in vivo* functional studies of I3 in other organisms which demonstrated the indispensable role of I3 in cell survival and division. Studies carried out in yeast showed that the deletion of I3 (Ypi1) is lethal and its conditional suppression leads to the inhibition of cell growth of mid-mitosis (Garcia-Gimeno *et al.*, 2003; Pedelini *et al.*, 2007). More recently, genetic studies in *Arabidopsis thaliana* revealed that I3 disruption delayed the progression of embryogenesis and arrested the development at an early stage. In addition, the reduction of I3 expression by RNA interference led to a significant decrease in fertility (Takemiya *et al.*, 2009). Unfortunately, all attempts we made to induce RNA interference responses in *P. falciparum* have so far been unsuccessful (personal observations). In order to further examine the role of PfI3, we decided to determine whether it could function in yeast since both PP1 and I3 are highly conserved in this organism. We therefore used a conditional yeast strain deficient in the expression of I3 (Ypi1 gene product). Heterologous complementation experiments using PfI3 did not allow recovery of the growth of deficient yeast while homologous complementation did rescue the same strain. The absence of recovery did not

seem to be related to a defect in the interaction of PfI3 with yeast PP1. Our observation supports the idea that the PfI3-yeast PP1 complex is not able to fulfill similar functions to those accomplished by the homologous yeast complex and suggests that the action of I3 on PP1 in *P. falciparum* may be different from those described in other eukaryotes. The failure to generate *P. falciparum* mutants with a disrupted *PfI3* locus is not an absolute proof of the essential nature of the PfI3 protein and additional approaches are required. A few *Plasmodium* conditional protein expression systems have been reported, but none of these have been proven to be reproducible for regulating *Plasmodium* protein expression (de Koning-Ward and Gilson, 2009; Ward *et al.*, 1994)

It has been shown that human, plant and yeast I3 were able to regulate PP1 *in vitro* by inhibiting its activity towards different non-specific substrates including phosphorylase a, pNPP or myelin basic protein (Garcia-Gimeno *et al.*, 2003; Takemiya *et al.*, 2009; Zhang *et al.*, 1998; Zhang *et al.*, 2008). To further assess the regulatory role of PfI3, we determined whether the PfPP1 activity could be influenced by PfI3. In a previous study, we showed that recombinant PfPP1 is catalytically active and can hydrolyse pNPP (Daher *et al.*, 2006a). Surprisingly, the inclusion of PfI3 at nanomolar concentrations to the reaction significantly increased the PfPP1 activity. This increase did not occur when PfI3 was replaced by mutated PfI3W45A, indicating the importance of the W45 not only in the binding of PfI3, but also in the control of PP1 activity. Under the same conditions,, mammalian I3 and yeast Ypi1 proteins significantly decreased the activity of PfPP1, ruling out any methodological artefact. These results indicate that, at least *in vitro*, PfI3 has opposite effects on PfPP1 to other inhibitor 3 homologs. However, precautions should be taken in drawing conclusions concerning the *in vivo* role of I3 in *P. falciparum* and the determination of its exact function will await the further development of optimized conditional mutagenesis systems.

Having shown the ability of PfI3 to bind to PfPP1 and to regulate its activity, we next explored the localization of PfI3 by expressing it in *P. falciparum* as a fusion protein with fluorescent reporter molecule. Interestingly, fluorescence microscopy of parasites transfected with PfI3-GFP construct revealed a specific localization which overlapped the DNA staining, clearly pointing to a nuclear localization of PfI3 (Fig. 8). Examinations of blood parasites at different stages of growth demonstrated that there is no variation during the parasite cycle in its nuclear localization. These findings are in accordance with those reported for yeast where I3 was shown to be localized in the nucleus, like its PP1 partner (Bharucha *et al.*, 2008). Our previous studies, using the subcellular fractionation of blood parasites allowed the detection

of PfPP1 both in cytoplasm and nuclear extracts (Daher *et al.*, 2006a). Together, these observations suggest that PfI3 could have a modulatory effect on PfPP1 activity toward nuclear substrates.

The findings reported in this study, combined with previous observations on the expression of a second potential nuclear regulator (PfLRR1) of PfPP1 which we identified as a negative regulator (Daher *et al.*, 2006a), suggest that the regulation of PfPP1 must be coordinated and tightly controlled in the nucleus of *P. falciparum*. It is worthy of note that converging studies evidenced a major role for PP1 and its regulators in mitosis of many organisms (De Wulf *et al.*, 2009). Recent work by Wu *et al* on Hela and *Xenopus* cells (Wu *et al.*, 2009) suggests the implication of PP1 in a framework in which PP1 is blocked by inhibitor-1 during metaphase and a subsequent activation of PP1 ensuring the completion of mitosis. Based on the view that any anomaly of PP1 activity in eukaryotic cells affects spindle organization and nuclear separation with overcondensed chromosomes, it seems very likely that the dephosphorylation of nuclear proteins directly or indirectly involved in molecular motors is vital and dynamically governed by PP1-regulator complexes. This is line with the fact that our attempts to express PfPP1-GFP episomaly were unsuccessful (2 different transfections, not shown), supporting the conclusion that blood parasites are unable to accept exogenous expression of PfPP1 and any potential changes in the level of its activity.

Further studies will evaluate the ability of small molecules targeting the interface of interaction PfPP1-PfI3 in order to follow up the phenotype of treated parasites. These explorations may help not only in deciphering the function of PfI3 but also in identifying inhibitors that can participate in a new strategy for drug discovery against malaria.

Experimental procedures

Materials

Plasmids pQE30, pGEX4T3, pETDuet and pACT2 were purchased from Qiagen, Life Sciences, Novagen and Clontech respectively. Plasmid pCAM-HA, pCAM-GFP and pCAM were kind gifts of Dr C. Doerig, Inserm EPFL, Switzerland). GST-Ypi1 and GST-I3 recombinant proteins were prepared as previously described (Garcia-Gimeno *et al.*, 2003; Lesage *et al.*, 2007) pWS93 to tag proteins with 3X-HA epitopes and pBTM116 vectors for yeast two hybrid experiments have been previously described (Song and Carlson, 1998), (Vojtek,A.B., Cooper,J.A. and Hollenberg, S.M. 1997; The yeast two hybrid system. Oxford University Press). Plasmid construction pWS93-Ypi1 has been previously described (Garcia-Gimeno *et al.*, 2003), and. pBTM116-Glc7 was generated by subcloning the *BamHI* fragment obtained by digestion of pGAD-Glc7 plasmid previously reported (Tu and Carlson, 1995). The encoding region of PfI3 amplified with primers P15 and P16 (supplemental Table I), cloned initially in TA vector and sequenced, was cloned into BamHI-SalI sites and into EcoRI-SalI sites of pWS93 and PBTM116 vectors respectively. With respect to PfPP1, the encoding region amplified with primers P17 and P18 (supplemental Table I), initially cloned in pGBKT and sequenced, was cloned into *EcoRI-BamHI* sites and *SfiI-XhoI* sites of pBTM116 and pACT2 vectors respectively.

Monoclonal anti HA and anti-Myc antibodies were purchased from Roche and Invitrogen respectively.

Preparation of parasites

P. falciparum 3D7 clone was grown according to Trager and Jensen (Trager and Jensen, 1976), in RPMI-1640 medium with 10% human AB^+ serum, in the presence of O^+ erythrocytes. Cultures were maintained at 37°C in a humidified atmosphere (5 % CO_2, 5 % O_2 and 90 % N_2). Parasites were synchronized by a double sorbitol treatment as previously described (Vernes *et al.*, 1984). In order to isolate total RNA or proteins, parasitized erythrocytes were saponin lysed (Umlas and Fallon, 1971) and either resuspended in Trizol (Invitrogen) or in phosphate buffered saline containing EDTA-free protease inhibitor cocktail (Roche). For some experiments, infected red blood cells were purified using Percoll-sorbitol density gradients with slight modifications (Ginsburg *et al.*, 1987). Protein extracts were prepared from saponin-isolated parasites by resuspending the pellet in lysis buffer 1 (50mM Tris-HCl pH 7.4, 0.1 % SDS, 0.05% sodium deoxycholate and protease inhibitors cocktail) or lysis buffer 2 (50mM Tris pH 7.4,150mM

NaCl, 20 mM MgCl2, 1mM EDTA, 1mM DTT, 0.5% Triton X-100, 1% NP40, and protease inhibitors cocktail (Roche)) followed by 5 consecutive freeze/thawing cycles with intermediate homogenising steps using a micro-pestle and 0.7 mm glass beads (Sigma) and subsequent centrifugation at 13 000 rpm for 30 min at 4°C.

Cloning of full-size open reading frame and analysis of PfI3

All primers used throughout this study are listed in supplementary Table I. The encoding region of *PfI3* was initially obtained from first-strand cDNA derived from mRNA prepared from unsynchronized blood cultures of *P. falciparum* 3D7. The PCR was performed with the P1 and P2 primers using the advantage 2 PCR kit (Clontech). To confirm the stop codon, 3' RACE was carried out using the SMART kit (Clontech). The 3' end was obtained using the forward primer F2 and the adapter primer according to the manufacturer's instructions. To determine the start codon, 4 forward primers (P3, P4, P5 and P6) derived from the 5' upstream genomic region were tested in PCR on cDNA with the reverse R2 primer derived from the coding region. P1 and P2 primers amplified one PCR product of the expected size. PCR products were cloned in TA cloning vector (Invitrogen) and sequenced. Comparative analysis of the PfI3 protein was performed by DNA Star and ClustalW, and using the Pfam database (http://www.sanger.ac.uk)

Generation of P. falciparum transgenic parasites

The *PfI3* disruption plasmid (pCAM-*PfI3*) was generated by inserting a PCR product corresponding to a 5' portion from the *PfI3* sequence (550 bp) into the pCAM-BSD vector which contains a cassette conferring resistance to blasticidin. The insert was obtained using 3D7 genomic DNA as template and the oligonucleotides P19 and P20, which contain PstI and BamHI sites respectively. Attempts to check the accessibility of the *PfI3* locus were performed by transfecting wild 3D7 parasites with 3' tagging constructs. To this end, the 3′ end of the *PfI3* sequence (650 bp, omitting the stop codon) was amplified by PCR using 3D7 genomic DNA and the primers P21 and P22 containing PstI and BamHI restriction sites respectively. The 3' tagging plasmids were generated by inserting the PCR product into PstI and BamHI sites of the pCAM-BSD-hemagglutinin (HA) or GFP plasmids. Transfections were carried out by electroporation of ring stage 3D7 parasites with 75-100 µg of plasmid DNA, according to Sidhu et al. (Sidhu *et al.*, 2005). To select transformed parasites, 48h after transfection, Blasticidin (Invivogen) was added to a final concentration 2.5 µg/ml. Resistant parasites appeared after 3-4 weeks and were maintained under drug selection. Populations of

stably transfected parasites were obtained after 6 weeks. To enrich the populations for integrants, 3 to 4 cycles of on/off drug were applied.

Genotype and phenotype analysis of p. falciparum transfectants.

To confirm that transfected parasites contained the right constructs, plasmid rescue was carried out. Genomic DNA extracted (KAPA Express Extract, kapaBioSystems) from wild or transfected parasites were used to transform *E. coli* DH5α cells (Invitrogen). Plasmid DNA was then purified from bacterial clones and digested with PstI and BamHI.

Genotypes of *PfI3* knock-out parasites were analyzed by PCR on genomic DNA using standard procedures with the primers number P27 (derived from the 5' non-translated region and not present in the construct) and P168 specific for the pCAM-BSD vector. Genotypes of *PfI3* knock-in were analyzed using the primer P19 and P639 or P635 (reverse primer corresponding to HA or GFP respectively). The expression of PfI3-HA or -GFP fusion protein was checked by western blotting. Live parasites potentially expressing PfI3-GFP were analysed by fluorescence microscopy as described below.

Recombinant protein expression and purification

The full-length coding region of PfI3 obtained by PCR with the primers P7-P8 or P9-P10 was subcloned in pQE30 and pGEX4T3 respectively. For the expression of PfPP1 obtained with the primers P11 and P12, the pETDuet expression system was used. The restriction sites are mentioned in Table 1. Before cloning in expression vectors, all PCR products were subcloned in a TA cloning vector and verified by sequencing for the absence of any modification introduced by Taq polymerase.

To obtain the PfI3W45A mutant construct, we performed a PCR-based site-directed mutagenesis strategy using the constructions pQE30-PfI3 or pGEX4T3-PfI3 as templates, the primers P13 and P14 and Isis Proofreading DNA polymerase (Qbiogene). The PCR conditions consisted of 30 s at 95°C followed by 16 cycles at 95°C (30 s), 50°C (1 min) and 72°C (5 min). The parental DNA plasmid was then digested with DpnI and an aliquot was used to transform XL10-Gold Ultracompetent cells (Stratagene). Mutated plasmids, checked by sequencing for the replacement of W45 by alanine were used for the expression of PfI3 W45A recombinant protein.

Protein expression was carried out in the *E. coli* M15 strain for the pQE30 construct and the BL21 strain for pGEX4T3 and pETDUET constructs. The expression of PfI3 and PfI3W45A was carried out in the presence of 1 mM IPTG at 37°c for 3 hr. For the expression of PfPP1,

the culture was induced overnight at 16°C in the presence IPTG at 0.5mM and 2mM $MnCl_2$. Cells were harvested in sonication buffer (50 mM Tris, 1% Triton X-100, lysozyme 1mg/ml, 1 mM DTT and protease inhibitor cocktail). His- or GST-tagged proteins were purified according to manufacturer's instructions by Ni^{2+} chelation chromatography or glutathione agarose beads respectively (Sigma). With respect to the PfI3-His protein, the extract was loaded on a 1 ml nickel-NTA resin column (HiTrap, GE Healthcare). Washing steps were performed with a buffer containing 50 mM sodium phosphate (pH 7.8), 300 mM NaCl and 20 mM imidazole. Elution was done with a gradient from 50 to 250 mM imidazole. The eluted proteins were dialyzed against 50 mM Tris pH 7.4, NaCl 150mM. Under these conditions, the purity checked by SDS-PAGE followed by Coomassie-blue staining was >95%. The PfI3 recombinant protein was further subjected to peptide mass fingerprint by MALDI-TOF mass spectrometry to confirm its identity.

Preparation of isotope-labelled PfI3 protein and NMR spectroscopy

M13 bacteria containing pQE30-PfI3 construct were grown in minimal medium (M9) supplemented with $^{15}NH_4Cl$ with or without labelled [^{13}C]-Glucose as the nitrogen or carbon sources, respectively. Purification was performed by heating the cell extract for 15 min at 75°C and centrifugation, followed by Ni-affinity chromatography (Hiprep Ni-NTA, GE Healthcare) on the supernatant. The purified protein was then exchange to 50 mM ammonium bicarbonate buffer, using a Hiprep desalting column (HR16/60, GE Healthcare) before lyophilisation. Proteins were resuspended in NMR buffer for data acquisition, at 200 µM for the 3D-experiment series and 100 µM for 2D [^{1}H,^{15}N]-HSQC.

NMR buffer is 25 mM Trisd11 pH 6.8, 25 mM NaCl, 2.5 mM EDTA, 1 mM D_4-TMSP (TriMethyl Silyl Propionate) as proton chemical shifts internal reference and 5% D_2O. Additionally, 1 mM of Tri-(Hydroxypropyl) phosphine (THP) was used as reductor in the 3D-experiments that required several days of data acquisition, while 2.5 mM DTT (Dithiothreitol) was used for [^{1}H,^{15}N]-HSQC. NMR spectra were recorded at 20 °C on a Bruker DMX600 spectrometer equipped with a triple resonance cryogenic probe head (Bruker, Karlsruhe, Germany). Classical pairs of 3D HNCACB, HN(CO)CACB, HNCO, HN(CA)CO and HN(CA)N were processed using Bruker TOPSPIN 2.1 and used for the assignment of the backbone CA, CO, ^{15}N and $^{1}H_N$ atoms and CB.

NMR mapping of the interaction of PfI3 with PfPP1 was performed by comparison of [^{1}H,^{15}N]-HSQC spectra acquired with 2048 and 256 points in the direct and indirect dimensions, respectively. 64 scans were used for the control experiment with PfI3 alone (100 µM) and 256 scans for the interaction mapping experiment. The increased scan number in the

latter case is necessary to compensate for dilution due to the addition of an equivalent volume of 100 µM PfPP1 in phosphatase buffer to half of the [^{15}N]-PfI3 sample, resulting in an equimolar concentration of 50 µM each. To further ensure adequate comparison of these [^{1}H,^{15}N]-HSQC, intensities were normalized in each spectrum by the average value of the resonances of residues 110, 112 and 114 that are intense and not affected by the interaction. Peak picking and intensity measurements were performed using bruker TOPSPIN 2.1 software (Bruker, Germany).

GST Pull-down Assay and Immunoblot assays

Preparation of recombinant proteins for pull down assays was essentially as described above. Briefly, the *E. coli* extracts containing GST-PfI3 or GST-PfI3W45A (corresponding to a culture of 250 ml) were allowed to bind to glutathione beads overnight at 4°C. Beads were then washed twice with PBS and twice with binding buffer (50mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP40 and protease inhibitor cocktail). The beads were then incubated for 45 min at room temperature under gentle rotation in 200 µl of binding buffer containing 25 µg BSA and 10 µg of purified PfPP1. Beads were recovered by centrifugation, washed 6 times in the binding buffer, resuspended in SDS/PAGE buffer and loaded on a 12% denaturing polyacrylamide gel. GST alone was used as a control. Proteins retained by the affinity column were detected by immunoblot using anti-GST or anti-His monoclonal antibodies.

Measurement of binding of PfI3

Binding of recombinant PfI3 and PfI3W45A proteins to PfPP1 was assessed by an ELISA based assay. Plates were coated with 100 µl per well at 10µg/ml of either PfI3 or PfI3W45A protein in PBS overnight at 4°C. Following washings with PBS-tween 0.1%, the plates were blocked with PBS containing 0.5% gelatine for 1 hour at room temperature. Coated-plates were then incubated with different concentrations of biotinylated PfPP1 (labelled with biotin-NHS according to the manufacturer's instructions (Calbiochem)) in PBS- Tween 0.1% at 37°C for 2 hrs. After 5 washes with PBS-Tween 0.1%, binding was detected using streptavidin-HRP. After a period incubation of 30 min and 5 washes, TMB substrate (Uptima) was added and the reaction stopped using 2N HCl. The OD was measured on an ELISA plate reader at 450 nm. In these experiments, BSA was used as control. The statistical significance was calculated with the Mann-Whitney U test for nonparametric data. P values < 0.05 are considered significant.

Functional complementation of *S. cerevisiae*

For the complementation assays we constructed the conditional null mutant W303 *ypi1Δ::KANMX*4 [pGAL-HA-Ypi1], where the expression of Ypi1 is under the control of the *GAL1* promoter. In this way, when the cells are grown in media with galactose as carbon source they grow normally whereas when they are shifted to media containing glucose as carbon source they become unviable because they are Ypi1 depleted.

This strain was transformed with the plasmids indicated in the corresponding assay to overexpress different proteins and was grown during three days in media containing either Galactose or Glucose as carbon source.

Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in rich medium (YPD) or synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids (Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, pp. 164–165, Cold Spring Harbor Laboratory,Cold Spring Harbor, NY), containing the indicated carbon sources.

Assays for PfPP1 and effect of PfI3

The activity of PfPP1 with *p*-nitro-phenylphosphate (pNPP) as substrate was assayed exactly as previously described (Daher *et al.*, 2006a). To investigate the role of PfI3 on PfPP1 activity, different amounts of PfI3 were added to PfPP1 and preincubated for 30 min at 37°C before testing the PfPP1 phosphatase activity. Results are presented as mean of increase or decrease of phosphatase activity in comparison to PP1 incubated in the reaction buffer.

Localisation of PfI3.

For an episomal expression of PfI3-GFP, the full-length coding region of *PfI3* was amplified by PCR the primers P22 and P23 containing *XhoI* and *KpnI* restriction sites respectively. The PCR fragment was cloned into TOPO-TA cloning vector (Invitrogen) and its nucleotide sequence was verified. The PCR product was then subcloned in frame with GFP into the pARL vector (Yonne Kuhn Traffic 2010 11: 236-249) (Kind gift of Dr C. Sanchez Heidelberg, Germany) digested with *XhoI* and *KpnI.* The plasmid carries the human *dhfr* gene for selection with WR99210. Populations of stably transfected parasites were obtained after 6 weeks. Live parasites were analysed and images were recorded by fluorescence microscopy (Leitz DM RB equipped with a cool SNAP MPS52 camera).

1

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Figure legends

Fig.1. Nucleotide and deduced amino acid sequences of *P. falciparum* inhibitor 3 (PfI3). **A.** Amino acids are numbered to the right of the sequence. The sequences of 5' and 3' untranslated regions were obtained using primers derived from genomic DNA followed by PCR on cDNA and by performing 3'RACE respectively. **B.** Analysis of amino acid sequence of PfI3. PfI3 was aligned with the mammalian (Inhibitor 3) and yeast I3 (Ypi1) homologs using the Megalign program (DNAstar). The identical residues are shown in green. The box contains the KVVRW sequence which fits with the consensus sequence [K/R]-X0-1-[V/I]-{p}-[F/W], known as the RVXF motif and required for binding with PP1.

Fig.2. Expression of the PfI3 gene product by *P. falciparum*. **A.** Purified His-fusion PfI3 separated by 15% SDS-PAGE and blotted onto nitrocellulose (Red Ponceau staining, Lanes 1-3). **B**. Immunoblot analysis of recombinant PfI3 with rat prebleed sera (lane 1), with rat anti-PfI3 antisera (lane 2) and with mAb anti-His (lane 3) showed a single band at ~ 20kDa, indicating an anomalous electrophoretic migration of PfI3 (expected size 13kDa). The identity of the purified recombinant PfI3 has been further confirmed by MALDI-TOF mass spectrometry**. B.** Detection of endogenous PfI3 in total proteins extracted from asynchronous cultures of *P. falciparum*. Total protein extracts (10mg) pre-cleared on Ni-NTA sepharose beads were incubated overnight with 6xHis-tagged PfPP1 affinity Ni-NTA column. After washings, proteins eluted with SDS-PAGE loading buffer were migrated and blotted to nitrocellulose. The blots were probed with preimmune serum (lane1), anti-PfI3 (lane2) or with anti-His mAb antibodies (lane3). The blots were revealed as described in Materials and Methods.

Fig.3. NMR mapping of the PfI3 site interacting with PfPP1. Overlayed [^{1}H-^{15}N]-HSQC of ^{15}N-PfI3, free (Gray spectrum) and in presence of an equimolar amount of PfPP1 (Red spectrum). Resonances are annotated. Interaction induces broadening of numerous resonances; see for example the isolated G73 resonance of free PfI3, in the upper part of the spectrum. (The ratio of the intensity of a given resonance in the free PfI3 spectrum (Gray) and in the 1:1 PfI3:PfPP1 spectrum (red), normalized on the average value of the intensities of resonances of residues 110, 112 and 114 are reported along the PfI3 sequence.

Fig.4. Interaction studies of PfI3 with PfPP1 *in vitro*. **A.** GST-Pull down assays. Glutathione-agarose beads coupled with GST alone (lane1), GST-PfI3 bound to beads (lane2) or GST-PfI3W45A bound beads (lane3) were incubated with 6xHis-tagged PfPP1. After washes, proteins bound to the beads were separated by 15% SDS-PAGE and blotted to nitrocellulose. Immunoblot analysis was performed with anti-His mAb (upper blot) and mAb anti-GST antibodies (lower blot) providing loading controls for bound GST and GST-fusion proteins. **B.** Quantification of the binding capacity of PfPP1 to PfI3 using an ELISA based technique. Increased quantities of biotinylated PfPP1 were added to wells coated with recombinant PfI3 or PfI3W45A proteins (1µg/well) Results representative experiments carried out with 2 different batches. Bars indicate SEM. * $p< 0.05$, ** $p< 0.001$ when compared either to BSA or PfI3W45A.

Fig.5. Targeted gene disruption and HA-tagging of the *PfI3* locus. (**A**), The gene-targeting construct for gene disruption by single homologous recombination using the pCAM-BSD, and the locus resulting from integration of the knock-out construct. (**B**), Epitope tagging of PfI3 by knock-in strategy. Insertion of an HA epitope tag at the Cterminus of *PfI3* by single homologous recombination (knock-in). The locations of the primers (P19, P20, P21, P22 P27, P167, P168 and P639) used for PCR analysis are indicated as well as the blasticidin-resistance cassette (BSD). **C,** Plasmid rescue experiments showing the presence of pCAM-PfI3 (lane1), pCAM-PfI3-2HA (lane2) constructs in transfected parasite culture. **D,** Analysis of pCAM-PfI3 transfected 3D7 culture by PCR; lanes 1, 2 and 3 correspond to DNA extracted from transfected parasites; lanes 4, 5 and 6 correspond to DNA extracted from wild-type parasites. Lanes 1 and 4 represent the detection of a portion of the wild type locus (PCR with P19 and P20), lanes 2 and 5 represent the detection of episomal DNA (PCR with P167 and P168) and lanes 3 and 6 represent the detection of the integration at the 5' end of the insert (PCR with P27 and P168). The absence of amplification of a PCR product using genomic DNA prepared from transfected parasite culture and using P27 and P168 as primers indicates the lack of homologous recombination (lane 3). **E**. Analysis of pCAM-PfI3-2HA transfected 3D7 culture by PCR; lanes 1, 2 and 3 correspond to DNA extracted from transfected parasites; lanes 4, 5 and 6 correspond to DNA extracted from wild-type parasites. Lanes 1 and 4 represent the detection of a portion of the wild type locus (PCR with P21 and P22), lanes 2 and 5 represent

the detection of episomal DNA (PCR with P167 and P639) and lanes 3 and 6 represent the detection of the integration at the 3' end of the insert (PCR with P19 and P639). The amplification of a PCR product at ~ 600pb using genomic DNA prepared from transfected parasites indicates the homologous recombination and integration of the 2-HA tag construct in endogenous *PfI3* (lane 3). **F**. Immunoblot analysis of total extracts of transfected 3D7 with pCAM-BSD-PfI3-2HA (lane1) and wild 3D7strain transfected (lane2) 3D7 culture mAb anti-HA antibody.

Fig..6. Study of PfI3 function using the yeast model. Complementation assays using the conditional null *ypi*Δ mutant W303 *ypi1Δ::KANMX*4 [pGAL-HA-Ypi1], were done by transforming this strain with the plasmids expressing the proteins indicated in the Fig. (HA: pWS93, HA-PfI3: pWS-PfI3, HAYpi1: pWS-Ypi1) The transformants were grown during three days in selective media with Glucose (GLU) or Galactose (GAL). The Fig. is representative of the results obtained assaying at least four different transformants Anti HA immunoblot is representative of the expression of the HA-tagged PfI3 or Ypi1.

Fig.7. Effect of PfI3 and PfI3W45 on PfPP1 phosphatase activity. Recombinant PfPP1 at 71nM (Fig. 7.A**)** or at 143nM (Fig. 7.B) were pre-incubated for 30 min at 37°C with different concentrations of PfI3 or PfI3W45A before the addition of pNPP. O Represents the relative phosphatase activity in the presence of different concentrations of recombinant 6xhis-tagged PfI3. ● Represents the relative phosphatase activity in the presence of different concentrations of recombinant 6xHis-tagged PfI3W45A. Fig. 7C represents the inhibition of Pf-PP1 phosphatase activity by Inhibitor 3 and Ypi1. Pf PP1 at 122 nM was preincubated for 10 min with different amounts of GST-Inh3 (○) or GST-Ypi1(●) before the addition of pNPP. Results presented as % of relative increase or decrease are means ± SEM for three independent experiments performed in duplicate.

Fig.8. Expression of PfI3 gene products by transfected *P.falciparum*. **A,** Schematic representation of the pARL2-PfI3-GFP used for episomal expression of PfI3. The construct contains the complete open reading frame of PfI3 in fusion with GFP. **B,** Plasmid rescue experiments showing the presence of pARL2-PfI3-GFP constructs in transfected parasite

culture **C.** Immunoblot analysis of pARL2-PfI3-GFP transfected *P. falciparum*. Protein extracted from wild-type parasites (lane1) or from transfected parasites (lane2) were subjected to western-blotting and probed with anti-GFP antibodies. **D,** Expression and localization of PfI3-GFP throughout the erythrocytic cell cycle of *P. falciparum.* Parasites were transfected as described in Material and Methods section and live transfectants were analysed by fluorescence microscopy.

A

```
CTAAAAAATATATTGTAGGGTTGTTTGAATTTATAATATAATTATATGTATATATATATATA
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TTCTATCTTTTTATATATAATGTGTCCTATGCATTCATCATCCACAACCACTACTACGTATG
                    M  C  P  M  H  S  S  S  T  T  T  T  T  T   14

TTCAAGATACGAATACGCAAAATGATACTAATGAAAACTCTAGTACAATTGTAAGAATATTA
V  Q  D  T  N  T  Q  N  D  T  N  E  N  S  S  T  I  V  R  I  L  35

AAACTGGCTCCACAAAAAGTTGTTAGATGGGATGAGAACACTATAGATAATGAAAATGCTCA
 K  L  A  P  Q  K  V  V  R  W  D  E  N  T  I  D  N  E  N  A  Q 56

AAAGAAGTCATCCAAAGTATGCTGCATTTATCACAAACCAAAAAATTTTGGTGAAAGTTCTG
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 S  C  K  K  D  N  P  N  N  D  K  N  E  E  L  E  L  K  K  *    116

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AAAAAAA
```

B

```
PfI3          MCPMHSSSTTTTTTVQDTNTQNDTNENSSTIVRILK------- 36
Inhibitor3...M--AEAGAGLSETVTETTVTVTTEPENRSLTIKLRK------- 34
Ypi1.........M--SGNQMAMGSEQQQTVGSRTVSVEEVPAVLQLRATQDPPRS 41

PfI3.............---LAPQKVVRWDENTIDNENAQKKSSKVCCIYHKPKNFGESS 76
Inhibitor3...---RKPEKKVEWTSDTVDNEHMGRRSSKCCCIYEKPRAFGESS 74
Ypi1.........QEAMPTRHNVRWEENVIDNENMNKKKTKICCIFHPQNEDEEEC 86

PfI3.........DSESDLDSDVEQPDTQKKCNSS-CKKDNPNNDKNEE------- 111
Inhibitor3...T-ESDE-------EEEEGCGHTHCVRGHR---KGRRRATLGPT 107
Ypi1.........NHHSD------DDGSSSSGSSSESENEKDLDFNERRQRRLER 123

PfI3.............----LELKK.......................... 116
Inhibitor3...PTTPPQPPDPSQPPPGPMQH.............. 126
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Fig. 2.

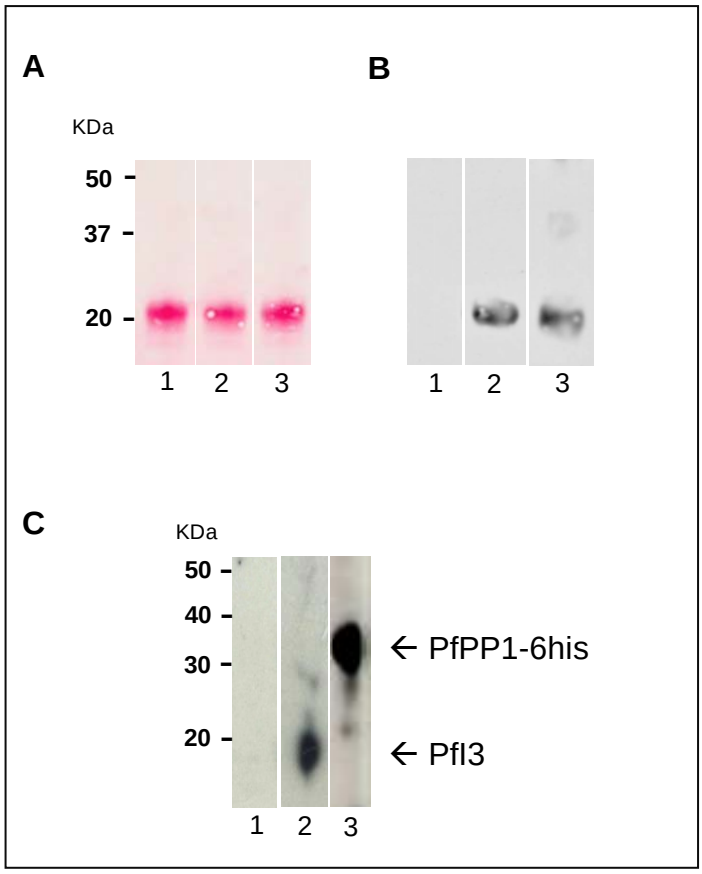
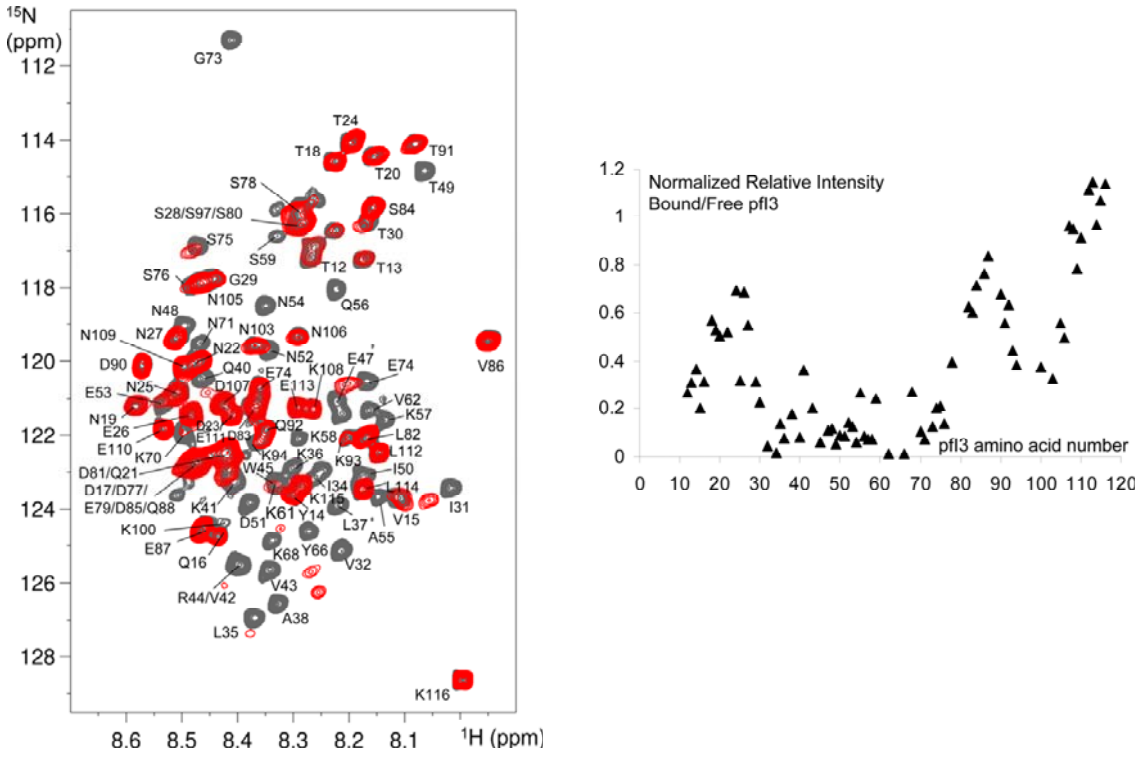


Fig. 3.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116
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120
122
124
126
128
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8.5
8.4
8.3
8.2
8.1
1H (ppm)
G73
T24
T18
T91
T20
T49
S78
S28/S97/S80
S84
T30
S75
S59
T12
T13
S76
G29
N105
N54
Q56
N48
N71
N109
N27
N103
N106
N22
N52
E47
E74
V86
D90
Q40
E74
K108
E53
N25
D107
E113
V62
K57
N19
E26
D23/E111/D83
Q92
K58
L82
E110
K70
K94
K36
K93
L112
D81/Q21
W45
I50
D17/D77/
E79/D85/Q88
K41
I34
K116
L114
I31
K61
Y14
L37'
V15
K100
D51
A55
E87
Q16
K68
Y66
V32
V43
R44/V42
A38
L35
K116
1.2
1
0.8
0.6
0.4
0.2
0
Normalized Relative Intensity
Bound/Free pfl3
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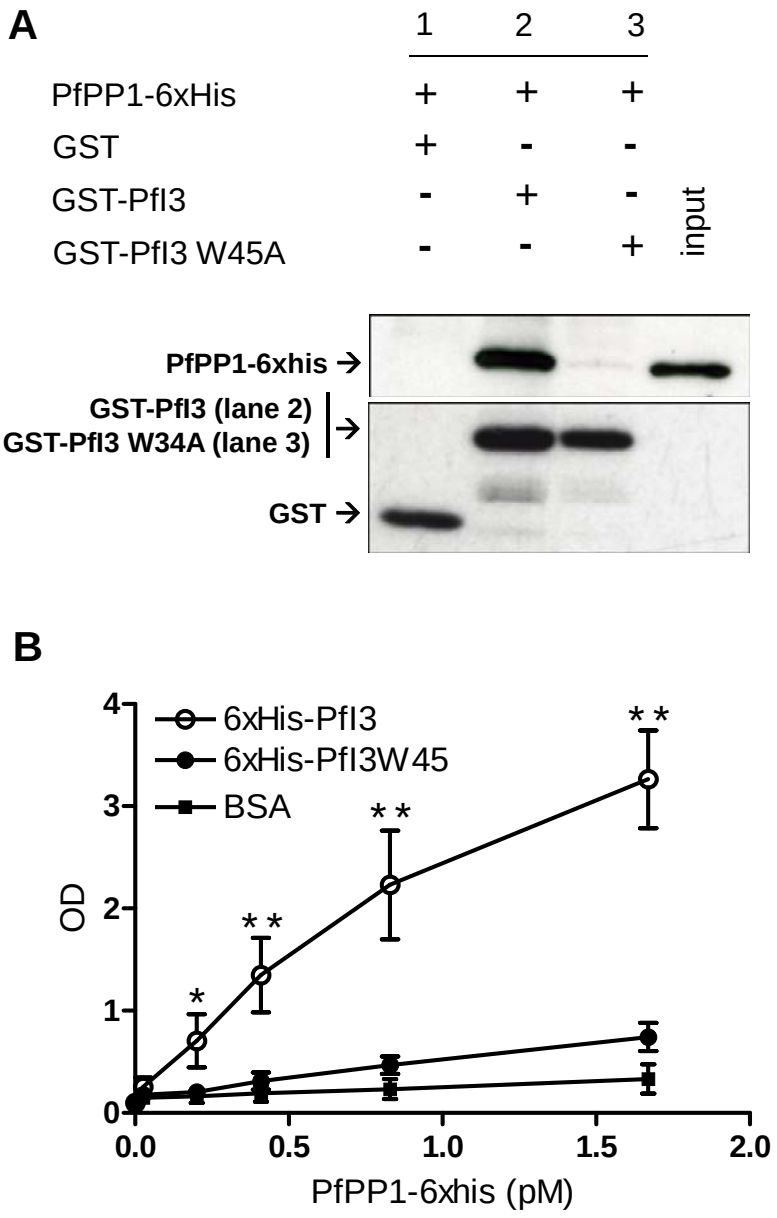
20
30
40
50
60
70
80
90
100
110
120

Fig. 4.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+
+
GST
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-
-
GST-PfI3
-
+
-
GST-PfI3 W45A
-
-
+
input
PfPP1-6xhis →
GST-PfI3 (lane 2)
GST-PfI3 W34A (lane 3)
→
GST →
B
6xHis-PfI3
6xHis-PfI3W45
BSA
OD
PfPP1-6xhis (pM)
0
1
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3
4
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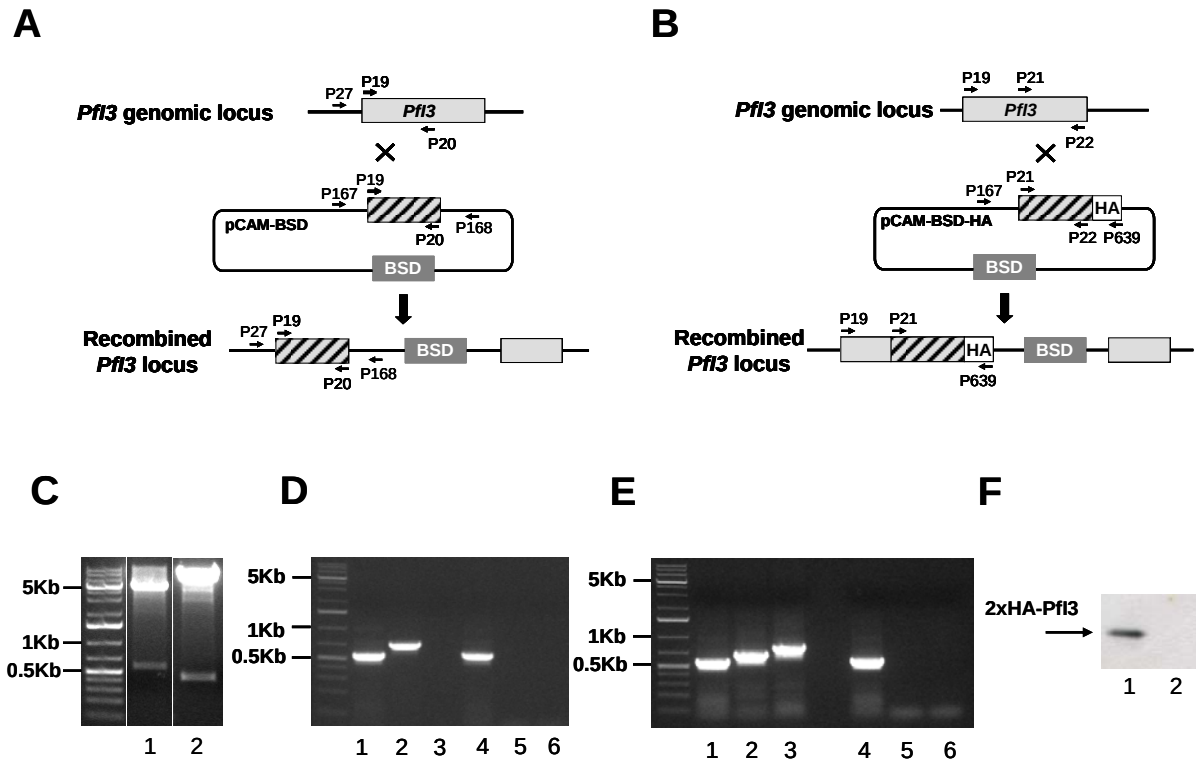
0.5
1.0
1.5
2.0
*
* *
* *
* *

Fig. 5.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Pfl3
P20
P167
P19
pCAM-BSD
P20
P168
BSD
Recombined
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P27
P19
BSD
P20
P168
B
Pfl3 genomic locus
P19
P21
Pfl3
P22
P167
P21
HA
pCAM-BSD-HA
P22
P639
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Pfl3 locus
P19
P21
HA
BSD
P639
C
5Kb
1Kb
0.5Kb
1 2
D
5Kb
1Kb
0.5Kb
1 2 3 4 5 6
E
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1Kb
0.5Kb
1 2 3 4 5 6
F
2xHA-Pfl3
1 2

Fig. 6.

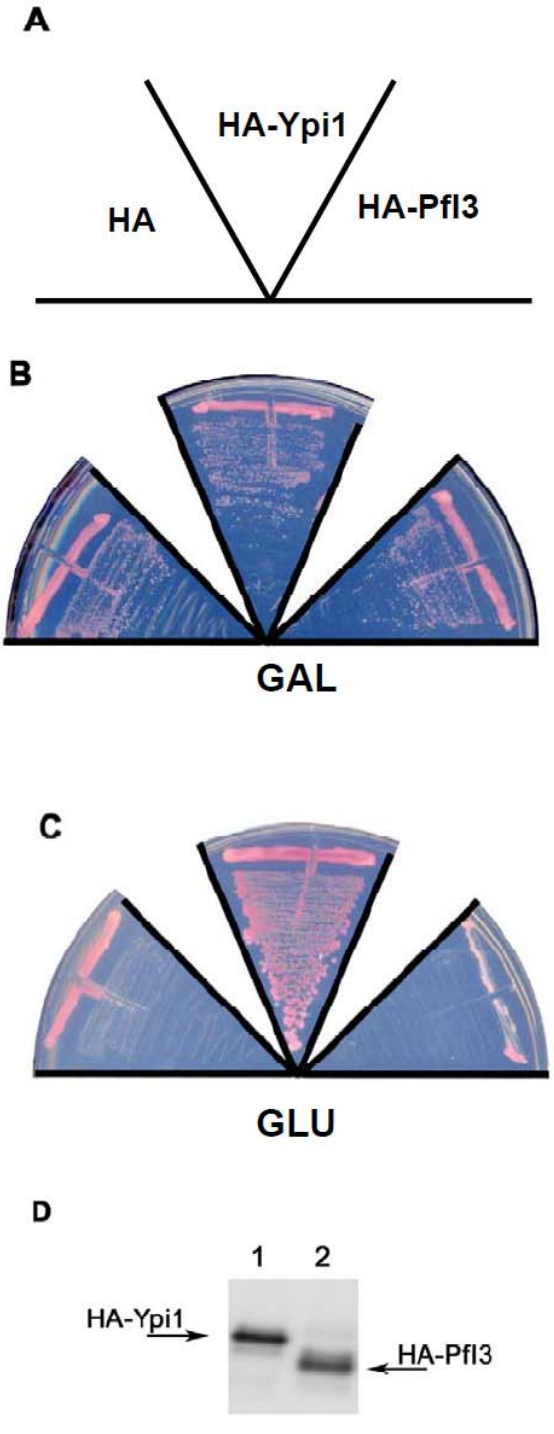
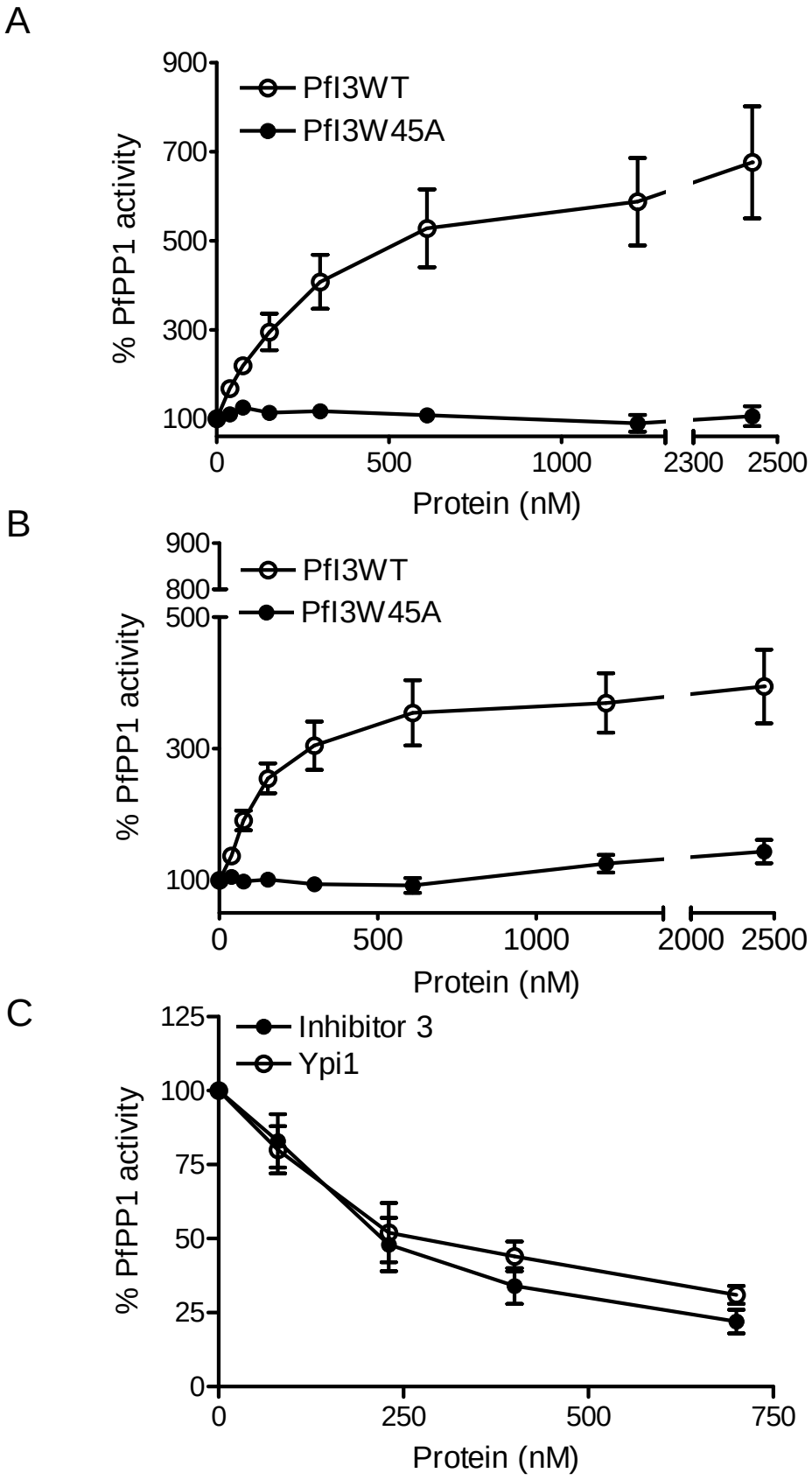


Fig.7.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500
300
100
PfI3WT
PfI3W45A
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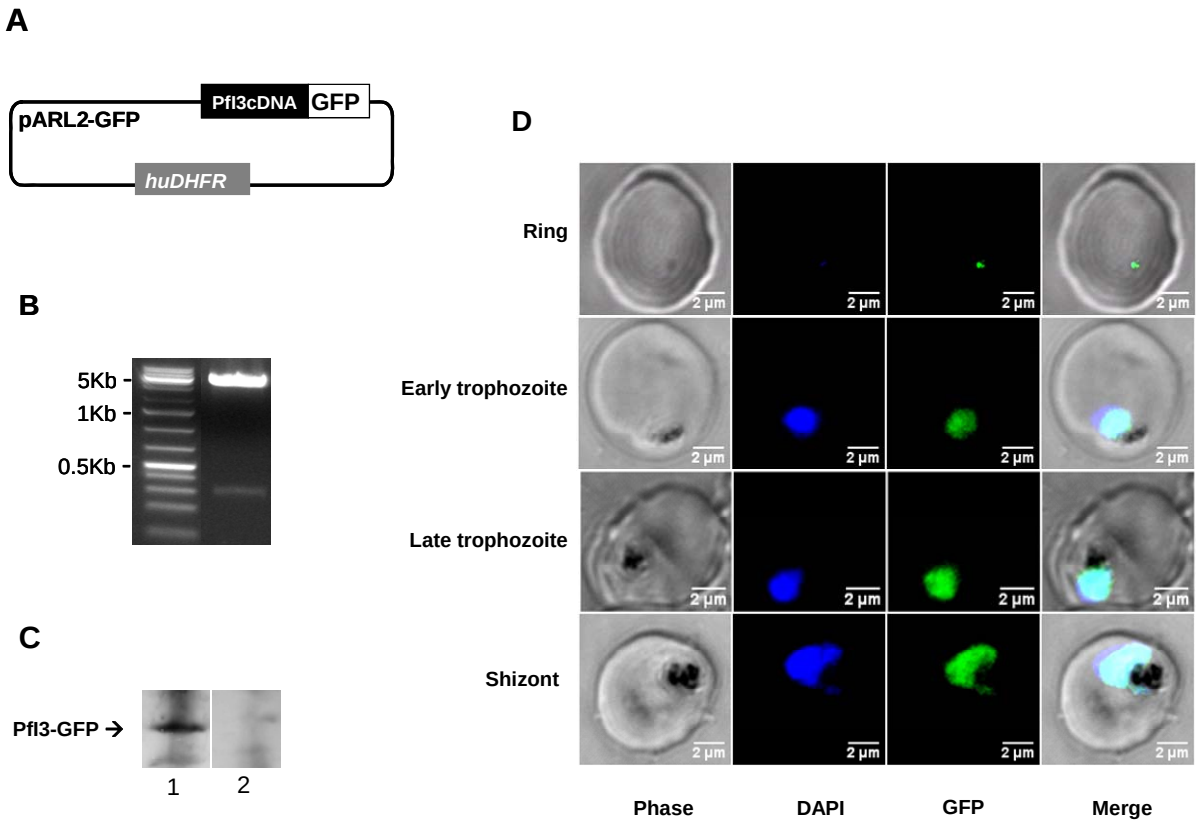
500
1000
2300
2500
Protein (nM)
B
900
800
500
300
100
PfI3WT
PfI3W45A
% PfPP1 activity
0
500
1000
2000
2500
Protein (nM)
C
125
100
75
50
25
0
Inhibitor 3
Ypi1
% PfPP1 activity
0
250
500
750
Protein (nM)

Fig. 8.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Pfl3cDNA
GFP
huDHFR
B
5Kb
1Kb
0.5Kb
C
Pfl3-GFP
1
2
D
Ring
Early trophozoite
Late trophozoite
Shizont
2 μm
Phase
DAPI
GFP
Merge

Table I

β-gal assay	GAD	GAD-PfI3	GAD-PfPP1
LexA	-	-	-
LexA-PfPP1	-	+	
LexA-Glc7	-	+	
LexA-PP1α	-	-	
LexA-PfI3	-		+++
LexA-Ypi1	-		+++

Table 1

CTY.5d strain was transformed with each pair of plasmids indicated in the table. The empty vectors (pBTM116 and pACT2) were used as controls Transformants were grown on selective media plates and transfer to nitrocellulose filters to carry out beta-galactosidase assays. At least six transformants of each pair were checked by this assay. The intensity of the interaction is specified as folllows: -: no interaction; +: low interaction, +++: High interaction.