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1 **The multifaceted role of Protein Phosphatase 1 in *Plasmodium***

2 Jamal Khalife<sup>1\*</sup>, Aline Fréville<sup>1,2,P</sup>, Bénédicte Gnangnon<sup>1,P</sup> and Christine Pierrot<sup>1</sup>

3

4 <sup>1</sup> Center for Infection and Immunity of Lille - Biology of Apicomplexan Parasites - UMR  
5 9017 CNRS - U1019 INSERM - Université de Lille - Institut Pasteur de Lille

6 <sup>2</sup> current address: Department of Infection Biology, London School of Tropical Medicine and  
7 Hygiene, London, United Kingdom.

8 \*Correspondence: [jamal.khalife@pasteur-lille.fr](mailto:jamal.khalife@pasteur-lille.fr) (J. Khalife)

9 <sup>P</sup> : AF and BG equally contributed to this review

10

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12

13 **Abstract**

14 Protein Phosphatase type 1 (PP1) forms a wide range of Ser/Thr specific phosphatase  
15 holoenzymes which contain one catalytic subunit (PP1c), present in all eukaryotic cells,  
16 associated with variable subunits known as regulatory proteins. It has recently been shown  
17 that regulators take a leading role in the organization and the control of PP1 functions. Many  
18 studies have addressed the role of these regulators in diverse organisms including humans and  
19 investigated their link to diseases. In this review we summarize recent advances on PP1c in  
20 *Plasmodium*, its interactome and regulators. As a proof of concept, peptides interfering with  
21 the regulator binding capacity of PP1c were shown to inhibit the growth of *P. falciparum*,  
22 suggesting their potential as drug precursors.

23

## 1 **Reversible protein phosphorylation: An Overview**

2 The processes of protein phosphorylation and dephosphorylation are involved in most aspects  
3 of cell biology by contributing to growth, development and division. These central roles are  
4 ensured by protein **kinases** (see Glossary) and **phosphatases** that add and remove phosphate  
5 groups respectively from serine, threonine and tyrosine residues of proteins in eukaryotes and  
6 have a huge impact on start and stop decisions of their functions by affecting structures and  
7 shapes. The rapidly acquired and deep understanding of how kinases control diverse cellular  
8 functions at the molecular levels and their direct connection to cancer due to mutations,  
9 dysregulation or overexpression has brought these enzymes to the forefront of the search for  
10 effective inhibitors of kinases for cancer chemotherapy. The progress and development of  
11 inhibitors towards kinases have been highlighted in two recent reviews in which most of the  
12 FDA-approved inhibitors are discussed [1, 2].

13 The involvement of phosphatases in diverse cellular functions began to emerge through the  
14 discovery of natural toxins with inhibitory activities against these enzymes and converging  
15 studies using diverse biochemical and biological approaches underline the specific and key  
16 roles played by several phosphatases [3]. Among serine/threonine phosphatases, Protein  
17 Phosphatase type 1 (PP1) is one of the most broadly and best studied. It is estimated that PP1  
18 accounts for most of dephosphorylation processes with specific activities despite the identity  
19 of the central region of its catalytic subunit with other phosphatases [4-6]. PP1 has been  
20 shown to be essential in cell cycle progression through its ability to regulate the level of  
21 phosphorylation of major mitotic kinases [7-9]. More recent studies established that PP1 loss  
22 of function induces termination defects of transcription [10, 11]. PP1 holoenzyme formation  
23 results from the association of the catalytic subunit (PP1c) with variable partners (designated  
24 as **PIPs** for Phosphatase type 1-Interacting Proteins), known as regulatory subunits, which  
25 direct dephosphorylation of hundreds of substrates at the right place and right time [3].  
26 Interestingly, along with the fact that interfering with the function of PP1c is lethal, the  
27 deregulation of PP1c/PIPs interactions has been shown to be involved in several human  
28 diseases [12]. In consequence, drug targeting of these holoenzymes is now being more  
29 carefully interrogated and considered although it is still challenging. A recent study based on  
30 the inhibition of a PP1c/PIP interaction characterized a lead that showed efficacy in a mouse  
31 model of Huntington's disease [13, 14]. Powerful arguments from mouse genetic studies that  
32 support the need to pursue targeting the PP1 holoenzymes have been elegantly reported in a  
33 recent review, suggesting that promising ways to develop selective and potent drugs involve  
34 the disruption of interactions between PP1c and PIPs [12].

1 In this review, we summarize recent advances in the role of phosphatases during the malaria  
2 parasite life cycle and focus on PP1c and its PIPs. We also discuss the associated structural  
3 and functional networks and potential means to interfere with these functions for medical  
4 benefits.

5

## 6 **Phosphatases in *Plasmodium*: Where do we stand?**

7 *Plasmodium* species affecting humans are responsible for an estimated 228 million malaria  
8 cases worldwide, mainly in Africa where they cause major health, social and economic  
9 problems. *P. falciparum* remains accountable for the greatest part of this burden [15]. A better  
10 understanding of *Plasmodium* biology remains essential to refuel the research strategies and  
11 to reveal druggable pathways to tackle the development of this deadly parasite.

12 *Plasmodium* has an elaborate and fascinating life cycle to adapt and to survive within  
13 different hosts and tissues [16]. It is transmitted back and forth between a vertebrate  
14 (intermediate host) and an *Anopheles* mosquito (definitive host), in which a bottleneck phase  
15 is rapidly followed by a period of replication, leading to the generation of thousands of  
16 progeny ready to spread the infection. One of the most intriguing features of *Plasmodium*  
17 development is the closed mitosis strategy used for replication, which involves the formation,  
18 during **schizogony** and **sporogony**, of a **syncytium** followed by **budding** and **cytokinesis**  
19 [17, 18]. Equally interesting is the **gametogenesis** where three rounds of rapid DNA  
20 replication occur within minutes to generate 8 fertile male gametes (**Figure 1**). As those  
21 processes rely on highly flexible, adaptable and parasite-specific mechanisms, they are of  
22 great interest for the malaria scientific community aiming to uncover *Plasmodium*-specific  
23 druggable pathways.

24 Recently, with the massive technological improvement of reverse genetics, multi-omics tools,  
25 as well as the availability of databases to speed up data mining, major breakthroughs have  
26 been achieved in deciphering the role of phosphatases in the *Plasmodium* life cycle.  
27 Functional screens and characterization of engineered parasite lines led to the identification of  
28 crucial phospho-signaling pathways affecting *Plasmodium* specific development [19-23].

29 Although some of the phosphatases identified in *Plasmodium* are conserved across eukaryotic  
30 phyla which were reported in a recent review [23], others belong to subfamilies restricted to  
31 apicomplexans and plants (Kelch-like domain containing protein phosphatase (PPKL) and  
32 Shewanella-like phosphatases (SLP)) or are parasite-specific such as the N-terminus EF-hand  
33 motif protein phosphatase (EFPP) [24]. Functional analyses conducted on the unique member  
34 of the PPKL subfamily revealed its involvement in **ookinete** shape and motility and further

1 transmission in the mosquito [25, 26]. In *Plasmodium berghei* (*Pb*, a rodent malaria parasite,  
2 exploited as a model for *P. falciparum* infection), SLP1 has been shown to be crucial to  
3 microneme formation and ookinete development. Ultimately, its deletion resulted in a  
4 complete inhibition of oocyst formation [27]. In *P. falciparum*, SLP2 has been found enclosed  
5 in vesicles at the apex of merozoites and was first proposed to be involved in the invasion  
6 processes [28] although recent studies suggest that the protein is more likely dispensable  
7 during the whole life cycle of both *P. berghei* and *P. falciparum* [19, 29].

### 9 ***Pf*PP1c, cornerstone of dephosphorylation processes**

10 PP1c emerged after the divergence of Archaea and eukaryotes and was among the first  
11 phosphatases biochemically characterized and cloned. It contains a single polypeptide chain  
12 with a biologically active domain [30, 31] and was reported as one of the most active  
13 phosphatases with its ability to increase the dephosphorylation rate by a factor of  $10^{21}$  [32].  
14 PP1c is extremely well conserved as illustrated not only at the protein sequence identity level  
15 [33] but also by its capacity to rescue phenotypes linked to a PP1c loss-of-function via cross-  
16 complementation between diverse organisms, including *Plasmodium* [33-35].

17 Unlike the human genome, which contains three *PP1c* genes encoding four isoforms ( $\alpha$ ,  $\beta/\delta$   
18 and  $\gamma$ ), the *Plasmodium* genomes encode only one PP1 catalytic subunit [23]. *Pf*PP1c is 80%  
19 identical (88% similar) to *Hs*PP1c and its predicted secondary and overall tertiary structures  
20 are also well conserved, with 9  $\alpha$ -helices and 11  $\beta$ -strands (**Figure 2**). The catalytic site is  
21 located at the surface of the protein as described for *Hs*PP1c [36, 37]. Surprisingly,  
22 *Plasmodium* PP1c lacks a portion of 18 AA at the Ct side when compared to its human  
23 counterpart. This part contains a threonine residue (Thr320) whose phosphorylation is  
24 dynamically controlled, playing an extremely important role in the regulation of mammalian  
25 PP1c activity and in particular in cell mitosis entry [38, 39]. Hence this type of regulation by  
26 PP1c does not seem to take place in *Plasmodium*.

27 *Pf*PP1c is expressed throughout the parasite **Intraerythrocytic Development Cycle (IDC)** with  
28 an upregulation of its expression from the trophozoite up to the schizont stage. It is localized  
29 in the parasite nucleus and cytoplasm [40-42]. This was further confirmed in *P. berghei* where  
30 *Pb*PP1c was additionally found to be expressed in sexual and liver stages [19, 43]. *Pf*PP1c has  
31 also been localized at Maurer's clefts during the IDC [44]. The major role of *Pf*PP1c in the  
32 dephosphorylation processes was first suggested via the use of phosphatase inhibitors,  
33 including okadaic acid, calyculin and microcystin [33, 45, 46]. Subsequently, various reverse  
34 genetic screens have suggested the essentiality of *PP1c* for the completion of the parasite IDC

1 in *P. falciparum* [47] and *P. berghei* [19, 48]. The use of two inducible knockdown (KD)  
2 approaches confirmed the critical role of *PfPP1c* for the timely regulation of parasite asexual  
3 development [41]. The knock down of *Pfpp1c* in rings significantly impaired DNA  
4 replication, resulting in the formation of multinucleate schizonts with a reduced number of  
5 nuclei and a delayed IDC. At both early or late blood stages, the absence of *PfPP1c* led to a  
6 blockage of egress as **exonemes** failed to excrete the protease *PfSUB1* that orchestrates early  
7 stages of egress, and ultimately the rupture of the parasitophorous vacuole membrane [41,  
8 49]. Finally, as microneme discharge is prevented in these parasites, the egress-to-invasion  
9 transition was also impaired [41].

10 To identify potential proteins (de)phosphorylated in a *PfPP1c*-dependent manner a phospho-  
11 proteomic approach has been implemented in parasites in which *PfPP1c* was knocked down.  
12 The analysis revealed hyperphosphorylation of the Ser-29 *PfHistone 3* which corresponds to  
13 the human ortholog Ser-28, a PP1-targeted site for mitotic exit and metaphase [50, 51].  
14 Additionally, potential substrates as diverse as chromatin factors, AP2 transcription factors or  
15 vacuolar-proteins-sorting family (VSP) members have been identified. Although additional  
16 investigations are required to confirm the identified substrates, the analysis pointed out some  
17 exciting candidates such as *PfHECT1* or *GC $\alpha$*  (guanylyl cyclase alpha). *PfHECT1* is an  
18 uncharacterized protein containing a C-terminal HECT E3 protein-ubiquitin ligase domain  
19 which had been previously identified *in silico* as a potential *PfPIP* [20]. *PfGC $\alpha$* , on the other  
20 hand, is a well-known effector essential for cGMP production and stimulation of *PfPKG*, a  
21 critical component of egress required for *PfSUB1* discharge from exonemes [52-54]. Using a  
22 chemical-genetics assay, the authors demonstrated the essential ability of *PfPP1c* to regulate  
23 these proteins and their egress-related activity. They also revealed the involvement of host  
24 serum phospholipid PtdC (phosphatidylcholine) through the dephosphorylation by *PfPP1c* of  
25 a phospholipid transporter domain situated at the N-terminal of *GC $\alpha$* . In sum, the authors  
26 proposed a model where *PfPP1c* is a central regulator for egress, essential for balancing  
27 environmental signals and intra-cellular pathways to ensure timely and proper parasite  
28 propagation in the blood [41]. An additional conditional KD study conducted in *P. berghei*  
29 showed that *PbPP1c* did not play any important role in sporozoites and liver stages, despite  
30 being expressed, but seemed critical for the establishment of blood infection after *pp1c*KD  
31 sporozoite injection in mice [43]. Since the *pp1* genomic locus was specifically disrupted  
32 when the parasites were developing into sporozoites, no information can thus be drawn about  
33 upstream development occurring in mosquitoes.

34

## 1 **Regulatory subunits: Spatiotemporal orientation of the function of PP1c**

2 Converging evidence from numerous studies strongly supports the model that the PP1c  
3 platform acts in a highly and regulated manner through its combinatorial capacity to interact  
4 with many PIPs. PIPs are structurally unrelated and expressed at key points during the cell  
5 cycle. They can thus orient the “dephosphorylation power” of PP1c in time and space, by  
6 regulating the localization and/or the activity of the enzyme [55, 56]. PIPs bind the  
7 phosphatase surface grooves via docking motifs including the RVxF motif, which was  
8 originally the best-defined binding site for PP1, along with other motifs (**Box 1**) [57, 58].  
9 Also, ensuring the functional diversity of PP1 holoenzymes towards substrates prevents PP1c  
10 from being deleterious to the cell [59, 60]. In vertebrates, at least 200 PIPs have been  
11 identified [61] while it has been estimated that as many as 650 distinct PP1 complexes may  
12 exist [62].

13

## 14 ***Conserved Plasmodium PIPs reveal parasite specific features***

15 Despite the essential role of *PfPP1c*, little was initially known about its regulation in  
16 *Plasmodium*. Based on comparative sequence analyses, our earlier work revealed that *P.*  
17 *falciparum* expresses only four well-known conserved *PfPP1c*-regulators described in  
18 mammals: *PfLRR1* (Leucine Rich repeat 1), *PfI2* (Inhibitor 2), *PfI3* (Inhibitor 3), and *Pfeif2β*  
19 (eukaryotic initiation factor 2β) [40, 63-65]. At the functional level, several lines of evidence  
20 have indicated that yeast or human SDS22 (*PfLRR1* orthologs) and inhibitor 3, known as PP1  
21 inhibitors *in vitro* [66], are involved in a common pathway regulating cell division by  
22 stabilizing kinetochore spindle attachment and proper chromosome segregation [67-71].  
23 Regarding Inhibitor 2, its implication in the regulation of mitosis, and more specifically in  
24 cytokinesis, has been reported [72, 73]. Interestingly, a recent proteomic approach aiming to  
25 explore *PfPP1c* signaling events revealed a significant accumulation of *PfLRR1* as well as  
26 *PfI2* in a *P. falciparum* strain in which PP1c was depleted [41]. The functional significance of  
27 such an accumulation on the progress of IDC is still unknown. A targeted knock out approach  
28 suggested the essentiality of the 4 *PfPIP*s during its IDC [40, 63-65]. However, a more recent  
29 study based on saturation mutagenesis aiming to identify *P. falciparum* essential genes  
30 nuanced these results, as it suggested that *PfI2* and *PfI3* are non-essential during IDC. It is,  
31 however, worth noting that *PfI2* may have a function during blood stage development as  
32 insertions in its gene were associated with a slow growth phenotype [47].  
33 Biochemical and structure-activity relationship studies revealed the ability of *PfLRR1*, *PfI2*,  
34 *PfI3*, and *Pfeif2β* to physically bind *PfPP1c* and to regulate its activity [40, 63-65]. In the case

1 of PP1c-*PfLRR1* binding, pepscan analysis revealed that only one synthetic peptide derived  
2 from Leucine-Rich Repeats (LRRs) was involved in this binding [74]. Unexpectedly, the  
3 peptide corresponding to the C-terminal LRR cap domain, previously known as crucial for the  
4 stabilization and integrity of the protein [75], was also shown to directly interact with PP1c  
5 [74]. At this point, it is important to emphasize that a recent work on its human ortholog,  
6 SDS22, showed a different pattern of binding which involves six LRR motifs, without  
7 reporting a role of the C-terminal LRR cap domain [76]. In *Plasmodium*, this needs further  
8 work on the structural interactions and raises an interesting question about the role of the  
9 *PfLRR1* cap domain on the function of PP1c.

10 With respect to *PfI2*, *PfI3*, and *Pfeif2β*, their primary amino acid sequences did show the  
11 presence of RVxF consensus motifs. As expected, biochemical and mutational analysis  
12 showed a main contribution of this motif to *PfPP1c* binding. The 3D structures of *PfI2* and  
13 *PfI3* have been solved by NMR spectroscopy alone and in the presence of *PfPP1c*, confirming  
14 the interaction via the RVxF motif in solution. Interestingly, *PfI2* presents a more complex  
15 interaction pattern which involves an additional FxxR/KxR/K (also referred to in this paper as  
16 Fxx) motif also described in PP1c binding anti-apoptotic proteins and ion transporters [77-  
17 79]. Divergences were also observed in *PfI2* at the primary sequence level when compared to  
18 its human ortholog in which the RVxF considerably deviates from the consensus sequence.  
19 The protein is also around 30% shorter than its mammalian ortholog and does not possess a  
20 SILK motif, which has been showed to play a critical role in both PP1c binding and  
21 regulation in mammals [57, 80]. Based on these observations, the mode of binding of *PfI2* to  
22 *PfPP1c* differs from its human ortholog. Also, it should be noted that the nature of the x  
23 residue in the RVxF motif could play a role in the stabilization of the interaction as previously  
24 reported [81]. This x residue is not conserved between *P. falciparum* conserved PIPs and their  
25 human counterparts. More importantly, in the case of *PfI3*, despite its sequence similarity  
26 with its yeast and human orthologs, it seems to have a distinct regulatory role on *PfPP1c* as  
27 attested by a stimulation rather than an inhibition of the phosphatase activity [64]. All these  
28 differences in the binding and regulation of PP1c highlight some intriguing specific  
29 characteristics of *Plasmodium* PP1 holoenzymes.

30

### 31 ***Insights into Plasmodium specific PIPs***

32 *Plasmodium* PP1c interactome -. To decipher the signaling pathways regulated by this  
33 phosphatase, the *Plasmodium* PP1c interactome has been explored using different and  
34 complementary approaches. A first study, based on **yeast two-hybrid (Y2H)** and *in silico*



1 RVxF motif screenings, reported 134 and 55 *Pf*PIPs respectively [20]. Additionally, a recent  
2 proteomics study carried out in *P. berghei* schizonts led to the identification of 178 potential  
3 PIPs [82] among which 19 proteins have been already shown to physically interact with PP1c  
4 either by Y2H approach and/or binding assays using recombinant proteins in *P. falciparum*  
5 (supplemental **Table S1**) [20, 40, 63]. Strikingly, 80 PIPs (*i.e.* 70% of the proteins for which  
6 data are available) may be essential for *P. berghei* IDC completion, as assessed by the lack of  
7 viable knockout parasites [48] (**Figure 3A**). More details of the phenotypes of the potential  
8 *Pb*PIPs are depicted in **Figure 1** and supplemental **Table S2**. A GO term analysis of *Pb*PIPS  
9 showed significant enrichment of distinct biological pathways (mostly proteasome assembly,  
10 protein folding and translational processes) which confirms and extends to *Plasmodium* the  
11 diverse functions of the PP1 holoenzyme (supplemental **Table S3**).

12 Comparative studies revealed that 31.5% of the potential *Pb*PIPs do not have homologs in  
13 human and thus can be considered as promising drug targets if the binding with the  
14 phosphatase can be confirmed. In this context, two proteins previously identified as *Pf*PIPs in  
15 Y2H screening whose expression is restricted to *Plasmodium* were further investigated.  
16 Biochemical and reverse genetics approaches determined that the first, designated RCC-PIP,  
17 can bind/transport not only *Pf*PP1c but also the kinase *Pf*CDPK7 [83]. The second PIP,  
18 *Pf*GEXP15, is a regulator that increases *Pf*PP1c activity [82]. The KO line of its *P. berghei*  
19 ortholog failed to induce hyper-parasitemia and experimental cerebral malaria. The  
20 *Pbgexp15*-KO line was also unable to produce oocysts in mosquitoes, highlighting once again  
21 the role of *Plasmodium* PIPs in both asexual and sexual parasite stages.

22  
23 Potential *Plasmodium*-specific PP1c regulators and substrates - Assuming that the direct and  
24 physical interaction of regulators to PP1c is crucial for their functions, it was therefore  
25 necessary to examine their mode of binding. In this context, it is interesting to note that 93  
26 *Pb*PIPs identified from the IP/MS interactome [82] have an RVxF motif defined by Wakula et  
27 al. [84] (**Box 1**), with 16 of them fitting with the less sensitive but more specific consensus  
28 sequence defined by Meiselbach et al. [85]. Enrichment analysis showed that the RVxF motif  
29 conforming to the Meiselbach sequence was significantly enriched (fold enrichment: 1.86,  
30 hypergeometric P-value: 0.01). Fifty-one PIPs had at least one additional interaction motif  
31 (SILK, Fxx) (**Figure 3B, Box 1**). Further analysis showed that 14 PIPs have a highly specific  
32 RVxF motif [85] and at least one secondary interaction motif, and two PIPs show a less  
33 specific RVxF motif [84] together with a Fxx and a SILK motif (supplemental **Table S4**).  
34 This strongly suggests that these 16 PIPs can interact directly with *Pb*PP1c and regulate its

1 activity. Overall, this provides insights into the formation of multiple PP1 complexes in  
2 *Plasmodium* and explains why PP1 can be associated with several signaling pathways.  
3 It has been previously reported that several vertebrate PIPs are also PP1 substrates [62]. In an  
4 attempt to find out potential *Plasmodium* PP1c substrates among the PIPs identified above,  
5 the phosphorylation status of their *P. falciparum* orthologs was examined from  
6 phosphoproteomics data collected in *Pfpp1c*-knockdown parasites [41]. This led to the  
7 identification of 4 PIPs showing an increase in their level of phosphorylation following  
8 *PfPP1c* depletion (supplemental **Table S5**). Furthermore, two of them possess three PP1c  
9 binding motifs (supplemental **Table S4**), suggesting that they could belong to a  
10 regulator/substrate group.

11  
12 PP1c complexes as target for the design of innovative of anti-malaria peptides - The above  
13 analyses define an emerging and robust theme in the *Plasmodium* PP1 field, where PP1c  
14 forms complexes with diverse (regulatory) PIPs whose interactions are crucial for the correct  
15 functions of this enzyme during the lifecycle of blood stage parasites. From this point of view,  
16 it is rational to attempt to interfere with these interactions and evaluate how this impacts  
17 parasite growth. Synthetic peptides corresponding to the RVxF motifs of *PfI2* and *PfI3*, able  
18 to bind *PfPP1c* *in vitro*, successfully induced a drastic inhibition of *P. falciparum* growth *in*  
19 *vitro* [63] while peptide corresponding to Fxx motif of *PfI2* showed slight but significant  
20 growth inhibition [65, 78]. In a more recent study, the peptides derived from *PfLRR1*  
21 revealed the capacity of one peptide to inhibit both blood stage parasite growth and SPZ  
22 development in hepatocytes [74]. Together, these results provide proof of concept that the  
23 PP1c/PIP interfaces are relevant targets for the design of new antimalarials. This could be  
24 achieved via the development of specific small molecules that mimic the primary or  
25 secondary PP1c docking motifs.

26

## 27 **Concluding Remarks**

28 The idea that PP1 is a nonspecific housekeeping phosphatase, which was initially based on *in*  
29 *vitro* data obtained with its catalytic subunit PP1c, is no longer tenable. The high  
30 combinatorial capacity of the catalytic subunit with hundreds of PIPs makes it a highly  
31 regulated and specific phosphatase. From *Plasmodium* PP1c interactome studies, there are  
32 many distinct complexes with restricted substrate specificity known to be involved in major  
33 and essential pathways in *Plasmodium*. Despite the recent advances, more in-depth analyses  
34 are required to understand the multifaceted role of PP1 holoenzymes during *Plasmodium* life

1 cycle, as well as in other Apicomplexa (see outstanding questions). In a short-term outlook,  
2 obtaining structural data on the interaction of PP1c with PIPs will reveal how PP1 can be  
3 efficiently targeted with small molecules. In a long-term outlook, it will become important to  
4 determine the activity of various PP1c-PIP complexes toward different substrates, including  
5 those which will be identified in *Plasmodium*. This will help defining specific molecular  
6 mechanisms and pathways. This might in turn contribute to the elaboration of innovative  
7 ways to control parasite development, as has been shown via the use of interfering peptides  
8 that inhibit *P. falciparum* growth.

9  
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### 21 **Box 1. Structural basis of interaction with PP1c**

- 22 • PP1c Interacting Proteins (PIPs) are structurally unrelated
- 23
- 24 • The PP1 binding code is specific, universal, degenerate, overlapping, non-exclusive and  
25 dynamic [60, 61]:
- 26 ▪ Specific: PIPs contain a combination of multiple binding motifs, and thus bind  
27 PP1c in a specific way.
  - 28 ▪ Universal: given the high degree of conservation of PP1c.
  - 29 ▪ Degenerate: binding motifs come in many sequence variants, which bind PP1c  
30 with a given affinity.
  - 31 ▪ Overlapping: different binding motifs can target the same site on PP1c (but 2  
32 variants cannot bind at the same time).
  - 33 ▪ Non-exclusive: more than one PIP can bind PP1c simultaneously (as long as they  
34 have one non-shared binding motif).



- 1           ▪ Dynamic: the competition between PIPs and PP1c, as well the post-translational  
2           modification of binding motifs [7], can impact the formation of holoenzymes.  
3
- 4   • The RVxF motif is the most widely occurring binding motif in PIPs  
5 PIPs can be PP1c regulators, substrates, or both. 70% of PIPs possess an RVxF motif,  
6 which makes it the main binding site to PP1c [60, 62]:
- 7           ▪ The RVxF motif serves as an anchor for the initial binding of the PIP (it is  
8           therefore a primary binding site). It brings the PIP close to PP1c and facilitates  
9           binding of secondary interaction sites with lower binding affinity which may in  
10          turn modulate the enzymatic activity and/or substrate specificity of the  
11          phosphatase [60, 86, 87].
- 12          ▪ Table I: Overview of RVxF consensus sequences

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|---|--|
| <p><b>Wakula RVxF consensus sequence:</b><br/> [RK] X<sub>0-1</sub> [VI] {P} [FW]</p>   | <p><b>High sensitivity</b> (recapitulates 90% of existing RVxF motifs) <b>but low specificity</b> (randomly present in 25% of proteins) [84].</p>  |
| <p><b>Meiselbach RVxF consensus sequence:</b><br/> These basic residues are the first anchor point of the motif to PP1c<br/> Nonpolar aromatic residues are critical at this position for maximal affinity<br/> (HRK) [ACHKMNQRSTV] (V) [CHKNQRST] (FW)<br/> This residue is preferentially a Valine to bind a hydrophobic pocket on PP1c</p> | <p><b>Low sensitivity</b> (result of targeted mutagenesis analysis to improve our understanding of the function of each residues) <b>but its specificity is 10 times Wakula sequence's</b> [85].</p> |
| <p><b>Hendrickx RVxF binding mode:</b><br/> [K<sub>54</sub>R<sub>34</sub>L<sub>4</sub>] [K<sub>28</sub>R<sub>26</sub>S<sub>10</sub>T<sub>9</sub>A<sub>8</sub>M<sub>3</sub>V<sub>3</sub>H<sub>4</sub>N<sub>3</sub>]<br/> [V<sub>94</sub>I<sub>6</sub>] {FIMYDP} [F<sub>83</sub>W<sub>17</sub>]</p>   | <p>Combination of the above sequences. Both <b>high sensitivity and specificity</b> [87].</p>  |
| <p><b>Caption</b><br/> [HRK] = either Histidine, Arginine or Lysine at this position in the motif<br/> X = any residue at this position in the motif<br/> {P} = any residue but Proline at this position in the motif</p>   |  |

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- 14   • -Examples of secondary binding motifs (i.e. that bind PP1c after a primary binding site  
15   like RVxF anchored the protein partner to the phosphatase) include SILK (consensus  
16   sequence [GS]IL[KR]{DE}) [57], myosin phosphatase N-terminal element (MyPhoNE -  
17   consensus sequence RxxQ[VIL][KR]x[YW]) [58, 87], Fxx (consensus sequence  
18   Fxx[RK]x[RK]), and HYNE.  
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## Figure Legends

**Figure 1. Schematic illustration of *Plasmodium* life cycle showing proteins with known functions identified as potential PIPs in *P. berghei*.** *Plasmodium* is transmitted between its intermediate and definitive hosts via mosquito bites. Sporozoites are injected in the skin of intermediate hosts by infectious mosquitoes. Some reach the bloodstream and, ultimately, the liver where they will infect hepatocytes. After growth and replication (hepatic schizogony) in the liver, merozoites (merozoite stages contained in the hepatocyte membrane) migrate to the bloodstream. Thousands of merozoites are released upon merozoite lysis. These stages are only infectious to erythrocytes. Upon invasion, each merozoite gives a ring which will then mature into a trophozoite and then to a schizont. Each schizont can divide into about 16 merozoites which will be released in the bloodstream upon erythrocyte lysis, thus inducing/sustaining an intra-erythrocytic cycle that will lead to a rise in parasitemia and malaria symptoms. A portion of trophozoites can also differentiate into transmissible stages, called **gametocytes**. These sexual stages are activated in the mosquito midgut into gametes. Following fertilization, the zygote differentiates into an ookinete able to cross the midgut barrier and develop there into an oocyst. Upon maturation, oocysts give hundreds of sporozoites which will migrate to the mosquito salivary glands, thus allowing them to be easily transmitted to the next intermediate host via mosquito bites. The stage specific function of *Plasmodium berghei* PIPs are also presented (see supplemental **Table S2** for details and references). This scheme has been created using PowerPoint from Microsoft Office 365.

**Figure 2. The high level of conservation of PP1c between *Plasmodium* and Human.** Since human PP1c (*HsPP1c*) has never been entirely crystallized (i.e. available crystal structures do not cover the last 30 AA of the protein), its tertiary structure was predicted using I-Tasser (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). The model for *PfPP1c* tertiary structure was uploaded from SwissModel (<https://swissmodel.expasy.org/repository/uniprot/Q8ILV1?csm=9CE0B7C29FEFEFC6>). Both models were superimposed using the MatchMaker tool from Chimera (version 1.14). This superimposition illustrates the high level of conservation between the two proteins, except for their C-terminal extremities. *HsPP1c* and *PfPP1c* are shown in brown and blue respectively; the C-terminal extremity of *HsPP1c* has been circled in purple.

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**Figure 3. Feature analysis of the PIPs identified in *P. berghei* schizonts.** **A.** Pie chart of the essentiality of the PIPs in *P. berghei* for the parasite IDC, as determined in the PlasmoGEM study [48]. Essential, Dispensable and Slow are for relative growth rate of 0.1, 1.0 and between 0.1 and 1.0 respectively. **B.** Venn diagram of the distribution of *P. berghei* PIPs according to their PP1c binding motifs. RVxF\_W and RVxF\_M stand for RVxF consensus sequences as defined by Wakula et al [84] and Meiselbalch et al [85] respectively and Fxx stands for FxxR/KxxR/K motif. The consensus sequences of binding motifs are detailed in **Box 1.** This diagram has been created using R version 3.6.3 and the eulerr package.