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

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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.

#### 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 support the need to pursue targeting the PP1 holoenzymes have been elegantly reported in a 32 33 recent review, suggesting that promising ways to develop selective and potent drugs involve 34 the disruption of interactions between PP1c and PIPs [12].

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

5

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

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

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

Although some of the phosphatases identified in *Plasmodium* are conserved across eukaryotic phyla which were reported in a recent review [23], others belong to subfamilies restricted to apicomplexans and plants (Kelch-like domain containing protein phosphatase (PPKL) and Shewanella-like phosphatases (SLP)) or are parasite-specific such as the N-terminus EF-hand motif protein phosphatase (EFPP) [24]. Functional analyses conducted on the unique member of the PPKL subfamily revealed its involvement in **ookinete** shape and motility and further transmission in the mosquito [25, 26]. In *Plasmodium berghei* (*Pb*, a rodent malaria parasite, exploited as a model for *P. falciparum* infection), SLP1 has been shown to be crucial to microneme formation and ookinete development. Ultimately, its deletion resulted in a complete inhibition of oocyst formation [27]. In *P. falciparum*, SLP2 has been found enclosed in vesicles at the apex of merozoites and was first proposed to be involved in the invasion processes [28] although recent studies suggest that the protein is more likely dispensable during the whole life cycle of both *P. berghei* and *P. falciparum* [19, 29].

8

### 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<sup>21</sup> [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 HsPP1c 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 HsPP1c [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 PfPP1c 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

in *P. falciparum* [47] and *P. berghei* [19, 48]. The use of two inducible knockdown (KD) 1 2 approaches confirmed the critical role of *Pf*PP1c 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 *Pf*SUB1 that orchestrates early 7 stages of egress, and ultimately the rupture of the parasitophorous vacuole membrane [41, 49]. Finally, as microneme discharge is prevented in these parasites, the egress-to-invasion 8 9 transition was also impaired [41].

To identify potential proteins (de)phosphorylated in a PfPP1c-dependent manner a phospho-10 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 GCa (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 *Pf*PKG, 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 *Pf*PP1c 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 GCa. In sum, the authors proposed a model where PfPP1c is a central regulator for egress, essential for balancing 26 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 *Pb*PP1c 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 pplcKD 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.

#### **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 from being deleterious to the cell [59, 60]. In vertebrates, at least 200 PIPs have been 10 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 Pfeif2ß 19 (eukaryotic initiation factor 2B) [40, 63-65]. At the functional level, several lines of evidence 20 have indicated that yeast or human SDS22 (*Pf*LRR1 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 *Pf*PIPs 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 Pf13, and Pfeif2B 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 stabilization and integrity of the protein [75], was also shown to directly interact with PP1c 4 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 *Pf*LRR1 cap domain on the function of PP1c.

With respect to PfI2, PfI3, and Pfeif2ß, their primary amino acid sequences did show the 10 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 *Pf*I3 have been solved by NMR spectroscopy alone and in the presence of *Pf*PP1c, confirming 14 the interaction via the RVxF motif in solution. Interestingly, *Pf*I2 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 Pf12 to 22 *Pf*PP1c 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 Pf13, 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 <u>*Plasmodium* PP1c interactome</u> -. 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 PfPIPs 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 PfPP1c but also the kinase PfCDPK7 [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 PbPIPs 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 RVxF motif [85] and at least one secondary interaction motif, and two PIPs show a less 32 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

activity. Overall, this provides insights into the formation of multiple PP1 complexes in
 *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 phosphoproteomics data collected in *Pfpp1c*-knockdown parasites [41]. This led to the 6 7 identification of 4 PIPs showing an increase in their level of phosphorylation following 8 *Pf*PP1c 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 *Pf*PP1c 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

The idea that PP1 is a nonspecific housekeeping phosphatase, which was initially based on *in vitro* data obtained with its catalytic subunit PP1c, is no longer tenable. The high combinatorial capacity of the catalytic subunit with hundreds of PIPs makes it a highly regulated and specific phosphatase. From *Plasmodium* PP1c interactome studies, there are many distinct complexes with restricted substrate specificity known to be involved in major and essential pathways in *Plasmodium*. Despite the recent advances, more in-depth analyses 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 determine the activity of various PP1c-PIP complexes toward different substrates, including 4 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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13

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19			
20			
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	<ul> <li>Specific: PIPs contain a combination of multiple binding motifs and thus bind</li> </ul>		
20	PD1c in a specific way		
21	<ul> <li>Universal, given the high degree of concernation of DD1.</li> </ul>		
20	Oniversal: given the high degree of conservation of PPTC.		
29	• Degenerate: binding motifs come in many sequence variants, which bind PPTc		
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	<ul> <li>Non-exclusive: more than one PIP can bind PP1c simultaneously (as long as they</li> </ul>		
34	have one non-shared binding motif).		

- Dynamic: the competition between PIPs and PP1c, as well the post-translational modification of binding motifs [7], can impact the formation of holoenzymes.
- 2 3

1

#### 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

Wakula RVxF consensus sequence: [RK] X <sub>0-1</sub> [VI] {P} [FW]	<b>High sensitivity</b> (recapitulates 90% of existing RVxF motifs) <b>but low specificity</b> (randomly present in 25% of proteins) [84].		
Meiselbach RVxF consensus sequence: These basic residues are the first anchor point of the motif to PP1c for maximal affinity (HRK)[ACHKMNQRSTV][V][CHKNQRST][FW] This residue is preferentially a Valine to bind a hydrophobic pocket on PP1c	Low sensitivity (result of targeted mutagenesis analysis to improve our understanding of the function of each residues) but its specificity is 10 times Wakula sequence's [85].		
Hendrickx RVxF binding mode: [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> ] [V <sub>94</sub> I <sub>6</sub> ] {FIMYDP} [F <sub>83</sub> W <sub>17</sub> ]	Combination of the above sequences. Both <b>high sensitivity</b> and <b>specificity</b> [87].		
Caption[HRK] = either Histidine, Arginine or Lysine at this position in the motifX = any residue at this position in the motif{P} = any residue but Proline at this position in the motif			

<sup>14</sup> -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 sequence [GS]IL[KR]{DE}) [57], myosin phosphatase N-terminal element (MyPhoNE -16 17 consensus sequence RxxQ[VIL][KR]x[YW]) [58, 87], Fxx (consensus sequence 18 Fxx[RK]x[RK]), and HYNE. 19

1

#### 2 Figure Legends

3

4 Figure 1. Schematic illustration of *Plasmodium* life cycle showing proteins with known 5 functions identified as potential PIPs in P. berghei. Plasmodium is transmitted between its 6 intermediate and definitive hosts via mosquito bites. Sporozoites are injected in the skin of 7 intermediate hosts by infectious mosquitoes. Some reach the bloodstream and, ultimately, the 8 liver where they will infect hepatocytes. After growth and replication (hepatic schizogony) in 9 the liver, merosomes (merozoite stages contained in the hepatocyte membrane) migrate to the bloodstream. Thousands of merozoites are released upon merosome lysis. These stages are 10 11 only infectious to erythrocytes. Upon invasion, each merozoite gives a ring which will then 12 mature into a trophozoite and then to a schizont. Each schizont can divide into about 16 13 merozoites which will be released in the bloodstream upon erythrocyte lysis, thus 14 inducing/sustaining an intra-erythrocytic cycle that will lead to a rise in parasitemia and 15 malaria symptoms. A portion of trophozoites can also differentiate into transmissible stages, 16 called gametocytes. These sexual stages are activated in the mosquito midgut into gametes. 17 Following fertilization, the zygote differentiates into an ookinete able to cross the midgut 18 barrier and develop there into an oocyst. Upon maturation, oocysts give hundreds of 19 sporozoites which will migrate to the mosquito salivary glands, thus allowing them to be 20 easily transmitted to the next intermediate host via mosquito bites. The stage specific function 21 of Plasmodium berghei PIPs are also presented (see supplemental Table S2 for details and 22 references). This scheme has been created using PowerPoint from Microsoft Office 365.

23

24 Figure 2. The high level of conservation of PP1c between *Plasmodium* and Human. 25 Since human PP1c (HsPP1c) has never been entirely crystallized (i.e. available crystal 26 structures do not cover the last 30 AA of the protein), its tertiary structure was predicted using 27 I-Tasser (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The model for *Pf*PP1c tertiary 28 SwissModel structure was uploaded from 29 (https://swissmodel.expasy.org/repository/uniprot/Q8ILV1?csm=9CE0B7C29FEFEFC6).

30 Both models were superimposed using the MatchMaker tool from Chimera (version 1.14). 31 This superimposition illustrates the high level of conservation between the two proteins, 32 except for their C-terminal extremities. *Hs*PP1c and *Pf*PP1c are shown in brown and blue 33 respectively; the C-terminal extremity of *Hs*PP1c has been circled in purple.

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.