PTRAMP, CSS and Ripr form a conserved complex required for merozoite invasion of *Plasmodium* species into erythrocytes

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39 *P. knowlesi, P. cynomolgi.*

40

Summary

42 Invasion of erythrocytes by members of the *Plasmodium* genus is an essential step of the parasite

43 lifecycle, orchestrated by numerous host-parasite interactions. In *P. falciparum* Rh5, with

44 PfCyRPA, PfRipr, PfCSS, and PfPTRAMP, forms the essential PCRCR complex which binds

45 basigin on the erythrocyte surface. Rh5 is restricted to *P. falciparum* and its close relatives;

46 however, PTRAMP, CSS and Ripr orthologs are present across the *Plasmodium* genus. We

47 investigated PTRAMP, CSS and Ripr orthologs from three species to elucidate common features

48 of the complex. Like *P. falciparum,* PTRAMP and CSS form a disulfide-linked heterodimer in

49 both *P. vivax* and *P. knowlesi* with all three species forming a complex (PCR) with Ripr by binding

50 its C-terminal region. Cross-reactive antibodies targeting the PCR complex differentially inhibit

51 merozoite invasion. Cryo-EM visualization of the *P. knowlesi* PCR complex confirmed predicted

52 models and revealed a core invasion scaffold in *Plasmodium* spp. with implications for vaccines

53 targeting multiple species of malaria-causing parasites.

54

Introduction

55

There are more than 200 species of *Plasmodium* that infect a diverse range of hosts including primates, rodents, reptiles and birds. At least six species, including *Plasmodium falciparum, P. vivax* and *P. knowlesi,* have the ability to infect humans. *P. falciparum* is the most lethal species to infect humans, while *P. vivax* is the most widespread globally[1,2]. *P. knowlesi* is confined mainly to regions of Southeast Asia and is transmitted from macaques by zoonotic infection[3,4]. The *Plasmodium* genus can be divided into three main subgenera or clades that include *Laverania* (includes *P. falciparum*), *Plasmodium* (includes most human infective species such as *P. vivax* and *P. knowlesi*) and *Vinckeia* (primarily rodent infective species).

The three clades of the *Plasmodium* genus represent distinct evolutionary branches distinguished by geographic distribution and severity of disease; however, they can also exhibit distinct host cell selectivity. *P. vivax* has a strict preference for invasion of reticulocytes in the blood whereas *P. falciparum* can invade both reticulocytes and the more mature normocytes[5]. While the core machinery for invasion of reticulocytes and normocytes by *P. vivax* and *P. falciparum*, such as the parasite actomyosin motor, is conserved, there are distinct ligand-receptor interactions that provide selectivity for host cell invasion (reviewed in [6]). In *P. falciparum,* many of these ligands are dispensable for invasion[7]. The notable exception is reticulocyte-binding protein homologue 5 (Rh5)[8,9] which is an essential *P. falciparum* ligand that binds to the receptor basigin on human erythrocytes[10]. Rh5 can play a role in host tropism through polymorphisms in the protein and differential binding to basigin of other non-human primates[8,11].

In *P. falciparum* Rh5 functions in a complex of five proteins that include CyRPA (Cysteine Rich Protective Antigen)[12], Ripr (Rh5 interacting protein)[13], PTRAMP (*Plasmodium* thrombospondin-related apical merozoite protein)[14,15], and CSS (cysteine-rich, small, secreted)[16] that has been termed the PCRCR complex[17-19]. PTRAMP and CSS form a disulfide-linked heterodimer that tethers the PCRCR complex to the merozoite membrane via the transmembrane domain of PTRAMP[18]. All proteins in the PCRCR complex are functionally essential for *P. falciparum* merozoite invasion of human erythrocytes and, crucially, antibodies and nanobodies that bind to individual proteins can inhibit merozoite invasion[18,20-25]. Conditional gene knockouts of each PCRCR protein in *P. falciparum* display the same phenotype whereby the merozoites can interact with the erythrocyte surface and produce the strong deformation of the host membrane typical during normal invasion; however, the merozoite fails to internalize[18,26]. The PCRCR

86 complex has been hypothesized to capture and anchor the increased membrane surface contact

87 formed between the merozoite and erythrocyte membrane that is created during strong deformation

88 driven by the merozoites actomyosin motor[18]. This facilitates the establishment of the moving

89 junction and is followed by the downstream events of invasion and ultimately the internalization

90 of the merozoite into the erythrocyte[27,28].

91 Despite being essential for *P. falciparum* invasion, Rh5 orthologs are absent in species

92 outside of the *Laverania* subgenus[29,30]. Consequently, utilization of basigin as a host receptor for

93 invasion is not universal, as demonstrated for both *P. knowlesi* and *P. vivax*[16], suggesting that other

94 parasite ligand-receptor interactions facilitate host cell attachment in other *Plasmodium* spp. Non-

95 *Laverania* species do, however, possess homologues of other components of the *P. falciparum*

96 PCRCR complex and *P. knowlesi* orthologs of PTRAMP, CSS and Ripr have been shown to be

97 essential for merozoite invasion using conditional gene knockouts[16] and more recently a high-

98 resolution transposon mutagenesis screen for growth[31,32]. It has been suggested that PkPTRAMP,

99 PkCSS and PkRipr form a complex and that PkPTRAMP provides the means for erythrocyte

100 binding[16]. The PkCyRPA homologue was also identified, and while it has been shown to be

101 essential for parasite growth, it does not appear to be part of this complex in *P. knowlesi*[16].

102 Here we hypothesized that PTRAMP, CSS and Ripr form a common basis for invasion

103 complexes across *Plasmodium* spp. We leveraged recent insights into the PCRCR complex to

104 characterize these proteins in several species of *Plasmodium* to elucidate the conserved features of

105 merozoite invasion complexes. Our findings revealed a conserved PCR trimeric complex common

106 to all *Plasmodium* clades that forms a core invasion scaffold. Additionally, cross-reactive

107 antibodies targeting PTRAMP, CSS, and Ripr were identified that differentially inhibit merozoite

108 invasion of erythrocytes, including an antibody targeting Ripr that inhibited both *P. knowlesi* and

109 *P. falciparum* growth. Identification of a conserved molecular scaffold presents an attractive

110 approach for the development of vaccines targeting multiple species of malaria-causing parasites.

111

112 **Results**

113 **PTRAMP, CSS and Ripr are conserved in all clades of *Plasmodium***

114 We first confirmed that the proteins constituting the *P. falciparum* PCRCR complex are

115 conserved in other *Plasmodium* spp. by searching for orthologs and found that PTRAMP, CSS and

116 Ripr were present in all subgenera **(Fig. 1a)**. This conservation contrasts with Rh5 which is only

117   present in *P. falciparum* and other *Laverania* species. Whilst CyRPA is relatively conserved, none

118   of the species within the *Vinckeia* subgenus possess a CyRPA ortholog. This suggests PTRAMP,

119   CSS and Ripr (PCR) form a conserved three-membered complex present across all *Plasmodium*

120   spp.

121   AlphaFold 3 was used to predict the structure of the PCR complex of different *Plasmodium*

122   spp. to understand the assembly of the three proteins **(Fig. 1b, Extended data Fig. 1)**[33,34]. This

123   revealed a common architecture of the PCR complex in which PTRAMP and CSS together form

124   a platform that is bound by the C-terminal end of Ripr. The PfCSS crystal structure aligned with

125   the AlphaFold 3 prediction of PfPCR with an RMSD of 0.711 Å, suggesting that no conformational

126   changes are required within PfCSS to facilitate PfRipr binding **(Fig. 1b)**.

127   The predicted engagement of Ripr with both PTRAMP and CSS in the AlphaFold 3 models

128   was similar in all *Plasmodium* spp. but was inconsistent with existing biophysical data for *P.*

129   *falciparum* which had suggested that PfCSS alone was sufficient for PfRipr binding[18]. However,

130   this interaction was low affinity with K_D of CSS binding to Ripr in the low micromolar range[18,19].

131   In contrast, the PCR model predicted significant interaction of both PTRAMP and CSS with Ripr

132   for *P. falciparum*, *P. vivax* and *P. knowlesi*. PfPTRAMP, PfCSS and PfRipr all contain multiple

133   predicted N-linked glycan motifs and whilst there is very limited glycosylation in *P. falciparum*

134   these proteins were expressed in insect cells and therefore are predicted to undergo extensive

135   glycosylation **(Fig 1c)**[35]. To further investigate PfPTRAMP-PfCSS (PfPC) - PfRipr binding, we

136   recombinantly expressed glycosylation-modified variants that either completely lacked glycans or

137   had significantly reduced glycosylation **(Fig 1c, d)**.

138   Using biolayer interferometry (BLI), we determined that non-glycosylated forms of PfRipr

139   and PfPC interacted with a K_D of 160 nM, representing a ~10-fold stronger interaction compared

140   to previous measurements (1-4 μM)[18,19] and equivalent to the affinity measured between Rh5 and

141   CyRPA (179 nM) **(Fig. 1e)**[36]. The enhanced binding affinity of non-glycosylated proteins suggests

142   that N-linked glycans on PfRipr and PfPC, added during heterologous expression, had interfered

143   with their interaction interface in previous studies. Despite removal of a majority of the

144   PfPTRAMP glycans monomeric PfPTRAMP showed no binding to PfRipr **(Supp. Fig. 1)**. These

145   results suggest that the complete PfPC heterodimer is necessary for high-affinity PfRipr binding.

146

147   **The *P. vivax* orthologs of PTRAMP and CSS form a disulfide-linked heterodimer**

148 To investigate whether the disulfide-linked PC heterodimer is conserved as the basis for
149 Ripr binding across *Plasmodium* species, as predicted by AlphaFold 3[34], we co-expressed *P. vivax*
150 PTRAMP and CSS orthologs in mammalian cells. The resulting PvPC heterodimer could be
151 separated into its component monomers through reduction of the intermolecular disulfide bond
152 **(Fig. 2a)**. Nanobodies were raised against the purified PvPC heterodimer to enable further
153 structural and biophysical characterization **(Supp. Fig. 2)**. Crystallization of the PvPC heterodimer
154 was achieved by truncating the predicted disordered N-terminal repeat region of PvCSS and adding
155 nanobody D7 **(Fig. 2b, Table S1)**. The resulting structure confirmed that PvCSS adopts the
156 previously characterized two-domain degenerate 6-Cys fold seen in PfCSS **(Fig. 2c, Extended
157 data Fig. 2)**[18].

158 No electron density was observed for either the growth-factor domain (GFD) or the
159 thrombospondin repeat (TSR) domain of PvPTRAMP, despite space being available within the
160 crystal lattice **(Extended data Fig. 2c)**. This suggests that the majority of PvPTRAMP was
161 insufficiently stabilized within the crystal lattice to produce coherent diffraction. Nevertheless,
162 clear electron density extended from PvCSS cysteine 122, the predicted site of disulfide formation
163 with PvPTRAMP **(Fig. 2d)**. Modelling of PvPTRAMP residues 42 to 53, revealed the structural
164 basis for PvPC heterodimerization **(Fig. 2e, f)**. Specifically, PvPTRAMP forms an interrupted β-
165 strand that extends across both β-sheets of the CSS D1 domain, establishing multiple backbone
166 interactions **(Fig. 2c, f, Table S2)**.

167 Alignment of available PTRAMP and CSS sequences showed that the two cysteines
168 involved in heterodimerization are conserved across most species **(Supp. Fig. 3, 4)**. One exception
169 is *Plasmodium inui*, which has tyrosine and serine substitutions in PTRAMP and CSS, respectively
170 **(Supp. Fig. 3, 4)**. Nevertheless, AlphaFold[33] modelling predicts a similar interface between
171 PTRAMP and CSS in this region **(Extended data Fig. 1d)**.

172

PvRipr binds the PvPC heterodimer to form a high affinity complex

174 Biophysical analysis revealed that PvPC binds to PvRipr with high affinity ($K_D = 28.8 \pm$
175 3.9 nM) **(Fig. 3a, Extended data Fig. 3a, b)**. While monomeric PvPTRAMP was sufficient for
176 binding, it showed approximately 10-fold weaker affinity ($K_D = 292.5 \pm 25.6$ nM) **(Fig. 3b)**. No

177 interaction was detected between PvRipr and PvCSS **(Fig. 3c)**, and none of the PvPCR components

178 bound to PvCyRPA at the tested concentrations **(Fig. 3d)**. Mass photometry analysis confirmed

179 the formation of a stable PvPCR complex, with PvPC and PvRipr each displaying monodisperse

180 peaks when analyzed individually **(Extended data Fig. 3c)**. Formation of the PvPCR complex,

181 following incubation of PvPC and PvRipr, was evidenced by the emergence of a higher molecular

182 weight peak corresponding to a mass of 207 ± 29 kDa which is consistent with a 1:1:1 complex of

183 PTRAMP:CSS:Ripr **(Fig. 3e)**. The formation of a stable complex was further validated by the co-

184 elution of PvPC and PvRipr in size-exclusion chromatography **(Extended data Fig. 3d)**.

185

The C-terminus of Ripr is sufficient for PTRAMP-CSS binding in multiple species of *Plasmodium*

188 Previous studies have demonstrated that a three-membered PTRAMP-CSS-Ripr complex

189 is involved in *P. knowlesi* invasion[16]. We confirmed that PkPTRAMP and PkCSS form a disulfide-

190 linked heterodimer analogous to those observed in *P. falciparum* and *P. vivax* **(Fig. 4a)** and found

191 that the PkPC heterodimer exhibited high-affinity binding to PkRipr ($K_D = 0.6 \pm 0.1$ nM) **(Fig. 4b,**

192 **Extended data Fig. 4a, b).** Monomeric PkPTRAMP, but not monomeric PkCSS, was sufficient

193 for this interaction **(Fig. 4c, d)**. Like its *P. vivax* orthologs, PkPCR formed a stable complex as

194 demonstrated by mass photometry, with a molecular weight of 206 ± 13 kDa consistent with a

195 1:1:1 stoichiometry **(Extended data 4, Fig. 4e)**.

196 We performed a comparative biophysical analysis of PC-Ripr binding to identify the

197 minimal regions of Ripr required for complex formation. Several truncations in the Ripr tail region

198 were generated for *P. falciparum*, *P. vivax*, and *P. knowlesi* proteins and assessed for their ability

199 to bind their cognate PC heterodimer **(Extended data Fig. 5)**[19]. The tail region of Ripr, which

200 encompasses epidermal growth factor (EGF)-like domains 5-10 and the C-terminal domain (CTD),

201 was sufficient for heterodimer binding in all three species with no observable impact on affinity

202 **(Fig. 4f, Extended data Fig. 5)**[19]. A shorter construct containing only EGFs 9 and 10 plus the

203 CTD also retained the ability to bind the heterodimer **(Fig. 4f)**. Interestingly, complete removal of

204 all EGF domains, leaving only the CTD, abolished binding for *P. falciparum* proteins but not for

205 *P. vivax* or *P. knowlesi* **(Fig. 4f)**. These results demonstrate that heterodimer binding requires only

206 a discrete region of Ripr, with some species-specific differences in the minimal binding

207 requirements.

Anti-PCR antibodies are cross-reactive and differentially inhibit *Plasmodium* spp. invasion

Plasma samples from *P. falciparum*[37], *P. vivax*[38] and *P. knowlesi*[39,40] infected individuals from Thailand (Tha Song Yang) and Malaysia (Sabah) were assessed to determine the extent of patient antibody response to the components of the PCR invasion complexes (**Extended data Fig. 6**). IgG antibodies were assessed one week after clinical presentation and compared with malaria-naïve negative controls (IgG temporal kinetics from clinical presentation, one week, and one month post infection are shown in **Extended data Fig. 6**). Significant IgG antibody reactivity was detected for *P. falciparum* patients against the PCRCR complex components with the exception of PfCyRPA (**Fig. 5a**). Antibodies from *P. vivax* patients showed reactivity to the PvPCR components PvPC, PvCSS and PvRipr, but not PvPTRAMP (**Fig. 5a**). Similarly, antibodies from *P. knowlesi* patients showed reactivity to PkPC, PkCSS and PkRipr but not PkPTRAMP. Overall, there was a consistently low response to monomeric PTRAMP compared to other antigens and a consistently high response to monomeric CSS suggesting that the antibody response to the PC heterodimer is predominantly against CSS. High antibody responses were also observed for Ripr from all species and is consistent with Ripr being immuno-dominant as reported previously[41]. Furthermore, antibody responses to CSS and Ripr were broadly cross-reactive, with the antigens of all three species cross-reacting with sera from individuals independent of infective species (**Extended data Fig. 6**).

We sought to investigate the potential of antibodies and nanobodies to inhibit growth of multiple *Plasmodium* species, given the serological cross-reactivity observed. Monoclonal antibodies (mAbs) and nanobodies generated against PvPC and PvRipr were evaluated for cross-reactivity with their *P. falciparum* and *P. knowlesi* orthologs (**Extended data Fig. 7, 8, Supp. Fig. 5**). All tested antibodies bound to PkPC and PkRipr, with three out of seven showing cross-reactivity across all three species (**Extended data Fig. 7, 8, Fig. 5b**)[42]. All anti-PvPC nanobodies were cross-reactive with PkPC however this cross-reactivity was much lower against PfPC with only one out of eight binding PfPC (**Supp. Fig. 5**). Growth inhibition assays (GIAs) were performed against *P. knowlesi* to assess the inhibitory potential of anti-PvPC nanobodies and anti-PvPCR antibodies. Initial screening revealed that two anti-Ripr antibodies (5B3 and 5B4) and one anti-PC antibody (2D9) inhibited parasite growth at 0.5 mg/mL (**Fig. 5c**). This inhibition was dose-dependent, with 5B3 and 5B4 showing half-maximal effective concentrations (EC$_{50}$) of 77 µg/mL and 520 µg/mL respectively, while 2D9 exhibited an EC$_{50}$ of 657 µg/mL (**Fig. 5d**). The cross-reactive mAb 5B3 also inhibited *P. falciparum* growth, albeit with a significantly higher EC$_{50}$ of

240 3 mg/mL **(Fig. 5e)**. This reduced inhibitory effect may be attributed to varying affinities of 5B3

241 for different Ripr orthologs **(Extended data Fig. 8e)**. Neither 4E2 nor 4H10 affected *P. falciparum*

242 parasite growth, consistent with their lack of inhibitory activity against *P. knowlesi*. The mAb

243 4H10, which binds to the Ripr tail region and competes for PvPC binding **(Fig. 5b, Extended data**

244 **Fig. 8c)**, showed no inhibitory activity. This suggests that the PCR complex forms prior to

245 merozoite surface exposure, as observed previously for the PCRCR complex[18].

246 Following screening of the antibodies in *P. knowlesi* and *P. falciparum* GIAs **(Fig. 5c-e,**

247 **Supp. Fig. 6)**, we assessed their potential inhibitory effect on *P. vivax* merozoite invasion and

248 parasite growth in *ex vivo* GIAs. Assays performed on Cambodian *P. vivax* parasites revealed no

249 inhibitory effect for any of the tested antibodies **(Fig. 5f)**. To validate the *P. vivax* results, we

250 evaluated a subset of these antibodies for their ability to inhibit growth in the closely related species

251 *P. cynomolgi*[43]. The data from these assays were consistent with the *P. vivax* findings, confirming

252 that none of the antibodies could inhibit parasite growth in either of these two species **(Fig. 5g)**.

253 These results demonstrate that while antibodies against the PCR complex may exhibit cross-

254 reactivity across recombinant PCR complexes from multiple species of *Plasmodium*, this cross-

255 reactivity does not necessarily correlate with growth inhibitory capacity. As these antibodies were

256 raised against the *P. vivax* protein, these results either suggest minor functional differences

257 between the complexes of *P. falciparum* and *P. knowlesi* compared to *P. vivax* and *P. cynomolgi*

258 or that the PCR components are less critical for invasion of *P. vivax* and *P. cynomolgi*.

259

260 **Cryo-EM analysis of PkPCR supports AlphaFold predictions**

261 To provide more confidence in the predicted models of the PCR complexes, and to

262 understand how inhibitory antibodies may function, we carried out cryo-electron microscopy

263 (cryo-EM) experiments on the PkPCR complex. Cryo-EM analysis of the PkPCR[tail] complex

264 revealed an overall shape consistent with the AlphaFold prediction **(Fig. 6a, Extended data Fig.**

265 **9)**. The addition of the antigen-binding fragment (Fab) of 5B3 allowed unambiguous assignment

266 of the orientation of the two-dimensional (2D) classes **(Fig. 6a)**. Furthermore, comparison of Fab

267 bound and unbound classes showed no discernible differences in the PCR complex which

268 confirmed that 5B3 binds to the tail region of Ripr without interfering with complex formation

269 **(Fig. 5b, Extended data Fig. 8, 9)**. This suggests that parasite inhibition by 5B3 likely has a direct

270 effect on Ripr function during invasion rather than on the complex as a whole. The cryo-EM data,

271 combined with the PvPC crystal structure, strongly support the predicted PCR complex structure

272 **(Fig. 6b)**. In this model, the PTRAMP-CSS heterodimer is formed by an intermolecular disulfide

273 bond. This heterodimer engages Ripr via two interfaces: PTRAMP clinching the CTD of Ripr, and

274 the D2 domain of CSS interacting with EGF 9 of Ripr **(Fig. 6b)**. The remaining mass of Ripr likely

275 extends below the PCR complex, where it may interact with other invasion proteins (such as

276 PfCyRPA) or potentially with erythrocyte proteins.

277 While PfPC lacks the ability to bind erythrocytes directly, it enhances Rh5 binding when

278 incorporated into the PCRCR complex[18]. Previous studies have shown that PkPTRAMP can bind

279 erythrocytes; however, these experiments were performed with monomeric PkPTRAMP and not

280 heterodimeric PkPC[16]. We performed flow-cytometry based erythrocyte binding assays to assess

281 whether PkPC or the PkPCR complex bound to erythrocytes. Neither PkPC nor PkPCR showed

282 significant binding to erythrocytes relative to the positive control, PfRh5 **(Extended data Fig. 10a,**

283 **b)**. Considering that *P. vivax* invades reticulocytes exclusively, we extended our investigation to

284 include reticulocyte binding assays. Similarly, we observed no binding of PvPC or PkPC to

285 reticulocytes **(Extended data Fig. 10c, d)**. Collectively our analysis found no evidence of

286 erythrocyte or reticulocyte binding by PkPC, PvPC, or the PkPCR complex.

287 We therefore hypothesize that PTRAMP, CSS and Ripr form a core invasion scaffold in

288 *Plasmodium* parasites. This scaffold provides the basis for the assembly of species-specific

289 complexes that are adapted for binding a diverse set of host erythrocyte receptors **(Fig. 6c)**. In *P.*

290 *falciparum* this complex incorporates CyRPA and Rh5 which facilitate invasion via the essential

291 interaction with basigin. The equivalent proteins in *P. vivax* and *P. knowlesi* that are responsible

292 for erythrocyte binding are yet to be identified. If findings from *P. falciparum* are applicable to

293 these species, these interactions will be an essential step in merozoite invasion.

294

Discussion

296 The highly conserved nature of PTRAMP, CSS, and Ripr across the *Plasmodium* genus,

297 combined with their demonstrated essential roles in both *P. falciparum*[18] and *P. knowlesi*[16]

298 invasion, positions these proteins as compelling targets for understanding fundamental

299 mechanisms of merozoite invasion. Recent advances in protein structure prediction through

300 AlphaFold[33] have enabled a comprehensive comparative analysis of these proteins across three

301 clinically significant *Plasmodium* species: *P. falciparum*, *P. knowlesi*, and *P. vivax*. Our cross-

302 species structural and functional analyses reveal that PTRAMP, CSS, and Ripr form a conserved
303 invasion scaffold in *Plasmodium* parasites that serves as a foundation for the assembly of species-
304 specific protein complexes **(Fig. 6c)**. These complexes appear to be evolutionarily adapted for
305 engaging diverse host erythrocyte receptors. In *P. falciparum*, this complex includes CyRPA and
306 Rh5, which mediate the essential interaction with the host receptor basigin. While the equivalent
307 erythrocyte-binding proteins in *P. vivax* and *P. knowlesi* remain unidentified, the conservation of
308 this core scaffold suggests that analogous essential receptor-ligand interactions likely govern
309 invasion in these species.

310 Structural analysis of PvPC revealed a critical intermolecular disulfide bond between
311 PvPTRAMP and PvCSS. The essentiality of this linkage was previously established in *P.*
312 *falciparum* invasion[18], and the evolutionary conservation of these cysteine residues across
313 *Plasmodium* species strongly suggests that PTRAMP-CSS heterodimerization represents a
314 fundamental feature throughout the genus. Optimization of recombinant PfPC heterodimer
315 revealed a much tighter interaction with PfRipr than previously reported[18,19], aligning with the
316 structural architecture predicted by AlphaFold[33]. Our biochemical studies demonstrated that the
317 heterodimeric PkPC forms a high-affinity complex with PkRipr, corroborating earlier pull-down
318 mass spectrometry data from *P. knowlesi* parasites[16] and providing robust evidence for the
319 biological significance of this complex *in vivo*. The formation of this trimeric complex extends
320 beyond *P. knowlesi* and *P. falciparum*, as we also demonstrated its assembly in *P. vivax*, providing
321 evidence for a conserved molecular feature across multiple *Plasmodium* species.

322 Previous studies have shown that while PfRipr's core region interacts with CyRPA to form
323 the RCR complex, its C-terminal tail mediates PfPC binding[17,19]. Our findings have further refined
324 this understanding by demonstrating that only a small domain within the Ripr tail is required for
325 PC heterodimer binding. In *P. falciparum*, this binding region encompasses EGFs 9 and 10 and
326 the CTD of PfRipr. Notably, in both *P. vivax* and *P. knowlesi*, the CTD alone is sufficient for PC
327 heterodimer binding, indicating evolutionary divergence in these interactions across *Plasmodium*
328 species. The observation that both PvPC and PkPC can bind Ripr^{CTD}, coupled with the finding that
329 PvPTRAMP and PkPTRAMP alone are sufficient for Ripr binding, suggests that the PTRAMP-
330 Ripr^{CTD} interaction serves as the primary interface driving complex formation.

331 Analysis of antibodies from patients infected with *P. falciparum*, *P. knowlesi*, or *P. vivax*
332 has revealed significant cross-reactivity of antibodies to CSS and Ripr across these *Plasmodium*

333   species. It is unlikely that these patients had previously been recently infected with all three

334   *Plasmodium* species, particularly given the low transmission in these settings, suggesting that

335   antibodies generated against the PCR complex targeted conserved epitopes. This raised the

336   potential for cross-species antibody-mediated inhibition of invasion. Indeed, analysis of

337   monoclonal antibodies identified the Ripr-binding mAb 5B3, which exhibited cross-inhibitory

338   activity against both *P. knowlesi* and *P. falciparum*, but not *P. vivax* or *P. cynomolgi*. Interestingly,

339   this mAb was raised against PvRipr, suggesting functional differences with the PCR complex

340   between these *Plasmodium* species that may render the conserved epitope on PvRipr and PcRipr

341   inaccessible to the antibody in the full complex. It is also possible that the essentiality of the

342   complex differs between species. The high degree of conservation of the complex and its

343   components across the *Plasmodium* genus would make this conclusion unlikely. The differential

344   inhibition is unlikely to be due to antibody affinity but may reflect differences in how Ripr

345   functions during invasion of different host cells. The identification of cross-species neutralizing

346   antibodies is an attractive finding for vaccinology[44,45]; however, the inhibitory activity of such

347   naturally acquired cross-reactive antibodies is yet to be fully explored. The conservation of the

348   PCR complex makes it an attractive target for such an approach.

349   The considerable length of Ripr (>150 Å), while largely uninvolved in PCR complex

350   formation, may be important for enabling the PCR/PCRCR complex to bridge the gap between the

351   merozoite surface and host cell membrane during invasion **(Fig 6c)**[19]. This model is supported by

352   previous studies showing that antibodies targeting EGF domains 6, 7, and 8 within the Ripr tail

353   effectively inhibit parasite growth *in vitro*[24,41]. Our findings suggest these antibodies may function

354   by preventing adequate extension of Ripr between the two membranes, thereby disrupting receptor

355   engagement. Understanding the structural basis of Ripr inhibition will be crucial for elucidating

356   its role within the PCR complex and its potential as a therapeutic target.

357

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Author Contributions

BAS designed experiments, expressed proteins, performed and analyzed biophysical experiments, solved the crystal structure with assistance from SWS, analyzed cryo-EM data, and wrote the manuscript. PSL, LBFD, KHL and SD performed parasite growth inhibition assays and analyzed data. XX and NCJ purified proteins and performed biophysical assays. AA and PSL performed serological assays and analyzed data. TW, MJG, NMA, JS and RJL organized and collected patient plasma and clinical data for antibody analysis. AL carried out cryo-EM data collection. RJL, MJG, MTD, JP, DWW, SWS designed and interpreted experiments. AFC and SWS designed and interpreted experiments and wrote the manuscript. All authors read and edited the manuscript.

Competing interests

The authors have no conflicts of interest to declare.

Fig. 1. PTRAMP, CSS, and Ripr are common to all clades of *Plasmodium*.
a. Comparison of PCRCR orthologs in the *Plasmodium* genus, showing that PTRAMP, CSS, and Ripr are common to all species, whereas Rh5 is restricted to the *Laverania* subgenus, and CyRPA is absent from the rodent-infective species (*Vinckeia*). **b.** AlphaFold 3 predictions of PTRAMP, CSS, and the Ripr tail show a conserved architecture is predicted for each species. For the *P. falciparum* predicted complex, the previously published structure for PfCSS is superimposed in dark green. Regions that are predicted to be disordered, and therefore have low model confidence, are shown as transparent. The transmembrane domain and signal peptide have been removed from PTRAMP and CSS for clarity **c.** Domain diagrams for PfPTRAMP, PfCSS and PfRipr showing the predicted N-linked glycosylation sites[35] (blue) that were either mutated (red) or the sequon truncated (grey). Grey regions indicate the stretches of sequence either processed (signal peptides) or removed for recombinant expression, in the case of PfPTRAMP (transmembrane domain and cytoplasmic tail). **d.** SDS-PAGE of non-glycosylated PfPC in both non-reduced (NR) and reduced (R) conditions. **e.** A representative biolayer interferometry sensorgram of non-glycosylated PfRipr binding to non-glycosylated PfPC showing data (yellow) and 1:1 model best fit (black).

Fig. 2. Structure of the intermolecular disulfide bond between PvCSS and PvPTRAMP.

a. SDS-PAGE of recombinantly expressed PvPTRAMP, PvCSS and PvPC heterodimer. **b.** Domain diagram of PvCSS showing the disordered repeat region at the N-terminus. **c.** Crystal structure of $PvCSS_{115-381}$(green) and $PvPTRAMP_{42-53}$(pink) with the disulfide formed between them in space-filling atomic depiction (yellow). **d.** Detail of the intermolecular disulfide bond between PvCSS and PvPTRAMP. Density is contoured at 1.0 σ and density extends to a range of 1.8 Å. **e.** A model of PvCSS and $PvPTRAMP_{42-53}$, showing the region of the unbiased electron density omit map that was attributed to PvPTRAMP. Density is represented as in d). **f.** The interface between $PvPTRAMP_{42-53}$ and PvCSS. All intermolecular hydrogen bonds formed are represented in grey.

Fig. 3. The PvPC heterodimer forms a stable complex with PvRipr. **a.**, **b.** and **c.** Representative biolayer interferometry sensorgrams of PvPC, PvPTRAMP, PvCSS, and PvRipr binding assays. Dilution series data are shown in color and 1:1 model best fit is shown in black. Representative sensorgrams of PvPTRAMP, PvRipr and PvPC binding to PvCyRPA at an analyte concentration of 5 μM. Data could not be reliably fit with a model, and so no best fit has been shown. **e.** Mass distribution of PvPC and PvRipr after pre-incubation as measured by mass photometry. Histogram data are shown in grey and Gaussian curve fit in black.

Fig. 4. A small region of Ripr is sufficient for PTRAMP-CSS binding in *P. falciparum, P. vivax,* and *P. knowlesi*. a. SDS-PAGE of recombinantly expressed PkPTRAMP, PkCSS and PkPC heterodimer **b-d**. Representative biolayer interferometry sensorgrams of PkPC, PkPTRAMP, PkCSS and PkRipr binding assays. Dilution series data are shown in color and 1:1 model best fit is shown in black. **e.** Mass distribution of PkPC and PkRipr after pre-incubation as measured by mass photometry. Histogram data are shown in grey and Gaussian curve fit in black. **f.** The ability of Ripr and Ripr truncations to bind to PTRAMP-CSS in *P. falciparum, P. vivax* and *P. knowlesi*. The table shows the truncations used and their dissociation constant (K_D, in nM, with standard error of the mean (SEM)) for binding their cognate heterodimer. N.B indicates no binding.

Fig. 5. Cross-reactive antibodies targeting PTRAMP, CSS, and Ripr exhibit differential inhibition in multiple species of *Plasmodium*. a. IgG antibodies in human patients with *Plasmodium* infections. Fold-change peak week one antibody response relative to the

436   seropositivity cut-off (mean of negative controls + 2x standard deviation). The panels are facetted

437   by species of the recombinant protein (*P. falciparum*, *P. knowlesi* and *P. vivax*). The colored dots

438   represent the plasma samples in which the proteins were assayed. **b**. Purified mouse monoclonal

439   antibodies mapped by binding region. Open text represents antibodies that are able to bind both *P.*

440   *vivax* and *P. knowlesi,* and closed text represents cross-reactivity between *P. vivax, P. knowlesi*

441   and *P. falciparum*. Bold line indicates that 4H10 blocks PvPC binding to Ripr **c.** *P. knowlesi*

442   growth inhibition assay of anti-PvPC and anti-PvRipr biologics. The non-inhibitory and PfCSS

443   nanobody H2 was included as a negative control[18]. Three independent experiments were

444   performed, and the mean and SEM are shown in black. Antibodies and nanobodies were tested at

445   a final concentration of 0.5 mg/mL. Data points are colored according to antigen, with CSS in

446   green, PTRAMP in pink and Ripr in yellow **d.** Growth inhibition dilution series for inhibitory

447   antibodies in *P. knowlesi*. Growth inhibition (%) is the mean of four independent experiments.

448   Error bars represent standard deviation. **e.** Growth inhibition dilution series for inhibitory

449   antibodies in *P. falciparum*. Growth inhibition (%) is the mean of four independent experiments

450   for 5B3, and two independent experiments for 4E2 and 4H10. Error bars represent standard

451   deviation. **f.** *Ex vivo* growth inhibition assay of *P. vivax* parasites. Antibodies were tested at a final

452   concentration of 0.5 mg/mL. Anti-Duffy antigen receptor for chemokines (DARC) mouse

453   monoclonal antibody 2C3 was used as a positive control[46]. Data are from six independent

454   experiments. Error bars show mean and SEM. Data points colored as in c. **g.** *P. cynomolgi* growth

455   inhibition assay of anti-PvPC and anti-PvRipr antibodies. Antibodies were tested at a final

456   concentration of 0.5 mg/mL. Three independent experiments were performed, and the mean and

457   SEM are shown in black. Data points are colored as in c.

458

459   **Fig. 6. PTRAMP, CSS, and Ripr form a core invasion scaffold in *Plasmodium* spp. a.** Cryo-

460   EM 2D class averages of the PkPCR[tail] complex. Addition of the 5B3 Fab fragment shows distinct

461   density at the end of the Ripr tail (white triangle). Inset shows the PkPCR[tail] complex colored

462   according to the predicted model **b.** AlphaFold 3 predicted model (left) and diagram (right) of

463   PkPCR[tail]. Transmembrane domains, signals sequences and large disordered regions have been

464   omitted for clarity. Inset shows the alignment of the PvPC (purple and dark green) structure and

465   the PkPC predicted structure (pink and light green) **c.** Model of the PCRCR complex of *P.*

466   *falciparum* and PCR complexes of *P. vivax,* and *P. knowlesi.* PTRAMP, CSS and Ripr form a

467   conserved three-membered complex that serves as a scaffold for an invasion complex. In *P.*

468 *falciparum* this complex involves CyRPA and Rh5. In *P. vivax* and *P. knowlesi* this complex likely

469 contains other components (dashed grey) that have yet to be identified, which engage with the

470 erythrocyte membrane via host-cell specific receptors.

471

Materials and Methods

472

473

474 **Recombinant protein expression**

475 All gene sequences used were retrieved from the VEuPathDB (accessed through

476 www.plasmodb.org)[47] from reference strains (3D7 for *P. falciparum,* PvP01 for *P. vivax (*with the

477 exception of PvRBP2b for which the *Sal*-1 sequence was used)*,* and strain H for *P. knowlesi*). All

478 genes were synthesized by Genscript (Singapore) unless otherwise stated.

479

480 *P. falciparum*

481 Recombinant PfRh5, PfCyRPA, PfPTRAMP, PfPC and PfRipr constructs were produced as

482 described previously, with some changes made to the synthetic gene constructs used for

483 expression[18]. PfPTRAMP, comprising residues 31 to 307, was subcloned into the pAcGP67a

484 vector with a C-terminal C-tag. Four potential N-linked glycosylation sites were removed, at

485 positions Asn112, Asn149 and Asn155 by mutation to Gln, and at position Asn195 by mutation of

486 Thr197 to Ala, to produce PfPTRAMP_31-307_4x. To produce a large amount of pure monomeric

487 PfPTRAMP, another construct was made that contains all of the same mutations and also contains

488 Cys60Ser mutation to prevent disulfide formation, termed PfPTRAMP_31-307_4xC60S. PfCSS

489 was subcloned into the pAcGP67a vector with a C-terminal FLAG-tag preceded by a TEV protease

490 cleavage site. This construct has all six potential N-linked glycosylation sites removed at positions

491 Asn74, Asn88, Asn192, Asn234, Asn261 and Asn283 by mutation of Ser76, Thr90, Ser194,

492 Thr236 and Thr263 to Ala and Asn283 to Gln, to produce PfCSS_21-290_6x[18]. The previous

493 PfRipr construct[18] was altered with the following mutations: Thr966Ala-Ser1023Ala yielding the

494 construct PfRipr_20-1086_2xA. PfRipr^tail^(aa 717-1086), PfRipr^EGF 9,10,CTD^(aa 899-1086) and

495 PfRipr^CTD^(aa 981-1086), were all synthesized by Genscript and purified in an identical fashion to

496 PfRipr.

497

498 *P. vivax*

499 The *pvptramp* gene (PVP01_1436800, aa 21-297) excluding the transmembrane and cytoplasmic

500 domains was subcloned into a modified pTRIEX2 vector that contains an N-terminal Small

501 Ubiquitin-like Modifier (SUMO)-Flag tag followed by a Tobacco Etch Virus (TEV) protease

502 cleavage site (from here on termed SFT). Potential N-glycosylation sites were assessed and one

503 site, Asn115, was removed by mutation of Ser117 to Ala. This yielded SFT_PvPTRAMP_21-

504 297_S117A. This construct was then further cloned to incorporate a C-terminal Avitag, yielding

505 SFT_PvPTRAMP_21-297_S117A_Avi. Both constructs were expressed in Human Embryonic

506 Kidney (HEK) Expi293F cells (Life Technologies) as secreted soluble proteins. Transient

507 transfection was carried out as per the manufacturer's protocol and the culture medium harvested

508 5-6 days post-transfection. The proteins were purified via multiple rounds of binding and eluting

509 using Anti-Flag M2 Affinity Gel (Merck) and 100 µg/mL of Flag peptide (Genscript) in HBS (20

510 mM HEPES pH 7.2, 150 mM NaCl). The eluted fractions were pooled and incubated with TEV

511 protease (1 mg of TEV for every 10 mg of protein) overnight at 4°C. His-tagged TEV was removed

512 by applying the protein solution to nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen)

513 and collecting the flowthrough. The flowthrough was then concentrated on a 10,000 dalton (Da)

514 Molecular Weight Cut-Off (MWCO) Amicon Ultra-15 Centrifugal Filter (Merck) and applied to

515 an S75 Increase 10/300 column (Cytiva) connected to an Akta Pure (Cytiva) to separate the TEV-

516 cleaved PvPTRAMP from the SUMO-Flag tag. Peak fractions were assessed for purity via sodium

517 dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and pure fractions were pooled

518 and concentrated. Glycerol was added (10% v/v final) to the concentrated protein and then

519 aliquoted and flash frozen in liquid nitrogen (LN$_2$).

520

521 The full-length *pvcss* gene (PVP01_1344100, aa 22-381) was subcloned into a modified pTRIEX2

522 vector with a C-terminal Flag-tag preceded by a TEV protease cleavage site (from here on termed

523 TF). N-glycosylation sites were predicted and three potential sites, Asn114, Asn180, Asn352, were

524 mutated via three mutations: Ser116Ala, Thr182Ala and Ser354Ala. This yielded PvCSS_22-

525 381_S116A_T182A_S354A_TF. This construct was further cloned to remove a predicted N-

526 terminal repeat region to aid in crystallisation. This yielded the construct PvCSS_115-

527 381_S116A_T182A_S354A_TF. These constructs were expressed as soluble secreted proteins in

528 HEK Expi293F cells (Life Technologies) as above.

529

530 To generate disulfide-linked PvPTRAMP-PvCSS (PvPC), PvPTRAMP and PvCSS were co-

531 expressed in HEK Epi293F cells (Life Technologies) at a ratio of 50:50 PvPTRAMP:PvCSS for

532 full length CSS and a ratio of 40:60 PvPTRAMP:PvCSS for PvCSS_115. The expression and

533 purification were carried out as above with size-exclusion chromatography performed using either

534 an S200 Increase 10/300 GL (Cytiva) or S200 16/600 HiLoad (Cytiva).

535

536 The full-length *pvripr* gene (PVP01_0816800, aa 22-1074) was subcloned into pAcGP67a with a

537 C-terminal 6xHis-tag yielding PvRipr_22-1074_His and expressed in Sf21 cells using the

538 flashBAC ULTRA baculovirus system (Oxford Expression Technologies). After initial

539 transfection, the P1 virus was amplified and titrated several times to produce a P3 virus. This P3

540 virus was used for large scale expression. The proteins were expressed as soluble secreted proteins.

541 The culture medium was harvested 3 days after the addition of P3 virus. The media was

542 concentrated via tangential-flow filtration with a 3,000 Da MWCO (Merck) to reduce the volume

543 ~10-15 fold. This resultant concentrate was then dialysed into TBS (20 mM Tris pH 8.5, 150 mM

544 NaCl) overnight at 4°C. Imidazole was then added to a final concentration of 10 mM and the

545 culture media passed over Ni-NTA Agarose resin (Qiagen), washed with TBS + 20 mM imidazole,

546 and eluted in TBS + 500 mM imidazole. The eluted protein was then concentrated on a 30,000 Da

547 MWCO Amicon Ultra-15 Centrifugal Filter (Merck) and applied to an S200 Increase 10/300 GL

548 column (Cytiva) connected to an Akta Pure (Cytiva). Peak fractions were pooled, concentrated

549 and flash frozen as above. PvRipr truncations (PvRipr^{tail}(aa 665-1074), PvRipr$^{EGF\,9,10,CTD}$(aa 844-

550 1074), PvRipr^{CTD}(aa 972-1074)) were synthesized via the Genscript mutagenesis service. The

551 truncated constructs were subcloned into the pAcGP67a vector and purified in an identical manner

552 to the full-length construct.

553

554 PvRipr$^{EGF\,6-8}$(aa 717-843) was synthesized and subcloned into pET28a yielding a construct with

555 an N-terminal 6x His tag followed by a TEV site. Expression was carried out in *Escherichia coli*

556 *(E. coli)* strain SHuffle® T7 (New England Biolabs) grown in terrific Broth with 40 µg/mL of

557 kanamycin. One litre of culture was grown in incubators at 37°C and shaking at 180 revolutions

558 per minute (rpm) until an optical density at 600 nm (OD 600) of around 1.0 was reached. Isopropyl

559 ß-D-1-thiogalactopyranoside (IPTG)(Astral) was then added to a final concentration of 1mM, and

560 protein expression was continued at 16°C for 16-18 hours. Cells were then harvested via

561 centrifugation and the pellet resuspended in TBS pH 8.5, and with cOmplete

562 ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche). The

563 resuspended cells were then sonicated, and the cellular extract clarified by centrifugation at 30,000

564 x*g* for 30 minutes at 4°C. Imidazole was added to the clarified supernatant to a final concentration

565   of 10 mM and then passed over pre-equilibrated Ni-NTA resin, washed with TBS pH 8.5 + 20 mM

566   imidazole, and then eluted in TBS pH 8.5 containing 500mM imidazole. The eluted protein was

567   then concentrated on a 10,000 MWCO Amicon Ultra-15 Centrifugal Filter (Merck) and injected

568   onto an S200 16/600 HiLoad (Cytiva) equilibrated in TBS pH 8.5. Peak fractions were then

569   concentrated, supplemented with glycerol to a final concentration of 10% (v/v) and flash frozen in

570   liquid nitrogen.

571

572   The *pvcyrpa* gene (PVP01_0532400, aa 24-362) was subcloned into pTRIEX2-TF vector which

573   yielded PvCyRPA_22-362_TF. Two predicted N-glycoslyation sites, Asn78 and Asn282, were

574   removed with the following mutations: Thr80Ala, Thr284Ala. The protein was expressed in HEK

575   Expi293F cells and purified in an identical manner to PvPTRAMP above.

576

577   PvRBP2b (PVX_094255, aa 161-1454) was purified as described previously[48].

578

579   ***P. knowlesi***

580   The *pkptramp* gene (PKNH_1437600, aa 21-297) excluding the transmembrane and cytoplasmic

581   domains was subcloned into a modified pTRIEX2-SFT as was done for PvPTRAMP. Potential N-

582   glycosylation sites were assessed and two sites, Asn115 and Asn261, were removed by mutation

583   of Ser117 and Ser263 to Ala. This yielded SFT_PkPTRAMP_21-297_S117A_S263A. A

584   PkPTRAMP construct expressing a C-terminal Avitag for biotinylation was made using PCR and

585   restriction digests to yield SFT_PkPTRAMP_21-297_S117A_S263A-Avi. Expression of

586   PkPTRAMP and PkPTRAMP-Avi was carried out in an identical manner to PvPTRAMP

587   described above.

588

589   The *pkcss* gene (PKNH_1353400, aa 22-362) was subcloned into pTriEX2-TF. Five potential N-

590   glycosylation sites, Asn96, Asn161, Asn175, Asn243 and Asn333 were removed via five

591   mutations: Ser98Ala, Thr163Ala, S177Ala, S245A and S335A. This yielded PkCSS_22-362_

592   S98A_T163A_S177A_S245A_S335A _TF. Expression of PkCSS was carried out in an identical

593   manner to PvCSS above.

594

595   Expression of the PkPC heterodimer was carried out in an identical manner as described for PvPC.

596   The ratio of PkPTRAMP:PkCSS deoxyribonucleic acid (DNA) used was 60:40 when

597   PkPTRAMP-SFT and PkCSS-TF were being used, and 50:50 when PkPTRAMP-Avi was being

598   used.

599

600   The *pkripr* gene (PKNH_0817000, aa 22-1096) was subcloned into pAcGP67a with a C-terminal

601   6xHis-tag yielding PkRipr_22-1096_His, as per the PvRipr construct. Truncations of the full-

602   length construct were made by Genscript using the mutagenesis service, to produce PkRipr^{tail}(aa

603   669-1096), PkRipr$^{EGF\ 9,10,CTD}$(aa 848-1096) and PkRipr^{CTD}(aa 994-1096). All PkRipr constructs

604   were expressed and purified in an identical manner to the equivalent PvRipr constructs.

605

606   For all constructs containing an Avitag, *in vitro* biotinylation was carried out as previously

607   described[49].

608

609   **Antibodies and nanobodies**

610   One alpaca was subcutaneously immunized six times 14 days apart with 130 μg (800 μg total) of

611   recombinant PvPC. GERBU FAMA (GERBU Biotechnik GmbH, Heidelberg, Germany) was used

612   as an adjuvant. Whole blood was collected three days after the last immunization for the

613   preparation of lymphocytes. Nanobody library construction was carried out according to

614   established methods[50]. Briefly, alpaca lymphocyte mRNA was extracted and amplified by reverse

615   transcription PCR (RT-PCR) with nanobody-encoding, gene-specific primers. This produced a

616   library of nanobody cDNA sequences that contained approximately 10^8 sequences. The sequences

617   that were cloned into the pMES4 phagemid vector were amplified in *E. coli* TG1 strain and

618   subsequently infected with M13KO7 helper phage for downstream recombinant phage expression.

619   Handling of the alpaca for scientific purposes was approved by Agriculture Victoria, Wildlife and

620   Small Institutions Animal Ethics Committee, project approval No. 26-17.

621

622   Biopanning was performed over two rounds with 1 μg of immobilized antigen as previously

623   described[50]. Ninety-four positive clones were taken for further screening via enzyme-linked

624   immunosorbent assay (ELISA). Clones showing positive binding by ELISA (n = 93) were

625   sequenced. Of these, 71% were full length Variable Heavy domain of Heavy chain (VHH) (n =

626   66).

627

628 Nanobodies were expressed in the periplasm of *E. coli* WK6 cells as described previously (6).
629 Briefly, bacteria (250 mL) were grown in Terrific Broth at 37°C to an OD 600 of 0.7. The cultures
630 were then induced with 1 mM IPTG (Astral) and grown overnight at 28°C. Cells were harvested
631 and resuspended in PBS containing 20% sucrose and 20mM imidazole to rupture the periplasm.
632 EDTA was added to a final concentration of 5 mM, and the cells were incubated on ice. $MgCl_2$
633 was then added to a final concentration of 10 mM, and the periplasmic extract was harvested via
634 centrifugation. The nanobodies were purified via standard Ni-NTA purification methods.
635
636 Monoclonal antibodies were raised in mice as per the Walter and Eliza Hall Animal Ethics
637 Committee approved procedures. All monoclonal antibodies were produced by the WEHI
638 Antibody Facility. Mice were injected with 80-180 µg of protein three times and then boosted once
639 with 30-60 µg. After cloning of hybridomas, the supernatants were tested via ELISA and BLI.
640 Based on these results, several hybridomas for each antigen were selected for further scale up of
641 purified immunoglobulin G (IgG).
642

643 **Structure prediction**
644 Prediction of PCR complexes from multiple *Plasmodium* species was done using the AlphaFold 3
645 server[34].
646

647 **Biolayer interferometry (BLI)**
648 Biolayer interferometry (BLI) experiments were carried out on an Octet Red96e (Sartorius) at
649 25°C. For kinetics analysis ligands were immobilized onto either anti-penta-His (His1K),
650 streptavidin (SAX or SAX2) or Ni-NTA (NTA) biosensors (Sartorius) depending on the affinity
651 tag present on the protein (His-tag or biotinylated Avitag). Ligands were diluted to 10-40 µg/mL
652 in 1x kinetics buffer (PBS, pH 7.4, 0.1% (w/v) bovine serum albumin (BSA), 0.02% (v/v) Tween-
653 20) prior to immobilisation. Biosensors were initially dipped in kinetics buffer for 30-60 seconds
654 to establish a baseline signal, and then dipped into wells containing the ligand, followed by another
655 30-60 second baseline. After the second baseline step, the ligands were then dipped into wells
656 containing two-fold dilution series of analyte. Association was measured for 120 seconds and then
657 the biosensors were dipped into kinetics buffer to measure the dissociation for another 120 seconds.
658 Data were analysed using Sartorius Data Analysis software 11.0. Kinetic curves were fitted using
659 a 1:1 binding model.

660

661 Competition studies for anti-PvPC nanobodies were performed using Ni-NTA (NTA) biosensors

662 (Sartorius) with His-tagged nanobodies as the ligand (diluted to 5 µg/mL in kinetics buffer). After

663 a 30 second baseline step, the biosensors were dipped into wells containing an irrelevant nanobody

664 that does not bind to PvPC to quench the biosensor and ensure no free sites are present for the

665 downstream steps. Following a second baseline step, the biosensors were dipped into PvPC diluted

666 to 500 nM in kinetics buffer. After loading of PvPC onto the biosensors, a final baseline step was

667 performed before the biosensors were dipped into either secondary nanobody (at 10 µg/mL diluted

668 in kinetics buffer) or PvRipr (at 200 nM diluted in kinetics buffer). Data were analysed using

669 Sartorius' Data Analysis software 11.0 and the epitope bins were assessed by normalization and

670 manual curation.

671

672 Antibody kinetics were determined similarly to the above methods. Anti-Mouse IgG Fc Capture

673 (AMC) biosensors (Sartorius) were used to immobilize mouse monoclonal antibodies at a

674 concentration of 5-20 µg/mL in kinetics buffer. Antibody competition studies were carried out in

675 a similar manner to the nanobodies, however anti-pentaHis (His1K) biosensors were used.

676

Protein crystallization

678 PvPC_115 was purified as above. PvPC and nanobody D7 were co-complexed with the nanobody

679 at 3x molar excess. The free nanobody was separated from the PvPC-nanobody complex by size-

680 exclusion chromatography on an S200 Increase GL 10/300 (Cytiva) in HBS. Peak fractions were

681 pooled and concentrated to ~5-6 mg/mL and set up in coarse screen sitting drop crystal trays at the

682 Monash Macromolecular Crystallisation Facility. Needle-like crystals formed after ~9 days in

683 0.2M ammonium sulfate ($(NH_4)_2SO_4$) and 20% (w/v) polyethylene glycol (PEG) 3,350. Further

684 in-house optimization of conditions yielded large crystals in 0.2M $(NH_4)_2SO_4$ and 16% (w/v) PEG-

685 3,350. Crystals were looped in mother liquor containing 10% (v/v) glycerol and flash frozen in

686 liquid nitrogen. Diffraction data were collected with the MX2 beamline at the Australian

687 Synchrotron (Clayton, Australia) at 100 K (λ = 0.9537 Å). Statistics are in Table 1.

688

Structure determination and model building

690 Diffraction data were processed with the XDS package[51] before being scaled and merged using

691 Aimless[52] in the CCP4 suite[53]. The program Matthews[54] was used to estimate the number of

692   molecules in the asymmetric unit. An AlphaFold 2[33] model of PvCSS constituting residues 115-

693   381 was used as a search model for molecular replacement using Phaser[55]. After 3 copies of PvCSS

694   were fitted, additional searches were performed with a nanobody structure. To ensure the best fit

695   possible, a BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) search was

696   performed with nanobody D7 to find the structure with the highest sequence similarity for

697   molecular replacement searches. This search yielded a nanobody (PDB: 7N0R), which was used

698   as a search model with the complementarity determining region 3 (CDR3) sequence removed[56].

699   The structure was then iteratively refined in Phenix[57] and assessed and modified with Coot[58]. Clear

700   density extended from the unpaired cysteine in PvCSS, C122, that was not accounted for by either

701   PvCSS or nanobody D7. Due to the crystals being set up with the PvPC heterodimer, it was

702   reasonable to conclude that this density belonged to PvPTRAMP. PvPTRAMP residues 41-53

703   were built into the electron density *de novo* independently for each of the three molecules in the

704   asymmetric unit. Refinement and model statistics are described in Table 1. For analysis of the

705   contacts formed between PvPTRAMP and PvCSS, and PvCSS and D7, the program Contact (part

706   of CCP4 suite)[53] was used in conjunction with PISA server (https://www.ebi.ac.uk/pdbe/pisa/) and

707   are summarized in Tables 2 and 3. Nanobodies were renumbered according to the Kabat

708   numbering system, as determined by ANARCI[59].

709

710   **Mutiple sequence alignment**

711   Multiple sequence alignments were computed using ESPript 3.0[60].

712

713   **Mass photometry**

714   Mass photometry experiments were carried out on a Two^MP mass photometer (Refeyn). Each well

715   was focused after the addition of 10 μL of filtered PBS. Once focused, 10 μL of protein at either

716   50 nM (*P. knowlesi*) or 100 nM (*P. vivax*) was added, mixed, and events were recorded for one

717   minute using AcquireMP (Refeyn). Raw data processing was done in DiscoverMP (Refeyn) and

718   the data exported and presented using Prism v9 (GraphPad). In-house recombinant mouse mixed

719   lineage kinase-like (MLKL), human glutamine synthetase and human catalase were used for the

720   construction of a calibration curve.

721

722   **Human samples**

723   Human plasma samples were utilized from three cohorts of *Plasmodium* infected patients along

724   with three cohorts of malaria-naïve negative controls. Patients infected with *P. vivax* were recruited

725   from Tha Song Yang, Thailand, during the year 2014, as previously described[38], with a subset of

726   34 included in the current study. Patients infected with *P. falciparum*[37] and *P. knowlesi*[39,40] were

727   recruited from Sabah, Malaysia, during the years 2010-2018 and 2012-2014, respectively. *P.*

728   *falciparum* (n = 31) and *P. knowlesi* (n = 33) infected patients were included in the current study.

729   Plasma samples were assayed from time of clinical presentation, 1 week later, and 1 month later.

730   28 malaria-naïve samples from the Melbourne Volunteer Biospecimen Donor Registry (VBDR)

731   and 29 malaria-naïve samples from the Thai Red Cross (TRC) were utilized to create seropositivity

732   cut-offs. Individuals from the TRC donated blood in Bangkok, a malaria-free region of Thailand,

733   and had not had malaria diagnosed in the year prior nor had they travelled to endemic regions in

734   the prior three years, as previously described[61]. An additional set of afebrile healthy controls

735   (n=30) were assayed from Sabah, Malaysia; however, these individuals may have had prior

736   *Plasmodium* infections and were thus not utilized to create the seropositivity cut-off.

737

738   Ethical approval for sample use was provided by WEHI Human Research Ethics Committee

739   (14/02), with original study approval in Thailand (Faculty of Tropical Medicine, Mahidol

740   University, MUTM 2014-025-01 and 02) and Malaysia (Menzies School of Health Research,

741   HREC 12-1815, 16-2544, 10-1431, 12-1807). All individuals gave informed consent and/or assent

742   to participate in the studies.

743

744   **Multiplexed antibody assay**

745   Recombinant *Plasmodium* proteins were coupled to unique regions of magnetic, fluorescent, Bio-

746   Plex microbeads (Bio-Rad) following the manufacturer's instructions and as previously

747   described[62]. Briefly, 200 µL of microbeads were washed then activated for 20 minutes with sulfo-

748   N-hydrosuccinimide (50 mg/mL) and N-ethyl-N-(3-dimethylaminopropyl) carbodiimide (EDC)

749   (50 mg/mL) in monobasic sodium phosphate (pH 6.2). Following further washing, the activated

750   microspheres were resuspended in PBS with 1-6 µg of *Plasmodium* protein. After overnight

751   incubation, the coupled beads were washed and then stored in PBS-TBN (PBS, 0.1% (w/v) BSA,

752   0.02% (v/v) TWEEN-20, 0.05% (w/v) sodium azide, pH 7) at 4 °C until further use. Microbeads

753   were always kept protected from light.

754

Plasma samples were diluted in PBT (1X PBS, 1% (w/v) BSA, 0.05% (v/v) Tween-20) at a dilution of 1:100. For *P. vivax* and *P. falciparum* antigens, plasma samples from hyper-immune individuals from PNG were used as a positive control. For *P. knowlesi* antigens, plasma samples from acutely infected *P. knowlesi* patients were used as the positive control. Both positive control pools were used to create a modified reference standard curve, starting at 1:50 with a 5 point 5-fold serial dilution. Diluted samples (50 µL) all controls and patients) were added to black flat-bottom 96-well plates and mixed with 50 µL of the coupled-antigen bead mixture (0.1 µL of each coupled antigen per well in PBT), then incubated for 30 minutes. The plate was washed and then 100 µL of 1:100 phycoerythrin (PE)-conjugated anti-human secondary antibody (Jackson Immunoresearch) was added and incubated for a further 15 minutes. Plates were washed then resuspended in PBT before being read on a MAGPIX instrument. Median fluorescent intensity was converted to arbitrary relative antibody units (RAU) using the standard curves, to adjust for plate-plate variation[61].

Statistical analysis

An antigen-specific seropositivity cut-off was set as the mean of the negative controls (VBDR + TRC) plus two times the standard deviation. Data are presented as the fold change of the mean peak week 1 antibody response relative to the seropositivity cut-off. RAU values of samples and control cohorts are shown in Extended Data.

Growth inhibition assays

P. knowlesi growth inhibition assays were undertaken using *P. knowlesi* YH1 parasites over 2 cycles of growth (~64 hrs) using standard conditions[63]. Antibodies were initially screened at 0.5 mg/mL for inhibitory activity before 2-fold serial dilution dose response curves were undertaken to define potency for inhibitory antibodies. Parasitemia was determined using flow cytometry (BD Acurri) after staining with 10 mg/mL of ethidium bromide, with data analysed using FlowJo software (BD Life Sciences). *P. knowlesi* growth in the presence of antibodies was compared to that of untreated control wells to define growth inhibitory activity. All experiments were performed a minimum of three times with duplicate wells unless stated otherwise.

P. falciparum growth inhibition assays were performed as described previously[18].

787 *P. vivax* growth was analysed using an *ex vivo* invasion assay performed as described previously
788 with slight modification[64]. *P. vivax* samples were collected in 2023 from infected individuals in
789 Kampong Speu, Western Cambodia. *P. vivax* infection was determined using rapid diagnostic
790 testing (CareStartTM Malaria Pf/pan rapid diagnostic tests, Accessbio) or microscopy and species-
791 specific PCR to ensure monoinfection. Venous blood was collected in lithium heparin tubes and
792 immediately sent on ice to the Malaria Research Unit at Institute Pasteur, Cambodia. There,
793 erythrocytes were separated from the plasma, and the plasma was discarded. Erythrocytes were
794 then suspended in warm Roswell Park Memorial Institute (RPMI) medium before leukocyte
795 depletion using a nonwoven fabric filter. The work presented here was approved by the National
796 Ethics Committee for Health Research in Cambodia (192NECHR, July 11, 2022). All patients
797 and/or their parents/guardians provided informed written consent for samples to be taken and used
798 for these purposes.

799

800 Infected erythrocytes were enriched using a potassium chloride (KCl)-Percoll density gradient and
801 then transferred into culture in supplemented Iscove′s Modified Dulbecco′s Medium
802 (IMDM)(Gibco) (supplemented with 0.5% (w/v) Albumax II (Gibco), 2.5% (v/v) heat-inactivated
803 human serum, 25 mM HEPES (Gibco), 20 µg/mL gentamicin (Sigma) and 0.2 mM hypoxanthine
804 (C-C Pro)). The stage of the parasite culture was then assessed via thin blood smear. In the case of
805 a majority ring culture, parasites were allowed to mature through to the schizont stage (~40 hours)
806 before starting the experiment. If the culture consisted mainly of trophozoites, the experiment was
807 carried out after 18-20 hours. The enriched schizonts were then mixed 1:1 with reticulocytes
808 (previously enriched from cord blood or adult peripheral blood from malaria-naïve donors) and
809 pre-labelled with Celltrace Far Red Dye for quantitation. The cultures were incubated with either
810 500 µg/mL (anti-tetanus toxin 43038) or either 100 µg/mL or 500 µg/mL (monoclonal antibodies
811 and nanobodies) of biologics in a volume of 50 µL in 384-well plates. Cells were stained with
812 Hoechst 33342 to stain parasite DNA and parasitemia was quantified via flow cytometry, with
813 new infections being defined as Far Red/Hoechst double-positive cells. For quantitation, data were
814 normalized against parasites mock treated with PBS. Observed control invasion rates ranged from
815 0.46 – 5.3% (median = 0.7%).

816

817 *P. cynomolgi* assays were performed as previously described[43]. Both *P. falciparum* and *P. knowlesi*
818 GIAs were carried out in parallel to *P. cynomolgi* assays to serve as positive controls for antibody

819   inhibition. *P. cynomolgi* strain Berok R9 was maintained in rhesus red blood cells (Emory Primate

820   Center) at 2% hematocrit in RPMI 1640 with 10% human O+ serum and gassed (1% O_2, 5% CO_2,

821   94% N_2) at 37°C. The invasion assay was set up with 0.2% hematocrit and 2-3% schizontemia in

822   30 µl volumes in 384-well plates using antibodies 2D9, 4H10, 5B3, 5B4, IgG control and heparin

823   (positive control) After 12 h of incubation, the parasite DNA was stained with Vybrant™

824   DyeCycle™ Violet (Invitrogen), and 100,000 cells were analyzed via a Cytek-Northern Lights

825   flow cytometer. Invasion was measured by the percentage of newly parasitized erythrocytes

826   (CellTrace Far Red+/Vybrant™ DyeCycle™ Violet+). Inhibition was assessed relative to control

827   wells without antibodies. Data analysis was done using GraphPad Prism v10.

828

829   **Electron microscopy**

830   All electron microscopy was carried out at the Bio21 Ian Holmes Imaging Centre, University of

831   Melbourne.

832

833   For negative staining, purified PkPCR^tail+5B3 Fab (at ~0.1 mg/mL) was applied to formvar and

834   carbon coated, glow discharged copper grids (300 mesh, ProSciTech). Four microlitres of protein

835   was incubated for one minute, then blotted off, washed twice in water, and then stained with 1%

836   (w/v) uranyl acetate for two minutes before being blotted and dried thoroughly. The grids were

837   then imaged on a Tecnai F30 operating at 200kV. Two-dimensional classification was performed

838   in Cryosparc (v4.4.1)[65].

839

840   For cryo-electron microscopy, freshly purified PkPCR^tail and PkPCR^tail+5B3 Fab at 0.5-1 mg/mL

841   was applied to glow discharged UltrAuFoil (Quantifoil Micro Tools GmbH) grids (300 mesh,

842   R1.2/1.3) or HexAuFoil (Quantifoil Micro Tools GmbH) and then blotted for 5 seconds with a

843   blot force of 7 (UltrAuFoil) or 10 (HexAuFoil) before being plunged into liquid ethane using a

844   Vitrobot Mark IV (Thermo Fisher Scientific) operating at 4°C and 100% humidity. Grids were

845   screened for good ice quality on an FEI Talos Arctica (Thermo Fisher Scientific) operating at 200

846   kV. Grids showing sufficient thin and amorphous ice were then transferred to an FEI Titan Krios

847   G4(Thermo Fisher Scientific) for data collection. Data were collected using an acceleration voltage

848   of 300 kV and a Falcon 4i detector (Thermo Fisher Scientific) using EPU automation software.

849   Data were collected over three sessions, two for PkPCR^tail (from two independent grids) and one

850   for PkPCR^tail+5B3. Pixel sizes used for collection were 0.506 Å/pixel with a total dose of 50 $e^-/Å^2$

851 for PkPCR^{tail} and 0.808 Å/pixel with a total dose of 40 e$^-$/Å^2 for PkPCR^{tail}+5B3 and all datasets

852 were collected with a nominal defocus range of -0.5 µm to -2 µm. CryoSPARC (v4.4.1-v4.6.2)[65]

853 was used for all data processing. Gain and motion corrected, and contrast transfer function (CTF)-

854 estimated movie stacks were curated to select for good CTF fit and to remove micrographs that

855 showed signs of significant drift, contained obvious frost contamination, or had no visible particles.

856 This resulted in 8,730 and 826 movie stacks for session one and two, respectively, for PkPCR^{tail}

857 and 3,285 movie stacks for PkPCR^{tail}+5B3. The curated datasets were then used in multiple rounds

858 of automated picking and 2D class averaging. For PkPCR^{tail} 11,955 particles corresponded to the

859 'front view', and 3,067 particles corresponded to the 'side view'. For PkPCR^{tail}+5B3 13,478

860 particles corresponded to the 'front view', and 2,694 particles corresponded to the 'side view'.

861 Whilst clear features were visible in these small number of classes, severe orientation bias and the

862 small, flat, and elongated shape of the particles precluded three-dimensional reconstruction.

863

864 **Reticulocyte enrichment for flow cytometric binding assays**

865 Cord blood was obtained through a material transfer agreement (MTA, ID# M19/110) with the

866 Bone Marrow Donor Institute (BMDI) at the Royal Children's Hospital in Melbourne, Australia

867 under the human ethics project "14/09, Malaria parasite growth and invasion into reticulocytes"

868 which was approved by the Walter and Eliza Hall Institute Human Research Ethics Committee

869 (HREC). Cord blood was passed through a RC High Efficiency Leucocyte Removal Filter

870 (Haemonetics Australia) and then centrifuged at 2000 x *g* for five minutes to separate blood from

871 serum. The blood was then washed three times in 1x human tonicity PBS (HTPBS) before being

872 made up to 50% hematocrit. This 50% solution was then layered on top of a 70% (v/v) Percoll

873 cushion (GE Healthcare). Centrifugation for 25 minutes at 2100 x*g* separated the mature

874 erythrocytes from the reticulocytes, with the reticulocytes forming a thin band at the interface

875 between buffer and Percoll. Reticulocytes were stored in 1x HTPBS at 4°C.

876

877 **Flow cytometry-based erythrocyte binding assays**

878 For assays using mature erythrocytes, erythrocytes were washed twice in PBS and then made up

879 to a density of approximately 1 x 10^7 cells/mL in PBS + 1% (w/v) BSA (PBS-BSA). Each sample

880 used 100 µL of this suspension. Erythrocytes were centrifuged, the supernatant was removed, and

881 the cells were resuspended in a solution containing freshly prepared recombinant proteins in PBS-

882 BSA. Individual proteins were prepared at a final concentration of 2 µM (except for PfRh5, which

883   was prepared at 400 nM), and complexes were mixed with an equimolar amount of protein to a

884   final concentration of 2 μM. After a 45-minute incubation at room temperature, the samples were

885   centrifuged, washed, and then incubated with primary antibodies, either 5A9 (anti-Rh5), 4E2 (anti-

886   PC) or 5E11 (anti-Ripr). After a 45-minute incubation the cells were again centrifuged and then

887   incubated with Alexa-488 anti-mouse fluorescent antibody at a dilution of 1:100. After a 45-

888   minute incubation, cells were washed twice in PBS and then resuspended before analysis on an

889   Attune NxT flow cytometer (Thermo Fisher Scientific). For each sample 50,000 events were

890   recorded. The data were then analysed in FlowJo^{TM} v10.7 Software (BD Life Sciences). Antibody

891   background was subtracted from the positive population recorded in the presence of recombinant

892   protein and this background-subtracted value has been plotted in the summary figures.

893

894   For assays involving reticulocyte-enriched cord blood, erythrocytes were made up in 1x HTPBS

895   + 1% (w/v) BSA (HTPBS-BSA) to a density of approximately 1 x 10^7 cells/mL. Each sample used

896   100 μL of this suspension. Reticulocytes were centrifuged (2000 x *g* for one minute), the HTPBS-

897   BSA removed, and then resuspended in a solution containing recombinant proteins in HTPBS-

898   BSA and incubated at room temperature for 45 minutes. PvPC and PkPC were used at a final

899   concentration of 2 μM. Samples were centrifuged after which the protein solution removed, and

900   cells were washed once with HTPBS-BSA and then incubated with 4E2 (anti-PC) at a

901   concentration of 0.05 mg/mL or polyclonal sera (anti-RBP2b) at a concentration of 12.5 μg/mL.

902   After a 45-minute incubation, the cells were again centrifuged, and the antibody solution was

903   removed. Cells were washed once as before and then stained with Alexa-647 (either anti-rabbit or

904   anti-mouse) at a dilution of 1:100. After 45 minutes the reticulocytes were again washed and

905   incubated with 50 μL of thiazole orange (BD Retic-Count, BD Biosciences) for 30 minutes. Finally,

906   the reticulocytes were centrifuged, the Retic-Count solution removed, and cells were washed with

907   1x HTPBS two times before analysis on an Attune NxT flow cytometer (Thermo Fisher Scientific).

908   For each sample 50,000 events were recorded. The data were then analysed in FlowJo^{TM} v10.7

909   Software (BD Life Sciences). This involved gating reticulocytes and then applying a quadrant gate

910   according to the thiazole orange staining and the background staining of the antibody in

911   combination with the Alexa 647. This antibody background was subtracted from the positive

912   population recorded in the presence of recombinant protein: this background-subtracted value has

913   been plotted in the summary figures. Positive binding is determined by the double positive

914   population in the upper right-hand quadrant.

915

Data visualization

All data visualization was done in University of California, San Francisco (UCSF) ChimeraX versions 1.2-1.8 (https://www.cgl.ucsf.edu/chimerax/)[66]. PyMOL was utilised for structure alignment and calculation of root mean square deviation (RMSD) (https://www.pymol.org/)[67].

Data availability

The crystal structure reported in this manuscript has been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 9NSD).

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Reference List

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1. Weiss DJ, Lucas TCD, Nguyen M, et al. Mapping the global prevalence, incidence, and mortality of *Plasmodium falciparum*, 2000-17: a spatial and temporal modelling study. *Lancet*. Jul 27 2019;394(10195):322-331. doi:10.1016/s0140-6736(19)31097-9

2. Battle KE, Lucas TCD, Nguyen M, et al. Mapping the global endemicity and clinical burden of *Plasmodium vivax*, 2000-17: a spatial and temporal modelling study. *The Lancet*. 2019;394(10195):332-343. doi:10.1016/S0140-6736(19)31096-7

3. Singh B, Kim Sung L, Matusop A, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet*. Mar 27 2004;363(9414):1017-24. doi:10.1016/s0140-6736(04)15836-4

4. Cox-Singh J, Davis TM, Lee KS, et al. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis*. Jan 15 2008;46(2):165-71. doi:10.1086/524888

5. Galinski MR, Medina CC, Ingravallo P, Barnwell JW. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell*. 1992/06/26/ 1992;69(7):1213-1226. doi:https://doi.org/10.1016/0092-8674(92)90642-P

6. Cowman AF, Tonkin CJ, Tham W-H, Duraisingh MT. The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. *Cell Host & Microbe*. 2017;22(2):232-245. doi:10.1016/j.chom.2017.07.003

945   7.   Lopaticki S, Maier AG, Thompson J, et al. Reticulocyte and Erythrocyte Binding-Like
946   Proteins Function Cooperatively in Invasion of Human Erythrocytes by Malaria
947   Parasites. *Infection and Immunity*. 2011;79(3):1107-1117. doi:10.1128/iai.01021-10

948   8.   Hayton K, Gaur D, Liu A, et al. Erythrocyte Binding Protein PfRH5 Polymorphisms
949   Determine Species-Specific Pathways of *Plasmodium falciparum* Invasion. *Cell Host &*
950   *Microbe*. 2008;4(1):40-51. doi:10.1016/j.chom.2008.06.001

951   9.   Baum J, Chen L, Healer J, et al. Reticulocyte-binding protein homologue 5 - an essential
952   adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J*
953   *Parasitol*. Feb 2009;39(3):371-80. doi:10.1016/j.ijpara.2008.10.006

954   10.   Crosnier C, Bustamante LY, Bartholdson SJ, et al. Basigin is a receptor essential for
955   erythrocyte invasion by *Plasmodium falciparum*. *Nature*. 2011/12/01
956   2011;480(7378):534-537. doi:10.1038/nature10606

957   11.   Wanaguru M, Liu W, Hahn BH, Rayner JC, Wright GJ. RH5–Basigin interaction plays a
958   major role in the host tropism of *Plasmodium falciparum*. *Proceedings of the National*
959   *Academy of Sciences*. 2013;110(51):20735-20740. doi:10.1073/pnas.1320771110

960   12.   Reddy KS, Amlabu E, Pandey AK, Mitra P, Chauhan VS, Gaur D. Multiprotein complex
961   between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium*
962   *falciparum* erythrocyte invasion. *Proceedings of the National Academy of Sciences*.
963   2015;112(4):1179-1184. doi:10.1073/pnas.1415466112

964   13.   Chen L, Lopaticki S, Riglar DT, et al. An EGF-like Protein Forms a Complex with PfRh5
965   and Is Required for Invasion of Human Erythrocytes by *Plasmodium falciparum*. *PLOS*
966   *Pathogens*. 2011;7(9):e1002199. doi:10.1371/journal.ppat.1002199

967   14.   Thompson J, Cooke RE, Moore S, Anderson LF, Janse CJ, Waters AP. PTRAMP; a
968   conserved *Plasmodium* thrombospondin-related apical merozoite protein. *Mol Biochem*
969   *Parasitol*. Apr 2004;134(2):225-32. doi:10.1016/j.molbiopara.2003.12.003

970   15.   Green JL, Hinds L, Grainger M, Knuepfer E, Holder AA. *Plasmodium* thrombospondin
971   related apical merozoite protein (PTRAMP) is shed from the surface of merozoites by
972   PfSUB2 upon invasion of erythrocytes. *Molecular and Biochemical Parasitology*.
973   2006/11/01/ 2006;150(1):114-117. doi:https://doi.org/10.1016/j.molbiopara.2006.06.010

974   16.   Knuepfer E, Wright KE, Kumar Prajapati S, et al. Divergent roles for the RH5 complex
975   components, CyRPA and RIPR in human-infective malaria parasites. *PLOS Pathogens*.
976   2019;15(6):e1007809. doi:10.1371/journal.ppat.1007809

977   17.   Wong W, Huang R, Menant S, et al. Structure of *Plasmodium falciparum* Rh5-CyRPA-
978   Ripr invasion complex. *Nature*. Jan 2019;565(7737):118-121. doi:10.1038/s41586-018-
979   0779-6

980   18.   Scally SW, Triglia T, Evelyn C, et al. PCRCR complex is essential for invasion of human
981   erythrocytes by *Plasmodium falciparum*. *Nature Microbiology*. 2022/12/01
982   2022;7(12):2039-2053. doi:10.1038/s41564-022-01261-2

983   19.   Farrell B, Alam N, Hart MN, et al. The PfRCR complex bridges malaria parasite and
984   erythrocyte during invasion. *Nature*. 2024/01/01 2024;625(7995):578-584.
985   doi:10.1038/s41586-023-06856-1

986   20.   Chen L, Xu Y, Healer J, et al. Crystal structure of PfRh5, an essential *P. falciparum*
987   ligand for invasion of human erythrocytes. *eLife*. 2014/10/08 2014;3:e04187.
988   doi:10.7554/eLife.04187

989   21.   Wright KE, Hjerrild KA, Bartlett J, et al. Structure of malaria invasion protein RH5 with
990   erythrocyte basigin and blocking antibodies. *Nature*. Nov 20 2014;515(7527):427-30.
991   doi:10.1038/nature13715

992   22.   Chen L, Xu Y, Wong W, et al. Structural basis for inhibition of erythrocyte invasion by
993   antibodies to *Plasmodium falciparum* protein CyRPA. *eLife*. 2017/02/14 2017;6:e21347.
994   doi:10.7554/eLife.21347

995   23.   Favuzza P, Guffart E, Tamborrini M, et al. Structure of the malaria vaccine candidate
996   antigen CyRPA and its complex with a parasite invasion inhibitory antibody. *eLife*.
997   2017/02/14 2017;6:e20383. doi:10.7554/eLife.20383

998   24.   Healer J, Wong W, Thompson JK, et al. Neutralising antibodies block the function of
999   Rh5/Ripr/CyRPA complex during invasion of *Plasmodium falciparum* into human
1000   erythrocytes. *Cellular Microbiology*. 2019;21(7):e13030. doi:10.1111/cmi.13030

1001 25.   Ragotte RJ, Pulido D, Lias AM, et al. Heterotypic interactions drive antibody synergy
1002   against a malaria vaccine candidate. *Nature Communications*. 2022/02/17
1003   2022;13(1):933. doi:10.1038/s41467-022-28601-4

1004 26.   Volz Jennifer C, Yap A, Sisquella X, et al. Essential Role of the PfRh5/PfRipr/CyRPA
1005   Complex during *Plasmodium falciparum* Invasion of Erythrocytes. *Cell Host & Microbe*.
1006   2016;20(1):60-71. doi:10.1016/j.chom.2016.06.004

1007 27.   Weiss GE, Gilson PR, Taechalertpaisarn T, et al. Revealing the Sequence and Resulting
1008   Cellular Morphology of Receptor-Ligand Interactions during *Plasmodium falciparum*
1009   Invasion of Erythrocytes. *PLOS Pathogens*. 2015;11(2):e1004670.
1010   doi:10.1371/journal.ppat.1004670

1011 28.   Geoghegan ND, Evelyn C, Whitehead LW, et al. 4D analysis of malaria parasite invasion
1012   offers insights into erythrocyte membrane remodeling and parasitophorous vacuole
1013   formation. *Nature Communications*. 2021/06/15 2021;12(1):3620. doi:10.1038/s41467-
1014   021-23626-7

1015   29.   Otto TD, Gilabert A, Crellen T, et al. Genomes of all known members of a *Plasmodium*
1016   subgenus reveal paths to virulent human malaria. *Nat Microbiol*. Jun 2018;3(6):687-697.
1017   doi:10.1038/s41564-018-0162-2

1018   30.   Sundararaman SA, Plenderleith LJ, Liu W, et al. Genomes of cryptic chimpanzee
1019   *Plasmodium* species reveal key evolutionary events leading to human malaria. *Nature*
1020   *Communications*. 2016/03/22 2016;7(1):11078. doi:10.1038/ncomms11078

1021   31.   Elsworth B, Ye S, Dass S, et al. The essential genome of *Plasmodium knowlesi* reveals
1022   determinants of antimalarial susceptibility. *Science*. 2025;387(6734):eadq6241.
1023   doi:doi:10.1126/science.adq6241

1024   32.   Oberstaller J, Xu S, Naskar D, et al. Supersaturation mutagenesis reveals adaptive
1025   rewiring of essential genes among malaria parasites. *Science*. 2025;387(6734):eadq7347.
1026   doi:doi:10.1126/science.adq7347

1027   33.   Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with
1028   AlphaFold. *Nature*. 2021/08/01 2021;596(7873):583-589. doi:10.1038/s41586-021-
1029   03819-2

1030   34.   Abramson J, Adler J, Dunger J, et al. Accurate structure prediction of biomolecular
1031   interactions with AlphaFold 3. *Nature*. 2024/06/01 2024;630(8016):493-500.
1032   doi:10.1038/s41586-024-07487-w

1033   35.   Gupta R, Brunak S. Prediction of glycosylation across the human proteome and the
1034   correlation to protein function. *Pac Symp Biocomput*. 2002:310-22.

1035   36.   Triglia T, Scally SW, Seager BA, Pasternak M, Dagley LF, Cowman AF. Plasmepsin X
1036   activates the PCRCR complex of *Plasmodium falciparum* by processing PfRh5 for
1037   erythrocyte invasion. *Nature Communications*. 2023/04/19 2023;14(1):2219.
1038   doi:10.1038/s41467-023-37890-2

1039   37.   Grigg MJ, William T, Piera KA, et al. *Plasmodium falciparum* artemisinin resistance
1040   monitoring in Sabah, Malaysia: *in vivo* therapeutic efficacy and kelch13 molecular
1041   marker surveillance. *Malaria Journal*. 2018/12/10 2018;17(1):463. doi:10.1186/s12936-
1042   018-2593-x

1043   38.   Longley RJ, Sripoorote P, Chobson P, et al. High Efficacy of Primaquine Treatment for
1044   *Plasmodium vivax* in Western Thailand. *Am J Trop Med Hyg*. Nov 2 2016;95(5):1086-
1045   1089. doi:10.4269/ajtmh.16-0410

1046   39.   Grigg MJ, William T, Barber BE, et al. Age-Related Clinical Spectrum of *Plasmodium*
1047   *knowlesi* Malaria and Predictors of Severity. *Clin Infect Dis*. Jul 18 2018;67(3):350-359.
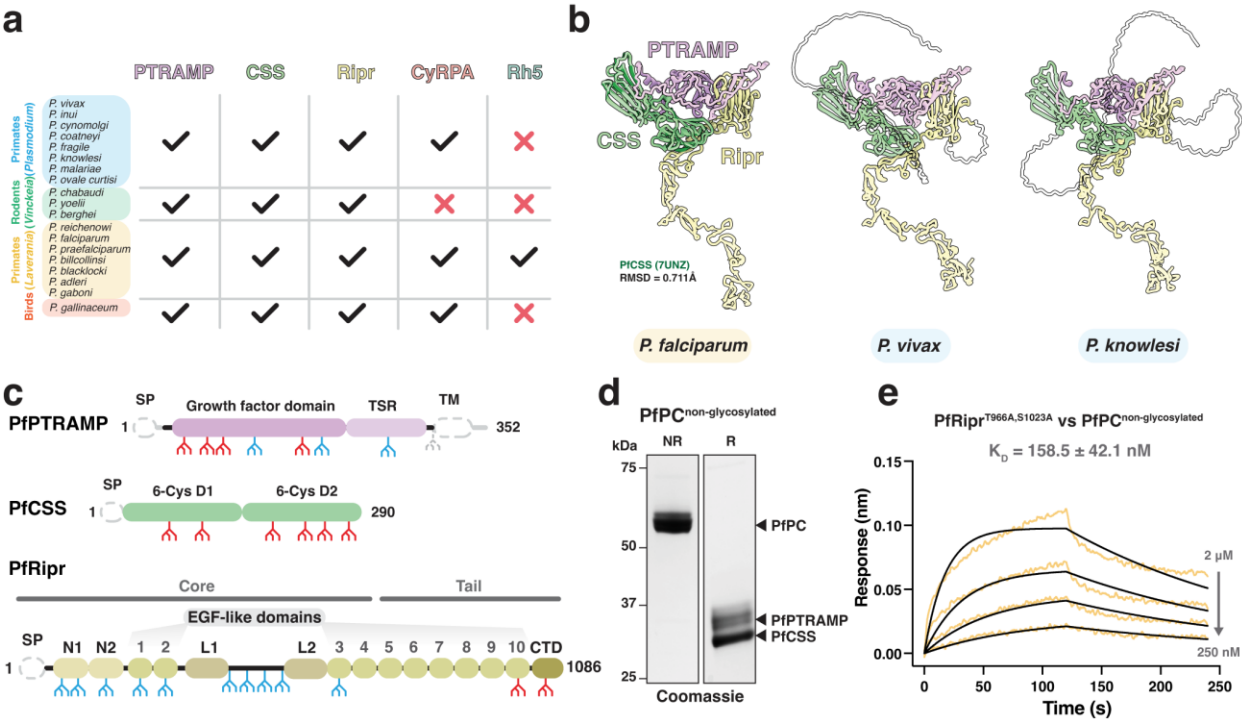1048   doi:10.1093/cid/ciy065

1049   40.   Longley RJ, Grigg MJ, Schoffer K, et al. *Plasmodium vivax* malaria serological exposure
1050   markers: Assessing the degree and implications of cross-reactivity with *P. knowlesi*. *Cell*
1051   *Rep Med*. Jun 21 2022;3(6):100662. doi:10.1016/j.xcrm.2022.100662

1052   41.   Williams BG, King LDW, Pulido D, et al. Development of an improved blood-stage
1053   malaria vaccine targeting the essential RH5-CyRPA-RIPR invasion complex. *Nature*
1054   *Communications*. 2024/06/07 2024;15(1):4857. doi:10.1038/s41467-024-48721-3

1055   42.   Muh F, Kim N, Nyunt MH, et al. Cross-species reactivity of antibodies against
1056   *Plasmodium vivax* blood-stage antigens to *Plasmodium knowlesi*. *PLOS Neglected*
1057   *Tropical Diseases*. 2020;14(6):e0008323. doi:10.1371/journal.pntd.0008323

1058   43.   Dass S, Kundu P, Naskar D, et al. Miniaturized assay to evaluate *Plasmodium cynomolgi*
1059   and *P. knowlesi* as models for prioritizing *P. vivax* vaccine targets. *The Journal of*
1060   *Infectious Diseases*. 2025;doi:10.1093/infdis/jiaf136

1061   44.   Mitran CJ, Yanow SK. The Case for Exploiting Cross-Species Epitopes in Malaria
1062   Vaccine Design. Review. *Frontiers in Immunology*. 2020-February-27
1063   2020;11doi:10.3389/fimmu.2020.00335

1064   45.   Drew DR, Wilson DW, Weiss GE, et al. Defining species-specific and conserved
1065   interactions of apical membrane protein 1 during erythrocyte invasion in malaria to
1066   inform multi-species vaccines. *Cell Mol Life Sci*. Feb 27 2023;80(3):74.
1067   doi:10.1007/s00018-023-04712-z

1068   46.   Wasniowska K, Petit-LeRoux Y, Tournamille C, et al. Structural characterization of the
1069   epitope recognized by the new anti-Fy6 monoclonal antibody NaM185-2C3. *Transfusion*
1070   *Medicine*. 2002;12(3):205-211. doi:https://doi.org/10.1046/j.1365-3148.2002.00373.x

1071   47.   Amos B, Aurrecoechea C, Barba M, et al. VEuPathDB: the eukaryotic pathogen, vector
1072   and host bioinformatics resource center. *Nucleic Acids Research*. 2021;50(D1):D898-
1073   D911. doi:10.1093/nar/gkab929

1074   48.   Gruszczyk J, Kanjee U, Chan L-J, et al. Transferrin receptor 1 is a reticulocyte-specific
1075   receptor for *Plasmodium vivax*. *Science*. 2018;359(6371):48-55.
1076   doi:10.1126/science.aan1078

1077   49.   Fairhead M, Howarth M. Site-specific biotinylation of purified proteins using BirA.
1078   *Methods Mol Biol*. 2015;1266:171-84. doi:10.1007/978-1-4939-2272-7_12

1079   50.   Pardon E, Laeremans T, Triest S, et al. A general protocol for the generation of
1080   Nanobodies for structural biology. *Nat Protoc*. Mar 2014;9(3):674-93.
1081   doi:10.1038/nprot.2014.039

1082   51.   Kabsch W. XDS. *Acta Crystallogr D Biol Crystallogr*. Feb 2010;66(Pt 2):125-32.
1083   doi:10.1107/s0907444909047337

1084   52.   Evans PR, Murshudov GN. How good are my data and what is the resolution? *Acta*
1085   *Crystallogr D Biol Crystallogr*. Jul 2013;69(Pt 7):1204-14.
1086   doi:10.1107/s0907444913000061

1087   53.   Collaborative Computational Project N. The CCP4 suite: programs for protein
1088   crystallography. *Acta Crystallogr D Biol Crystallogr*. Sep 1 1994;50(Pt 5):760-3.
1089   doi:10.1107/s0907444994003112

1090   54.   Kantardjieff KA, Rupp B. Matthews coefficient probabilities: Improved estimates for unit
1091   cell contents of proteins, DNA, and protein-nucleic acid complex crystals. *Protein Sci*.
1092   Sep 2003;12(9):1865-71. doi:10.1110/ps.0350503

1093   55.   McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser
1094   crystallographic software. *Journal of Applied Crystallography*. 2007;40(4):658-674.
1095   doi:doi:10.1107/S0021889807021206

1096   56.   Ye Q, Lu S, Corbett KD. Structural Basis for SARS-CoV-2 Nucleocapsid Protein
1097   Recognition by Single-Domain Antibodies. Original Research. *Frontiers in Immunology*.
1098   2021-July-26 2021;12doi:10.3389/fimmu.2021.719037

1099   57.   Adams PD, Afonine PV, Bunkóczi G, et al. PHENIX: a comprehensive Python-based
1100   system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr*. Feb
1101   2010;66(Pt 2):213-21. doi:10.1107/s0907444909052925

1102   58.   Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta*
1103   *Crystallogr D Biol Crystallogr*. Dec 2004;60(Pt 12 Pt 1):2126-32.
1104   doi:10.1107/s0907444904019158

1105   59.   Dunbar J, Deane CM. ANARCI: antigen receptor numbering and receptor classification.
1106   *Bioinformatics*. 2015;32(2):298-300. doi:10.1093/bioinformatics/btv552

1107   60.   Gouet P, Robert X, Courcelle E. ESPript/ENDscript: extracting and rendering sequence
1108   and 3D information from atomic structures of proteins. *Nucleic Acids Research*.
1109   2003;31(13):3320-3323. doi:10.1093/nar/gkg556

1110   61.   Longley RJ, White MT, Takashima E, et al. Development and validation of serological
1111   markers for detecting recent *Plasmodium vivax* infection. *Nat Med*. May 2020;26(5):741-
1112   749. doi:10.1038/s41591-020-0841-4

1113   62.   Mazhari R, Brewster J, Fong R, et al. A comparison of non-magnetic and magnetic beads
1114   for measuring IgG antibodies against *Plasmodium vivax* antigens in a multiplexed bead-
1115   based assay using Luminex technology (Bio-Plex 200 or MAGPIX). *PLoS One*.
1116   2020;15(12):e0238010. doi:10.1371/journal.pone.0238010

1117   63.   Wilson DW, Crabb BS, Beeson JG. Development of fluorescent *Plasmodium falciparum*
1118   for in vitro growth inhibition assays. *Malaria Journal*. 2010/06/03 2010;9(1):152.
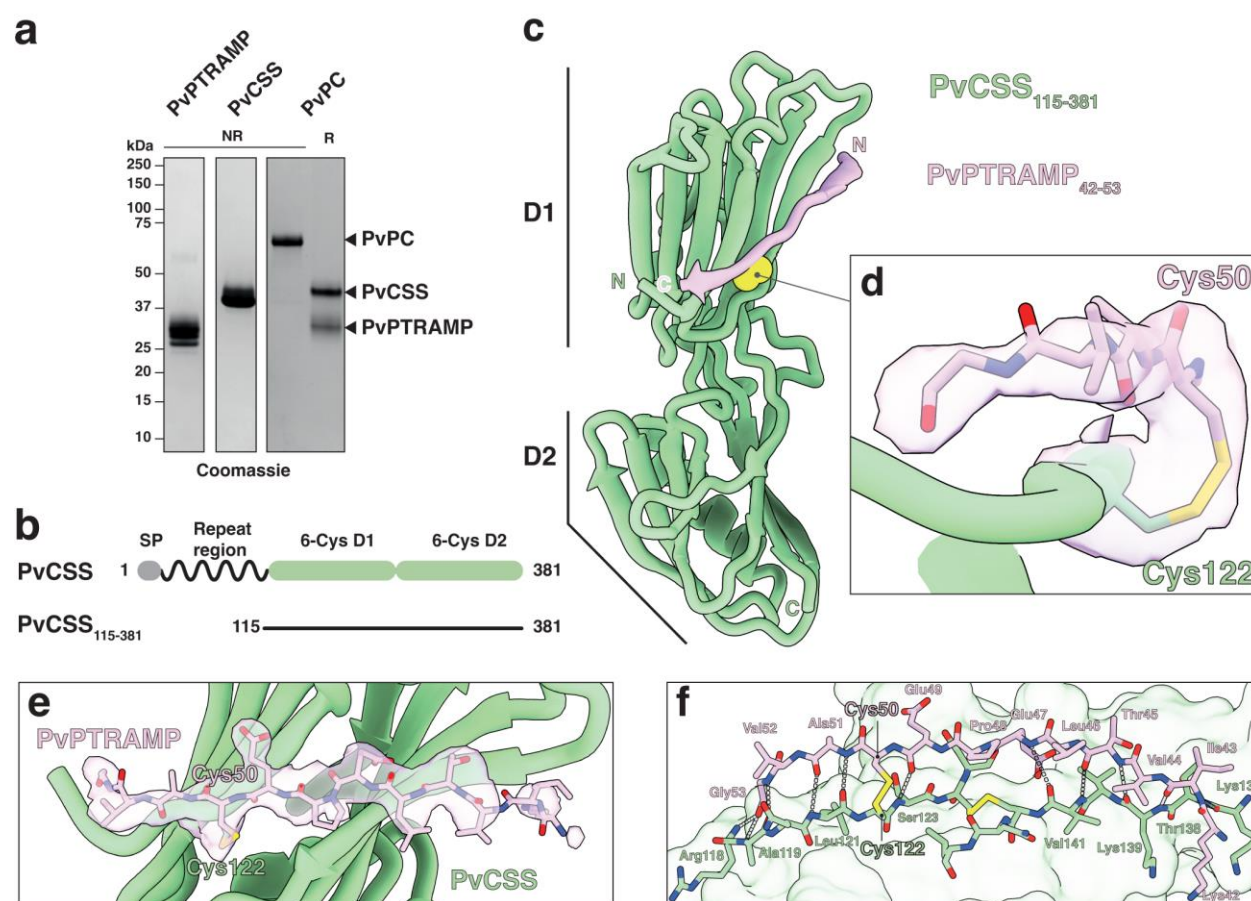1119   doi:10.1186/1475-2875-9-152

1120   64.   Popovici J, Roesch C, Carias LL, et al. Amplification of Duffy binding protein-encoding
1121   gene allows *Plasmodium vivax* to evade host anti-DBP humoral immunity. *Nature*
1122   *Communications*. 2020/02/19 2020;11(1):953. doi:10.1038/s41467-020-14574-9

1123   65.   Punjani A, Rubinstein JL, Fleet DJ, Brubaker MA. cryoSPARC: algorithms for rapid
1124   unsupervised cryo-EM structure determination. *Nature Methods*. 2017/03/01
1125   2017;14(3):290-296. doi:10.1038/nmeth.4169

1126   66.   Pettersen EF, Goddard TD, Huang CC, et al. UCSF ChimeraX: Structure visualization for
1127   researchers, educators, and developers. *Protein Sci*. Jan 2021;30(1):70-82.
1128   doi:10.1002/pro.3943

1129   67.   Schrödinger L, & DeLano, W. PyMOL. 2020;

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1132 **Main figures**

1133 **Figure 1**

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1136 **Figure 2.**

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1139 **Figure 3.**

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1142   **Figure 4.**

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1145   **Figure 5.**

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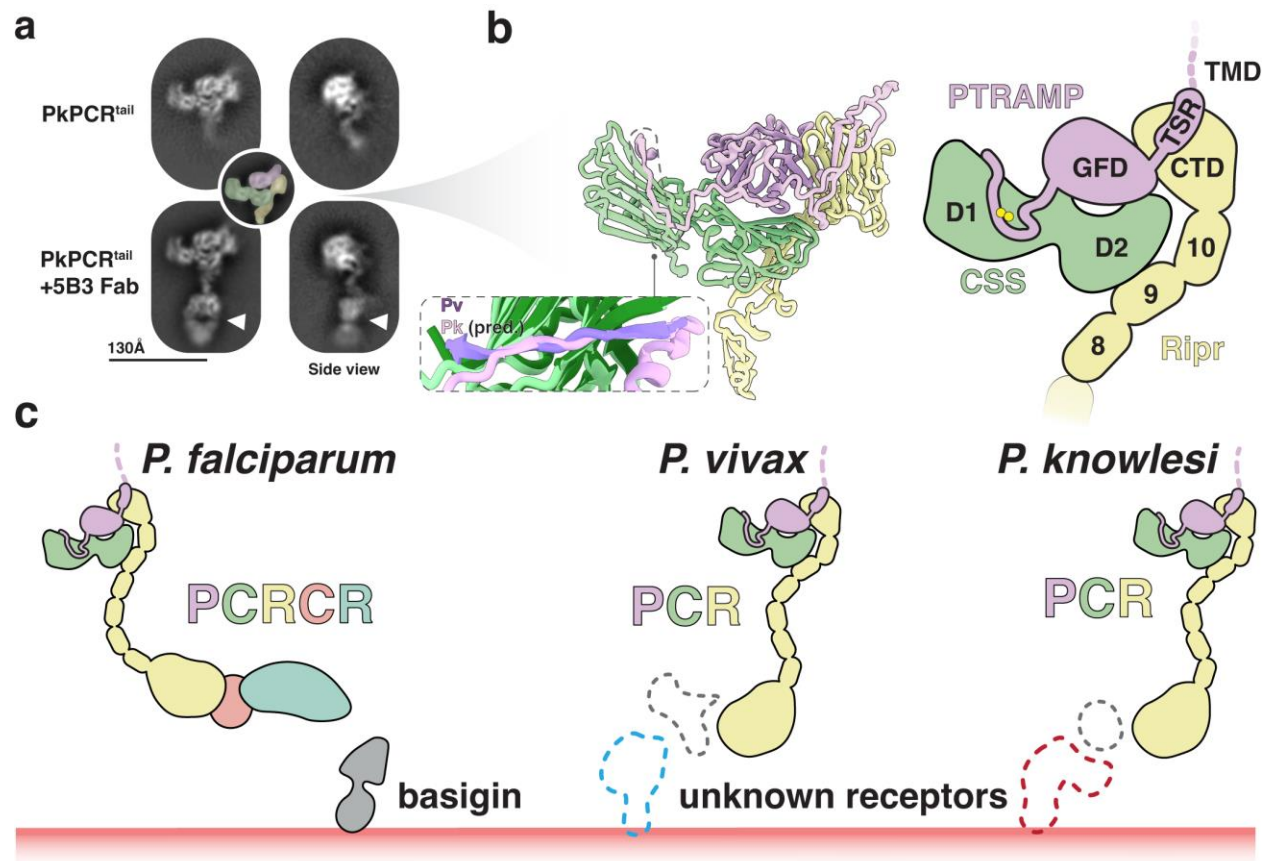
1148   **Figure 6.**

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Extended Data

1 # **PTRAMP, CSS and Ripr form a conserved complex required for merozoite**

2 # **invasion of *Plasmodium* species into erythrocytes**

3

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5 Sheena Dass[6], Xiao Xiao[1,2], Nicolai C. Jung[1,2], Anju Abraham[1], Matthew J. Grigg[7,8], Nicholas
6 M. Anstey[7,8], Timothy William[8,9], Jetsumon Sattabongkot[10], Andrew Leis[1,2], Rhea J.
7 Longley[1,2,10], Manoj T. Duraisingh[6], Jean Popovici[4,5], Danny W. Wilson[3,11,12], Stephen W.
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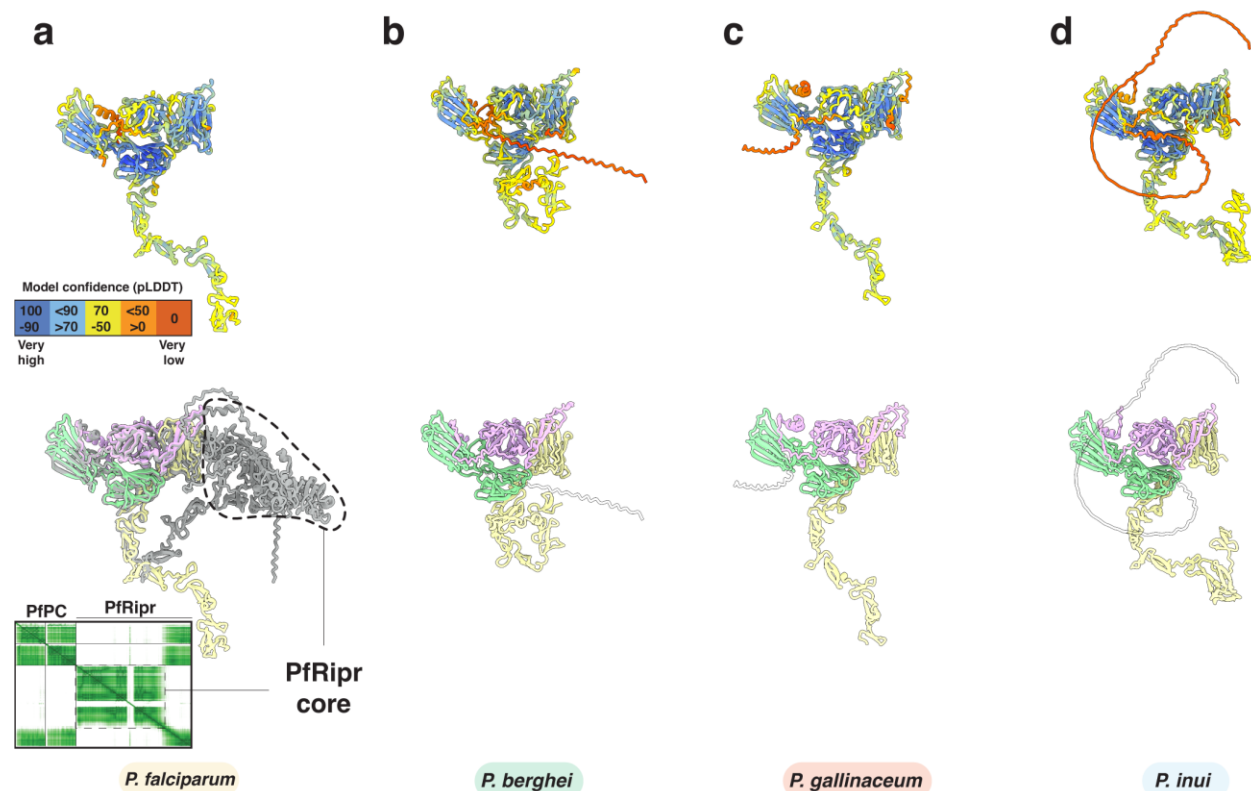28 [12] Institute for Photonics and Advanced Sensing (IPAS), University of Adelaide, 5005, SA,
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30

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34

Extended Data

35 **Extended Data**

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37 **Extended Data figure 1. AlphaFold 3 predicts a PTRAMP, CSS, Ripr complex for all major**

38 **clades of *Plasmodium*. a-d.** AlphaFold 3 predictions of PTRAMP, CSS and Ripr from several

39 species of *Plasmodium*. Top row: Models colored by confidence (pLDDT). Bottom row: models

40 colored as follows: PTRAMP (pink), CSS (green) and Ripr (yellow). The prediction for *P.*

41 *falciparum* shows the alignment with full length Ripr, where the core forms an ordered structure.

42 Predicted alignment error (PAE) plot is shown as inset. All models have transmembrane domains

43 and signal peptides omitted for clarity.

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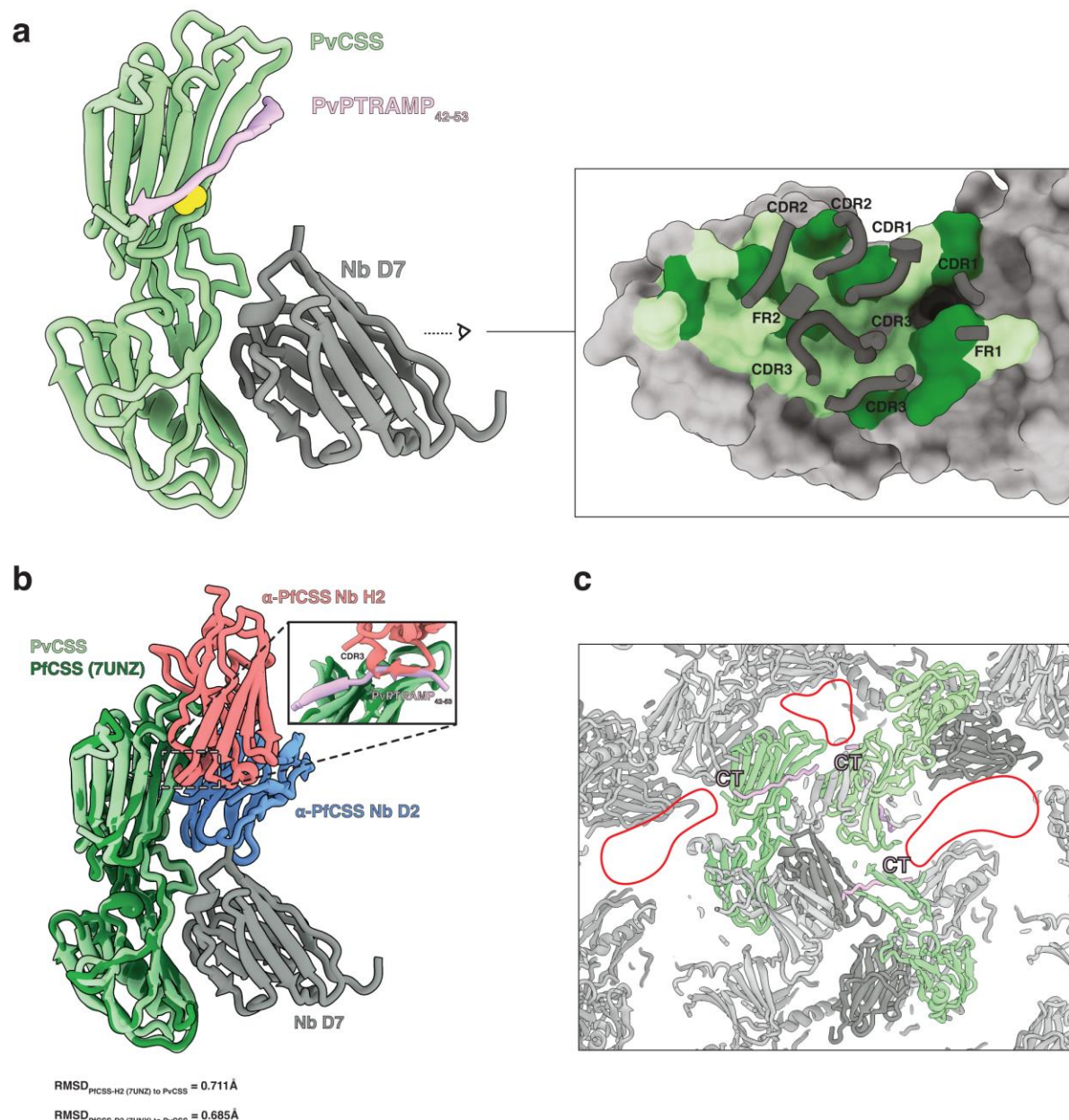
Extended Data

Extended Data figure 2. Comparison of PvCSS crystal structure with PfCSS structures. a. Binding interface between nanobody D7 and PvCSS. The detail shows the entire interface. PvCSS is grey and the region of D7 binding is shown in pale green, with hydrogen bonds and salt bridges shown in forest green. Regions of D7 involved in the interaction are shown and consist of complementarity determining regions (CDR) and framework regions (FR). Regions of D7 not involved in binding are omitted for clarity. **b.** Overlay of PfCSS-H2 (H2 in red) and PfCSS-D2 (D2 in blue) crystal structures with PvCSS-PvPTRAMP-D7 (D7 in grey) crystal structure. Inset shows PvPTRAMP$_{42-53}$ occupies the same region as the CDR3 loop of H2, explaining how it

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Extended Data

57   competes with PfPTRAMP binding to PfCSS[1]. **c.** Crystal packing of PvCSS-PvPTRAMP-D7. The

58   asymmetric unit is shown in color (PvCSS in green, PvPTRAMP in pink and D7 in dark grey) and

59   other symmetry related copies in light grey. Red circles show pockets within the crystal lattice that

60   are adjacent to the C-terminal (CT) of PvPTRAMP$_{42-53}$ that likely accommodate the GFD and TSR

61   domains of PvPTRAMP.

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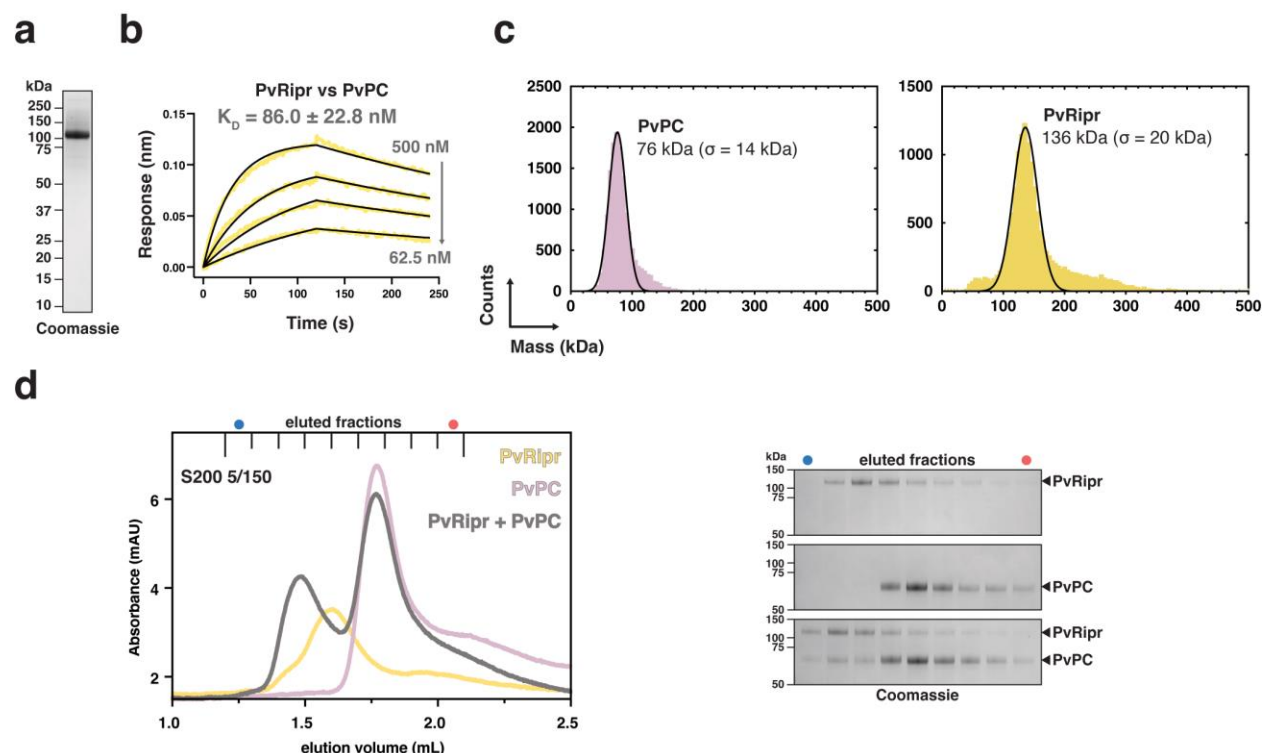
Extended Data figure 3. The PvPCR complex can be reconstituted *in vitro* using recombinant proteins. a. Representative SDS-PAGE of recombinant full-length PvRipr. **b.** Representative biolayer interferometry sensorgram of PvRipr vs PvPC. Data are in yellow and 1:1 model best fit in black. **c.** Mass distribution plots of PvPC (pink) and PvRipr (yellow) as determined by mass photometry. Histogram data are in color and the Gaussian curve fit in black. **d.** Size-exclusion chromatography of PvPC (pink), PvRipr (yellow) and PvPCR (grey), with SDS-PAGE results of the co-complexation, showing co-elution of PvPC and PvRipr.

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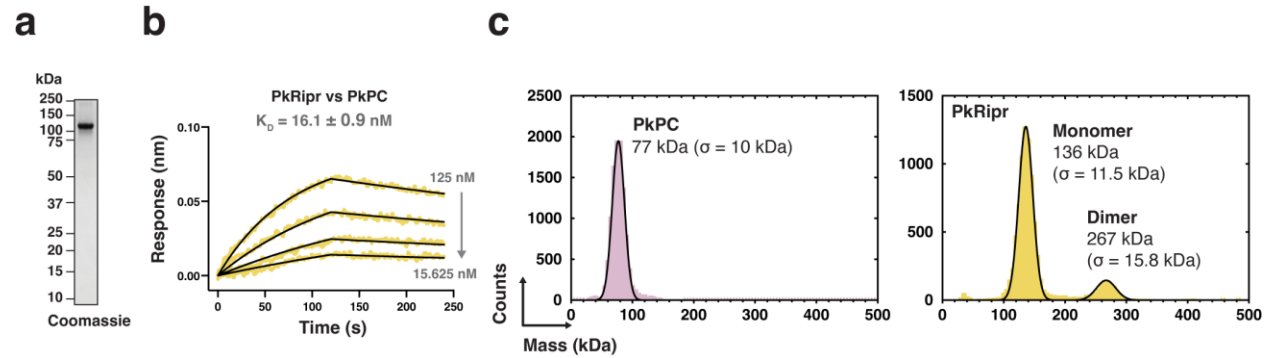
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Extended Data figure 4. Biophysical analysis of PkPC and PkRipr. a. SDS-PAGE of recombinant full-length PkRipr. **b.** Representative biolayer interferometry sensorgram of PkRipr vs PkPC. Data are in yellow and 1:1 model best fit in black. **c.** Mass distribution plots of PkPC (pink) and PkRipr (yellow) as determined by mass photometry. Histogram data are in color and the Gaussian curve fit in black.

Extended Data
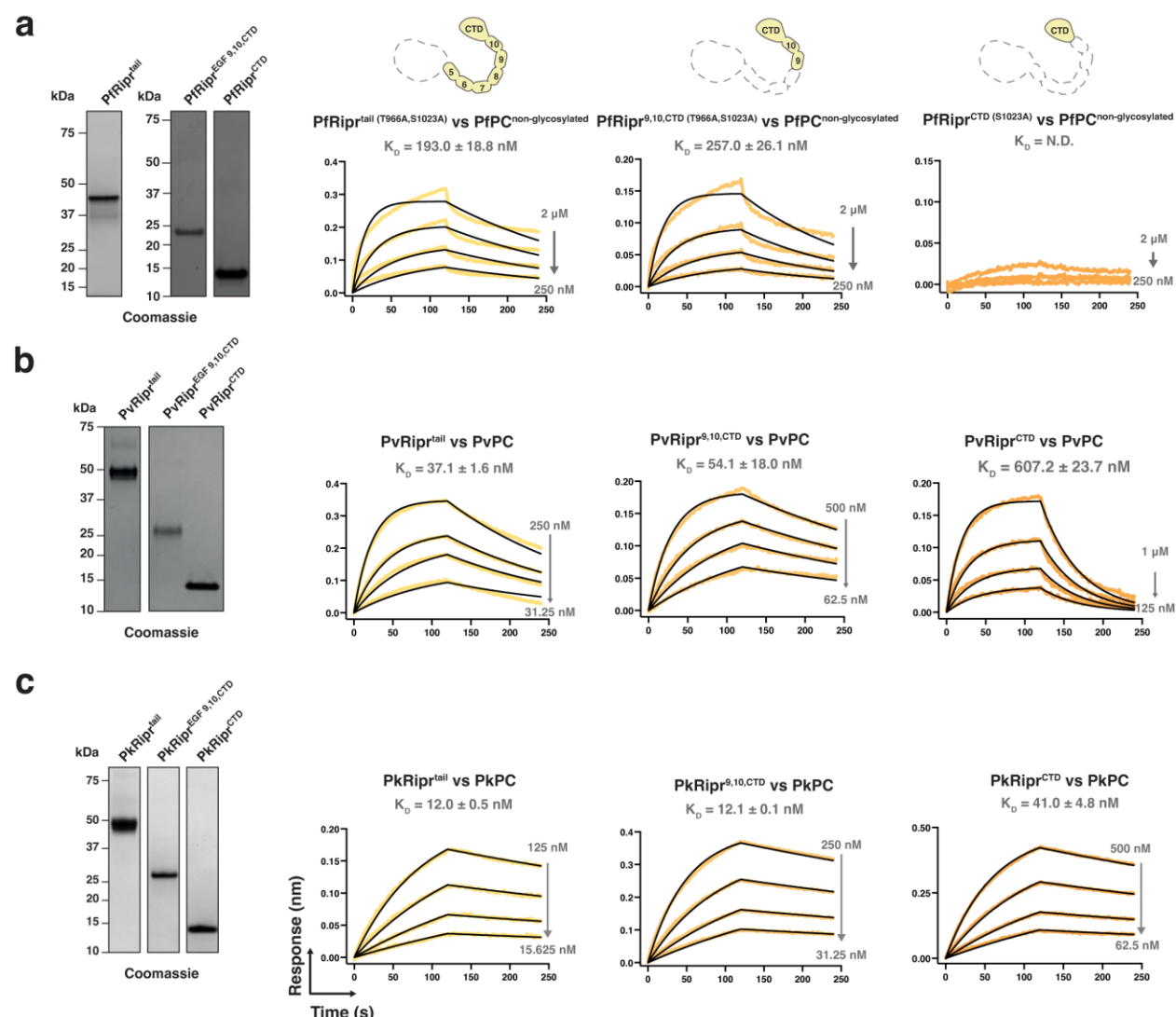
Extended Data figure 5. Biophysical analysis of Ripr truncations. a. SDS-PAGE of recombinant PfRipr truncations and representative biolayer interferometry sensorgrams of Ripr truncations vs PfPC. PfRipr^{tail} and PfRipr$^{EGF\ 9,10,CTD}$ harbor T966A and S1023A mutations and PfRipr^{CTD} harbors S1023A mutation. **b.** SDS-PAGE of recombinant PvRipr truncations and representative biolayer interferometry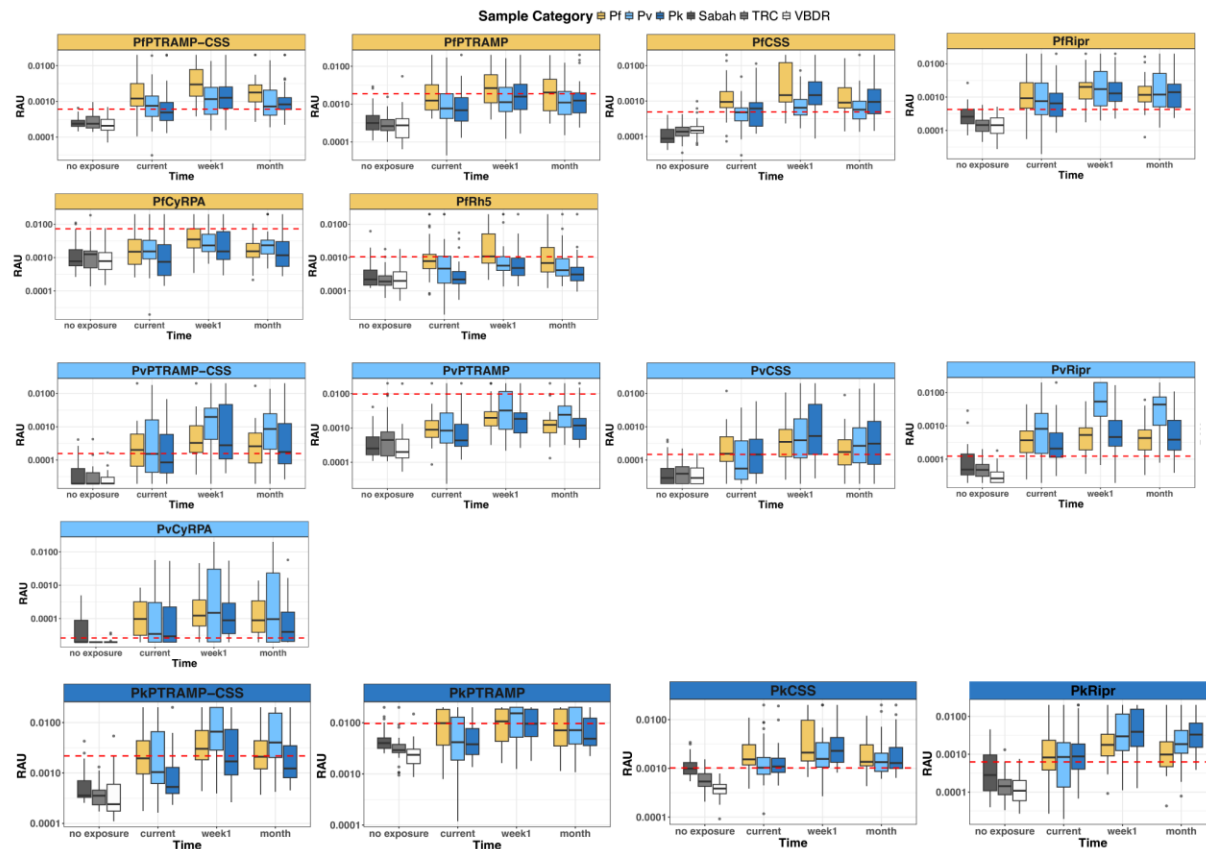 sensorgrams of Ripr truncations vs PvPC. **c.** SDS-PAGE of recombinant PkRipr truncations and representative biolayer interferometry sensorgrams of Ripr truncations vs PkPC. For all sensorgrams, data are in color and 1:1 model best fit in black where applicable. Schematics of the Ripr truncations are shown above.
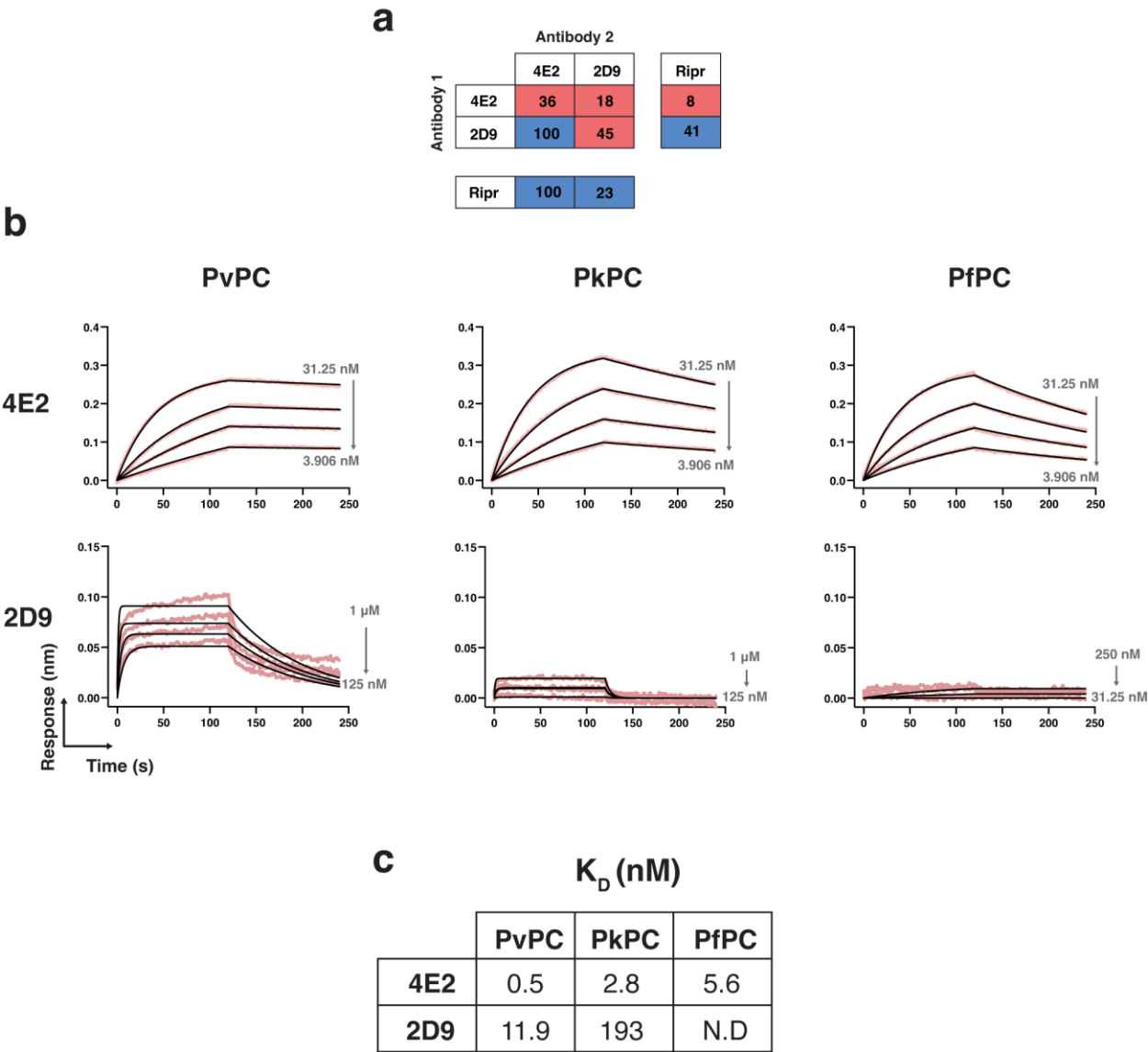
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Extended Data figure 6. Serological assessment of invasion antigens. Antibody kinetics in longitudinal plasma samples collected from individuals with *P. falciparum* (yellow), *P. vivax* (light blue), *P. knowlesi* (dark blue) infections at three timepoints (current (n=95), week 1 (n=98) and a month (n=98)) on a logarithmic scale. Samples from Volunteer Biospecimen Donor Registry (white, n =28) and Thailand Red Cross (light grey, n = 29) was used to set the sero-positivity cut-off (red dotted line), which is mean of these samples + 2 x standard deviation. Samples from healthy (non-febrile) individuals from Sabah Malaysia (dark grey, n=30) are not included in the sero-positivity calculation as their previous exposure status to any of the *Plasmodium* species is unknown. Boxplots display the median (horizontal line), interquartile range (IQR) (the box), largest and smallest values (1.25 x IQR, whiskers) and outliers are displayed as points.

Extended Data

Extended Data figure 7. Characterization of anti-PvPC mouse monoclonal antibodies. a. Competition binning of anti-PvPC monoclonal antibod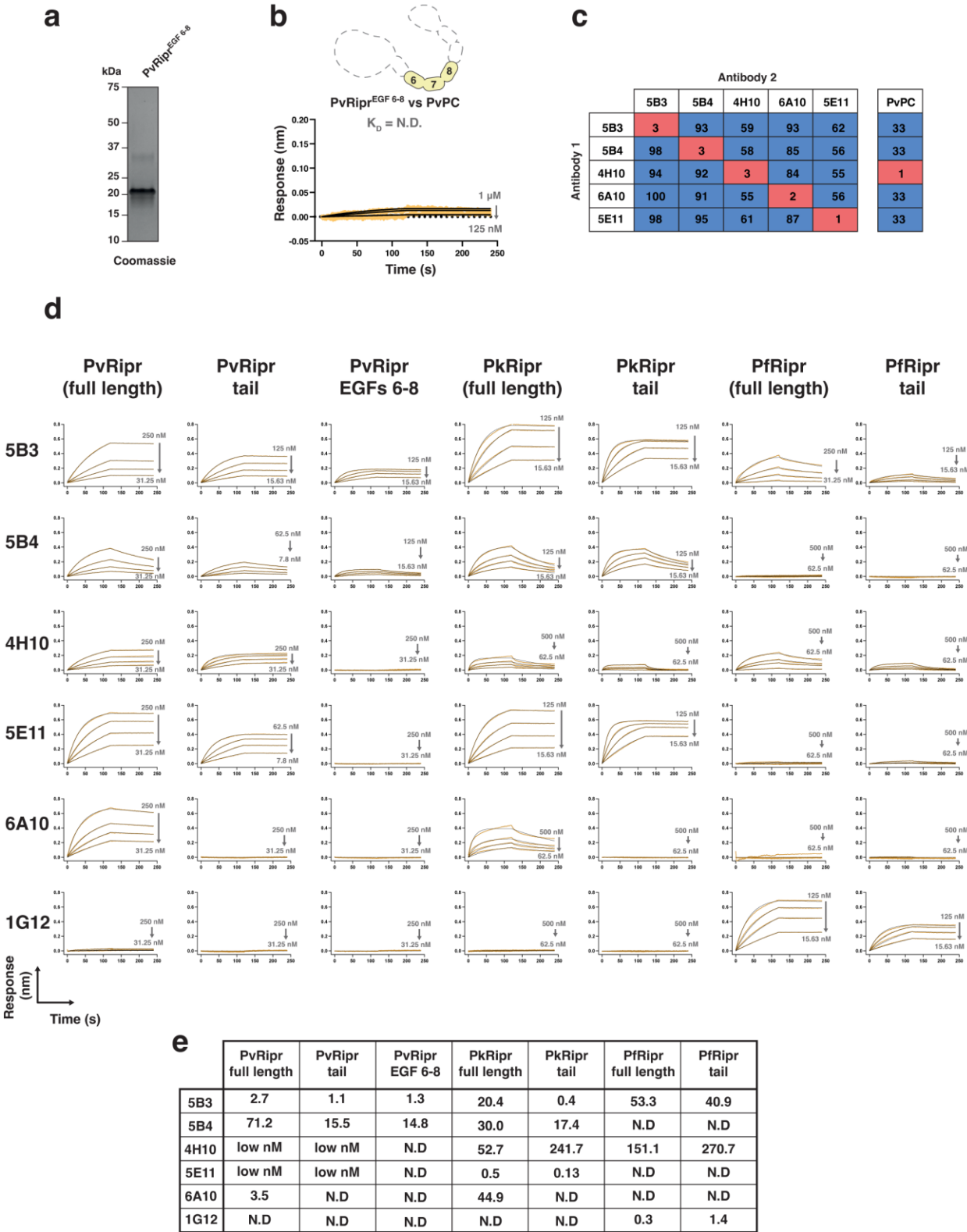ies and PvRipr. **b.** Representative biolayer interferometry sensorgrams of antibody binding to PvPC, PkPC, and PfPC. Data are in color and 1:1 model best fit in black. **c.** Table of K_D values, in nM, for the curves in b. N.D = not determined

Extended Data

a

b

PvRipr$^{EGF 6-8}$ vs PvPC

K_D = N.D.

c

	Antibody 2						
Antibody 1		5B3	5B4	4H10	6A10	5E11	PvPC
	5B3	3	93	59	93	62	33
	5B4	98	3	58	85	56	33
	4H10	94	92	3	84	55	1
	6A10	100	91	55	2	56	33
	5E11	98	95	61	87	1	33

d

e

|	PvRipr full length	PvRipr tail	PvRipr EGF 6-8	PkRipr full length	PkRipr tail	PfRipr full length	PfRipr tail
5B3	2.7	1.1	1.3	20.4	0.4	53.3	40.9
5B4	71.2	15.5	14.8	30.0	17.4	N.D	N.D
4H10	low nM	low nM	N.D	52.7	241.7	151.1	270.7
5E11	low nM	low nM	N.D	0.5	0.13	N.D	N.D
6A10	3.5	N.D	N.D	44.9	N.D	N.D	N.D
1G12	N.D	N.D	N.D	N.D	N.D	0.3	1.4

Extended Data figure 8. Characterization of anti-PvRipr mouse monoclonal antibodies. a. SDS-PAGE of recombinant PvRipr^{EGF6-8}. **b.** Biolayer interferometry sensorgram of PvRipr^{EGF6-8}

Extended Data

113   vs PvPC showing no interaction between the two proteins. **c.** Competition binning of anti-PvRipr

114   antibodies. **d.** Representative biolayer interferometry sensorgrams of antibody binding to PvRipr,

115   PkRipr and PfRipr. The anti-PfRipr antibody 1G12 was included as a control [2]. Data are in color

116   and 1:1 model best fit in black. **e.** Table of K_D values, in nM, for the curves in d. N.D. = not

117   determined.

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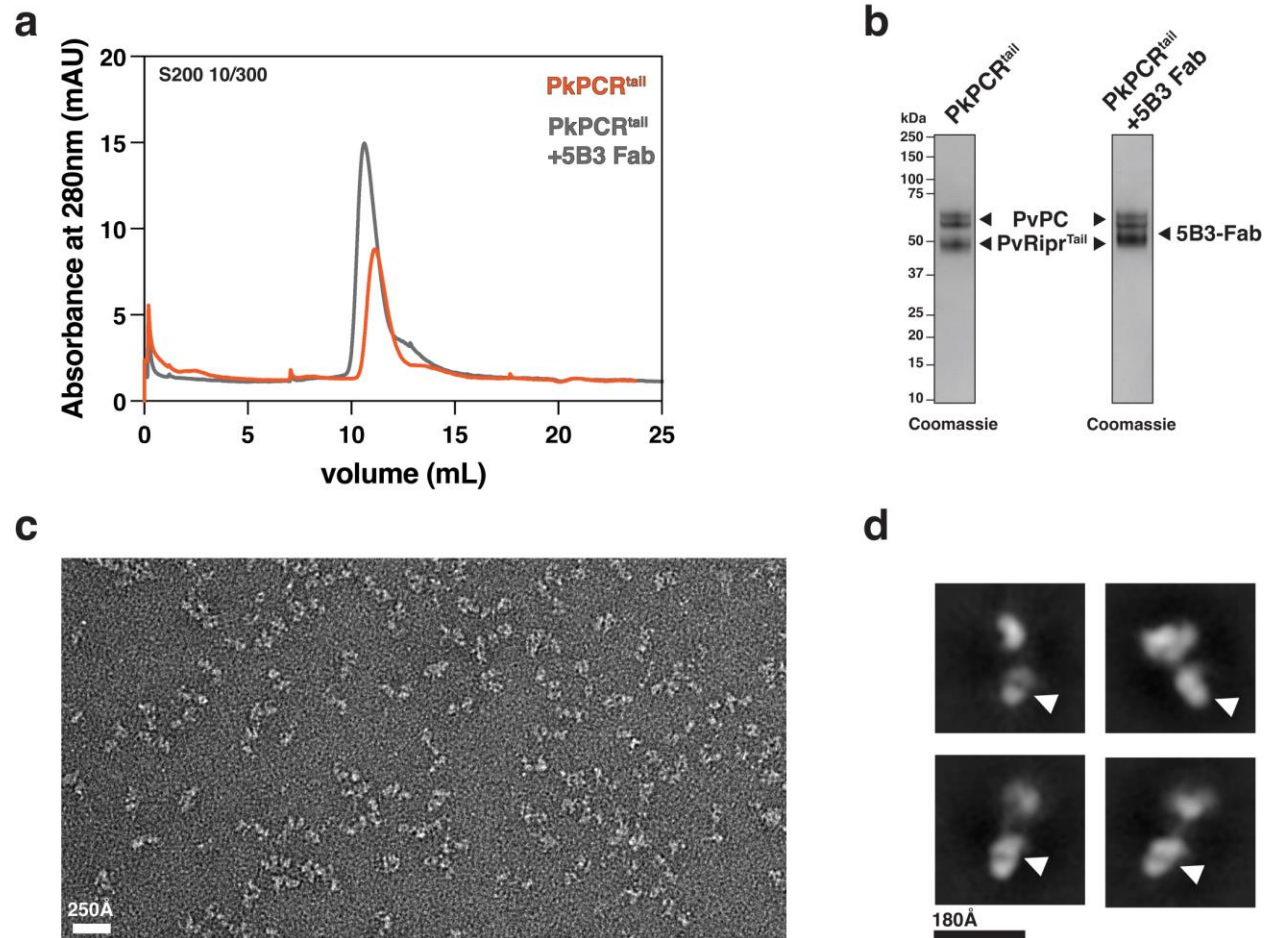
Extended Data figure 9. Negative stain electron microscopy of PkPCR^{tail}+5B3 Fab shows an elongated structure. a. Size-exclusion chromatography chromatograph of PkPCR^{tail} and PkPCR^{tail}+5B3 Fab. **b.** Non-reducing SDS-PAGE of peak fractions of purified complexes from a. 5B3 Fab and Ripr^{tail} have approximately the same molecular weight. **c.** Representative electron micrograph of negatively stained PkPCR^{tail}+5B3 Fab particles. **d.** Two-dimensional class averages of PkPCR^{tail}+5B3 Fab. White arrows show density attributable to 5B3-Fab.

Extended Data

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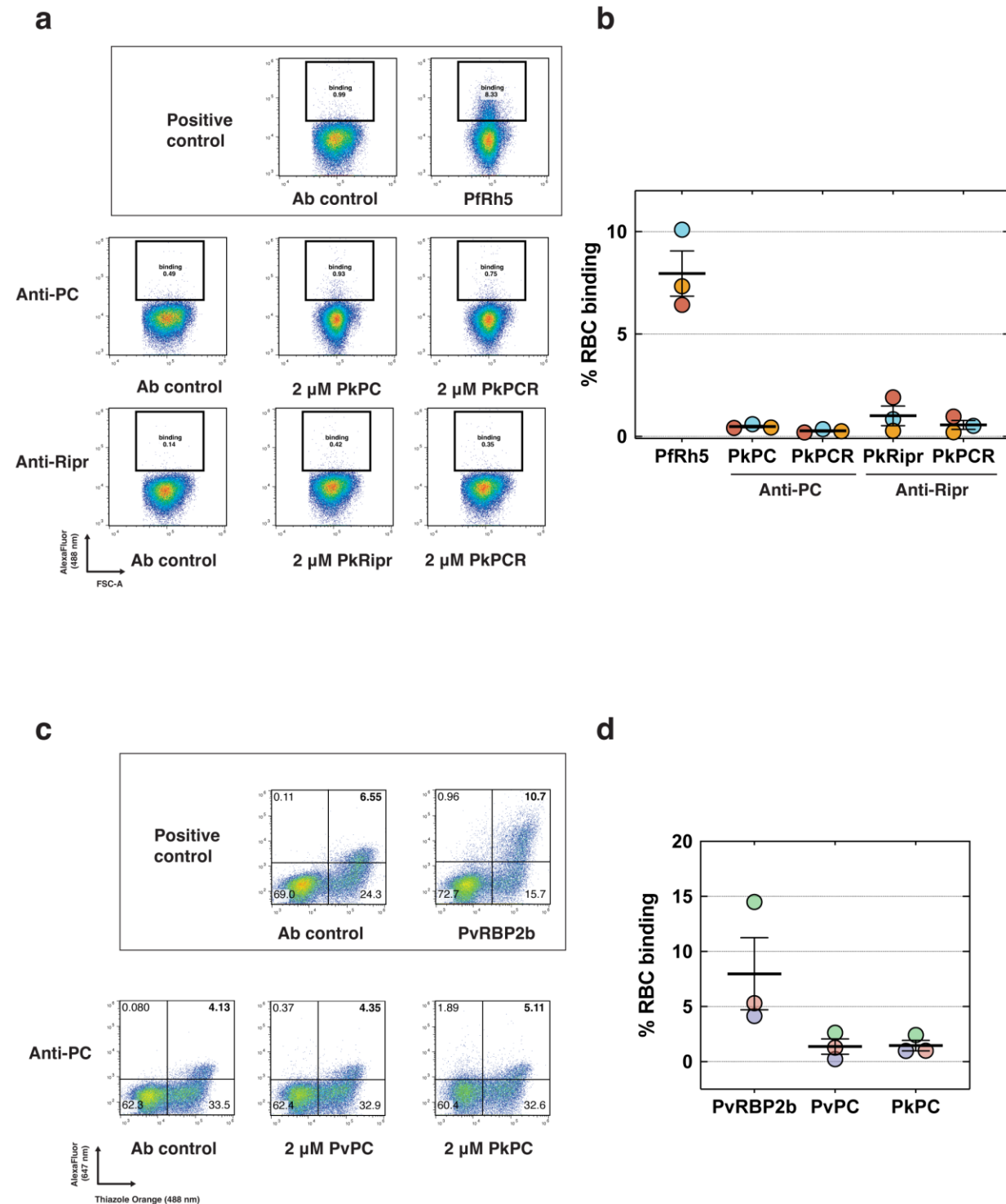
Extended Data figure 10. PvPC, PkPC and PkPCR do not bind erythrocytes. a. Representative flow cytometry plots of PkPC, PkRipr, and PkPCR incubated with erythrocytes and detected with either anti-PC or anti-Ripr antibodies. PfRh5 was used as a positive control for erythrocyte binding. **b.** Quantitation of binding events from three independent experiments.

Extended Data

133   Individual experimental replicates are depicted as colored dots, and the mean is shown with

134   standard error of the mean (SEM). **c.** Representative flow cytometry plots of PvPC and PkPC

135   incubated with reticulocyte enriched cord blood and detected with anti-PC antibody. PvRBP2b

136   was used as a positive control for reticulocyte binding. **d.** Quantitation double positive binding

137   events of three independent experiments. Individual colored dots represent a replicate performed

138   with a single donor's blood. Mean is shown with SEM.

139

140   **Reference List**

141

142   1.   Scally SW, Triglia T, Evelyn C, et al. PCRCR complex is essential for invasion of human

143   erythrocytes by *Plasmodium falciparum*. *Nature Microbiology*. 2022/12/01 2022;7(12):2039-

144   2053. doi:10.1038/s41564-022-01261-2

145   2.   Healer J, Wong W, Thompson JK, et al. Neutralising antibodies block the function of

146   Rh5/Ripr/CyRPA complex during invasion of *Plasmodium falciparum* into human erythrocytes.

147   *Cellular Microbiology*. 2019;21(7):e13030. doi:10.1111/cmi.13030

148