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1   **Cryo-EM structure of an essential *Plasmodium vivax* invasion complex**

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Summary

Plasmodium vivax is the most widely distributed malaria parasite that infects humans[1]. It exclusively invades reticulocytes and successful entry depends on the specific interactions between *P. vivax* reticulocyte-binding protein 2b (PvRBP2b) and transferrin receptor 1 (TfR1)[2]. The PvRBP2b-TfR1 invasion pathway is essential for *P. vivax* entry into human reticulocytes, as TfR1-deficient erythroid cells are refractory to invasion by *P. vivax,* and anti-PvRBP2b monoclonal antibodies inhibit reticulocyte-binding and block *P. vivax* invasion in field isolates[2]. Here we report a high-resolution structure of a ternary complex with PvRBP2b bound to human TfR1 and transferrin, at 3.7 Å resolution by cryo-electron microscopy. Mutational analyses show that PvRBP2b residues involved in complex formation are conserved, providing a promising design strategy to engineer antigens that may be strain-transcendent against *P. vivax* infection. Functional analyses of TfR1 highlight how *P. vivax* has hijacked TfR1, which is an essential housekeeping protein, by binding to sites that govern host specificity without affecting its cellular function of transporting iron. Crystal and solution structures of PvRBP2b in complex with antibody fragments characterize the inhibitory epitopes. Our results establish a structural framework for understanding how *P. vivax* reticulocyte-binding protein engages its receptor and the molecular mechanism of inhibitory monoclonal antibodies, providing important information for the design of novel vaccine candidates.

Main text

68   Red blood cell invasion is an essential step in the malaria parasite's lifecycle

69   and depends on interactions between parasite invasion ligands and their cognate red

70   blood cell receptors[2–9]. *Plasmodium vivax* invades reticulocytes[10,11] and a family of

71   parasite adhesins called the *P. vivax* reticulocyte-binding protein (PvRBP) play a

72   crucial role in reticulocyte recognition[12–14]. One major invasion pathway for *P. vivax*

73   entry into reticulocytes is mediated by the specific interaction between a member of

74   this protein family, PvRBP2b, and the essential housekeeping protein transferrin

75   receptor 1 (TfR1)[2].

76   We expressed and purified a recombinant fragment of PvRBP2b

77   encompassing residues 161-969 (PvRBP2b$_{161-969}$) in complex with TfR1 and Tf, and

78   reconstructed a three-dimensional electron density map using cryo-electron

79   microscopy (cryo-EM) (Extended Data Fig. 1 and Extended Data Table 1). The

80   electron density corresponding to residues 161-167 and 634-969 could not be

81   observed. The remaining residues 168 to 633 (PvRBP2b$_{168-633}$) adopt a highly

82   elongated, predominantly α-helical fold that can be subdivided into two domains

83   denoted as N- and C-terminal domains with an angle of ~130° between them (Fig.

84   1a). The N-terminal domain encompassing residues 168-460 and helices α1 to α7,

85   can be superimposed with the crystal structure of the corresponding fragment (PDB

86   accession number 5W53)[2] with root mean square deviation (RMSD) of 0.84 Å over

87   279 aligned Cα atoms (Extended Data Fig. 2a). The structure of the C-terminal

88   domain encompassing residues 461-633 was determined *de novo* and consists of a

89   bundle of three long helices denoted as α8, α9 and α10 (Fig. 1a). The N-terminal

90   domain is predominantly positively charged, in contrast to the negatively charged C-

91   terminal domain (Extended Data Fig. 2b).

92   The cryo-EM structure of the PvRBP2b-TfR1-Tf ternary complex was

93   determined to an overall resolution of 3.7 Å (Fig. 1b and Extended Data Fig. 1). The

94   size of the resolved structure is approximately 150 Å in its longest dimension, and

95   120 and 140 Å in the other two dimensions (Fig. 1b). The PvRBP2b-TfR1-Tf

96   complex consists of the homodimeric TfR1 (residues 120-760) and its corresponding

97   two molecules of iron bound-Tf (residues 1-679), with two molecules of PvRBP2b

98   bound on either side (Fig. 1c). We performed analytical ultracentrifugation

99   experiments using a fixed amount of TfR1-Tf in the presence of increasing

100   concentrations of PvRBP2b (Extended Data Fig. 2c-e). These data are consistent with

101   the binding of two molecules of PvRBP2b to the TfR1-Tf complex with macroscopic

102   dissociation constants in the mid-nanomolar to low-micromolar range. We determined

103   another structure with only one PvRBP2b protein bound to the TfR1-Tf binary

104   complex allowing us to observe the conformational changes induced in the receptor

105   upon ligand binding (Extended Data Fig. 3a-e). Additional classification further

106   separated the two-ligand complex into two subclasses, which indicate an intrinsic

107   flexibility of the N-terminal domain (Extended Data Fig. 3f, g). Overall, binding of

108   PvRBP2b results in small conformational changes confined to the apical domain of

109   TfR1 that mostly involve rearrangement of the loops on the tip of the domain.

110   TfR1 is a single pass type II transmembrane protein, with a cytoplasmic

111   domain and a large extracellular domain[15]. The extracellular domain has a protease-

112   like domain which resides proximal to the membrane, a helical domain responsible

113   for dimerization, and an apical domain of unknown function (Fig. 1c). Co-crystal

114   structures have been solved of either human TfR1 in complex with Tf or HFE, or with

115   the entry glycoprotein of a New World hemorrhagic arenavirus, Machupo virus

116   (MACV GP1)[16–19]. The cryo-EM structure of the PvRBP2b-TfR1-Tf complex shows

117   that PvRBP2b interacts with the TfR1-Tf binary complex through three principal

118   sites; i) the apical domain of TfR1, ii) the protease-like domain of TfR1 and iii) the N-

119   terminal region of Tf (Fig. 2a-c and Extended Data Table 2). When bound to

120   PvRBP2b, TfR1 shows a more extensive buried surface area (~1,271 Å^2) compared to

121   Tf (~386 Å^2) (Fig. 2a). We mapped naturally occurring field polymorphisms using

122   data from the MalariaGEN *P. vivax* Genome Variation project onto the PvRBP2b$_{168-}$

123   $_{633}$ structure (Fig. 2d,e)[2,20]. Our results show that residues in PvRBP2b that interact

124   with TfR1 and Tf are mostly conserved, but reside within a region that is under

125   balancing selection[2,20].

126   We performed site-directed mutagenesis to identify residues at the PvRBP2b-

127   TfR1 interface critical for complex formation (Extended Data Fig. 4a, b). We mutated

128   the following residues on PvRBP2b that interact with TfR1, R359, E530, D531,

129   Y538, Y542, E556, K563, K600, Y604, to alanine. These residues were chosen as

130   they were involved in either the formation of salt bridges or as part of the three-

131   tyrosine motif (Fig. 2c). We also mutated four PvRBP2b residues that interact with

132   Tf, Y186, K297, R304 and N428 to alanine. Of all the aforementioned mutants, only

133   PvRBP2b$_{Y542A,}$ PvRBP2b$_{K600A}$ and PvRBP2b$_{Y604A}$ showed a ~80% reduction in

134   binding to reticulocytes compared to wildtype (Fig. 3a).

135   We examined the ability of these PvRBP2b mutant proteins to form a stable

136   complex with TfR1-Tf using analytical SEC. As a control, we used TfR1 with a

137   deletion in glycine 217 (TfR1ΔG217) which is known abolish complex formation[2].

138   While TfR1ΔG217 is able to bind Tf, ternary complex formation with PvRBP2b was

139   not detected compared to wildtype (Fig. 3b-d). For all PvRBP2b mutants, only

140   PvRBP2b$_{Y542A,}$ PvRBP2b$_{K600A}$ and PvRBP2b$_{Y604A}$ were defective in forming a

141   complex with TfR1-Tf, as indicated by an overlap of elution profiles with

5

142 TfR1ΔG217 (Fig. 3b, c). We examined if stacking interactions mediated by the three-

143 tyrosine motif at PvRBP2b residues Y538, Y542 and Y604, were important for

144 complex formation (Fig. 2c, top middle panel). Our results indicate the hydrogen

145 bonds formed by residue Y542 and the stacking interactions formed by aromatic side

146 chains of Y538 and Y604 play an important role in PvRBP2b interaction with TfR1

147 (Extended Data Fig. 4c). To examine the importance of the salt bridge at residue

148 PvRBP2b$_{K600}$ (Fig. 2c, top right panel), we generated both PvRBP2b$_{K600E}$ and

149 PvRBP2b$_{K600M}$ mutants, which have similar side chain lengths but lack the ability to

150 form salt bridges. Both these mutants were defective in complex formation,

151 supporting the importance of the salt bridge formed by the residue K600 (Extended

152 Data Fig. 4c). These results indicate that PvRBP2b residues Y542, K600 and Y604 are

153 critical for reticulocyte-binding and complex formation.

154   We mutated the following residues on TfR1, E149, R208, Y211, E214, E294,

155 K574 and E578, to alanine. We also modified S142 to glycine to mimic a common

156 polymorphism in TfR1 that is prevalent in Asian populations, and N348 to alanine as

157 it is a critical determinant for MACV GP1 engagement with TfR1[17,21]. The overall

158 structure of TfR1 is unperturbed by these single site mutations based on circular

159 dichroism analyses and the observation that all TfR1 mutants retain their ability to

160 bind Tf (Extended Data Fig. 4d-f). Of all the aforementioned mutants, only

161 TfR1$_{Y211A}$, TfR1$_{E294A}$ and TfR1$_{E149A}$ mutants were not able to bind to PvRBP2b, as

162 indicated by an overlap of elution profiles with TfR1ΔG217 (Fig. 3d).

163   Our structure-function analyses pinpoint three key interactions important for

164 complex assembly; the interactions formed by PvRBP2b$_{Y542}$ and TfR1$_{Y211}$, a salt

165 bridge formed between PvRBP2b$_{K600}$ and TfR1$_{E294}$ and a second salt bridge between

166 TfR1$_{E149}$ and PvRBP2b$_{R359}$ (Fig. 3e). Y211 is localized in the strand βII-2 of TfR1's

167 apical domain and is a critical residue for entry of New World hemorrhagic

168 arenaviruses[22]. Furthermore, $TfR1_{Y211}$ and $TfR1_{E149}$ are only present in human TfR1

169 but not mouse TfR1, providing an explanation of how *P. vivax* invasion is not

170 permissive into rodent reticulocytes (Extended Data Fig. 5a, b)[21,23]. We show that

171 PvRBP2b does not bind to TfR1-Tf binary complexes containing either mouse TfR1

172 or mouse Tf, indicating that PvRBP2b displays specificity for human TfR1 and

173 human Tf (Extended Data Fig. 5c). While the majority of PvRBP2b interactions

174 involve TfR1, we propose that interactions with Tf are important for complex stability

175 as only the PvRBP2b-TfR1-Tf ternary complex was isolated by size exclusion

176 chromatography and visualized by cryo-EM techniques. These results show that

177 PvRBP2b binds to TfR1 residues not involved with Tf binding and identifies TfR1

178 residues implicated in *P. vivax* specificity for human reticulocytes.

179   Anti-PvRBP2b monoclonal antibodies, 3E9, 6H1 and 10B12, inhibit

180 PvRBP2b binding to reticulocytes and block the invasion of *P. vivax* into

181 reticulocytes[2]. We identified an additional mAb, 4F7 that inhibits PvRBP2b binding

182 to reticulocytes (Fig. 4a, Extended Data Fig. 6a). As expected, non-inhibitory anti-

183 PvRBP2b mAb 8G7 and anti-PfRh4 mAb 10C9 did not affect PvRBP2b binding (Fig.

184 4a)[2]. We obtained crystal structures for PvRBP2b-3E9, PvRBP2b-4F7 and PvRBP2b-

185 6H1 complexes refined to 2.53, 2.66 and 3.34 Å respectively, while a model for

186 PvRBP2b–10B12 was derived from SAXS analysis (Fig. 4b-d, Extended Data Fig.

187 6b-f, Extended Data Table 3 and Extended Data Table 4). 3E9 binds on the side of the

188 N-terminal domain of PvRBP2b to a region localized between three helices α4, α5

189 and α6 (Fig. 4b,c). The binding sites for 6H1 and 4F7 antibody fragments overlap and

190 they bind close to the tip of the N-terminal domain of PvRBP2b, forming interactions

191 with the residues localized in helix α2 and α3 as well as to the loop interconnecting

7

192   helices α3 and α4 (Fig. 4b, c). 10B12 binds at the tip of PvRBP2b at a distinct site

193   from 6H1 and 4F7 (Fig. 4b, c). When superimposed on the ternary complex structure

194   (Fig. 4c, d), 3E9 sterically clashes with TfR1 and Tf molecules. Superimposition of

195   4F7, 6H1 and 10B12 crystal structures onto the ternary complex structure shows that

196   this collection of antibodies does not inhibit by blocking the sites of interaction with

197   TfR1 or Tf, but rather through steric hindrance with the reticulocyte membrane (Fig.

198   4d). While all these antibodies target the N-terminal domain of PvRBP2b, future work

199   will need to determine if antibodies against the C-terminal domain will be effective at

200   blocking PvRBP2b interaction with TfR1.

201   The cryo-EM single particle analysis of the PvRBP2b-TfR1-Tf complex

202   identified crucial interfaces between PvRBP2b and TfR1 required for complex

203   formation. While *P. vivax* harbors more global genetic diversity relative to *P.*

204   *falciparum*, we show that PvRBP2b residues crucial for complex formation are

205   conserved across >200 field isolates from Asia Pacific[20,24]. The crystal structures of

206   the inhibitory antibody fragments with PvRBP2b provide mechanistic insight on the

207   modes of action that involve steric hindrance with either the receptor or the

208   reticulocyte membrane. These high-resolution structures provide a solid structural

209   framework for the rational design of a promising vaccine candidate to block *P. vivax*

210   invasion into reticulocytes.

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215   human malaria parasite. *Lancet Infect. Dis.* **9,** 555–566 (2009).

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Figure Legends

312 **Figure 1.** The cryo-EM structure of PvRBP2b-TfR1-Tf ternary complex at 3.7 Å

313 resolution. a, Structure of PvRBP2b$_{168-633}$ in two orthogonal views with the secondary

314 structure labeled. b, The two-ligand PvRBP2b-TfR1-Tf ternary complex in two

315 orthogonal views. TfR1, Tf, PvRBP2b and ferric ions (Fe^{3+}) are in green, cyan, violet

316 and as red spheres. c, The ternary complex relative to the reticulocyte membrane.

317 PvRBP2b is divided into N-terminal (violet) and C-terminal (blue) domains. The

318 apical, helical and protease-like domains of TfR1 are shown in green, yellow and red,

319 with the transmembrane (TM) and cytoplasmic domains of TfR1 indicated. Tf (cyan)

320 with Fe^{3+} as red spheres.

321 **Figure 2.** The PvRBP2b-TfR1-Tf interface. a, Surface representation of the

322 PvRBP2b-TfR1-Tf complex. The apical, helical and protease-like domains of TfR1

323 are in green, yellow and red. Tf, N- and C-terminal domains of PvRBP2b are in cyan,

324 violet and blue. TfR1 and Tf residues involved in the interaction with N- and C-

325 terminal domain of PvRBP2b are in violet and blue. For PvRBP2b, the footprints of

326 TfR1 and Tf are highlighted in green, red and cyan. b, Residues of PvRBP2b that bind

327 the receptor with hydrophobic interactions, hydrogen bonds and salt bridges are in

328 yellow, green and red. c, The interaction sites between PvRBP2b, TfR1 and Tf.

329 Residues forming important contacts are shown as sticks and labeled. Proteins are

330 colored according to the domain organization and as in a. d, Polymorphic residues

331 labeled as pink spheres are mapped on the structure of PvRBP2b shown in two

332 orthogonal views. Regions interacting with TfR1 and Tf are shown in green and cyan.

333 e, Schematic of the PvRBP2b sequence. Residues involved in binding and forming

334 hydrogen bonds and salt bridges are indicated with arrows above the sequence and

335   color-coded as in the left panel. Polymorphic residues are indicated with pink arrows

336   below the sequence.

337   **Figure 3.** Structure function analyses of PvRBP2b and TfR1 residues involved in

338   reticulocyte binding and complex formation. a, PvRBP2b mutant proteins analyzed

339   using reticulocyte-binding assay. Mean ± S.E.M, n = 4, open circles represent

340   biological replicates. b, Analytical SEC for PvRBP2b mutants that bind to TfR1

341   residues. c, Analytical SEC for PvRBP2b mutants that bind to Tf residues. d,

342   Analytical SEC for TfR1 mutants. e, Schematic diagrams summarizing the mutational

343   analyses of PvRBP2b and TfR1. Mutants which showed no defect, moderate defect

344   and complete defect were colour coded as green, orange and red respectively. The key

345   interactions between PvRBP2b and TfR1 are highlighted as a dashed line for stacking

346   interactions or double lines for salt bridges. All analytical SEC in b and d were

347   performed once.

348   **Figure 4.** Structural modes of inhibition for anti-PvRBP2b antibodies. a, PvRBP2b

349   binding in the presence of anti-PvRBP2b mAbs 3E9, 4F7, 6H1, 8G7, 10B12 and anti-

350   PfRh4 mAb 10C9. Normalized binding results where PvRBP2b binding in the

351   absence of mAbs was arbitrarily assigned to be 100%. Mean ± S.E.M, n = 4, open

352   dots represent biological replicates. b, Crystal structures of PvRBP2b (purple) bound

353   to inhibitory antibody fragments of 3E9, 4F7 and 6H1, and SAXS-based model for

354   10B12 antibody fragment complex. c, Surface representation of PvRBP2b molecule

355   with TfR1 (green), Tf (cyan) and the inhibitory antibodies interaction sites

356   highlighted. d, Superimposition of the structures of 3E9, 4F7, 6H1 and 10B12

357   antibodies fragments on the cryo-EM structure of the PvRBP2b-TfR1-Tf complex.

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Methods

Expression and purification of recombinant PvRBP2b and TfR1 mutants. Protein expression and purification for wildtype PvRBP2b, wildtype TfR1 and Tf were performed as described previously[2]. Mouse Tf was purchased from Sigma-Aldrich. Restriction free cloning was used to generate the alanine mutants in both PvRBP2b and TfR1 with all sequences verified using the Melbourne Translational Genomics Center. Expression and purification of all mutant proteins were performed as for the wildtype proteins. The complex between PvRBP2b, TfR1 and Tf was prepared by mixing equimolar amounts of each protein to a final concentration of 1.0 mg/ml in buffer containing 20 mM NaHEPES pH 7.5, 100 mM NaCl and 50 mM $NaHCO_3$. The sample was flash-frozen in liquid nitrogen after the addition of 10% (v/v) glycerol, and stored at -80°C. Circular dichroism experiments were performed as described previously[2] to compare the secondary structure of wildtype vs mutant proteins.

Cryo-EM sample preparation and data acquisition. Prior to grid preparation, an aliquot of protein was thawed on ice immediately followed by glycerol removal using a 0.5 µl 100 kDa MWCO Amicon filtration unit (Millipore, Darmstadt Germany) at 4°C and 2,000 x g for 5 cycles. 3.2 µl of sample in glycerol-free buffer (20 mM NaHEPES pH 7.5, 100 mM NaCl and 50 mM $NaHCO_3$) was applied to a glow-discharged 400-mech quantifoil 1.2/1.3 Au grid (Quantifoil, Großlöbichau Germany) then rapidly plunge-frozen into a liquid ethane bath on a Vitrobot (FEI company, part of Thermo Fisher Scientific Hillsboro, OR). Data were acquired at a sampling rate of 1.35 Å/pixel on a FEI Titan Krios at 300 kV equipped with spherical aberration corrector and a post-energy filter K2 direct electron detector. Each exposure was recorded with total dose of ~80 electron/$Å^2$ at a frame rate of 0.3 s per frame.

384 **Image processing and reconstruction.** Motion and drift in each image stack was

385 measured, corrected, and dose-weighted by unblur and sum_movie at 1.65 electron/Å^2

386 per frame[25]. 3,000 particles were manually picked in EMAN2[26] followed by 2D class

387 averages calculation in Relion. The preliminary 2D class averages were used to

388 generate a template for automated particle picking. CTF determination for each drift-

389 corrected micrograph was calculated by CTFFIND4[27]. We used Relion1.4 and

390 Relion2.0[28] for particle picking, extraction, 2D and 3D classifications, and high-

391 resolution refinement to derive final reconstructions for one-ligand and two-ligand

392 bound complexes. 3D classification was performed without imposing any

393 symmetry. As a result, one-ligand and two-ligand subclasses were identified. One-

394 ligand complex continued to be refined without symmetry applied. The two-ligand

395 class was refined independently in both C1 and C2 symmetry, and both refinements

396 yielded nearly identical high-resolution reconstructions (3.74 vs. 3.68Å). The

397 difference between C1 and C2-applied maps is negligibly minor indicating transient

398 heterogeneity was not detected under the current resolution limit. Therefore, C2

399 symmetry was applied in local refinements and post-processing to generate the final

400 reconstructed map of the two-ligand complex.

401 **Model building and refinement.** The program Chimera[29] was initially employed to

402 dock the available crystal structures into the electron density maps obtained from

403 cryo-EM. We used the following models: for TfR1 PDB accession code 1CX8[15], for

404 Tf PDB accession code 3QYT[30] and for the N-terminal domain of PvRBP2b (residues

405 169-470) PDB accession code 5W53[2]. For Tf, we observed some conformational

406 differences between the model and electron density and to facilitate the fitting we

407 divided the molecule into three parts consisting of subdomain N1, N2 and C-lobe.

408 Model building and refinement was continued manually in COOT[31]. The C-terminal

409   domain of PvRBP2b (residues 471-633) was built *de novo*. The high resolution of this

410   region allowed unambiguous assignment of the side chains in the region interacting

411   with TfR1. We did not observe the electron density corresponding to residues 161 till

412   167 and 634 till 969.

413   The model of the ternary complex was refined in Phenix[32] using real space

414   refinement. For the two-ligand complex, non-crystallographic symmetry (NCS) was

415   imposed on the corresponding chains of identical molecules. For one-ligand complex,

416   no NCS restrains were used. No secondary structure restraints were initially applied to

417   the model. The final assignment of secondary structures was performed using the

418   DSSP program[33] integrated in Chimera[29]. The quality of the final models was

419   validated using MolProbity[34] and EMRinger[35]. Electron density maps were visualized

420   using Chimera[29].

421   **Determination of field polymorphisms**. We determined naturally occurring field

422   polymorphisms using data from the May 2016 release of the MalariaGEN *P.*

423   *vivax* Genome Variation project. We selected all variants that passed filters and had a

424   non-reference allele frequency among all samples of > 0.1. These variants can be

425   accessed using the URL:

426   https://www.malariagen.net/apps/pvgv/index.html?dataset=pvivax_stable&workspace

427   =workspace_1&view=cc42817a-d8dd-11e7-a74a-

428   22000a4d9adb&state=table_variants.

429   **Antibodies production.** Anti-PvRBP2b mouse monoclonal antibodies were prepared

430   at the Walter and Eliza Hall Institute Monoclonal Antibody Facility as described

431   previously[2]. 24 hybridomas were selected based on displaying the strongest antibody

432   response to PvRBP2b$_{161-1454}$ as measured by ELISA. These hybridoma supernatants

433   were screened for the ability to inhibit PvRBP2b$_{161-1454}$ binding to reticulocytes.

434   Selected hybridomas producing inhibitory antibodies were cloned by limiting dilution

435   in multi-well plates aiming for one cell or less per well. The sub-cloned cell

436   supernatants were screened by ELISA against PvRBP2b$_{161-1454}$. Two or more rounds

437   of limiting dilution cloning were generally required before the hybridomas were

438   deemed monoclonal. The antibodies were purified from monoclonal hybridoma

439   supernatants with Protein A Sepharose. All monoclonal antibodies used for protein

440   crystallization have been sequenced by GenScript Company.

441   **Flow cytometry-based reticulocyte-binding assay.** Enriched reticulocytes were

442   resuspended in phosphate buffered saline (PBS) to a final volume of 1 x 10^7 cells/ml.

443   Recombinant proteins were incubated at 0.02 mg/ml in 50 µl of the resuspended

444   reticulocytes for one hour at room temperature. All washes were performed in PBS

445   supplemented with 1% (w/v) bovine serum albumin (BSA) and spun at 4,000 x g for 1

446   minute. All antibody incubations were performed at room temperature for one hour.

447   Binding assays were washed once and incubated with the respective rabbit polyclonal

448   antibodies (12.5 µg/ml). After washing, Alexa Fluor 647 chicken anti-rabbit

449   secondary antibody (1:100; Life Technologies) was added. After a final wash, 100 µl

450   thiazole orange (TO) (Retic-Count Reagent; BD Biosciences) was added and

451   incubated for half an hour. Reticulocytes with bound proteins were resuspended in

452   200 µl PBS and analysed on the LSR II flow cytometer (BD Biosciences). 50,000

453   events were recorded and results were analysed using FlowJo software (Three Star).

454   The background signal from a rabbit polyclonal antibody and Alexa Fluor 647

455   conjugated antibody control (without protein) was subtracted from all binding results.

456   To enable comparison between biological repeats, the percentage binding of the

457   protein in the presence of mAbs was divided by the percentage binding with no mAbs

18

458   and multiplied by 100 to obtain the percentage binding relative to the no inhibitor

459   control.

460   **Analytical size exclusion chromatography.** Liquid chromatography was performed

461   using ÄKTA pure 25 M1 chromatographic system (GE Healthcare). Proteins were

462   mixed in equimolar ratio one hour prior to the analysis and the same amount of

463   protein was used throughout the experiment. 100 μl was injected onto Superdex 200

464   Increase 10/300 GL column (GE Healthcare) equilibrated with the buffer containing

465   20 mM NaHEPES pH 7.5, 100 mM NaCl, 50 mM $NaHCO_3$ at 0.75 ml/min of buffer

466   flow. The absorbance of the eluent was monitored at 280 nm. Eluates were collected

467   in 0.5 ml fractions and analyzed using SDS-PAGE. All steps were performed at room

468   temperature.

469   **Analytical ultracentrifugation.** Sedimentation velocity experiments were performed

470   using an XL-I analytical ultracentrifuge (Beckman Coulter) equipped with UV/Vis

471   scanning optics. Samples were prepared containing a constant concentration of 2.5

472   μM TfR1-Tf complex and $PvRBP2b_{161-969}$ at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0,

473   8.0, and 16.0 μM.   Buffer reference (20 mM NaHEPES pH 7.5, 100 mM NaCl, 50

474   mM $NaHCO_3$) and sample solutions were loaded into 12 mm double-sector cells with

475   quartz windows and the cells were mounted in an An-50Ti 8-hole rotor.

476   Centrifugation was conducted at 35,000 rpm (98,780 x g) at 20°C, and radial

477   absorbance data were collected at 250 nm in continuous mode. Data were fitted to a

478   continuous sedimentation coefficient distribution [$c(s)$] model and distributions were

479   integrated between 9 S and 13 S using SEDFIT[36] to determine the integrated

480   absorbance signal. Buffer density and viscosity were calculated using SEDNTERP[37].

481   **Fab fragment purification and formation of Fab-PvRBP2b complexes.** Mouse

482   monoclonal antibodies were digested using papain (Sigma-Aldrich) for two hours at

483   room temperature. For 6H1 and 10B12, L-cysteine (Sigma-Aldrich) was added to

484   cleave F(ab')$_2$ fragments. An excess of iodoacetamide (Sigma-Aldrich) was added to

485   halt the reaction and the samples were passed through a protein A column (GE

486   Healthcare. The flow-through was concentrated and applied on a S75 Superdex

487   16/600 column (GE Healthcare) equilibrated with a buffer containing 20 mM

488   NaHEPES pH 7.5 and 150 mM NaCl. Protein samples were flash-frozen in liquid

489   nitrogen after addition of 10% (v/v) glycerol, and stored at -80°C until further

490   processing.

491   Recombinant PvRBP2b fragment PvRBP2b$_{169-470}$ was mixed with molar

492   excess of individual Fab fragments and incubated overnight at 4°C to allow complex

493   formation and purified using SEC. Fractions containing the complexes were

494   concentrated down using Vivaspin 15 Turbo centrifugal concentrators (Sartorius) with

495   a 5 kDa molecular weight cut-off in a buffer containing 20 mM NaHEPES pH 7.5 and

496   150 mM NaCl and used for crystallization.

497   **Protein crystallization.** Crystallization trials were performed at the CSIRO

498   Collaborative Crystallization Center using two different protein concentrations, 7.5

499   and 15 mg/ml. Crystals of 3E9 in complex with the N-terminal domain of PvRBP2b

500   were obtained in 0.2 M KBr, 15% (w/v) PEG 4,000 and 0.05 M sodium citrate/citric

501   acid pH 5.5. Crystals of 4F7 Fab fragment alone were obtained in 20% (w/v) PEG

502   4,000, 0.05 M sodium citrate/citric acid pH 5.5 and 20% (v/v) 2-propanol. Crystals of

503   4F7 in complex with the N-terminal domain of PvRBP2b were obtained in 0.2 M

504   trisodium citrate and 20% (w/v) PEG 3,350. Crystals of 10B12 Fab fragment alone

505   were obtained in 20% (w/v) PEG 4,000, 0.1 M sodium citrate/citric acid pH 5.5 and

506   20% (v/v) 2-propanol. Crystals of 6H1 in complex with the N-terminal domain of

507   PvRBP2b were obtained in 0.2 M (NH$_4$)$_2$SO$_4$ and 20% (w/v) PEG 3,350.

Data collection and structure solution. Crystals were cryo-protected in a reservoir solution supplemented with 20% (v/v) glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at MX2 beamline at the Australian Synchrotron Facility in Clayton, Australia at 0.9537 Å wavelength using ADSC Quantum 315r detector. Data were integrated using iMosflm[38]. Scaling and merging were performed using program Aimless from the CCP4 package[39]. Cell content was analyzed using the program Matthews[40]. Molecular replacement for the data collected for 4F7 Fab fragment alone was performed using Phaser[41] with an unrelated antibody as a model (PDB accession code 5EN2). The model obtained together with the structure of the N-terminal domain of PvRBP2b (PDB accession code 5W53) were used to solve structures of 3E9, 4F7 and 6H1 Fab complexes. The crystals for 10B12 contained only the Fab fragment. Initial models were rebuilt automatically using the program AutoBuild[32] followed by a manual improvement using the program Coot[14]. The structures were refined using the program Phenix Refine[32] and included TLS (translation/libration/screw) motions that were generated using the TLSMD web server[42]. Crystallographic data collection and refinement statistics for all structures are summarized in Extended Data Table 3. Figures were prepared using either Pymol (http://www.pymol.org) or Chimera[29].

Small angle X-ray scattering. SAXS experiments were performed as described previously[2,14]. We docked the crystal structures of the N-terminal domain of PvRBP2b and 10B12 Fab fragment into the SAXS envelope of the 10B12-PvRBP2b complex using program Collage from the Situs Program Package[43]. Due to the symmetry of the SAXS envelope and the Fab fragment, we could not unambiguously assign the identity of the heavy and light chains within the model.

533 **Data availability**

534 The cryo-EM maps have been deposited in the Electron Microscopy Data Bank with

535 accession codes EMD-7783, EMD-7784 and EMD-7785. The coordinates of the

536 atomic models have been deposited in the Protein Data Bank under accession codes

537 PDB 6D03, PDB 6D04 and PDB 6D05 for the PvRBP2b-Tf-TfR1 one-ligand

538 complex, two-ligand complex subclass 1 and subclass 2, respectively. Coordinates

539 and structure factors have been deposited in the Protein Data Bank under accession

540 codes 6BPA (PvRBP2b-3E9 complex), 6BPB (4F7 Fab alone), 6BPC (PvRBP2b-4F7

541 complex), 6BPD (10B12 Fab alone) and 6BPE (PvRBP2b-6H1 complex).

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589 **Figure Legends for Extended Data**

590 **Extended Data Figure 1**. Details of the cryo-EM data collection and analysis. a,

591 Coomassie-stained reducing SDS-PAGE gel of the proteins used for the sample

592 preparation. M - molecular weight marker (molecular weight of protein standards is

593 given in kDa). For gel source data, see Supplementary Figure 1. b, Representative

594 micrograph of the sample after drift correction and dose-weighing. c, Representative

595 2D class averages. 2D class averages exhibit different projections corresponding to

596 each orientation. d, 3D classification effectively separated two groups of populations.

597 On the left: TfR1-Tf complex with bound one PvRBP2b molecule. On the right:

598 TfR1-Tf complex with bound two PvRBP2b molecules. TfR1-Tf is shown in blue and

599 PvRBP2b in pink. Scale bar is displayed as labeled. e, Local resolution estimation

600 diagram of the final refined maps. On the left: One-ligand complex. On the right:

601 Two-ligand complex. Resolution keys are labeled from 3.5 to 6.0 Å. f, Resolution

602 estimation of the cryo-EM map. Fourier-shell-correlation (FSC) plot showing the

603 resolutions at 0.143 FSC (drawn in a dashed line) for both complexes determined by

604 gold-standard method. g, Representative EM density for different parts of C-terminal

605 domain of PvRBP2b. Some of the residues are also indicated. h, FSC curves of the

606 final refined model versus the final cryo-EM map (full dataset, blue), of the outcome

607   of model refinement with a half map versus the same map (red), and of the outcome

608   of model refinement with a half map versus the other half map (green). For panels a to

609   c, the experiment was performed once.

610   **Extended Data Figure 2**. Detailed structural analyses of PvRBP2b and sedimentation

611   velocity analysis of the formation of the ternary PvRBP2b$_{161-969}$-TfR1-Tf complex. a,

612   Superimposition of the cryo-EM based structure of PvRBP2b with the previously

613   reported crystal structure of the N-terminal domain (PDB accession number 5W53).

614   b, Two orthogonal views of PvRBP2b shown in surface representation and colored

615   according to the potential on the surface of the molecule. Electrostatic surface

616   potential was calculated using program APBS integrated in Chimera with the

617   nonlinear Poisson-Boltzmann equation and contoured at ±5 kT/e. Negatively and

618   positively charged surface areas are colored in red and blue, respectively. c,

619   Continuous sedimentation coefficient [c(s)] distributions for the TfR1-Tf complex

620   (2.5 μM complex) in the presence of increasing concentrations of PvRBP2b$_{161-969}$

621   (0.25-16 μM). The configuration and surface charge properties of the ternary complex

622   suggest that this particle may display non-ideal sedimentation, possibly contributing

623   to the shift in sedimentation coefficient observed. Thus, the integrated absorbance

624   signal in the fast sedimenting peak of the distributions was analysed as a function of

625   PvRBP2b$_{161-969}$ concentration. d, Here the TfR1-Tf complex was assumed to sediment

626   as a single, stable species. These data are consistent with the binding of two

627   molecules of PvRBP2b$_{161-969}$ to the TfR1-Tf complex with macroscopic dissociation

628   constants in the mid-nanomolar to low-micromolar range. e, Residuals for the best fit

629   of the raw radial absorbance sedimentation velocity data to a c(s) distribution model

630   for 2.5 μM of TfR1-Tf in the presence of (respectively from top to bottom): 0.25 μM,

631   0.5 μM, 1.0 μM, 2.0 μM, 4.0 μM, 8.0 μM and 16.0 μM of PvRBP2b$_{161-969}$.

632   **Extended Data Figure 3**. Structure of the one-ligand complex and conformational

633   changes induced in TfR1 upon PvRBP2b binding. a, Overall view of the one-ligand

634   complex. Proteins are shown in ribbon representation. Molecule of TfR1 interacting

635   with PvRBP2b is colored according to domain organization with protease-like domain

636   in red, apical domain in green and helical domain in yellow. The other molecule of

637   TfR1 that remains unliganded is colored in wheat. Tf is colored in cyan with ferric

638   ions Fe^{3+} shown as red spheres. The N- and C-terminal domains of PvRBP2b are

639   shown in violet and blue, respectively. b, Superimposition of the unliganded TfR1

640   molecule from one-ligand complex on the ligand-bound TfR1 from two-ligand

641   complex. The color-code as in a. The movement of the apical domain has been

642   schematically indicated by a black arrow. For clarity, both Tf molecules have been

643   omitted. c, Closer view of the superimposition shown in panel b. The residues that are

644   affected the most by PvRBP2b binding are shown as sticks and labeled. d, The

645   superimposition of TfR1 in complex with Tf and PvRBP2b (colored according to the

646   domain) and TfR1 in the complex with Tf only (in grey, PDB accession number

647   3S9L). The r.m.s.d. between 1,090 aligned atoms Cα is 0.81 Å. The movement of the

648   apical domain is schematically represented with black arrows. e, Superimposition of

649   Tf in cryo-EM structure of PvRBP2b-TfR1-Tf complex (in cyan) with the crystal

650   structure of holo-Tf in Tf-TfR1 complex (on the left, in wheat, PDB accession

651   number 3S9L) or with the crystal structure of holo-Tf (in the middle, in green, PDB

652   accession number 3V83) or with the structure of apo-Tf (on the right, in orange, PDB

653   accession number 2HAV). Iron ions are shown as red spheres. R.m.s.d. values are

654   indicated above each superimposition. f, Two orthogonal views of the

655   superimposition between two subclasses for two-ligand complex. Molecules are

656   shown in cartoon representation and colored in violet and cyan for subclass 1 and 2,

26

657   respectively. The most important difference between the two subclasses is the

658   movement of the N-terminal domain of PvRBP2b, which has been indicated with

659   black arrows. g, Close view of the PvRBP2b-Tf interaction site. The N2 subdomain of

660   Tf in the cryo-EM structure (in cyan) has been superimposed with the crystal

661   structures for holo-Tf in closed conformation (in wheat), holo-Tf in partially open

662   conformation (in green) and apo-Tf in open conformation (in orange). The movement

663   of the N1 subdomain has been schematically highlighted with the black arrows.

664   **Extended Data Figure 4**. Site-directed mutagenesis of PvRBP2b and TfR1. a, SDS-

665   PAGE gels of purified PvRBP2b mutant recombinant proteins. Two micrograms of

666   each protein were loaded onto a 4-12% NuPAGE gradient gel under reducing

667   conditions and stained with Coomassie Brilliant Blue. Molecular mass marker (M)

668   indicated in kDa. b, Circular dichroism spectra of recombinant PvRBP2b mutants. c,

669   Analytical SEC (left to right panel) of complex formation between PvRBP2b mutants

670   at residues Y538, Y542, K600 and Y604 with TfR1-Tf. The same representative

671   SDS-PAGE gels are used for wildtype PvRBP2b protein and for TfR1 ΔG217 mutant

672   protein SEC analyses. d, SDS-PAGE gel of purified TfR1 mutant recombinant

673   proteins. Two micrograms of each protein were loaded onto a 4-12% NuPAGE

674   gradient gel under reducing conditions and stained with Coomassie Brilliant Blue.

675   Molecular mass marker (M) indicated in kDa. e, Analytical SEC of TfR1 mutants in

676   complex with Tf. f, Circular dichroism spectra of recombinant TfR1 mutants. For all

677   panels, the experiments were performed once. For gel source data, see Supplementary

678   Figure 1.

679   **Extended Data Figure 5**. PvRBP2b interacts mostly with the residues specific for

680   human TfR1. a, Schematic representation of TfR1 sequence colored according to the

681   domain (apical in green, protease-like in red and helical in yellow). The limits of the

682   particular domains are indicated above the schematic. The most important residues

683   forming either hydrogen bonds or salt bridges with PvRBP2b are indicated below

684   with the black arrows and labeled. b, Comparison between Machupo virus GP1 and

685   PvRBP2b binding sites. Human TfR1 is shown in the center in surface representation

686   and colored according to the domain organization. The residues interacting with

687   PvRBP2b are colored in violet and those with Machupo virus GP1 in dark blue. The

688   residues overlapping between the two sites are colored in magenta. The surrounding

689   panels show fragments of the alignment between TfR1 sequences from different

690   species (human, mouse, rat, hamster, cat and dog). The amino acid residues are

691   colored according to their chemical properties (acidic residues shown in red, basic in

692   blue, polar in magenta, cysteines in yellow and hydrophobic in a scale of grey with

693   the intensity proportional to the residue hydrophobicity). The fragments of the

694   alignment corresponding to the apical and protease-like domains of TfR1 are

695   additionally shaded in green and red, respectively. The residues interacting with

696   PvRBP2b are indicated with stars above the alignment and colored according to the

697   type of the interaction (hydrogen bonds in green, salt bridges in red, other as empty

698   stars). The Machupo virus GP1 interaction site is indicated with a dark blue line. The

699   position of G217 deletion as well as the G142S polymorphism have been indicated

700   with black arrows above the alignment. c, Analytical SEC results showing

701   PvRBP2b$_{161-1454}$ interaction with mouse Tf and TfR1. For gel source data, see

702   Supplementary Figure 1. The elution profiles for the separate components as well as

703   for the mix between them are shown on the top. Bottom panels presents the SDS-

704   PAGE gels for the particular fractions. Left panel: human Tf and human TfR1, middle

705   left panel: mouse Tf and human TfR1, middle right panel: human Tf and mouse TfR1,

706 right panel: mouse Tf and mouse TfR1. The same representative SDS-PAGE gel is

707 used for PvRBP2b protein SEC analyses. All analytical SEC were performed once.

708 **Extended Data Figure 6**. Reticulocyte-binding and SAXS data analyses for the N-

709 terminal domain of PvRBP2b in complexes with four different inhibitory Fab

710 fragments. a, PvRBP2b$_{161-1454}$ binding in either the absence of mAbs or the presence

711 of anti-PvRBP2b mAbs (3E9, 4F7, 6H1, 8G7 and 10B12) or anti-PfRh4 mAb (10C9),

712 analysed by flow cytometry. Dot plots of PvRBP2b$_{161-1454}$ binding (y-axis) to

713 reticulocytes stained with thiazole orange (TO, x-axis). This experiment was repeated

714 independently four times with similar results. b, Arbitrarily offset scattering intensity

715 profiles for the PvRBP2b-Fab complexes. The background-subtracted SAXS data are

716 shown as black open circles representing natural logarithm of mean intensity lnI(q) as

717 a function of momentum transfer q in Å-1 (a.u. - arbitrary unit). The theoretical

718 scattering profiles (solid lines, PvRBP2b-3E9 in blue, PvRBP2b-4F7 in red,

719 PvRBP2b-6H1 in green and PvRBP2b-10B12 in yellow) calculated from the crystal

720 structures were fitted to the experimental scattering data using CRYSOL. c, Guinier

721 plots for qRg ≤ 1.3 showing that neither high-molecular-mass aggregates nor inter-

722 particle interference contributes measurably to scattering and the data are of high

723 quality (colors as in b). d, Pair-wise inter-atomic distance distribution function, P(r)

724 (colors as in b). e, Two orthogonal views of *ab initio* bead models represented as grey

725 spheres, superimposed with the crystal structure of the corresponding antibody

726 complexes. For PvRBP2b-10B12 complex, program COLLAGE was used to rigid

727 body fit two crystal structures, the N-terminal domain of PvRBP2b (PDB 5W53) and

728 the structure of the 10B12 Fab fragment alone. f, Table summarizing SAXS data

729 collection and analysis. The radius of gyration (Rg) and initial scattering intensity I(0)

730 were approximated using the Guinier equation with program PRIMUS. The Rg and

731   maximum particle dimension Dmax were calculated from the P(r) analysis using

732   program GNOM. The normalized spatial discrepancy parameter (NSD) indicates the

733   similarity between 20 independently generated *ab initio* models. The c parameter

734   provides the assessment of the fit of the average model to the experimental data

735   calculated using program CRYSOL. The structural parameters and their associated

736   errors are those derived by least-squares curve-fitting analysis of the scattering data as

737   formulated within the listed software packages.

738   **Extended Data Table 1.** Cryo-EM data collection, refinement and validation

739   statistics.

740   **Extended Data Table 2.** Summary of interactions between PvRBP2b and TfR1-Tf.

741   The distance measurements are based on molecules B, C and F in two-ligand complex

742   subclass 1.

743   **Extended Data Table 3**. Data collection and refinement statistics for PvRBP2b

744   complexes with Fab fragments. X-ray diffraction data were collected on single

745   crystals. [*]Values in parentheses are for highest-resolution shell.

746   **Extended Data Table 4.** Summary of interactions between PvRBP2b and

747   monoclonal antibodies 3E9, 4F7 and 6H1 Fab fragments. The distance measurements

748   are based on molecules A, B and C.

749

750

751

752

753

754

755

756

757

758

a

C

C

α8

α10

α7

α9

α5

α3

N

α6

α2

α4

α1

N ▭ C

168   633

b

~ 140 Å

~ 120 Å

~ 150 Å

TfR1

PvRBP2b

90°

Tf

Fe³⁺

c

C-terminal

apical   helical

protease
-like

N-terminal   stalk

membrane

TfR1
cytoplasmic domain

TfR1
TM domain

a

protease-like
apical
helical
PvRBP2b footprint
C-terminal
TfR1 footprint ~1,271 Å²
N-terminal
Tf
Tf footprint ~386 Å²

b

D531
E530
Y604
Y538
Y542
K600
E556
R359
K563
Y186
N428
K297
R304

c

α10
α9
D531
R208
E530
βII-1-βII-2 loop

K371
Y211
L212
βII-2
V213
K344
αII-2
α10
Y538
α9
Y542
S541
Y604

βII-1
βII-2
αII-2
K600
E214
E294
α10
α9

K574
αI-9
E578
E556
K563
α10
α9

α7
α10
E149
α5
R359
αI-2
αI-3

K297
α1-β2 loop
α3
V29
R304
S28
α7

β1-β2 loop
E265
Y186
N428
S32
α7
α12

d

TfR1 footprint
E497
N591
D558
K363
Q564
D366
G382
R242
K288
R217
K309
Tf footprint
90°

e

PvRBP2b residues involved in TfR1 and Tf binding

E556   K563
Y542   S586
S541   S590
Y538   K600
D531   D603
Y186   K297 R304 R359 K363   N428   E530   Y604
                                         N527

168   477   633

N-terminal   C-terminal

R217 R242 K288 K309 K363   G382   E497   D558   N591
                         D366   Q564

PvRBP2b polymorphic residues in field isolates

a

% reticulocyte binding

PvRBP2b mutants TfR1 site | PvRBP2b mutants Tf site

x-axis labels: WT, E530, D531, Y538, Y542, E556, K563, K600, Y604 | Y186, K297, R304, N428

b

A_{280} (mAU)

Legend: WT, E530, D531, Y538, Y542, E556, K563, K600, Y604, ΔG217

Labels (lower panel): K563, WT, E530, D531, Y538, E556, K600, Y604, Y542, ΔG217

c

A_{280} (mAU)

Legend: WT, Y186, K297, R304, N428, ΔG217

Labels (lower panel): WT, N428, R304, K297, Y186, ΔG217

d

A_{280} (mAU)

Legend: WT, S142G, E149, R208, Y211, E214, E294, N348, K574, E578, ΔG217

Labels (lower panel): N348, E578, S142G, WT, R208, E214, K574, E294, E149, Y211, ΔG217

e

PvRBP2b

E556 K563
Y538
D531
Y538
E530

Y186   K297 R304   N428   E556 K563   Y604

| 168 | N-terminal | 477 | C-terminal | 633 |

E149 ━━━━━ R359
Y211 ┅┅┅┅┅ Y542
E294 ━━━━━ K600

TfR1

| 120 | 188 | apical | 384 | protease-like | 606 | helical | 760 |

S142G   R208   E214   N348   K574 E578

a

% reticulocyte binding (relative to no mAb control)

no mAb · 3E9 · 4F7 · 6H1 · 8G7 · 10B12 · 10C9

b

3E9 light chain

3E9 heavy chain

4F7 heavy chain

4F7 light chain

6H1 heavy chain

6H1 light chain

10B12 Fab

c

PvRBP2b₁₆₈₋₆₃₃

90°

TfR1 footprint

Tf footprint

6H1 Fab

4F7 Fab

3E9 Fab

10B12 Fab

d

PvRBP2b

90°

TfR1

Tf

3E9 Fab

membrane

4F7 Fab

10B12 Fab

6H1 Fab