Structure of endogenous Pfs230:Pfs48/45 in complex with potent malaria transmission-blocking antibodies

Ezra T. Bekkering[1]*, Randy Yoo[2,3]*, Sophia Hailemariam[2,3]*, Fabian Heide[2], Danton Ivanochko[2], Matthew Jackman[2], Nicholas I. Proellochs[1], Rianne Stoter[1], Oscar T. Wanders[1], Renate C. van Daalen[1], Maartje R. Inklaar[1], Carolina M. Andrade[1], Pascal W.T.C. Jansen[4], Michiel Vermeulen[4,5], Teun Bousema[1], John L. Rubinstein[2,3,6], Taco W.A. Kooij[1], Matthijs M. Jore[1#], Jean-Philippe Julien[2,3,7#]

Affiliations

[1]Department of Medical Microbiology, Radboud University Medical Center, The Netherlands
[2]Program in Molecular Medicine, The Hospital for Sick Children Research Institute, Toronto, ON, Canada.
[3]Department of Biochemistry, University of Toronto, Toronto, ON, Canada.
[4]Department of Molecular Biology, Faculty of Science, Oncode Institute, Radboud University Nijmegen, Nijmegen, The Netherlands
[5]Division of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[6]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada
[7]Department of Immunology, University of Toronto, Toronto, Ontario, Canada

*These authors contributed equally.
#Corresponding authors.
matthijs.jore@radboudumc.nl
jean-philippe.julien@sickkids.ca

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Summary

The Pfs230:Pfs48/45 complex is essential for malaria parasites to infect mosquitoes and forms the basis for current leading transmission-blocking vaccine candidates, yet little is known about its molecular assembly. Here, we used cryogenic electron microscopy to elucidate the structure of the endogenous Pfs230:Pfs48/45 complex bound to six potent transmission-blocking antibodies. Pfs230 consists of multiple domain clusters rigidified by interactions mediated through insertion domains. Membrane-anchored Pfs48/45 forms a disc-like structure and interacts with a short C-terminal peptide on Pfs230 that is critical for Pfs230 membrane-retention *in vivo*. Analyses of Pfs48/45- and Pfs230-targeted antibodies identify conserved epitopes on the Pfs230:Pfs48/45 complex and provides a structural paradigm for complement-dependent activity of Pfs230-targeting antibodies. Altogether, the Pfs230:Pfs48/45 antibody-complex structure presented improves our understanding of malaria transmission biology and the mechanisms of action of transmission-blocking antibodies, informing the development of next-generation transmission-blocking interventions.

Introduction

Nearly half of the human population is at risk for malaria infection, making malaria one of the largest public health concerns (1). The parasite that is responsible for the majority of fatal human cases, *Plasmodium falciparum*, is efficiently transmitted by *Anopheles* mosquitoes throughout the population, with low parasite numbers being sufficient for mosquito infections (2) and studies reporting basic reproductive numbers R_0 over 100 (3). Human-to-mosquito transmission is mediated by mature male and female gametocytes that are taken up by an *Anopheles* mosquito during a bloodmeal (4). Inside the mosquito midgut, male and female gametocytes rapidly activate and egress from their red blood cells (RBCs) as micro- and macrogametes, respectively. Microgametes fuse with macrogametes creating a zygote that forms the basis for further parasite development in the mosquito, eventually resulting in an infectious mosquito that can further spread the parasite and its related disease (5).

Two sexual-stage surface proteins essential for *Plasmodium* transmission, Pfs230 (6) and Pfs48/45 (7), are expressed in both gametocytes and gametes. Pfs48/45 and Pfs230 knockout (KO) parasites showed markedly lower oocyst formation rates, demonstrating that these proteins play a critical role in establishing mosquito infection (8, 9)**.** Based on rodent models, it has been hypothesized that these proteins are only essential for male fertility (9, 10), but this remains to be demonstrated for *P. falciparum*. Pfs48/45 is predicted to have a GPI-anchor and localizes together with Pfs230 to the parasite plasma membrane (PPM) (11). Pfs230, without a recognizable GPI anchor or transmembrane motif, is thought to associate with the PPM by forming a stable heterodimeric complex with Pfs48/45, as shown in co-immunoprecipitation studies (12, 13). This is further supported by the observation that Pfs48/45 KO parasites lose Pfs230 surface retention (8). Both proteins are part of the 6-Cysteine (6-Cys) protein family, characterized by the presence of 6-Cys domains: Immunoglobulin-like folds that contain up to six cysteines that can form disulfide bonds (14, 15). Pfs230 contains fourteen 6-Cys domains, whereas Pfs48/45 contains three (**Figure 1A**). Structural information on the full Pfs230:Pfs48/45 structure has been limited; two full-length structures of recombinant Pfs48/45 have been reported, adopting two distinct conformations – a disc-like (16) and an extended (17) conformation – whereas the structural characterization of Pfs230 has been limited to just the first two domains of the protein (18-22). Additionally, there is a lack of structural understanding of how Pfs230 and Pfs48/45 interact with one another.

The human-to-mosquito transmission stages form a developmental bottleneck for the malaria parasite and are therefore an attractive target for interventions seeking malaria elimination (23), such as transmission-blocking vaccines (TBVs). Indeed, modeling studies have suggested that TBVs could synergistically increase the effectiveness of vaccines targeting other parasite stages (24-26). Importantly, both Pfs230 and Pfs48/45 were recognized in the early 1980s as the targets for antibodies that had potent human-to-mosquito transmission-reducing activity (TRA) (11, 13, 27). Since then, multiple studies have shown that naturally infected individuals can acquire Pfs230 and Pfs48/45-targeted antibodies that are associated with high-level TRA in serum (*e.g.* (28-33)) and these antibodies can block transmission when purified (28). The structure, epitopes, and TRA of these naturally acquired antibodies have been partially characterized at the monoclonal antibody level (17, 22, 34). Domain 1 of Pfs230 (Pfs230D1) and domain 3 of Pfs48/45 (Pfs48/45D3) are the target of the most potent monoclonal antibodies (mAbs) with TRA that can potently block human-to-mosquito transmission (*e.g.* (17, 19, 21, 22, 34-37)). Initial reports suggested that other domains of Pfs230 and Pfs48/45 were not able to elicit comparably potent transmission-blocking antibodies (37). Recently at least three other Pfs230 domains, and Pfs48/45D1, have been identified as targets for potent transmission-blocking antibodies, but structural insight into these epitopes is lacking in comparison to Pfs230D1 and Pfs48/45D3 (34, 38-41).

90
91 Regardless of the domain they target, the vast majority of the potent α-Pfs230 antibodies is complement-
92 dependent (19, 22, 38, 42), although some antibodies retain TRA at very high concentrations in the
93 absence of complement (18, 36). In contrast, α-Pfs48/45 antibodies can be neutralizing at low
94 concentrations in a complement-independent manner (17, 20, 34, 35, 40, 43). The rationale behind what
95 drives antibody potency for both α-Pfs230 and α-Pfs48/45 antibodies remains unclear. As both proteins
96 are capable of eliciting highly potent antibodies, they have been targeted as lead antigens and are
97 currently the most advanced and promising TBVs in development (44, 45). Indeed, immunogens based off
98 these two proteins have been extensively evaluated in preclinical and phase I/II clinical trials (46-49).
99 However, these vaccines did not block transmission completely and it remains unclear whether they can
100 induce long-lasting immunity. Structure-function relationships of how potent antibodies target the
101 Pfs230:Pfs48 complex would not only enhance our understanding of *P. falciparum* transmission biology
102 and antibody mechanism of actions, but also aid in the design and development of next-generation
103 malaria vaccines more fully capable of blocking transmission to reach elimination goals.
104
105 Here, we report the molecular structure of the endogenous Pfs230:Pfs48/45 heterodimer in complex with
106 six antibodies with potent TRA as solved by cryogenic electron microscopy (cryo-EM). Cumulatively, our
107 data provide critical molecular insights into the Pfs230:Pfs48/45 heterodimer complex, moving towards
108 deepening our understanding of *P. falciparum* transmission biology and opportunities to block this
109 process through next-generation biomedical interventions.
110

Results

112
113 **Elucidation of the endogenous Pfs230:Pfs48/45 complex structure**
114
115 We set out to determine the cryo-EM structure of the Pfs230:Pfs48/45 complex by purifying it from
116 mature *P. falciparum* gametocytes. Using the inducible gametocyte producing parasite line NF54/iGP2
117 (50), we fused *pfs230* (Pf3D7_0209000) C-terminally with a 3xFLAG-C-tag coding sequence, yielding
118 parasite line iGP2$^{230-tag}$ (**Figure S1A-D**). iGP2$^{230-tag}$ gametocytes showed normal Pfs230 and Pfs48/45
119 localization and retained normal *in vitro* exflagellation and mosquito infectivity in standard membrane
120 feeding assays (SMFA; **Figure S1E-F**). The Pfs230:Pfs48/45 complex was purified using anti-FLAG resin.
121 Pfs230 and Pfs48/45 were the highest enriched proteins from this purification as detected by LC-MS/MS
122 (**Figure 1B-D**). Two high-molecular weight bands were observed in Blue-Native Polyacrylamide Gel
123 Electrophoresis (BN-PAGE). Both bands were stained by α-Pfs230 antibodies in immunoblotting assays,
124 but only one band was stained by α-Pfs48/45 antibodies, attributing these two bands to the
125 Pfs230:Pfs48/45 complex and Pfs230 alone, respectively (**Figure 1E-F**). BN-PAGE analysis of eluted protein
126 incubated with a molar excess of different fragment of antigen-binding (Fab) fragments of transmission-
127 blocking antibodies, targeting a range of conformational epitopes on Pfs48/45 and Pfs230, confirmed the
128 native structure of the eluted proteins (**Figure S1G**). While α-Pfs230 Fabs shifted both protein species
129 upwards, α-Pfs48/45 Fabs only affected the upper band, further ascertaining the top band as the
130 Pfs230:Pfs48/45 complex.
131
132 We posited that Fabs could be used as fiducial markers to help distinguish in a cryo-EM structure the
133 seventeen highly similar 6-Cys domains found in the Pfs230:Pfs48/45 complex. As such, we obtained a
134 size exclusion chromatography (SEC)-purified Pfs230:Pfs48/45 heterodimer in complex with six Fab
135 fragments, using the structurally delineated RUPA-97 (22), LMIV230-01 (21), and RUPA-44 (34), and the
136 yet-to-be-structurally delineated 2A2 (40, 42, 51), 18F25 (38, 51), and RUPA-71 (17, 34) inhibitory

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137 antibodies (**Figure 1G, Figure S1H**). Cryo-EM data of this Pfs230:Pfs48/45:6Fab complex was collected at
138 0°, 35°, and 40° tilt. 2D class averages from this dataset clearly revealed the presence of all components
139 of the complex: Pfs230 bound to four Fabs (LMIV230-01, RUPA-97, 2A2, 18F25) in complex with Pfs48/45
140 bound to two Fabs (RUPA-44 and RUPA-71) (**Figure 1H**).

142 The highest resolution structural information was derived by generating four locally refined maps
143 corresponding to more rigid components of the complex: Pfs230D1-6 bound to RUPA-97, LMIV230-01,
144 and 2A2 at a global resolution of 3.6 Å; Pfs230D7-8 bound to 18F25 at a global resolution of 3.8 Å;
145 Pfs230D9-14 bound to Pfs48/45 at a global resolution of 4.7 Å; and Pfs230D13-14 bound to Pfs48/45,
146 RUPA-44, and RUPA-71 at a global resolution of 3.5 Å (**Figure 1H, Figures S2-3, Table 1**).

148 **Insertion domains are responsible for Pfs230 domain organization**

150 Molecular models were built into each of the locally refined maps to elucidate structural features of the
151 interdomain topologies and dispositions of potent mAbs. The models derived from the maps of Pfs230D1-
152 6:RUPA-97:LMIV230-01:2A2 and Pfs230D7-8:18F25 (**Figure 2A, Table 1**) were initialized from
153 experimentally determined crystal structures and predicted AlphaFold (AF) models, which were refined
154 into maps with sufficiently high resolution to model sidechain positions (**Table 1, Table S3**). For the 4.7 Å
155 resolution map of Pfs230D9-14:Pfs48/45, we report the backbone positions of a compositely refined
156 Pfs230D9-12 structure in the presence of the Pfs230D13-14:Pfs48/45 structure decerned from the 3.5 Å
157 resolution map (**Figure 2A, Table 1**). AF models of Pfs230D9-D12 were initially docked and manually
158 rearranged into the Pfs230D9-14:Pfs48/45 map. A template-guided inference strategy in AF2 followed by
159 refinement using map-constrained energy minimizations resulted in the final model. We attribute the
160 lower local resolution of Pfs230D9-12 in our Pfs48/45:Pfs230D9-14 map to intrinsic flexibility between the
161 D12 and D13 domains (**Figure S3**).

163 Each tandem structural unit of Pfs230 consists of A- and B-type 6-Cys domains (**Figure S4A**). Most A-type
164 domains feature a 5-on-5 β-sandwich whereas B-type domains feature a 4-on-5 β-sandwich fold (**Figure**
165 **S4B**). A key differentiating feature of these folds is the way in which the first two β-strands are arranged.
166 In the A-type folds (i.e. Pfs230 D1, D3, D5, D7, D9, D11, and D13), the first canonical β1 strand is split
167 between the two β-sheets (β1 and β1') of the 6-Cys β-sandwich fold before transitioning into the β2
168 strand. In contrast, in the B-type folds (i.e. Pfs230 D2, D4, D6, D8, D10, D12 and D14), the first two β-
169 strands (β1 and β2) are part of the same β-sheet (**Figure S4B**).

171 While small deviations from the canonical 6-Cys domains exist, primarily near the perpendicular β-hairpin
172 (**Figure 2B-C, Figure S4C-D**), most of the structural diversity that distinguishes the different Pfs230 6-Cys
173 domains lie in long sequence insertions in loop regions between the β-strands. These regions were
174 previously coined as insertion domains (IDs) (52, 53) and are present in Pfs230D3, D6, D7, D10 and D11
175 (**Figure 2B**). Many of these IDs (i.e. Pfs230D3, D6, D10, and D11-ID2) contribute to interdomain contacts
176 (**Figure 2C-G**), while others (i.e. Pfs230D11-ID1 and D7) were not observed to interact with other domains
177 (**Figure 2G-H**). The presence of these IDs mediates the formation of domain clusters in Pfs230 (**Figure 2I-**
178 **J**).

180 The Pfs230D6-ID is the most extensive insertion domain (**Figure 2D**) (res. 1538-1670) and forms structural
181 elements that interface with a β-hairpin in Pfs230D5 (res. 1371-1388) and interacts with the Pfs230D3-ID
182 (res. 983-1067) and the Pfs230D2-3 linker (res. 983-1067) (**Figure 2I**). A portion of this structural fold
183 localizes near Pfs230D3 and these elements altogether pack against the base of Pfs230D2, holding the
184 Pfs230D1-D6 domain cluster together. Similarly, the Pfs230D10-ID (res. 2265-2319) localizes to the same

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185 region as Pfs230D11-ID2 (res. 2491-2566) near Pfs230D9. Here, the Pfs230D11-ID2 forms a β-hairpin that
186 interacts with a β-sheet of Pfs230D9 – effectively forming an extended β-sheet consisting of two different
187 domains (**Figure 2J**). The resolution of the map from which this part of the model was derived is low (4.7
188 Å), especially in the Pfs230 section of the map, making the modeling of Pfs230D11-ID2 more challenging
189 (**Figure S3D**). Nonetheless, the portion of the ID that interacts with Pfs230D9 (res: 2514-2525) is
190 consistently predicted to interact with Pfs230D9 across multiple AF runs and density accounting for this
191 region is present in our map with a local resolution of approximately 6-7 Å (**Figure S4E**). This in conjunction
192 with the integrative modeling approach utilized provides confidence in the backbone positions of these
193 interactions. In summary, Pfs230 consists of four domain clusters (e.g. Pfs230D1-6, D7-8, D9-12, and D13-
194 14) that are coordinated closely by IDs, with evident flexibility between the four domain clusters.
195
196 **Pfs230 interacts with Pfs48/45 through its C-terminus**
197
198 The interaction site between Pfs48/45 and Pfs230 was delineated at 3.4 Å resolution (**Figure 3A, Figure
199 S3C, Table 1**). Our structure indicates that Pfs230 engages with all three domains of Pfs48/45 through its
200 terminal 6-Cys domains, Pfs230D13 (296 Å^2) and Pfs230D14 (1,376 Å^2) (**Figure 3B**). Pfs48/45 adopts a disc-
201 like conformation in our structure with Pfs230, with Pfs48/45D1 (1057 Å^2) and Pfs48/45D3 (478 Å^2)
202 accounting for the majority of contacts with Pfs230, while Pfs48/45D2 (112 Å^2) contributes more modestly
203 (**Figure 3B, Figure S5A, Table S2**). Pfs230 binds to Pfs48/45 on the opposite face of the disc conformation
204 compared to the C-terminus of Pfs48/45, where the GPI linker is located for attachment to the PPM
205 (**Figure 3B**). Previously, two different conformations of Pfs48/45 have been observed in the absence of
206 Pfs230 binding, a compact, disc-like structure solved using X-ray crystallography (16) and an elongated
207 Pfs48/45 structure determined by cryo-EM (17). A comparison of these models with our Pfs230-bound
208 Pfs48/45 structure indicates very similar arrangements of Pfs48/45D1-2 (RSMD = 0.92-1.08 Å) (**Figure
209 S5B**). However, the position of Pfs48/45D3 relative to Pfs48/45D1-2 varies considerably more. There is
210 almost no overlap between Pfs48/45D3 from Pfs230-bound Pfs48/45 and the elongated Pfs48/45
211 structure while modest positional differences are observed between Pfs48/45D3 of the two disc-like
212 Pfs48/45 structures (RMSD = 4.12 Å; **Figure S5B**).
213
214 The C-terminus of Pfs230 (Pfs230Cterm; res. 3108-3118) – a sequence extending out of the canonical 6-
215 Cys domain – is integral for mediating the Pfs230:Pfs48/45 interaction (**Figure 3C**). These residues occupy
216 a groove of the Pfs48/45 disc and form extensive interactions with loop 45-49 and strand 65-76 of
217 Pfs48/45D1, loop 246-250 of Pfs48/45D2, and loop 377-380 of Pfs48/45D3 (**Figure 3B**). While accounting
218 for fewer interactions than Pfs230D14, Pfs230D13 residues 2866-2871 and the intervening loop between
219 Pfs230D13 and Pfs230D14 (res. 2977-2984) support complexation by engaging with a portion of a
220 Pfs48/45D3 extended loop (res. 348-371) (**Figure 3D**). Additionally, the Pfs48/45 N-terminus wraps around
221 Pfs48/45D1-2 and through residues 41-50 forms interactions with loops 2993-2998 and 3108-3114 and
222 strand 3007-3014 of Pfs230D14 (**Figure 3B**). While the N-terminal region of Pfs48/45 remained largely
223 unresolved in previous structural studies of Pfs48/45 without Pfs230 implying flexibility (16, 17), this
224 region is well-resolved in the Pfs230-bound structure and reveals that unlike other A-type domains,
225 Pfs48/45D1 features a different β1 orientation and connectivity (**Figure S5C**), localizing to the interface.
226 An analysis of single nucleotide polymorphisms (SNP) across *P. falciparum* revealed that the interface
227 between Pfs48/45 and Pfs230 is highly conserved (**Figure S5A and S5D**).
228
229 Corroborating these structural findings, a recombinantly produced protein construct spanning Pfs230D13-
230 14-Cterm bound to female gametes on which Pfs48/45 is anchored, while similarly produced constructs
231 spanning either Pfs230D1 or Pfs230D10 could not (**Figure S6A**). To further confirm the interaction
232 between Pfs230D13-14-Cterm and Pfs48/45, two transgenic parasite lines carrying a truncated *pfs230*

6

gene were created (**Figure S6B-E**). Pfs230$^{\Delta D13/14}$ parasites express Pfs230 up until Pfs230D12, while Pfs230 expressed in Pfs230$^{\Delta Cterm}$ gametocytes only lacks the last 29 C-terminal residues (res. 3107 – 3135) seen as central to the Pfs230:Pfs48/45 interface in our cryo-EM structure. Wildtype Pfs230 is expected to localize on the outside of the PPM, facing into the parasitophorous vacuole, and is expected to co-localize with PPM-bound Pfs48/45. Western blot analysis shows that late-stage Pfs230$^{\Delta D13/14}$ and Pfs230$^{\Delta Cterm}$ gametocytes still contain (truncated) Pfs230. However, saponin-treated parasites with a disrupted parasitophorous vacuole membrane (PVM) lose all Pfs230, demonstrating that Pfs230 is no longer retained on the PPM (**Figure S6F-G**). In fixed immunofluorescence microscopy, full-length and truncated Pfs230 proteins localized in close proximity to Pfs48/45, suggesting that Pfs230 protein trafficking is unaffected in both mutant parasite lines (**Figure S6H**). Interestingly, both truncated Pfs230 proteins seemed to localize slightly more towards the outside of the cell compared to the PPM-tethered full-length Pfs230, (**Figure S6H**). When the PVM was naturally disrupted upon activation of macrogametes (**Figure 3E, Figure S6I**) or chemically disrupted in saponin-treated gametocytes (**Figure 3F**), Pfs230 remained bound on the PPM surface in wildtype parasites in gamete binding and suspension immunofluorescence microscopy assays. In contrast, both Pfs230$^{\Delta D13/14}$ and Pfs230$^{\Delta Cterm}$ gametocytes and gametes lacked surface-bound Pfs230 in these experiments (**Figure 3E-F, Figure S6I**). The α-Pfs48/45 signal was found to be slightly higher for Pfs230-truncated parasites in both microscopy and flow cytometry experiments. Together, our structural and *in vivo* experiments demonstrate that the C-terminal region of Pfs230 is essential for the formation of the Pfs230:Pfs48/45 complex.

Structural characterization of potent Pfs48/45D1-targeting antibody RUPA-71

The X-ray crystal structure of unliganded RUPA-71 was solved at 2.3 Å resolution (**Table S3**) to use as a high-resolution model to fit and refine into the 3.4 Å resolution Pfs230D13-14:Pfs48/45:RUPA-44:RUPA-71 cryo-EM map (**Figure 4A-B, Table 1**). RUPA-71 predominantly interacts with strand 126-135 of Pfs48/45D1, with additional contacts on Pfs48/45D1 loop 88-90 (**Figure 4A-B, Table S4**). This is consistent with the previous epitope mapping derived from HDX-MS (17). Heavy chain Complementarity-Determining Region 1 (HCDR1; 108 Å^2), HCDR2 (66 Å^2), HCDR3 (472 Å^2), and Kappa chain CDR1 (KCDR1; 52 Å^2) of RUPA-71 all contact Pfs48/45D1, with the extended HCDR3 (20 aa) contributing most key contacts (**Figure 4A, Table S4**). The epitope of RUPA-71 is highly charged with several negative and positive patches. RUPA-71 binds to these regions through salt bridges formed between RUPA-71 KCDR1 residue K^{32} and HCDR1-3 residues D^{31} and R^{98} and E_{129}, K_{89}, K_{101}, E_{130}, and D_{132} of Pfs48/45D1 (**Figure 4A, Table S4**). Noticeably, the HCDR3 of RUPA-71 forms a β-hairpin that forms a β-sheet-like structure with two β-strands of Pfs48/45D1 (res. 126-143) (**Figure 4B**). In addition to intra-chain hydrogen bonds, this β-sheet is held together by five backbone hydrogen bonds between RUPA-71 G^{96}, R^{98}, and Y^{100} and D_{132}, E_{130}, and I_{128} of Pfs48/45D1 (**Figure 4B, Table S4**). Sequence analysis across parasite field isolates revealed only five rare SNPs within the RUPA-71 epitope: K33T (0.015%), K33N (0.007%), S90R (0.007%), K101T (0.029%), and S119T (0.007%), making this epitope highly conserved (**Figure S7A**) (40). When compared to RUPA-58 which is the only other D1 antibody that has been structurally characterized to date, RUPA-71 binds to a largely separate epitope, although with some overlap in residue contacts (K_{88}, K_{89}, S_{90}, K_{101}, D_{132}, and R_{140}) that would result in steric clashes (**Figure S7B**) (17)**.** RUPA-58 and RUPA-71, as well as other antibodies that compete with them, require low concentrations to inhibit in the SMFA (IC$_{80}$ < 10 μg/ml), indicating that mAbs that bind to this region of Pfs48/45D1 (res. 86-101 and 126-139) are potently inhibitory (17).

Pfs48/45 epitope accessibility in the Pfs230:Pfs48/45 complex

Next, we investigated how the Pfs230 interaction with Pfs48/45 might affect the accessibility of Pfs48/45-targeting antibodies. An overlay of structures of previously characterized antibodies with the Pfs230-

281 bound structure revealed that Pfs48/45D1, Pfs48/45D2 and Pfs48/45D3-1b epitope antibodies approach
282 from the side of the Pfs48/45 disc and are accessible when Pfs230 is bound (**Figure 4C-E**). In contrast,
283 many antibodies that target the Pfs48/45D3-1a epitope, including highly potent antibodies TB31F and
284 RUPA-29, would have considerable clashes with both Pfs230D13 and Pfs230D14 in this conformation
285 (**Figure 4F-G**) (17).
286
287 To determine whether these antibodies can bind to Pfs48/45 in the context of the Pfs230:Pfs48/45
288 complex, we used the BN-PAGE-based mobility shift assay to study the changes in migratory pattern of
289 Fab-bound endogenous Pfs230:Pfs48/45 complex. To this end, the purified complex was incubated with
290 tenfold molar excess Fab derived from a range of both potent and non-potent TRA antibodies, spanning
291 almost all currently known epitopes on the complex (**Figure 1A**). As expected, α-Pfs230 Fabs shifted both
292 bands upwards, with the exception of 15C5, which targets epitope III on Pfs230D1 that is shielded by
293 Pfs230D2 and thus incompatible with binding full-length Pfs230 (**Figure 4H**) (19, 22). None of the other
294 tested α-Pfs230 Fabs, targeting epitopes on Pfs230D1, Pfs230D4 and Pfs230D7, disrupted the
295 Pfs230:Pfs48/45 complex, in agreement with the relative orientation of Pfs230 on Pfs48/45 in our
296 structure. The α-Pfs48/45 Fabs did not shift the lower free Pfs230 band, but did affect migration of the
297 upper band. While some Fabs i) bound to the intact complex (*e.g.* RUPA-44, RUPA-117), others ii)
298 disrupted the Pfs230:Pfs48/45 complex (*e.g.* RUPA-58 and RUPA-50), or iii) showed a heterogeneous
299 effect resulting in Fab-bound and unbound Pfs230:Pfs48/45, as well as disrupted protein complex (*e.g.*
300 TB31F, RUPA-47) (**Figure 4H**). The non-potent 10D8, targeting Pfs48/45-D2, could not bind to the
301 Pfs230:Pfs48/45 complex. To confirm the observation that some but not all Pfs48/45-targeted antibodies
302 could disrupt the Pfs230:Pfs48/45 complex, we tested whether a subset of these Fabs could displace
303 Pfs230 on the female gamete surface in a competition assay. In agreement with the BN-PAGE results,
304 RUPA-50, RUPA-57, and RUPA-58 significantly decreased Pfs230 on the gamete surface in a dose-
305 dependent manner, while *e.g.* TB31F, RUPA-46, and RUPA-89 Fabs showed no displacement of Pfs230
306 (**Figure S7C**).
307
308 Intriguingly, TB31F could still partially bind to the Pfs230:Pfs48/45 complex in BN-PAGE (**Figure 4H**) and
309 did not displace Pfs230 on the gamete surface (**Figure S7C**), despite showing significant steric clashes in
310 our Pfs230-bound Pfs48/45 structure. In this context, it is interesting to note again that it has previously
311 been described how Pfs48/45 can adopt a disc-like conformation (similar to the structure reported here
312 in complex with Pfs230), but also more extended conformations where Pfs48/45D3 can reorient from the
313 rest of the protein (17). Given that Pfs48/45D1-2 contributes 1169 $Å^2$ of a total 1647 $Å^2$ buried surface
314 area within the Pfs48/45-Pfs230 interface, it is conceivable that Pfs48/45D1-2 is sufficient for Pfs230
315 binding, allowing for Pfs48/45D3 to retain a range of motion in the complex. A superimposition of
316 Pfs48/45D3 antibodies onto the previously solved extended conformation of Pfs48/45 with Pfs230D13-
317 14 aligned to its Pfs48/45D1-2 binding site demonstrates that Pfs48/45D3-1a epitope would be accessible
318 in this arrangement (**Figure 4G**). This elongated conformation can also accommodate binding for most
319 anti-Pfs48/45 antibodies that target epitope 1b or Pfs48/45D1-2 (**Figure S7D**). Together, this could
320 indicate that Pfs230-bound Pfs48/45 retains flexibility, and that this mobility may allow for the binding of
321 antibodies that would otherwise be blocked in the Pfs230-bound disc-like conformation of Pfs48/45.
322
323 Interestingly, the ability of Fab fragments to induce partial disruption of the Pfs230:Pfs48/45 complex
324 does not seem to be associated with TRA of the corresponding antibody in SMFAs (**Figure 4H, Figure S7C,**
325 **Table S1**). This is illustrated by Pfs48/45D3-1a targeting antibodies RUPA-57 (IC_{80} > 50 μg/ml) and RUPA-
326 50 (IC_{80} < 2 μg/ml) that are both able to fully dissociate the complex *in vitro* and partially *in vivo*, but have
327 very different potencies. Also, the very potent TB31F (IC_{80} < 2 μg/ml) and the non-potent RUPA-89 (IC_{80}
328 >100 μg/ml) show only partial dissociation *in vitro* and no displacement of Pfs230 *in vivo*, but again have

8

329   very different potencies. Together, these data strongly suggest that Pfs230:Pfs48/45 complex disruption
330   is not a generalizable mode-of-action for functional α-Pfs48/45 and α-Pfs230 antibodies.
331
332   **Structural delineation of potent Pfs230D4 and D7 epitopes**
333
334   Our cryo-EM studies reveal potent epitopes outside of Pfs230D1 that had not been previously structurally
335   elucidated. Fab 2A2 could be resolved in the Pfs230D1-6 map, while Fab 18F25 could be resolved in the
336   Pfs230D7-8 map (**Figure 2A, Table 1**). X-ray crystal structures of unliganded 2A2 and 18F25 Fabs were
337   solved to 1.2 Å and 1.6 Å resolution (**Table S3**), respectively and docked and refined into the maps. Our
338   cryo-EM structure reveals that 2A2 targets Pfs230D4 (**Figure 5A**), confirming the epitope location
339   previously proposed based on the differential binding and TRA of mAb 2A2 against *P. falciparum* strains
340   that contain SNPs in Pfs230D4 (40). 2A2 buries 824 $Å^2$ on Pfs230D4, primarily making contacts through
341   the HCDR1/3 and KCDR2/3 loops as well as the N-terminus of the heavy chain (**Table S4**). Most residues
342   contacted by the antibody lie in the canonical perpendicular β-hairpin of Pfs230D4, and a moderate
343   number in the loop region between β9 and β10 (**Figure 5A, Table S4**). A subset (H1159D, Y1194S, Q1196E,
344   N1209Y, Q1250K) of the SNPs found in the strains of *P. falciparum* against which 2A2 had diminished TRA
345   localize to the epitope (**Figure 5B**) (45). In addition, analysis of the MalariaGEN Pf7 database reveals that
346   more SNPs differing from the 3D7 strain are found in the epitope (**Figure 5B**) (54). Some of the SNPs are
347   quite rare with frequencies less than 1% (Q1255E, K1259I, K1261N, Q1196E, E1198V, L1208F, and
348   N1209Y), while others are seemingly more common (Q1250K – 86.3%, Y1194S – 59.3%, and H1159D –
349   53.3%).
350
351   The epitope of mAb 18F25 localizes to Pfs230D7 (**Figure 5C**) (38). 18F25 buries 515 $Å^2$ on Pfs230D7
352   through all CDRs of the antibody (**Table S4**). Our structure reveals that 18F25 primarily engages with the
353   Pfs230D7-ID (**Figure 2H and 2K**) found in between β4 and β5, with a small amount of interactions localized
354   to the β8-β9 loop (**Figure 5C, Table S4**). Unlike the 2A2 epitope and Pfs230D4 overall, both the 18F25
355   epitope and Pfs230D7 are more conserved (**Figure 5D**). Only two SNPs in Pfs230D7 identified from the
356   MalariaGEN Pf7 database have frequencies higher than 10% (Y1829N – 69.4% and I1870V – 13.5%) and
357   these are not contained within the 18F25 epitope (54). Only two residues within the epitope are reported
358   with mutations in the database. V1801I, V1801L and V1801A, are conservative mutations that have a
359   combined frequency of 5.8% (5.7%, 0.01%, and 0.09%, respectively). The relatively conservative D1803N
360   mutation has only been observed in 0.01% of strains. Together, elucidation of the 18F25 and 2A2 epitopes
361   on Pfs230D4 and D7 provide much-needed structural insights into Pfs230-directed antibody response
362   outside of Pfs230D1.
363
364   **Potent anti-Pfs230 antibodies target membrane distal epitopes**
365
366   mAb 2A2, while showing strain-dependent TRA, was highly potent in SMFAs utilizing the NF54 strain (IC_{80}
367   = 1.9 μg/ml (40)). The potency was on par with the highly potent RUPA-97 that targets Pfs230D1 (IC_{80} =
368   0.7 μg/mL (22)), suggesting that a common feature of these mAbs might be responsible for the high
369   potency. These two mAbs, despite binding different domains, bind to the same side of Pfs230 with a
370   similar angle of approach (**Figure 5E**). mAb 18F25 (IC_{80} < 30 μg/ml (38)) also binds to the same face of
371   Pfs230, while differing slightly in its angle of approach (**Figure 5E**). Interestingly, Pfs230D1-directed mAbs
372   of lower potency, such as RUPA-38 (IC_{80} > 100 μg/mL) (22), bind to the opposite face of where RUPA-97,

9

373 2A2, and 18F25 bind to Pfs230 (**Figure 5E**). This trend is generally applicable to α-Pfs230D1 mAbs
374 published to date (**Figure S8**) (19).
375
376 These Pfs230-targeting mAbs have been demonstrated to be complement-dependent for their TRA (22,
377 38-40, 42). Previously, it has been shown that for efficient C1q deposition on an antigenic surface, the first
378 step in the classical complement activation pathway, IgGs need to form into ordered hexameric antibody
379 structures (55, 56). Intriguingly, the epitopes of potent mAbs RUPA-97, 2A2, and 18F25 are localized to
380 the opposite face of where Pfs48/45 and presumably the PPM would be (**Figure 5E**). In contrast, the
381 epitope of non-potent RUPA-38 is localized near the presumed location of the PPM (**Figure 5E**). This
382 correlation led us to hypothesize that the angle of approach of the antibody is a determinant of Pfs230-
383 directed antibody potency, as antibodies that are near the PPM might be prevented from efficient
384 recruitment of complement components and thus complement activation (**Figure 5E**). To test this
385 hypothesis, we selected five different mAbs targeting Pfs230D1 with different potencies (RUPA-97 (IC_{80} =
386 0.7 µg/mL (22)), rh4F12 (IC_{80} = 55 µg/mL (22)), LMIV230-01 (IC_{80} = 48 µg/mL (22)), RUPA-38 (IC_{80} > 100
387 µg/mL) (22)), and LMIV230-02 (IC_{80} > 1,000 µg/mL (21)), expressed these as IgGs of the same complement-
388 fixing subclass, and tested antibody binding, C1q recruitment and C3 deposition on live female NF54
389 gametes. All antibodies except LMIV230-02 bound gametes with similar intensity (**Figure 5F**), which is in
390 agreement with previously determined *in vitro* affinities (22). In contrast, the highly potent antibody
391 RUPA-97 and moderately potent antibodies rh4F12 and LMIV230-01 were able to recruit C1q and deposit
392 C3 on the surface, while the non-potent RUPA-38 was not able to do so efficiently (**Figure 5G-H**). These
393 findings, as implied by the Pfs230:Pfs48/45 complex structure, suggest that the angle of approach relative
394 to the PPM might be a critical determinant for potency in complement-dependent Pfs230-directed
395 antibodies.
396

Discussion

398
399 Targeting human-to-mosquito transmission, a developmental bottleneck in the *Plasmodium* life cycle, is
400 a promising intervention strategy for malaria control and elimination. Here, we reveal the structural
401 disposition of the heterodimer complex Pfs230:Pfs48/45, which is essential for *P. falciparum* transmission
402 and a major target for malaria transmission-blocking vaccine development. Using advances in molecular
403 parasitology and cryo-EM, we were able to circumvent recombinant expression by purifying the
404 approximately 410 kDa large, membrane-bound Pfs230:Pfs48/45 complex from its native source, late-
405 stage *P. falciparum* gametocytes. Previously, similar methods have been used to elucidate the structure
406 of the *Plasmodium* Translocon of Exported Proteins (PTEX) complex (57) and the soluble RhopH complex
407 (58, 59), both crucial protein complexes within the *Plasmodium* life cycle non-amenable to standard
408 recombinant protein production. Using genome engineering to enable the purification of endogenous
409 proteins for cryo-EM studies has also found traction in research on other organisms, including human cells
410 (60), and bacterial pathogens like *Pseudomonas spp.* (61) and *Mycobacteria spp.* (62). This highlights the
411 opportunities that this approach, combined with recent advances in the field of structural biology
412 including bottom-up structural proteomic platforms such as cryoID (63) and protein folding prediction
413 software such as AlphaFold 3 (64), may offer to the many other *Plasmodium* proteins refractory to
414 recombinant expression.
415
416 Our structure reveals how insertion domains present within Pfs230 6-Cys domains mediate inter-domain
417 interactions. Regions without ID-mediated interdomain contacts show increased flexibility, which results
418 in the Pfs230 structure being segmented into four rigid domain clusters of relative flexibility in relation to
419 one another (Pfs230D1-6, Pfs230D7-8, Pfs230D9-12 and Pfs230D13-14; **Figure 6, Figure S3**). ID-mediated

420   inter-domain contacts have also been observed in the Pf12-Pf41 heterodimer complex, which are two
421   other malarial proteins that are composed of 6-Cys domains (52, 53). Additionally, inter-protein contacts
422   between Pfs230 and Pfs48/45 appear to be mediated by regions outside of the canonical 6-Cys fold; we
423   have shown how the C-terminal extension of Pfs230 is critical for binding, and observed that the N-
424   terminal extension of Pfs48/45 also localized to this interface. This suggests that regions outside of the
425   canonical 6-Cys fold may have been introduced to provide functional roles by mediating interactions,
426   which may be a generalizable property of 6-Cys domain family of proteins.

427

428   Although it is currently unknown what exact cellular process the Pfs230:Pfs48/45 complex is involved in,
429   knockout studies in *P. falciparum* and the rodent malaria parasite *Plasmodium berghei* suggest the
430   Pfs230:Pfs48/45 complex is involved in microgamete-to-macrogamete adhesion and microgamete-to-RBC
431   adhesion (8-10). It is tempting to speculate that flexibility between Pfs230 domain clusters might play a
432   role in its cellular function. The presented structure will enable the design of domain clusters with more
433   precision than previously possible for the generation of genetically modified parasite lines that can
434   interrogate Pfs230 function. While the C-terminal Pfs230 domains are involved in the Pfs48/45-based
435   membrane anchoring onto the PPM, the N-terminal domains are free to interact with other elements. The
436   previous descriptions of some functional complement-independent α-Pfs230D1 antibodies suggest that
437   Pfs230D1 might be involved in some protein interactions that are disrupted upon binding of these
438   antibodies (18, 36). Interestingly, the addition of recombinant Pfs230Pro-D1-2 was previously shown to
439   inhibit the formation of exflagellation centers (18), further suggesting that the N-terminus of Pfs230 might
440   be involved in cell-to-cell adhesion. On the Pfs48/45 side, we showed that functional activity of
441   complement-independent Pfs48/45 antibodies does not directly correlate with their ability to disrupt the
442   Pfs230:Pfs48/45 complex. This implies that some Pfs48/45 targeting antibodies may prevent interactions
443   of Pfs48/45 with other proteins and that Pfs48/45 does not merely serve as Pfs230 membrane retention.
444   Future studies will continue to provide insights into interaction partners of the Pfs230:Pfs48/45 complex
445   and uncover the roles of Pfs230 and Pfs48/45 in parasite transmission.

446

447   Here, we found that Pfs230 binds to the GPI-anchored Pfs48/45 through its C-terminal peptide. It has
448   been reported that Pfs230 can be membrane-bound in the absence of Pfs48/45 in a small subpopulation
449   of female macrogametes via a currently unknown mechanism (20). We did not observe any Pfs230$^{\Delta Cterm}$
450   macrogamete or gametocyte still harboring membrane-bound Pfs230, suggesting that the presumed
451   Pfs48/45-independent membrane-binding of Pfs230 is mediated through the same Pfs230 C-terminal
452   peptide. Previously, co-immunoprecipitation experiments have suggested the existence of additional
453   interacting partners in the Pfs230:Pfs48/45 complex, including a large complex consisting of six adhesion-
454   linked *Pf*CCp proteins, the PPM-bound and macrogamete-specific protein Pfs25, and the WD40-repeat
455   protein PfWLP1 (65-67). Interestingly, we could not detect enrichment for any of these proteins after
456   FLAG-tag based co-immunoprecipitation performed on lysates from late-stage iGP2$^{230-tag}$ gametocytes
457   (**Figure 1D**), although we cannot exclude that differences between the protocols used might have
458   disrupted more labile interactions. Future studies using the iGP2$^{230-tag}$ line might reveal additional targets
459   for TBVs by fully characterizing the protein-interaction network of Pfs230:Pfs48/45, throughout all sexual
460   parasite stages.

461

462   Next to characterizing the full-length structure of the Pfs230:Pfs48/45 heterodimer, we also molecularly
463   characterized the epitope of multiple transmission-blocking antibodies. The potent Pfs48/45D1 antibody,
464   RUPA-71, binds an adjacent epitope to the previously characterized mAb, RUPA-58 (17). All highly potent
465   Pfs48/45D1 antibodies compete with either RUPA-58 or RUPA-71, indicating that binding to this
466   conserved portion of Pfs48/45D1 is associated with transmission-reducing activity (17). Additionally, we
467   structurally characterized potent epitopes outside of Pfs230D1 found on Pfs230D4 and Pfs230D7 targeted

468 by murine mAbs, 2A2 and 18F25, respectively. While mAb 2A2 is notably potent when used against the
469 NF54 strain (40, 51), the epitope is highly polymorphic with many Pfs230D4 SNPs observed in field isolates
470 (40) and described in the MalariaGEN Pf7 database (54). In contrast, both Pfs230D7 and the 18F25 epitope
471 are highly conserved and may thus be an ideal domain for subunit vaccine development. However, in
472 comparison to 2A2 and highly potent Pfs230D1-bin I binders, 18F25 exhibits approximately 10-fold lower
473 potency. The ability to design Pfs230 probes based on the full-length structure will now enable discovery
474 efforts seeking more potent antibodies. In addition, the structure of the full Pfs230:Pfs48/45 heterodimer
475 complex reported here with structure-function relationships of antibody inhibition, now enables a more
476 fulsome opportunity for structure-guided immunogen design, which has been transformative in
477 vaccinology during the development of *e.g.* SARS-CoV-2, and respiratory syncytial virus vaccines (68, 69).
478
479 Immunogens based on Pfs230 and Pfs48/45 have already shown promising results in clinical trials (46-49),
480 though the induced inhibitory activity was incomplete and the durability of the response remains
481 uncertain. These findings suggest that current TBV candidates can be further optimized, for example by
482 including Pfs230 domains that were previously thought to be unable to induce functional transmission-
483 blocking antibodies (38-40), or by rational structure-based immunogen design (70, 71). Currently, in the
484 case of Pfs230 domains, Pfs230D1, D4, D7, and D12 have been demonstrated to elicit potent antibodies
485 (22, 38-40). Interestingly, these Pfs230 domains share a common feature in that they are membrane
486 distal, suggesting that domains present on this side should be prioritized for subunit vaccine development.
487 By this logic, Pfs230D8 and D9 may also be relevant targets (**Figure 6**). To date, most Pfs48/45-based
488 interventions focus on Pfs48/45D3, but Pfs48/45D1 epitopes may be beneficial inclusions into future
489 Pfs48/45-based immunogens. Future immunogen design efforts should integrate inter-domain structural
490 elements to present well-folded epitopes. Approaching immunogen design through stabilizing structural
491 clusters instead of individual domains will help eliminate the presentation of non-neutralizing epitopes
492 that are absent endogenously, as previously evidenced by the elicitation of Pfs230D1-binIII mAbs (15C5,
493 LMIV230-02, and 230AS-26) and 230AL-20 by a Pfs230D1-only antigen (**Figure S8**). The presented
494 Pfs230:Pfs48/45 structure might also allow for future protein-engineering campaigns that graft multiple
495 potent epitopes onto a single chimeric immunogen (72), or use stabilizing mutations in conjunction with
496 display on nanocages (73). Additionally, advances in mRNA vaccination approaches combined with precise
497 immunogen design might allow for the *in situ* expression of stable, native Pfs230:Pfs48/45 co-assemblies.
498 Ultimately, the aim will be to create the most potent next-generation TBVs possible to aid in meeting the
499 ambitious goals of malaria elimination and eradication.
500
501 **Limitations of the study**
502
503 One limitation of our structure is the low resolution obtained for the Pfs230D9-12 domains (global
504 resolution of 4.7 Å). This prevented us from building an atomic model for these domains, limiting the
505 information we could confidently extract from this part of the map. Instead, we combined this low-
506 resolution map with an AF-based modeling approach to obtain a backbone model with reasonable
507 confidence regarding the domain orientations in this region of Pfs230. The flexibility of the different
508 structural units in Pfs230, in conjunction with the flexibility within membrane-anchored Pfs48/45,
509 suggests Pfs230 likely has a considerable range of motion on the parasite surface (**Figure 6 and S3**). Future
510 studies should look to elucidate whether this flexibility plays an important role in the function of
511 Pfs230:Pfs48/45. Finally, we purified the Pfs230:Pfs48/45 from late-stage gametocytes instead of
512 gametes, due to practical reasons. After gametogenesis, however, the Pro-domain of Pfs230 is
513 proteolytically cleaved (74), and therefore our structure does not fully recapitulate the mature protein
514 species. How these additional Pro-domain residues add onto the structure and if these influence the
515 overall Pfs230:Pfs48/45 conformation, remains currently unknown.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jean-Philippe Julien (jean-philippe.julien@sickkids.ca) upon reasonable request.

Materials availability

All unique reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- Previously unpublished antibody sequences are available in Table S6. Crystal and cryo-EM structures have been deposited in the Protein Data Bank and are publicly available as the date of publication. EMDB and PDB IDs are listed in Table 1 and Table S3. The proteomics dataset has been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (75) with the dataset identifier PXD060716.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

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568

Authors contribution

570 **Conceptualization**: E.T.B., R.Y., S.H., M.M.J., J-P.J.; **Investigation**: E.T.B., R.Y., S.H., N.I.P., R.S., O.T.W.,
571 R.C.v.D., M.R.I., C.M.A., P.W.T.C.; **Formal Analysis**: E.T.B., R.Y., S.H., F.H, D.I., M.J.; **Resources**: M.V., T.B.,
572 J.L.R.; **Writing – Original Draft**: E.T.B., R.Y., S.H., M.M.J., J-P.J.; **Writing – Review & Editing**: E.T.B., R.Y.,
573 S.H., F.H., D.I., M.J., N.I.P., C.M.A., T.B., J.L.R., T.W.A.K., M.M.J., J-P.J.; **Supervision**: M.V., T.B., J.L.R.,
574 T.W.A.K., M.M.J., J-P.J.; **Funding acquisition:** E.T.B., M.M.J., J-P.J.
575

Declaration of interests

577 The authors declare no competing interests.

Main Figures

Pfs230D1-6:RUPA-97:LMIV230-01:2A2 **Pfs230D7-8:18F25** **Pfs230D9-14:Pfs48/45** **Pfs230D13-14:Pfs48/45:RUPA-44:RUPA71**
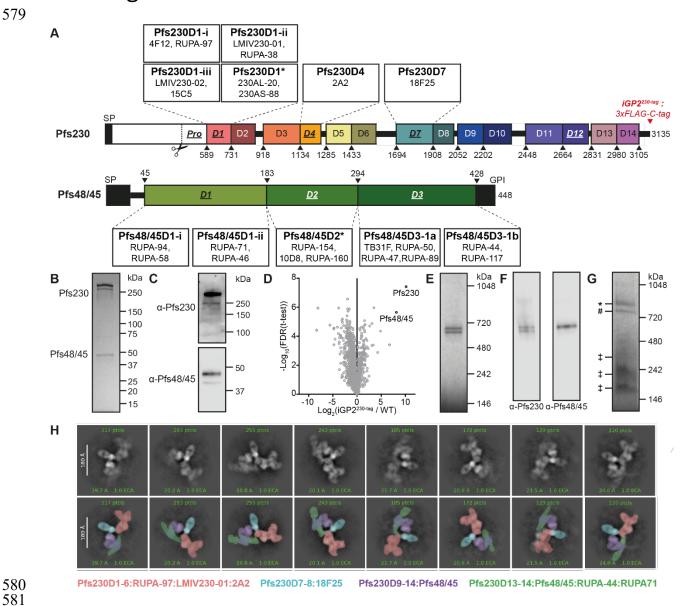
Figure 1: Purification of Pfs230:Pfs48/45:6Fab complex from *P. falciparum* gametocytes. (A) Schematic illustration of Pfs230 and Pfs48/45 domains (not to scale), epitopes found within these domains, and examples of mAbs targeting these epitopes (see also **Table S1**). Asterisks indicate multiple additional distinct epitopes at the indicated domain. Domains that contain known transmission-blocking epitopes are indicated by underlined italic. Arrows indicate amino acid number of the approximate start of the domain as determined by (15). SP = Signal peptide, GPI = Glycosylphosphatidylinositol anchor site. (B-C) Coomassie Blue (B) and immunoblot (C) staining of a denaturing SDS-PAGE gel of FLAG-tag purified Pfs230:Pfs48/45 complex. (D) Mass spectrometry analysis of FLAG-tag purified Pfs230:Pfs48/45 complex. The volcano plot shows the $\log_2$ fold change plotted against the $\log_{10}$ fold false discovery rate, comparing FLAG-tag co-immunoprecipitation of iGP2$^{230\text{-tag}}$ versus NF54/WT parasites. (E-G) Blue-Native PAGE analysis, showing (E) Coomassie blue and (F) α-Pfs230 and α-Pfs48/45 immunoblot staining of neat purified Pfs230:Pfs48/45 complex, or (G) Coomassie blue staining of eluted complex incubated with molar excess of six Fabs (RUPA-97, LMIV230-01, 2A2, 18F25, RUPA-44, and RUPA-71). Symbols: * = Pfs230:Pfs48/45:6Fab ; # = Pfs230:4Fab ; ‡ = excess Fab fragment. (H) Eight

15

595   representative 2D class averages generated from cryo-EM of HPLC-SEC purified Pfs230:Pfs48/45:6Fab
596   complex. Structures are annotated in the lower row, with orange highlighting Pfs230D1-6:RUPA-97:LMIV230-
597   01:2A2, blue Pfs230D7-8:18F25, purple Pfs230D-13-14:Pfs48/45, and green Pfs230D13-14:Pfs48/45:RUPA-
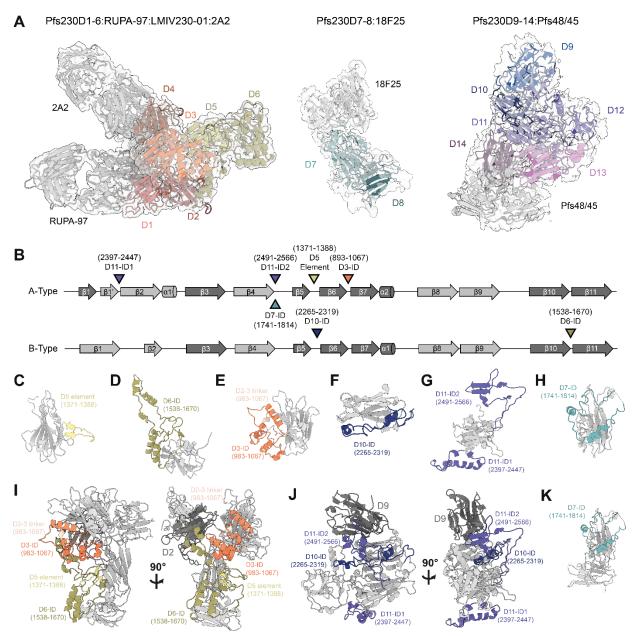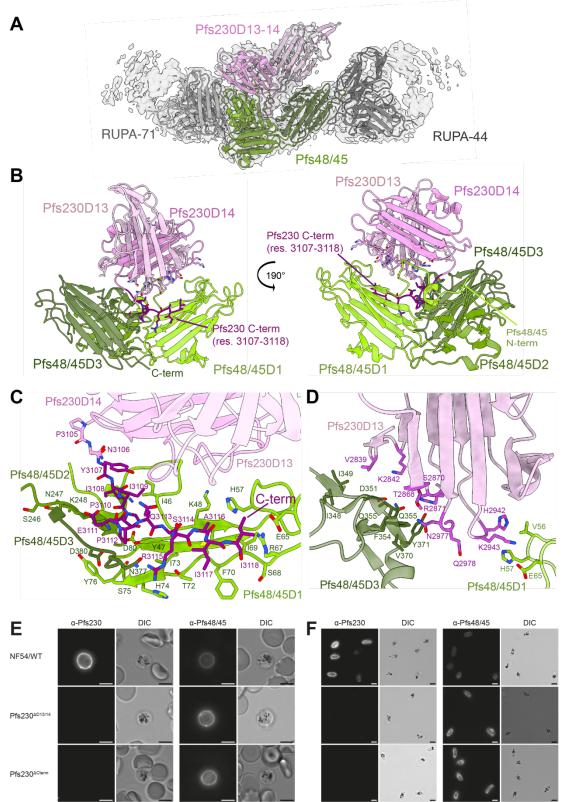598   44:RUPA-71. See also **Figures S1-3** and **Table S1.**

Figure 2 Structural Basis of Pfs230 6-Cys Domain Clustering. (A) Models of Pfs230D1-6:RUPA-97:LMIV230-01:2A2, PfsD7-8:18F25, and Pfs230D9-14:Pfs4845 shown in their respective maps (rendered at 4.5 Å radius around the models). Domains are coloured as in Figure 1. (B) Secondary structural elements of A- and B-type domains, with insertion domains (ID) or non-canonical structural element (D5 element) found in respective domains indicated by coloured arrows and with residue range in brackets. (C) D5 structural element, (D) Pfs230D6-ID, (E) D2-D3 linker (light orange) and D3-ID, (F) D10-ID, (G) D11-ID1 and –ID2, (H) and D7-ID are coloured as done previously with the rest of the domain coloured in silver. (I) The Pfs230D2-D3 linker (light orange), D3-ID (orange), D5 structural element (yellow), and D6-ID (dark yellow) interact with one another forming a structural fold that packs against the base of Pfs230D2 (dark grey). (J) The Pfs230D10-ID (blue) and Pfs230D11-ID2 (purple) interact with one another, positioning the D11-ID2 to form an extended β-sheet with D9 (dark grey). (K) Pfs230D7-8 (silver) and Pfs2230D7-ID (cyan) lack interdomain contact. See also **Figure S4**.
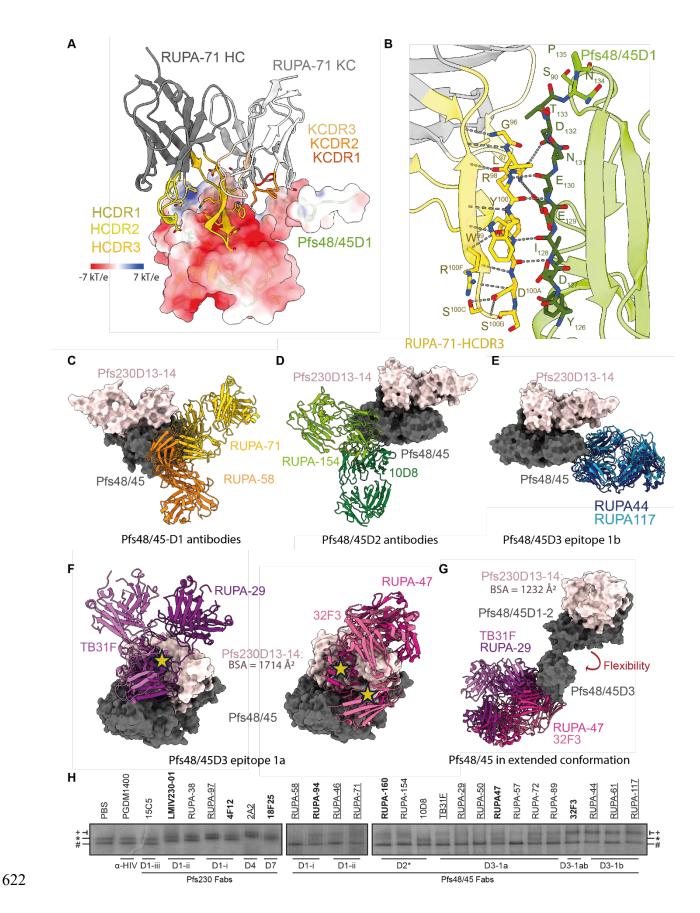
17

Figure 3: Molecular description of the Pfs230 and Pfs48/45 interaction. (A) Overview of the structure of Pfs48/45 (yellow green, olive drab, and dark olive drab for domains 1-3, respectively) bound to Pfs230D13-14 (plum), RUPA-71 Fab (dark grey), and RUPA-44 Fab (light grey) with the cryo-EM map shown in grey. (B) Pfs48/45 bound to Pfs230D13-14 with key Pfs230 contact residues shown as sticks and the C-terminal region of Pfs230 highlighted in

616   dark magenta. (C-D) binding interactions between Pfs48/45 and Pfs230D14-Cterm (C) and Pfs230D13 (D) with key
617   residue contacts shown as sticks. (E-F) Representative suspension immunofluorescence microscopy images of
618   NF54/WT, $230^{\Delta D13/14}$, and $230^{\Delta Cterm}$ female macrogametes (E) and saponin-treated stage V gametocytes (F). Parasites
619   were incubated with 15 µg/ml 18F25-DyLight488 (α-Pfs230) or 45.1-DyLight488 (α-Pfs48/45). All images per
620   experiment were taken and analyzed with the same settings. Scale bar represents 5 µm. See also **Figure S5-6, Table**
621   **S2**.

622

623 **Figure 4: Molecular characterization of anti-Pfs48/45 epitopes in the Pfs230-bound state of Pfs48/45**. (A)
624 Structure of RUPA-71 bound to Pfs48/45D1 depicted as surface and coloured by electrostatic potential calculated by
625 Adaptive Poisson-Boltzmann Solver (APBS) and visualized in ChimeraX. (B) RUPA-71 HCDR3 (gold) bound to
626 Pfs48/45D1 (yellow green) with key residues shown as sticks and hydrogen bonds or electrostatic interactions
627 indicated by dashed lines. (C-G) Model of Pfs48/45 (dark gray, depicted in surface) bound to Pfs230D13-14 (misty
628 rose, depicted in surface) in the disc-like (C-F) or extended (G) conformation bound to (C) Pfs48/45D1 binders RUPA-
629 71 (gold), RUPA-58 (orange, PDB ID: 8U1P)); (D) Pfs48/45D2 binders 10D8 (dark green, PDB ID: 7ZXF), RUPA-
630 154 (yellow green, PDB ID: 8U1P)); (E) Pfs48/45D3-1B binders (RUPA-44 (dark blue), RUPA-117 (sky blue, PDB
631 ID: 7UNB)); and (F-G) Pfs48/45D3-1a binders (TB31F (orchid, PDB ID: 6E63), RUPA-29 (dark magenta, PDB ID:
632 7UXL), RUPA-47 (violet red, PDB ID: 7UNB), and 32F3 (pale violet red, PDB ID: 7ZWI)). Clashes are indicated
633 with yellow stars. (**H**) Blue Native PAGE-based mobility shift assay of FLAG-tag purified Pfs230:Pfs48/45 complex,
634 incubated with a tenfold molar excess of individual anti-Pfs230 or anti-Pfs48/45 Fab fragments. The first lane shows
635 the Pfs230:Pfs48/45 complex with the addition of a mock liquid (PBS) as a reference, and PGDM1400, an anti-HIV
636 envelope protein targeted Fab, was included as negative control. Left panel shows anti-Pfs230 Fabs, right panels show
637 anti-Pfs48/45 Fabs. Fabs are sorted by antibody epitope and potency (underlined: $IC_{80} < 10$ µg/ml; bold: $IC_{80} = 10$-
638 100 µg/ml, others: $IC_{80} > 100$ µg/ml; See also Table S1). Note that due to the nature of Blue Native PAGE, the
639 hydrodynamic radius and therefore the angle of approach can heavily influence the extend of Fab-induced mobility
640 retardation. The symbols #, *, and + denote Pfs230, Pfs230:Pfs48/45, and Pfs230:Pfs48/45:Fab, respectively. See also
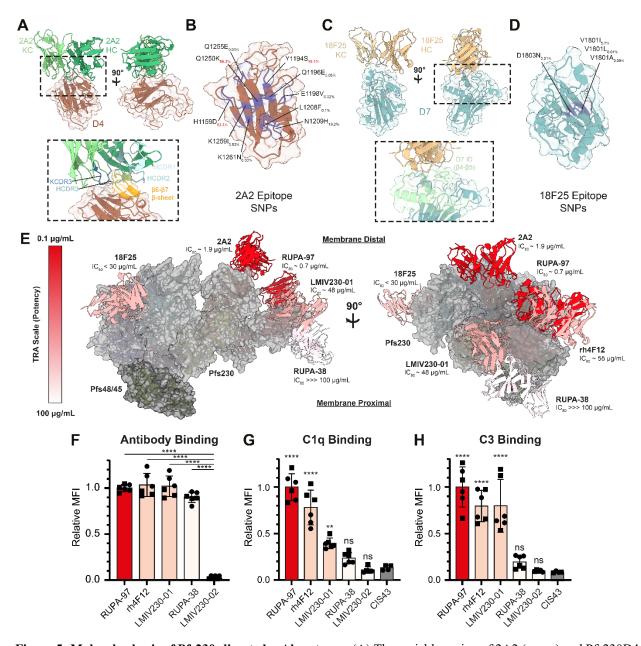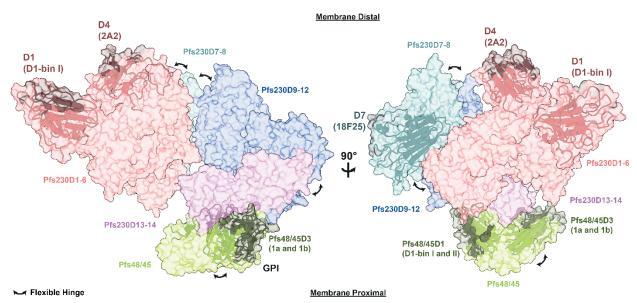641 **Figure S7, Table S1 and S3**.

Figure 5: Molecular basis of Pfs230-directed mAb potency. (A) The variable region of 2A2 (green) and Pfs230D4 (orange) are shown with key contacts (inset) made by the HCDR3, KCDR1, KCDR2, and KCDR3 (shades of blue) of mAb 2A2 with the $\beta 6$-$\beta 7$ perpendicular β-hairpin (yellow) of Pfs230D4 highlighted. (B) SNPs of Pfs230D4 identified in the MalariaGEN Pf7 database (54) are indicated in purple and labeled. The frequency of the SNP is shown as a subscript with values exceeding 1% coloured in red. (C) The variable region of 18F25 (tan) and Pfs230D7 (cyan) are shown. Key contacts made by 18F25 with Pfs230D7 (inset) include the ID of Pfs230D7 (light green). (D) SNPs of Pfs230D7 identified in the MalariaGEN Pf7 database (54) are indicated in purple and labeled. The frequency of the SNP is shown as a subscript. (E) The binding sites of 18F25, LMIV230-01, 2A2, RUPA-97, RUPA-38, and rh4F12 shown in the context of Pfs230 (surface: gray, ribbons: domains coloured as done in Figure 1 and 2) and Pfs48/45 (surface: black, ribbons: domains coloured as done in Figure 1). The antibodies are coloured in accordance with their IC_{80} values or TRA via heat map with darker colours indicating higher TRA or potency (left scale). The membrane distal side corresponds to the top of the panel and the membrane proximal side at the bottom of the panel. (F-H) Anti-Pfs230D1 mAb binding (F), or antibody-mediated human C1q recruitment (G) and human C3 deposition (H) at 1 µg/ml on the surface of wildtype NF54 macrogametes as determined by flow cytometry. CIS43, an anti-PfCSP antibody, was included as a negative control for the C1q and C3 deposition assays. Mean fluorescence intensity (MFI)

22

658   values were normalized against the mean of RUPA-97 replicates, to allow averaging across two independent
659   experiments (depicted as different symbols) with two to three technical replicates each. Mean ± standard deviation is
660   shown. Statistical analysis was done using an ordinary one-way ANOVA with either a Tukey's multiple comparisons
661   test with a single pooled variance comparing all groups to each other (F, only showing significant comparisons) or
662   Dunnett's multiple comparisons test with a single pooled variance comparing all groups to CIS43 (G-H). ns = not
663   significant; **=p<0.01, ****=p<0.0001. See also **Figure S8** and **Table S1**.

Figure 6. Structural model of the Pfs230:Pfs48/45 complex and potent epitopes. Domain clusters of Pfs230 and Pfs48/45 are indicated (Pfs230D1-6: red, Pfs230D7-8: teal, Pfs230D9-12: blue, Pfs230D13-14: pink, and Pfs48/45: green). Flexible hinges within the complex are indicated as a double-sided arrow. Domains targeted by potent mAbs ($IC_{80} < 30$ µg/mL) are shown as ribbons with the surfaces of these epitopes targeted by these antibodies coloured in a darker shade. Surfaces of epitopes are based off RUPA-97 (Pfs230D1-bin I), 2A2, 18F25, RUPA-58 (Pfs48/45D1-bin I), RUPA-71 (Pfs48/45D1-bin II), TB31F (Pfs48/45D3-1a), and RUPA-44 (Pfs48/45D3-1b).

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Main Tables

Table 1. CryoEM data collection and refinement statistics.

Data Collection	Pfs230D1-6: RUPA-97:LMIV1:2A2	Pfs230D7-8:18F25	Pfs230D9-14:Pfs48/45	Pfs230D13-14:Pfs48/45: RUPA-44:RUPA-71
Electron microscope	Titan Krios G3	Titan Krios G3	Titan Krios G3	Titan Krios G3
Camera	Falcon 4i Direct Electron Detector, SelectrisX energy filter	Falcon 4i Direct Electron Detector, SelectrisX energy filter	Falcon 4i Direct Electron Detector, SelectrisX energy filter	Falcon 4i Direct Electron Detector, SelectrisX energy filter
Voltage (kV)	300	300	300	300
Nominal magnification	130,000	130,000	130,000	130,000
Calibrated physical pixel size (Å)	0.93	0.93	0.93	0.93
Total exposure (e^-/Å^2)	50-53	50-53	50-53	50-53
Number of frames	30	30	30	30
Image Processing				
Motion correction software	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4
CTF estimation software	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4
Particle selection software	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4
3D map classification and refinement software	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4	cryoSPARC v4
Micrographs used	12,192	10,363	12,192	12,192
Particle images selected	1,167,622	237,721	1,942,561	1,942,561
Global resolution (Å)	3.6	3.8	4.7	3.4
Particle images contributing to final maps	70,113	165,992	138,915	146,814
Model Building				
Modeling software	Coot, phenix.real_space_refine, ISOLDE, Rosetta	Coot, phenix.real_space_refine, ISOLDE, Rosetta	Coot, phenix.real_space_refine, ISOLDE, Rosetta	Coot, phenix.real_space_refine, ISOLDE, Rosetta
Number of residues built	2430	546	1476	1156
RMS (bonds)	0.002	0.002	0.002	0.003

25

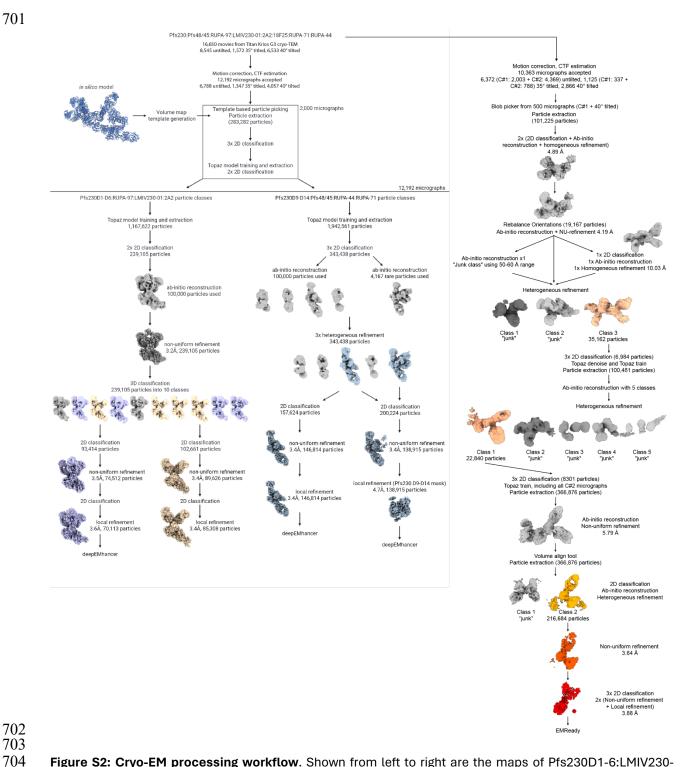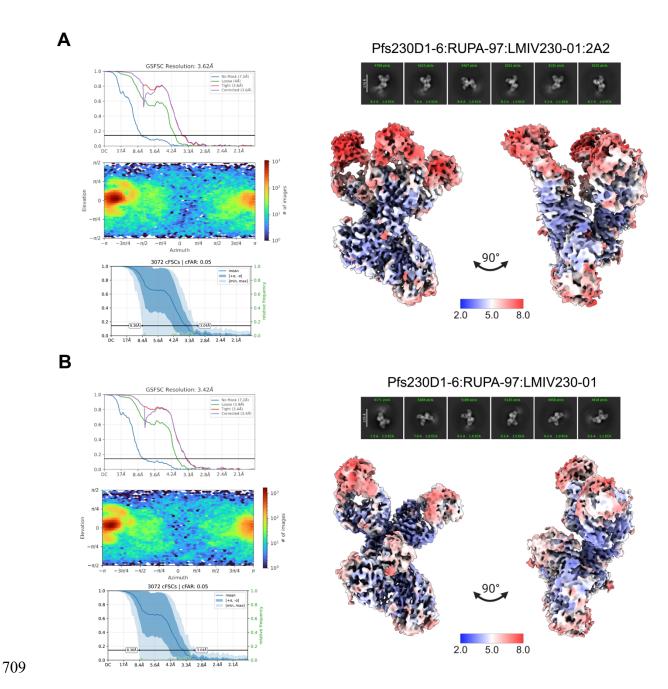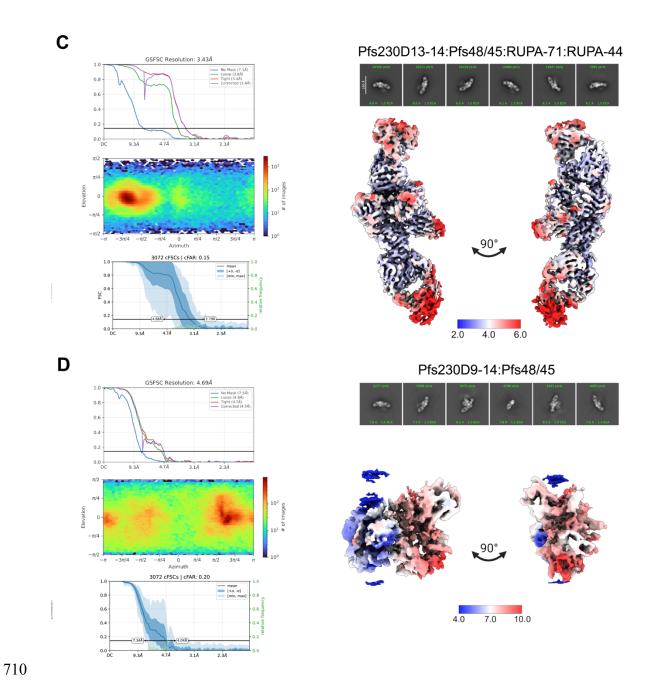|				
RMS (angles)	0.582	0.629	0.534	0.550
Ramachandran outliers (%)	0.04	0	0	0
Ramachandran allowed (%)	4.0	5.8	5.1	6.0
Ramachandran favoured (%)	96.0	94.2	94.9	94
Rotamer outliers (%)	0.3	0	0.2	1.3
Clashscore	6.3	5.2	4.3	10.3
MolProbity score	1.63	1.67	1.65	2.03
EMRinger Score	1.14	0.60	0.83	2.26
PDB, EMDB codes	9N5H, EMD-48921	9N5O, EMD-48941	9N5K, EMD-48924	9N5I, EMD-48922

Supplementary Figures

Figure S1: The iGP2[230-tag] parasite line allows for purification of endogenous Pfs230:Pfs48/45 complex recognized by transmission-blocking antibodies. (A) Schematic overview of the genomic modification to obtain the iGP2[230-tag] parasite line. HDR = Homology Directed Repair, HR = homology region, 3xFLAG = triple FLAG tag, C-tag = EPEA* tag, 3'UTR = bidirectional 3'untranslated region of PBANKA_142660, H2B = promoter of Pf3D7_1105100. (B) Genomic integration PCR to confirm 5' and 3' integration and the absence of wildtype parasites. Primers and expected PCR product are indicated in (A). (C) Immunofluorescence microscopy images of paraformaldehyde/glutaraldehyde-fixed and permeabilized iGP2 wildtype and iGP2[230-tag] stage V gametocytes.

27

686   Parasites were stained for Pfs230 (green, RUPA-55), FLAG-tag (magenta, M2), and Pfs48/45 (45.1), and DAPI (blue).
687   Scalebar represents 5 µm. ESID = Electronically Switchable Illumination and Detection brightfield image. (D)
688   Western blot analysis of iGP2 and iGP2$^{230\text{-tag}}$ to confirm FLAG-tag and C-tag integration. (E) Exflagellation of stage
689   V iGP2 (white) and iGP2$^{230\text{-tag}}$ (gray) gametocytes. Bars represent mean ± standard deviation of number of
690   exflagellation centers per ml from four independent cultures (dots). No statistical significance was found using an
691   unpaired t-test. (F) Transmission of iGP2 (white) and iGP2$^{230\text{-tag}}$ (gray) to mosquitoes in two independent standard
692   membrane feeding experiments. Dots represent the number of oocysts per midgut *Anopheles stephensi* mosquitoes
693   (n=20 per experiment) in two independent standard membrane feeding assays using iGP2 (white) and iGP2$^{230\text{-tag}}$ (gray)
694   parasites. Statistical analysis was done using a Mann-Whitney test with Holm-Šídák's multiple comparisons test (α =
695   0.05). ns = not significant. (G) Coomassie blue stained Blue Native PAGE gel of purified Pfs230:Pfs48/45 (and free
696   Pfs230) incubated with molar excess of the following individual Fab fragments: RUPA-44 (Pfs48/45D3-1a), RUPA-
697   71 (Pfs48/45D1-ii), RUP-97 (Pfs230D1-i), LMIV230-01 (Pfs230D1-ii), 2A2 (Pfs230D4), or 18F25 (Pfs230D7). (H)
698   Normalized absorption at 280 nm during high-pressure liquid size-exclusion chromatography of Pfs230:Pfs48/45
699   (black) or Pfs230-Pfs48/45:6Fab (red) complexes. Dotted lines indicate fractions of eluted Pfs230:Pfs48/45:6Fab that
700   were used for cryo-electron microscopy. Related to **Figure 1**.

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702
703
704 **Figure S2: Cryo-EM processing workflow**. Shown from left to right are the maps of Pfs230D1-6:LMIV230-
705 01:RUPA-97:2A2, Pfs230D1-6:LMIV230-01:RUPA-97, Pfs230D13-14:Pfs48/45:RUPA-44:RUPA-71, the focused
706 refinement for Pfs230D9-14:Pfs48/45, and the Pfs230D7-8:18F25 map. Abbreviations for the two data
707 collection sessions are abbreviated in the Pfs230D7-8:18F25 workflow (C#1: collection C#2: collection 2).
708 Related to **Figure 1**.

A

Pfs230D1-6:RUPA-97:LMIV230-01:2A2

B

Pfs230D1-6:RUPA-97:LMIV230-01

709

C

Pfs230D13-14:Pfs48/45:RUPA-71:RUPA-44
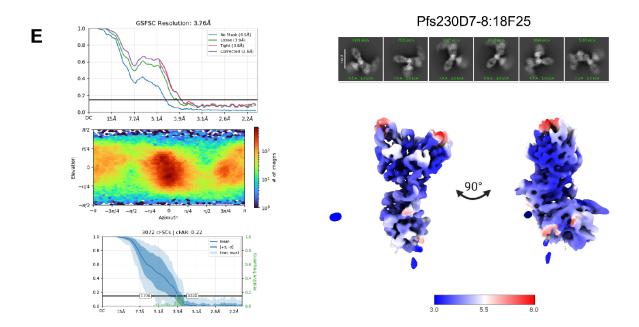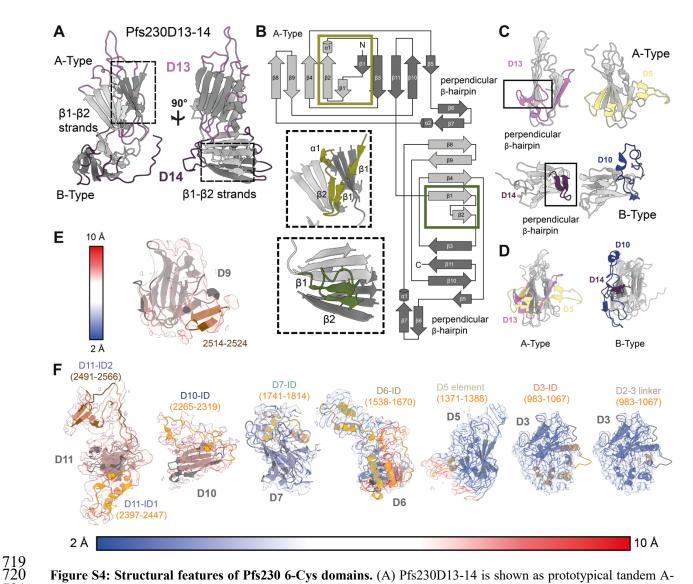
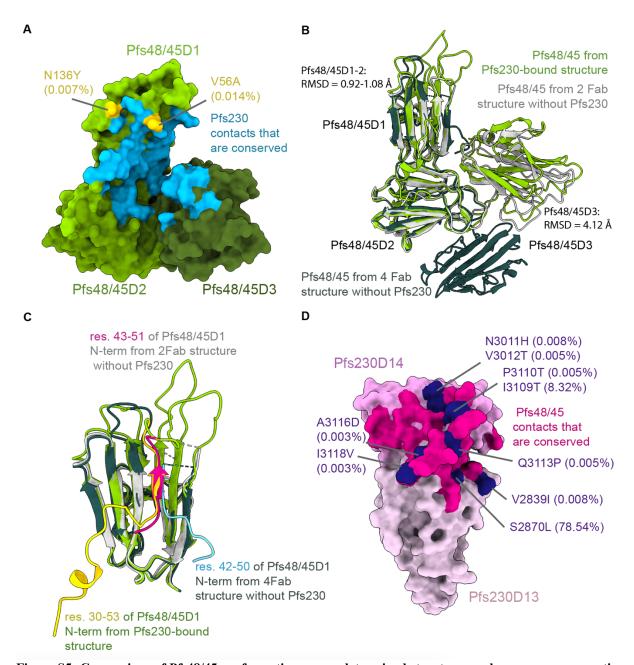**D**

Pfs230D9-14:Pfs48/45

710

Figure S3: Cryo-EM map validation data. Shown are the structures of (A) Pfs230D1-6:RUPA-97:LMIV230-01:2A2; (B) Pfs230D1-6:RUPA-97:LMIV230-01; (C) Pfs230D13-14:Pfs48/45:RUPA-44:RUPA-71; (D) focused refinement for Pfs230D9-14:Pfs48/45; and (E) Pfs230D7-8:18F25. The Fourier shell correlation curves following a gold-standard refinement with correction for the effects of masking for varying masks, the viewing direction particle distribution, and cFAR plot and score are shown (left panel). Additionally, representative 2D classes and cryo-EM maps coloured by local resolution are shown for each processed map (right panel). Related to **Figure 1**.

Figure S4: Structural features of Pfs230 6-Cys domains. (A) Pfs230D13-14 is shown as prototypical tandem A- and B-type 6-Cys domains of Pfs230. Loops of D13 and D14 are coloured according to the colouring scheme in Figure 1. The first β-sheet of the A-type and B-type domains is coloured distinctly (light gray) from the second β-sheet (gray). The distinguishing features of the A- and B-type domains (β1 to β2 strands) are indicated through the dashed box. (B) Secondary structure topology of the two 6-Cys domains are shown with distinguishing features of A- (olive) and B-type (green) domains (β1 to β2 strands) indicated by box (inset) with a 3D representation of these differences shown. (C-D) Comparison of prototypical tandem 6-Cys domains (D13 and D14) to D5 and D10 respectively, (C) side by side and (D) overlaid, highlighting the structural differences of D5 and D10 at the perpendicular β-hairpin region. The regions that are structurally distinct are coloured according to the scheme in Figure 1. (E) Pfs230D9 (gray) and a portion of the Pfs230D11-ID2 (2514-2524, brown) overlaid on top of the map which is coloured according to local resolution (left scale). (F) Pfs230 domains that harbour IDs (Pfs230D11, D10, D7, D6, D5, and D3, gray) or structural elements contributing to ID clustering (Pfs230D3 and D5) are shown (Pfs230D11-ID2: brown, rest: orange) as ribbon representations with the map overlaid (shown at 5 Å radius around the models) and coloured according to local resolution (scale bar below). Related to **Figure 2.**

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Figure S5: Comparison of Pfs48/45 conformation across determined structures and sequence conservation of Pfs230:Pfs48/45 binding site. (A) Structure of Pfs48/45 depicted as surface (yellow green, olive drab, and dark olive drab for Pfs48/45D1-3, respectively) with the Pfs230 binding site shown in sky blue and single nucleotide polymorphisms present within the epitope shown in gold. (B) Overlay of Pfs48/45 structures from Pfs48/45-Pfs230-6Fab complex (yellow green), Pfs48/45-4Fab complex (dark slate grey, PDB ID: 8U1P), and Pfs48/45-2 Fab complex (light grey, PDB ID: 7ZXF). (C) Overlay of N-terminal region of Pfs48/45-Pfs230-6Fab complex (green, yellow N-term), Pfs48/45-4Fab complex (dark slate grey, light blue N-term, PDB ID: 8U1P), and Pfs48/45-2 Fab complex (light grey, dark violet N-term, PDB ID: 7ZXF). (D) Pfs230D13 (thistle) and D14 (plum) shown as surface with the Pfs48/45 binding site in dark pink and SNPs in indigo. Related to **Figure 3.**
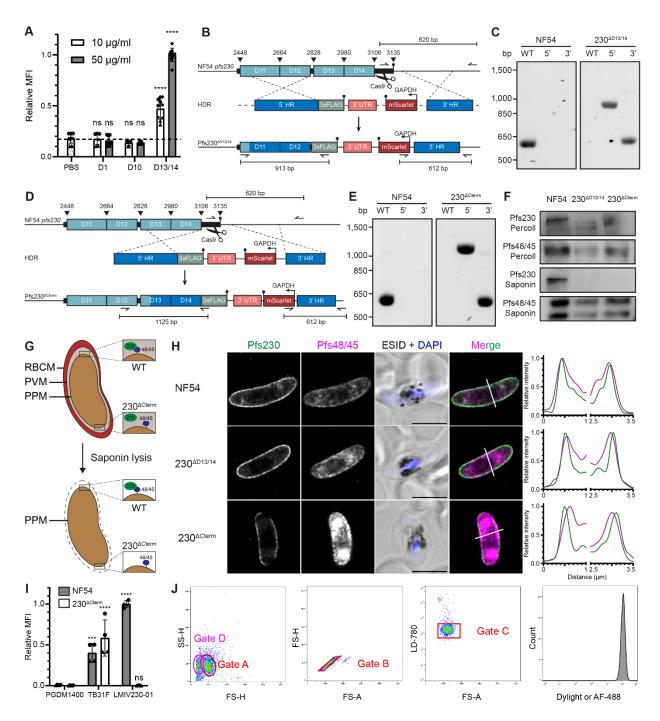
34

Figure S6: Pfs230:Pfs48/45 interactions are mediated by the C-terminal region Pfs230D13-14-Cterm *in vivo*.
(A) Female gamete binding assay with recombinantly purified Pfs230D1, Pfs230D10 and Pfs230D13/14-Cterm at 10 µg/ml (white bar) or 50 µg/ml (gray bar). Bars show mean MFI (Mean Fluorescence Intensity) of two to four independent experiments with two to three technical replicates each. Error bars show standard deviation. Individual data points are shown, with different symbols for each independent experiment. Data was normalized against the mean of the highest value (50 µg/ml D13-14) in each independent experiment to allow for comparison across multiple experiments. Statistical analysis was done by comparing each group to the PBS control using an ordinary one-way ANOVA with Dunnett's multiple comparisons test with a single pooled variance. (B-E) Schematic overview of genomic integration of Pfs230$^{\Delta D13/14}$ (B) and Pfs230$^{\Delta Cterm}$ (D), and the corresponding diagnostic integration PCR (C, E). HDR = Homology Directed Repair, HR = homology region, 3xFLAG = triple FLAG tag, 3'UTR = bidirectional
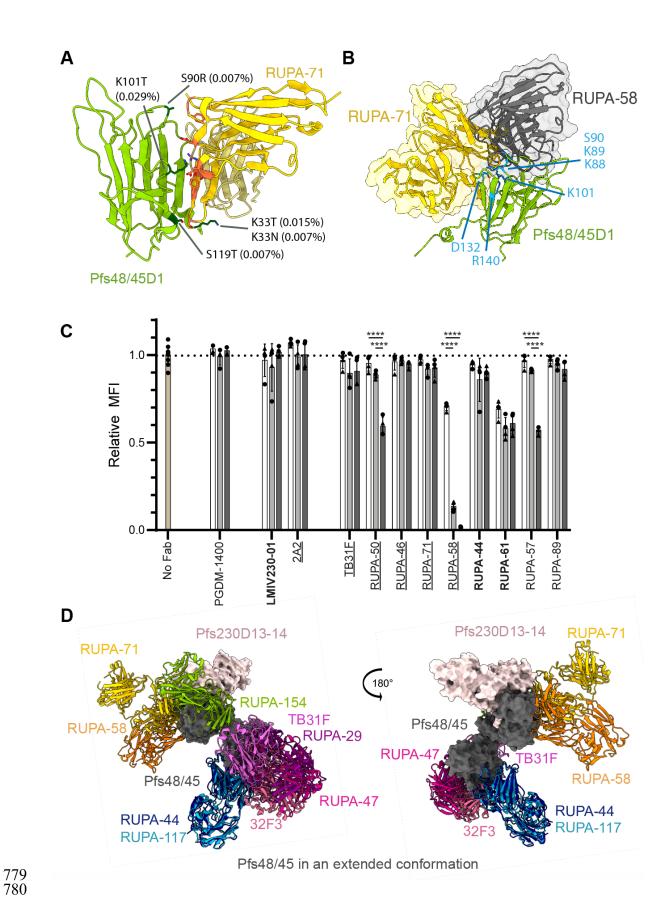
757 3'untranslated region of PBANKA_142660, GAPDH = promoter of Pf3D7_1462800. (F) Western blot analysis of
758 Pfs230 (18F25) and Pfs48/45 (32F3) expression in wildtype, Pfs230$^{\Delta D13/14}$ and Pfs230$^{\Delta Cterm}$ late-stage gametocytes,
759 isolated with Percoll (intact RBC and PVM membrane) or saponin (permeabilized RBC and PVM membrane). (G)
760 Schematic illustration of localization of the Pfs230:Pfs48/45 complex on the parasite plasma membrane (PPM), and
761 the effect of saponin on the red blood cell membrane (RBCM) and parasite vacuole membrane (PVM). (H)
762 Representative immunofluorescence microscopy images of paraformaldehyde/glutaraldehyde-fixed NF54 wildtype,
763 Pfs230$^{\Delta D13/14}$ and Pfs230$^{\Delta Cterm}$ stage V gametocytes. Parasites were stained for Pfs230 (green, RUPA-55), Pfs48/45
764 (magenta, 45.1), and DNA (DAPI, blue). Right inset shows intensity plot profile analysis along the indicated section,
765 normalized against maximum value along the profile per signal. Scale bar is 5 µm. ESID = Electronically Switchable
766 Illumination and Detection brightfield image. (I) Binding assay with wildtype (white bar) and Pfs230$^{\Delta Cterm}$ (gray bar)
767 female gametes, testing the binding of PDGM1400 (α-HIV-1 envelope glycoprotein), TB31F (α-Pfs48/45), and
768 LMIV230-01 (α-Pfs230) antibodies at 1 µg/ml. Bars show the mean relative MFI of two independent experiments
769 with two technical replicates each, error bars show standard deviation. MFI was normalized against the average of
770 LMIV230-01 in the independent experiments. MFI values were compared within each parasite line to the PGDM1400
771 control using an ordinary two-way ANOVA and Šídák's multiple comparison's test with a single pooled variance. ns
772 = not significant ; ***=p<0.001, ****=p<0.0001. (J) Exemplary plots to provide an overview for the gating strategy
773 of macrogamete flow cytometry experiments. Live gametes (gate A) were gated based on side-scatter (SS) and forward
774 scatter (FS). Single cells (gate B) were selected based on FS area (FS-A) versus height (FS-H), after which live cells
775 (gate C) were selected based on absence of LD efluor780 staining. The geometric mean fluorescence intensity of the
776 Dylight/AlexaFluor-488 channel of the resulting population was then used as the mean fluorescence intensity. Note
777 that both gate A and D (containing dead gametes) were used in the case of the complement deposition assays, when
778 no live/dead stain was used. Related to **Figure 3** and **Methods**.

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781 **Figure S7: RUPA71 epitope comparison and Pfs48/45-targeted Fab-induced dissociation of the Pfs230:Pfs48/45**
782 **complex *in vivo*.** (A) RUPA-71 (yellow) bound to Pfs48/45 (yellow green) with Pfs48/45 residues with single
783 nucleotide polymorphism in dark green and RUPA-71 residues that contact them in coral. (B) Overlay of Pfs48/45D1
784 (yellow green, cartoon) bound to antibodies RUPA-71 (gold) and RUPA-58 (dim gray; PDB ID: 8U1P). Pfs48/45
785 residues that contact both antibodies are indicated in blue. (C) Live female *P. falciparum* NF54 macrogametes were
786 incubated with increasing concentrations of Fab fragments (white bar: 1 µg/ml; light grey: 10 µg/ml; dark grey: 100
787 µg/ml), after which Pfs230 surface retention was measured by determining the binding of 18F25-DyLight488 by flow
788 cytometry. MFI was normalized against the no Fab control to allow for averaging across experiments (two biological
789 replicates with two technical replicates each). Bars depict mean ± standard deviation, different symbols depict different
790 biological replicates. Anti-Pfs48/45 Fabs are sorted on potency as determined by the IC_{80} value (Underlined: IC_{80} <
791 10 µg/ml ; Bold: IC_{80} = 10-100 µg/ml, Others: IC_{80} > 100 µg/ml, see Table S1). Statistical analysis to test for a dose-
792 dependent reduction in 18F25-488 binding by comparing all concentrations per individual Fab using an ordinary two-
793 way ANOVA with a Šídák's multiple comparisons test with a single pooled variance as one family. Only significant
794 comparisons are shown. **** = p<0.0001. (D) Model of Pfs48/45 (dark gray, depicted in surface) bound to
795 Pfs230D13-14 (misty rose, depicted in surface) in the extended conformation overlayed with RUPA-71 (gold), RUPA-
796 58 (orange, PDB ID: 8U1P), , RUPA-154 (green, PDB ID: 8U1P)), RUPA-44 (dark blue), RUPA-117 (sky blue, PDB
797 ID: 7UNB), TB31F (orchid, PDB ID: 6E63), RUPA-29 (magenta, PDB ID: 7UXL), RUPA-47 (violet red, PDB ID:
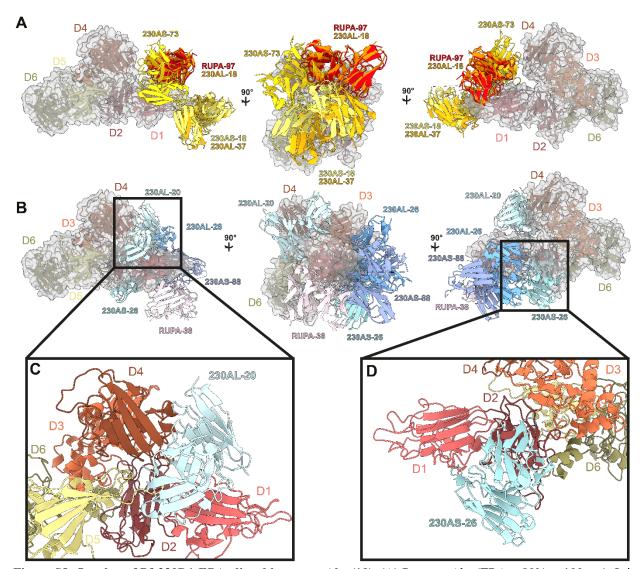798 7UNB), and 32F3 (pale violet red, PDB ID: 7ZWI)). Related to **Figure 4.**

799
800 **Figure S8. Overlay of Pfs230D1-EPA-elicted human mAbs** (19). (A) Potent mAbs (TRA > 80% at 100 μg/mL in
801 SMFA; shades of yellow and orange) are shown overlaid on Pfs230D1-6 (surface: gray, ribbons: coloured as done in
802 Figure 1) compared to RUPA-97 (red). (A) Non-potent mAbs (TRA < 80% at 100 μg/mL in SMFA; shades of blue)
803 are shown overlaid on Pfs230D1-6 compared to RUPA-38 (light pink). Zoom in of (C) 230AL-20 and (D) 230AS-26
804 showcasing steric clashes that occur with Pfs230D4 and Pfs230D2, respectively – as examples of mAbs elicited via a
805 single Pfs230 domain subunit vaccine exposing non-native epitopes that would be incompatible with full-length
806 Pfs230 binding. Related to **Figure 5**.
807

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Supplementary Tables

Table S1. Overview of antibodies used or described in these studies. Shown are the targeted epitopes, structures in the RSCB/PDB database (structures in bold: this work), and estimates of the IC_{80} value (antibody concentration required to achieve 80% TRA in Standard Membrane Feeding Assay). * Estimate value from limited number of observations. ** No blocking in High-Throughput membrane feeding assay at 100 µg/ml (34). Related to **Figures 1, 4, and 5.**

Target	Antibody	Epitope	IC_{80} (µg/ml)	Reference	Structure
Pfs230	RUPA-97	D1 – i	0.7	Ivanochko[22]	7UVQ / **9N5H**
	4F12	D1 – i	55	MacDonald[36], Ivanochko[22]	6OHG
	LMIV230-01	D1 – ii	48	Coelho[21], Ivanochko[22]	7UFW / 7JUM / **9N5H**
	RUPA-38	D1 – ii	>>100	Ivanochko[22]	7UVI
	LMIV230-02	D1 – iii	>>100	Coelho[21], Ivanochko[22]	7UVS
	15C5	D1 – iii	>>100	Lee, Ivanochko[22]	7UVQ
	2A2	D4	1.9	Roeffen[51], De Jong[40]	**9N5H, 9N89**
	18F25	D7	10 to 30 *	Roeffen[51], Inklaar[38]	**9N5O, 9N6U**
Pfs48/45	RUPA-58	D1-i	7.5	Kucharska[17]	8U1P
	RUPA-94	D1-i	<100 *	Fabra-García[34]	-
	RUPA-46	D1-ii	2 to 10 *	Fabra-García[34]	-
	RUPA-71	D1-ii	2 to 10 *	Fabra-García[34]	**9N5I, 9N7K**
	RUPA-154	D2	103.1	Kucharska[17]	8U1P
	RUPA-160	D2	± 10 *	Fabra-García[34]	-
	10D8	D2	>350 *	Ko[16]	7ZXF/7ZXG/7ZWM
	TB31F	D3 - 1a	0.86	Kundu[43], Kucharska[17], Ko[16]	6E63/7ZXF/6H5N
	RUPA-29	D3 - 1a	0.4 to 2 *	Fabra-García[34]	7UXL
	RUPA-50	D3 - 1a	0.4 to 2 *	Fabra-García[34]	-
	RUPA-47	D3 - 1a	10 to 100 *	Fabra-García[34]	7UNB
	RUPA-57	D3 - 1a	>100**	Fabra-García[34]	-
	RUPA-72	D3 - 1a	>100**	Fabra-García[34]	-
	RUPA-89	D3 - 1a	>100**	Fabra-García[34]	-
	32F3	D3 - 1ab	<25 *	Vermeulen[27], Ko[16]	7ZWI/7ZWF/7ZWM
	RUPA-61	D3 - 1b	± 10 *	Fabra-García[34]	-
	RUPA-44	D3 - 1b	11	Fabra-García[34]; Kucharska[17]	7UXL / **9N5I**
	RUPA-117	D3 - 1b	± 10 *	Fabra-García[34]	7UNB
Negative controls	PGDM1400	HIV Env.	n/a	n/a	n/a
	CIS43	PfCSP	n/a	n/a	n/a

Table S2: Pfs230 – Pfs48/45 residue contact table. Related to **Figure 3**.

Pfs48/45 Domain	Pfs48/45 Residue	BSA (Å^2)	Pfs230 Residue contacts
Domain 1	ASN38	18.0	3108
	GLU40	25.2	3011, 3013
	ILE41	59.9	3011, 3112, 3013
	SER 42	87.6	2986, 2997, 2998, 3011,3012
	GLY43	33.5	2997
	PHE44	15.0	2997
	ILE45	85.7	2984, 3108-3110
	GLY46	15.8	3110
	TYR47	65.2	3110-3114
	LYS48	49.4	2982, 2997, 3114
	ASN50	16.6	2993
	GLU54	47.5	2990, 2993, 3001
	GLY55	9.4	2993
	VAL56	90.1	2942, 2991, 2993, 2994
	HIS57	78.1	2942, 3116, 3118
	GLU65	34.2	2943, 3118
	ARG67	30.1	3118
	SER68	20.4	3117-3118
	ILE69	24.9	3116-3118
	PHE70	55.7	3115-3117
	CYS71	24.5	3114-3116
	THR72	49.9	3113-3115
	ILE73	15.8	3114
	HIS74	24.1	3115
	SER75	8.1	3112
	ASP80	16.6	3110
	ASN136	38.9	3001
	TYR137	17.5	2999
Domain 2	ASN247	13.0	3111
	LYS248	70.9	3109-3111
	ILE249	10.7	3108
	ILE250	17.4	3108
Domain 3	ILE348	27.8	2839
	ILE349	11.3	2839
	PHE354	48.1	2871, 2980
	GLN355	24.8	2870
	ILE369	53.2	2868, 2870, 2977
	VAL370	8.5	2977
	TYR371	10.9	2870, 2871, 2978-2980
	ASP373	46.6	2980, 3113
	ASN377	31.1	3115
	GLY379	25.3	3111, 3112
	ASP380	25.8	3111, 3112
	GLU385	12.4	3079

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Table S3. Data collection and refinement statistics for the RUPA-71, 2A2, and 18F25 Fab crystal structures. Related to **Figures 4-5.**

	RUPA-71 Fab	2A2 Fab	18F25 Fab
Wavelength (Å)	0.95357	0.920194	0.920194
Space group	P $2_1 2_1 2_1$	P $2_1 2_1 2_1$	P $2_1 2_1 2_1$
Cell dimensions			
a, b, c (Å)	64.66, 74.55, 204.9	59.97, 76.68, 91.87	37.64, 81.80, 140.53
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	48.85 - 2.28 (2.36 –2.28)	28.51 - 1.20 (1.21 -1.20)	34.19 - 1.60 (1.63 - 1.60)
No. molecules in ASU	2	1	1
No. unique observations	46,105 (4434)	254,175 (8275)	109,994 (5231)
Multiplicity	15.4 (15.2)	3.5 (3.6)	3.3 (3.3)
R_{meas}[1] (%)	39.3 (229.3)	8.1 (68.5)	86.2 (74.4)
R_{pim}[2] (%)	13.7 (80.7)	4.2 (35.6)	4.6 (40.2)
<I/sigma I>	7.5 (1.7)	8.9 (2.1)	9.8 (1.8)
$CC_{1/2}$ (%)	99.1 (56.1)	99.7 (71.6)	99.8 (55.6)
Completeness (%)	100 (100)	99.5 (98.2)	99.4 (98.6)
Refinement Statistics			
Non-hydrogen atoms	7037	3708	4056
Macromolecule	6776	3343	3432
Heteroatom	52	0	20
Water	209	427	604
R_{work}[3]/ R_{free}[4] (%)	19.1/ 24.1	18.6 / 19.4	18.8 / 21.3
Rms deviations from ideality			
Bond lengths (Å)	0.009	0.004	0.006
Bond angle (°)	0.99	0.78	0.83
Ramachandran plot			
Favoured regions (%)	98.0	98.3	97.4
Allowed regions (%)	2.0	1.7	2.6
B-factors (Å^2)			
Wilson B-factor	36.5	9.8	15.2
Average B-factors	41.4	12.8	17.1
PDB code	9N7K	9N89	9N6U

*Values in parentheses are for the highest-resolution shell.
[1]$R_{meas} = \Sigma hkl [N/(N-1)]1/2 \Sigma i \mid Ihkl, i - <Ihkl> \mid / \Sigma hkl <Ihkl>$.
[2]$R_{pim} = \Sigma hkl [1/(N-1)]1/2 \Sigma i \mid Ihkl, i - <Ihkl> \mid / \Sigma hkl <Ihkl>$.
[3]$R_{work} = \Sigma ||F_o| - |F_c||/\Sigma|F_o|$.
[4]R_{free} is calculated for 5% of the data that was not included in refinement.

829 **Table S4: 2A2, 18F25, and RUPA-71 contacts table.** Related to **Figures 4 and 5**.

Pfs230 Residue	Structural Element	BSA (Å²)	2A2 HC Residue	Structural Element	BSA (Å²)	2A2 KC Residue	Structural Element	BSA (Å²)
HIS1159	D4: β2-β3 loop	37.1	ASN33	HCDR1	16.0	SER1	N-term / FR1-IMGT	27.1
GLU1160	D4: β3	13.6	ASN35	FR2-IMGT	2.3	ILE2	N-term / FR1-IMGT	7.9
TYR1194	D4: β5	27.2	TRP47	FR2-IMGT	21.6	GLN27	KCDR1	72.7
GLN1196	D4: β5	67.5	ASN50	FR2-IMGT	19.2	SER28	KCDR1	8.2
GLU1198	D4: β5-β6 loop	20.1	ASP52	HCDR2	21.4	ASN30	KCDR1	6.2
TYR1205	D4: β6	33.6	HIS55	HCDR2	57.7	ASP91	KCDR3	8.1
LYS1206	D4: β6-β7 loop	51.1	GLY57	HCDR2	23.1	TYR92	KCDR3	80.0
GLY1207	D4: β6-β7 loop	21.4	THR58	HCDR2	11.2	SER93	KCDR3	41.9
LEU1208	D4: β6-β7 loop	169.6	THR59	FR3-IMGT	60.3	SER94	KCDR3	74.0
ASN1209	D4: β6-β7 loop	107.2	TYR60	FR3-IMGT	7.9	PRO95	KCDR3	12.4
SER1210	D4: β7	53.5	ASN61	FR3-IMGT	1.0	LEU96	KCDR3	26.3
VAL1211	D4: β7	43.9	GLN62	FR3-IMGT	51.2			
ASP1212	D4: β7	0.4	LYS65	FR3-IMGT	14.3			
THR1215	D4: α1	31.4	SER99	HCDR3	0.1			
GLN1250	D4: β10	32.6	THR100	HCDR3	0.9			
THR1252	D4: β10	8.9	LEU101	HCDR3	19.9			
GLN1255	D4: β10-β11	4.3	TYR102	HCDR3	69.5			
VAL1256	D4: β11	8.0	GLY103	HCDR3	16.5			
VAL1257	D4: β11	69.7	SER105	HCDR3	32.1			
LYS1259	D4: β11	40.9						
LYS1261	D4: β11	15.1						
Pfs230 Residue	Structural Element	BSA (Å²)	18F25 HC Residue	Structural Element	BSA (Å²)	18F25 KC Residue	Structural Element	BSA (Å²)
LYS1751	D7: β4-β5 loop (D7-ID)	35.7	TYR32	HCDR1	33.3	THR30	KCDR1	15.8
VAL1752	D7: β4-β5 loop (D7-ID)	46.5	TRP33	HCDR1	10.2	ASN31	KCDR1	10.0
ILE1754	D7: β4-β5 loop (D7-ID)	26.6	TYR52	HCDR2	23.3	ASP32	KCDR1	32.2
GLU1756	D7: β4-β5 loop (D7-ID)	35.4	ASP54	HCDR2	8.8	TYR49	FR2-IMGT	24.3
VAL1801	D7: β4-β5 loop (D7-ID)	19.7	ASP56	HCDR2	12.1	SER50	KCDR2	3.6
LEU1802	D7: β4-β5 loop (D7-ID)	79.2	ARG58	HCDR2	10.5	TYR53	KCDR2	32.0
ASP1803	D7: β4-β5 loop (D7-ID)	52.0	ARG94	HCDR3	1.5	TYR55	FR3-IMGT	7.2
ASN1804	D7: β4-β5 loop (D7-ID)	6.7	LEU96	HCDR3	27.3	HIS91	KCDR3	27.5
THR1806	D7: β4-β5 loop (D7-ID)	31.6	TYR97	HCDR3	42.3	TYR92	KCDR3	84.9
PHE1807	D7: β4-β5 loop (D7-ID)	114.5	LEU98	HCDR3	69.8	SER93	KCDR3	1.3
GLU1808	D7: β4-β5 loop (D7-ID)	40.0						
LYS1809	D7: β4-β5 loop (D7-ID)	1.8						
LYS1856	D7: β8-β9 loop	24.8						
ASP1857	D7: β8-β9 loop	0.6						
Pfs48/45 Residue	Structural Element	BSA (Å²)	RUPA-71 HC Res.	Structural Element	BSA (Å²)	RUPA-71 KC Res.	Structural Element	BSA (Å²)
LYS33	D1: N-term	28.2	GLU2	N-term / FR1-IMGT	47.1	LYS32	KCDR1	40.3
SER35	D1: N-term	13.8	GLY26	HCDR1	19.3	LEU46	FR2-IMGT	20.0
SER36	D1: N-term	10.97	PHE27	HCDR1	14.0	TYR49	FR2-IMGT	56.8

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LYS88	D1: β3-β4 loop	235.1	THR28	HCDR1	26.0	THR56	FR3-IMGT	78.8
LYS89	D1: β3-β4 loop	40.3	ASP31	HCDR1	24.4	SER67	FR3-IMGT	39.2
SER90	D1: β3-β4 loop	45.4	TYR32	HCDR1	24.7			
LYS101	D1: β4-β5 loop	81.1	ASP52C	HCDR2	48.3			
GLN104	D1: β4-β5 loop	61.0	GLU53	HCDR2	17.5			
SER119	D1: α1	20.8	ARG94	HCDR3	13.6			
TYR126	D1: α1-β7 loop	47.0	GLY96	HCDR3	23.8			
GLU127	D1: β7	41.9	LEU97	HCDR3	60.2			
ILE128	D1: β7	41.9	ARG98	HCDR3	86.1			
GLU129	D1: β7	61.1	TRP99	HCDR3	58.6			
GLU130	D1: β7	76.2	TYR100	HCDR3	96.1			
ASN131	D1: β7	51.4	ASP100A	HCDR3	35.2			
ASP132	D1: β7-β8 loop	71.4	SER100B	HCDR3	68.9			
THR133	D1: β7-β8 loop	113.1	ASP101	HCDR3	29.5			
ASN134	D1: β7-β8 loop	24.3						
PRO135	D1: β7-β8 loop	25.5						
ARG140	D1: β8	5.7						

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831 **Table S5: Oligonucleotides used in this study.** Red or green nucleotides depict restriction sites used for cloning.
832 Underlined nucleotides were used for overlap-PCR. Blue nucleotides highlight the CRISPR/Cas9 guide RNA
833 sequence. Orange nucleotides are three sequential guanosine nucleotides used for template switching, as described in
834 (76). Related to **Methods.**

Name	Function	Sequence
HDR template - primers		
p1	5'HR(-A) Pfs230-tag FW	cagttCGTCTCtggagCCACCATATGTACATAAAGATATACATTTCTCATTAG
p2	5'HR-A Pfs230-tag RV	aGCcCTactcTGAGGTTCTGGGATTATATAATTAGGATCAAATGTG
p3	5'HR Pfs230-tag RV	gtcaaCGTCTCataccAAGCTTTCTCAAGTATTTTG
p4	3'HR Pfs230-tag FW	cagttCGTCTCtgcacTCGTAAATAATTAATCAAACATATATATAATCAAAAGG
p5	3'HR Pfs230-tag RV	cagttCGTCTCtatggGGACTCTAAGATATTTCTTTCGCTAATCC
p6	H2B_mScarlet_biUTR-A FW	cagttGGTCTCTggagCGTCTCttaagCTAAATGGATGATTCCCCTCTTTGCAATATG
p7	H2B_mScarlet_biUTR-A RV	CAGTTggtctctTGACatttcatatttgcatgtgcatattaaaagtatcg
p8	H2B_mScarlet_biUTR-B FW	cagttggtctctGTCAgtctctgcataattttcgctatttaaatatat
p9	H2B_mScarlet_biUTR-B RV	cagttGGTCTCTatggCGTCTCtGTGCgTTCGAATCAACTCCGTTCAGTTAAAAATATTAC
p10	5'HR Pfs230ΔD13-14 FW	CAGTcgtctcTagagGGGATGATGTACATTTATTTTATCCTCCTC
p11	5'HR Pfs230ΔD13-14 RV	CAGTcgtctcTatccaccTGTTTTTTTTTCATCTATTTTTATTTTATCCATTGTAC
p12	5'HR Pfs230ΔCterm FW	CAGTcgtctcTagagCAATTGGTAAAGATATATGTAAATATGATGTTACTAC
p13	5'HR Pfs230ΔCterm RV	CAGTcgtctcTatccaccATTAGGATCAAATGTGATTCGTATAGTATAATTTG
p4	3'HR Pfs230Δ FW	See p4
p14	3'HR Pfs230Δ RV	cagtCGTCTCtcggtGGACTCTAAGATATTTCTTTCGCTAATCC
p15	GAPDH_swap FW	cagtGCTAGCGAAAAGAATTAAAAAGCCGAAGAAAAAATATATG
p16	GAPDH_swap RV	cagtGAGCTCGAGCTATGAAAAACATGGGTGTG
p17	C-terminal 3xFLAG FW	cagtGAATTcgtctcTggatCTGGTGATTACAAAGATCATGATGG
p18	C-terminal 3xFLAG RV	cagtAAGCTTcgtctcTcttaGTCATGATCCTTGTAATCTATATCGTG
HDR template - oligonucleotides		
p19	5'HR-B anneal Pfs230 FW	CTAATTATATAATCCCAGAACCTCAgagtAGgGCtATCATTAAATATGTAGATCTGCAAGATAAAAATTTTGCAAAATACTTGAGAAAGCTTggtaTGAGACGttgac
p20	5'HR-B anneal Pfs230 RV	gtcaaCGTCTCataccAAGCTTTCTCAAGTATTTTGCAAAATTTTTATCTTGCAGATCTACATATTTAATGATaGCcCTactcTGAGGTTCTGGGATTATATAATTAG
p21	FLAG-C-tag FW	gatccgtctctGGTACATCTGGTGATTACAAAGATCATGATGGAGATTATAAAGATCACGATATAGATTACAAGGATCATGACGGTTCAGGTGAACCAGAAGCATAAgtGAGACG
p22	FLAG-C-tag RV	aattCGTCTCacTTATGCTTCTGGTTCACCTGAACCGTCATGATCCTTGTAATCTATATCGTGATCTTTATAATCTCCATCATGATCTTTGTAATCACCAGATGTACCagagacg
CRISPR/Cas9 guide plasmid oligonucleotides		
p23	Guide Pfs230 Cterm FW	tattgTTAATGATGGCTCTTGATTG
p24	Guide Pfs230 Cterm RV	aaacCAATCAAGAGCCATCATTAAc
p25	Guide Pfs230 D13 FW	tattgATTCTATTACATTATCAAGA
p26	Guide Pfs230 D13 RV	aaacTCTTGATAATGTAATAGAATc
Template-switch oligonucleotide for hybridoma sequencing		
p27	RT-PCR template switch FW	*AAGCAGTGGTATCAACGCAGAGTACAT*rGrGrG
p28	RT-PCR for mIgK RV	TTGTCGTTCACTGCCATCAATC
p29	RT-PCR for mIgH RV	AGCTGGGAAGGTGTGCACAC
p30	Universal ISPCR primer FW	AAGCAGTGGTATCAACGCAGAG

| p31 | mIgK RV | ACATTGATGTCTTTGGGGTAGAAG |
| p32 | mIgH RV | GGGATCCAGAGTTCCAGGTC |

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836 **Table S6: Amino acid sequence of variable chains of mAbs 18F25 and 2A2.** Related to **Methods.**

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mAb	Region	Amino acid sequence
18F25	Heavy chain (VDJ)	QVQLQQSGAELARPGASVKLSCKASGYTFTNYWMQWVKQRPGQGLEWFGAIYPGDGD TRYTQKFKGKATLTADKSSSTAYMQLSSLASEDSAVYYCARSLYLGAMDYWGQGTSVTVSS
	Light chain (VJ)	DIVMTQSHKFMSTSVGDRVSITCKASQDVTNDVAWYQQKPGQSPKLLIYSAS YRYTGVPDRFTGSGSGTDFIFTISSVQAEDLAVYYCQQHYSAPPTFGGGTKLEIK
2A2	Heavy chain (VDJ)	EVQLQQSGPELEKPGASVKISCKASGYSITDYNMNWVKLSNGKSLEWIGNIDPSHGGTTY NQKFKGKATLTVDKSSSTAYMQLKSLTSEDSAVYHCARSTLYGNSAMDCWGQGTSVTVSS
	Light chain (VJ)	SIVMTQTPKFLLVPAGDRVTITCKASQSVNNDVTWYQQKPGQSPKLLIYYASN RYTGVPDRFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYSSPLTFGAGTKLEL

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Methods

1103

Plasmodium falciparum genome modification plasmids

1104

To create the Cas9 guide plasmids, a pair of complementary guide-encoding oligonucleotides 5'-TTAATGATGGCTCTTGATTG-3' (targeting Pfs230 C-terminus to create iGP2$^{230\text{-tag}}$ / 230$^{\Delta D13/D14}$ / 230$^{\Delta Cterm}$) and 5'-ATTCTATTACATTATCAAGA-3' (targeting Pfs230D13 to create 230$^{\Delta D13/14}$) were treated with T4 polynucleotide kinase, annealed, and ligated into BbsI-digested pMLB626 (a kind gift from Marcus Lee) (77).

1110

To generate the iGP2$^{230\text{-tag}}$ homology-directed repair (HDR) template, partial 5' and full 3' homology regions (HR) were PCR amplified from NF54 genomic DNA (**Table S5**). Synonymous mutations to shield the Cas9 guide region in the full 5'HR were inserted by overlap PCR using the partial 5'HR and two annealed custom-made oligonucleotides (Sigma-Aldrich, **Table S5**). A selection cassette containing the i) PfH2B promoter, ii) the *mScarlet* gene, and iii) a bidirectional 3'UTR (PBANKA_142660) was PCR amplified from an in-house plasmid (78) as two separate amplicons to remove internal restriction sites. These two amplicons were cloned into pGGASelect using a BsaI-HFv2 Golden Gate reaction, and further sub-cloned using AatII and BamHI-HF into a pUC19-based plasmid that had all internal type IIS restriction enzymes removed via site-directed mutagenesis, yielding pRF0508. The final HDR template was created using the NEBridge Golden Gate Assembly Kit (BsmBI-v2), combining i) pGGAselect, ii) the 5' HR amplicon, iii) two annealed oligonucleotides coding for a tandem affinity purification tag (GTSG-(3xFLAG)-GSG-EPEA-stop) (**Table S5**), iv) pRF0508, and v) the 3' HR amplicon.

1123

To generate HDR templates to make Pfs230$^{\Delta D13/14}$ and Pfs230$^{\Delta Cterm}$, the respective 5'HRs (Pfs230$^{\Delta D13/D14}$ spanning Pfs230D11-12, Pfs230$^{\Delta Cterm}$ spanning Pfs230D13-14) and 3'HR were PCR amplified from NF54 genomic DNA (**Table S5**). The selection cassette was modified by swapping the PfH2B promoter in pRF0508 for the PfGAPDH promoter (Pf3D7_1462800). The HDR templates were created in a golden gate reaction combining i) pGGAselect, ii) respective 5'HR amplicon, iii) PCR amplicon coding for (GGSG-3xFLAG-stop), iv) GAPDH selection marker, and v) the 3' HR amplicon.

1130

All PCR reactions were performed using Primestar® GXL DNA polymerase (Takara Bio), all other cloning enzymes were obtained via New England Biolabs. Sequences were confirmed by Sanger Sequencing (Baseclear).

1134

Plasmodium falciparum culturing, transfections, and gametocyte production

1136

P. falciparum cultures were maintained at 37 °C with 3% O_2 and 4% CO_2, in complete medium (RPMI1640 medium with 25 mM HEPES, 25 mM NaHCO$_3$, 10% human type A serum) with 5% O$^+$ human red blood cells (Sanquin, The Netherlands) (79). Asexual NF54/iGP2 and NF54/iGP2$^{230\text{-tag}}$ parasites were cultured in the presence of 2.5 mM D-(+)-glucosamine hydrochloride (Sigma #1514). Parasites were synchronized using sorbitol treatment before transfection or gametocyte induction (80).

1142

For transfection, 80 μg of HDR plasmid was linearized overnight, ethanol precipitated and co-transfected with 80 μg of Cas9 guide plasmid by ring-stage transfection as described previously (81, 82). Briefly, plasmids were resuspended in cytomix (10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.6, 120 mM KCl, 0.15 mM CaCl$_2$, 5 mM MgCl$_2$, 25 mM HEPES, 2 mM EDTA) and added to a 3% ring-stage NF54/iGP2 (to generate iGP2$^{230\text{-tag}}$) or NF54 (to generate 230$^{\Delta D13/D14}$ / 230$^{\Delta Cterm}$) culture. Parasites were electroporated (310 V, 950 μF), allowed to recover for 4 h, after which transfected parasites were selected using 2.5 nM WR99210 (Jacobus Pharmaceuticals) for five days. To obtain single-cell clones for iGP2$^{230\text{-tag}}$, late-stage parasites were stained

1150 using 1 μg/ml Rhodamine123 solution, and single-cell sorted using a FACSAria III Cell Sorter (BD
1151 Biosciences) in a round-bottom 96 well plate containing 100 μl complete medium with 3% haematocrit.
1152 To obtain an isogenic population for 230$^{\Delta D13/D14}$ and 230$^{\Delta Cterm}$, parasites were sorted based on mScarlet
1153 expression using a Cytoflex SRT Benctop Cell Sorter (Beckman Coulter). Successful integration was
1154 confirmed by diagnostic PCR (**Table S5**).

1156 To induce gametocytogenesis, a 1% early trophozoite culture (±24 hours post invasion) was induced with
1157 AlbuMAX medium (RPMI1640 supplemented with 25 mM HEPES, 25 mM NaHCO$_3$, and 0.5% AlbuMAX-II
1158 (Gibco, #11021-045)), in a previously described automatic shaker setup with media changes every 12 h
1159 (83, 84). Additionally, glucosamine was removed from iGP2-based cultures to further increase gametocyte
1160 induction. 36 h post induction until completion, cultures were maintained in complete medium. From day
1161 4 – 8 post induction, gametocytes were cultured in the presence of 20 units/ml heparin (Sigma, #H3393)
1162 to eliminate asexual parasite growth. To obtain late-stage gametocyte saponin pellets, gametocytes were
1163 harvested on day 13 post induction by incubating the cells for 10 min in a 10x pellet volume ice-cold 0.06%
1164 (w/v) saponin in PBS supplemented with 1x Complete™ Protease Inhibitor (PI) Cocktail (Roche), and
1165 subsequent centrifugation at 3,000xg for 5 min at 4 °C. Pellets were washed three times in ice-cold PBS
1166 with 1x PI, flash frozen and stored at -70 °C.

1168 **Western blot**

1170 Saponin pellets from late-stage gametocyte cultures were resuspended in lysis buffer (10 mM Tris-HCl (pH
1171 8.0), 150 mM NaCl, 1% (w/v) sodium deoxycholate, and 1x PI) for 15 min at RT. Lysate was cleared by
1172 centrifugation, and supernatant was supplemented with 1x NuPAGE LDS buffer, and heated to 56 °C for
1173 15 min. Samples were loaded on 4-12% Bis-Tris SDS-PAGE gels (SurePAGE), using Precision Plus Dual Color
1174 (BioRad) as a reference. After electrophoresis, proteins were transferred to 0.45 μm Immun-Blot PVDF
1175 membrane (Bio-Rad) using the Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in
1176 PBS with 3% BSA, before incubation with primary antibody diluted in PBS-T with 1% BSA. The following
1177 primary antibodies were used: 18F25 (mouse anti-Pfs230 (38), 5 μg/ml), 32F3 (mouse anti-Pfs48/45 (27),
1178 5 μg/ml), M2 (mouse anti-FLAG, F1804 Sigma, 1:2000), CaptureSelect™ (biotinylated single domain
1179 antibody fragment anti-C-tag, Thermofisher, 1:1000). After washing, blots were incubated with one of the
1180 following secondary antibodies: HRP-conjugated rabbit anti-mouse (P0260, Dako, 1:2000), HRP-
1181 conjugated streptavidin (890803, R&D Systems, 1:500). Blots were developed with Clarity Western ECL
1182 substrate (BioRad) and imaged on an ImageQuant™ LAS 4000 (GE Healthcare).

1184 **Immuno-fluorescence microscopy**

1186 Heparin-treated stage V gametocytes (day 13) were fixed (4% EM-grade paraformaldehyde, 0.0075%
1187 glutaraldehyde in PBS) for 20 min on pre-warmed poly-L-lysine coated coverslips at 37 °C. Cells were
1188 permeabilized with 0.1% Triton X-100, washed in PBS and blocked using 3% BSA in PBS for 1 h. Primary
1189 and secondary antibodies were incubated for 1 h at RT, with PBS washes in between. Primary antibodies
1190 were diluted in 1% BSA in PBS, and included M2 (mouse anti-FLAG, F1804 (Sigma), 1:500), RUPA-55 mAb
1191 (human anti-Pfs230 (22), 5 μg/ml), and 45.1 mAb (rat anti-Pfs48/45 (27), 5 μg/ml). Secondary antibodies
1192 (goat anti-mouse 594 (A11031), goat anti-human 488 (A11013), and chicken anti-rat 674 (A21472)
1193 (ThermoFisher)) were all diluted 1:500 in PBS with 1 μM DAPI. Coverslips were mounted using
1194 Vectashield® Antifade Mounting Medium (Vector Laboratories). Images were taken on a Zeiss LSM900
1195 Airyscan confocal microscope using a 63x oil objective with 405, 488, 561, and 633 nm laser excitation
1196 and an Electronically Switchable Illumination and Detection Model (ESID) for transmitted light. Images
1197 were processed for airyscan, using the Zeiss Zen Blue software and analysed using FIJI software (85).

55

Suspension Immunofluorescence Microscopy (SIFA)

For gamete SIFAs, heparin-treated stage V gametocytes (day 13 post-induction) were collected, spun down to remove the culture medium, and resuspended in half the culture volume of FCS. After incubation with gentle agitation at RT for 1 h, parasites were spun down and washed in ice-cold PBS. For saponin-treated gametocytes, heparin-treated stage V gametocytes (day 13 post-induction) were collected, spun down, and resuspended in half the volume of ice-cold PBS supplemented with 0.06% Saponin and 1x protease inhibitor. After 10 min incubation on ice, saponin-treated gametocytes were harvested by centrifugation and washed three times in ice-cold SIFA buffer (PBS supplemented with 0.5% FCS). All subsequent steps were performed at 4 °C.

Both gametes and gametocytes were incubated for 1 h in SIFA buffer with 15 µg/ml mAb 18F25 or 45.1, labeled with LYNX Rapid Plus DyLight488 Antibody Conjugation Kit (Bio-Rad). After washing in ice-cold SIFA buffer, parasites were resuspended in ice-cold PBS and deposited in pre-cooled poly-L-lysin-coated µ-slide 8 well Ibidi chambers. Parasites were allowed to settle at 4 °C, after which the Ibidi chamber was transferred to a room-temperature Axio Observer 7 Inverted LED microscope equipped with a Colibri 7 LED source and Axiocam 705 mono (Zeiss). Images were taken with a 63x oil objective using the 475 nm LED module and a Differential Interference Contrast enhanced brightfield channel. Images for different parasite lines were taken on the same day with the same microscope settings, and were analyzed with the same settings in FIJI software (85).

Exflagellation assay and standard membrane feeding assay

Day 15 cultures of non-heparin treated gametocytes were used to test exflagellation. Cultures were mixed 1:1 with 50 µM xanthurenic acid in complete media, and incubated at room temperature in a dark, humid chamber for 15 min. The number of exflagellation centres per ml was determined by bright-field microscopy using a Neubauer chamber. To assess mosquito infectivity, day 15 non-heparin treated gametocytes were added to the bloodmeal of *Anopheles stephensi* mosquitoes (colony maintained at Radboudumc (Nijmegen, The Netherlands)), as described previously (86). Mosquito midguts from 10 - 20 mosquitoes were dissected 6-8 days after the blood meal, stained with mercurochrome, and oocysts were counted by microscopy.

Affinity purification of Pfs230:Pfs48/45 complex from stage V gametocytes

Frozen stage V gametocyte pellets were resuspended in solubilization buffer (25 mM HEPES pH 7.4, 150 mM KCl, 10% glycerol, 1x PI, and 0.25% (v/v) DDM), and rotated at 4 °C for 1 h. The cell suspension was centrifuged at 18,000xg at 4 °C for 30 min, after which the pellet was solubilized a second time following the previous steps. The combined supernatants were applied to anti-FLAG® M2 affinity resin (Merck), and incubated overnight at 4 °C. The resin was extensively washed in Wash buffer (25 mM HEPES pH 7.4, 150 mM KCl, 10% glycerol, 1x PI, and 0.02% DDM), after which proteins were eluted using 5x resin volume of elution buffer (25 mM HEPES pH 7.4, 150 mM KCl) supplemented with 0.02% DDM, and 150 µg/ml 3xFLAG peptide (Merck)). For cryo-EM studies, the eluted proteins were mixed with a fivefold molar excess of RUPA-97, LMIV230-01, 2A2, 18F25, RUPA-71, and RUPA-44 Fabs and incubated on ice for 30 min. After concentrating the protein fraction, the Fab-bound complex was separated from unbound Fabs by high-pressure size exclusion chromatography using a Bio SEC-3 300 Å column (Agilent, 5190-5213), pre-equilibrated with elution buffer.

Expression and purification of Fabs, mAbs, and recombinant proteins

Expression and purification of individual Fabs was performed as described previously (22, 34). Variable light and heavy chains were gene synthesized (GeneArt), and cloned into a custom pcDNA3.4 expression vector directly upstream of the constant chains. The variable chain sequences for 32F3, TB31F, LMIV-230s, and all RUPA mAbs were described previously (22, 34, 43). Variable sequences for 18F25 (**Table S6**) were determined by a previously published workflow for hybridoma sequencing (76). In short, RNA was obtained from 18F25.1 hybridoma cells by Trizol purification, which was used as template for RT-PCR with heavy/kappa constant chain specific reverse primer and a universal template-switching oligonucleotide (**Table S5**). The obtained cDNA was used for a PCR, and obtained PCR products were inserted into a TOPO vector, and sequenced by Sanger Sequencing (Baseclear, The Netherlands). Variable sequences for 2A2 were sequenced by Genmab (**Table S6**). For the IgGs produced for complement-fixation assays, sequences of the variable chain of α-Pfs230D1 IgGs were described previously (22), and ordered as synthetic products subcloned into custom-made pcDNA3.4 vectors directly upstream of Igγ1-CH1-CH3 or Igκ-CL1.

Fab and IgG purification were done in a similar manner. Plasmids coding for Fab/IgG heavy chain and light chain were co-transfected at a 1:1 molar ratio using PEI MAX® transfection reagent (PolySciences) into Freestyle 293-F or 293-S cells (ThermoFisher). Cells were cultured in FreeStyle 293 Expression medium (Gibco) at 37 °C and 8% CO_2 while shaking for seven days, after which the supernatant containing secreted recombinant protein was collected. After filtering, the supernatant was used for affinity purification using a HiTrap Kappaselect column (Cytiva) for recombinant Fab, or the HiTrap Protein G HP column (Cytiva) for recombinant IgG, pre-equilibrated in 1x PBS. In both cases, recombinant protein was eluted in 100 mM glycine (pH 2.2). Fabs were further purified by cation-exchange chromatography using a MonoS column (Cytiva) with a buffer of 20 mM sodium acetate (pH 5.6) across a 0-1.0 M potassium chloride gradient.

Single Pfs230 domains Pfs230D1 and Pfs230D10 were expressed and purified as described previously (39). Coding sequences for the double Pfs230 domain D13-14-Cterm (residue 2831-3105) were ordered and synthesized as *Drosophila melanogaster* codon-optimized sequences (Baseclear). The synthetic gene was subcloned into pExpreS2.2 plasmid (ExpreS2ion Biotechnologies), in frame with an N-terminal BiP signal peptide – His_6 tag. The expression plasmid was used to transfect *D. melanogaster* S2 cells (ExpreS2ion Biotechnologies) to generate stable cell lines as described previously (39). Pfs230D13-14-Cterm-containing supernatant was loaded directly on a 1 ml cOmplete™ His-Tag Purification Column (Roche). The column was washed with PBS, and Pfs230D13-14-Cterm was eluted using PBS with 250 mM imidazole. After overnight dialysis against PBS, the sample was concentrated and further purified by size-exclusion chromatography using a Superdex200 Increase column (Cytiva), using PBS as running buffer.

Blue Native Polyacrylamide Gel Electrophoresis

For BN-PAGE, the elution fraction from FLAG-based affinity purification, containing purified Pfs230:Pfs48/45 protein complex, was used. To test Fab binding to the complex, a tenfold molar excess of Fab fragment was added to the purified Pfs230:Pfs48/45 complex, and incubated on ice for 30 min. Protein samples were supplemented with a home-made 1x loading solution (final concentration 10% glycerol, 0.025% Coomassie Blue G-250), and loaded on a NativePAGE™ 3-12% Bis-Tris protein gels (ThermoFisher) following manufacturer's protocols. NativeMark™ Unstained Protein Standard was used as marker. After electrophoresis, gels were either stained with Coomassie InstantBlue® protein stain (Abcam) or incubated in 0.1% SDS for western blotting. For western blotting, proteins were transferred to 0.45 µm Immun-Blot PVDF membrane (BioRad) using the Trans-Blot Turbo Transfer system (BioRad). After transfer, proteins were fixed to the membrane using 8% acetic acid. Membranes were air-dried, washed

1294 in pure methanol and re-hydrated using MQ. Blocking, antibody incubation and detection were performed
1295 as described above.
1296
1297 **Mass spectrometry**
1298
1299 Three batches of 40 ml heparin-treated, late-stage gametocyte cultures (iGP2$^{230\text{-tag}}$ and iGP2 WT) of
1300 comparable gametocytaemia were harvested and lysed in parallel, as described as above for purification
1301 of the endogenous Pfs230:Pfs48/45 complex. Protein concentration in parasite lysate was determined
1302 using the PierceTM BCA Protein Assay kit. 120 µg of protein extract in 300 µl solubilization buffer (25 mM
1303 HEPES pH 7.4, 150 mM KCl, 10% glycerol, 1x PI, and 0.25% (v/v) DDM) was applied on 10 µl anti-FLAG®
1304 M2 affinity resin, and incubated overnight. The resin was washed in ice-cold wash buffer (25 mM HEPES
1305 pH 7.4, 150 mM KCl, 10% glycerol, 1x PI, and 0.02% DDM) three times, and consequently washed three
1306 times in ice-cold PBS (Gibco). After harvesting, beads were resuspended in 50 µl elution buffer (100 mM
1307 Tris-HCl pH 8.0, 2 M Urea, 10 mM DTT), and incubated for 20 min at 25 °C with shaking. 50 mM
1308 iodoacetamide was added to alkylate cysteines, after which samples were kept in the dark for 10 min at
1309 25 °C. After adding 0.25 µg sequencing grade trypsin (Promega), samples were incubated at 25 °C for 2 h
1310 with gentle shaking. Samples were spun down, supernatants were collected, and the beads were
1311 resuspended in 50 µl fresh elution buffer. After 5 min incubation on a shaker, the samples were spun
1312 down again and the supernatant combined with the previous elution fraction. Another 0.1 µg of fresh
1313 trypsin was added to the combined supernatants, which was left to digest overnight at 25 °C. The following
1314 day, samples were concentrated and purified on C18 StageTips (87). Samples were analyzed on a Obitrap
1315 Exploris 480 mass spectrometer (ThermoScientific) ran in Top20 mode (with dynamic exclusion enabled
1316 for 45 s), operated with an online Easy-nLC 1000. A gradient of buffer B (80% acetonitrile, 0.1% formic
1317 acid) was applied for 60 min. Raw data was analyzed using Maxquant software (version 2.1.4.0), and
1318 analyzed against a *Plasmodium* database (PlasmoDB, downloaded 21-10-2022). LFQ, iBAQ and match
1319 between runs were enabled, and deamidation (NQ) was added as additional variable modification. The
1320 resulting output was filtered using Perseus (version 1.0.15), removing potential contaminants, reverse
1321 hits, and proteins with less than two unique peptides or with less than three valid values in at least one
1322 group. Missing values were imputed using default settings, and a t-test was performed to identify outliers.
1323 Data was visualized using GraphPad Prism.
1324
1325 **Cryo-EM data collection and image processing**
1326
1327 The Pfs230:Pfs48/45:RUPA-97:LMIV230-01:2A2:18F25:RUPA-71:RUPA-44 Fab complex was concentrated
1328 to 0.8 mg/ml, and 1.8 µl sample was deposited on homemade holey gold grids (88) that were glow-
1329 discharged in air for 15 s. Excess sample was blotted away for 1.7 s using a Leica EM GP2 Automatic Plunge
1330 freezer at 4 °C and 90% humidity, and plunge-frozen in liquid ethane. Data were collected on a Thermo
1331 Fisher Scientific Titan Krios G3 equipped with a Selectris X energy filter (slit width: 10 eV) and Falcon 4i
1332 camera, operated at 300 kV and automated with the EPU software. Data was collected at a magnification
1333 of 130,000× with a calibrated pixel size of 0.93 Å. Exposures were collected for 6.5 s as movies with a
1334 camera exposure rate of ~7.11 or ~7.15 e⁻ per pixel per s, and total exposure of ~50 or ~53.7 electrons/Å^2
1335 and recorded in Electron Event Representation mode (89). Before processing, movies were fractionated
1336 into 40 frames. A total of 8193, 1572, and 6885 raw movies were obtained at 0º, 35º, and 40º tilt,
1337 respectively.
1338
1339 The collected cryo-EM data was processed using CryoSPARC v4.6.0 (90). The movies from the 35° tilted,
1340 40° tilted, and untilted data collections were corrected for motion in patches, and contrast transfer
1341 function (CTF) parameters were estimated in patches. Resulting micrographs were curated using

1342 thresholds for average defocus values (4000-35,000 Å), CTF fit resolution (2.0-5.0 Å), and relative ice
1343 thickness (0.95-1.10). An *in silico* model of the Pfs230:Pfs48/45:RUPA-97:LMIV230-01:2A2:18F25:RUPA-
1344 71:RUPA-44 complex was built from AF models and arranged based on views seen from 2D classes. The
1345 resulting model was used to generate particle templates corresponding to 40 evenly distributed
1346 projections which were low-pass filtered to 20 Å to avoid model bias. Initially, particle images were
1347 selected from a subset of 2000 curated micrographs, which were then extracted in 450x450 pixel boxes
1348 and subjected to 2D classification. Classes where the average showed clear views of the complex were
1349 used to train a Topaz particle selection model (91), with subsequent 2D classification steps used to clean
1350 the dataset of selected particle images. To focus on the various subsections of the full complex, 2D class
1351 average images showing the appropriate domains were used as templates to select more particles from
1352 the full dataset of 12,192 curated micrographs. Further Topaz models were trained on specific regions
1353 (Pfs230D1-6:RUPA-97:LMIV230-01:2A2, Pfs230D9-14:Pfs48/45:RUPA-71:RUPA-44) of the full complex,
1354 after which particles images were selected and extracted with the trained models. Initial junk particle
1355 image removal was performed with multiple rounds of 2D classification, followed by *ab initio* 3D
1356 reconstruction. Non-particle images and poorly behaved particle images were removed with a
1357 combination of heterogeneous refinement, 3D classification, and 2D classification. Selected particle
1358 images were used to generate 3D maps with reconstructions in non-uniform refinement (92) jobs without
1359 enforced symmetry (C1). The resulting maps were further improved by local refinement and post-
1360 processing with DeepEMhancer (93). Heterogeneity for 2A2 Fab binding could be distinctly observed, such
1361 that two maps of Pfs230D1-6:LMIV230-01:RUPA-97 were obtained: with and without 2A2 Fab. A detailed
1362 processing flow path and experimental map details are available in the supplementary document (**Figure**
1363 **S2, S3A-S3D**).
1364
1365 For the Pfs230D7-8:18F25 map, particle images from a subset of 500 micrographs were used from one set
1366 of collections of the untilted (2003), 35° tilted (337), and 40° tilted (2866) micrographs for blob picking
1367 after motion correction, CTF estimation, and curation of the micrographs (average defocus = 1000-30,000
1368 Å; CTF fit resolution < 5 Å; relative ice thickness = 0.954-1.1; astigmatism < 4000 Å). The 101,225 particle
1369 stack was cleaned using multiple rounds of 2D classification, ab initio reconstruction, and homogeneous
1370 refinement (4.89 Å). The rebalance orientations job was used, followed by ab initio reconstruction and
1371 non-uniform refinement (4.19 Å). A map containing the interface between Pfs230 and 18F25 Fab was
1372 solved by cleaning the particle stack with 2D classification, followed by ab initio reconstruction and
1373 homogeneous refinement (10.03 Å). This map, along with the 4.19 Å map and a junk class generated with
1374 ab initio reconstruction were used as input for heterogeneous refinement, resulting in a class containing
1375 18F25 with 35,162 particles. The resulting particle stack was cleaned with multiple rounds of 2D
1376 classification, with the final particle stack used for Topaz training on denoised micrographs. Ab initio
1377 reconstruction with five classes was run using the resulting 100,481 particle stack, followed by
1378 heterogeneous refinement. The particle stack from the class containing 18F25 was cleaned using 2D
1379 classification, then used again for Topaz training on all micrographs (6372 untilted, 1125 35° tilted, 2866
1380 40° tilted). To ensure the particles were centered on 18F25, ab initio reconstruction, followed by non-
1381 uniform refinement was run with the resulting 366,876 particle stack at a box size of 600 pix and Fourier
1382 crop of 300 pix (5.79 Å), followed by centering of the particles using volume alignment tools and re-
1383 extraction of the particles to 300 pix boxes. 2D classification without re-centering, 2 class ab initio
1384 reconstruction, and heterogeneous refinement were used to obtain a clean 216,684 particle stack. A
1385 further three rounds of 2D classification and two rounds of non-uniform refinement and local refinement
1386 resulted in a 3.88 Å map using 106,349 particles. Post-processing was performed in EMReady to prepare
1387 the map for model building. A detailed processing workflow and experimental map details are available
1388 in the supplementary document (**Figure S2, S3E**).
1389

X-Ray Crystallography and Structural Determination

Fractions containing purified 2A2, 18F25, and RUPA-71 Fab following cationic exchange chromatography were pooled and concentrated at 16.8 mg/mL, 27.3 mg/mL, and 10 mg/ml, respectively in preparation for crystallization trials. RUPA-71 Fab was mixed in a 1:1 ratio with reservoir conditions from a JCSG Top96 screen (0.2 µL + 0.2 µL) using the sitting drop vapor diffusion method. RUPA-71 crystals then formed in a drop containing 0.2 M (NH4)$_2$SO$_4$, 0.1 M MES 6.5 pH, and 30 %w/v PEG MME 5K. For the 2A2 and 18F25 Fab crystallization trials, reservoir conditions from a JCSG Top96 screen and Fab were mixed at a 1:1 volumetric ratio (0.3 µL + 0.3 µL) resulting in 0.6 µL drops. Approximately 48 h after setting drops at room temperature, 2A2 crystals were obtained from a drop containing reservoir solution: 30% (w/v) PEG 4K. 18F25 crystals were obtained from a drop containing reservoir solution: 0.2 M Na$_2$SO$_4$ and 20% (w/v) PEG 3350. The 2A2 and 18F25 crystals were cryo-protected with 25% glycerol (v/v) and 15% polyethylene glycol 200 (PEG200), respectively. Crystals of all three samples were flash-frozen in liquid nitrogen. Data collection on 2A2 and 18F25 was performed at the 17-ID-1 beamline at the Brookhaven National Laboratory Synchrotron Light Source. RUPA-71 data collection occurred at the CMCF-ID beamline at the Canadian Light Source. Datasets were processed using autoproc (94) and xds (95). A molecular replacement solution was obtained using PhaserMR (96). Model building and refinement was performed using Coot (97) and phenix.refine (98). Inter- and intra-molecular contacts were determined using PISA (99) and manual inspection. Structural figures were generated using UCSF ChimeraX (100).

Cryo-EM model building

Starting structural models were obtained by manually fitting previously determined structural models (PDB: 8U1P, Pfs48/45-D1D2; 7UXL, Pfs48/45-D3:RUPA-44; 7UVQ, Pfs230-D1:RUPA-97; and 7UFW, Pfs230-D1D2:LMIV230-01), novel crystal structures (Fabs of 18F25, RUPA-71, and 2A2), and models generated by AlphaFold2 (101) and AlphaFold3 (64) into the experimentally determined maps. Structural refinements were performed using Isolde (102), PyRosetta (103), and PHENIX (104); models were manually checked and improved with Coot (97). Images generated using ChimeraX (100). Access to all software was supported through SBGrid (105).

Single nucleotide polymorphism detection

Single nucleotide polymorphisms were obtained from the MalariaGEN Catalogue of Genetic Variation in Pf version 7 (54). Genotype calls for chromosomes 2 and 13 (Pf3D7_02_v3 and Pf3D7_13_v3, respectively) were downloaded (https://ngs.sanger.ac.uk/production/malaria/Resource/34/Pf7_vcf/). Bcftools (106) was used to subset calls and calculate the allele frequencies between nucleotide positions 369,351 and 380,156(+) on chromosome 2 that coincide with Pfs230 and between nucleotide positions 1,875,452 and 1,878,087(-) on chromosome 13 that coincide with Pfs48/45. SNPs calls below MalariaGEN's quality filter (Low_VQSLOD) were removed before identification of missense variants.

Gamete binding assay with flow cytometry

Heparin treated stage V gametocytes (day 13 – 16 post induction) were collected and resuspended in FCS with half the volume of the original culture volume. Gamete maturation was induced for 30 min at room temperature while shaking, after which samples were centrifuged at 2,000xg at 4 °C. The pellet was resuspended in PBS and placed on a layer of 12.4% Nycodenz (Serumwerk Bernburg) and centrifuged for 30 min at 3,500xg without break at 4°C. The top layer containing female gametes was harvested and washed in PBS before continuing.

50,000 female gametes per well were deposited in a V-bottom non-treated 96-well plate (Costar). All gamete incubations were carried out at RT in PBS supplemented with 2% Fetal Calf Serum (FCS, Gibco) and 0.02% sodium azide, and washed in ice-cold PBS. All secondary antibody incubations included eBioscience™ Fixable Viability Dye eFluor™ 780 (Invitrogen, 1:1000). For the Pfs230 domain binding experiment (**Figure S6A**), gametes were incubated for 1 h with recombinant proteins and washed in PBS. Afterwards, gametes were incubated with mouse anti-his tag antibody (Sigma-Aldrich, A7058, 1:200) for 1 h, washed, and stained with chicken-anti-mouse 488 secondary antibodies (Invitrogen, A21200, 1:200) for 30 min. For the antibody gamete binding experiment (**Figure S6H, Figure 5G**), gametes were incubated with 1 μg/ml antibody for 1 h, washed, and stained with goat-anti-human 488 secondary antibody (Invitrogen, A11013, 1:200). For the Fab competition experiment (**Figure S7C**), gametes were incubated for 1 h with Fabs. After washing in PBS, gametes were stained with 18F25 antibody labeled with LYNX Rapid Plus DyLight488 Antibody Conjugation Kit (Bio-Rad, 5 μg/ml). Finally, for complement deposition assay, gametes were incubated with 1 μg/ml antibody for 1 h and washed. Gametes were then incubated with 10% Normal Human Serum (Sanquin, The Netherlands) and incubated for 45 min at RT. After washing, parasites were first stained with polyclonal goat anti-human C1q (CompTech, 1:5,000) or anti-human C3 (CompTech, 1:10,000), washed, and finally stained with donkey-anti-goat 488 secondary antibody (Invitrogen, A11055, 1:200). After staining, gametes were resuspended in PBS and fluorescence was measured for approximately 2,000 gametes with the Gallios™ 10-color system (Beckman Coulter), and analyzed with FlowJo (BD, version v10.10.0). For all gamete binding experiments only live gametes were analyzed, except for the complement deposition assays for which both live and dead gametes were included in the analysis (gating strategy in **Fig S6J**). Statistical analyses were performed using GraphPad Prism (version 10.4.1), and are described in the figure legends.