PCRCR complex is essential for invasion of human erythrocytes by *Plasmodium falciparum*

The most severe form of malaria is caused by *Plasmodium falciparum*. These parasites invade human erythrocytes, and an essential step in this process involves the ligand PfRh5, which forms a complex with cysteine-rich protective antigen (CyRPA) and PfRh5-interacting protein (PfRipr) (RCR complex) and binds basigin on the host cell. We identified a heteromeric disulfide-linked complex consisting of *P. falciparum* Plasmodium thrombospondin-related apical merozoite protein (PfPTRAMP) and *P. falciparum* cysteine-rich small secreted protein (PfCSS) and have shown that it binds RCR to form a pentameric complex, PCRCR. Using *P. falciparum* lines with conditional knockouts, invasion inhibitory nanobodies to both PfPTRAMP and PfCSS, and lattice light-sheet microscopy, we show that they are essential for merozoite invasion. The PCRCR complex functions to anchor the contact between merozoite and erythrocyte membranes brought together by strong parasite deformations. We solved the structure of nanobody–PfCSS complexes to identify an inhibitory epitope. Our results define the function of the PCRCR complex and identify invasion neutralizing epitopes providing a roadmap for structure-guided development of these proteins for a blood stage malaria vaccine.
pfrh5, pfcss and PfRh5 are essential for growth of P. falciparum. a–c, Inducible knockdown of PfRh5 (a), PfPTRAMP (b) and PfCSS (c) expression. Rapa minus and plus rapamycin. HA-tagged PfRh5, PfPTRAMP and PfCSS were detected using anti-HA antibodies. Molecular weight markers (kDa) are shown on the right. Below each panel is a diagram of the protein with the position of the HA tag (red) marked with an antibody symbol. The relevant PMX and SUB2 protease cleavage sites are shown for PfRh5. Signal peptide sequence (SP) at N-terminus and transmembrane sequence (TM) are grey. The predicted (p) size of each processed polypeptide is shown. d–f, Representative experiments showing P. falciparum parasitemia over time plus (red lines) and minus (green lines) rapamycin for inducible knockdown of PfRh5 (3D7–Rh5iKO) (d), PfPTRAMP (3D7–PTRAMPiKO) (e) and PfCSS (3D7–CSSiKO) (f). Intraerythrocytic developmental cycle (IDC). Hours post invasion (hpi). Also shown in Extended Data Fig. Ig–i is a second independent representative experiment.

Results

PfPTRAMP and PfCSS are essential for invasion

Immunoprecipitation of PfRh5, CyRPA and PfRipr from P. falciparum and analysis using mass spectrometry identified PfPTRAMP and PfCSS as components of the RCR complex (Extended Data Fig. 1a–f). To analyse the function of PfPTRAMP, PfCSS and PfRh5, each corresponding gene (pfptramp, PF3D7_1218000, pfcss, PF3D7_1404700 and pfrh5, PF3D7_0424100) was placed under conditional control15. Conditional deletion of Pfptramp, pfcss and pfrh5 resulted in substantial decreases in protein expression (Fig. 1a–c). It was also shown that expression levels of PfRh5, CyRPA and PfRipr were not affected by the knockdown of PfPTRAMP or PfCSS (Extended Data Fig. 2). Analysis of parasite growth showed they were not able to expand, indicating the function of each protein was essential (Fig. 1d–f and Extended Data Fig. Ig–i) and that the schizont to ring stage transition was blocked, consistent with these proteins being required for invasion (Fig. 2a).

PCRCR captures surface contact of merozoite and erythrocyte

PfPTRAMP, PfCSS and PfRh5 function was analysed using LLSM to quantitate interaction of merozoites with erythrocytes (Fig. 2b–h and Supplementary Table 1)1,9,12. 3D7–PTRAMPiKO, 3D7–CSSiKO and 3D7–Rh5iKO grew efficiently and invaded erythrocytes (Fig. 2b). The interactions of 21 merozoites were visualized for each parasite (63 observations), and of these 32 merozoites (9, 12 and 11 for each parasite line) successfully invaded with most showing a Ca2+ flux (Fig. 2b, Supplementary Video 1). The -50% invasion frequency for merozoites interacting with erythrocytes accords well with previous studies13,14.

3D7–Rh5iKO, 3D7–PTRAMPiKO and 3D7–CSSiKO were grown in rapamycin and merozoites imaged interacting with erythrocytes. Between 11 and 15 merozoites were imaged for each parasite line (41 observations) (Supplementary Table 1), and of these none invaded (Fig. 2b and Supplementary Video 2). The phenotype observed was identical for PfPTRAMP, PfCSS and PfRh5 conditional knockout merozoites.
Therefore, PPI TRAMP and PfCSS are essential and presumably function at the same step as PRRh5 and the RCR complex in merozoite invasion. To characterize merozoite–erythrocyte interactions, we developed a semi-automated method for quantitation of surface contact between the parasite and host cell that we termed parasite-associated host membrane (PAM), which was used to quantitate deformations (Fig. 2c–h). PAM of 3D7–RhSIKO, 3D7–PTRAMPiko and 3D7–CSSiko merozoites during invasion increased rapidly over the first 10 s (Fig. 2c), and this defined host membrane deformations (Fig. 2d). After 10–25 s the magnitude of PAM showed a second phase of increase and reached a level where the parasite became fully wraped, which was maintained after internalization. The plateauing between the end of deformation and internalization was consistent with parasite ‘recoiling’, often concurrent with Ca\(^{2+}\) flux. This ‘recoil’ phase was a dip in PAM before internalization, in the example of single merozoite interactions (Fig. 2d). However, this feature was obscured in averaged PAM from multiple merozoites due to timing variabilities for pre-internalization deformations and recoiling (Fig. 2c).

In contrast, rapamycin-treated 3D7–RhSIKO, 3D7–PTRAMPiko and 3D7–CSSiko merozoites had a significantly decreased PAM (surface area) of the parasite interacting with the host membrane and an extended period of moderate to weak deformations (Fig. 2c–e). During the first 10 s of interaction, deformation was the same as parental merozoites. The magnitude of deformation then dipped significantly after the first 10 s, went through rounds of increasing and decreasing surface contact (PAM) and in some cases fell below the deformation threshold as the parasite continued to make unsuccessful attempts to invade (Fig. 2d,e). Rapamycin-treated merozoites displayed similar maximum and average deformations compared with invading parental merozoites (Fig. 2f,g). However, total deformation time showed a highly significant increase for merozoites lacking PRRh5, PPI TRAMP or PfCSS function (Fig. 2h).

In the absence of the function of these proteins, the parasite rebounds from the strong deformation to a baseline with a minimal degree of PAM at the apical end. Therefore, the function of PRRh5, PPI TRAMP and PfCSS is not required for establishment and maintenance of the initial merozoite apical interaction with the erythrocyte that precedes and is required for strong deformations. However, it is required to capture and hold the increased membrane surface contact formed between the merozoite and erythrocyte membranes created by strong deformations. In addition, merozoites are capable of multiple rounds of strong deformations mediated by generation of force from the parasite pushing into the host cell membrane.

PPI TRAMP and PfCSS are present on invading merozoites Subcellular localization of PPI TRAMP and PfCSS during merozoite invasion was determined and compared with the RCR complex. PPI TRAMP and PfCSS were concentrated at the merozoite apical end abutting the erythrocyte membrane during invasion with strong overlap in co-localization with each other and CyRPA; however, there were areas with weaker overlap (Fig. 3a–c). To determine the subcellular localization of PfCSS and PPI TRAMP during merozoite invasion, co-localization experiments were performed with RON4. RON4 shows a ring fluorescence surrounding the parasite that corresponds to the moving junction. PfCSS and PPI TRAMP localization was posterior to RON4 in the merozoite (Fig. 3d,e) consistent with a surface location where they would be removed by SUB2 sheddase as the junction extends to the posterior of the merozoite during invasion. PfCSS and PPI TRAMP were located on the surface of invading merozoites as shown using Triton X-100 (TX-100)-treated and untreated parasites (Fig. 3f–i).

PPI TRAMP forms a disulfide bonded heterodimer with PfCSS To determine whether PPI TRAMP and PfCSS form a complex, we first immunoprecipitated PfCSS–HA (hemagglutinin) resulting in enrichment of PPI TRAMP as detected by mass spectrometry (Extended Data Fig. 3). Second, immunoprecipitation of PfCSS–HA with anti-HA antibodies revealed a 65 kDa band under non-reducing conditions that migrated at 34 kDa (p34) when reduced (Fig. 4a). The reciprocal experiment with anti-PPI TRAMP monoclonal antibody (mAb) 1D9 detected the same 65 kDa band under non-reducing conditions and at 30–32 kDa when reduced. Third, conditional knockdown of PPI TRAMP expression disrupted the 65 kDa band so that the 33 kDa PfCSS protein was predominantly observed (Extended Data Fig. 4). Taken together, these data show PPI TRAMP and PfCSS form a disulfide linked heterodimer.

PPI TRAMP–CSS bind Ripr and enhance PRRh5 erythrocyte binding PPI TRAMP, PfCSS and PPI TRAMP–CSS heterodimer were used to test their ability to bind the RCR complex and human erythrocytes. Initially, we determined whether PPI TRAMP and PfCSS were proteolytically cleaved to ensure that the equivalent of the mature processed proteins was expressed. PfCSS was not processed; however, PPI TRAMP was cleaved by plasminogen X (PMX) (Extended Data Fig. 4). PfCSS and PPI TRAMP constructs were designed, and the monomeric and PPI TRAMP–CSS dimer were expressed and purified to homogeneity (Fig. 4b).

Binding of PPI TRAMP–CSS and PfCSS to PRRh5 was detected, with a moderate affinity of equilibrium dissociation constant (\(K_d\)) 3.6 ± 0.9 μM and Kd 1.1 ± 0.07 μM, respectively (Fig. 4c,d). The monomers of PRRh5 or CyRPA showed no binding to PPI TRAMP–CSS (Fig. 4e). The ability of PfCSS to bind to PRRh5 indicated it bound PfRipr and that PPI TRAMP does not contribute to this interaction (Fig. 4c,d).

Finally, PPI TRAMP bound to PfCSS at a lower affinity of 19 ± 5 μM (Fig. 4f), suggesting that while the interacting surface between these
two proteins is complementary, the disulfide bond is critical for formation and stability of the heterodimer. Our finding that the unpaired cysteine residue (C30) in PfCSS (see below), proposed to form the disulfide bound heterodimer, was essential for growth supported the functional importance of PTRAMP–CSS (Extended Data Fig. 1).

PTRAMP, PfCSS, PTRAMP–CSS and PTRAMP–CSS–Ripr were incubated with human erythrocytes, and no direct binding was detected (Fig. 4g and Extended Data Fig. 5a). However, when PTRAMP–CSS was added with the RCR complex, significant binding was detected. Consistent with our previous studies, PfRipr and CyRPA bound erythrocytes only as part of the tripartite RCR complex. While PfRipr33 bound erythrocytes either alone or in the RCR complex, it bound most efficiently in the pentameric complex with PCRCR (Fig. 4g). When added to human erythrocytes, PCRCR was not sufficient to induce a basal increase in Ca2+ in vitro as shown previously for RCR (Extended Data Fig. 5b). These results show PCRCR enhances the ability of PfRipr to bind the receptor basigin on erythrocytes.

**PfCSS and PTRAMP nanobodies inhibit merozoite invasion.** We generated nanobodies to PfCSS and PfPTRAMP and determined the binding affinities, binding sites and ability to block binding to PfRipr (Fig. 5a, Extended Data Fig. 6, and Supplementary Tables 2 and 3). Anti-PfPTRAMP nanobodies H8 and H10 bound to distal sites on PTRAMP–CSS and did not block binding to PfRipr (Fig. 5a and Extended Data Fig. 6b). Anti-PfCSS nanobodies bound to three distinct sites on PfCSS. The first site comprised 12 out of the 14 nanobodies tested, and all competed with PfRipr for binding to PfCSS (Fig. 5a and Extended Data Fig. 6c). Within this bin, nanobodies bound to three overlapping epitopes. The second and third sites were distinct from the PfRipr binding site and comprised nanobodies H2 and D2. H2 competed with PTRAMP for binding to PfCSS. D2 nanobody bound to a site distal to the PfRipr and PTRAMP binding sites and did not block binding of PTRAMP–CSS to PfRipr (Extended Data Fig. 6d).

The anti-PfCSS nanobody D2 inhibited parasite growth with potency comparable with IG12 anti-PfRipr mAb (Fig. 5b)29. PTRAMP nanobodies H8 and H10 showed inhibitory activity, with the former nanobody showing over 80% inhibition, whereas anti-CSS (2D2) and anti-PTRAMP (ID9, 3D8) mAbs did not inhibit growth. Inhibition of growth was dose dependent for nanobody D2 (anti-PfCSS) and H8 (anti-PfPTRAMP) with a half maximal effective concentration (EC50) of 283 μg ml−1 and 288 μg ml−1, respectively (Fig. 5c). D2 and H8 nanobody–Fc fusion proteins also inhibited growth to similar levels as the nanobodies and were used to show specificity to PTRAMP–CSS in merozoites (Extended Data Fig. 7). D2–Fc and H8–Fc recognized recombinant PTRAMP–CSS in non-reducing conditions, suggesting they bind conformational epitopes. In addition, D2–Fc recognized PTRAMP–CSS in merozoites. The ability of D2 and H8 nanobodies and nanobody–Fc fusions to inhibit growth showed PTRAMP–CSS was exposed on the merozoite surface and plays an essential role in invasion. Nanobodies blocking binding of PfCSS to PTRAMP and PfRipr did not inhibit growth suggesting the PTRAMP–CSS–Ripr complex was pre-formed in micronemes before exposure on the surface during invasion and consistent with co-localization of PfCSS and PfPTRAMP with CyRPA in mature schizonts (Extended Data Fig. 8). LLSM was used to confirm D2 and H8 nanobodies inhibited merozoite invasion of erythrocytes (Fig. 5d–g and Supplementary Table 1)31,32.

Using anti-PfCSS D2 nanobody, 23 merozoites were imaged interacting with erythrocytes, and of these 3 invaded. For H8 nanobody, 12 merozoites were imaged interacting with the erythrocyte membrane, and none invaded. Consequently, D2 and H8 nanobodies blocked merozoite invasion to ~87% and 100%, respectively, in accordance with growth inhibition assays (Fig. 5c).

Parental merozoites deformed the membrane, and 42–44% successfully invaded (Fig. 5d,e and Supplementary Table 1). For D2 and H8 nanobody-treated merozoites, the PAM plateaued as observed for merozoites lacking PfRipr5, PTRAMP or PfCSS function (Fig. 2). Maximum and average deformation was the same for parental and D2 or H8 nanobody-treated merozoites indicating these activities were normal when PfCSS or PTRAMP function was inhibited (Fig. 5d,g). In contrast, total deformation time was significantly increased consistent with merozoites mediating rounds of deformation in repeated attempts to invade, as observed for those lacking PfRipr5, PTRAMP or PfCSS function (Fig. 5d,g). Therefore, D2 and H8 nanobodies inhibit PfCSS and PTRAMP function, respectively, and block the function of PCRCR in invasion.

**Structure of PfCSS–nanobody complexes.** Crystal structures of nanobodies D2 (inhibitory) and H2 (non-inhibitory) in complex with PfCSS were determined to a resolution of 4.13 Å and 2.00 Å, respectively (Supplementary Table 4). Analysis of the PfCSS sequence revealed similarity to the Plasmodium 6-Cys protein family, with 8 of the 11 cysteines conserved among five double domain P.falciparum 6-Cys proteins (Extended Data Fig. 9)33,34. Indeed, PfCSS adopts two ‘degenerate’ 6-Cys domains, denoted here as D1 and D2 (Fig. 6a, b). Both domains contain a β-sandwich fold with a mix of five on four parallel and antiparallel β-sheets. The D2 domain has an α-helix between residues 213 and 229 that replaces a β-sheet and loop present in other 6-Cys proteins (Fig. 6c)35,36. The eight conserved cysteines are paired to adopt the characteristic C1–C2 and C4–C5 6-Cys motifs in both D1 and D2 domains (Fig. 6a, b). An interdomain disulfide bond between residues C80 and C276 appears to rigidify the position of the two domains (root mean square deviation (rmsd) of PfCSS between two crystal structures, 0.49 Å). Importantly, C30 was solvent exposed and available for pairing to form a disulfide linkage with PTRAMP (Extended Data Fig. 3).

Consistent with the competition binding data, D2 and H2 nanobodies bind to non-overlapping sites in PfCSS (Fig. 6a). D2 nanobody contacts both the D1 and D2 domains with most contacts targeted to one face of the β-sheet of the D1 domain (buried surface area (BSA) of 655 Å2) and the remaining to a loop in the D2 domain (BSA of 175 Å2) (Supplementary Table 5). The D2 CDR3 contributes more than half the total BSA (421 Å2), with CDR1, CDR2, FR2 and FR3 contributing the rest (Fig. 6d). CDR3 and FR3 of D2 form interactions with an N-linked glycan on Asn88 of PfCSS, with a BSA of 378 Å2 (Extended Data Fig. 10), which was glycosylated in the recombinant protein. Therefore, binding of D2 nanobody to a glycan deficient PfCSS–CSS construct was determined using biolayer interferometry. Although affinity for the glycan deficient PfCSS was tenfold lower, it showed notable binding (Kd = 73 nM compared with Kd = 7.5 nM), confirming D2 nanobody can bind to a ‘parasite-like’ PTRAMP–CSS heterodimer (Extended Data Fig. 10).

The non-inhibitory nanobody H2 interacts solely with the D1 domain of PfCSS (total BSA of 750 Å2) (Fig. 6e). It binds near the solvent-exposed Cys30, which we predict forms a disulfide bond with RON4. RON4 was used as a marker of the tight junction and allowed to differentiate between early, mid, and late invasion events. F-1, PfCSS–HA (f,g) or HA–PTRAMP (h,i) invading merozoites were fixed and either permeabilized (TX-100, f,h) or not (no TX-100, g,i) before staining for HA and CyRPA. Positive signal in the absence of permeabilization suggests that the labelled proteins are exposed at this stage, allowing for the access of antibodies. Arrows show signal overlap. 4′,6-diamido-2-phenylindole (DAPI). Differential interference contrast (DIC). Scale bars, 2 μm.
PfPTRAMP, consistent with the inability of H2 to bind PfPTRAMP–CSS. H2 binds in a side on orientation, with most contacts mediated by CDR3 (BSA 447 Å²); however, the CDR2, FR2 and FR3 all contribute (Fig. 6e and Supplementary Table 6). The CDR1 does not interact with PfCSS. Notably, the first six residues of PfCSS are missing from the crystal structure and are likely flexible. In the D2 nanobody–PfCSS crystal structure, these residues form the first β-strand of a β-sheet involved in the sandwich fold. However, in the H2–PfCSS crystal structure, this β-strand was replaced by the H2 CDR3, highlighting the flexibility of this β-strand in the absence of PfPTRAMP (Extended Data Fig. 10c).
Fig. 4 | PfPTRAMP and PfCSS form a functional heterodimer and a complex with PfRipr, CyRPA and PfRh5 to enhance PfRh5 binding to erythrocytes.

a. The *P. falciparum* line PfCSS–HA was used for immunoprecipitation (IP) from merozoite supernatants of PfCSS using anti-HA antibodies (left) and PfPTRAMP using monoclonal antibody 1D9 (right) under non-reduced (NR) and reduced (Red.) conditions. The positions of the PTRAMP–CSS heterodimer, PfCSS–HA and PfPTRAMP (p32 and p30) proteins detected are arrowed. Shown are cartoons of PfCSS and PfPTRAMP with the position of antibody epitopes, the processing by PMX and SUB2 and the polypeptides detected. b. Size exclusion chromatography profiles for PTRAMP–CSS (black), PfPTRAMP (blue) and PfCSS (green) from a Superdex 200 Increase 10/300 GL column. Absorbance (A). SDS–PAGE of the final purified PfPTRAMP, PfCSS and PTRAMP–CSS proteins in reducing (R) and non-reducing (N) conditions. The molecular weight markers are shown on the left in kDa. c–f. Representative sensorgrams and 1:1 model best fit (black). c. PfRipr binding to PTRAMP–CSS (Ripr versus PTRAMP–CSS). d. PfRipr binding to PfCSS (Ripr versus CSS). e. PTRAMP–CSS does not bind to PfRh5 or CyRPA (PTRAMP–CSS versus Rh5 and CyRPA). f. PfPTRAMP binding to PfCSS (PTRAMP versus CSS). g. Fluorescence-Activated Cell Sorting (FACS) analysis of different combinations of PfPTRAMP (P), PfCSS (C), PfRipr (R), CyRPA (Cy) and PfRh5 (Rh) binding to erythrocytes. N = 3; experiments were performed at least 3 times with biologically independent samples and were reproducible. Error bars represent s.e.m. Statistical significance was determined by an ordinary one-way analysis of variance with Tukey’s multiple comparisons test. Exact *P* values are shown in the figure where applicable.
The sequence diversity of D2, H2 and H8 nanobodies in PfCSS and PIPTRAMP was analysed and found to be largely conserved (https://plasmodb.org/plasmo/app) (Extended Data Fig. 10). PfCSS has six polymorphisms, and PIPTRAMP has one polymorphism where the minor allele has a frequency of ~5%. Notably, all PfCSS residues contacted by D2 and H2 nanobodies are conserved among all sequences (Extended Data Fig. 10). In addition, as PIPTRAMP had one polymorphic site, it is likely that H8 binds to a conserved epitope (Extended Data Fig. 10). The conserved nature of PfCSS and PIPTRAMP make them attractive targets for rational design of a vaccine eliciting strain-transcending antibodies that inhibit invasion.

**Discussion**

The near isotropic data obtained from the high spatiotemporal resolution of LLSM imaging has provided a unique view of merozoite invasion providing a quantitative understanding of the surface contact between the pathogen and host cell. This showed the PCRCR complex was responsible for capturing and anchoring the increased membrane surface contact formed between the merozoite and erythrocyte membranes created by strong deformations. This results in an irreversible interaction between the merozoite and erythrocyte and a stable platform for activation of the next steps for invasion and internalization of the merozoite into the erythrocyte.

PTRAMP–CSS was exposed on the surface of the invading merozoite and binds to the RCR complex, and we propose a model where it anchors the PCRCR complex to the parasite membrane through the transmembrane domain of PIPTRAMP to provide a platform for PIRh5 binding to basigin on the erythrocyte (Fig. 6f). PCRCR function was not required for initial interactions of the merozoite and binding at the apical tip that abuts the erythrocyte membrane after reorientation. Nor was it required for weak or strong deformations of the erythrocyte membrane driven by the merozoite actomyosin motor. The most likely mediators of the interaction at the merozoite tip are the EBA and PfRh protein families (excluding PfRh5). A consequence of these strong deformations would be an increase in surface area of the merozoite membrane in proximity with the membrane of the erythrocyte allowing the PCRCR complex to bind basigin across a broad area of the parasite, activating the insertion of PIRipr and PIRh5 into the erythrocyte membrane and providing an anchor on the parasite membrane (Fig. 6f). This would provide the ‘velcro’ that ties the membranes together, so the apical end of the merozoite remains embedded in the deformed host cell providing a stable and irreversible platform. When PCRCR function was inhibited, the interaction of the merozoite tip with the erythrocyte membrane and the strong deformations still occur; however, the lack of the ‘velcro’ to tie the parasite and host membrane together resulted in the merozoite bouncing back and proceeding through cycles of deformation until the energy driving the actomyosin motor would be depleted followed by parasite detachment (Fig. 6f).

Stabilization of merozoite–erythrocyte interactions would provide a base to establish the pore that allows Ca entry into the erythrocyte and enable proteins to be injected under the erythrocyte membrane for formation of the moving junction by AMA1 and the RON complex. However, while PCRCR function is required for these next steps, it is not directly involved. Once the moving junction has been established, it is propelled across the merozoite membrane towards the posterior of the parasite. The PCRCR complex would be released from the surface by processing of PIPTRAMP near the transmembrane domain by the protease SUB2 (Fig. 6f). This would free the PCRCR ‘velcro’ attachment between the parasite and host cell, allowing the moving junction to slide along the membranes to the posterior end for membrane sealing and completion of invasion and internalization.

Previously, it has been shown that the protein P113 binds to the N-terminus of PIRh5 and postulated that its glycosylphosphatidylinositol anchor bound the RCR complex to the merozoite membrane. However, recent studies have shown that P113 function was not required for *P. falciparum* growth and is unlikely to be the membrane anchor for the RCR complex.

PfCSS is a cryptic 6-Cys protein comprising two ‘degenerate’ 6-Cys domains, and this protein family typically mediates extracellular protein–protein interactions, consistent with PfCSS binding to both PIPTRAMP and PIRipr. PIPTRAMP–CSS heterodimer formation would occur in the endoplasmic reticulum and then be trafficked to the micromeres where PfCSS could interact with PIRipr and CyRPA and form a tetrameric complex (PCRCR). These proteins would then be exposed to PIRh5 at merozoite invasion as the micromeres empty their contents into the neck of the rhoptries allowing the PCRCR complex to form and spread onto the merozoite surface. The inability of nanobodies that block binding of PIRipr to PfCSS and PIPTRAMP binding to PfCSS to inhibit merozoite invasion was consistent with formation of the PCRCR complex before exposure on the merozoite.

Identification of invasion inhibitory nanobodies to PfCSS and PIPTRAMP showed that these proteins have an essential role in the PCRCR complex and that they are exposed on the merozoite surface during invasion. The mechanism of D2 and H8 nanobody inhibition of PfCSS and PIPTRAMP remains to be determined, but it is possible that they either block insertion of PIRipr and PIRh5 into the erythrocyte membrane or inhibit three-dimensional changes in the PCRCR complex required for function. Indeed, the RCR complex binds with higher affinity to basigin, and the PCRCR complex shows even more efficient binding. This would suggest that conformational changes to the PCRCR complex occur during interaction between basigin and PIRh5 that alter the affinity of binding to basigin. PIRh5 has a mobile structure, and formation of the RCR and PCRCR complex could lock in a conformation that binds more efficiently to basigin.

The finding that nanobodies blocking Ripr–CSS or PTPRAMP–CSS binding are non-neutralizing was consistent with these proteins associating before merozoite egress. Similar findings have been reported for CyRPA–Rh5 blocking antibodies; however, a CyRPA–Rh5 blocking antibody was capable of inhibiting parasite growth which is somewhat at odds with this finding. Previous studies have investigated whether...
polyclonal antibodies to PfCSS or PfPTRAMP could neutralize merozoite invasion; however, they showed no inhibitory activity \(^{35,36}\). An explanation, and consistent with our observations for the PfCSS and PfPTRAMP nanobodies, is that polyclonal responses are skewed to non-inhibitory immunodominant epitopes. Identification of invasion inhibitory epitopes on PfCSS and PfPTRAMP, both of which are highly conserved in \(P. falciparum\), provides the molecular basis for rational design of immunogens.

**Methods**

**Parasite, insect cell culture and antibodies**

3D7 \(P. falciparum\) parasites were obtained from David Walliker, Edinburgh University. Asexual blood stage parasites were grown in vitro culture as described\(^{37}\).

SF21 insect cells were cultured in Insect-XPRESS protein-free with l-glutamine (Lonza, 10036636) medium at 28 °C. Expi293F cells were grown in Expi293 expression medium (ThermoFisher) at 37 °C, 8% CO\(_2\), 120 r.p.m.

In this study, we used: rat mAb, anti-HA (Roche 3F10, catalogue number 11867423001, lot 47877600); mouse mAbs, 1D9 and 3D8 anti-PfPTRAMP (this study), rat mAb 2D2 anti-PfCSS (this study), mouse mAbs 5B12, 7A6 and 8B9 anti-CyRPA\(^{38}\), 5A9 and 6H2 PfRh5\(^{10}\), mouse mAb 1G12 anti-Ripr\(^19\), rabbit anti-RON4 polyclonal\(^{39}\); rat pAb KM81 anti-PfCSS (this study); and rabbit pAb R1541 anti-Ripr\(^19\).

The mouse mAbs 1D9 and 3D8 that bound PfPTRAMP and the rat mAbs 2D2 mAb and pAb KMS1 that bound PfCSS were made at the WEHI Antibody Facility as described in Supplementary Materials and Methods.

The following secondary antibodies labelled with Alexa 488/594 fluorophores (Life Technologies) and HRP antibodies were used: chicken anti-mouse 594 (catalogue number A21201, lot 42099 A), donkey anti-rat 488 (catalogue number A21208, lot 2310102), and goat anti-rabbit 594 (catalogue number A21423, lot 40000000).
chicken anti-rabbit 594 (catalogue number A21442, Lot 2110863), goat anti-mouse 488 (catalogue number A11001), goat anti-rabbit (catalogue number A11008). Peroxidase affinity pure goat anti-human IgG (H+L) (catalogue number 109-035-088, Jackson Immuno Research).

Transgenic parasites and rhesus and microneme secretion assay
Transgenic parasite lines were made using CRISPR–Cas9 with methods and oligonucleotides listed in Supplementary Materials and Methods.

Crosslinking, immunoprecipitation and mass spectrometry analysis
Parasites used for anti-HA antibody immunoprecipitation with and without cross-linking were synchronized and allowed to develop to schizonts; this is described in the Supplementary Materials and Methods.

Live imaging with LLSM
A standard protocol was developed to ensure that parasites were at the same stages for each experiment. Two 30 ml dishes of asynchronous culture were synchronized with 5% sorbitol, as described. In brief, the culture medium was removed, and the cells were incubated with five volumes of 5% sorbitol in a water bath at 37 °C for 8 min. The sorbitol was then washed off and fresh culture medium added back to the synchronized culture. This synchronization step was repeated 3 days after the first synchronization, and 10 nM rapamycin was added to one of the culture dishes after the second synchronization to induce pfhr5 (3D7–RhS10K), pfptramp (3D7–PTRAMP10K) and pfcss (3D7–CSS10) gene deletion in the relevant parasite lines. Two days after the second synchronization, late-stage parasites were isolated from the culture by magnet purification using LS columns attached to MACS MultiStand (Miltenyi Biotech).

Erythrocytes were resuspended on 0.5% haematocrit in RPMI-HEPES supplemented with 0.2% sodium bicarbonate and 5 mM sodium pyruvate (Gibco 11360070). To load uninfected erythrocytes with calcium indicator and stain the plasma membrane, the cells were incubated with 10 μM Mitotracker Red CMXRos (Invitrogen M7512) and 0.25 mM CaCl2 and 10 μM Trolox (Santa Cruz 53188-07-1). With the 588 nm excitation, emitted fluorescence was split using a 594 nm dichroic (Semrock) before passing through a FP594 nm filter (Chroma) on camera A and 525/50 nm (Chroma) filter on camera B. This allowed simultaneous detection of Fluo-4 AM signals by camera B at 500–550 nm range and DI-4-ANEPPDHQ signals by camera A for wavelengths longer than 594 nm. With the 589 nm excitation, emitted fluorescence from Mitotracker Red CMXRos was detected on camera A with the same detection range as previous. All data were acquired in an imaging chamber (Okolabs) set to 36 °C and 5% humidified CO2.

For deconvolution, point spread functions were measured using 100 nm Tetrasperk beads on the surface of a 5 mm coverslip. Data were de-skewed and de-convolved using LLSpy, a Python interface for processing LLSM data. Deconvolution was performed using a Richardson–Lucy algorithm with 15 iterations with the point spread functions generated for each excitation wavelength.

PAM plotting
Parasite–erythrocyte interactions were characterized by plotting the amount of surface contact at each timepoint for each event. The analysis was performed using Imaris (version 9.7.2, Bitplane) with Tracking module. A surface called ‘Erythrocytes’ was first created from the erythrocyte membrane channel with smoothing and absolute intensity setting. The threshold was adjusted accordingly to achieve reasonable values for parasite surface area (4–9 μm2), and 0.5 μm seed point value was used to split touching parasites. Next, a masked erythrocyte membrane channel was created from the erythrocyte surface by setting the voxel value inside the surface to 1 and outside the surface to 0. From the ‘All parasites’ surface, parasites that interact with the erythrocyte were then selected, by either automated tracking or manual selection, and duplicated into individual surfaces called ‘Parasite 1’, ‘Parasite 2’ and so on. For each parasite, all parts of the surface were selected and then unified and made into a single track. Finally, values of the ‘Intensity Sum’ from the masked erythrocyte membrane channel and the ‘Area’ at each timepoint were extracted from each parasite surface and exported to Microsoft Excel. The ‘Intensity Sum’ values represent the number of voxels in the erythrocyte membrane channel in contact with the parasite surface. The PAM values were then plotted from the Intensity Sum and normalized by the Area.

P. falciparum schizont supernatant and merozoite preparations and analysis
Merozoite and supernatant preparations for SDS–PAGE and immunoblot analysis were performed as previously described. Synchronized late trophozoite cultures were passed over LD magnetic columns (Miltenyi Biotech) to remove uninfected erythrocytes. Eluted parasites were adjusted to 5 × 106 schizonts per ml and 150 μl added per well of a 96-well flat-bottomed culture dish. The assay dishes were further cultured for 16 h and a representative well smeared for Giemsa staining to ensure either that rupture had occurred normally (control well) or that rupture had been blocked when inhibitors were added. Parasites from each condition were spun at 10,000 × g for 10 min to collect the merozoite pellet and supernatant fractions. Proteins from both fractions were extracted with reducing sample buffer and separated on 4–12% or 3–8% acrylamide gel (NuPAGE, Invitrogen). When inhibitors WM4...
and WM382 were at 40 nM and 2.5 nM final concentrations, respectively, a control dish without any protease inhibitor was also included. Parasites were eluted from columns with complete RPMI 1640 culture medium to which the appropriate inhibitor at the same concentration had been added.

**Expression and purification of PfCSS, PfPTRAMP, PTPRAMP- CSS heterodimer, PfRipr, CyRPA and PfRh5**

The gene for the PipM cleaved ectodomain of PTPRAMP (residues 42 to 309) was subcloned into a modified pTRIEX2 vector with N-terminal SUMO and Flag tags followed by a Tobacco etch virus (TEV) protease cleavage site. One potential N-linked glycosylation site at Asn195 was removed by mutation of Thr197 to Ala. The construct was expressed in Sf21 insect cells and secreted into the medium as a soluble protein. The supernatant was purified by ANTI-FLAG M2 Affinity Gel (Merck) and size exclusion chromatography (S200 Increase 10/300 GL, Cytiva). Fractions containing PTPRAMP were pooled and cleaved with TEV protease for 16 h at 4 °C. His-tagged TEV was removed via NiNTA agarose resin (Qiagen), and PTPRAMP was further purified via another size exclusion chromatography (S200 Increase 10/300 GL, Cytiva). For biopanning anti-PfPTRAMP nanobodies and their kinetic characterization, a PTPRAMP (42–309) construct with a C-terminal Avitag was generated and specifically biotinylated. In addition, a PTPRAMP construct comprising residues 25 to 309 with a C-terminal His tag was used for bilayer interferometry binding studies to PICSS; however, the purification was the same.

The gene for PICSS (residues 20 to 290) was subcloned into a modified pTRIEX2 vector with a C-terminal Flag tag preceded by a TEV protease cleavage site. The construct was expressed in Sf21 insect cells and purified similarly to PTPRAMP. The construct used in binding and crystallization had been added.

To generate disulfide-linked PTPRAMP–CSS, PTPRAMP (42–309) and PICSS (20–290) constructs were co-expressed in Sf21 insect cells and secreted into the medium as a soluble protein. The supernatant was purified by ANTI-FLAG M2 Affinity Gel (Merck) and size exclusion chromatography (S200 Increase 10/300 GL, Cytiva). As both PTPRAMP and PICSS constructs contain a Flag tag, some free PfPTRAMP and PfCSS were removed by mutation of Thr197 to Ala. The construct was expressed via transient transfection into Sf21 cells and secreted into the medium as a soluble protein. The supernatant was purified by CaptureSelect C-tagXL Affinity Matrix (ThermoFisher) and eluted with 20 mM Tris pH 7.5, 2 M MgCl₂. The sample was further purified via size exclusion chromatography, using a S200 Increase 10/300 GL (Cytiva).

**Bilayer interferometry studies**

Bilayer interferometry experiments were conducted at 25 °C to determine the affinity and epitope bins of selected proteins and nanobodies for PTPRAMP–CSS, PTPRAMP and PfCSS. For protein–protein binding kinetic studies, either PfRipr or PTPRAMP was diluted into kinetics buffer (PBS, pH 7.4, 0.1 % (w/v) BSA, 0.02 % (v/v) Tween-20) at 20 µg ml⁻¹ and immobilized onto Anti-Penta-His (His1K) biosensors (Sartorius). Following a 60 s baseline step, biosensors were dipped into wells containing twofold dilution series of either PTPRAMP–CSS or PICSS. Sensors were then dipped back into kinetics buffer to monitor the dissociation rate. For nanobody–PTPRAMP binding kinetic studies, nanobodies were diluted in kinetics buffer to 5 µg ml⁻¹ and immobilized onto NiNTA (NTA) biosensors (Sartorius). Following a 60 s baseline step, biosensors were dipped into wells containing twofold dilution series of either PTPRAMP–CSS or PICSS. Sensors were then dipped back into kinetics buffer to monitor the dissociation rate. For nanobody–PfCSS binding kinetic studies, biotinylated PTPRAMP or PTPRAMP–CSS were immobilized onto High Precision Streptavidin (SAX) biosensors (Sartorius). Following a 60 s baseline step, biosensors were dipped into wells containing twofold dilution series of anti-PTPRAMP nanobodies.

For competition studies of the anti-PfCSS nanobodies, nanobodies were first diluted in kinetics buffer to 5 µg ml⁻¹ and immobilized onto Ni-NTA (NTA) biosensors (Sartorius). Following a 30 s baseline step, biosensors were dipped into wells containing a negative control nanobody that does not bind the proteins under analysis to quench the sensors. Following another 30 s baseline step, biosensors were dipped into either PICSS or PTPRAMP–CSS. Following a final 30 s baseline step, biosensors were then dipped into a secondary nanobody or PfRipr to assess competition. Due to the moderate affinity of the anti-PTPRAMP nanobodies, a premix format was employed. Nanobodies or PfRipr were first diluted to 10 µg ml⁻¹ and immobilized onto Anti-Penta-His (His1K) biosensors. Following a 30 s baseline step, biosensors were dipped into wells containing a negative control nanobody that does not bind the proteins under analysis to quench the sensors. Following another 30 s baseline step, biosensors were then dipped into PTPRAMP–CSS pre-incubated with a tenfold molar excess of competing secondary nanobody to assess competition.

Kinetics and competition data were analysed using Sartorius’ Data Analysis software 11.0. Kinetic curves were fitted to a 1:1 binding model. Mean kinetic constants reported are the result of two independent experiments. Data presented in Extended Data Fig. 6 represent the per cent of competing nanobody or PfRipr binding compared with the maximum competing nanobody response.

**Growth inhibition and flow cytometry of erythrocyte binding and Ca²⁺ flux**

One-cycle growth inhibition and erythrocyte binding assays were performed as described previously. The full methods are described in Supplementary Materials and Methods.
Three-dimensional structure determination of PfCSS–nanobody complexes

For crystallization studies, PTRAMP–CSS and PfCSS alone were mixed with D2 and H2 nanobodies, respectively, in a 1:2 molar ratio, and excess nanobody was purified away via size exclusion chromatography (Superdex 200 Increase 10/300 GL, Cytiva). Complexes were then concentrated to 5 mg ml⁻¹ and mixed 1:1 with mother liquor and set up in hanging or sitting drop crystallization experiments. D2 nanobody–CSS crystallized in 1.6 M ammonium sulfate, 0.1 M sodium chloride and 0.1 M sodium HEPES at pH 7.5 after 1 month and was cryoprotected in 15% (v/v) ethylene glycol. H2–PfCSS crystallized in 0.1 M bis-tris propane pH 6.0, 17.5% (v/v) PEG3350, 0.2 M sodium malonate in 24 h and was cryoprotected in 15% (v/v) ethylene glycol. Data were collected at the MX2 beamline at the Australian Synchrotron, processed and merged using XDS⁴⁴ and Aimless⁴⁵. The positions of the H2 nanobodies in the H2–PfCSS crystal structure were first determined by molecular replacement using the structure of nanobody VHH-α20⁴ from 6WAQ with its CDR3 removed⁴⁶. This solution was then used to build the two PfCSS molecules present in the asymmetric unit via AutoBuild⁴⁷. This PfCSS structure was then used as a model for molecular replacement in the low-resolution crystal structure of D2 nanobody–CSS, along with VH7-2 from 6WQ⁴⁸. PIPTRAMP was not present in the D2–PTRAMP–CSS crystal structure. Presumably, PIPTRAMP and PfCSS dissociated during crystallization, and only D2 nanobody–PfCSS crystallized after 1 month in the high salt crystallization condition. Refinement of the structures was carried out using phenix.refine⁴⁹ and iterations of refinement using Coot⁵⁰.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The crystal structures reported in this manuscript have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 7UNY, 7UNZ). The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE⁵¹ partner repository with the dataset identifier PXD036746.

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Extended Data Fig. 1 | PfPTRAMP and PfCSS interact with the PfRh5 complex and play an essential role for growth of *P. falciparum*. a–f. Volcano plots illustrating the log2 fold change (log2FC) of immunoprecipitated HA-tagged CyRPA, PfRipr, or PfRh5 versus 3D7 before and after cross-linking with DSP versus significance of the change (-log10 P value). Differential protein expression analysis was performed using Limma which involves a moderated t-test. Benjamini and Hochberg’s method was used to adjust the p-values for multiple testing. Proteins were deemed differentially regulated in the log2 fold change in protein expression was 1-fold and exhibited an adjusted p-value ≤ 0.05. Proteins that were significantly immunoprecipitated with PfCyRPA-HA, PfRipr-HA and PfRh5-HA were analysed further and this included PfPTRAMP, PfCSS and Apical Membrane Protein 1 (AMA1). In this study we concentrated on PfPTRAMP and PfCSS. We are testing a potential link of AMA1 with the function of the PCRCR complex and this will be published elsewhere. An additional protein that was significantly found in all three immunoprecipitation experiments was heat shock protein 70 (PF3D7_0917900) but this was not considered further because it is a highly expressed chaperone protein and frequently immuno-precipitates in experiments such as these. Proteins that immunoprecipitated significantly in less than the three conditions were analysed with respect to subcellular location, timing of expression and potential role in merozoite invasion and because they did not match these criteria were not analysed further. a. Immuno-precipitated HA-tagged CyRPA. b. Immunoprecipitated HA-tagged PfRipr. c. Immunoprecipitated HA-tagged PfRh5. d. Immunoprecipitated HA-tagged CyRPA after cross-linking proteins with DSP. e. Immuno-precipitated HA-tagged PfRipr after cross-linking proteins with DSP. f. Immunoprecipitated HA-tagged PfRh5 after cross-linking proteins with DSP. g. Parasitemia of *P. falciparum* parasites with rapamycin inducible knockdown of PfPTRAMP. h. Parasitemia of *P. falciparum* parasites with rapamycin inducible knockdown of PfCSS. i. Parasitemia of *P. falciparum* parasites with rapamycin inducible knockdown of PfRh5.
Extended Data Fig. 2 | PfCSS, PfRipr, CyRPA and PfRh5 are expressed at normal levels when PfPTRAMP expression is removed by conditional gene knockout. 3D7-PTRAMPiKO was grown without (Control) and with rapamycin (Rapa) to mature schizonts and merozoites and supernatants prepared and analysed by SDS-PAGE and immunoblots that were probed with mAbs, 1D9 (PfPTRAMP), 2D2 (PfCSS), 1G12 (PfRipr), 7A6 (CyRPA) and 6H2 (PfRh5). At bottom of each panel is structure of the relevant protein with the domain recognised by each mAb with respect to PMX protease processing. The PfPTRAMP protein had a HA tag towards the N-terminus (red). The grey domains correspond to the Signal Sequence (SP). The molecular weight of the processed polypeptides and position within each protein is shown. Supernatants were prepared from purified schizonts placed back in culture and allowed to egress. Merozoites were centrifuged as pellets and prepared for SDS-PAGE analysis.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | PfPTRAMP and PfCSS form a complex and the PfCSS C30 residue appears to be essential. a, Volcano plot illustrating the log₂ protein ratios of immunoprecipitated PfCSS-HA proteins versus 3D7 control as analysed by mass spectrometry analysis. Differential protein expression analysis was performed using Limma which involves a moderated t-test. Benjamini and Hochberg’s method was used to adjust the p-values for multiple testing. Proteins were deemed differentially regulated in the log₂ fold change in protein expression was 1-fold and exhibited an adjusted p-value ≤ 0.05. N = 3 biologically independent samples used. b, Scheme to construct *P. falciparum* lines that express PfCSS with the amino acid Cys30 mutated to Ser30 using CRISPR. Shown is the Cas9 cleavage site (red) near the protospacer adjacent motif and the resulting recombination event that replaces the endogenous *pfcss* gene with one mutated to encode Ser30. Both constructs included a HA-tag near the N-terminus of the PfCSS protein. In the grey box is the expected amino acid sequence expected after each insertion event. The HA-tagged *pfcss* gene that retained expression of C30 but inserted a HA-tag was successfully obtained and confirmed by sequencing (bottom panel). Parasites from multiple transfections with the construct that was identical to the former but would result in mutation of C30 to S30 was not successfully obtained. Therefore, we conclude that the C30 amino acid and the disulfide bond with PfPTRAMP was essential.
Extended Data Fig. 4 | Conditional knockdown of PfPTRAMP and PfCSS confirms they form a heterodimer. a and b. Merozoites (Meros) and supernatants (Supn) probed with 1D9 monoclonal antibody to detect PfPTRAMP (a) and 2D2 monoclonal antibody to detect PfCSS (b) from 3D7 iKO PfPTRAMP and 3D7 iKO PfCSS in the absence (control) or presence of rapamycin (Rapa) and proteins run on SDS/PAGE under non-reducing (NR) or reducing (Reduced) conditions and an immuno-blot performed. Position of detected PTRAMP−CSS heterodimer and PfPTRAMP and PfCSS monomers are arrowed. Cartoons below panels show structure of PfPTRAMP and PfCSS, the position of the monoclonal epitopes and the processing due to PMX and SUB2. SP, Signal sequence. TM, transmembrane.

c. Aspartic protease PMX processes PfTRAMP at the N-terminus. P. falciparum parasites that express a HA-tagged PfPTRAMP were probed with anti-HA or a specific monoclonal (1D9) to detect processed polypeptides. Molecular weight markers are shown in kDa on the left and predicted (p) sizes of the processed proteins on the right. Cartoon of PfPTRAMP is shown below the panels. Antibody symbol shows the position of the HA-tag (red) and the domain to which the monoclonal antibody 1D9 binds. Signal sequence SP and transmembrane TM (grey). SUB2 protease and the identified PMX cleavage site are shown with an arrow. Below the protein is the predicted molecular weights of each processed polypeptide.

d. WM4 and WM382 inhibitors block processing of PfPTRAMP. PMX inhibitor WM4 used at 40 nM, dual PMX and PMIX inhibitor WM382, 2.5 nM. On the left are the predicted (p) molecular weights of each detected polypeptide. The molecular weight markers shown on the right in kDa. PfPTRAMP detected using the monoclonal antibody 1D9. A cartoon of the PfPTRAMP protein is shown below. An antibody symbol shows the position of 1D9 binding to the domain. Signal sequence SP and transmembrane TM (grey). SUB2 protease and the identified PMX cleavage site are shown with an arrow. Below the protein is predicted molecular weight due to WM4 and WM382 inhibition of PMX processing. Western blot in all panels were performed at least twice.
Extended Data Fig. 5 | Gating strategy for detection of erythrocyte binding Ca<sup>2+</sup> uptake by flow cytometry. **a**, Example scatter plots of the gating strategy for the erythrocyte binding assay showing unstained erythrocytes and erythrocytes incubated with PCRCR and detected with anti-PfRipr polyclonal sera and Alexa-488. The erythrocyte population was gated with SSC-A and FSC-A (top), then doublets were excluded using FSC-H and FSC-A (middle). For determining complex binding to erythrocytes, a cutoff of >10<sup>3</sup> was used (bottom). Gating was performed in an identical manner for all other antibody and antigen combinations. **b**, Kinetic plot of A23187 stimulation of erythrocytes pre-loaded with Fluo-4 AM. A titration of A23187 shows the responsiveness of the Fluo-4 AM labelled erythrocytes to the calcium ionophore (top). The mean fluorescence intensity for the Fluo-4 AM signal is plotted. Kinetic plot of PCRCR addition to erythrocytes in comparison to 0.5 μM A23187 stimulation (bottom).
Extended Data Fig. 6 | Characterization of α-PfPTRAMP and α-PfCSS nanobodies. a. Representative sensorgrams and 1:1 model best fit (black) for nanobody binding to PFTRAMP, PFCSS and PTRAMP-CSS, determined by biolayer interferometry. A 2-fold dilution series was used, starting at 2500 nM (brown), 1250 nM (red), 625 nM (orange), 313 nM (yellow) and 156 nM (wheat) for α-PfPTRAMP nanobodies and 250 nM (light pink), 125 nM (purple), 62.5 nM (cyan), 31.25 nM (teal) and 15.63 nM (pink) for α-PfCSS nanobodies. Epitope binning of α-PfPTRAMP nanobodies against b. PTRAMP-CSS and α-PfCSS nanobodies against c. PFCSS or d. PTRAMP-CSS. Primary nanobodies tested are listed in the left column, while secondary competing nanobodies are listed at the top. Data indicate the percent of competing nanobody or PfRipr binding compared to the maximum competing nanobody response. Boxes are coloured on a sliding scale, with red representing competition and blue representing non-competition. Nanobodies are coloured according to their epitope bins as in Fig. 5a.
Extended Data Fig. 7 | Nanobody-Fc fusion proteins specific to PfPTRAMP and PfCSS inhibit parasite growth and recognize PTRAMP-CSS in merozoites.

a. Growth inhibition of parasites by D2 and H8 nanobodies and nanobody-Fc fusion proteins. Nanobodies and nanobody-Fc fusion proteins were tested at the EC₅₀ concentration for growth inhibition of *P. falciparum*; D2 at 283 μg/ml and D2-Fc at 1.68 mg/mL or 21.2 μM; H8 at 288 μg/mL and H8-Fc at 1.72 mg/mL or 21.7 μM. Data is shown from one independent experiment, performed in triplicate. Error bars indicate standard error of the mean.

b. Recombinant PTRAMP-CSS were probed with D2-Fc or H8-Fc fusion proteins under reduced (R) and non-reduced (NR) conditions. Western blot experiment has been performed at least twice.

c. PfCSS-HA merozoites were probed with D2-Fc under non-reduced conditions to detect PTRAMP-CSS. Western blot experiment was performed once.

Data is shown from one independent experiment, performed in triplicate. Error bars indicate standard error of the mean. Recombinant PTRAMP-CSS were probed with D2-Fc or H8-Fc fusion proteins under reduced (R) and non-reduced (NR) conditions. Western blot experiment has been performed at least twice. PfCSS-HA merozoites were probed with D2-Fc under non-reduced conditions to detect PTRAMP-CSS. Western blot experiment was performed once.
Extended Data Fig. 8 | Co-localisation of PfCSS, PfPTRAMP and CyRPA in late schizonts of *P. falciparum* by immunofluorescence and super resolution microscopy. 

**a.** Localisation of PfCSS-HA (green) detected with anti-HA antibodies compared to PfPTRAMP detected using mAb 3D8 (purple).

**b.** Localisation of PfCSS-HA (green) detected with anti-HA antibodies compared to CyRPA detected using mAb 8B9 (purple).

**c.** Localisation of PfPTRAMP (green) detected with anti-HA antibodies compared to CyRPA detected using mAb 8B9 (purple). Shown for all panels is merge+DAPI where nuclei have been stained and PfCSS, PfPTRAMP and DAPI channels have been merged. Fourth panels on the right includes merge of all as well as DIC (differential interference contrast). The far right panels indicate co-localisation of each protein across the broken white line of the merge+DAPI panels. The scale bar in merge + DIC panels is 2 μM and is relevant for all panels.
Extended Data Fig. 9 | Amino acid sequence comparisons of the 6-cys family members including PfCSS, Pf12, Pf12p, Pf36, Pf41 and Pf52. Conserved cysteine residues are shown in dark red. The C30 cysteine in PfCSS that likely forms the disulfide bond with PfPTRAMP is marked in green. Less conserved residues are marked in light red.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Nanobody recognition of PfCSS and sequence conservation of PfCSS and PfPTRAMP. a. D2 contacts an N-linked glycan on Asn88 of recombinant PfCSS. Interacting residues are shown as sticks. b. Representative sensorgram and 1:1 model best fit (black) for D2 binding to non-glycosylated PTRAMP-CSS determined by biolayer interferometry. A 2-fold dilution series was used, starting at 500 nM (light pink), 250 nM (lilac), 125 nM (purple), 62.5 nM (cyan). c. Superposition of the D1 domains from the D2-PfCSS and H2-PfCSS structures showing the β-strand is replaced by the H2 CDR3. d. Weblogo representation of PfCSS sequence diversity from 212 sequences from the PlasmoDB55. D2 and H2 interacting residues are denoted with teal and pink circles, respectively. e. Weblogo representation of PfPTRAMP sequence diversity from 214 sequences from the PlasmoDB.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ □ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Software and code

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The crystal structures reported in this manuscript have been deposited in the Protein Data Bank, www.rcsb.org (PDB codes 7UNY, 7UNZ). The mass spectrometry
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical method was used to predetermine sample size. Instead, sample sizes were chosen according to best practices in the field and previous studies (Wong et al. Nature 2019, Geoghegan et al. Nat Comms 2021, Ragotte et al. Nat Comms 2022). |
| Data exclusions | No data were excluded. |
| Replication | Monitoring of P. falciparum parasitemia of the inducible knockdowns was performed in duplicate in two independent experiments. The number of infection events recorded by lattice light sheet microscopy is detailed in Supplementary Table 1. BLI kinetic experiments were performed at least twice. Erythrocyte binding assays were performed in triplicate on separate days. All growth inhibition assays were performed in three independent experiments, with data points representing the mean from one experiment, performed in triplicate, except in Extended Data Fig 7a, which was performed once in triplicate. All attempts at replication were successful. |
| Randomization | Randomization was not relevant to this study as no subjective judgements were required about which data to include, exclude or measure. |
| Blinding | The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| [x] Antibodies | Involved in the study |
| [x] Eukaryotic cell lines | [x] ChIP-seq |
| [ ] Palaeontology and archaeology | [x] Flow cytometry |
| [x] Animals and other organisms | [x] MRI-based neuroimaging |
| [x] Human research participants | [ ] Clinical data |
| [x] Clinical data | [ ] Dual use research of concern |
| [x] Dual use research of concern | |

Antibodies

Antibodies and monoclonal antibodies were raised in rabbits and mice and all procedures approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. Immunization and handling of the alpaca for scientific purposes was approved by Agriculture Victoria, Wildlife and Small Institutions Animal Ethics Committee, project approval No. 26-17.

In this study, we used the following antibodies: rat mAb, anti-HA (Roche 3F10, Cat.: 11867423001, Lot: 47877600); mouse mAbs, 1D9 and 3D8 anti-PfPTRAMP (this study), mouse mAbs SB12, 7A6 and 8B9 anti-CyRPA (Chen et al, Elife 2017), 5A9 and 6H2 PfRh5 (Chen et al, Plos Path 2011), mouse mAb 1G12 anti-Ripr (Healer et al, Cell Microbiol 2019), rabbit anti-RON4 polyclonal (Richard et al, JBC, 2010); rat pAb KM81 anti-PfCSS (this study), rabbit pAb R1541 anti-Ripr (Healer et al, Cell Microbiol 2019).

The mAbs 1D9 and 3D8 that bound to PfPTRAMP were raised in mice at the WEHI Antibody Facility, by immunising with recombinant PfPTRAMP expressed and purified from insect cells. Briefly, PfPTRAMP (N25-K309) between the end of the signal sequence and the start of the transmembrane domain was recombinantly expressed in insect cells (Genscript) and cloned into an insect cell expression vector bearing an N-terminal gp67 signal peptide, a SUMO tag, a FLAG tag and a tobacco etch virus (TEV) protease cleavage site.

The 2D2 mAb and pAb KM81 that bound to PfCSS were made in rats, at the WEHI Antibody Facility, by immunising with PfCSS recombinant protein expressed and purified from insect cells. PfCSS (Q21-K290) after the end of the signal sequence was recombinantly expressed in insect cells (Genscript) and cloned into an expression vector bearing an N-terminal gp67 signal peptide and a C-terminal proteosome cleavage site.

proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 59 partner repository with the dataset identifier PXD (reviewer token: Username: reviewer@ebi.ac.uk Password: ). P. falciparum sequences were derived from PlasmoDB (https://plasmodb.org/plasmo/app). The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
Validation

Rat mAb, anti-HA (Roche 3F10, Cat.: 11867423001) validated by the supplier by western blot.

Chicken anti-mouse 594 (Cat.: A21201, Lot: 42099A) validated by the supplier by IFA.

Donkey anti-rat 488 (Cat.: A21208, Lot: 2310102) validated by the supplier by IFA.

Chicken anti-rabbit 594 (Cat.: A21442, Lot: 210863) validated by the supplier by IFA.

Goat anti-mouse 488 (Cat.: A11001) validated by the supplier by IFA and flow cytometry.

Goat anti-rabbit 488 (Cat.: A11008) validated by the supplier by IFA and flow cytometry.

Peroxidase Affinity Pure Goat Anti-Human IgG (H+L) (Cat No. 109-035-088) validated by the supplier by western blot and ELISA.

anti-PPTRAMP mAb 1D9 was validated by western blot in Figure 4a and Extended Data Figures 2 and 4. anti-PPTRAMP mAb 3D8 was validated by IFA in Figure 3 and flow cytometry in Figure 4g.

anti-PfCSS mAb 2D2 was validated by western blot in Extended Data Figures 2 and 4. Rat pAb KM81 anti-PfCSS was validated by flow cytometry in Figure 4g.

Anti-CyRPA mAb 5B12 was validated in Chen et al., Elife 2017 by GIA and in this paper by flow cytometry. Anti-CyRPA mAb 889 from Chen et al., Elife 2017 was validated in this paper by IFA. Anti-CyRPA mAb 7A6 was validated in Chen et al., Elife 2017 and this paper by western blot.

Anti-Rh5 mAb 5A9 was validated in Chen et al., Plos Path 2011 by western blot and in this paper by flow cytometry in Figure 4g. Anti-Rh5 mAb 6H2 was validated in Chen et al., Plos Path 2011 and this paper by western blot (Extended Data Figure 2).

Anti-Ripr mAb 1G12 was validated in Healer et al., Cell Microbiol 2019 by western blot, SPR and GIA and in this paper by western blot in Extended Data Figure 2 and GIA in Figure 5b. Anti-Ripr polyclonal Ab R1541 was validated in Healer et al., Cell Microbiol 2019 by western blot and in this paper by flow cytometry in Figure 4g.

Anti-RON4 polyclonal Ab was validated in Richard et al., JBC 2010 by western blot and IFA and in this paper by IFA in Figure 3.

### Eukaryotic cell lines

#### Policy information about cell lines

**Cell line source(s)**

- 3D7 and CS2 P. falciparum lines
- SF21 cells (Thermofisher Scientific)
- Exp293F™ cells (Thermofisher Scientific)
- O+ erythrocyte (Australian red-cross bloodbank, South Melbourne, Australia)

**Authentication**

The P. falciparum lines are periodically sequenced for other projects. This serves as an authentication that they are the expected versions of the P. falciparum lines. SF21 and Exp293F™ cell lines were purchased or obtained with the certificate of analysis.

**Mycoplasma contamination**

All cell lines are tested periodically for Mycoplasma infection and were negative.

**Commonly misidentified lines**

(See ICLAC register)

N/A

### Animals and other organisms

#### Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

**Laboratory animals**

- Two female Balb/C mice received their first immunisation at 8-9 weeks of age. Two female Wistar rats received their first immunisation at 8 weeks of age. Animals were housed in open top cages with irradiated feed, autoclaved bedding, were checked daily, dark/light cycle 12 hrs - 7pm-7am dark - 7am-7pm light, temperature set to 21C, ranging from 18-24C, humidity approximately 40% but uncontrolled.

- Two female alpacas were immunized with recombinant PPTRAMP and PfCSS for the generation of nanobodies.

**Wild animals**

No wild animals were used in this study.

**Field-collected samples**

No field-collected samples were used in this study.

**Ethics oversight**

Antibodies and monoclonal antibodies were raised in rats and mice and all procedures approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. Immunization and handling of the alpaca for scientific purposes was approved by Agriculture Victoria, Wildlife and Small Institutions Animal Ethics Committee, project approval No. 26-17.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a group is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Erythrocyte binding assays were performed as described with some minor changes. Briefly, Erythrocytes were made up to a final density of 1 x 10^7 cells/ml in 1X PBS − 1% (w/v) BSA (PBS/BSA). All incubations were in 100 μl volume and washed in PBS/BSA at room temperature unless otherwise stated. Recombinant proteins were used at 0.1 nM final concentration. Complexes were made at equimolar ratios of 400 nM and incubated at room temperature for 1 hr for complex formation. Each sample was prepared using 100 μl of resuspended erythrocytes which were centrifuged at 2,000 x g for 1 min, supernatant removed, and the preincubated protein complexes of PBS/BSA added. After a 1 hr incubation on a roller, cells were centrifuged at 2,000 x g and washed once before a primary antibody was added. All antibodies were used at a final concentration of 0.2 ng/ml. After incubation for 1 hr, cells were washed once, and an Alexa Fluor 488 (Life Technologies) conjugated secondary anti-mouse, anti-rabbit, or anti-rat added at 1:100 dilution. Cells were washed twice in PBS and resuspended in 500 μl, followed by analysis with a LSR II flow cytometer (BD Life Sciences). Five-thousand events were recorded, and results analyzed using FlowJoTM 10.7.8 Software (BD Life Sciences). For quantitation, the background signal of erythrocytes incubated with only primary and secondary antibodies was subtracted from the signal of erythrocytes incubated with recombinant protein and relevant primary and secondary antibodies divided by the total number of events and then multiplied by 100 to achieve a percentage binding value. Statistical analysis was performed in Prism® (GraphPad) using an ordinary one-way ANOVA with multiple comparisons.

Analysis of Ca^2+ flux across the erythrocyte membrane was performed as described previously. PCR was performed at 30 μM and diluted into the erythrocytes to 1 μM to test stimulation of Ca^2+ flux. An LSR II flow cytometer (BD Life Sciences) was used for analysing samples and the results analysed in FlowJoTM 10.7 Software (BD Life Sciences) using the kinetics package.

Instrument

LSR II flow cytometer (BD Life Sciences).

Software

FlowJoTM 10.7 Software (BD Life Sciences) using the kinetics package.

Cell population abundance

N/A

Gating strategy

The erythrocyte population was gated with SSC-A and FSC-A, then doublets were excluded using FSC-H and FSC-A. For determining complex binding to erythrocytes, a cutoff of >1,000 was used. Gating was performed in an identical manner for all other antibody and antigen combinations.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.