Short communication, Parasitology International; Simon et al. 2015

Supplemental data

Material and Methods

Gene Identification. The following gene identifiers are assigned to the proteins investigated in this study (www.plasmodb.org) (Aurrecoechea et al., 2009): PfCCp1, PF3D7_1475500; PfCCp2, PF3D7_1455800; PfCCp3, PF3D7_1407000; PfCCp4, PF3D7_0903800; PfCCp5, PF3D7_0109100; PfFNPA, PF3D7_1451600; Pfs48/45, PF3D7_1346700; Pfs230, PF3D7_0209000; Pfs25, PF3D7_1031000.

Antibodies. The following antibodies were used in this study: mouse antisera against PfCCp1rp1, PfCCp1rp6, PfCCp2rp3, PfCCp3rp3, PfCCp4rp1, PfCCp5rp4, PfFNPArp1, and Pf39rp1 (Scholz et al., 2008; Simon et al., 2009); Pfs230 region C (Williamson et al., 1995), and Pfs48/45 (ATCC); rabbit antisera against Pfs25 (ATCC); goat antibody against the GST-tag (Amersham Biosciences) or mouse antibody against the MBP-tag (Sigma-Aldrich).

Parasite culture. For the study, either *P. falciparum* WT strain NF54 (ATCC) or line Pfs230-delta2 (Eksi et al., 2002) was used. The parasites were cultivated *in vitro* in human A^+ erythrocytes as described (Ifediba and Vanderberg, 1981). The RPMI1640/HEPES medium (Gibco) was complemented with 10% v/v heat-inactivated human serum, 50 µg/ml hypoxanthine (Sigma-Aldrich) and 10 µg/ml gentamicin (Gibco) and cultures were kept in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C. Human A^+ serum and erythrocytes were purchased from the Department of Transfusion Medicine, University Hospital Aachen, Germany. For cultivation of Pfs230-delta2, pyrimethamine at a final concentration of 502 µM was added to the medium and the cultures were cultivated with or without pyrimethamine in intervals of 3 weeks to remove potential revertants. For enrichment of gametocytes, cultures were harvested and enriched by 80/65/50/35% v/v Percoll gradients (GE Healthcare Life Sciences) as described (Kariuki et al., 1998) and parasites were collected at the 50/35% v/v Percoll gradient interfaces. Gametocyte activation was induced by addition of 100 µM xanthurenic acid at room temperature (RT).

Co-immunoprecipitation assay. Pellets of enriched non-activated gametocytes and gametocytes at 30 min post-activation of *P. falciparum* WT strain 54 were resuspended in 0.5% w/v saponin and 0.5% nonidet p-40 in PBS, homogenized and sonicated for 1 min as described (Simon et al., 2009). The homogenate was pelleted at 16,000g and the supernatant was pre-purified by incubation with 5% v/v pre-immune mouse or rabbit sera followed by 20 µl protein G-beads (Santa Cruz Biotechnology) for 30 min each at 4°C. After centrifugation at 3,400g, the supernatant was incubated for 1 h at 4°C with 5% v/v mouse immune sera against PfCCp proteins, Pfs230, Pfs48/45, and Pf39, or polyclonal rabbit antisera against Pfs25, followed by incubation with 20 µl protein G-beads for 1 h or overnight at 4°C. Beads were centrifuged, washed 5 times with PBS and mixed with an equal volume of SDS-loading buffer for SDS-PAGE. Precipitated proteins were analyzed via Western blot as described below.

Western blot analysis. Non-activated gametocytes of *P. falciparum* WT strain NF54 or line Pfs230-delta2 were enriched by Percoll gradient purification as described above. For generation of activated

gametocyte lysates, enriched mature gametocytes were washed twice with supplemented RPMI medium, incubated for 30 min at RT in 100 µM xanthurenic acid-containing medium and washed again with PBS. Mature non-activated or activated gametocytes were resuspended in PBS and SDS-PAGE loading buffer as described (Scholz et al., 2008). For studying the effect of inhibition of Pfs230 processing during gametocyte activation, gametocytes were pre-incubated for 2 h with 1 mM of the metalloprotease inhibitor 1,10-phenanthroline at 37°C prior to activation. Proteins were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) according to the manufacturer's instructions. Non-specific binding was blocked by incubation of the membranes in Tris-buffered saline containing 5% w/v skim milk and 1% w/v bovine serum albumin fraction V, followed by immune recognition for 2 h at RT using mouse or rabbit immune sera specific for the PfCCp proteins, Pfs48/45, Pfs230, Pfs25, or Pf39. Tagged proteins were either detected by goat anti-GST or mouse anti-MBP antibody. Afterwards membranes were washed, incubated for 1 h at RT with a goat anti-mouse or goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) and developed in a solution of nitroblue tetrazolium chloride (NBT) and 5-brom-4-chlor-3-indoxylphosphate (BCIP; Sigma-Aldrich). Scanned blots were processed using Adobe Photoshop CS software.

Affinity chromatography co-elution binding assay. MBP-tagged Pfs230 region C (MBP-Pfs230-C) and GST-tagged PfCCp1rp6, PfCCp3rp3 and Pf39rp1 were expressed in *E. coli* using vectors pGEX-4T1 (Amersham Bioscience) or pIH902 as described previously (Williamson et al., 1995; Scholz et al., 2008; Simon et al., 2009; Ngwa et al., 2013). GST-fusion proteins were purified from bacterial extracts using glutathione-sepharose according to the manufacturer’s protocol (GE Healthcare) and MBP-tagged recombinant proteins were purified using amylose resin (New England Biolabs) as described previously (Kern et al., 2014; Wirth et al., 2014). The regions of the recombinant proteins are indicated in Fig. S1. Co-elution binding assays were conducted as described previously (Simon et al., 2009) with following modifications of the procedure: Recombinant MBP-Pfs230-C was used as bait and was bound to amylose-resin columns according to the manufacturer´s protocol (New England Biolabs). Eluted proteins were analyzed by SDS-PAGE followed by Western blot analysis using goat anti-GST and mouse anti-MBP antibodies.

References

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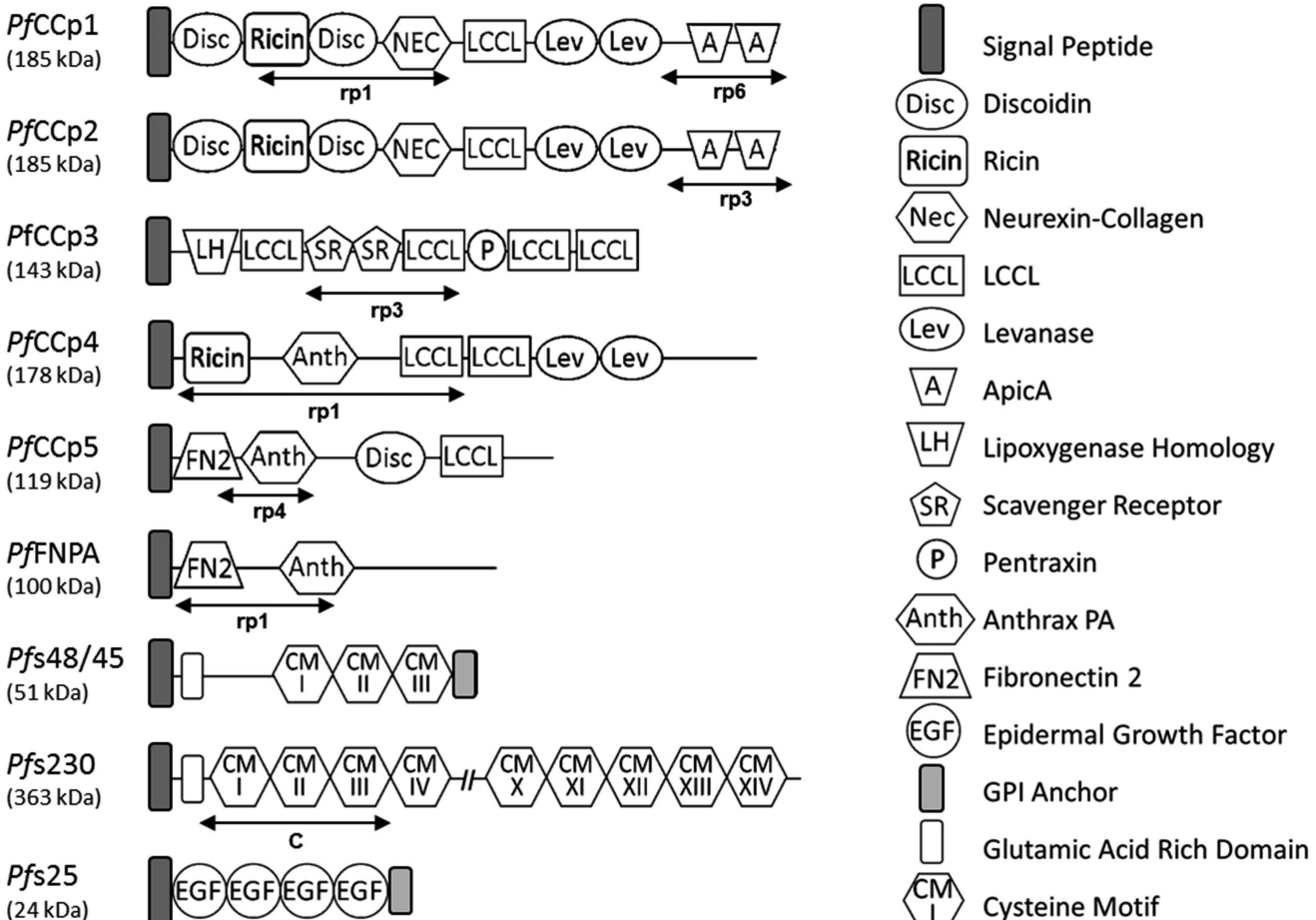
Figure Legends

Figure S1: Schematic of the domain structures of the MPC components.
Schematic of the domain structures of the PfCCp protein family, the cysteine-rich motif proteins Pfs48/45 and Pfs230 and the EGF domain protein Pfs25. The underlined regions denote the recombinant proteins used for generation of antisera and for co-elution binding assays (modified from Kuehn et al., 2010).

Figure S2. Protein-protein interactions between MPC components.
Lysates of WT strain NF54 mature non-activated (Gc) and activated (aGc) gametocytes (30 min p.a.) were subjected to co-immunoprecipitation assay, using mouse antisera against PfCCp5 and PfFNPA (bait), followed by Western blot analysis using the same antibodies as well as mouse antibodies against PfCCp1-3 and Pfs230 to detect the precipitated proteins (prey). Bands of immunoprecipitated proteins migrated at the expected molecular weights of 185 kDa (PfCCp1, PfCCp2), 143 kDa (PfCCp3), 119 kDa (PfCCp5), 100 kDa (PfFNPA), 360 and 300 kDa (Pfs230 unprocessed versus processed); an additional processed form of PfCCp2 migrated at 80 kDa. The intensities of the precipitated protein bands are indicated (++, strong; +, regular; -, negative). Smeared protein bands migrating at ~ 55 and 20 kDa resemble the heavy and light chains of the mouse antibody used for precipitation.

Figure S3. Protein-protein interactions between recombinant PfCCp proteins and Pfs230.
Affinity chromatography co-elution binding assays were performed using recombinant MBP-tagged Pfs230 region C (Pfs230-C-MBP) as bait and two selected GST-tagged recombinant PfCCp proteins, PfCCp1rp6-GST and PfCCp3rp3-GST, for prey. Eluate fractions E1-3 were subjected to Western blot analysis. Immunoblotting with goat anti-GST antibody detected the respective recombinant PfCCp peptides in the eluates. The buffer of the last washing step (W4) was used as negative control in the Western blots. For additional negative control, recombinant GST-tagged Pf39 (Pf39rp1-GST) was used as prey (neg. control 1). Further, buffer without protein was used and demonstrates the presence of an unspecific protein reacting with the anti-GST antibody (marked with an asterisk in the eluates; neg. control 2), which probably has bacterial origin. Immunoblotting with mouse anti-MBP antibody confirmed the presence of Pfs230-C-MBP in the eluates.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rp3
PfCCp3
(143 kDa)
LH
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P
rp3
PfCCp4
(178 kDa)
Anth
rp1
PfCCp5
(119 kDa)
FN2
rp4
PfFNPA
(100 kDa)
Pfs48/45
(51 kDa)
CM I
CM II
CM III
Pfs230
(363 kDa)
CM IV
CM X
CM XI
CM XII
CM XIII
CM XIV
C
Pfs25
(24 kDa)
EGF
Signal Peptide
Discoidin
Ricin
Neurexin-Collagen
LCCL
Levanase
ApicA
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Scavenger Receptor
Pentraxin
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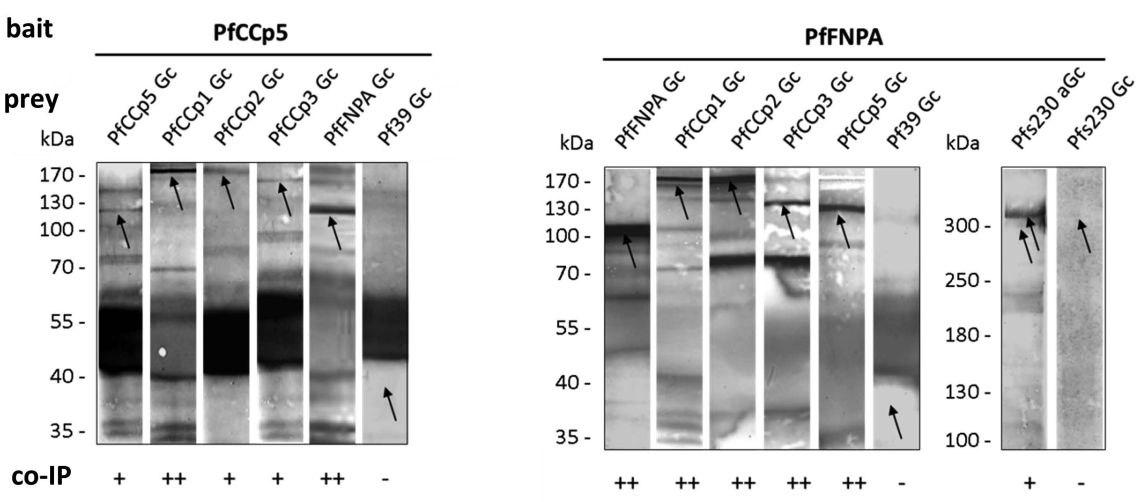
Fibronectin 2
Epidermal Growth Factor
GPI Anchor
Glutamic Acid Rich Domain
Cysteine Motif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PfCCp2 Gc
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Pf39 Gc
PfCCp5 Gc
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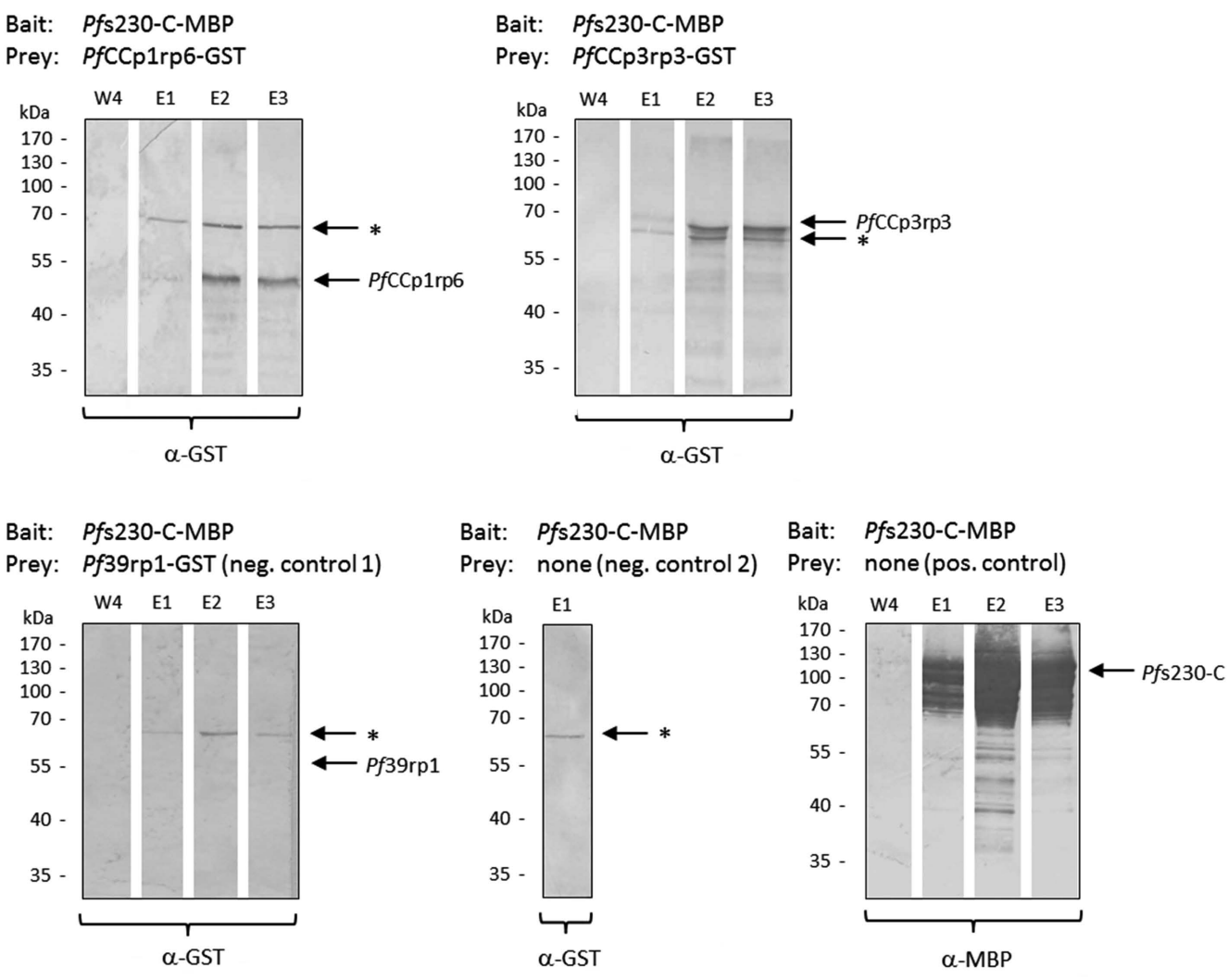
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Prey: PfCCp1rp6-GST
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PfCCp1rp6
α-GST
Bait: Pfs230-C-MBP
Prey: PfCCp3rp3-GST
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Bait: Pfs230-C-MBP
Prey: Pf39rp1-GST (neg. control 1)
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35 -
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Pf39rp1
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Bait: Pfs230-C-MBP
Prey: none (neg. control 2)
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Bait: Pfs230-C-MBP
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Pfs230-C
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