The *Plasmodium falciparum* protein VCAP1 controls Maurer's cleft morphology, knob architecture and PfEMP1 trafficking.

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Running Head: VCAP1 is required for virulence complex assembly

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26 **Abstract**

27 The malaria parasite, *Plasmodium falciparum*, traffics the virulence protein, *P. falciparum*
 28 erythrocyte membrane protein 1 (*PfEMP1*) to the surface of infected red blood cells (RBCs) via
 29 membranous organelles, known as the Maurer's clefts. We developed a method for efficient
 30 enrichment of Maurer's clefts and profiled the protein composition of this trafficking organelle. We
 31 identified 13 previously uncharacterised or poorly characterised Maurer's cleft proteins. We
 32 generated transfectants expressing GFP-fusions of 7 proteins and confirmed their Maurer's cleft
 33 location. Using co-immunoprecipitation and mass spectrometry we have generated a protein
 34 interaction map of proteins at the Maurer's clefts. We identified two key clusters that may function
 35 in the loading and unloading of *PfEMP1* into and out of the Maurer's clefts. We focus on a putative
 36 *PfEMP1* loading complex that includes the newly characterised virulence complex assembly protein
 37 1 (VCAP1). Disruption of VCAP1 causes Maurer's cleft fragmentation, aberrant knobs, ablation of
 38 *PfEMP1* surface expression and loss of the *PfEMP1* directed adhesion. Δ VCAP1 parasite lines have
 39 a growth advantage compared to wildtype parasites; and the infected RBCs are more deformable
 40 and more osmotically fragile.

41

42 **Importance**

43 The trafficking of the virulence antigen *PfEMP1* and its presentation at the knob structures at the
 44 surface of parasite infected RBCs is central to severe adhesion related pathologies such as cerebral
 45 and placental malaria. This work adds to our understanding of how *PfEMP1* is trafficked to the
 46 RBC membrane by defining the protein-protein interaction networks that function at the Maurer's
 47 clefts controlling *PfEMP1* loading and unloading. This work adds significantly to our understanding
 48 of virulence protein trafficking and will provide crucial knowledge that will be required to
 49 determine the mechanisms underpinning parasite driven host cell remodelling, parasite survival
 50 within the host and virulence mechanisms.

51 Introduction

52 Each year, *Plasmodium falciparum* causes ~200 million cases of illness in humans and more than
53 400,000 deaths, mostly of children under the age of five (1). In the asexual blood stage of infection,
54 parasites invade red blood cells (RBCs) and develop through the so-called ring, trophozoite
55 (growing) and schizont (dividing) stages, eventually releasing invasive merozoites that continue the
56 blood cycle. During this cycle the parasite induces marked changes to the host RBC, including the
57 elaboration of new organelles in the RBC cytoplasm, known as the Maurer's clefts and the
58 establishment of protrusions at the RBC membrane, known as knobs. The knobs comprise a spiral
59 protein structure supported by the knob-associated histidine-rich protein, (KAHRP) (2). The knob
60 acts as a scaffold for the presentation of the major virulence antigen *P. falciparum* erythrocyte
61 membrane protein 1 (*PfEMP1*). This virulence complex has an important role in adhesion of
62 infected RBCs to endothelial cell receptors. Rigid mature stage-infected RBCs sequester away from
63 the circulation, thus avoiding recognition and removal during passage through the spleen (3).
64 *PfEMP1* is exported into the host RBC cytoplasm via a translocon at the parasitophorous vacuole
65 membrane (4, 5). The current model suggests that *PfEMP1* is trafficked to Maurer's clefts as a
66 soluble, chaperoned complex (6, 7), where it is inserted into the membrane bilayer (8), before
67 repackaging for vesicle-mediated trafficking to the RBC surface (9).
68 Maurer's clefts are roughly disc-shaped cisternae, with a diameter of ~500 nm (10). In the ring
69 stage (up to ~20 h post-invasion), they are mobile in the RBC cytoplasm but later become tethered
70 to the RBC membrane skeleton (11). Remodelled host actin filaments and parasite-derived tethers
71 connect the Maurer's clefts to the RBC membrane (12, 13). However, the precise role of the
72 Maurer's clefts in protein trafficking remains unclear, and their composition is only partly defined
73 (14). In particular, there is very limited information about interactions between proteins of the
74 Maurer's clefts and with their virulence-associated cargo (15, 16).
75 In this work we developed a method for enriching "mobile" Maurer's clefts from parasites at 14-18
76 h post-invasion. We provide a detailed analysis of the Maurer's cleft proteome, identifying and

77 validating several novel resident proteins. Using super-resolution optical microscopy, we define the
 78 spatial organisation of new and established Maurer's clefts proteins. We use co-
 79 immunoprecipitation (co-IP) and mass spectrometry to describe a network of protein interactions,
 80 identifying two *PfEMP1*-interacting complexes, one of which we propose is responsible for loading
 81 *PfEMP1* into the Maurer's clefts. We show that deletion of a partially characterised Maurer's cleft
 82 component (*PF3D7_1301700*, *CBP2/GEXP07*), which we have renamed the virulence complex
 83 assembly protein 1 (*VCAP1*), leads to defective *PfEMP1* trafficking, altered Maurer's cleft
 84 architecture, aberrant knob formation and a loss of parasite adhesion to endothelial ligands. These
 85 changes are accompanied by decreased cellular rigidity, increased osmotic fragility and a marked
 86 growth advantage.

87

88 **Results**

89 **Enrichment of Maurer's clefts from infected RBCs**

90 Maurer's clefts are parasite-derived membrane-bound structures that remain mobile within the RBC
 91 cytoplasm up until ~20 h post-invasion (Movie S1). Leveraging this biology, we developed a
 92 protocol for purifying Maurer's clefts from parasite-infected RBCs at 14-18 h post-invasion.
 93 Fluorescence microscopy of transfectants expressing GFP-tagged ring exported protein 1 (*REX1*-
 94 GFP; (17)), confirms a typical Maurer's cleft profile (Fig 1A). *REX1*-GFP transfectant-infected
 95 RBCs were hypotonically lysed, and the suspension was extruded through a 27-gauge needle,
 96 adjusted to isotonicity, pre-cleared, and incubated with GFP-Trap beads (Fig 1B). Fluorescence
 97 microscopy of the beads (Fig S1A) confirms the presence of GFP-containing structures with
 98 dimensions consistent with a mixture of intact (~500 nm) and fragmented Maurer's clefts. Fractions
 99 from the total cell lysate, the cleared input and the unbound and bound fractions were analysed by
 100 Western blot (Fig 1C; full-length blots in Fig S1B). Due to the high haemoglobin content in the
 101 total, input and unbound fractions the samples were diluted 1:10 before electrophoresis; as a result,
 102 *REX1*-GFP and *SBP1* were not detected in these fractions. *REX1*-GFP and the known Maurer's

103 cleft protein, skeleton-binding protein 1 (SBP1;(18, 19), were highly enriched in the bound fraction,
104 while spectrin, a component of the host cytoskeleton was not detected, consistent with a high level
105 of enrichment. The parasitophorous vacuole membrane (PVM) protein, exported protein-1 (EXP1)
106 and the parasite cytoplasmic protein, glyceraldehyde-3 phosphate dehydrogenase (*Pf*GAPDH), were
107 also absent (Fig 1C).

108

109 **Mass spectrometry analysis of the enriched Maurer's clefts**

110 The captured Maurer's clefts were released from the beads, digested with trypsin and analysed by
111 LC-MS/MS. Proteins were deemed significant if two or more peptides were detected in the
112 Maurer's cleft (REX1-GFP) sample and were 3-fold enriched compared to the 3D7 control, in 2
113 separate experiments (Fig 1D; Data set 1). Five well-characterised Maurer's cleft proteins were
114 identified, namely REX1 (20), *Pf*EMP1 trafficking protein 1 (PTP1 (21)), skeleton binding protein
115 1 (SBP1 (22)), small exported membrane protein 1 (SEMP1 (15)) and membrane-associated
116 histidine-rich protein 1 (MAHRP1 (23)). A tether protein, membrane-associated histidine-rich
117 protein 2 (MAHRP2, (13)) was also identified.

118 Six partially characterised proteins that have been reported previously to be Maurer's cleft-located
119 were identified, namely the parasite-infected erythrocyte surface protein 2 (PIESP2 (24), sporozoite
120 and liver stage tryptophan-rich protein (25), *Pf*J23 (14), *PF3D7_0301700*, *PF3D7_1148900*,
121 *PF3D7_0702500* and *PF3D7_0601900* (15, 25, 26).

122 We identified *PF3D7_1301700* and *PF3D7_0113900*, for which there are conflicting reports with
123 respect to the cellular location, ranging from the RBC cytosol in gametocytes (27), to the Maurer's
124 clefts in asexual stages (28), to the RBC surface (29). These proteins have been previously referred
125 to as gametocyte exported protein 7 (GEXP07) / CX3CL1-binding protein 2 (CBP2) and GEXP10/
126 CBP1 (27, 29). Based on functional data described below we now refer to *PF3D7_1301700* as the
127 virulence complex assembly protein 1 (VCAP1).

128 A further 7 proteins were identified for which no location data has been reported previously. All but
 129 one protein (calcyclin-binding protein) has a predicted secretory signal or transmembrane segment
 130 and 5 have a Plasmodium export element (PEXEL) motif, which predicts export of parasite proteins
 131 to the host RBC (30). Four of these proteins have been successfully genetically disrupted: PTP5,
 132 PTP6, *PF3D7_1353100* and *PF3D7_0501000* (21). The remaining proteins *PF3D7_0702300*,
 133 *PF3D7_0811600*, *PF3D7_1002000*, *PF3D7_1035800*, and sporozoite threonine and asparagine-
 134 rich protein (STARP) do not have characterised locations. Over 100 peptides were observed for
 135 *PfEMP1* itself (Data set 1), consistent with a number of studies showing that *PfEMP1* is highly
 136 enriched at the Maurer's clefts (8, 31, 32), with only a sub-population reaching the RBC membrane.
 137 Some RBC proteins were also identified in the enriched Maurer's clefts, including Annexin A4 and
 138 A11 and Copine-3, which are calcium-dependent phospholipid-binding protein involved in
 139 membrane remodelling (33), as well as VPS28, TSG101, syntaxin and VAC14 - proteins that are
 140 involved in membrane binding, vesicle trafficking and lipid biogenesis (Table S1; Data set 1).

141

142 **GFP-tagging of proteins confirms their Maurer's cleft location**

143 To determine or confirm the locations of a number of the identified proteins, we generated
 144 transfectants expressing GFP fusions of five uncharacterised or partially characterised proteins
 145 (VCAP1, GEXP10/CBP1, PTP5, PTP6 and *PF3D7_1353100*) and two proteins that were
 146 previously reported to be Maurer's cleft-located (REX1 (17) and MAHRP1), each under the control
 147 of the *CRT* (chloroquine resistance transporter) promoter (Fig 2). An immunoblot probed with anti-
 148 GFP confirms the expression of chimeric proteins that migrate close to the calculated molecular
 149 masses (Fig S1C). Using live-cell microscopy, we showed that each of the GFP-tagged proteins
 150 localises to puncta in the host RBC cytoplasm, consistent with Maurer's cleft association (Fig 2).
 151 Immunofluorescence microscopy of samples co-labelled with antibodies recognising GFP and
 152 REX1 confirmed that the proteins are directed to the Maurer's clefts (Fig S2A). A previous study
 153 suggested that VCAP1 and GEXP10 are present at the RBC surface (29). While the presence of a

154 small sub-population at the RBC surface or in the RBC cytoplasm cannot be ruled out, our
155 immunofluorescence microscopy shows that the bulk of the protein is located at the Maurer's clefts
156 (Fig 2; Fig S2A).

157

158 **Co-IP of Maurer's cleft proteins reveals a compartmentalised network of protein interaction** 159 **clusters**

160 We performed co-IP experiments on our GFP-tagged transfectants. Western analysis confirmed the
161 enrichment of the GFP-tagged bait proteins (Fig S2B-D). In each of the lines analysed the bait
162 protein and several interacting proteins were identified (Table S2-8; Data sets 2-8). The protein
163 interaction networks were analysed using Navigator (Fig 3A,B, S3) (34). When combined with data
164 from other studies that performed IPs with epitope-tagged PTP1, SEMP1 and *PfEMP1*, a complex
165 map of interactions is revealed (Fig S3B) (15, 16, 31). We identified 2 protein clusters that each
166 contains Maurer's cleft proteins that are known to have roles in *PfEMP1* trafficking, connecting to
167 uncharacterised proteins and to *PfEMP1* itself (Fig 3A, B).

168 For example, previous work showed that MAHRP1 is needed for efficient loading of *PfEMP1* into
169 the Maurer's clefts (35). We found that MAHRP1-GFP co-precipitated VCAP1 and GEXP10, while
170 VCAP1-GFP and GEXP10-GFP co-precipitated MAHRP1 (Table S2-4; Data sets 2-4). These
171 interactions were confirmed by Western blotting (Fig S2E). GEXP10-GFP and MAHRP1-GFP also
172 precipitated PIESP2, while VCAP1-GFP precipitated *Pf322*, consistent with its known Maurer's
173 cleft location. VCAP1-GFP and GEXP10-GFP also precipitated *PfEMP1* (Table S2-4; Data sets 2-
174 4), suggesting a potential role for these proteins in *PfEMP1* trafficking.

175 3D-Structured Illumination Microscopy (3D-SIM) provides an 8-fold increase in volume resolution,
176 which enhances the analysis of compartments, such as the Maurer's clefts, that have dimensions
177 close to the resolution limit of conventional microscopy. 3D-SIM reveals that REX1 is located
178 around the perimeter of the clefts, while MAHRP1 exhibits a more central location (Fig 3C, S4A),
179 in agreement with our previous report (11). VCAP1-GFP and GEXP10-GFP are concentrated in the

180 central region of the Maurer's cleft surrounded by REX1 (Fig 3D,E, S4B). The profile for
181 antibodies recognising the ATS region of *PfEMP1* exhibits partial overlap with that for GEXP10-
182 GFP (Fig S4C).

183 The interaction analysis revealed a second protein cluster containing REX1-GFP, PTP5-GFP and
184 *PF3D7_1353100* (Fig 3B). REX1-GFP co-precipitated *PfEMP1* (Table S5; Data set 5), consistent
185 with the known role for REX1 in trafficking *PfEMP1* from the Maurer's clefts to the RBC
186 membrane (36, 37). A co-IP with PTP5-GFP co-precipitates REX1, PIESP2 and *PfJ23* (Table S6;
187 Data sets 6). These data point to the existence of a protein interaction network comprised of
188 *PfEMP1*, REX1, PTP5 and PIESP2, potentially functioning at the cleft periphery (Fig 3B). REX1-
189 GFP and *PF3D7_1353100*-GFP also co-precipitated the tether protein, MAHRP2 (Table S7; Data
190 set 7).

191 3D-SIM imaging of PTP5-GFP and *PF3D7_1353100*-GFP cell lines reveals a dotted pattern at the
192 periphery of the Maurer's cleft cisternae, partially overlapping or alternating with the REX1 signal
193 (Fig 3F, G, S4B). Similarly, *PfEMP1* (ATS labelling) partly overlaps with REX1 and *PfEMP1* at
194 the cleft periphery (Fig S4C).

195 Components of the PVM translocation machinery were observed in IPs with VCAP1-GFP
196 (HSP101, PTEX150), suggesting interactions with these proteins during the export processes. IPs
197 with PTP6-GFP showed a number of interacting proteins including MESA, STARP and three
198 proteins of unknown function, *PF3D7_0702500*, *PF3D7_1002000* and *PF3D7_0301700*. This
199 protein interaction network showed no connectivity to the clusters described above (Table S8; Data
200 set 8).

201

202 **VCAP1 gene knockout results in altered trafficking of exported proteins and a growth** 203 **advantage**

204 As we were interested in identifying proteins that may be involved in trafficking *PfEMP1*, we
205 selected VCAP1 and GEXP10 (which IP each other and *PfEMP1*) as candidates for genetic

206 disruption. These genes have been previously reported to be refractory to deletion using
 207 conventional approaches (21, 28). In this work, we used a CRISPR-Cas9 approach to disrupt these
 208 genes in CS2 parasites (Fig S5). We were unable to obtain a knockout of GEXP10 from two
 209 separate attempts, supporting a recent genome-wide transposon screen which suggested that
 210 GEXP10 is essential (38). By contrast, a knockout was obtained for VCAP1, with disruption of the
 211 native locus confirmed by PCR (Fig S5).

212 We examined the Δ VCAP1 infected RBCs by immunofluorescence microscopy, using antibodies to
 213 REX1 and KAHRP (Fig 4A). An increase in the number of REX1-positive puncta was observed
 214 suggesting a change in Maurer's cleft morphology (Fig 4A). The labelling pattern for KAHRP also
 215 appeared more punctate (Fig 4A). We next analysed the growth of the Δ VCAP1 parasite line
 216 compared with the CS2 parent parasites. The Δ VCAP1 parasite line grows faster than the parent
 217 line with a more than 2-fold difference observed after four parasite cycles (Fig 4B).

218

219 **Knockout of VCAP1 results in altered Maurer's cleft morphology**

220 RBCs infected with the Δ VCAP1 parasite line were lightly fixed and permeabilised with the pore-
 221 forming toxin, Equinatoxin II (EqII; (39, 40)) to release haemoglobin and to permit the
 222 introduction of primary antibodies recognising REX1.

223 Transmission electron microscopy was performed on thin sections prepared from CS2 and
 224 Δ VCAP1 infected RBCs. The Maurer's clefts are observed as single slender cisternae with an
 225 electron-lucent lumen and an electron-dense coat in the wild type CS2 (Fig 4C, S6A). In Δ VCAP1
 226 infected RBCs, distorted and fragmented structures are observed (Fig 4C, S6A). These fragments
 227 label with immunogold labelled anti-REX1 antibodies, confirming a Maurer's cleft origin (gold
 228 arrows; Fig 4C). The 3D architecture of the Maurer's cleft fragments was examined using electron
 229 tomography (Fig 4D). The rendered images reveal the fragmented nature of the Maurer's clefts
 230 when compared to wildtype CS2. Each separate cleft structure is rendered in a different pastel hue;
 231 the RBC (red) and the tethers (magenta) are also displayed (Fig 4D). The morphology changes are

232 best appreciated in translations through the virtual sections of the tomogram and rotations of the
233 rendered models (Movie S2-3).

234

235 **Δ VCAP1 parasite infected RBCs have altered knob morphology**

236 Scanning Electron Microscopy (SEM) of intact Δ VCAP1 infected RBCs reveals enlarged knobs
237 with a highly aberrant morphology, as well as knob clusters (Fig 5A). RBCs infected with Δ VCAP1
238 parasites have fewer knob structures (1.3 ± 0.2 per μm^2) than wildtype CS2 (5.5 ± 1 per μm^2) (Fig
239 5B). Thin section TEM confirmed the altered knob morphology (Fig S6B).

240 To investigate the architecture of the knobs in more detail we made use of a recently developed
241 method for imaging knobs at the cytoplasmic surface of infected RBCs (2). Infected RBCs were
242 tightly linked to lectin-coated slides and subjected to shearing. The remnant membrane discs were
243 fixed, dehydrated, gold-coated, and then imaged using high-resolution SEM. The spectrin-actin
244 network is evident as bright skeletal elements over dark patches of background membrane (Fig 5C).
245 The knobs appear as raised, dimpled disk-shaped structures that are closely integrated into the
246 skeletal meshwork (Fig 5C, arrows). About 75% of the knobs were malformed in the Δ VCAP1
247 infected RBCs, presenting as asymmetrical, enlarged and clustered knob structures (Fig 5D, S7A).
248 The knobs in the Δ VCAP1 infected RBC membranes exhibit a larger average diameter (90 ± 1 nm)
249 than CS2 wild type parent (80 ± 1 nm), and a broader size distribution (Fig 5E).

250

251 **Knockout of VCAP1 ablates *Pf*EMP1 surface expression and binding to chondroitin sulphate-**

252 **A**

253 To determine if *Pf*EMP1 trafficking was affected in the Δ VCAP1 infected RBCs we performed
254 immunofluorescence microscopy using antibodies directed towards the conserved acidic terminal
255 segment (ATS) of *Pf*EMP1. Wildtype CS2 infected RBCs exhibit characteristic localisation of
256 *Pf*EMP1 at the Maurer's clefts, while *Pf*EMP1 labelling is much weaker in the Δ VCAP1 infected
257 RBCs (Fig 6A, S7B).

258 We next examined if *PfEMP1* is displayed at the surface of Δ VCAP1 infected RBCs by using a
 259 trypsin cleavage assay (8, 32). In this assay whole infected RBCs are treated with trypsin (T) or
 260 with trypsin plus inhibitor (i), before Triton X-100 solubilisation. Only the Triton-insoluble (RBC
 261 membrane-embedded) *PfEMP1* population survives the extraction process. In the protease-inhibited
 262 CS2 control (i), RBC membrane-embedded full-length *PfEMP1* is evident, with an apparent
 263 molecular mass of ~270 kDa (Fig 6B, top blue arrow). It is evident that the pool of RBC
 264 membrane-embedded *PfEMP1* is decreased in the Δ VCAP1 infected RBCs (Fig 6B, top blue
 265 arrow). In the trypsin-treated CS2 sample (T) the surface pool of *PfEMP1* is depleted and two
 266 cleavage products of ~75 kDa and ~60 kDa (blue arrows) which correspond to the membrane
 267 embedded conserved ATS region of *PfEMP1* were observed. These cleavage products are absent in
 268 the Δ VCAP1 infected RBC samples consistent with a loss of *PfEMP1* at the RBC membrane (Fig
 269 6B). A cross-reaction of the *PfEMP1* antibody with RBC spectrin at ~220 kDa is observed as well
 270 as a number of additional bands of variable intensity which likely represent spectrin breakdown
 271 products were present in all samples (21).

272 To further examine surface presentation of *PfEMP1*, we used a rabbit polyclonal serum that
 273 recognises VAR2CSA on the surface of CS2 parasite-infected RBCs (41). A strong signal was
 274 observed in CS2 parasites, which was reduced by 90% in the Δ VCAP1 infected RBCs (Fig 6C).

275 The CS2 parent line expresses a fixed *PfEMP1* variant, VAR2CSA, which binds to chondroitin
 276 sulphate-A. We investigated infected RBC binding to immobilised chondroitin sulphate-A under
 277 physiologically relevant flow conditions. RBCs infected with Δ VCAP1 parasites show a 95% lower
 278 level of adhesion compared to the CS2 parent (Fig 6D). This provides further evidence that in the
 279 absence of VCAP1, parasites are unable to traffic *PfEMP1* to the RBC surface.

280

RBCs infected with Δ VCAP1 parasites have increased deformability and fragility

282 We examined the ability of CS2 and Δ VCAP1 infected RBCs to filter through a bed of beads
 283 designed to mimic splenic fenestrations. RBCs infected with Δ VCAP1 parasites synchronised to 20-

284 24 h post invasion (the window during which knob assembly and host cell remodelling occurs (2))
 285 exhibited a 23% increase in the filterability of the parasites compared to CS2 controls (Fig 6E).
 286 We next examined the sensitivity of infected RBCs to osmotic lysis. We found that Δ VCAP1
 287 infected RBCs are more sensitive to sorbitol-induced swelling than RBCs infected with wildtype
 288 CS2 (Fig 6F).

290 Discussion

291 The suggestion that Maurer's clefts play a role in transporting virulence proteins from the parasite
 292 to the RBC membrane was first put forward nearly 40 years ago (42), however our understanding of
 293 the repertoire of cleft-associated proteins and their functions remains incomplete. A previous study
 294 examined the proteins present in the ghost fraction of trophozoite stage-infected RBCs and
 295 characterised two novel Maurer's clefts proteins (14). More recently, proteomics approaches have
 296 been used to define the "exportomes" of both *P. yoelii* and *P. berghei* (43, 44). In this study, we
 297 built on this work and defined the composition of Maurer's cleft in *P. falciparum* during the late
 298 ring stage of infection (14 – 18 h post-invasion). During this time window PfEMP1 is trafficked to
 299 Maurer's clefts and a sub-population is transferred to the RBC membrane (8). A technical
 300 advantage of this time window is that the Maurer's clefts are still mobile in the RBC cytoplasm,
 301 allowing separation from host and other parasite components.

302 Making use of a previously generated REX1-GFP tagged line (17), we used GFP-Trap to affinity
 303 purify clefts released by hypotonic lysis. Mass spectrometry analysis identified enriched peptides
 304 from 18 proteins. Of these, 11 were previously shown to be Maurer's cleft-located proteins, and 7
 305 were potentially novel residents.

306 We also observed enrichment of host cell proteins in the Maurer's clefts. The most highly enriched
 307 protein was Annexin A4, which is involved in vesicle aggregation and the formation of lipid rafts
 308 (45). Other enriched human proteins include Annexin A11, which binds to the calcium sensor,
 309 calyculin (33, 46) and Copine-3 (a calcium-dependent phospholipid-binding protein), as well as

310 VPS28, TSG101 (a component of the ESCRT-I complex), syntaxin-7 (a SNARE component) and
 311 VAC14 (a scaffold protein involved in phosphatidylinositol 3,5-bisphosphate synthesis). All of
 312 these proteins are involved in membrane trafficking. It is possible these proteins play roles in
 313 Maurer's cleft sculpting, vesicle formation or *PfEMP1* trafficking.

314 We generated transfectants expressing GFP fusions of five putative Maurer's cleft proteins and two
 315 known resident proteins. We confirmed that all of the tagged proteins were located at the Maurer's
 316 clefts. The cell lines were then employed in an investigation of protein-protein interactions using
 317 GFP-Trap IP and protein identification by mass spectrometry. This analysis identified a network of
 318 interactions. We identified hubs where newly identified or poorly characterised Maurer's clefts
 319 proteins participate in networks with known *PfEMP1* trafficking proteins and importantly *PfEMP1*
 320 itself.

321 For example, we identified a network involving *PfEMP1*, REX1, PTP5 and the tether protein
 322 MAHRP2. This is consistent with previous evidence that PTP5 co-precipitates with GFP-tagged
 323 *PfEMP1* and that REX1 and PTP5 are needed for *PfEMP1* trafficking to the RBC surface (21, 31,
 324 37). We confirmed the interaction between REX1 and *PfEMP1*, consistent with a previous study
 325 showing that GFP-tagged *PfEMP1* co-precipitates REX1 and vice versa (31). Our previous work
 326 showed that REX1 plays a role in trafficking *PfEMP1* from the clefts to the RBC membrane. Using
 327 3D-SIM microscopy we previously showed that REX1 is located at the edges of the Maurer's clefts
 328 (11, 36, 37). In this work, we showed that PTP5 also locates at the cleft periphery, partially
 329 overlapping or intercalated with REX1 and *PfEMP1*. Collectively, these data points to a protein hub
 330 that plays a role in the trafficking of *PfEMP1* from the Maurer's clefts to the RBC membrane.
 331 Interestingly two members of this peripherally located hub, REX1 and *PF3D7_1353100*, co-
 332 precipitate MAHRP2. MAHRP2 is located on parasite-derived structure called tethers that have
 333 been proposed to link Maurer's clefts to the RBC membrane (13).

334 A second cluster comprises MAHRP1, VCAP1, GEXP10 and PIESP2. 3D-SIM microscopy
 335 revealed that MAHRP1, VCAP1 and GEXP10 have an overlapping physical location in the central

336 region of the Maurer's clefts. Previous work has shown that MAHRP1 disruption prevents
337 trafficking of *PfEMP1* to the Maurer's clefts (35), suggesting a possible role for this protein hub in
338 loading *PfEMP1* into the Maurer's clefts.

339 We further characterised two proteins from this cluster, VCAP1 and GEXP10. These proteins show
340 sequence similarity (32% identity) and are the only members of the Hyp8 gene family of exported
341 Plasmodium proteins (47). They both contain a signal peptide and a PEXEL motif and have no
342 orthologues in other Plasmodium species. The two proteins were initially reported to be exported to
343 the RBC cytosol in gametocytes (27), but another report identified VCAP1 as a Maurer's cleft-
344 associated protein in asexual stages (28). Yet another antibody-based study reported that VCAP1
345 and GEXP10 are located at the RBC surface in the asexual blood stage (29) and are implicated in
346 binding of infected RBCs to the chemokine CX3CL1. That study referred to them as CX3CL1-
347 Binding Proteins 2 and -1 (CBP2 and CBP1, respectively). In our work, we show that both VCAP1
348 and GEXP10 are present at the Maurer's cleft with no detectable GFP tagged protein in the RBC
349 cytosol or at the RBC membrane. We therefore classify VCAP1 and GEXP10 as Maurer's cleft
350 resident proteins but it remains possible they also occupy other locations at levels below the limit of
351 detection in our analysis.

352 Previous attempts to genetically disrupt VCAP1 and GEXP10 were unsuccessful, suggesting these
353 may be essential (21, 28). Using CRISPR-Cas9 and homology directed repair we were able to
354 successfully generate a Δ VCAP1 parasite line but were unable to generate a GEXP10 knockout.
355 The inability to knock-out GEXP10 is consistent with data from the recent saturation-level
356 mutagenesis study (38) which showed that GEXP10 is indispensable, based on the lack of *piggyBac*
357 transposon insertion sites (Mean Insertion Score of 0.26).

358 The Δ VCAP1 parasite line exhibited significant ultrastructural changes, with cleft fragmentation
359 and swelling. VCAP1 has two predicted transmembrane domains, as for the previously
360 characterised 2TM-MC Maurer's cleft protein family. The 2TM-MC proteins have been reported to
361 be oriented with the N- and C-terminal regions facing the RBC cytoplasm (48). If VCAP1 adopts an

equivalent orientation, its 26-amino acid loop (between the two transmembrane segments) would face the Maurer's cleft lumen. It is possible that this loop sequence is involved in interactions that stabilise the Maurer's clefts, and bring the two lamellae close together. Knock-out of VCAP1 also leads to the formation of enlarged aberrant knobs and knob clusters, sometimes located at the ends of membrane protrusions. Similarly, SEM imaging of the cytoplasmic face of infected RBC membranes reveals doughnut-shaped knob structures that are much larger in the Δ VCAP1 infected RBCs than in the CS2 parent.

The aberrant knob and Maurer's cleft morphology is associated with a defect in the delivery of *Pf*EMP1 to the RBC membrane, as confirmed by trypsin cleavage assays and flow cytometry using antibodies to the ectodomain of *Pf*EMP1. As a consequence, the binding of the infected RBCs to chondroitin sulphate-A was completely abrogated.

It is interesting to consider how the loss of a Maurer's cleft-resident protein could result in aberrant knob morphology. While an earlier study using a chimeric KAHRP-GFP fusion (with a non-natural signal sequence) suggested that KAHRP might be trafficked to the knobs via the Maurer's clefts (49), more recent studies do not support this model. Thus, it seems unlikely that Maurer's cleft-located VCAP1 plays a direct role in KAHRP trafficking. We considered the possibility that there is a small population of VCAP1 located at the RBC membrane, as previously proposed (29) which may participate in knob assembly. However, we were unable to detect GFP-tagged VCAP1 at the RBC membrane or in the RBC cytoplasm.

An alternative possibility is that VCAP1 is required for the trafficking of an unknown protein required for knob assembly. A recent ultrastructural study visualised the knob complex as a spiral structure connected by multiple links to the RBC membrane skeleton and coated by an electron-dense layer that likely represents KAHRP (50). An earlier cryo-electron tomography study revealed branched actin filaments (each ~500 nm in length) that connect the Maurer's clefts to the RBC membrane skeleton in the region of the knobs (12). Recent work from our laboratory suggests that knobs are generated by the trafficking of individual KAHRP-containing modules to the RBC

388 membrane skeleton followed by assembly of KAHRP modules into ring-shaped complexes that sit
389 at the base of the physical knob structure (2). The composition of the spiral core remains unknown.
390 One possibility is that correctly formed Maurer's clefts may be needed for delivery of a knob
391 assembly factor.

392 The Δ VCAP1 parasite line exhibited a growth advantage compared to wildtype CS2, perhaps
393 consistent with the general observation that knob-minus parasites outgrow knob-plus parasites in
394 culture. The abnormal knob morphology is associated with increased deformability, suggesting that
395 the reduced number of these aberrant knobs is unable to contribute the strain hardening effects
396 associated with wildtype knobs (51). Of interest, we found that Δ VCAP1 infected RBCs are more
397 sensitive to osmotic changes, suggesting that the abnormal knobs weaken the membrane structure.

398 The current model for *Pf*EMP1 trafficking suggests that it is trafficked across the RBC cytoplasm as
399 a chaperoned complex and then loaded into the membrane at the Maurer's clefts (52). Forward
400 trafficking delivers *Pf*EMP1 into the RBC membrane bilayer from where it moves laterally to the
401 knobs to form the mature virulence complex (2). The exact mechanism of *Pf*EMP1 unloading and
402 trafficking is not known but it has been speculated that *Pf*EMP1-loaded vesicles bud from the
403 Maurer's clefts and traffic along remodelled actin filaments or tethers. In the Δ VCAP1 parasite
404 infected RBCs we observe a dramatic fragmentation of the Maurer's clefts. We propose that this
405 compromises correct trafficking of both *Pf*EMP1 and a knob assembly factor. VCAP1 is a basic
406 protein located in the central region of the Maurer's clefts. It is possible that VCAP1 interacts with
407 the acidic C-terminal segment of *Pf*EMP1 and facilitates loading into the Maurer's cleft membrane.
408 It may then transfer *Pf*EMP1 to REX1 (at the Maurer's cleft periphery) for onward delivery. Further
409 studies are required to fully elucidate the role of VCAP1 in virulence protein trafficking.

410 In summary, we have developed a novel method for enriching Maurer's clefts from late ring stage
411 *P. falciparum*-infected RBCs that avoids host cell membrane contamination. We identified a
412 number of novel Maurer's cleft proteins that expand the repertoire of resident proteins locating at
413 these structures. We characterised different protein hubs that may function in the trafficking of

414 *PfEMP1* and determined their spatial organisation. In particular, we identified interacting proteins
 415 located in the central region of the Maurer's clefts that may be involved in loading *PfEMP1* into the
 416 Maurer's cleft membrane. In the absence of one of these proteins, VCAP1, the Maurer's clefts swell
 417 and fragment, the knobs enlarge and clump and *PfEMP1* trafficking to the RBC membrane fails.
 418 RBCs infected with VCAP1-disrupted parasites are more deformable, more sensitive to sorbitol
 419 lysis and the parasites possess a growth advantage. A more detailed understanding of the steps in
 420 the export pathway may reveal new strategies to target the parasite by blocking the delivery of
 421 *PfEMP1* to the infected RBC surface.

422

423 **Materials and Methods**

424 ***Plasmodium falciparum* culture**

425 Parasites were cultured as previously described (53). Briefly, *P. falciparum* cell lines were cultured
 426 in 5% human O+ red blood cells (Australian Red Cross blood service) in RPMI-GlutaMAX-
 427 HEPES (Invitrogen) supplemented with 5% (v/v) human serum (Australian Red Cross blood
 428 service), 0.25% (w/v) AlbuMAX II (Invitrogen), 200 μ M hypoxanthine, 10 mM D-glucose (Sigma)
 429 and 20 μ g/ml gentamicin (Sigma). Mature stage parasites were enriched from parasite culture using
 430 Percoll purification or magnetic separation (54, 55). Ring stage parasites were enriched by
 431 treatment with 5% D-sorbitol (56). Knob positive parasites were enriched by flotation on
 432 Gelofusine (Braun) as previously described for gelatin (57). Transfectants containing the pGLUX
 433 plasmids were maintained in the presence of 5 nM WR99210 (Jacobus Pharmaceuticals). The
 434 Δ VCAP1 parasite line was maintained on 2 nM DSM1 (BEI Resources).

435

436 **Plasmid construction and transfection**

437 To create the GFP-tagged transfectant parasites used in this study, the full-length genes (minus the
 438 stop codon) were amplified from 3D7 gDNA using primers incorporating *XhoI* and *KpnI* restriction

enzyme cleavage sites at the 5' and 3' ends of the gene (Table S9). Sequence verified DNA was then directionally cloned into the *Xho*I and *Kpn*I sites of the pGLUX vector. To generate the VCAP1 knockout cell lines two regions of homology within the 5' and 3' of the VCAP1 locus were PCR amplified using the primers as outlined in Table S9. The HR1 was cloned into the pUFTK plasmid containing a yeast dihydroorotate dehydrogenase cassette which confers resistance to DSM1 using the *Avr*II and *Nco*I sites and HR2 was cloned into the *Spe*I and *Sac*II site of the plasmid. The guide RNA was selected using CHOP CHOP and cloned into the *Btg*ZI linearised pAIO-Cas9 vector containing a hDHFR selection cassette (58). Transfections were performed as previously described (59).

448

449 **Live cell and immunofluorescence microscopy**

Infected red blood cell smears were fixed in an acetone and methanol solution at -20°C for 10 min. Wells were marked with a hydrophobic PAP pen, then washed three times with PBS. Primary antibodies were diluted in 3% (w/v) bovine serum albumin (BSA) and were added to the wells for 1 h at room temperature and subsequently washed three times with PBS. The primary antibodies used in this study were: mouse anti-REX1 (1:1000, (20)), rabbit anti-REX1 (1:1000, (20)), mouse anti-GFP (1:300, Roche), rabbit anti-GFP (1:300, (60)), mouse anti-MAHRP1 (1:300, (23)), mAb anti-ATS (1:100, (19)), mouse anti-SBP1 (1:300, (18)), rabbit anti-HA (1:300, Sigma Aldrich). Secondary anti-mouse or anti-rabbit AlexaFluor® 488, 568 or 647 antibodies (1:300 in 3% (w/v) BSA, Life Technologies) were added to the wells for 1 h at room temperature, then wells were washed 3 times with PBS. Each well was then treated with DAPI (4',6-diamidino-2-phenylindole) and PPD antifade (p-phenylenediamine) prior to mounting and sealing. For live-cell fluorescence microscopy, parasites were suspended in RPMI and mounted on a coverslip. Samples were imaged on a DeltaVision Elite Restorative Widefield Deconvolution Imaging System (GE Healthcare). Samples were excited with solid state illumination (Insight SSI, Lumencor). The following filter sets with excitation and emission wavelengths were used: DAPI Ex390/18, Em435/48; FITC,

Ex475/28, Em523/26; TRITC, Ex542/27, Em594/45; Cy5 Ex 632/22, 676/34 nm. A 100x UPLS Apo (Olympus, 1.4NA) oil immersion objective lens was used for imaging. For 3D Structured Illumination microscopy (3D-SIM), the DeltaVision OMX V4 Blaze was used (GE Healthcare). Samples were imaged using a 60X Olympus Plan APO N (1.42 NA) oil immersion lens. The following laser emission and band pass filter sets were used: Ex488, Em528/48; Ex568, Em609/37 or Ex642, Em683/40 nm. Images were processed using the FIJI ImageJ software (61).

471

472 **SDS-PAGE and immunoblotting of protein samples**

473 For immunoblotting, samples were resuspended in Bolt™ LDS sample buffer and 50 mM
474 dithiothreitol (DTT) and separated on Bolt™ 4-12% Bis-Tris gel at 200V for 32 min. Separated
475 samples were transferred to a nitrocellulose membrane using an iBlot® gel transfer device.
476 Membranes were blocked in 3% (w/v) skim milk for 1 h at room temperature. The membranes were
477 then probed for 16 h at 4 °C with the following primary antibodies in 3% (w/v) skim milk in PBS:
478 mouse anti-GFP (1:1000, Roche), rabbit anti-SBP1 (1:1000, (18)), mAb anti-ATS (1:100, (19)),
479 mouse anti-REX1 (1:1000, (20)), mouse anti-EXP1 (1:1000), rabbit anti-spectrin (1:1000, Sigma
480 Aldrich), mouse anti-MAHRP1 (1:1000, (23)), rabbit anti-GAPDH (1:1000), rabbit anti-HA
481 (1:1000, Sigma Aldrich). Membranes were washed 3 times in 0.05% (v/v) Tween-20 in PBS for 10
482 min. Secondary antibodies (anti-mouse or anti-rabbit) conjugated to horseradish peroxidase (HRP,
483 Promega) were diluted 1:25,000 in 3% (w/v) skim milk in PBS and were incubated with
484 membranes for 1 h at room temperature. Membranes were again washed 3 times in 0.05% (v/v)
485 Tween-20 in PBS, then once in PBS before being incubated with Clarity™ ECL reagents (Bio-Rad)
486 and visualised on a ChemiDoc imaging system (Bio-Rad).

487

488 **Enrichment of Maurer's clefts**

489 Parasites were synchronised to a 4 h window by Percoll purification followed by sorbitol lysis. At
490 14-18 h post-invasion, parasites (~15% parasitaemia) were harvested and washed in PBS. Infected

red blood cells were lysed on ice with chilled hypotonic buffer (1 mM HEPES/NaOH, Roche cOmplete EDTA-free protease inhibitor, pH 7.4). The lysed infected red blood cell solution was passaged through a 27-gauge needle 10 times and was then made isotonic by the addition of 4X assay buffer (200 mM Hepes-NaOH, 200mM NaCl, 8 mM EDTA (pH 7.4). The solution was centrifuged at 2500 g for 10 min at 4 °C, the supernatant was collected and centrifuged again at 2500 g for 10 min at 4 °C. The supernatant (containing the Maurer's clefts) was then pre-cleared with Pierce™ Protein A agarose beads (Thermo Scientific) for 30 min at 4 °C on a mixing wheel. The Protein A agarose beads were pelleted by centrifugation and the supernatant was incubated with GFP-Trap® agarose beads (Chromotek) for 4 h at 4 °C on a mixing wheel. The beads were then collected and used for downstream applications.

501

502 **Co-IP with GFP-Trap agarose beads**

Parasite-infected RBCs were purified using a VarioMACS™ (Miltenyi Biotec) magnet or by floatation in 70% (v/v) Gelofusine® (Braun) in PBS. The infected RBCs were washed in RPMI and then solubilised on ice for 30 min in 10 times pellet volumes of IP buffer (1% (v/v) Triton X-100, 150 mM NaCl, 50 mM Tris, 8 mM EDTA) with Complete™ protease inhibitor cocktail (Roche). The Triton X-100-insoluble material was pelleted twice by centrifugation (10 min at 16,000 g). The supernatant was pre-cleared with Pierce™ Protein A agarose beads (Thermo Scientific) for 1 h at 4 °C on a mixing wheel. The Protein A beads were pelleted and an aliquot of the supernatant was taken as the input fraction. The remainder of the supernatant was incubated with washed GFP-Trap agarose beads (Chromotek) for 16 h at 4 °C. The GFP- Trap beads were then washed 5 times in IP buffer.

513

514 **Mass spectrometric analysis of Maurer's clefts and co-IPs**

Agarose beads were washed twice in 1 mM Tris-HCl prior to elution of bound proteins. Samples were prepared for mass spectrometric analysis as previously described (31). Briefly, proteins were

517 eluted from GFP-Trap beads by the addition of 20% (v/v) trifluoroethanol in formic acid (0.1%, pH
518 2.4) and were incubated at 50°C for 5 min. The eluate was reduced with 5 mM TCEP (Thermo
519 Fischer Scientific) and neutralised with TEAB (tetraethylammonium bromide). Samples were
520 digested with trypsin (Sigma) overnight at 37 °C.

521 Samples were analysed by ESI LC-MS/MS on Orbitrap Elite (purified Maurer's clefts) or Q
522 Exactive (co-IPs) mass spectrometers. Mass spectra (ProteoWizard) were searched against a custom
523 database containing the *Plasmodium falciparum* 3D7 and UniProt human proteomes. Searches were
524 performed on MASCOT (Matrix Science), with the following parameters: precursor ion mass
525 tolerance of 10 ppm, fragment ion mass tolerance of 0.2 Da, trypsin as the cleavage enzyme, three
526 allowed missed cleavages and allowing for oxidation. The network map of protein interactions was
527 created using NAViGaTOR 2.3 software, networks were redrawn in Adobe Illustrator. The mass
528 spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the
529 PRIDE (62) partner repository with the dataset identifier PXD014873. (Temporary
530 Username: reviewer30396@ebi.ac.uk Password: thU0KxPW).

531

532 **Infected RBC binding assay under flow conditions**

533 Ibidi μ -Slide 0.2 channel slides were incubated with 100 μ l chondroitin sulfate A (100 μ g/ml,
534 Sigma) in 1% (w/v) BSA in PBS overnight at 37°C. Channels were blocked with 1% (w/v) BSA in
535 PBS for 1 h at room temperature then gently flushed with warm bicarbonate-free RPMI 1640
536 (Invitrogen). Mid-trophozoite stage cultures were diluted to 3% parasitemia and 1% haematocrit in
537 bicarbonate-free RPMI 1640 and pulled through the channel at 100 μ l/min for 10 min at 37°C.
538 Unbound cells were washed out of the channel at 100 μ l/min for 10 min at 37°C. Adherent cells
539 were counted at 10 points along the axis of the channel.

540

541 **VAR2CSA ectodomain labelling and analysis via flow cytometry**

Mid-trophozoite cultures were diluted to 3% parasitemia, 0.4% hematocrit and plated into a 96 well plate. Cells were washed 3 times in 1% (w/v) BSA in PBS and incubated with a rabbit polyclonal anti-VAR2CSA for 30 min at 37°C (R1945 1:50, Elliott et al., 2005) or a 1% (w/v) BSA in PBS control. Cells were washed in 1% BSA/PBS between all subsequent incubations and all antibodies were diluted in 1% BSA/PBS. Cells were incubated with mouse anti-rabbit IgG (Dako; 1:100) for 30 min at 37°C, then mouse anti-GFP (Alexa Fluor-488) under the same conditions. Following tertiary labelling, cells were washed 3 times in 1% (w/v) BSA in PBS. Nuclei were stained with SYTO61 as described previously (Klonis et al., 2011) and analysed via flow cytometer (BD FACSCanto II). SYTO61 positive cells were gated and the Alexa Fluor-488 fluorescence intensity measured. The results are displayed as the percentage of fluorescence relative to wild type controls.

Trypsin cleavage of surface exposed *Pf*EMP1

Late stage infected RBCs were purified and incubated with 20 volumes of 1 mg/ml TPCK-treated trypsin in PBS (Sigma) or trypsin and soybean trypsin inhibitor (5 mg/ml, Sigma) at 37°C for 1 h. Trypsin activity was quenched with a 15 min incubation with trypsin inhibitor at room temperature for 15 min. Cells were subsequently solubilised in 1% Triton X-100 in PBS containing 1x cOmplete protease inhibitor (Roche) and incubated on ice for 20 min. The sample was centrifuged at 16,000 x g at 4°C for 10 min and the resulting pellet washed in the triton solution 2 additional times. The resulting triton-insoluble pellet was solubilized in 2% SDS in PBS and mixed on a rotating wheel for 40 min. This SDS-soluble fraction was then separated by SDS-PAGE and transferred to a nitrocellulose membrane for western blotting.

Parasite growth assay

Parental CS2 and Δ VCAP1 cell lines were synchronised to a 2 h window and diluted to 1.5% late stage parasitemia. Every 48 h infected red blood cells were taken from culture and stained with SYTO-61 nuclear stain and parasitemia was calculated by flow cytometry as previously described

(Klonis et al., 2011). The cultures were then diluted by the same dilution factor. Parasitaemia was recorded over 4 cycles and normalised to the parental CS2 line.

Sorbitol sensitivity assay

Mid-trophozoite infected red blood cells at 2-5% parasitemia were incubated at 37°C with one of 12 solutions between 0.1% (w/v) and 5.5% (w/v) D-sorbitol. Parasitemia was measured via flow cytometry following incubation with nuclear stain SYTO-61 and 30,000-50,000 events recorded per well as previously described (Klonis et al., 2011). Parasitemia was normalised to an internal RPMI control for each cell. This data was then used to calculate the IC50 values for each separate experiment. The IC50 values from each experiment are plotted.

Microbead filtration

Spleen mimic filtration was performed to assess the deformability properties of parasite infected RBC (63). Synchronous parasites at 22 h post-invasion were prepared at 6% parasitemia and 1% hematocrit in 1% (w/v) AlbuMAX II in 1X PBS and flowed through the microbead bed using a syringe pump at a flow rate of 60mL/min. The data presented is the percentage of parasites present in the flow through relative to starting parasitemia. The mean and standard error are plotted, from 3 separate experiments. A Students t-test was used to evaluate statistical significance.

Membrane shearing

Glass coverslips were cleaned with acetone and 50% methanol prior to treatment with 3-aminopropyl triethoxysilane (APTES), bis-sulfosuccimidyl suberate and addition of the ligand erythroagglutinating phytohemagglutinin (PHAE) as previously described (64). Infected RBCs were immobilised on the functionalised glass slides, then sheared by applying a hypotonic buffer (5 mM Na₂HPO₄/NaH₂PO₄, 10 mM NaCl, pH 8) from a 30 mL syringe (23-G needle) at an angle of ~20° (65). The membrane disks were placed in PBS prior to down-stream imaging.

594

595

596

597 **Scanning Electron Microscopy**

598 Whole infected RBC cell samples bound to glass coverslips were immersed in 0.05%
 599 glutaraldehyde in PBS for 20 minutes prior to fixation in 2.5% glutaraldehyde for 2 h at room
 600 temperature. Sheared membranes were prepared for scanning electron microscopy as previously
 601 described (2). In brief, sheared cells were fixed immediately after lysis with 2.5% glutaraldehyde in
 602 PBS for 2 h at room temperature. Both whole and sheared cell samples were then washed
 603 thoroughly with deionised water and dehydrated via sequential 5 min incubations in 20%, 50%,
 604 70%, 80%, 90%, 95% and (3x) 100% ethanol before critical point drying in a Leica CPD300 critical
 605 point dryer. Samples were stored under desiccation and gold coated immediately before imaging.
 606 Coverslips were gold coated for 40 s or 75 s at 25 mA using a Dynavac sputter coating instrument
 607 to a thickness of ~0.2 nm or ~0.4 nm for sheared and whole cells respectively. Images were
 608 acquired with the ETD detector of an FEI Teneo SEM in Optiplan mode, at a working distance of 5
 609 mm, a beam current of 50 pA and a 2-kV accelerating voltage.

610

611 **Immuno-electron Tomography**

612 Infected red blood cells (20 – 30 h post invasion) were magnet purified, washed in PBS, and fixed
 613 in 10 pellet volumes of 2% (v/v) *PFA* in PBS for 20 min at room temperature. Cells were washed
 614 then permeabilised in 10 pellet volumes of PBS with 1 HU equinotoxin II for 6 min. After washing
 615 cells were fixed again in 2% *PFA*/PBS for 5 min, washed, then blocked in suspension for 1 h with
 616 3% BSA/PBS. Cells were incubated with anti-REX1 (1:50) for 2 h, washed, then incubated with a
 617 gold secondary for 1 h (1:15, Aurion protein A EM grade 6 nm gold JA806-111). Cells were
 618 washed in 3% (w/v) BSA in PBS then in PBS to remove the BSA.

619 Cell pellets were resuspended and fixed in 2.5% glutaraldehyde at 4°C for at least 1 h, prior to pre-
 620 embedding in low melting point agarose. Cells were then post fixed in 1% potassium ferricyanide
 621 ($K_3[Fe(CN)_6]$) reduced osmium tetroxide (OsO_4) solution for 1 h. Blocks were then rinsed 5 x 3
 622 min in ddH₂O then dehydrated by sequential incubation 5 min each in 20%, 50%, 70%, 80%, 90%,
 623 95%, and 100% ethanol. Blocks were incubated in 100 % ethanol twice more 5 min each, followed
 624 by ethanol/acetone solution (1:1) incubation for 30 min, then 100% acetone for 30 min. Acetone
 625 was substituted for acetone/1% thiocarbohydrazide resin (1:1) for 2 h and infiltrated with 100%
 626 resin 2 x 12 h and polymerised in oven at 60°C.

627 Resin blocks were trimmed and sectioned on ultramicrotome (Leica EM UC7, Leica Microsystems,
 628 Wetzlar, Germany) and both 70 nm and 300 nm sections were prepared for imaging and electron
 629 tomography, respectively. The sections were then post stained by 4% uranyl acetate in water for 10
 630 mins and Reynold's lead citrated for 10 min. The 300 nm sections were overlayed with 10 nm
 631 fiducial gold particles on both sides of the grid. Imaging and electron tomography were performed
 632 on FEI Tecnai F30 electron microscope (FEI company, Hillsboro, US) at an accelerating voltage of
 633 300kV. The tilt series were acquired for every 2° in the range between -70° to 70° tilts. Virtual
 634 sections were reconstructed from raw tilt series in IMOD using weighted back projection algorithm
 635 (66).

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649

650 **Figure legends**

651 **Fig 1. Enrichment and analysis of Maurer's clefts from *P. falciparum* REX1-GFP infected** 652 **RBCs.**

653 (A) Live cell fluorescence of an RBC infected with a REX1-GFP expressing parasite at 14-18 h
654 post-invasion. GFP (green), DIC and merged images are shown. Scale bar 5 μ m. (B) Schematic
655 overview of the GFP-Trap Maurer's cleft enrichment method. Infected RBCs (14 – 18 h post-
656 invasion) are hypotonically lysed, subjected to differential centrifugation, then the REX1-GFP-
657 labelled Maurer's clefts are captured with GFP-Trap beads. (C) Western blot assessing enrichment
658 of Maurer's clefts from REX1-GFP infected RBCs. Total = complete infected RBC lysate; Input =
659 fraction applied to the GFP-Trap beads; Unbound = supernatant after GFP-Trap bead incubation;
660 Beads = material bound to beads. Molecular masses are in kDa. (D) Summary of proteins identified
661 in the mass spectrometric analysis of enriched REX1-GFP Maurer's clefts. See Data set 1 for more
662 detail. Proteins chosen for GFP tagging are indicated in green text. MC = Maurer's clefts; RBC =
663 red blood cell; Surface = red blood cell surface; N/A = Not Available; PEXEL = Plasmodium
664 export element; PNEP = PEXEL negative exported protein.

665

666 **Fig 2. Live cell fluorescence analysis of GFP-tagged exported proteins.**

667 Predicted native protein length (amino acids) and schematic representations of six proteins that
668 were selected for GFP-tagging are shown on the left. Grey = signal sequence, blue =
669 transmembrane domain, yellow = PEXEL motif, green = GFP tag. Live cell fluorescence and DIC

670 microscopy of transfectants expressing GFP-tagged putative Maurer's cleft proteins reveals
671 fluorescent puncta in the RBC cytoplasm. Scale bar = 5 μ m.

672

673 **Fig 3. Maurer's clefts proteins interact to form distinct protein clusters.**

674 (A) Protein interaction maps highlighting a putative *Pf*EMP1 loading hub comprising VCAP1,
675 MAHRP1 and GEXP10 and (B) a putative unloading hub comprising REX1, PTP5, SEMP1 and
676 *Pf*3D7_1353100. Green nodes are the GFP-tagged proteins. Double thickness edges indicate
677 reciprocal co-precipitation (See Fig S3 for full network maps). (C) 3D-SIM analysis of REX1-GFP-
678 infected RBCs fixed and labelled with anti-GFP (green) and anti-MAHRP1 (red). (D-G) 3D-SIM
679 analysis of transfectant-infected RBCs expressing GFP-tagged VCAP1, GEXP10, PTP5 and
680 *Pf*3D7_1353100 that were fixed and labelled with anti-GFP (green) and anti-REX (red) antibodies.
681 Maximum projections of Z-stacks are displayed. Scale bars = 3 μ m, zoom scale bar = 3 μ m.

682

683 **Fig 4. Knockout of VCAP1 alters parasite growth and Maurer's cleft architecture.** (A)

684 Infected-RBCs were fixed and probed with anti-MAHRP, and counterstained with anti-REX1.
685 Nuclei were stained with DAPI (blue). CS2 = parent line. Projections of Z stacks are shown. Scale
686 bar = 5 μ m. (B) Parasite growth assay measuring proliferation over 4 asexual cycles. Growth was
687 assessed by staining infected RBCs with SYTO61 and subsequent flow cytometry analysis. The
688 data have been normalised and expressed relative to the Δ VCAP1 parasite line (n=4). (C) EqtII-
689 permeabilised wildtype CS2 and VCAP1 knockout transfectants were labelled with antibodies
690 recognising REX1, followed by immunogold labelling and prepared for electron microscopy.
691 Images have been cropped around Maurer's clefts (white arrows) - see Fig S6A for uncropped
692 micrographs. Gold arrows point to gold particles. Scale bar 100 nm. (D) Rendered 3D models of
693 Maurer's clefts generated from electron tomograms. Red = RBC; magenta stalk = tether; pastel
694 hues = independent clefts. Scale bar = 100 nm. Translations through the virtual sections of the
695 tomograms and rotations of the rendered models can be seen in Movies S2-3.

696

697 **Fig 5. Examination of the Δ VCAP1 infected RBCs reveals altered knob morphology.** (A) Mid-
698 trophozoite stage infected-RBCs were fixed in 2.5% glutaraldehyde/PBS and prepared for SEM of
699 the exterior surface. Scale bar = 1 μ m. (B) Quantification of the number of knob like structures
700 observed in the external SEM. Data is the mean number of structures per μ m². (n = 15 cells per
701 sample, minimum 3 areas per cell, Student's t-test P<0.0001). (C) Late trophozoite stage-infected
702 RBCs were immobilised onto glass slides. Shearing under hypotonic conditions leaves remnant
703 membrane discs that were fixed, dehydrated, gold-coated and imaged using SEM. Knobs (K) are
704 arrowed. Scale bar = 1 μ m; zoom 1 scale bar = 500 nm; zoom 2 = 50 nm. (D) Quantification of the
705 knob morphologies observed by internal SEM imaging. These are defined as normal knobs (blue),
706 asymmetrical knobs (orange), large knobs (green) and knob clusters (yellow). Example images of
707 these morphologies are shown in Fig S7A. (E) Graph showing the frequency distribution of knob
708 sizes from wildtype CS2 and Δ VCAP1 infected RBCs. A bin size of 10 nm was used; n = 760
709 (Δ VCAP1), 1098 (CS2).

710

711 **Fig 6. Genetic knockout of VCAP1 lead to a loss of *Pf*EMP1 at the RBC surface and impaired**
712 **adhesion.** (A) Wildtype CS2 and VCAP1 knockout-infected RBCs were fixed and probed with
713 anti-*Pf*EMP1 (green) and counterstained with anti-REX1 (red). Nuclei were stained with DAPI
714 (blue). CS2 = parent line. Projections of Z stacks are shown. Scale bar = 5 μ m. (B) Whole infected
715 RBCs were treated with either trypsin (T), or trypsin + trypsin inhibitor (i), solubilised in Triton X-
716 100 and the resultant pellet representing RBC membrane embedded *Pf*EMP1 subjected to western
717 blotting and probed with anti-ATS. Full length *Pf*EMP1 (350 kDa) and ATS bands are indicated
718 with blue arrows. Bands at ~240 kDa represent cross reaction with spectrin (red arrow). A ~70 kD
719 cross-reactive species can be seen in all samples. (C) Flow cytometry analysis of infected RBCs
720 labelled with antibodies to the external domains of *Pf*EMP1. Experiments were performed in
721 triplicate on 3 separate occasions. Errors bars represent SEM, Student's t-test, P = 0.0002. The

relative mean fluorescence intensity (rMFI) normalised to CS2 controls is shown. (D) Mid-trophozoite stage-infected RBCs were examined for their ability to bind to chondroitin sulfate-A under physiological flow conditions. The average number of adherent infected-RBCs in 10 fields of view was assessed in 3 independent experiments. Values were normalized to the parent line, CS2. (F) Spleen mimic filtration showing a significant increasing in filterability for the Δ VCAP1 infected RBCs. Experiments were performed in triplicate on 3 separate occasions. Student's t-test, $P = 0.0006$. Error bars represent SEM. (G) Analysis of susceptibility to lysis in sorbitol solutions. The IC_{50} values for triplicate experiments performed on 5 separate occasions are shown. The line represents the median.

731

732 **Supplementary Figure Legends**

733 **Fig S1. Live-cell microscopy of REX1-GFP bound to GFP-Trap beads and full-length** 734 **Western blots of GFP-tagged transfectants.**

735 (A) Fluorescence microscopy of the beads reveals structures with dimensions consistent with a
736 mixture of intact (~500 nm) and fragmented Maurer's clefts. Scale bar = 5 μ m. (B) Full uncropped
737 Western blot images for assessing enrichment of Maurer's clefts from REX1-GFP infected RBCs.
738 These blots were cut horizontally in some cases to simultaneously probe for proteins of different
739 sizes. Total = complete infected RBC lysate; Input = fraction applied to GFP-Trap beads; Unbound
740 = supernatant after GFP-Trap bead incubation, Beads = material bound to beads. Molecular masses
741 are in kDa. (C) Western blot of saponin-lysed infected RBCs from GFP-tagged transfectant parasite
742 lines. Green arrowheads indicate predicted size of the mature full-length protein. Molecular masses
743 are in kDa.

744

745 **Fig S2. Immunofluorescence microscopy and Western blots of GFP-tagged proteins**

746 (A) The GFP-tagged transfectant-infected RBC smears fixed in acetone/methanol (9:1) at -20°C.
747 Samples were labelled with anti-GFP (green) to localise the GFP-tagged protein and anti-REX1

(red) to label the Maurer's clefts. Parasite nuclei were stained with DAPI (blue). Scale bars = 5 μ m.

(B-E) Infected RBCs were solubilised in 1% Triton X-100 and subjected to SDS-PAGE. Input loading was 4% of the pellet loading. Green arrowheads indicate the full-length GFP-tagged proteins. Blue arrowheads indicate co-precipitated proteins. (B) 3D7 wildtype, REX1-GFP; (C) PTP6-GFP; (D) GEXP10-GFP, VCAP1-GFP, MAHRP1-GFP (M1), *PF3D7_1353100*-GFP (*PF13*) IPs, probed with anti-GFP antibody. (E) GEXP10-GFP, VCAP1-GFP and MAHRP1-GFP (M1) IPs probed with anti MAHRP1 antibodies. Input (In) and IP fractions (IP) are shown. Molecular masses are in kDa.

Fig S3. Complete network map of co-IP experiments

(A) Connectivity map of the GFP tagged bait proteins and their interactions with proteins identified in the co-IP experiments. (B) Connectivity map combining IP data for SEMP1, PTP1 and *PfEMP1* from the literature. Green nodes are the GFP-tagged proteins. Blue nodes are exported interacting proteins. Purple nodes represent proteins within the parasitophorous vacuole. Double width lines represent reciprocal IP.

Fig S4. Whole cell analyses of GFP tagged transfectants by 3D-SIM.

(A) REX1-GFP-infected RBCs were fixed and labelled with anti-GFP (green) and anti-MAHRP1 (red). (B) The remaining GFP-tagged transfectant-infected RBCs were fixed and labelled with anti-GFP (green) and anti-REX (red). Average projections of z-stacks are displayed. The merged image and zoom as displayed in Fig 3 are shown. Merge scale bars = 3 μ m. Zoom scale bars = 3 μ m. (B) 3D-SIM imaging of GEXP10-GFP and REX1-GFP (red) parasites co-stained with ATS antibodies (*PfEMP1*) (green). Merged images and a zoom of the merge are shown. Merge scale bars = 3 μ m. Zoom scale bars = 1 μ m.

Fig S5. Generation and validation of the Δ VCAP1 parasite line. (A) Gene disruption and PCR validation schematic. GOI = gene of interest; HR1 = homology region 1; HR2 = homology region

2; crossed lines indicate crossover event. Arrows, primers; bracketed lines, expected amplicon size.
(B) PCR amplicons, letters in reference to primers in part B; KO = knockout; first lane features the
marker. (C) Long term growth assay. Cumulative growth rates of wild type CS2 and Δ VCAP1
parasite lines over 4 parasite lifecycles. n=4.

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782 **Fig S6. Whole cell images showing SEM and TEM of CS2 and Δ VCAP1 infected RBCs**

783 (A) TEM of Equinatoxin treated RBCs highlighting the fragmented Maurer's clefts. (B) Thin
784 section TEM. EQII permeabilised cells large section view of the zoomed Maurer's clefts shown in
785 fig 4 C. Parasite features are labelled. K = knobs; MC = Maurer's Clefts. Scale bars 500 nm.

786

787 **Fig S7. Knob morphology examples and Immunofluorescence microscopy of *Pf*EMP1 location 788 in Δ VCAP1 infected RBCs**

789 (A) Scanning electron microscopy images of normal, large, clustered and asymmetrical knobs.
790 Scale bars = 100 nm. (B) Enhanced exposure images of Δ VCAP1 infected RBCs from figure 6A.
791 Anti-*Pf*EMP1 (green) and counterstained with anti-REX1 (red). Nuclei were stained with DAPI
792 (blue). Projections of Z stacks are shown. Scale bar = 5 μ m.

793

794 **Movie S1. MC mobility movie**

795 Movie showing the mobility of the Maurer's clefts (Green) in 14-18 h post-invasion parasite
796 infected RBCs.

797

798 **Movie S2. CS2 electron tomography translation and rendered model**

799 Part 1: Movie translating through a reconstructed z-stack for CS2 infected red blood cells that were
 800 equinatoxin II permeabilised and probed with anti-REX1 (1:50) followed by Aurion protein A (EM
 801 grade 6 nm gold JA806-111, 1:15). Part 2: 360° rotation around the y axis of CS2 Maurer's clefts
 802 3D structure reconstructed and rendered in IMOD. Red = RBC; fuchsia stalk = tether; pastel hues =
 803 independent clefts; scale bar = 100 nm.

804

805 **Movie S3. Δ VCAP1 electron tomography translation and rendered model**

806 Part 1: Movie translating through a reconstructed z-stack for Δ VCAP1 infected red blood cells that
 807 were equinatoxin II permeabilised and probed with anti-REX1 (1:50) followed by Aurion protein A
 808 (EM grade 6 nm gold JA806-111, 1:15). Part 2: 360° rotation around the y axis of Δ VCAP1
 809 Maurer's clefts 3D structure reconstructed and rendered in IMOD. Red = RBC; fuchsia stalk =
 810 tether; pastel hues = independent clefts; scale bar = 100 nm.

811

812 **Tables**

813 All tables are available for download from figshare using the following link.

814 <https://melbourne.figshare.com/s/ab61640da1d1849f0887>

815

816 **Data sets**

817 All data sets are available for download from figshare using the following link.

818 <https://melbourne.figshare.com/s/2cdf8cc5b787d6b8ea67>

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820 **References**

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