1 Virulence determinant, PTP7, controls vesicle budding from the Maurer's clefts, adhesin protein 2 trafficking and host cell remodeling in *Plasmodium falciparum*

3 Olivia M. S. Carmo¹, Gerald J. Shami¹, Dezerae Cox¹, Boyin Liu¹, Adam J. Blanch¹, Snigdha Tiash¹,
4 Leann Tilley^{1#}, Matthew W.A. Dixon^{2,3#*}

5 ¹Department of Biochemistry and Pharmacology, Bio21 Molecular Science and Biotechnology
6 Institute, University of Melbourne, Parkville, Australia; ²Department of Infectious Diseases, Peter
7 Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Australia;
8 ³Division of Infectious Diseases and Immune Defence, Walter and Eliza Hall Institute of Medical
9 Research, Parkville, Australia.

10 [#]Equal senior authors.

11 *For correspondence. Matthew Dixon. Department of Infectious Diseases, Peter Doherty Institute
12 for Infection and Immunity, University of Melbourne, Melbourne, Australia. Email:
13 matthew.dixon@unimelb.edu.au.

14 Abstract

15 Presentation of the variant antigen, *Plasmodium falciparum* erythrocyte membrane protein 1
16 (EMP1), at knob-like protrusions on the surface of infected red blood cells, underpins *P. falciparum*
17 malaria pathogenicity. Here we describe a protein PF3D7_0301700 (PTP7), that functions at the
18 nexus between the intermediate trafficking organelle, the Maurer's cleft, and the infected red blood
19 cell surface. Genetic disruption of PTP7 leads to accumulation of vesicles at the Maurer's clefts,
20 grossly aberrant knob morphology, and failure to deliver EMP1 to the red blood cell surface. We
21 show that an expanded low complexity sequence in the C-terminal region of PTP7, found only in the
22 *Laverania* clade of *Plasmodium*, is critical for efficient virulence protein trafficking.

23 **Author Summary**

24 We describe a malaria parasite protein involved in virulence factor trafficking (PTP7) that moves
 25 between different compartments in the host red blood cell cytoplasm in a stage-dependent manner.
 26 Upon disruption of the PTP7 locus, the Maurer's cleft trafficking compartments become decorated
 27 with vesicles; the knobby protrusions on the host red blood cell surface are depleted and distorted;
 28 and trafficking of the virulence protein, EMP1, to the host red blood cell surface is ablated. We
 29 provide evidence that a region of PTP7 with low sequence complexity plays an important role in
 30 driving fission of vesicles from the Maurer's clefts.

31

32 **Introduction**

33 *Plasmodium falciparum* causes more than 200 million malaria infections every year, killing more
 34 than 400,000 people [1]. Central to the ability of *P. falciparum* to maintain an infection and cause
 35 disease, is the invasion and remodelling of host red blood cells (RBCs). Maturation of the parasite
 36 inside the RBC is accompanied by striking changes in the surface topology of the infected RBC and
 37 a marked loss of cellular deformability [2,3]. One key modification is the assembly of ~90 nm
 38 diameter structures, called knobs, at the infected RBC surface [4]. The knob structure acts as an
 39 elevated platform at the RBC surface for presentation of the major virulence protein *P. falciparum*
 40 erythrocyte membrane protein-1 (EMP1), which mediates binding of infected RBCs to endothelial
 41 ligands [5–8].

42 The trafficking of EMP1 and other virulence determinants beyond the confines of the parasite is
 43 mediated by the *Plasmodium* translocon of exported proteins (PTEX) that is present in the
 44 parasitophorous vacuole membrane (PVM), with help from a second complex termed the exported
 45 protein interacting complex (EPIC) [9–12]. Once exported across the PVM, EMP1 is thought to be

trafficked across the RBC cytoplasm as a soluble, chaperoned complex; and inserted into the membrane bilayer of the Maurer's clefts - an intermediate trafficking compartment in the RBC cytoplasm [12–14]. The mechanisms controlling EMP1 insertion into the Maurer's clefts and its subsequent forward trafficking and delivery to the RBC membrane remains unclear. Of interest, coated electron-dense vesicles (EDVs) and uncoated vesicle-like structures have been observed in the host cytoplasm and have been shown to contain EMP1 [15–17]. These EMP1-containing vesicles have been reported to associate with remodeled RBC actin filaments and membranous structures, called tethers, that link the RBC membrane to the Maurer's clefts in later stages of intraerythrocytic development [15,16,18].

An interesting feature of exported *Plasmodium* proteins is that they are enriched in repetitive sequences with low sequence diversity [19,20]. For example, glutamine and asparagine repeats are present in about 30% of *P. falciparum* proteins [21,22]. Proteins with low-complexity sequences have been shown to play critical roles in vesicle trafficking in other eukaryotes [23,24]. Thus, it is possible that low complexity regions in exported plasmodium proteins help regulate protein trafficking and host cell remodeling.

We recently reported a method for enrichment of Maurer's clefts that enabled profiling of the protein composition and identification of protein networks within this organelle [25]. That work identified several key protein clusters that play an important role in EMP1 trafficking through the Maurer's clefts. Among the clusters was an uncharacterised protein, PF3D7_0301700, that interacts with the Maurer's cleft resident protein, *Pf*EMP1 Trafficking Protein 6 (PTP6). PTP6 has been previously implicated in EMP1 trafficking and was shown to locate peripherally within the Maurer's clefts [25,26]

Here we provide a detailed characterization of PF3D7_0301700, showing that it associates with the Maurer's clefts as well as chaperone-containing structures known as J-dots, and EMP1-containing

EDVs, by light and electron microscopy and immuno-precipitation. Upon deletion of PF3D7_0301700, vesicles accumulate at the Maurer's clefts; the knobs become larger and fewer in number; EMP1 delivery to the RBC membrane fails; and cytoadhesion to an endothelial cell ligand is ablated. Removing the low complexity C-terminal region of PF3D7_0301700 show that this region is essential for correct vesicle formation and EMP1 trafficking. Taken together, the data show that PF3D7_0301700 plays a critical role in vesicle-mediated trafficking of EMP1 to the RBC membrane. We have given it the name, *P. falciparum* EMP1 Trafficking Protein-7 (PTP7), to reflect its critical role in EMP1 trafficking.

Results

PTP7 migrates between different compartments in the host RBC cytoplasm

The PTP7 (PF3D7_0301700) sequence contains a predicted plasmodium export element (PEXEL) (KSLAE), a recessed signal sequence (SS), an acidic N-terminal region, a central transmembrane domain and a basic C-terminal region containing 34 consecutive asparagine residues (Fig 1A). The mature protein has a predicted molecular mass of 26,954 Da.

We used selection linked integration (S1 Fig) to generate transfectants expressing PTP7 tagged endogenously with a 2xFK506-binding protein (FKBP)-GFP-2xFKBP (3D7-PTP7-GFP^{sand}) [27]. Correct integration of the tag was verified by PCR (S1 Fig) and maintenance of EMP1 trafficking was confirmed by trypsin digest (S1 Fig). Immunoblotting, using anti-FKBP antibodies, revealed a band with an apparent molecular mass of ~130 kDa (Fig 1B). This molecular mass is larger than the predicted 108 kDa, which may be due to the large number of basic residues in the protein, or alternatively, due to non-skipping of the 2TA peptide [28,27,29].

Live cell microscopy of the PTP7-GFP^{sand} within the infected RBC revealed a population of punctate structures in the host cell cytoplasm (Fig 1C). To confirm the location of PTP7-GFP^{sand},

immunofluorescence microscopy was performed at four different windows from 16-30 hours post-invasion (hpi). In RBCs infected with 16-18 hpi parasites, PTP7-GFP^{sand} fluorescence overlaps with that of the Maurer's cleft resident protein, REX1 (Fig 1D, S2 Fig), consistent with the Maurer's cleft location reported in a previous study [30]. However, in later stage parasites, the percentage co-occurrence of GFP and REX1 reduces (Fig 1E).

To investigate the sub-cellular location of PTP7-GFP^{sand} at the ultrastructural level, trophozoite stage-infected RBCs were permeabilized with Equinatoxin II and probed with anti-FKBP antibodies. Gold particles are observed at the Maurer's clefts and at vesicle-like structures, located between the Maurer's clefts and the RBC membrane (Fig 1F, white arrows), consistent with the association of PTP7 with multiple compartments.

The protein localization observed in light and electron microscopy was further interrogated by immunoprecipitation. Mid-trophozoite stage PTP7-GFP^{sand}-infected RBCs were solubilized in 1% Triton X-100 and interacting proteins precipitated using GFP-Trap[®] (S3 Fig). The proteins identified by mass spectrometry include PTP7 itself, as well as proteins reported to be associated with the Maurer's clefts (Membrane-associated histidine-rich protein-1, MAHRP1; PF3D7_0501000 and PF3D7_0601900) [31–33], the J-dot compartment (PF3D7_0801000 (0801) and gametocyte-exported protein-18, GEXP18, and an exported HSP40, PFE55) [34–36], EDVs (*Pf*EMP1-trafficking protein-2, PTP2) [37] and the RBC membrane skeleton (mature erythrocyte surface antigen, MESA) (S3 Fig). The PTP7-GFP co-precipitated pellet was subjected to immunoblotting. Probing with antibodies to FKBP, and an antiserum raised against PTP7 (91-233) (S3 Fig) confirmed pull-down of the bait. Probing with antibodies recognizing two of the interacting proteins, 0801 and MESA, confirmed their co-precipitation (S3 Fig). A network map of immunoprecipitated proteins, including data from a previous study, visualizes the protein-protein associations [25] (S3 Fig). Again, the data are consistent with PTP7 being present in multiple compartments.

117 Immunofluorescence microscopy using antibodies to PTP7-GFP^{sand} and the Maurer's cleft/EDV-
118 associated protein, PTP2, reveals partial overlap that increases slightly, later in development (Fig
119 1G). In late-stage parasites, PTP7-GFP^{sand} appears to be mainly associated with small puncta that
120 exhibit limited overlap with either the Maurer's cleft or EDV markers (Fig 1G, H). Interestingly, the
121 J-dot protein, 0801, shows an increasing level of co-occurrence with PTP7-GFP^{sand} as the parasite
122 matures (S2 Fig). There were no changes to the percentage co-occurrence for other exported
123 proteins tested including: EMP1, MAHRP1, and Membrane-associated histidine-rich protein-2
124 (MAHRP2) (S2 Fig).

125 ***Disrupting the PTP7 locus results in aberrant knob morphology***

126 The *ptp7* gene locus was disrupted in the CS2 parasite line using CRISPR/Cas9-directed cutting and
127 homology-directed repair (Fig 2A). Gene deletion was validated by PCR (Fig 2B); and loss of PTP7
128 protein in the Δ PTP7 transfectants was confirmed by immunoblotting (Fig 2C).

129 Immunofluorescence microscopy of Δ PTP7 trophozoite-infected RBCs revealed no obvious change
130 in the profiles of the Maurer's cleft markers (REX1 and MAHRP1) and the EDV marker (PTP2) (Fig
131 2D, E); or the RBC membrane skeleton associated proteins (PfEMP3, MESA and RESA; S4 Fig). In
132 contrast, the knob protein, knob associated histidine rich protein (KAHRP), exhibited a more
133 punctate profile when compared to wild type CS2, suggesting a disruption in knob distribution (Fig
134 2E). To investigate this further, we performed scanning electron microscopy of the external surface
135 of infected RBCs and the cytoplasmic face of infected RBC membranes. Characteristic knob
136 structures (76 ± 11 nm diameter) are observed on the wildtype CS2-infected RBCs, however Δ PTP7-
137 infected RBCs exhibited fewer larger knobs (108 ± 20 nm diameter) (Fig 2F-H). When imaged from
138 the cytoplasmic surface of CS2-infected RBCs, wildtype knobs appear as dimpled discs, with an
139 average diameter of 80 ± 14 nm (S5 Fig), as described previously [3]. By contrast, the Δ PTP7-infected
140 RBC knobs have an average diameter of 118 ± 24 nm (S5 Fig).

141 We employed electron tomography to investigate the organization of the spiral structure that
142 underlies the knobs [38]. The spiral is evident, but fragmented, in Δ PTP7-infected RBCs (Fig 2I, S6
143 Fig), compared to more complete spirals in the CS2 samples. Taken together, these data show that
144 both the distribution and the structure of knobs are severely compromised in the absence of PTP7.
145 Thin section transmission electron microscopy (TEM) confirmed the deformed nature of the knob
146 structures in Δ PTP7 parasites (S7 Fig).

147 ***Forward trafficking of EMP1 is PTP7-dependent***

148 We examined whether genetic disruption of PTP7 affects EMP1 trafficking and surface expression.
149 We assessed the ability of infected RBCs to adhere to the syncytiotrophoblast ligand, chondroitin
150 sulphate-A (CSA), under physiologically relevant flow conditions. The parent CS2 line shows efficient
151 binding to CSA (54 ± 7 infected RBCs/field of view), however, binding to CSA is completely ablated
152 in the Δ PTP7-infected RBCs (Fig 3A). To confirm the loss of surface displayed EMP1, we examined
153 the ability of an antibody recognizing the ectodomain of var2CSA to label EMP1 in intact infected
154 RBCs. A complete loss of surface labeling was observed in Δ PTP7-infected RBCs (Fig 3B). Lastly, we
155 assessed the surface accessibility of the EMP1 ectodomain to trypsin cleavage. After trypsinization
156 of intact infected RBCs, characteristic cleavage fragments are observed, confirming the presence of
157 surface displayed EMP1 (Fig 3C). In contrast, no cleavage products are observed in the Δ PTP7-
158 infected RBCs (Fig 3C). The presence of full-length skeleton binding protein 1 (SBP1), a Maurer's
159 cleft resident, confirms that the RBC membrane was not breached during trypsin treatment (Fig 3C,
160 bottom panel).

161 To determine where in the export pathway EMP1 was being trapped, we performed
162 immunofluorescence microscopy using an antiserum recognizing the conserved ATS region of
163 EMP1. This revealed Maurer's cleft labelling in both the CS2 and Δ PTP7-infected RBCs, suggesting
164 that EMP1 can still be trafficked to the Maurer's clefts (Fig 3D, left panels). To determine if the

165 amounts of EMP1 being trafficked to the Maurer's clefts had changed we performed a quantitative
166 analysis of the images. We looked at the co-occurrence of EMP1 and the Maurer's clefts marker,
167 REX1. A significant increase in the number of EMP1-containing clefts (Fig 3E, S8 Fig) and a total
168 increase in the amount of EMP1 at the clefts was observed in the knockout compared to the CS2
169 parasites (Fig 3G, S8 Fig). Additionally, this analysis revealed that the Δ PTP7-infected RBCs exhibited
170 fewer and larger clefts than CS2 controls (Fig 3H, I).

171 *Vesicles accumulate at the Maurer's clefts in PTP7-disrupted parasites*

172 Ultrastructural analysis of the Maurer's clefts was performed in infected RBCs that had been
173 permeabilized with Equinatoxin II to allow introduction of antibodies [16]. In wildtype parasites, the
174 Maurer's clefts are observed as single slender cisternae with an electron-lucent lumen and an
175 electron-dense coat. Upon immuno-labelling with anti-REX1, gold particles are observed at the cleft
176 periphery (Fig 4A top, zoom, white arrows). Occasional protrusions are observed that may represent
177 material budding from the Maurer's cleft surface. In Δ PTP7-infected RBCs, Maurer's clefts are still
178 present as indicated by the gold-labelled anti-REX1 (Fig 4A bottom, zoom, white arrows). However,
179 the clefts are decorated with numerous membrane-bound vesicle-like structures (Fig 4A bottom,
180 zoom), with an average diameter of 44 ± 17 nm (Fig 4A). The budding structures are morphologically
181 similar to previously described vesicle-like structures [16] but are much more numerous than in
182 wildtype parasites and remain in close proximity to the Maurer's clefts (Fig 4A, S1 Movie). The clouds
183 of REX1 labeled vesicles around the clefts agree with earlier observations of fewer, larger clefts in
184 Δ PTP7 (Fig 3H, I).

185 Immunolabelling with an antibody recognizing the ATS region of EMP1 [39] revealed gold particles
186 on the body of the clefts in wildtype parasites (Fig 4B top, white arrows); and on both the body of
187 the clefts and associated with the vesicles in the Δ PTP7-infected RBCs (Fig 4B bottom, white and

188 magenta arrows). These data suggest that these budding structures may be EMP1-trafficking
189 vesicles that fail to separate from the clefts in the absence of PTP7.

190 ***The C-terminal asparagine repeats are needed for PTP7 function***

191 The majority of the PTP7 sequence is highly conserved across *Plasmodium* species, however within
192 *P. falciparum* isolates and one clade of *P. gorilla* there has been the insertion of 34 asparagines near
193 the C-terminal end of the protein (see multiple sequence alignment in S9 Fig). To test the functional
194 significance of the C-terminal extension, we generated transfectants endogenously expressing one
195 of three truncated PTP7 species fused to a GFP reporter (Fig 5A). The first truncation has amino
196 acids 265-317 deleted, leaving the transmembrane domain and four amino acids as a linker. The
197 second cell line contains an internal deletion removing the asparagine repeats ($\Delta 278-310$), leaving
198 the C-terminal poly basic motif 'KKSKKN'. The final cell line expresses a truncation that removes the
199 poly basic motif ($\Delta 300-317$). These cell lines were validated by immunoblot, confirming the presence
200 of PTP7-GFP fusion proteins of expected sizes, whereas no signal is observed in the parent CS2 cell
201 line (Fig 5B). Detection of REX1 and KAHRP products by indirect immunofluorescence microscopy
202 confirmed the conservation of these subtelomeric genes (S10 Fig).

203 Scanning electron microscopy was performed on the truncations to determine if the knob
204 morphology was affected. Truncation of the C-terminal 17 amino acids (PTP7 $\Delta 300-317$) a minor effect
205 on knob density and knob diameter (Fig 5C-E). By contrast, deletion of the entire C-terminal region
206 (PTP7 $\Delta 265-317$) was associated with fewer, larger knobs (Fig 5C-E), recapitulating the phenotype
207 observed in the PTP7 knock-out line. Interestingly, internal deletion of the C-terminal asparagine
208 repeats, while keeping the C-terminal 'KKSKKN' motif (PTP7 $\Delta 278-310$), causes an intermediate
209 phenotype. The PTP7 $\Delta 278-310$ parasites exhibit sparser knobs with larger diameters (Fig 5C-E).

210 We next investigated if delivery of EMP1 to the RBC surface was affected in the truncations. Flow
 211 cytometry analysis revealed complete ablation of EMP1 at the surface of the PTP7 Δ 265-317 infected
 212 RBCs (Fig 6A). The PTP7 Δ 278-310 infected RBCs had a 63% reduction in surface EMP1 while the and
 213 PTP7 Δ 300-317 infected RBCs had a 32% reduction (Fig 6A). A trypsin cleavage assay was performed to
 214 confirm these results. Complete loss of EMP1 from the surface is observed in the PTP7 Δ 265-317
 215 infected RBCs, while faint trypsinized EMP1 bands are observed in both PTP7 Δ 278-310 and PTP7 Δ 300-317
 216 parasites, consistent with the reduction of surface-exposed EMP1 detected in the FACS experiments
 217 (Fig 6B). In a similar trend, the number of GFP-positive puncta is significantly reduced in PTP7 Δ 265-317
 218 and PTP7 Δ 278-310 parasites (Fig 1C, D, S10 Fig). These puncta counts suggest that the length of the C-
 219 terminal region of PTP7 affects its distribution.

220 To determine the effect of truncation of regions in the C-terminal domain of PTP7 on vesicular
 221 trafficking, thin section TEM was performed. Truncation of the entire C-terminal region leads to
 222 marked vesiculation of the Maurer's clefts as revealed in ultrastructural analysis (Fig 6C). The
 223 Maurer's clefts appear less affected when at least some of the PTP7 C-terminal domain is retained
 224 (PTP7 Δ 278-310 and PTP7 Δ 300-317; Fig 6C). The vesicle diameters are not significantly affected by PTP7
 225 mutations (Fig 6D); however, significantly more vesicles are observed within 100 nm of the PTP7 Δ 265-
 226 317 clefts, while the PTP7 Δ 278-310 and PTP7 Δ 300-317 parasites exhibited a non-significant increase,
 227 relative to the full-length control (Fig 6E). The accumulation of vesicles at the clefts is similar in the
 228 PTP7 Δ 265-317 and Δ PTP7 lines.

229 Discussion

230 The canonical protein trafficking system that is used by most eukaryotic cells to transport proteins
 231 to the plasma membrane is not present in mature human RBCs [40]. Thus, intraerythrocytic *P.*
 232 *falciparum* faces the challenge of exporting integral membrane proteins, such as the virulence
 233 antigen EMP1, to the RBC membrane, and inserting proteins into the lipid bilayer, in the absence of

234 host cell machinery. To meet this challenge, the parasite establishes organelles called Maurer's
235 clefts that are thought to function as intermediate trafficking compartments in the transport of
236 EMP1 to the surface [13,14]. Moreover, several studies have described vesicle-like structures in the
237 infected RBC cytoplasm [13,16,17,37,41–43]. However, the finding that EMP1 trafficking is GTP-
238 independent [44] suggests that the molecular machinery that the parasite employs to traffic
239 virulence proteins is divergent from that found in other cell types.

240 Here we characterized PTP7, a protein that was previously reported as a Maurer's cleft resident
241 [30]. Our analysis of the location of PTP7 relative to a range of other exported *P. falciparum* proteins
242 at different stages of the lifecycle suggests that PTP7 moves between different compartments. In
243 the early trophozoite stage, it is concentrated at or near the Maurer's cleft in a location that overlaps
244 with the cleft marker, REX1, the EDV marker PTP2, and the J-dot marker 0801. In later stage
245 parasites, PTP7 becomes more diffuse, but maintains partial co-occurrence with 0801.
246 Immunoprecipitation experiments confirm the association of PTP7 with the Maurer's cleft proteins,
247 MAHRP1, PF3D7_0501000 and PF3D7_0601900 [31–33]; the J-dot proteins, 0801, GEXP18, and
248 PFE55 [34–36]; the EDVs marker, PTP2 [37]; and the RBC membrane skeleton associated protein,
249 MESA. Taken together, the data suggest that PTP7 is located at the Maurer's clefts in the early stage
250 of EMP1 trafficking; but relocates to other trafficking compartments as the parasite develops. Of
251 note, MAHRP1, EDVs and J-dots have all been implicated in EMP1 trafficking [26,37,45,46].

252 To assess the function PTP7 plays in virulence protein trafficking, we generated a PTP7 knockout cell
253 line. Analysis of this cell line showed that PTP7 is not essential for growth of parasites in culture, in
254 agreement with a previous report [47]. However, in the absence of PTP7, EMP1 becomes stuck at
255 the Maurer's clefts and is not presented at the surface of the infected RBC. Interestingly, upon
256 deletion of PTP7, the Maurer's clefts became decorated with numerous vesicle-like structures that
257 appear to be in the process of budding from the cleft surface. Quantitative imaging revealed an

258 accumulation of EMP1 at the Maurer's clefts in the Δ PTP7 infected RBCs. Immuno-EM, using an
259 antibody to EMP1, reveals labeling of both vesicles and the Maurer's clefts. Taken together, this
260 suggests that the vesicles may be in the process of budding from the clefts, and that PTP7 is required
261 for correct vesicle fission and forward trafficking of EMP1 to the RBC membrane.

262 In addition to accumulation of vesicles at the clefts, the PTP7 knockout parasites also exhibit altered
263 knob morphology, with fewer, larger knobs being assembled at the RBC surface. The main structural
264 component of the knob complex, KAHRP, has been shown to traffic to the RBC membrane directly;
265 that is, not via the Maurer's cleft. Our data for the PTP7 knockout suggests convergence of the
266 KAHRP and EMP1 trafficking pathways during the final step, where EMP1 is loaded into the knobs
267 at the RBC membrane. One possibility is that the vesicles also transport a component needed for
268 correct assembly of knobs.

269 It is interesting to consider how PTP7, which has no ATP or GTP binding domain, could provide the
270 driving force for vesicle fission. In this context, the unusual asparagine repeat sequence in the C-
271 terminal region of PTP7 may be of functional significance. Recent studies suggest that membrane-
272 associated proteins with low complexity regions can induce membrane curvature (reviewed in [24]).
273 In a planar membrane, disordered regions of protein are constrained, thereby reducing the
274 conformational entropy. As a membrane curves, this constraint is relaxed and the gain in
275 conformational entropy enables curvature sensing [48] and can drive budding [49]. Fig 6H illustrates
276 a potential role of the low complexity domain in driving vesicle fission.

277 To test this hypothesis and to examine the role of the PTP7 asparagine repeats in the molecular
278 architecture of the infected RBC and in the EMP1 trafficking process, we generated transfectants in
279 which PTP7 was truncated. Complete removal of the C-terminal domain recapitulated the
280 phenotype seen in Δ PTP7; that is, numerous vesicles accumulate at the Maurer's clefts, while the
281 knobs showed aberrant morphology and EMP1 trafficking is ablated. The Maurer's cleft phenotype

282 was less dramatic if just the C-terminal basic motif was removed or if the asparagine repeats were
283 removed but the C-terminal polybasic motif was maintained. However, in all truncation mutants,
284 the trafficking of EMP1 to the surface was decreased suggesting that the length of the C-terminal
285 region also contributes to efficient EMP1 trafficking.

286 Conservation of PTP7 in the *Laverania* subgenus may be linked to the expansion of virulence protein
287 trafficking and/or host cell remodeling machinery in these species. The N-terminus of PTP7 is well
288 conserved in *gorilla*, *gaboni* and *reichenowi* orthologues, but the asparagine repeats are found only
289 in *P. falciparum* and a clade of *P. gorilla* [50]. This expansion of the PTP7 sequence may be associated
290 with increased virulence, which is supported by our truncation data showing that this region is
291 required for EMP1 trafficking.

292 Taken together, our data provide strong evidence that PTP7 and its interacting proteins play a
293 critical role in the budding of EMP1-containing vesicle-like structures from the Maurer's clefts and
294 their transfer to the host RBC membrane. Our data provide intriguing evidence for a key role of a
295 low complexity region in providing the driving force for vesicle fission. The promiscuous localization
296 and protein-protein interactions of PTP7 suggest that the routes of exported protein trafficking may
297 be more integrated and interdependent than previously thought. The suggestion that the vesicles
298 that bud from the Maurer's clefts contain an unidentified component that is needed for correct
299 assembly of the knob complex needs further exploration. An increased understanding of the
300 processes for trafficking virulence proteins may lead to new therapies to tackle malaria
301 pathogenesis.

302 **Materials and Methods**

303 **Ethics statement.** Red blood cells and serum were acquired from the Australian Red Cross Lifeblood
304 blood service. All blood products were anonymous and individual donors could not be identified.

305 This work was approved with the written consent of the University of Melbourne Human Research
306 Ethics Committee (approval number 1750526.3).

307 ***P. falciparum* culture.** Parasites were cultured as described previously [25]. Briefly, *P. falciparum*
308 cell lines were cultured in human O⁺ RBCs (Australian Red Cross) with RPMI 1640 medium with
309 GlutaMAX and HEPES (ThermoFisher), supplemented with 5% v/v human serum (Australian Red
310 Cross), 0.25% w/v AlbuMAX II (ThermoFisher), 10 mM D-glucose (Sigma), 200 μ M hypoxanthine
311 (Sigma) and 20 μ g/ml gentamicin (Sigma). Cultures were maintained at 5% haematocrit at 37°C in a
312 low oxygen environment (1% O₂, 5% CO₂, and 94% N₂). Parasitemia was monitored by thin blood
313 smears and Giemsa staining [51]. Cultures were synchronized at the ring stage by treatment with
314 D-sorbitol (Sigma) as described previously [52]. Gelatin floatation with 70% Gelofusine (Braun) was
315 used to maintain knob-positive populations of infected RBCs [53]. Transgenic parasites were
316 maintained in the presence of selection reagent: 4 nM or 5 nM WR (Jacobus Pharmaceuticals) for
317 hDHFR [54], 2 μ g/mL blasticidin S for blasticidin S resistance gene, 2 nM DSM1 for γ DHODH, and 400
318 μ g/ml G418 for neomycin phosphotransferase for SLI integration.

319 **Vector construction and generation of transgenic parasites.** For endogenous 3' tagging, the 3' 710
320 bp of the *ptp7* was amplified using SLI-sand-PTP7_fw and SLI-sand-PTP7_rv primers (S1 Table) and
321 directionally cloned into the NotI/AvrII restriction sites in the pSLI-sandwich plasmid (2xFKBP-GFP-
322 2xFKBP) [27]. Ring-stage parasites were transfected with 50-100 μ g of plasmid as previously
323 described [55]. Briefly, precipitated plasmid was resuspended in sterile TE buffer and Cytomix. 5-
324 10% ring-stage infected RBCs were resuspended in the DNA mix and transferred to a 2 mm
325 electroporation cuvette (BTX). Cells were electroporated at 310 V, 950 μ F, and ∞ resistance (Gene
326 Pulser Xcell™ Electroporation System, Bio-Rad), then washed in warm RPMI media and transferred
327 to culturing dishes. After neomycin selection, correct integration of the plasmid was verified by PCR
328 using the primers as described in S1 Fig and S1 Table.

329 To generate the plasmid for CRISPR/Cas9 mediated disruption of *ptp7*, 5' and 3' homology regions
 330 (HR; ~500 bp) were PCR-amplified from genomic DNA using the primers PTP7-HR1_fw, PTP7-HR1_rv
 331 for the HR1 and PTP7-HR2_fw, PTP7-HR2_rv for HR2 (S1 Table). The HR1 fragment was cloned into
 332 the AvrII/NcoI sites and the HR2 into the SpeI/SacII sites of the pUF-TK plasmid [56]. The pUF-TK
 333 vector was linearized by digestion with AvrII and used as the repair template. CRISPR/Cas9-
 334 mediated double-stranded breaks were guided by a single guide RNA and Cas9. The Cas9 target
 335 'ggttccaacacagtcacacg' was selected using CHOPCHOP and PTP7-sgRNA_top and PTP7-
 336 sgRNA_bottom oligonucleotides were annealed and cloned into the BsrGI site of pAIO using infusion
 337 cloning [56]. Both linearized repair template and guide plasmids were transfected simultaneously
 338 into CS2 parasites. Gene disruption was confirmed by PCR using primers yDHODH_ScrF and
 339 yDHODH_ScrR, and primers in the native 5' and 3' UTRs of the *ptp7* locus, PTP7-KO_ScrF and PTP7-
 340 KO_ScrR (Fig 2, S1 Table).

341 To generate the truncation parasite lines the DNA sequences encoding for amino acids 83-317, 83-
 342 264, 83-277, and 83-299 of PTP7 were PCR-amplified using the primers indicated (S1 Table) and
 343 directionally cloned into the NotI/MluI restriction sites in the pSLI-TGD plasmid to generate cell
 344 lines PTP7¹⁻³¹⁷, PTP7^{Δ265-317}, PTP7^{Δ278-310}, and PTP7^{Δ300-317} respectively [27]. Plasmids were
 345 transfected and integration selected using neomycin as described above.

346 **Protein sample preparation, gel electrophoresis and immunoblotting.** Whole parasite samples
 347 were prepared by lysis with saponin (Sigma). Cells were harvested and resuspended in 10 volumes
 348 of ice cold 0.03% saponin in PBS and incubated on ice for 10 min. Lysates were centrifuged at 2 000
 349 × *g* for 4°C for 5 min, the pellet was retained, and the supernatant discarded. The pellet was washed
 350 4 times in PBS containing cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche). Protein samples
 351 were mixed with 4X LDS buffer containing DTT (NuPAGE™ Thermofisher) and heated at 70°C for 10
 352 min prior to loading. Samples were separated by SDS-PAGE on either 4-12% Bis-Tris or 3-8% Tris-

353 Acetate gradient gels (NuPAGE™) run in MES, MOPS, or Tris-Acetate SDS running buffers (NuPAGE™)
354 following the manufacturers recommendations.

355 Protein gels were either dry transferred onto nitrocellulose membranes using the iBlot™ 2 device
356 (Invitrogen) or wet transferred onto PVDF membrane using the Mini Trans-Blot™ Cell (Bio-Rad). Wet
357 transfers were conducted overnight at 4°C with ice cold transfer buffer (44 mM Tris, 30 mM glycine,
358 0.03% SDS, 20% v/v methanol) at 20-30 V. Membranes were blocked in 5% w/v skim milk in PBS
359 with 0.5% Tween20 (mPBST) for at least 1 h at room temperature or overnight at 4°C. Membranes
360 were probed with the relevant primary antibody resuspended in 5% mPBST for either 90 min at
361 room temperature or overnight at 4°C. Primary antibodies used in this study were mouse αGFP
362 (1:2000, 0.4 mg/ml Roche), rabbit αREX1-repeat (1:1000, [57]), mouse αATS (1:100, 1B/98-6H1-1
363 gift from A. Cowman [28]), rabbit αFKBP (1:1000, Abcam, ab24373), mouse αKAHRP (1:1000, mAb
364 18.2 obtained from the European Malaria Reagent Repository, donated by Dr. Jana McBride), mouse
365 αHA (1:500, HA-7 Sigma), rabbit αPTP7 (1:500), rabbit αSBP1^{N-term} (1:1000, [28]), rabbit αPTP2
366 (1:500, [26]), mouse αBiP (1:2000, Walter and Eliza Hall Institute of Medical Research, Australia),
367 rabbit αGAPDH (1:1000, [58]), rabbit αHSP70x (1:2000, gift from J. Przyborski [59]), rabbit α0801
368 (1:1000, gift from T. de Koning Ward and P. Gilson [9]), rabbit αMESA (1:1000, [60]). Membranes
369 were washed 3 times for at least 5 min each wash in PBST, then probed with anti-mouse or rabbit
370 secondary antibody conjugated to HRP (Merck AP181P, AP132P) for 60 min at room temperature.
371 Membranes were again washed 3 times in PBST before being incubated with Clarity™ ECL reagents
372 (Bio-Rad) or SuperSignal™ West Femto Maximum Sensitivity Substrate and visualized (see S11 Fig
373 for full length immunoblots).

374 **Immunoprecipitation and mass spectrometric analysis.** Mid-trophozoite stage-infected RBCs were
375 enriched by Percoll or gelatin floatation and washed twice in RPMI media. The cell pellet was
376 solubilized on ice for 30 min with 10 volumes of immunoprecipitation buffer (1% Triton X-100 in 50

377 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA and cOmplete™ EDTA-free Protease Inhibitor Cocktail
 378 (Roche)). The sample was mixed every 10 min. The samples were centrifuged at 16 000 × *g* for 10
 379 min at 4°C and the supernatants were transferred to new tubes. The supernatant was again
 380 centrifuged to pellet any remaining insoluble material. The lysates were pre-incubated with Protein-
 381 A Sepharose beads (Novagen) and allowed to mix for 30 min at 4°C. The lysate was again
 382 centrifuged, and the supernatant transferred into a microcentrifuge tube containing GFP-Trap®
 383 beads and mixed at 4°C for 4 h or overnight. Beads were washed 5 times in immunoprecipitation
 384 buffer and transferred to fresh tubes during the last wash. Samples were then either prepared for
 385 immunoblotting or mass spectrometry. In preparation for SDS PAGE analysis, the beads were
 386 resuspended in 4X LDS buffer containing DTT (NuPAGE™ Thermofisher) and warmed to 70°C for 10
 387 min. For mass spectrometry the beads were washed twice in 1 mM Tris-HCl pH 7.4. Formic acid
 388 (0.04% final) then 2,2,2-Trifluoroethanol (7% final) were added and the samples and incubated at
 389 50°C for 5 min. The sample was centrifuged, and the supernatant transferred to a fresh tube.
 390 Tetraethylammonium bromide (50 μM final) and Tris(2-carboxyethyl)phosphine hydrochloride (50
 391 μM final) were added to the sample and incubated for 10 min at 70°C. Trypsin (0.25 μg) was added
 392 and the samples were overnight at 37°C. Samples were then analyzed via tandem mass
 393 spectrometry. Samples were subjected to tandem mass spectrometry with electrospray ionization
 394 (ESI) LC-MS/MS on a Q-Exactive mass spectrometer and analyzed as previously described [25].

395 **EMP1 trypsin cleavage assay.** Cultures containing least 5% mid-trophozoite stage-infected RBCs
 396 were washed in 1xPBS and divided into 2 equal fractions. Cells were harvested at 300 × *g* for 5 min
 397 and resuspended in 10 volumes of warm 1xPBS (P) or 1xPBS containing 1 mg/ml TPCK-Treated
 398 Trypsin (Sigma) (T). Samples were incubated at 37°C for 1 h, inverting occasionally to prevent
 399 sedimentation. Trypsin inhibitor from Glycine max (soybean) (Sigma) was added to each sample to
 400 5 mg/ml final concentration and incubated at room temperature for 15 min. Cells were harvested

401 and the supernatant discarded. The pellet fraction was solubilized in 10 volumes of ice cold 1%
 402 TritonX-100 (Sigma) in PBS containing cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) and
 403 incubated on ice for 30 min. All subsequent steps contained 1x cOmplete™ EDTA-free Protease
 404 Inhibitor Cocktail (Roche). Samples were centrifuged at 16 000 × *g* for 10 min at 4°C and then the
 405 pellets were washed three times in the ice cold TritonX-100 solution. The pellet was solubilized in
 406 20 volumes of 2% SDS/PBS and mixed at room temperature for 30 min. The samples were
 407 centrifuged at 16 000 × *g* for 10 min, transferred to a new tube and centrifuged again. The
 408 supernatant was transferred to a new tube and prepared for immunoblotting.

409 **Antibody generation.** Polyclonal antibodies against PTP7 were generated by Genscript. Briefly, the
 410 mature N-terminal domain of PTP7 (89-317 aa) was expressed in *E. coli* as a His tagged recombinant
 411 protein, which was used to immunize rabbits. Total IgG was purified from the rabbit serum using
 412 protein A.

413 **Live cell and immunofluorescence microscopy.** For live-cell fluorescence microscopy, ~2 µl of *P.*
 414 *falciparum* culture was dispensed on a slide and flattened with a coverslip for immediate imaging.
 415 For indirect immunofluorescence assays, cells were either fixed in ice-cold acetone/methanol or
 416 formaldehyde/glutaraldehyde. Briefly thin blood smears were fixed with ice-cold acetone/methanol
 417 at a ratio of either 1:1 or 9:1 for 10 min and allowed to air dry. For formaldehyde-glutaraldehyde
 418 fixation, coverslips were coated with 0.05 mg/ml *Phaseolus vulgaris* erythroagglutinin (PHA-E) lectin
 419 (Sigma) and incubated in a humid environment for 15 min at 37°C. All subsequent incubations were
 420 conducted in a humid environment. Infected RBCs were washed in PBS, resuspended at 2%
 421 hematocrit and overlaid on the PHA-E coated slide. Cells were allowed to adhere for 15 min, then
 422 washed with PBS until a monolayer of cells was obtained. The slides were fixed in 4% formaldehyde
 423 0.0065% glutaraldehyde in PBS for 20 min. The slides were washed 3 times in PBS and permeabilized
 424 with 0.1% (v/v) TritonX-100 for 10 min. The slides were washed prior to the addition of the primary

antibodies diluted in 1xPBS containing 3% (w/v) bovine serum albumin. The antibodies were incubated on the slide for 1 h at room temperature. Primary antibodies used in this study include mouse α GFP (1:1000, 0.4 mg/ml, Roche), rabbit α GFP (1:500, [61]), rabbit α REX1-rpt (1:2000, [57]), mouse α ATS (1:100, 1B/98-6H1-1 gift from A. Cowman [28]), mouse α EMP3 (1:500, [62]), mouse α KAHRP (1:500, mAb 18.2 obtained from the European Malaria Reagent Repository, donated by Dr. Jana McBride), rabbit α Band 2.1 (1:500), rabbit α PTP7 (1:500), mouse α MAHRP1c (1:200, [63]), rabbit α MAHRP2 (1:500, [18]), rabbit α PTP2 (1:200, [26]), mouse α RESA (1:500), rabbit α MESA (1:200, [60]). Following incubation with the primary antibody the slides were washed 3 times in 1xPBS and the secondary antibodies added. Secondary anti-mouse or anti-rabbit Alex Fluor® 488 or 647 antibodies (1:1000 in 3% (w/v) BSA in PBS) were added to the wells for 1 h at room temperature and then the wells were washed 3 times with PBS. Each well was incubated with 2 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) for 15 min, then washed and mounted with p-phenylenediamine (PPD) antifade prior to sealing. Samples were imaged on a DeltaVision Elite restorative widefield deconvolution imaging system (GE Healthcare) as detailed in [25]. Briefly, samples were excited with solid state illumination (Insight SSI; Lumencor). The following filter sets (excitation [Ex] and emission [Em] wavelengths) were used: DAPI (Ex390/18 nm, Em435/48 nm), fluorescein isothiocyanate (FITC) (Ex475/28, Em523/26), tetramethyl rhodamine isocyanate (TRITC) (Ex542/27, Em594/45), and Cy5 (Ex632/22, 676/34 nm). A 100X UPLS Apo (Olympus, 1.4 NA) oil immersion lens objective was used for imaging. Images were processed using FIJI ImageJ software [64] and deconvolved with Huygens image analysis software (Professional version 19.04, Scientific Volume Imaging) using the CMLE algorithm with 50 iterations where indicated.

Scanning electron microscopy. Whole infected RBCs were fixed and imaged as previously described [25]. Scanning electron microscopy (SEM) imaging of the cytoplasmic surface of the host RBC membranes was performed on sheared infected RBC membranes. The membranes were prepared

as previously described [25]. Briefly, glass coverslips were cleaned with acetone and 50% methanol and functionalized with 3-aminopropyl triethoxysilane (APTES), bis-sulfosuccimidyl suberate, and erythroagglutinating phytohemagglutinin (PHAE). Mature infected-RBCs were immobilized on the functionalized glass slides and sheared by forceful application of a hypotonic buffer (5 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 10 mM NaCl, pH 8). For CS2 and ΔPTP7 cells, coverslips were coated with gold at 25 mA for 40 s and 75 s using a Dynavac sputter coating instrument to thicknesses of ~ 0.2 nm and ~ 0.4 nm for the sheared and whole cells, respectively. Otherwise, whole cells were gold-coated with a Safematic CCU-010 sputter coater to a thickness of 5 nm. Images were acquired with the T1 (A+B, for CS2 and ΔPTP7 whole cells) or the Everhart-Thornley detector in 'Optiplan' mode (for whole cells and sheared membranes) of an FEI Teneo SEM in using at a working distance of 5 mm, a beam current level of 50 pA, and 2 kV accelerating voltage.

Immunoelectron tomography. Infected RBC (20-32 hpi) were magnet purified, washed in PBS, and fixed in 10 pellet volumes of 2% (v/v) paraformaldehyde (PFA)/PBS for 20 min at room temperature. Cells were washed and then permeabilized in 10 pellet volumes of PBS with 1 hemolytic unit (HU) of Equinatoxin II for 6 min then washed again. Cells were then lightly fixed in 2% PFA/PBS for 5 min, washed, and incubated for 1 h with 3% BSA-PBS. The cells were incubated in one-volumes of the primary antibody (1:10) or no-primary control (3% BSA-PBS) for 2 h, washed, and then incubated with one-volumes of the gold secondary antibody for 1 h (1:15; Aurion protein A EM-grade 6-nm-diameter gold; catalog no. JA806-111). Cells were washed in 3% (w/v) BSA-PBS and then in PBS to remove the BSA.

Cell pellets were resuspended and fixed in 2.5% glutaraldehyde at 4°C for long term storage prior to embedding in agarose, fixed in 2% osmium tetroxide (w/v) then resin embedded, sliced, and imaged as described previously [25]. Alternatively, stored cells were post-fixed in 1% osmium tetroxide (w/v) and 1.5% potassium ferrocyanide (w/v) in 0.1 M PBS for 30 min at room temperature in

473 darkness. Cells were washed and incubated in 1% tannic acid (w/v) in 0.1 M PBS for 20 min. Cells
474 were washed in double-distilled H₂O and dehydrated in an ascending concentration series of
475 ethanol and acetone. Samples were progressively infiltrated with Procure 812 Epon-substitute resin
476 and polymerised at 60 °C for 48 hours. Ultrathin (70 nm) and semi-thin (300 nm) sections were
477 generated using a Leica EM Ultracut 7 ultramicrotome (Leica, Heerbrugg, Switzerland) and sections
478 were post-stained using 2% uranyl acetate and lead citrate respectively. Imaging and electron
479 tomography were performed on an FEI Tecnai F30 electron microscope (FEI Company, Hillsboro,
480 OR) at an accelerating voltage of 300 kV. The tilt series were acquired for every 2° in the range
481 between -70° and 70°. Virtual sections were reconstructed from the raw tilt series in IMOD using a
482 weighted back-projection algorithm [65].

483 **Infected RBC binding assay under flow conditions.** Cytoadherence of infected RBCs was performed
484 as described previously [25]. Briefly, Ibidi μ -Slide 0.2 channel slides were incubated with 100 μ l
485 chondroitin sulfate A (100 μ g/ml; Sigma) in 1% BSA/1xPBS overnight at 37°C. Channel slides were
486 blocked with 1% BSA/1xPBS for 1 h at room temperature prior to gentle washes with 37°C
487 bicarbonate-free RPMI 1640 (Invitrogen). Mature infected cultures (3% parasitemia and 1%
488 hematocrit) were harvested and resuspended in warm bicarbonate-free RPMI 1640. Samples were
489 pulled through the channel at 100 μ l/min for 10 min at 37°C to allow cytoadherence, then washed
490 for 10 min to remove unbound cells. Adhered cells were counted at 10 points along the axis of the
491 channel. The microscopy was performed on a DeltaVision Elite widefield imaging system (GE
492 Healthcare).

493 **VAR2CSA ectodomain labeling and analysis via flow cytometry.** Mid-trophozoite stage cultures
494 were diluted to 3-5% parasitemia and 0.4% hematocrit in loaded into a 96-well plate in duplicate
495 per condition (primary and no-primary controls). Cells were harvested at 90 sec at 528 $\times g$ (9 acc./
496 9 dec.) and washed once in 1% BSA/1xPBS. Cells were incubated with and incubated with 10 μ l of

497 rabbit polyclonal anti-VAR2CSA antibody for 30 min at 37°C (1:100, R1945 gift from S. Rogerson [39])
 498 or 1% BSA/1xPBS as a control. Following incubation, the cells were washed with 1% BSA/1xPBS prior
 499 to addition of Pellets were incubated with 10 µl of mouse anti-rabbit IgG (Sigma RG-96; 1:100) for
 500 30 min at 37°C. The samples were again washed in 1% BSA/1xPBS before addition of goat anti-
 501 mouse antibody conjugated to a fluorophore, which was incubated for 30 min at 37°C. For
 502 CS2/ΔPTP7 experiments, tertiary antibodies were conjugated to Alexa Fluor 488. For the GFP
 503 expressing PTP7 truncated cell lines, the antibodies were conjugated to Alexa Fluor 647. Where the
 504 tertiary antibody was conjugated to Alexa Fluor 488, cells were washed and stained with SYTO-61
 505 as described previously [66]. Where the tertiary antibody was conjugated to Alexa Fluor 647, cells
 506 were washed and incubated with the dsDNA stain Hoechst 33342 (1:2000) for 30 min at 37°C.
 507 Following dsDNA Hoechst labeling, the cells were washed 2 times in 1% (w/v) BSA/PBS then once in
 508 PBS prior to analysis. Flow cytometry was performed on a BD FACSCanto II with an integrated high
 509 throughput sampler. The following filter sets were used for fluorophore detection: Alexa Fluor 647
 510 and SYTO-61 (APC, 660/20 nm), Alexa Fluor 488 (FITC, 530/30 nm) and Hoechst 33342 (Pacific Blue,
 511 450/50 nm). A total of 50,000 events were collected and doublet discriminated. Infected cells were
 512 gated on SYTO-61/Hoechst positivity. The mean fluorescence intensities of Alexa Fluor 488/Alexa
 513 Fluor 647 fluorescence (*i.e.* EMP1 labeling) in these populations was calculated. Non-specific events
 514 the secondary-only antibody controls served as a guide for the FITC+/APC+ boundary.

515 **Statistical analysis.** Statistical tests and visualization were performed using GraphPad Prism 9.2.0
 516 (283) Macintosh Version by Software Mackiev © 1994-2021 GraphPad Software
 517 (www.graphpad.com). For comparisons between two groups only, P-values determined by Welch's
 518 t-test. For ≥2 groups, P-values determined by Brown-Forsythe and Welch ANOVA tests and
 519 Dunnett's multiple comparison test to the first timepoint or full-length protein. Only statistical
 520 comparisons with p-values < 0.05 are displayed.

Data availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [67] partner repository with the dataset identifier PXD027566 (not yet peer reviewed). All quantitative and qualitative microscopy data and analysis scripts are available from open-access Zenodo repositories (data DOI: 10.5281/zenodo.5146871; analysis scripts DOI: 10.5281/zenodo.5147885).

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Figures

Fig 1. PTP7 localizes to multiple compartments. (A) Schematic of the PTP7. Amino acid numbers are indicated. SS: Signal sequence. KSL/AE: export element; TM: transmembrane domain; 34xN: 34 consecutive asparagine amino acids. (B) Immunoblot of parent line (3D7) and PTP7-GFP^{sand} cell lysates probed with α FKBP. Loading control, α BiP, expected size of ~ 62 kDa. (C) Live cell microscopy of PTP7-GFP^{sand} infected RBCs showing native fluorescence (green) merged with bright field (BF). (D) Indirect immunofluorescence microscopy of paraformaldehyde-fixed PTP7-GFP^{sand} infected RBCs synchronized to a 2-hour window and sampled every 4 hours from 16 to 28 hpi. The infected RBCs were probed with anti-GFP (green), anti-REX1 (magenta), and DNA was stained with DAPI (blue). (E) Quantitation of the ratio of GFP positive puncta to total puncta. Data displayed are mean $\pm$ SD ($n = 85, 39, 34, \text{ and } 36$ respectively). (F) Representative immuno-TEM micrographs of 20-32 hpi infected RBCs permeabilized with Equinatoxin-II. Cells were probed for FKBP followed by immunogold secondary labeling. V: vesicles, MC: Maurer's cleft, RBCM: red blood cell membrane. White arrows highlight gold labelling. (G) Indirect immunofluorescence assays of infected RBCs probed with anti-GFP (green), anti-PTP2 (magenta) and the DNA stain DAPI (blue). (H) Quantitation and analysis of the number of GFP positive over total puncta. Data displayed are mean $\pm$ SD ($n = 48, 33, 10, 30$ in order listed in figure). P-values determined by Brown-Forsythe and Welch ANOVA tests and Dunnett's multiple comparison test.

Fig 2. Disruption of the PTP7 locus affects knob and cleft morphology. (A) Schematic outlining the gene disruption strategy. DSB: double stranded break; RNP: ribonucleoprotein; *yDHODH*: yeast dihydroorotate dehydrogenase; Gray: coding sequence; up-carrot: native intron; blue bulb and purple line: RNP and small guide RNA; HR1: homology region 1; HR2: homology region 2; crossing lines: homologous cross over events; arrows and letters A-D: primer locations. (B) PCR products of CS2 and CS2ΔPTP7 genomic DNA confirming disruption of the *ptp7* locus. (C) Immunoblot of cell lysates probed with αPTP7. Loading control αBiP, expected size of ~62 kDa. (D-E) Indirect immunofluorescence microscopy of acetone/methanol fixed cells. Bright field (BF) and DAPI stained DNA (blue) images are merged. Scale bar, 2 μm. (F) Scanning electron microscopy of the exterior surface of mid-trophozoite stage infected RBCs. (G) Knob density as knob count per square μm, averaged per image. Data displayed are mean ± SD (*n* = 13, 10). (H) Knob diameter measured along the major axis, averaged per image. Data displayed are mean ± SD (CS2 *n* = 13; ΔPTP7 = 10). (I) Electron tomograms of infected RBCs membranes reveal the spiral structure underlying the knob. P-values determined by Welch's t-test.

Fig 3. PTP7 disruption affects EMP1 distribution and surface presentation. (A) Infected RBCs were analyzed for their adherence to the var2CSA receptor chondroitin sulfate A (CSA). Infected CS2 and CS2 Δ PTP7 cell lines were passed through a channel slide coated with CSA under physiological flow conditions. The number of infected RBCs in 10 fields were recorded. Samples were run in technical triplicates. Data displayed are mean $\pm$ SD of each technical repeat (CS2 n = 3; Δ PTP7 n = 3). (B) Flow cytometry analysis of infected RBCs labeled with antibodies for the ectodomain of var2CSA followed by secondary antibodies and tertiary antibodies conjugated to Alexa Fluor 488. Samples were run in triplicate and the experiment was repeated 3 times. The relative mean fluorescence of events was calculated as the (FITC geometric mean $\times$ number of events $\times 10^5$) and averaged per cell line for each biological repeat. Data displayed are mean $\pm$ SD of each technical repeat (CS2 n = 3; Δ PTP7 n = 3, symbols indicate different biological repeats). (C) Trypsin cleavage assay. Membranes are probed with α ATS and α SBP1 as a control. P: PBS mock treatment; T: Trypsin treated samples; asterisk: spectrin cross-reactivity band; arrows: EMP1 cleavage products. Loading control and experimental control, α SBP1, expected molecular weight of ~ 50 kDa [28]. (D) Indirect immunofluorescence assays of cells fixed in acetone/methanol then probed with antibodies to the acidic terminal segment (ATS, green) and the Maurer's cleft protein REX1 (magenta) and stained for DNA using DAPI (blue). Example merged (left) and mask (right) images are shown, where EMP1 only (green), REX1 only (magenta) and both (white) objects are distinguished. Scale bar, 2 μm . (E) Quantitation of the percent of EMP1 positive REX1 labeled structures. Data displayed are mean $\pm$ SD (CS2 n = 44; Δ PTP7 n = 63). (F) A representative example of the cleft and RBC cytoplasm masks used to quantify fluorescence intensity. The same cell as shown in D is represented here. (G) Quantification of the mean fluorescence intensity of EMP1 at the Maurer's clefts. (H,I) Quantification of the number and size of the Maurer's clefts in the CS2 and Δ PTP7 infected RBCs. Data displayed are mean $\pm$ SD (CS2 n = 41; Δ PTP7 n = 68). P-values determined by Welch's t-test.

Fig 4. PTP7 disruption leads to an accumulation of vesicles at the clefts. (A-B) Immuno-electron micrographs of 20-32 hpi CS2 and Δ PTP7 infected RBCs permeabilized with Equinotoxin-II and probed for REX1 (A)(α REX1-repeats [57]) or EMP1 (B)(α R3031 [39]) followed by immunogold secondary labeling with protein-A EM grade 6 nm gold. K: knob, V: vesicles, MC: Maurer's cleft, RBCM: red blood cell membrane. (B) Immuno-electron micrographs of α R3031 (EMP1) labeled cells. Magenta arrows: gold labeled puncta, white arrows: gold labeled vesicles.

Fig 5. PTP7 C-terminus is required for wildtype knob morphology. (A) Schematic of the PTP7 primary sequence. Numbers indicate amino acid position. SS: Signal sequence; TM: transmembrane domain; N mono-repeats: asparagine repeats. (B) Immunoblot of parent line (CS2) and PTP7 C-terminally truncated cell lysates probed with α GFP. Expected sizes of GFP chimeras from left to right are 58, 51, 54, and 56 kDa. Loading control, α GAPDH, expected size of ~38 kDa. (D) Mid-trophozoite stage infected RBCs were fixed in 2.5% glutaraldehyde/PBS and prepared for SEM of the exterior surface. Scale bar indicated. (D-E) Quantitation of the (D) knob density as knob count per square μm^2 and (E) mean knob diameter in nm. Data displayed are mean $\pm$ SD ($n = 10$ per cell line). P-values determined by Brown-Forsythe and Welch ANOVA tests and Dunnett's multiple comparison test.

Fig 6. Full length C-terminus required for antigen delivery and wildtype cleft morphology. (A) Flow cytometry analysis of infected RBCs labeled with antibodies for the ectodomain of var2CSA followed by secondary antibodies and tertiary antibodies conjugated to Alexa Fluor 647. Samples were run in technical duplicates. Data displayed are mean fluorescence values $\pm$ SD for each biological repeat ($n = 4$ per cell line). (B) Trypsin cleavage assay of truncation lines. Membranes were probed with α ATS and the loading/experimental control α SBP1. P: PBS mock treatment; T: Trypsin treated samples; asterisk: spectrin cross-reactivity band; arrows: EMP1 cleavage products. Loading control and experimental control, α SBP1 expected molecular weight is ~ 50 kDa. (C) Equinatoxin-II permeabilized infected RBCs probed with α GFP followed by immunogold secondary labeling. (D-E) Quantitation of vesicles from immuno-electron micrographs. (D) Mean vesicle diameter per image. Data displayed are mean $\pm$ SD ($n = 32, 41, 24, 24$ respectively). (E) Number of vesicles within 100 nm of each cleft. Data displayed are mean $\pm$ SD ($n = 43, 73, 38, 42$ respectively). P-values determined by Brown-Forsythe and Welch ANOVA tests and Dunnett's multiple comparison test. (F) Model of PTP7 function at the cleft-vesicle interface. Left of dotted line: wildtype. Right of dotted line: PTP7 ^{Δ 265-317}. Low-complexity C-termini facing the RBC cytoplasm with wide hydrodynamic radii (blue halos). Steric hindrance (red dotted lines and double ended arrows) makes close intra-molecular proximity unfavorable. Steric hindrance between the halos is reduced when PTP7 is at the curved membrane boundaries of the cleft periphery or vesicles. Steric repulsion aids in the fission process, allowing for cargo transport to the RBC membrane.

863 **Supporting information**

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S1 Fig. Validation and characterization of PTP7-GFP. (A) Schematic illustrating selection linked integration of 2xFKBP-GFP-2xFKBP tag (GFP^{sand} tag) at the 3' end of the PTP7 locus. Dark gray rectangles: linkers; T: T2A skip peptide; Neo: neomycin selectable marker; hDHFR selectable marker; *PTP7: PTP7 homology region with 5' stop codon; crossed lines: homologous cross over event; letters (A-E) and half arrows: Primer locations. Adapted from [27]. (B) Confirmation of correct integration of the PTP7-GFP^{sand} plasmid. (C) Immunoblots of the TritonX-100 insoluble, SDS soluble fraction of surface trypsinized infected RBCs shown. 3D7c: parent line. High molecular weight band is full length EMP1 recognized by αATS. Bands annotated with an asterisk indicate spectrin cross-reactivity expected at approximately 225 kDa for polypeptides [68] and 65 kDa for spectrin degradation products [69]. Arrows indicate cleaved EMP1 species where intracellular ATS regions were protected during trypsin incubation. Loading control, αSBP1, is also an experimental control, as breaching of the infected red blood cell membrane during trypsinization would cleave the Maurer's cleft protein SBP1. SBP1 expected molecular weight of ~50 kDa [28].

S2 Fig. Two stages of PTP7 localization. Indirect immunofluorescence assays of paraformaldehyde fixed infected RBCs probed with the antibodies displayed. Cells were synchronized to a 2-hour window and measured every 4 hours from 16 to 28 hpi. Scale bar, 2 μ m. For quantitation of the ratio of PTP7 positive puncta to total puncta per antibody set, images were maximum projected and the parasitophorous vacuole signal was excluded. Data displayed are mean $\pm$ SD (0801 n = 45, 28, and 28; EMP1 n = 23, 42, 35 and 25; MAHRP2 n = 52, 30, 33 and 29; PTP2 n = 48, 33, 10, and 30; REX1 n = 85, 39, 34, and 36; MAHRP1 n = 34, 36, 43, and 30). P-values determined by Brown-Forsythe and Welch ANOVA tests and Dunnett's multiple comparison test.

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PlasmoDB ID	Protein name	Number of significant ms/ms spectra			
		Experiment 1		Experiment 2	
		85c	3D7	85c	3D7
PF3D7_0301700	Plasmodium exported protein, unknown function (85c)	2	0	11	1
PF3D7_0801000	Plasmodium exported protein, unknown function (PHISTc)	5	0	10	0
PF3D7_0731100	PfEMP1-trafficking protein (PTP2)	3	0	7	0
PF3D7_0213100	protein SIS1 (SIS1)	2	0	5	0
PF3D7_0402400	Plasmodium exported protein, unknown function (GEXP18)	2	0	5	0
PF3D7_0501100.1	Plasmodium exported heat shock protein 40, type II (PFE55)	13	0	4	0
PF3D7_0601900	conserved Plasmodium protein, unknown function	4	0	2	0
PF3D7_0501000	Plasmodium exported protein, unknown function	3	0	2	0
PF3D7_1370300	membrane associated histidine-rich protein (MAHRP1)	3	0	2	0
PF3D7_0500800	mature parasite-infected erythrocyte surface antigen (MESA)	2	0	2	0

S3 Fig. PTP7 associated with other protein trafficking structures. (A) Summary of proteins 3-fold enriched proteins identified in PTP7-GFP^{sand} immunoprecipitation using GFP-Trap®. (B) Immunoprecipitation of PTP7-GFP^{sand} parasite lysate using GFP-Trap® including I, input; P, post-GFP-Trap®; W, resin wash; E, eluate. Expected size for PTP7 tagged with 4xFKBP 1xGFP domains is 108 kDa. Blots probed with primary antibodies against αFKBP (B), PTP7 (C), α0801 (D) and αMESA (E) and detected with HRP conjugated secondary antibodies. (F) Network map of exported *P. falciparum* co-immunoprecipitants identified from PTP7-GFP bait, overlaid with network established in [25].

S4 Fig. IFA validation of PTP7 disrupted cell line. Indirect immunofluorescence assays of cells fixed at an acetone methanol ratio of 1:1 (A) or 9:1 (B) then probed with antibodies as indicated above each panel. Bright field (BF) and DAPI stained DNA (blue) images are merged. Green and magenta are used for the merged images of the two antibodies. Scale bar, 2 μ m.

S5 Fig. CS2 and CS2 Δ PTP7 internal scanning EM images. Early to mid-trophozoite cultures were adhered to lectin functionalized slides and sheared off with hypotonic buffer to reveal the cytoplasmic face of the infected red blood cell footprint which was then fixed, dehydrated, coated with gold, and imaged using scanning electron microscopy. Knobs are indicated by inverted. Data displayed are mean $\pm$ SD (CS2 n = 14; Δ PTP7 n = 12). P-values determined by Welch's t-test.

S6 Fig. Gallery of internal knob spiral tomograms. Poly lysine functionalized grids were incubated with infected RBCs then lysed and imaged. Tomograms reveal the spiral structure underlying the infected red blood cell membrane associated with knobs [38]. Scale bar, 20 nm.

S7 Fig. Transmission electron micrographs of CS2 and CS2 Δ PTP7. Mid-trophozoite stage infected red blood cells.

S8 Fig. Gallery of quantitation referenced in (Fig 4). (A) IFA channels probed with α ATS and α REX1 were Huygens deconvolved to resolve cytoplasmic α ATS puncta and clefts indicated by α REX1 signal. A FIJI Macro script counts the α ATS only, α REX1 only, and both particles to generate: a proof, including the merge image (color assignment as in (Fig 4A)) and results image with EMP1 only (green), REX1 only (magenta) and both (white) depicted. (B) Ratio of EMP1-only particles to clefts. Data displayed are mean $\pm$ SD (CS2 $n = 44$; CS2 Δ PTP7 $n = 63$). (C-H) Quantitation of α ATS signal distribution and cleft features. (C) Additional examples of the cleft and cytoplasm masks used to quantify the fluorescence intensities of these compartments. Bright field (BF) and DNA stain DAPI merged for reference. The 'Cleft mask' depicts where α ATS signal was classified as 'cleft'. The 'Cytoplasm' mask illustrates i) the internal object used to determine α ATS cytoplasmic signal and ii) the external space where α ATS signal was measured as a background mean gray value control. The merge illustrates the merged IFA channels (color assignment as in (Fig 4A)). Mean gray values in the cleft and cytoplasm compartments were background subtracted. Data analysis performed is indicated in the y-axis. Data displayed are mean $\pm$ SD ((D) CS2 $n = 42$; CS2 Δ PTP7 $n = 71$. (E) CS2 $n = 45$; CS2 Δ PTP7 $n = 69$. (F) CS2 $n = 41$; CS2 Δ PTP7 $n = 68$. (G) CS2 $n = 49$; CS2 Δ PTP7 $n = 72$. (H) CS2 $n = 49$; CS2 Δ 85c $n = 72$). P-values determined by Welch's t-test.

S9 Fig. PTP7 is conserved to *Laverania*, with asparagine repeats unique to two clades. A phylogenetic tree and multiple sequence alignment calculated using the NCBI Standard Protein BLAST with a full-length PF3D7_0301700 coding sequence query. A hierarchical tree and color-coded sequence alignment were visualized using the ETE toolkit. Distance scale indicated.

S10 Fig. Validation of PTP7 truncated cell lines. Indirect immunofluorescence assays of paraformaldehyde fixed infected RBCs probed with the antibodies displayed. Scale bar, 2 μm. (B) Live cell microscopy, native GFP fluorescence (green) merged with the BF (gray) image. (B-C) GFP-puncta quantification of live cell microscopy showing the mean GFP-puncta counts per cell normalized to parasite width (to control for age) for each of the truncations. Data displayed are mean ± SD (n = 110, 58, 26, 47 per cell line in the order displayed). P-values determined by Brown-Forsythe and Welch ANOVA tests and Dunnett's multiple comparison test.

S11 Fig. Full length immunoblots. Saturated pixels indicated in red.

956 **S1 Table. Oligonucleotide appendix.**

Primer name	RE site	Sequence
PTP7-HR1_fw	AvrII	CAGGCGCCAGCCTAGGggcaaaagatagtcaaaagaac
PTP7-HR1_rv	NcoI	ATCGATAACTCCATGGaagaatataggaatacacaagc
PTP7-HR2_fw	SpeI	AGATCTTCGGACTAGTgattcacctgttttaaatgcattac
PTP7-HR2_rv	SacII	CAATGGCCCCCTTCCGCGGcgataaccttcattcaaagtc
PTP7-sgRNA_top		TAAGTATATAATATTggtccaacacagtcacacgGTTTTAGAGCTAGAA
PTP7-sgRNA_bottom		TTCTAGCTCTAAAAACcgtgtgactgtgttggaaccAATATTATATACTTA
PTP7-KO_ScrF		caatggaaaaagttttaagcctatt
PTP7-KO_ScrR		ggattaaacaaatatcataagggatac
yDHODH_ScrF		atgttcaaatgcatccttac
yDHODH_ScrR		ttagcttatgtcaactctatcaatag
SLI-sand-PTP7_fw	NotI	GCGGCCGCTaagaattaataaatccttagc
SLI-sand-PTP7_rv	AvrII	CCTAGGatTTTTTTTgattttttattattattgttgta
SLI-PTP7_ScrF		gacggaagaagaataaaagg
pARL_ScrF		ggaattgtgagcggataacaatttcacacagg
SLI-linker_ScrR		cagatcttgatctcaatcctg
SLI-GFP_ScrR		aatagtctcgacctgaacacc
SLI-PTP7_fw	NotI	actatagaatactc gcggccgc TAAGAATTAATAAATCCTTAGC
SLI-TGD-PTP7_rv	MluI	AGCAGCACCTCTAGC acgcgt ATTTTTTTTTGATTTTTTATTATTATTG
SLI-TGD-PTP7Δ300-317_rv	MluI	AGCAGCACCTCTAGC acgcgt ATTATTATTGTTGTTGTTATTATTATTG
SLI-TGD-PTP7Δ278-310_rv	MluI	AGCAGCACCTCTAGC acgcgt ATTTTTTTTTGATTTTTT CTCTTTATGTTTGTAGTCAT
SLI-TGD-PTP7Δ265-317 rv	MluI	AGCAGCACCTCTAGC acgcgt TGCTAATATATAAAGTGCTAT

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