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**A protease cascade regulates release of the human malaria parasite *Plasmodium falciparum* from host red blood cells**

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27 Malaria parasites replicate within a parasitophorous vacuole (PV) in red blood cells (RBC). Progeny  
28 merozoites egress upon rupture of first the PV membrane (PVM) then poration and rupture of the RBC  
29 membrane (RBCM). Egress is protease-dependent<sup>1</sup>, but none of the effector molecules that mediate  
30 membrane rupture has been identified and it is unknown how sequential rupture of the two membranes  
31 is controlled. Minutes before egress, the parasite serine protease SUB1 is discharged into the PV<sup>2-6</sup> where  
32 it cleaves multiple substrates<sup>2,5,7-9</sup> including SERA6, a putative cysteine protease<sup>10-12</sup>. Here we show that  
33 *Plasmodium falciparum* parasites lacking SUB1 undergo none of the morphological transformations that  
34 precede egress and fail to rupture the PVM. In contrast, PVM rupture and RBCM poration occur normally  
35 in SERA6-null parasites but RBCM rupture does not occur. Complementation studies show that SERA6 is  
36 an enzyme that requires processing by SUB1 for its function. RBCM rupture is associated with SERA6-  
37 dependent proteolytic cleavage within the actin-binding domain of the major RBC cytoskeletal protein  $\beta$ -  
38 spectrin. We conclude that SUB1 and SERA6 play distinct, essential roles in a coordinated proteolytic  
39 cascade that enables sequential rupture of the two bounding membranes and culminates in RBCM  
40 disruption through rapid, precise, SERA6-mediated disassembly of the RBC cytoskeleton.

41

42 Malaria, caused by parasitic protozoa of the genus *Plasmodium*, causes over 400,000 deaths per  
43 annum. With widespread resistance to most antimalarial drugs, there is a need to better understand  
44 the biology of the parasite, especially the species responsible for most fatalities, *Plasmodium*  
45 *falciparum*. Asexual blood stage parasites proliferate within RBC. Following each cycle of intracellular  
46 development (lasting ~48 h in *P. falciparum*) the PVM and RBCM rupture to allow egress of merozoites  
47 which invade fresh RBC.

48 Egress comprises several rapidly successive steps. Following biogenesis of intracellular  
49 merozoites by segmentation of the mature schizont, the PVM becomes permeable, allowing mixing of  
50 the contents of the PV and residual RBC cytoplasm<sup>6</sup>. Within the ensuing minutes, the parasite cGMP-  
51 dependent protein kinase PKG is activated to trigger discharge of SUB1, a subtilisin-like protease, from  
52 merozoite secretory organelles called exonemes<sup>3,4</sup>. In the PV lumen, SUB1 proteolytically modifies

53 several merozoite surface and PV proteins<sup>2,5,7,8</sup>, including SERA6, which is cleaved to release a central  
54 domain with homology to papain-like (clan CA, family C1) cysteine peptidases<sup>10</sup>. Within ~10 minutes of  
55 SUB1 discharge, the PV abruptly swells whilst the entire infected RBC transforms from an irregular to a  
56 roughly spherical 'flower' or rounded-up structure<sup>13,14</sup>. The PVM then fragments into multilamellar  
57 vesicles, closely followed by collapse and poration (permeabilisation) of the RBCM<sup>6,14,15</sup>. Within seconds  
58 the RBCM ruptures, allowing merozoite release<sup>16</sup>. Inhibitors of PKG block SUB1 discharge and all stages  
59 of egress subsequent to the initial PVM permeabilisation step<sup>3,5,6,17</sup>. In contrast, the broad-spectrum  
60 cysteine protease inhibitor epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), which does not  
61 inhibit SUB1 activity<sup>3</sup>, prevents neither PVM rupture nor RBCM collapse and poration, but blocks the  
62 final step of RBCM rupture<sup>6,14,15,18</sup>.

63 We used the rapamycin (RAP)-inducible dimerizable Cre recombinase (DiCre) system<sup>19,20</sup> to  
64 conditionally excise either a segment of the *SUB1* gene encoding crucial catalytic residues, or the entire  
65 *SERA6* coding sequence (Fig. 1a). In each case, PCR (Fig. 1a) and Western blot (Fig. 1b and  
66 Supplementary Fig. 1) demonstrated rapid and efficient RAP-induced excision of the floxed DNA  
67 sequences and ablation of *SUB1* or *SERA6* expression. Immunofluorescence analysis (IFA) confirmed  
68 loss of SUB1 in 99.8% of schizonts (of 5,056 examined) by the end of the erythrocytic cycle (cycle 0) in  
69 which the parasites were RAP-treated (Fig. 1c). Both *SUB1*-null ( $\Delta$ *SUB1*) and  $\Delta$ *SERA6* parasites formed  
70 morphologically normal schizonts at the end of cycle 0, showing that neither gene is required for  
71 intracellular development (Fig. 1c). However, over the ensuing erythrocytic cycles there was a dramatic  
72 reduction in replication rates of the RAP-treated cultures (Fig. 1d). Monitoring over 8-10 erythrocytic  
73 cycles showed that the initially minor population of non-excised parasites gradually overgrew these  
74 cultures whilst the  $\Delta$ *SUB1* or  $\Delta$ *SERA6* parasites disappeared (Fig. 1e), indicating a severe defect. To  
75 further assess the impact of gene disruption we used a plaque assay<sup>12</sup> which captures successive rounds  
76 of replication by individual parasite clones. Substantial reductions in plaque numbers were observed in  
77 RAP-treated cultures (Fig. 1f and reference 12), and the few plaques generated were found to arise

78 from the small population of non-excised parasites (Supplementary Fig. 2 and reference 12). These  
79 results suggested that both the *SUB1* and *SERA6* genes are required for *in vitro* parasite growth.

80 To confirm that loss of viability was a consequence of gene disruption, plasmids for episomal  
81 expression of wild-type (WT) *SUB1* or *SERA6* transgenes were introduced into the (non-RAP-treated)  
82 *SUB1HA3:loxP* or *SERA6:loxP* parasites respectively. The resulting lines were RAP-treated to disrupt the  
83 chromosomal genes, then immediately analysed by plaque assay in comparison with RAP-treated  
84 control lines harbouring 'empty' plasmid. As shown in Fig. 1f, lines carrying episomal WT *SUB1* or *SERA6*  
85 transgenes produced significantly more plaques following disruption of the chromosomal genes than  
86 similarly-treated parasites harbouring empty plasmid. Parasites expanded from plaques produced by  
87 RAP-treated parasites carrying the episomal *SUB1* or *SERA6* transgenes had lost the respective  
88 chromosomal gene as expected and so were likely relying solely on the episomal gene copies  
89 (Supplementary Fig. 3). Crucially, the  $\Delta$ *SERA6* growth defect could not be rescued by a mutant *SERA6*  
90 transgene possessing an Ala substitution of the predicted catalytic Cys644 codon (expected to ablate  
91 enzyme activity<sup>10</sup>) (Fig. 1f). Similarly, the  $\Delta$ *SERA6* defect was not complemented by a *SERA6* mutant in  
92 which the *SUB1* processing sites flanking the papain-like domain were modified by Leu substitutions of  
93 the P1 and P2 residues upstream of each scissile bond; these mutations prevent *SUB1*-mediated  
94 cleavage<sup>10</sup> (Fig. 1f). Collectively, these findings confirmed that *SUB1* and *SERA6* are indispensable and  
95 indicated that *SERA6* is an enzyme that requires proteolytic processing by *SUB1* to perform its function.

96 To examine egress of  $\Delta$ *SUB1* and  $\Delta$ *SERA6* parasites, mature schizonts were incubated with the  
97 PKG inhibitor (4-[7-[(dimethylamino)methyl]-2-(4-fluorophenyl)imidazo[1,2- $\alpha$ ]pyridine-3-yl]pyrimidin-2-  
98 amine (compound 2; C2), which reversibly blocks development just prior to egress, resulting in  
99 accumulation of 'stalled' segmented schizonts. Wash-out of the inhibitor allows rapid progress to  
100 rupture, enabling live microscopic examination of multiple egress events over the ensuing ~10-30  
101 minutes<sup>3,5,9</sup>. As shown in Fig. 2a and Supplementary Movie 1,  $\Delta$ *SUB1* parasites underwent none of the  
102 morphological changes associated with egress, with no signs of PVM swelling, rounding up, apparent  
103 PVM rupture (as indicated by loss of differential interference contrast and increased mobility of the

104 intracellular merozoites), or RBCM rupture. Indeed, the  $\Delta SUB1$  phenotype was indistinguishable from  
105 that produced by continued incubation with C2, suggesting that SUB1 is required for all the egress-  
106 related transformations that follow PKG activation. This egress defect was completely rescued by the  
107 WT *SUB1* transgene (Supplementary Movie 2). As expected, proteolytic processing of the SUB1  
108 substrates SERA5<sup>2</sup>, SERA6 and merozoite surface protein-1 (MSP1)<sup>5,7</sup> was ablated in the  $\Delta SUB1$   
109 parasites (Fig. 2b). However, IFA using antibodies to AMA1, a protein released onto the merozoite  
110 surface from micronemes (which are distinct from the exonemes in which SUB1 is stored<sup>2,3</sup>) showed  
111 that microneme discharge occurred in the trapped  $\Delta SUB1$  merozoites (Fig. 2c). Like SUB1 discharge,  
112 AMA1 discharge is blocked by PKG inhibitors<sup>3</sup>, so this confirmed reversal of the C2-mediated PKG  
113 inhibition in these experiments.

114 *P. falciparum* is generally maintained in static culture. To address whether invasion-competent  
115 merozoites could be liberated by fluid shear stress, *SUB1HA3:loxP* schizonts were cultured overnight  
116 with fresh RBC under vigorously shaking conditions. As expected<sup>21</sup>, shaking enhanced increases in  
117 parasitaemia in mock-treated *SUB1HA3:loxP* cultures (Fig. 2d), likely due to more efficient merozoite  
118 dissemination and RBC invasion. However, shaking had no impact on the low proliferation rate of RAP-  
119 treated ( $\Delta SUB1$ ) parasites, indicating that SUB1 is essential for release of invasive merozoites.

120 In contrast to the  $\Delta SUB1$  phenotype,  $\Delta SERA6$  schizonts displayed normal rounding up as well  
121 as the increases in merozoite visibility and mobility thought to indicate PVM rupture; however, RBCM  
122 rupture did not occur (Fig. 2e and Supplementary Movie 3). Introduction of the complementing WT  
123 *SERA6* expression plasmid restored egress (Supplementary Movie 4). IFA confirmed microneme  
124 discharge in the  $\Delta SERA6$  schizonts (Fig. 2f) whilst Western blot revealed normal SUB1 activity (Fig. 2g).  
125 Similar to the  $\Delta SUB1$  parasites, shaking did not enhance the replicative capacity of  $\Delta SERA6$  parasites  
126 (Fig. 2h).

127 Upon PVM lysis, but just prior to RBCM rupture, the RBCM suddenly becomes permeable to  
128 the F-actin binding peptide phalloidin<sup>9,14,22</sup>. To definitively establish the fate of the PVM in the mutants  
129 and to examine the timing and efficiency of RBCM poration, *SUB1HA3:loxP* and *SERA6:loxP* parasites

130 were transfected prior to RAP-treatment with a plasmid for constitutive expression of the PVM protein  
131 EXP1<sup>23</sup> fused to mCherry, fluorescently labelling the PVM (Supplementary Fig. 4). Simultaneous DIC and  
132 fluorescence video-microscopy in the presence of fluorescent wheat germ agglutinin (which labels the  
133 RBCM) and fluorescent phalloidin, confirmed that neither PVM rupture nor RBCM poration took place  
134 in  $\Delta$ SUB1 parasites (Fig. 3a and Supplementary Movie 5). In contrast, RBCM poration occurred normally  
135 in the  $\Delta$ SERA6 parasites upon PVM rupture. Examination of the arrested  $\Delta$ SUB1 parasites by  
136 transmission electron microscopy (TEM) confirmed an intact PVM and RBCM indistinguishable from C2-  
137 arrested schizonts (Fig. 3b and Supplementary Fig. 5). In contrast, scanning EM (Fig. 3c) and TEM (Fig.  
138 3d and Supplementary Fig. 5) of arrested  $\Delta$ SERA6 parasites revealed merozoites and PVM fragments  
139 within an intact but collapsed and evacuated RBCM, as previously observed in WT parasites arrested  
140 by E64<sup>6</sup>.

141 Our observation that egress proceeded normally in the  $\Delta$ SERA6 parasites up to the point of  
142 RBCM rupture suggested that SERA6 mediates RBCM breakdown. To test this model and further dissect  
143 the  $\Delta$ SERA6 defect, we performed a proteomic comparison of mock- and RAP-treated *SERA6:loxP*  
144 schizonts. SDS PAGE (Fig. 4a) detected a high molecular mass species that appeared within 20 minutes  
145 in soluble fractions of mock-treated parasites allowed to undergo egress, identified as a truncated form  
146 of the major RBC cytoskeleton protein  $\beta$ -spectrin. Further quantitative tandem mass spectrometry (LC-  
147 MS/MS) analysis revealed the concomitant appearance of one or more lower molecular mass  
148 polypeptides comprising the N-terminal calponin homology (CH) domain of the  $\beta$ -spectrin actin-binding  
149 domain (ABD) (Fig. 4b and Supplementary Fig. 6). This suggested that RBCM rupture is associated with  
150 proteolytic cleavage of  $\beta$ -spectrin near its N-terminus and release of the cleavage products from the  
151 cytoskeleton (which is generally insoluble in aqueous buffers). Western blot (Fig. 4c) and pull-downs  
152 using spectrin-specific monoclonal antibodies (mAbs) combined with LC-MS/MS (Fig. 4d and  
153 Supplementary Fig. 7) confirmed and extended this, showing that RBCM rupture is accompanied by  
154 extensive SERA6-dependent cleavage of  $\beta$ -spectrin at two closely-spaced sites (Gln167-Glu168 and  
155 Gln165-Thr166) between the CH1 and CH2 domains of the ABD, releasing the CH1 domain as a ~17 kDa

156 fragment and resulting in solubilisation of the truncated  $\beta$ -spectrin along with some  $\alpha$ -spectrin. The  
157 released CH1 domain co-purified with human  $\beta$ -actin, likely also derived from the RBC cytoskeleton  
158 (Fig. 4d and Supplementary Fig. 8). No proteolytic cleavage of the other major RBC cytoskeletal  
159 components  $\alpha$ -spectrin and protein 4.1R was detectable, although limited SERA6-dependent cleavage  
160 of ankyrin was evident (Supplementary Fig. 9). Strikingly,  $\beta$ -spectrin cleavage was never observed in  
161  $\Delta$ SERA6 parasites, even upon mechanical, hypotonic, freeze-thaw or detergent-mediated rupture of  
162 the schizonts (Fig. 4e), showing that cleavage was not due to lysis *per se* and implicating SERA6 as the  
163 enzyme responsible. The  $\beta$ -spectrin CH1 domain mediates key interactions between each end of the  
164  $\alpha_2\beta_2$ -spectrin tetramers that constitute the bulk of the cytoskeleton, and short  $\beta$ -actin filaments  
165 (together with protein 4.1R) at the junctional complexes that link the RBC cytoskeleton to its plasma  
166 membrane, providing the latter with structural integrity (Fig. 4f)<sup>24-26</sup>. Cleavage is therefore predicted to  
167 unravel the cytoskeleton with resulting RBCM destabilisation (Fig. 4g). Spectrin tetramers also bind the  
168 plasma membrane through interactions with the ankyrin complex<sup>24</sup>, so the limited cleavage of ankyrin  
169 might facilitate release of the cleavage products and associated proteins from the cytoskeletal complex.

170 Our findings ascribe the physico-mechanical processes underlying malarial egress to two  
171 parasite proteases that act rapidly and sequentially within the same, PKG-regulated pathway. SUB1 is  
172 required for all the structural changes following PKG activation, including rounding up, PVM lysis, RBCM  
173 poration and RBCM rupture. SERA6 is not required for PVM rupture or RBCM poration, but  
174 accomplishes the final step of RBCM rupture primarily through targeted cleavage of  $\beta$ -spectrin at a site  
175 that is essential for cytoskeletal stability. PVM rupture is unlikely to be directly mediated by protease  
176 activity, so SUB1 may regulate this by activating one or more membrane lytic effectors that mediate  
177 PVM rupture, as well as perhaps RBCM poration. These could include pore-forming proteins or  
178 phospholipases, both implicated in egress of other parasite developmental stages<sup>22,27,28</sup>. That SERA6  
179 function requires SUB1-mediated processing is consistent with processing representing activation of  
180 SERA6, as previously suggested<sup>10</sup>. The striking similarity between the  $\Delta$ SERA6 phenotype and that  
181 produced by treatment with E64<sup>6,14,18</sup> supports this and suggests that SERA6 is the major target of E64



182 in schizonts. Importantly, our study proves that host RBC calpain-1, previously implicated by others in  
183 egress<sup>29</sup>, is not sufficient for RBCM rupture since its expression should be unmodified in  $\Delta$ SERA6  
184 parasites.

185 All *Plasmodium* species, including the other major pathogens *P. vivax* and *P. knowlesi*, express  
186 orthologues of SUB1 and SERA6. Drugs that inhibit these proteases, particularly if combined with  
187 inhibitors of PKG<sup>30</sup>, would target consecutive, interdependent steps in the egress pathway and so could  
188 form a new class of antimalarial designed to prevent parasite proliferation and disease.

## 189 **Methods**

### 190 **Reagents and antibodies**

191 Anonymised human blood was obtained from the UK National Blood Transfusion Service. The antifolate  
192 WR99210 was from Jacobus Pharmaceuticals (New Jersey, USA). Blasticidin, rapamycin and E64 (Sigma)  
193 were used as described previously<sup>9,19</sup>. Compound 2 was kindly provided by Dr Simon Osborne (LifeArc,  
194 SBC Open Innovation Campus, Stevenage UK); stocks (10 mM) were stored in DMSO at -20°C and used  
195 throughout at a final concentration of 1 µM. Alexa Fluor 488 phalloidin and Alexa Fluor 647-conjugated  
196 WGA was from Thermofisher. The β-spectrin-specific mAbs B-1, B-2 and VD4, the α-spectrin-specific  
197 mAb 17C7, and mAbs B-11 and 8C3 specific for protein 4.1R and ankyrin respectively were all from  
198 Santa Cruz Biotechnology. Monoclonal antibody 7.7 (anti-EXP2) was from the European Malaria  
199 Reagent Repository (<http://www.malariaeurope.eu/>), contributed by Jana McBride. The polyclonal  
200 anti-mCherry antibody (ab167453) was from Abcam. The *P. falciparum* MSP1-specific mAb 89.1 has  
201 been described previously<sup>31</sup>, as have rabbit antisera to *P. falciparum* SERA5<sup>9</sup>, SERA6<sup>10</sup>, SUB1<sup>32</sup> and  
202 AMA1<sup>33</sup>. Phusion high-fidelity DNA polymerase and restriction enzymes were from New England  
203 BioLabs, and a Rapid DNA Ligation Kit (Roche) was used for DNA ligation.

204

### 205 **Parasite maintenance, synchronization and transfection**

206 The DiCre-expressing *P. falciparum* clone 1G5DC<sup>19</sup> was maintained at 37°C in human RBC in RPMI 1640  
207 medium containing Albumax (Invitrogen) supplemented with 2 mM L-glutamine. Cultures were  
208 routinely monitored by microscopic examination of Giemsa-stained thin blood films and synchronised  
209 by standard procedures<sup>34</sup>. As required, mature schizonts were isolated by centrifugation over cushions  
210 of 70% (v/v) isotonic Percoll (GE Healthcare Life Sciences) as described<sup>34</sup>. Invasion assays were  
211 performed as previously described<sup>3,5</sup>, either in static culture or in a shaking incubator revolving at 225  
212 rpm. For transfection of plasmid constructs, ~10<sup>8</sup> Percoll-enriched schizonts were suspended in 100 µl  
213 of P3 primary cell solution containing 10 µg of DNA and electroporated with an Amaxa™ P3 primary cell  
214 4D Nucleofector™ X Kit L (Lonza), using program FP158 as previously described<sup>5</sup>. Growth medium was

215 supplemented ~20 h post transfection with WR99210 (2.5 nM) or blasticidin (2 µg/ml). Once sustained  
216 growth of drug-resistant parasites was observed, drug cycling was used to enrich for genomic  
217 integration of plasmid constructs as previously described<sup>2</sup>. Transgenic parasite clones *SUB1HA3:loxP*  
218 and *SERA6:loxP* were obtained by limiting dilution cloning in microplates at a 0.1-0.3 parasite per well.  
219 Parasite genomic DNA (gDNA) for genotype analysis was extracted using a Qiagen DNeasy Blood and  
220 Tissue kit and analyzed by PCR using Kappa 2G Fast HotStart ReadyMix (Kappa Biosciences).

221

### 222 Immunofluorescence and Western blot

223 For IFA, air-dried thin films of parasite cultures were fixed in paraformaldehyde, permeabilized, then  
224 probed with relevant primary antibodies as described previously<sup>10</sup>. Secondary Alexa Fluor 488- or 594-  
225 conjugated antibodies specific for human, rabbit or mouse IgG (Invitrogen), or Alexa Fluor 594-  
226 conjugated streptavidin (Invitrogen) were used at a dilution of 1:1000. Samples were mounted in  
227 Vectashield antifade mounting medium (Vector Laboratories) containing DAPI. Images were acquired  
228 using a Nikon Eclipse and NIS Elements software (Nikon, Japan), using identical exposure conditions for  
229 all samples being compared. Western blots were performed as described previously<sup>33</sup>. For detection of  
230 HA3-tagged SUB1, the rat anti-HA mAb 3F10 (Sigma) was used at a 1:1000 dilution, followed by biotin-  
231 conjugated anti-rat antibody (Roche) (1:8,000 dilution), then horseradish peroxidase (HRP)-conjugated  
232 streptavidin (Sigma) (1:10,000 dilution). Immobilon Western Chemiluminescent HRP Substrate  
233 (Millipore) was used according to the manufacturer's instructions, and blots were visualised and  
234 documented using a ChemiDoc Imager (Bio-Rad) with Image Lab software (BioRad).

235

### 236 Generation of integration, complementation and expression plasmids

237 *SUB1HA3:loxP* and *SERA6:loxP* parasite clones were generated by single crossover homologous  
238 recombination into the 1G5DC genome using integration plasmids pHH1\_SUB1HA3\_loxP and  
239 pHH1\_S6chimera\_loxP respectively. In both cases, correct transcriptional regulation of the modified  
240 gene was assured by placement of the 3' UTR of the *P. berghei* dihydrofolate reductase thymidylate

241 synthase (*PbDT*) gene downstream of the floxed coding sequence<sup>19</sup>. To target the *SUB1* gene, a chimeric  
242 gene fragment with a native 5' portion and a recodonised 3' segment was constructed by first  
243 amplifying the 5' sequence from *P. falciparum* 3D7 gDNA using primers JT-S1endo-F and JT-S1CO-R,  
244 whilst the recodonised region was amplified from plasmid pFastBac-sPfSUB1wt<sup>32</sup> using primers JT-  
245 S1CO-F and JT-S1synth-R. The amplicons were then fused in frame by inclusion of both in a fresh PCR  
246 reaction including primers JT-S1endo-F and JT-S1synth-R, and the product cloned into pGEM-T  
247 (Promega). A segment of this fragment was then excised using HindIII and KpnI and replaced with a  
248 similarly-digested *loxP*-containing synthetic intron (*loxPint*)<sup>20</sup> (Geneart). This intermediate vector was  
249 digested with HpaI and Xho I to liberate the *SUB1* sequence which was ligated into pHH1\_sera5\_LoxP1<sup>19</sup>  
250 digested with the same enzymes to generate pHH1\_SUB1HA3\_loxP. Integration of this construct into  
251 the 1G5DC *SUB1* locus by homologous recombination was detected by diagnostic PCR with primers  
252 JT111-1p and JT111-2p, and the floxed or excised *SUB1* locus was detected by PCR using primers JT111-  
253 1p and JT111-3p.

254 To generate pHH1\_S6chimera\_loxP, a chimeric *SERA6* gene fragment was excised from plasmid  
255 MWS36 by digestion with HpaI and NcoI. Full details of plasmid MWS36 will be provided in a separate  
256 manuscript (M. Shea and M. Blackman, in preparation). The chimeric *SERA6* sequence comprised a 920  
257 bp 5' portion of endogenous coding sequence starting from within the first intron followed by synthetic  
258 recodonised *SERA6* cDNA sequence extending to the stop codon. Plasmid pHH1\_sera5\_LoxP1 was  
259 digested with HindIII, 'blunted' with T4 DNA polymerase, then further digested with NcoI before ligation  
260 to the chimeric *SERA6* fragment excised from MWS36, generating pHH1\_S6chimera\_loxP. Integration  
261 of this construct by homologous recombination into the 1G5DC genome (which already contains a  
262 single genomic *loxP* site upstream of the *SERA6* locus<sup>19</sup>) was designed to introduce a second *loxP* site  
263 downstream of the *SERA6* stop codon. Correct integration was detected by diagnostic PCR using  
264 primers SERA6-5'UTRb and SERA6-37, whilst the floxed or excised *SERA6* locus was detected with  
265 primers S65'UTRb-2 and S6EndoEx2Rev.

266 For generation of plasmid constructs designed for transgenic expression of *SERA6*, a chimeric  
267 coding sequence and native *SERA6* promoter was excised from plasmid MW28 and ligated into Sall and  
268 NcoI digested plasmid pDC-mCherry-MCS (a modification of pDC2-mCherry<sup>9</sup>), giving rise to pDC2-  
269 wtSERA6 (WT *SERA6*). The chimeric *SERA6* sequence comprised 979 bp of putative promoter sequence  
270 upstream of the native *SERA6* ATG start codon, as well as 477 bp of the 5' segment of the coding  
271 sequence (including the first intron) and a synthetic recodonised *SERA6* cDNA<sup>10</sup> encoding the remainder  
272 of the ORF. Full details of plasmid MWS28 will be provided in a separate manuscript (M. Shea and M.  
273 Blackman, in preparation). Site-directed mutagenesis and sub-cloning steps were then used as  
274 previously described<sup>10</sup> to generate identical constructs containing di-Leu substitutions of the P1 and P2  
275 positions at the SUB1 processing sites 1 and 2 in *SERA6*, as well as a Cys644Ala substitution of the active  
276 site nucleophile, giving rise to plasmids pDC2-SERA6-uncleavable (Uncleavable *SERA6*) and pDC2-  
277 *SERA6*\_Alamut (Cys644Ala) respectively.

278 For transgenic expression of WT *SUB1* the pDC-mCherry-MCS plasmid was modified such that  
279 the blasticidin deaminase (BSD) drug selection cassette and mCherry reporter gene were expressed  
280 from a single promoter by the use of the ribosomal T2A skip peptide. To do this, the BSD cassette was  
281 excised from pDC2-mCherry\_MCS with ApaI and SacI and the backbone re-ligated. The mCherry ORF  
282 was then excised by digestion with AvrII and XhoI and replaced with a synthetic gBlock® (IDT)  
283 comprising the mCherry and BSD ORFs separated by the T2A sequence. This resulted in construct pDC2-  
284 mCherryT2ABSD\_MCS in which these ORFs remained under control of the constitutive *P. falciparum*  
285 calmodulin (*CAM*) promoter (which remained from the original mCherry expression cassette). This  
286 vector was then linearized with SnaBI. The *P. falciparum* *SUB1* promoter sequence was amplified from  
287 *P. falciparum* 3D7 gDNA using primers PfsUB1\_prom\_for\_infu and PfsUB1\_prom\_rev\_infu. Primers  
288 PfsUB1\_synth\_for\_infu and PfsUB1\_synth\_rev\_infu were used to amplify the recodonised synthetic  
289 *SUB1* ORF from pFastBac-sPfsUB1wt and primers PbDT3UTR-for\_infu and PbDT3UTR-for\_infu were  
290 used to amplify the *PbDT* 3' UTR from pDC2-mCherry\_MCS. Primers included complementary  
291 overhangs such that all 3 fragments could then be finally assembled into the linearized pDC2-

292 mCherryT2ABSD\_MCS backbone in a single step using an InFusion® HD Cloning Kit (Clontech),  
293 generating pDC2-mCherryT2ABSD\_wtSUB1 (WT *SUB1*).

294 For episomal transgenic expression of an EXP1mCherry fusion protein (to fluorescently label  
295 the PVM), a synthetic intronless DNA fragment encoding mCherry fused to the C-terminus of the *P.*  
296 *falciparum* EXP1 (PlasmoDB ID PF3D7\_1121600) via a polyglycine-alanine linker (GAGGGGGGGGA) was  
297 obtained from Geneart. This was sub-cloned into vector pCR-Blunt using the ZeroBlunt PCR cloning kit  
298 (Invitrogen). The resulting plasmid was digested with AvrII and XhoI before ligating the EXP1mCherry  
299 fragment into pDC-mCherry-MCS in the place of the mCherry ORF, generating pDC2-EXP1-mCherry.

300

### 301 **Parasitaemia quantitation by flow cytometry**

302 Parasites were fixed in 4% paraformaldehyde, 0.02% glutaraldehyde for 30 min at 37°C, diluted five-  
303 fold in phosphate-buffered saline, then stored at 4°C until required. Cells were stained with Hoechst  
304 33342 (diluted 1:10,000) for 30 min at 37°C, then parasitaemia determined using a Fortress or FACS  
305 Aria (BD) flow cytometer as previously described<sup>9</sup>. Briefly, samples were initially screened using forward  
306 and side scatter parameters and gated for RBC (Supplementary Fig. 10). Ultraviolet light with a violet  
307 filter (450/50 nm) was then used to determine the proportion of infected cells in 100,000 RBC.

308

### 309 **Time-lapse DIC and fluorescence video microscopy**

310 Viewing chambers for live microscopy were constructed as described previously<sup>3,5</sup> by adhering 22 x 64  
311 mm borosilicate glass coverslips to microscope slides. Mature Percoll-enriched schizonts were  
312 incubated for 3-4 h at 37°C in complete medium supplemented with C2 (1 µM), then ~5 x 10<sup>7</sup> schizonts  
313 were rapidly washed twice in gassed warm complete medium lacking C2, pelleting at 1,800 x g for 1  
314 min. The cells were suspended in 50 µl of the same medium and introduced into the pre-warmed  
315 viewing chamber on a temperature-controlled microscope stage held at 37°C on a Nikon Eclipse Ni-E  
316 wide-field microscope fitted with a Hamamatsu C11440 digital camera and Nikon N Plan Apo λ  
317 100x/1.45NA oil immersion objective. Images (DIC alone or simultaneous DIC and fluorescence) were

318 taken at 5-10 s intervals over a total of 20-60 min, then annotated and exported as TIFFs, AVI or  
319 QuickTime movies using Nikon NIS-Elements software.

320

### 321 **Parasite plaque assays**

322 Plaque assays were performed by dispensing parasite cultures (200  $\mu$ l at a haematocrit of 0.75%) into  
323 flat-bottomed 96-well microplates, as described<sup>12</sup>. Plates were imaged 14-16 days later to detect  
324 plaque formation, using an Epson Perfection V750 Pro high resolution flat-bed scanner in top-down  
325 transmission light mode. When required, parasites from wells containing a single plaque were  
326 expanded by transferring initially to round-bottomed microplate wells to aid medium changes, before  
327 further expansion into culture flasks.

328

### 329 **Scanning electron microscopy**

330 Mature arrested  $\Delta$ *SERA6* schizonts, or WT control schizonts allowed to reach the point of egress in the  
331 presence of E64 (50  $\mu$ M) were fixed in 2.5% glutaraldehyde, washed, osmicated (1% OsO<sub>4</sub> for 16 h),  
332 dehydrated, critical point dried and sputter coated with 5 nm gold for scanning EM. Images were  
333 collected on a JEOL JSM 7610F with 2.6 kV accelerating voltage.

334

### 335 **Transmission electron microscopy**

336 Mature schizonts were fixed for 5 min at 37°C in 2% formaldehyde ( $\Delta$ *SERA6* and E64-arrested schizonts)  
337 or 2% formaldehyde 1% glutaraldehyde ( $\Delta$ *SUB1* and C2-arrested WT schizonts), pelleted by  
338 centrifugation, mixed with 20% (w/v) dextran in complete medium containing bakers' yeast, then  
339 frozen using a HPM100 high-pressure freezer (Leica). Vitriified cells were freeze-substituted using a EM  
340 AFS2 (Leica) into Lowicryl HM20 resin (EMS) with 0.2% (w/v) uranyl acetate and cut into 250 nm or 120  
341 nm sections using a UC7 microtome (Leica). Sections were placed on glow-discharged carbon-coated  
342 copper London Finder grids (EMS) with 10 nm Protein A-Au fiducials (EMS) and post-stained with 0.2%  
343 (w/v) uranyl acetate and 4% (w/v) lead citrate. Images and tomograms were recorded using a Model

344 2040 dual-axis tomography holder (Fischione Instruments) on a Tecnai F20 200 kV field emission gun  
345 electron microscope (FEI) equipped with a DE20 camera (Direct Electron), or a Tecnai T12 120 kV field  
346 emission gun electron microscope (FEI) equipped with a 4kx4k Ultrascan 4000 CCD camera (Gatan).  
347 Dual-axis tilt series were acquired from -60° to +60° with an increment of 2° using SerialEM<sup>35</sup> and  
348 processed using IMOD<sup>36</sup> with nonlinear anisotropic diffusion filtering.

349

### 350 **Proteomic analysis and pull-downs**

351 Mature mock- or RAP-treated mature *SERA6:loxP* schizonts were incubated for ~4 h at 37°C in complete  
352 medium supplemented with C2, then washed once in gassed, warm protein-free medium containing  
353 C2 before rapidly washing twice in similar medium lacking C2, pelleting at 1,800 x g for 1 min. The cells  
354 were suspended at high density (~1 x 10<sup>9</sup>/ml) in warm protein-free medium lacking C2 and incubated  
355 for just 20 min at 37°C to allow progress to egress. The entire samples were then snap-frozen in liquid  
356 N<sub>2</sub>.

357 For global proteomic analysis, samples were thawed by the addition of an equal volume of ice-  
358 cold water containing protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich),  
359 followed by centrifugation at 16,000 x g for 10 min at 4°C to separate soluble and insoluble fractions.  
360 These were immediately fractionated by SDS PAGE on NuPAGE 4-12% Bis-Tris gels (Invitrogen). Gels  
361 were stained with Quick Blue Coomassie (Triple Red) then the entire gel cut into 24 equally-sized slices  
362 and proteins in excised slices subjected to tryptic digestion<sup>37</sup>. LC-MS/MS analysis was as described  
363 below.

364 For pull-down analysis, frozen schizont preparations were thawed in the presence of 1% (v/v)  
365 Nonidet® P40 (CAS 68412-54-4, Santa Cruz Biotechnology), 10 mM EDTA and protease inhibitors  
366 (cOmplete™ Protease Inhibitor Cocktail, Sigma-Aldrich) and extracted at 4°C for 1 h with intermittent  
367 vortexing. The extracts were clarified by centrifugation at 16,000 x g for 10 min at 4°C, filtered through  
368 0.22 µm PVDF centrifugal filter units (MilliporeSigma), then incubated with ~2 µg of the relevant anti-  
369 spectrin mAb for 1.5 h at 4°C with gentle mixing. Precipitation of immune complexes with Protein G



370 Sepharose™ 4 Fast Flow (GE Healthcare) followed the manufacturer's protocol. Bound complexes were  
371 subjected to SDS PAGE and stained bands of interest excised and analysed by tryptic digestion and LC-  
372 MS/MS.

373

#### 374 **Mass spectrometry (LC-MS/MS)**

375 Tryptic digests were chromatographically resolved using an Ultimate 3000 RSLCnano (Dionex) with an  
376 EASY-Spray column (2 µm particle size, PepMap C18, 100 Å pore size, 50 cm x 75 µm ID; Thermo  
377 Scientific). Spectra were acquired using an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific)  
378 acquiring the top 10 most intense ions in data dependent acquisition mode with CID fragmentation at  
379 35% normalised collision energy. For targeted work, data were acquired using an Orbitrap Fusion Lumos  
380 Tribrid mass spectrometer (Thermo Scientific) using a mixture of data dependent fragmentation and  
381 three targeted fragmentations (545.3006 Da, 659.8537 Da and 830.9363 Da for peptides FQIQDIVVQ,  
382 FQIQDIVVQTQ and FQIQDIVVQTQEGR, respectively) over a 3 sec cycle time. Dynamic exclusion was  
383 employed throughout to prevent repeat sampling of data dependent fragmentation.

384 Data were searched using Mascot (Matrix Science) against the UniProt database, using trypsin  
385 or semi-trypsin as the cleavage enzyme, with a fixed carbamidomethylation modification (+57.021 Da)  
386 and variable methionine oxidation (+ 15.994 Da). A 10 ppm mass tolerance filter was applied for  
387 peptides with charge states +2 or above. Mascot search results were imported into Skyline<sup>38</sup> to  
388 generate a spectral library for further quantitative processing of β-spectrin peptides.

389

#### 390 **Statistical analysis**

391 Prism 7 (GraphPad) was used for all statistical analysis. All experiments were performed at least twice,  
392 and statistical analysis was carried out by unpaired t-test of biological replicate or triplicate  
393 experiments. A p value of <0.05 was considered statistically significant.

394

395

396 **Data availability**

397 The data supporting the findings of this study are available within the paper and its Supplementary  
398 Information and are also available from the corresponding authors upon request.

399 **Figure legends.**

400 **Figure 1. SUB1 and SERA6 are essential for asexual blood stage *P. falciparum* growth.** **a**, Architecture of  
401 floxed loci in *SUB1HA3:loxP* and *SERA6:loxP* parasites. Introduced *loxP* sites (arrowheads), recodonised  
402 sequence (hatched), HA3 epitope and known (SUB1) or predicted (SERA6) catalytic residues are  
403 indicated. Outcomes of rapamycin (RAP)-induced DiCre-mediated excision and positions of primers  
404 (half arrows) used for diagnostic PCR are indicated (see [Supplementary Table 1](#) for primer sequences).  
405 Insets, PCR (representative of 4 independent experiments) confirming efficient gene excision by the  
406 end of cycle 0, ~44 h following mock-treatment (-RAP) or RAP-treatment (+RAP) of 'ring'-stage  
407 parasites. **b**, Western blots (representative of 2 independent experiments) showing ablation of SUB1  
408 and SERA6 expression in cycle 0 schizonts. **c**, Light microscopic and IFA images of mature cycle 0  
409 schizonts, showing normal parasite development and RAP-induced loss of SUB1HA3 expression  
410 (representative of 6 independent experiments). Loss of SERA6 expression could not be similarly  
411 confirmed by IFA due to C-terminal tagging of SERA6 being unsuccessful and the lack of suitable SERA6-  
412 specific antibodies. Scale bar, 5  $\mu$ m. DAPI, 4,6-diamidino-2-phenylindole. **d**, Replication of mock- and  
413 RAP-treated *SUB1HA3:loxP* and *SERA6:loxP* parasites over 2 erythrocytic cycles. Parasitaemia values  
414 (quantified by FACS) are averages from 2 biological replicates in different blood sources. Error bars,  
415  $\pm$ SD. **e**, PCR showing loss of  $\Delta$ *SUB1* (1 experiment) and  $\Delta$ *SERA6* (representative of 2 independent  
416 experiments) parasites and outgrowth of non-excised parasites upon extended passage of RAP-treated  
417 cultures. **f**, Dot plots showing relative plaque forming ability (ratio of plaque numbers produced by RAP-  
418 treated cultures to those produced by mock-treated cultures, x100) of *SUB1HA3:loxP* and *SERA6:loxP*  
419 parasites without or following transfection with the indicated episomal expression plasmids. Statistical  
420 significance was determined by two-tailed t-test: *SUB1HA3:loxP*: Empty vs WT  
421 complementation (t=7.702, d.f.=2, p=0.0164, 95% CI 4.636 to 16.73) n=2. *SERA6:loxP*: Empty vs WT  
422 complementation (t=19.65, d.f.=2, p=0.0026, 95% CI 68.39 to 107) n=2; WT vs Cys644Ala  
423 complementation (t=30.96, d.f.=4, p<0.0001, 95% CI -94.44 to -78.81 ) n=3; WT vs Uncleavable

424 complementation ( $t=13.8$ ,  $d.f.=4$ ,  $p=0.0002$ , 95% CI -98.14 to -65.25)  $n=3$ . In all plots, central horizontal  
425 bar, mean. Error bars,  $\pm SD$ . Significance levels:  $p \leq 0.001$ , \*\*\*;  $p \leq 0.01$ , \*\*;  $p \leq 0.05$ , \*.  
426

427 **Figure 2. SUB1 and SERA6 play distinct, sequential roles at egress. a,** Left, stills from time-lapse DIC  
428 microscopic examination of control (-RAP) and RAP-treated ( $\Delta$ *SUB1*) *SUB1HA3:loxP* schizonts following  
429 removal of C2; elapsed time indicated. Scale bar, 20  $\mu$ m. Right, quantitation of PVM rupture in control  
430 and RAP-treated *SUB1HA3:loxP* schizont populations, collated from 5 videos of each from 2  
431 independent experiments (total number of observed PVM rupture events in control parasites, 226).  
432 PVM rupture is normalised to that in the controls (100% egress). Statistical significance determined by  
433 two-tailed t-test: -RAP vs +RAP (t=13.84, d.f.=2, p=0.0052, 95% CI -113.2 to -59.5); p $\leq$ 0.01, \*\*. **b,**  
434 Processing of SUB1 substrates is ablated in  $\Delta$ *SUB1* parasites. Western blot of C2-blocked *SUB1HA3:loxP*  
435 schizonts, or 30 min after washing away C2. Processed forms of SUB1 substrates are arrowed. **c,**  
436 Microneme discharge in  $\Delta$ *SUB1* parasites. IFA of C2-arrested parasites compared to 30 min after  
437 washing away C2. Translocation of AMA1 to the intracellular merozoite surface is evident in the washed  
438 parasites. Scale bar, 10  $\mu$ m. **d,** Invasion by control and RAP-treated *SUB1HA3:loxP* parasites under static  
439 and shaking conditions. Statistical significance by two-tailed t-test: -RAP stationary vs -RAP shaking  
440 (t=5.233, d.f.=5, p=0.0034, 95% CI 2.666 to 7.813 ) n=4; +RAP start vs +RAP stationary (t=1.722, d.f.=5,  
441 p=0.1456, 95% CI -0.04104 to 0.2077) n=4; +RAP stationary vs +RAP shaking (t=0.4585, d.f.=5,  
442 p=0.6658, 95% CI -0.641 to 0.9193) n=4. Results shown are from 4 biological replicate experiments  
443 (some dots are overlaid). **e,** Left, time-lapse DIC microscopic stills of control and RAP-treated *SERA6:loxP*  
444 schizonts following C2 removal. Scale bar, 20  $\mu$ m. Right, quantitation of RBCM rupture. Data collated  
445 from 8 videos each of control and RAP-treated parasites, from 3 independent experiments (total  
446 number of observed rupture events in control parasites, 568). RBCM rupture is normalised to that in  
447 the controls (100% egress). Statistical significance by two-tailed t-test: -RAP vs +RAP (t=25.39, d.f.=4,  
448 p<0.0001, 95% CI -95.07 to -76.33) n = 3; p<0.001, \*\*\*. **f,** Microneme discharge in arrested  $\Delta$ *SERA6*  
449 parasites. IFA of C2-arrested parasites compared with 30 min after washing away C2. **g,** Disruption of  
450 the *SERA6* gene has no effect on processing of SUB1 substrates. **h,** Invasion efficiencies of the  $\Delta$ *SERA6*  
451 parasites under static and shaking conditions. Statistical significance by two-tailed t-test: -RAP  
452 stationary vs -RAP shaking (t=5.674, d.f.=4, p=0.0048, 95% CI 2.57 to 7.496) n=3; +RAP start vs +RAP

453 stationary ( $t=2.741$ ,  $d.f.=4$ ,  $p=0.0518$ , 95% CI -0.004807 to 0.7581)  $n=3$ ; +RAP stationary vs +RAP  
454 shaking ( $t=2.526$ ,  $d.f.=4$ ,  $p=0.0649$ , 95% CI -0.1348 to 2.855)  $n=3$ ;  $p\leq 0.01$ , \*\*. Results shown are from 3  
455 biological replicate experiments. In all plots, central bar, mean. Error bars,  $\pm$ SD. Experiments in panels  
456 **b**, **c**, **f** and **g** were repeated twice, with reproducible results.

457

458 **Figure 3. SUB1 is required for PVM disruption and RBCM poration, whereas the  $\Delta$ SERA6 phenotype**  
459 **mimics egress arrest with the cysteine protease inhibitor E64. a**, Stills from simultaneous time-lapse DIC  
460 and fluorescence microscopic examination of typical control WT,  $\Delta$ SUB1 and  $\Delta$ SERA6 schizonts at the  
461 indicated intervals following removal of the egress inhibitor C2. PVM rupture and RBCM poration  
462 (indicated by access of phalloidin to the host RBC cytoskeleton) occurs in the  $\Delta$ SERA6 parasites but not  
463 in the  $\Delta$ SUB1 parasites, whilst RBCM rupture occurs in neither mutant. Scale bar, 10  $\mu$ m. **b**, TEM  
464 micrographs of an arrested  $\Delta$ SUB1 schizont and a C2-arrested control cell, showing that the trapped  
465 merozoites are surrounded in both cases by an intact PVM and RBCM. Knob structures characteristic  
466 of the parasite-infected RBCM<sup>6</sup> are indicated on its outer surface (arrow heads). The black dots are gold  
467 fiducials added for tomography. Scale bar, 500 nm. **c**, SEM images of  $\Delta$ SERA6 schizonts before and 30  
468 min following C2 removal, showing collapse of the RBCM around the intracellular merozoites in the  
469 washed sample. Scale bar, 5  $\mu$ m. **d**, TEM micrographs of an arrested  $\Delta$ SERA6 schizont and an E64-  
470 arrested control cell, showing in both remnants of ruptured PVM (asterisks) adjacent to the trapped  
471 merozoites. Knobs are highlighted as above (arrow heads). Scale bar, 500 nm. All experiments were  
472 repeated twice, with reproducible results.

473

474 **Figure 4. RBCM rupture is associated with rapid, SERA6-dependent cleavage of host RBC cytoskeleton  $\beta$ -**  
475 **spectrin within its actin-binding domain. a**, SDS PAGE showing appearance upon egress of mock-treated  
476 (-RAP) *SERA6:loxP* schizonts of a high molecular mass species identified by Western blot and LC-MS/MS  
477 as truncated  $\beta$ -spectrin (reproducible in 15 independent experiments). **b**, Peptides (red) identified by  
478 LC-MS/MS (3 technical replicate runs from a single biological experiment) of tryptic digests of

479 polypeptide(s) enriched in the mock-treated *SERA6:loxP* schizont extract in the indicated region of the  
480 gel (~15-20 kDa), indicating egress-associated, SERA6-dependent cleavage of  $\beta$ -spectrin. A semi-tryptic  
481 peptide likely representing the C-terminus of the polypeptide(s) is in bold ([Supplementary Fig. 6](#) shows  
482 fragmentation spectra). Calculated mass of the  $\beta$ -spectrin sequence (UniProtKB P11277) from Thr2-  
483 Gln167 is 19,251 Da. CH1, CH2, calponin homology domains. **c**, Appearance of a ~17 kDa N-terminal  
484 fragment of  $\beta$ -spectrin (arrowed) upon egress of mock-treated *SERA6:loxP* schizonts (reproducible in 4  
485 independent experiments). **d**, Pull-down of cytoskeletal components from soluble extracts of egressing  
486 *SERA6:loxP* schizonts. Annotated species, including co-precipitating  $\beta$ -actin, were identified by LC-  
487 MS/MS or Western blot (reproducible in 3 independent experiments). Peptide fingerprinting of the ~17  
488 kDa  $\beta$ -spectrin CH1 domain was as in **b**. The presence of  $\alpha$ -spectrin and full-length and truncated  $\beta$ -  
489 spectrin in pull-downs from the -RAP extracts indicates their SERA6-dependent dissociation from the  
490 normally insoluble cytoskeleton. Antibody heavy and light chains, asterisked. **e**, Fate of  $\beta$ -spectrin in  
491 *SERA6:loxP* schizonts following washing away a C2 block (control) or with additional treatment by the  
492 indicated disruption methods. Cleavage never occurred in the absence of SERA6 (Western blot  
493 representative of 2 independent experiments). **f**, Architecture of RBC cytoskeleton spectrin  
494 heterotetramer, comprising 2 antiparallel  $\alpha\beta$ -spectrin heterodimers linked head-to-head (the right-  
495 hand dimer is abbreviated for clarity) which cross-link  $\beta$ -actin-containing junctional complexes<sup>24</sup>.  
496 Spectrin repeat domains are numbered. Other structural features and positions of epitopes recognised  
497 by mAbs B-1, B-2 and VD4 are indicated. **g**, Top, SERA6-dependent cleavage of  $\beta$ -spectrin should release  
498 each end of the  $\alpha\beta$ -spectrin tetramer from its cognate junctional complex. The cleaved CH1 domain  
499 may be released with actin still bound. Bottom, predicted global effect of SERA6-dependent cleavage  
500 on the cytoskeleton.

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### 623 **Author contributions**

624 J.A.T. performed all *P. falciparum* genetic manipulations and phenotype analysis. M.S.Y.T. performed  
625 phenotype analysis and parasite manipulation. F.H. performed parasite manipulation. G.V.B. and R.A.F.  
626 performed SEM. C.B., T.R.U. and V.L.H. performed and interpreted TEM. A.B., M.S.Y.T. and B.S.  
627 performed and interpreted proteomic analysis. J.A.T., M.S.Y.T, B.S., H.R.S. and M.J.B. conceived the  
628 study, designed experiments, interpreted results and wrote the manuscript.

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