

1                   **A lipocalin mediates unidirectional haem**  
2                   **biomineralization in malaria parasites**

3           Joachim M. Matz<sup>1,2,\*</sup>, Benjamin Drepper<sup>2</sup>, Thorsten B. Blum<sup>3</sup>, Eric van Genderen<sup>3</sup>,  
4           Alana Burrell<sup>4</sup>, Peer Martin<sup>2</sup>, Thomas Stach<sup>2</sup>, Lucy Collinson<sup>4</sup>, Jan Pieter  
5           Abrahams<sup>3,5,6</sup>, Kai Matuschewski<sup>2</sup> & Michael J. Blackman<sup>1,7</sup>

6    1    Malaria Biochemistry Laboratory, The Francis Crick Institute, London, UK

7    2    Department of Molecular Parasitology, Institute of Biology, Humboldt University, Berlin, Germany

8    3    Laboratory of Nanoscale Biology, Division of Biology and Chemistry, Paul Scherrer Institute,  
9    Villigen, Switzerland

10   4    Electron Microscopy Science Technology Platform, The Francis Crick Institute, London, UK

11   5    Center for Cellular Imaging and NanoAnalytics, Biozentrum, University of Basel, Basel,  
12   Switzerland.

13   6    Institute of Biology, Leiden University, Leiden, The Netherlands.

14   7    Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine,  
15   London, UK

16   \*    Corresponding and lead author: J.M.M., [joachim.matz@crick.ac.uk](mailto:joachim.matz@crick.ac.uk), <https://orcid.org/0000-0002-0575-0412>  
17

## 18 **ABSTRACT**

19 During blood stage development, malaria parasites are challenged with the detoxification of  
20 enormous amounts of haem released during the proteolytic catabolism of erythrocytic  
21 haemoglobin. They tackle this problem by sequestering haem into bioinert crystals known as  
22 haemozoin. The mechanisms underlying this biomineralization process remain enigmatic.  
23 Here, we demonstrate that both rodent and human malaria parasite species secrete and  
24 internalize a lipocalin-like protein, PV5, to control haem crystallization. Transcriptional  
25 deregulation of *PV5* in the rodent parasite *Plasmodium berghei* results in inordinate elongation  
26 of haemozoin crystals, while conditional *PV5* inactivation in the human malaria agent  
27 *Plasmodium falciparum* causes excessive multi-directional crystal branching. Although  
28 haemoglobin processing remains unaffected, PV5-deficient parasites generate less  
29 haemozoin. Electron diffraction analysis indicates that despite the distinct changes in crystal  
30 morphology neither the crystalline order nor unit cell of haemozoin are affected by impaired  
31 PV5 function. Deregulation of *PV5* expression renders *P. berghei* hypersensitive to the  
32 antimalarial drugs artesunate, chloroquine, and atovaquone, resulting in accelerated parasite  
33 clearance following drug treatment *in vivo*. Together, our findings demonstrate the  
34 *Plasmodium*-tailored role of a lipocalin family member in haemozoin formation and underscore  
35 the haem biomineralization pathway as an attractive target for therapeutic exploitation.

36 **KEYWORDS** *Plasmodium*, malaria, hemozoin, haem, heme, haemozoin, vacuole, lipocalin

## 37 **SIGNIFICANCE**

38 During blood stage development, the malaria parasite replicates inside erythrocytes of the  
39 vertebrate host, where it engulfs and digests most of the available haemoglobin. This results  
40 in release of the oxygen-binding prosthetic group haem, which is highly toxic in its unbound  
41 form. The parasite crystallizes the haem into an insoluble pigment called haemozoin, a  
42 process that is vital for parasite survival and which is exploited in antimalarial therapy. We  
43 demonstrate that the parasite uses a protein called PV5 in haemozoin formation and that  
44 interfering with PV5 expression can increase the parasite's sensitivity to antimalarial drugs  
45 during blood infection. An improved understanding of the mechanisms underlying haem  
46 sequestration will provide valuable insights for future drug development efforts.

## 47 INTRODUCTION

48 The devastating pathology of malaria is caused by infection of red blood cells with unicellular  
49 *Plasmodium* parasites which reside within an intraerythrocytic parasitophorous vacuole (PV)  
50 (1). Throughout blood stage development, the parasite ingests and catabolizes up to 80% of  
51 the host cell cytoplasm, facilitating amino acid acquisition and making sufficient room for  
52 parasite growth (2, 3). Haemoglobin is incorporated through endocytosis and then degraded  
53 by an array of functionally redundant proteases, a process which occurs in acidified lysosome-  
54 like organelles with species-specific morphology (4). In the rodent-infective parasite species  
55 *Plasmodium berghei*, one or more food vacuoles (FVs) give rise to small digestive vesicles  
56 (DVs) which only fuse at the very end of intraerythrocytic development (5). By contrast, the  
57 most virulent agent of human malaria, *Plasmodium falciparum*, directs all endosomal traffic to  
58 a single large FV, where proteolysis occurs (6).

59 Here, haemoglobin digestion is accompanied by the release of high levels of the  
60 porphyrin co-factor haem from the globin chains. Haem can damage proteins and lipids  
61 through various mechanisms, including the formation of free radicals (7). The unique  
62 challenge of haem detoxification is met by the parasite's capacity to sequester the released  
63 haem into a bioinert crystalline product called haemozoin (Hz), which accumulates in the FV  
64 or DVs. Haem is initially oxidised to yield haematin, which then dimerizes through the  
65 reciprocal coordination of iron and propionate moieties. This molecular unit then assembles  
66 into Hz crystals which typically take the form of triclinic high aspect ratio parallelograms (8-  
67 10). By the end of intraerythrocytic development, all the Hz crystals are contained within a  
68 central residual body, which is eventually released upon parasite egress from the host cell  
69 and which contributes to the inflammatory responses associated with acute malaria (11). The  
70 mechanism of Hz formation is highly debated. While several studies suggest a  
71 physicochemical and autocatalytic crystallization process (12-15), there have been reports of  
72 parasite proteins (16-18) and lipids (19-21) promoting Hz assembly *in vitro*.

73 The parasite's dependency on haem detoxification has long been exploited in  
74 antimalarial therapy with outstanding success. Aminoquinolines inhibit Hz formation *via* direct  
75 physical interactions with haematin and the crystal surface, eventually leading to the build-up  
76 of cytotoxic free haem (22-25). The aminoquinoline chloroquine was the front-line medication  
77 against malaria from the 1950s onward until the emergence of wide-spread drug resistance  
78 restricted its utility (26). Nonetheless, to this day, chloroquine remains among the most  
79 effective antimalarial drugs ever developed, highlighting the outstanding importance of haem

80 sequestration for *Plasmodium* survival. It is thus crucial for future drug development efforts to  
81 gain a better understanding of the mechanisms underlying this unique biomineralization event.

82           In this report, we demonstrate that a parasite-encoded lipocalin called PV5 is a central  
83 regulator of *H*<sub>z</sub> formation.

## 84 RESULTS

### 85 Malaria parasites encode a lipocalin-like protein, PV5

86 Employing a genome-wide *in silico* down-scaling approach, we previously identified an  
87 essential *P. berghei* PV protein, *PbPV5* (PBANKA\_0826700), which has orthologues in all  
88 other *Plasmodium* species, including *P. falciparum* (PF3D7\_0925900) (27). Inspection of the  
89 PV5 amino acid sequence revealed a striking similarity to members of the functionally diverse  
90 lipocalin family, barrel-shaped proteins capable of binding various hydrophobic ligands and/or  
91 protein interaction partners (28). The signature lipocalin fold comprises a short amino-terminal  
92 helix followed by eight consecutive barrel-forming  $\beta$ -strands, another  $\alpha$ -helix and one more  $\beta$ -  
93 strand (Fig. 1A) (29). In addition, PV5 harbours two preceding amino-terminal  $\beta$ -strands  
94 specific to *Plasmodium*, as well as a signal peptide. Multiple sequence alignments with  
95 lipocalins from phylogenetically distant organisms showed the presence of a highly conserved  
96 glycine and two aromatic amino acids within the structurally conserved region 1 (SCR1) of  
97 PV5, a hallmark of the extended calycin superfamily (Fig. 1A) (29). Among several structural  
98 homologues, the bacterial outer membrane lipocalin *Blc* from *Escherichia coli* was predicted  
99 to share the highest similarity with PV5. Homology modelling guided by the known *E. coli* *Blc*  
100 structure suggests that PV5 shares the overall architecture of the lipocalin family including the  
101 characteristic  $\beta$ -barrel (Fig. 1B). Together, the sequence signatures and predicted structural  
102 features support membership of *Plasmodium* PV5 in the calycin protein superfamily.

### 103 PV5 is trafficked to the parasite's digestive compartments

104 We first investigated the spatiotemporal expression of *PbPV5* during asexual blood stage  
105 development. Live fluorescence microscopy of transgenic *P. berghei* parasites expressing  
106 mCherry-3xMyc-tagged *PbPV5* confirmed that the protein localizes to tubular PV extensions  
107 during ring and trophozoite stages and surrounds individual merozoites in segmented  
108 schizonts (Fig. 1C) (27). In addition, a substantial fraction of the protein was restricted to the  
109 parasite cytoplasm. This was particularly prominent in schizonts, where intraparasitic *PbPV5*  
110 appeared to localize to the Hz-containing residual body (Fig. 1C). In merozoites, mCherry  
111 fluorescence was concentrated in a punctate intraparasitic region, perhaps signifying storage  
112 of *PbPV5* in the dense granules, as has been demonstrated for several other important PV  
113 proteins (30-32), but this fraction was minimal as compared to the protein contained in the  
114 residual body. Quantification of the mCherry-fluorescence intensity in live parasites indicated  
115 that *PbPV5* is much more abundant in mature parasite stages than in rings and merozoites,  
116 suggesting substantial levels of *de novo* synthesis throughout parasite maturation (Fig 1C).

117 In trophozoites, the intraparasitic fraction of *PbPV5* was associated with spherical  
118 structures at the parasite periphery (Fig. 1C) and microscopic examination of mechanically  
119 expanded free parasites revealed that these were the Hz-containing DVs (Fig. 1D). To test  
120 whether this localization is conserved across different *Plasmodium* species, we generated  
121 similar transgenic *P. falciparum* parasites expressing mCherry-tagged *PfPV5*. Here, a small  
122 fraction of the fusion protein consistently localized to the PV, whereas the majority of the  
123 fluorescent signal overlapped with the Hz crystals and with the signal of the acidotropic dye  
124 LysoSensor Blue DND-167, which accumulates in the acidic FV (Fig. 1E). In addition, we  
125 frequently observed smaller *PfPV5*-positive foci in the parasite cytoplasm, most likely  
126 reflecting early endosomal compartments (Fig. 1E). Subcellular fractionation of tagged *P.*  
127 *berghei* parasites revealed that *PbPV5* is freely soluble (Fig. 1F). These findings are in good  
128 agreement with the detection of *PfPV5* in the FV proteome of *P. falciparum* (33). Together,  
129 our observations suggest that in both *Plasmodium* species PV5 is first secreted into the PV  
130 and then internalized through endocytosis of host cell cytoplasm to accumulate in the matrix  
131 of the parasite's digestive compartments.

### 132 **Transcriptional deregulation of *PbPV5* impairs asexual parasite propagation *in vivo***

133 Our previous attempts to disrupt the genomic *PbPV5* locus resulted only in atypical integration  
134 of the targeting construct without perturbing the endogenous gene, which is indicative of  
135 essential functions during the asexual blood stage cycle *in vivo* (27). As an alternative genetic  
136 strategy to analyse *PbPV5* function, we sought to deregulate *PbPV5* expression by employing  
137 a promoter swap approach (Fig. 2A). Towards this aim, we generated parasites expressing  
138 the endogenous *PbPV5* gene from the promoters of *Plasmodium* translocon of exported  
139 proteins 88 (*PTEX88*) or heat shock protein 101 (*HSP101*), respectively (Fig. S1 A and B).  
140 Quantitative real-time PCR analysis of the mutants indicated that the knock-down efficiency  
141 was ~60% in asynchronous blood stages (Fig. S1C). Impaired growth prevented quantification  
142 of knock-down levels in synchronized *ex vivo* early blood stages. Strikingly, in the schizont  
143 stage, mutants exhibited significantly elevated *PbPV5* transcript levels, corresponding to ~3.4  
144 (*pv5::5'ptex88*) and ~5.6 times (*pv5::5'hsp101*) more than in WT schizonts (Fig. S1C).  
145 Therefore, using the promoter swap strategy, we succeeded in deregulating the physiological  
146 expression of *PbPV5* throughout the asexual replication cycle.

147 To investigate whether altered *PbPV5* transcription results in reduced parasite fitness,  
148 we examined asexual propagation of the mutants *in vivo*. Growth of the promoter swap  
149 mutants was significantly impaired, with the *pv5::5'ptex88* parasites growing at 80% and  
150 *pv5::5'hsp101* parasites at only 54% of the WT growth rate (Fig. 2B). These results underline  
151 the importance of correct *PbPV5* expression during asexual replication of the parasite *in vivo*.

## 152 **PbPV5 mutants form less haemozoin**

153 Inspection of Giemsa-stained thin blood films revealed striking morphological differences  
154 between WT parasites and the promoter swap mutants. During the trophozoite stage, the FV  
155 of the mutants was significantly swollen, visible as a large translucent area within the parasite  
156 cytoplasm close to the nucleus (Fig. 2C). Microscopic quantification revealed this area to be  
157 1.5-fold (*pv5::5'ptex88*) or 1.8-fold (*pv5::5'hsp101*) larger than in WT parasites (Fig. S2A).  
158 Vacuolar swelling was transient, as mature schizonts did not exhibit comparable abnormalities  
159 (Fig. 2C).

160 Another striking phenomenon was the low visibility of dark granular material in mutant  
161 trophozoites, when compared to WT (Fig. 2C). This lack of granularity was most noticeable in  
162 the *pv5::5'hsp101* mutant and became particularly apparent during the gametocyte stage,  
163 where pigment granules are usually very prominent. To validate this finding, we subjected  
164 mixed blood stage parasites to flow cytometry and measured the intensity of the side scattered  
165 light, a commonly used proxy for cellular granularity (Fig. 2D). In agreement with our  
166 microscopic analysis, the PbPV5 mutants displayed reduced side scattering and the  
167 phenotype was again more severe in the *pv5::5'hsp101* mutant.

168 Because we suspected a Hz formation defect in the mutants, we fixed intraerythrocytic  
169 parasites with methanol and subjected them to polarization microscopy, which exploits the  
170 birefringent properties of Hz to specifically visualize the crystals. Using this method, we  
171 detected a weaker signal for the mutants, which correlated with reduced visibility of dark  
172 pigment in brightfield (Fig. 2E). Enumeration of individual bright entities suggested the  
173 presence of fewer Hz-containing structures within the mutants (~80% of WT) (Fig. 2E).  
174 Quantification of the polarized light intensity also indicated that *pv5::5'ptex88* parasites form  
175 only 64% and the *pv5::5'hsp101* 61% of the Hz generated in WT parasites (Fig. 2F). Together,  
176 these observations show that perturbation of PbPV5 expression results in reduced haem  
177 biomineralization *in vivo*.

## 178 **Protracted haemozoin extension upon deregulation of PbPV5 expression**

179 We next aimed to examine how lower levels of Hz correlate with crystal size. To do this, we  
180 isolated Hz from mixed blood stage parasites and examined the material by scanning electron  
181 microscopy (SEM). This confirmed the characteristic high aspect ratio parallelogram  
182 morphology of WT Hz (Fig. 3 A and B). Strikingly, this was not the case for crystals isolated  
183 from the promoter swap mutants. Hz from both transgenic parasite strains exhibited highly  
184 irregular shapes and rough edges and showed only few of the distinctive Hz crystal vertices  
185 (Fig. 3B). Crystals from *pv5::5'ptex88* parasites most often had a pointed and canine tooth-



186 like appearance, extending from a single straight crystal face. These abnormalities were even  
187 more pronounced in the *pv5::5'hsp101* mutant, where in most cases there was a region of  
188 normal crystal morphology with 2 or 3 straight edges, corresponding to the {010}, {011} and  
189 {001} faces. From this regular parent crystal emerged an enormous outgrowth which usually  
190 surpassed the dimensions of the parent crystal (Fig. 3B). This outgrowth consistently grew at  
191 an obtuse angle of  $\sim 105^\circ$  in relation to the dominant *c* axis of the parent crystal, although  
192 accurate determination of the angle was complicated by the slightly bent and irregular shape  
193 of the outgrowth, which might be attributed to the space restrictions encountered in the *P.*  
194 *berghei* DVs. The outgrowth's angle was not reflected in the physiological morphology of Hz  
195 (Fig. 3 A and B) and at least two faces of the regular parent crystal appeared to be involved.  
196 Indeed, in most cases, the outgrowth emerged from sites where the {010} and {011} faces  
197 meet and always grew along a plane corresponding to one of the original crystal faces (Fig.  
198 3B). Other crystal formations were also observed, albeit at lower frequency, including some  
199 with multiple crystal branches and some with very rough surfaces (Fig. S2B). We observed  
200 similar crystal abnormalities *in situ* by transmission electron microscopy (TEM) of purified  
201 schizonts (Fig. 3C).

202         Despite the lower overall levels of Hz formed and the abnormal crystal morphology,  
203 we found that the mutants formed larger Hz crystals as indicated by the area exposed to the  
204 SEM electron beam (Fig. 3D). The *pv5::5'hsp101* mutant formed the largest crystals, which  
205 were  $\sim 180\%$  of WT size, while the *pv5::5'ptex88* crystals were at 130%. Importantly, the  
206 mutant Hz crystals displayed greater dimensions only in length but not in width owing to the  
207 unidirectional expansion of the outgrowth (Fig. 3D). Examination of the parent crystals from  
208 *pv5::5'hsp101* parasites showed that these were roughly half the size of whole WT crystals  
209 (Fig. 3D). The aspect ratios of WT crystals and the *pv5::5'hsp101* parent crystals were  
210 identical, together indicating that a period of normal crystallisation during earlier *pv5::5'hsp101*  
211 parasite development is followed by irregular crystal extension later on (Fig. 3E). Inspection  
212 of Hz from an unrelated slow-growing mutant (35) and from chloroquine-treated WT parasites  
213 ruled out a secondary effect of reduced parasite fitness or mortality on Hz morphology (Fig.  
214 S2 C and D).

215         To determine whether the crystalline order was affected by deregulation of PbPV5, we  
216 obtained electron diffraction patterns from WT- and *pv5::5'hsp101*-derived Hz (Fig. 3F) and  
217 analysed the maximum diffracted intensities in concentric bins as a function of resolution.  
218 There was no difference in the drop-off of diffracted intensity between WT and mutant and the  
219 peak positions of the maxima corresponded (Fig. 3G). Differences in the magnitude of  
220 individual peaks can be attributed to preferential orientation, especially of the *pv5::5'hsp101*-  
221 derived crystals, which most often come to lie at their {100} faces. Together, these data



222 indicate no differences in crystalline order or unit cell upon functional impairment of PbPV5  
223 and we conclude that altered crystal morphology is not caused by alternative nucleation into  
224 a different haematin polymorph. Thus, deregulation of PbPV5 leads to the formation of ordered  
225 elongated Hz crystals with a highly variable and abnormal habit.

## 226 **Loss of PV5 causes haemozoin branching in *P. falciparum***

227 To investigate the consequences of PV5 disruption, we generated a conditional PV5 knockout  
228 line of the human pathogen *P. falciparum*, allowing rapamycin-induced DiCre-mediated  
229 excision of the PfPV5 gene (Fig. 4A and Fig. S3A). Correct modification of the locus was  
230 indicated by diagnostic PCR and by the successful tagging of PfPV5 with a 3xHA tag, as  
231 demonstrated by Western blot and immunofluorescence analysis (Fig. 4 B and C, Fig. S3 B-  
232 D). 3xHA-tagged PfPV5 localized to the PV and to intraparasitic vesicular structures (Fig. S3C  
233 and D). Surprisingly, no signal was observed within the central FV, in marked contrast to the  
234 mCherry fusion protein (Fig. 1E) and despite previous evidence from FV proteomics (33). Most  
235 likely, the 3xHA-tag is obscured or processed in the FV.

236 Treatment of *pv5-3xHA:loxP* parasites with rapamycin led to efficient gene excision  
237 and complete loss of PfPV5 protein expression during the same intraerythrocytic cycle (Fig. 4  
238 B and C, Fig. S3D). This did not detectably affect parasite maturation but did result in a modest  
239 merozoite invasion defect upon rupture of the PfPV5-null schizonts, reducing parasite  
240 replication (Fig. S4). Extended monitoring of the rapamycin-treated parasites indicated an  
241 estimated fitness cost of ~40% (Fig. S4B). This is in good agreement with a proposed  
242 mutagenesis index score of 0.22 from a genome-wide piggy-Bac insertion mutagenesis  
243 screen (36). Accordingly, PfPV5, although not essential under standard *P. falciparum* culture  
244 conditions, is required for optimal parasite propagation *in vitro*.

245 To examine the effects of PfPV5 ablation on haem biomineralization, Hz was  
246 visualized and quantified by polarization microscopy. Rapamycin-treated *pv5-3xHA:loxP*  
247 parasites formed only 57% of the Hz observed in WT and DMSO-treated controls and  
248 individual crystals appeared to be globular rather than elongated (Fig. 4 D and E). In the  
249 absence of PfPV5, Hz released at parasite egress no longer formed clusters of separate  
250 slender crystals but rather appeared as aggregates which only occasionally fell apart into  
251 individual globular units (Fig. 4F). In good agreement, microscopic inspection of live parasites  
252 revealed that the characteristic twirling motion of Hz within the central FV was largely lost upon  
253 PfPV5 knockout (Fig. 4G and Movie S1).

254 The dramatic abnormalities in Hz crystal morphology resulting from ablation of PfPV5  
255 were even more evident by SEM analysis. While WT parasites formed crystals of the expected

256 brick-like morphology, individual Hz units from rapamycin-treated *pv5-3xHA:loxP* parasites  
257 appeared smaller and more globular (Fig. 4H and Table S1). The surfaces of these Hz units  
258 were covered in scales and stubby crystal buds. Individual crystals of comparable bud-like  
259 dimensions were not observed, indicating a branching rather than an aggregation  
260 phenomenon. In some instances, a lower number of crystal buds allowed the visualization of  
261 an ordered Hz core, suggesting that branching is initiated from a regular parent crystal (Fig.  
262 4H). We detected several morphological intermediates between slightly scaled Hz, highly  
263 branched crystal units and fused congregations (Fig. 4H). We frequently observed enormous  
264 aggregates of spherical proportions, mirroring the shape of the central FV (Fig. 4H). This  
265 suggested that hyperactive crystal branching in the absence of *PfPV5* caused individual  
266 studded Hz units to stick together and subsequently merge during Hz growth, which might  
267 explain the absence of motion in the parasite FV (Fig. 4H). Non-rapamycin-treated *pv5-*  
268 *3xHA:loxP* control parasites mainly formed regular Hz crystals, however 27.5% of the crystals  
269 exhibited a modest degree of branching (Fig. 4H and Table S1). Furthermore, crystal size and  
270 aspect ratio were reduced in comparison to WT parasites, suggesting a moderate functional  
271 impairment of 3xHA-tagged *PfPV5* (Fig. 4H and Table S1). Our data demonstrate that *PV5* is  
272 critical for the efficient sequestration of haem and for the ordered expansion of Hz crystals in  
273 *P. falciparum*.

#### 274 **Efficient haemoglobin processing in the absence of *PfPV5***

275 To exclude an indirect effect mediated by defective haemoglobin catabolism, we  
276 examined the haemoglobin content of saponin-released parasites. *PfPV5*-deficient ring stages  
277 and trophozoites contained normal quantities of internalized haemoglobin (Fig. 5A). Only  
278 mature segmented *pv5-3xHA:loxP* schizonts exhibited slightly elevated concentrations of  
279 residual haemoglobin upon induction (Fig. 5B). However, this was also observed in WT  
280 schizonts upon rapamycin treatment, suggesting a minor compound-specific effect.  
281 Furthermore, saponin treatment released normal amounts of haemoglobin from schizont-  
282 infected erythrocytes in the absence of *PfPV5*, indicating unaltered haemoglobin ingestion  
283 (Fig. 5B). As a positive control for perturbation of haemoglobin catabolism, we cultured WT  
284 parasites in the presence of the protease inhibitor E64. Vacuolar bloating, as readily detected  
285 by E64 treatment, was not visible in rapamycin-treated *pv5-3xHA:loxP* parasites (Fig. 5C). We  
286 also note that, unlike *PfPV5* deletion, E64 treatment produced no changes in the architecture  
287 of Hz (Fig. 5D). In addition, *PfPV5*-deficient parasites retained normal E64 sensitivity (Fig. 5  
288 E and F). Combined, these findings suggest that inhibition of haemoglobin catabolism does  
289 not directly translate into altered Hz morphology and that *PfPV5* is not involved in the overall  
290 consumption of host cell cytoplasm.

291 **PbPV5 expression influences antimalarial drug sensitivity *in vivo***

292 In the light of our evidence implicating PV5 in Hz formation, we next tested whether parasites  
293 with affected PV5 function display altered sensitivity towards chloroquine, a 4-aminoquinoline  
294 which is thought to inhibit haem biomineralization in *Plasmodium* (22-25). The absence of  
295 *PfPV5* did not detectably alter chloroquine sensitivity in cultured *P. falciparum* parasites (Fig.  
296 6A). In stark contrast to this, however, the *P. berghei* promoter swap mutants responded to  
297 drug treatment slightly earlier and disappeared from the circulation much more rapidly than  
298 WT parasites (Fig. 6B). A similar phenotype was observed upon treatment of infected mice  
299 with the artemisinin derivative artesunate, also previously been implicated in haem  
300 sequestration, although this remains contentious (37-41) (Fig. 6C). Most surprisingly, we also  
301 observed marked hypersensitivity of the *P. berghei* mutants towards atovaquone, a compound  
302 that targets the parasite's mitochondrial electron transport chain (Fig. 6D), as well as a slight  
303 but non-significant increase in sensitivity towards the antifolate sulfadoxin (Fig. 6E). The  
304 relative survival levels of *pv5::5'ptex88* and *pv5::5'hsp101* on the fourth day of drug treatment  
305 suggested the greatest degree of hypersensitivity towards chloroquine (2.1% and 1.4% of WT  
306 survival, respectively), followed by atovaquone (3.5% and 8.6%) and artesunate (4.4% and  
307 9.9%), and eventually sulfadoxin (20.5% and 27.8%). An unrelated slow-growing *P. berghei*  
308 mutant deficient in the maintenance of the mitochondrial membrane potential displayed normal  
309 sensitivity towards atovaquone using the same internally controlled assay (42), indicating that  
310 reduced parasite multiplication is unlikely to cause drug hypersensitivity. Collectively, these  
311 results suggest that interference with *PV5* expression critically enhances vulnerability of the  
312 parasite towards drug-mediated insult during *in vivo* blood infection.

## 313 DISCUSSION

314 In this work, we have demonstrated that Hz formation in malaria parasites involves a secreted  
315 calycin family member called PV5. While transcriptional deregulation of *PV5* in *P. berghei*  
316 resulted in protracted Hz elongation along a pre-existing crystal plane, multidirectional  
317 branching was observed in the complete absence of PV5 in *P. falciparum*. Species differences  
318 aside, the disparity between those two phenotypes can be explained by the unique  
319 transcriptional dynamics observed in the *P. berghei* mutants. Here, the hyperactive crystal  
320 elongation, that follows an initial phase of normal Hz growth, coincides with a substantial  
321 increase in PbPV5 transcript abundance during late parasite development. In their  
322 physiological context, PTEX88 and HSP101 are co-expressed as members of the same  
323 protein complex with HSP101 being more abundant than PTEX88 (34, 43), as supported by  
324 our qPCR analysis. Thus, the increased phenotypic severity in the *pv5::5'hsp101* mutant over  
325 the *pv5::5'ptex88* mutant indicates that the deficiencies in Hz formation can be attributed to  
326 late overexpression of PV5. Together with the chaotic Hz crystal branching observed in PV5-  
327 deficient *P. falciparum* parasites, this leads us to propose that PV5 acts as a facilitator of  
328 unidirectional Hz extension.

329 It is interesting to speculate on the mechanisms by which PV5 may partake in haem  
330 biomineralization. In laboratory crystallization experiments, the extent of crystallographic  
331 mismatch branching is highly dependent on the degree of solute supersaturation, with high  
332 levels favouring the generation of novel crystal nuclei on the surface of the parent crystal (44).  
333 By contrast, lower degrees of supersaturation usually promote the expansion of pre-existing  
334 crystals (44). It is conceivable that PV5 might reduce the extent of haem supersaturation by  
335 binding haem or haematin dimers, thereby moderating *de novo* Hz nucleation and promoting  
336 unidirectional crystal elongation. This could explain the Hz branching upon loss of PV5 in *P.*  
337 *falciparum* as well as the prolonged crystal elongation and reduced Hz production upon late  
338 upregulation of *PV5* in the *P. berghei* mutants. In support of this model, some lipocalin family  
339 members are known to specifically bind the haem degradation product biliverdin (45-47). Such  
340 interactions are unlikely to occur within the predicted PV5  $\beta$ -barrel due to spatial constraints.  
341 However, although experimental validation for this is currently lacking, potential ligand binding  
342 sites might be located at the predicted prominently exposed loop between the barrel's  $\beta$ -  
343 strands 5 and 6 (Fig. 1B) or at the extended amino-terminus.

344 The crystal branching characterising PV5-deficient *P. falciparum* parasites could also  
345 be elicited by non-haem impurities adsorbing onto the crystal surface where they would  
346 generate novel nucleation sites (44). In this alternative functional model, PV5 could act to bind

347 these impurities to create a vacuolar environment permissive for proper biomineralization.  
348 *PfPV5*-deficient parasites maintain an intact FV with a trans-vacuolar proton gradient as  
349 indicated by staining with Lysosensor Blue DND-167 (Fig. S5A). In the absence of indications  
350 for FV membrane damage, differences in leakage of impurities from the parasite cytoplasm  
351 can be dismissed. However, we cannot rule out the possibility that specific ions or organic  
352 compounds are more abundant in the vacuolar matrix of *PV5*-deficient parasites.

353 While the exact biophysical mechanisms that govern *PV5*-mediated Hz  
354 morphogenesis remain to be delineated, our results support a model of conventional  
355 crystallization within the aqueous milieu of the parasite's digestive compartments. The distinct  
356 changes in crystal morphology and branching behaviour in the *PV5* mutants concur with  
357 established mineralogical phenomena and are difficult to reconcile with a lipid or protein-  
358 mediated polymerization scenario.

359 The striking dual localization pattern of *PV5* is suggestive of initial secretion into the  
360 PV followed by endocytic uptake. Since we ruled out an involvement of *PV5* in haemoglobin  
361 ingestion and catabolism, the transient vacuolar swelling in the *P. berghei* mutants likely  
362 reflects a secondary effect mediated by the grave deficiencies in haem sequestration.

363 Our previous experiments had indicated that *PbPV5* is essential for the asexual blood  
364 stage development of *P. berghei* (27), and the deregulation of *PbPV5* transcription in the  
365 mutants described here indeed led to a striking fitness cost during *in vivo* infection. By contrast,  
366 complete ablation of *PfPV5* in *P. falciparum* only resulted in a moderate fitness loss *in vitro*.  
367 The apparent dispensability of *PfPV5* is therefore puzzling, but can be resolved by the notion  
368 that *in vitro* culture does not necessarily reflect all adversities encountered during host  
369 infection. For instance, Hz-mediated stiffening of the residual body in the absence of *PV5*  
370 might hinder the passage of infected cells through capillaries or through the inter-endothelial  
371 slits of the spleen, a scenario that would result in parasite elimination only during *in vivo*  
372 infection. Similarly, we observed enhanced drug susceptibility only in the *P. berghei* mutants.  
373 Thus, it appears plausible, that any imbalance in haem biomineralization in the absence of  
374 *PV5* is compensated for under optimal culture conditions and unfolds its adverse effects only  
375 during *in vivo* infection. We thus propose that the molecular mechanism by which *PV5*  
376 regulates Hz formation might involve host factors that are not encountered in a cell culture  
377 setting.

378 The observation of enhanced drug susceptibility in the *PbPV5* mutants is in agreement  
379 with the notion that aberrant haem biomineralization chemo-sensitizes parasites to partner  
380 drugs. Although oxidative stress and lipid peroxidation remained unchanged upon deletion of

381 PfPV5 (Fig. S5B), the reduced Hz formation efficiency in the PV5 mutants suggests elevated  
382 haem concentrations within the parasite mediating the fitness loss and drug hypersensitivity.

383 Together, we provide the first conclusive evidence for a parasite factor mediating Hz  
384 formation *in vivo*, called PV5. Since this *Plasmodium*-encoded member of the calycin  
385 superfamily also governs the parasites' vitality and susceptibility towards drug-mediated insult  
386 during blood infection, our observations reinforce Hz formation as an excellent pathway for  
387 therapeutic intervention. The investigation of the malaria parasite's haem detoxification  
388 machinery *in vivo*, as exemplified herein for PV5, will significantly improve our understanding  
389 of this unique haem biomineralization process and holds great promise for the development  
390 of novel malaria intervention strategies.



## 391 MATERIALS & METHODS

392 **Structure homology modelling.** Structure homology modelling was performed using the  
393 SWISS-MODEL server (48). *PfPV5* (residues 35-214) was aligned to the experimentally  
394 validated structure of *EcBlc* (residues 27-175, PDB ID: 3MBT) (49), resulting in a GMQE value  
395 of 0.39 and a QMEAN value of -5.48. Modelling was confirmed with I-TASSER (50), which  
396 also identified *EcBlc* (PDB ID: 2ACO) (51) as the most closely related structural analogue of  
397 *PfPV5* with a TM score of 0.75. Due to a lack of sequence similarity, the structure of the  
398 extended *PfPV5* amino-terminus was not modelled.

399 ***P. berghei* cultivation.** *P. berghei* parasites were propagated in SWISS mice under constant  
400 drug pressure with pyrimethamine (70 mg/l in drinking water, ingested *ad libitum*, MP  
401 Biomedicals) to avoid homology-induced reversion of the promoter swap mutants to the  
402 original WT genotype. This was routinely checked by diagnostic PCR of genomic DNA as  
403 shown in Figures S2 A and B. Drug pressure was withdrawn 5 days prior to experimentation  
404 to avoid secondary effects of pyrimethamine treatment. Pyrimethamine-resistant Berred WT  
405 parasites (52) were treated accordingly. All infection experiments were carried out in strict  
406 accordance with the German 'Tierschutzgesetz in der Fassung vom 22. Juli 2009' and the  
407 Directive 2010/63/EU of the European Parliament and Council 'On the protection of animals  
408 used for scientific purposes'. The protocol was approved by the ethics committee of the Berlin  
409 state authority ('Landesamt für Gesundheit und Soziales Berlin', permit number G0294/15).

410 *P. berghei* growth was determined with the previously described intravital competition  
411 assay (52). In short, 500 mCherry-fluorescent Berred WT and 500 GFP-fluorescent mutant  
412 blood stage parasites were co-injected intravenously and parasitaemia was analysed daily by  
413 flow cytometry. For drug sensitivity assays,  $5 \times 10^6$  WT and  $5 \times 10^6$  mutant parasites were co-  
414 injected intravenously. Drug treatment as well as daily flow cytometric parasite detection were  
415 commenced three days later (43). Mice were treated with curative doses of chloroquine (288  
416 mg/l in drinking water, ingested *ad libitum*, Sigma Aldrich), atovaquone (1.44 mg/kg body  
417 weight per day injected intraperitoneally; GlaxoSmithKline), artesunate (50 mg/kg body weight  
418 per day injected intraperitoneally; Sigma Aldrich) or sulfadoxin (1.4 g/l in drinking water,  
419 ingested *ad libitum*; Sigma Aldrich). Mixed blood stages and schizonts were purified by  
420 nycodenz gradient centrifugation (53).

421 ***P. falciparum* cultivation.** *P. falciparum* parasites were propagated in type AB+ human red  
422 blood cells at 90% N<sub>2</sub>, 5% CO<sub>2</sub> and 5% O<sub>2</sub> at 37°C in RPMI 1640 containing AlbuMAXII  
423 (Thermo Fisher Scientific) supplemented with 2 mM L-glutamine. Parasites were routinely



424 synchronized using a combination of percoll gradient centrifugation and sorbitol lysis and were  
425 treated with 100 nM rapamycin, various concentrations of E64 or equivalent volumes of  
426 dimethyl sulfoxide (DMSO) from the early ring stage onward. Growth assays were performed  
427 as described previously (54) and parasitaemia as well as DNA content were measured by flow  
428 cytometry using the nuclear dye SYBR Green (1:10,000; Thermo Fisher Scientific). For  
429 invasion assays, mature schizonts were incubated at 2% initial parasitaemia under static or  
430 shaking (120 rpm) conditions, as confirmed by flow cytometry. Parasitaemia was measured  
431 again 24 hours after inoculation and the fold-change was calculated. For drug- and inhibitor-  
432 response analyses, *pv5-3xHA:loxP* ring stage cultures at 0.5 – 2% parasitaemia were treated  
433 with varying concentrations of chloroquine or E64 in the presence of DMSO or rapamycin in  
434 a 96 well format. Parasitaemia was determined three days later and the fold-change was  
435 calculated. E64 was washed out after 36 hours to allow for reinvasion. In addition, nuclear  
436 SYBR Green fluorescence was determined by flow cytometry following 44 hours of continuous  
437 E64 treatment.

438 **Generation and validation of transgenic *Plasmodium* parasites.** To generate the PbPV5  
439 promoter swap mutants, the amino-terminal sequence of PbPV5 (PBANKA\_0826700) was  
440 PCR-amplified and cloned into the B3D+ vector using BamHI and SacII. The promoters of  
441 PbPTEX88 (PBANKA\_0941300) or PbHSP101 (PBANKA\_0931200) were then amplified and  
442 cloned in front of the start codon using BamHI (Figure S1A). Vectors were linearized with BstBI  
443 and transfected into GFP-fluorescent *P. berghei* Bergreen WT parasites, using standard  
444 protocols (53, 55, 56). Transgenic parasites were selected for with pyrimethamine and isolated  
445 by limiting dilution cloning. PbPV5 transcript abundance in the WT and the promoter swap  
446 mutants was measured by quantitative real-time PCR (qPCR) and normalized to *Pb18S* rRNA.

447 The conditional PfPV5 knockout line was generated using established Cas9-mediated  
448 techniques. In short, *P. falciparum* 3D7 parasites constitutively expressing DiCre (B11 line)  
449 were co-transfected with a pDC2 guide plasmid inducing Cas9-mediated double strand  
450 cleavage of the PfPV5 locus (PF3D7\_0925900), together with a linearized repair template (57-  
451 59) (Figure S3A). The template was generated by gene synthesis and contained 5' and 3'  
452 homology arms and a re-codonised 3xHA-tagged PfPV5 sequence. The endogenous intron  
453 of PfPV5 was replaced with an artificial intron containing a *loxP* site (58). A second *loxP*  
454 sequence was introduced behind the PfPV5 stop codon. For live imaging of PfPV5, mCherry  
455 was cloned into the conditional knockout vector in frame with the re-codonised PfPV5  
456 sequence using AatII. Transgenic parasites were selected for with WR99210 and cloned by  
457 limiting dilution, making use of a previously developed plaque assay (60). Primers used for

458 molecular cloning, diagnostic PCR, and qPCR are indicated in Figures 4A, S1A and S3A as  
459 well as in Table S2.

460 **Fluorescence microscopy.** The transgenic *P. berghei* parasite line *pv5-tag-GFP<sup>PV</sup>* (27) was  
461 imaged live using an AxioImager Z2 epifluorescence microscope equipped with an AxioCam  
462 MR3 camera (Zeiss). For mechanical parasite expansion, 1-2  $\mu$ l of infected blood were  
463 incubated under a 22 x 40 mm coverslip for several minutes until erythrocyte lysis became  
464 apparent. *P. falciparum* parasites were imaged on an Eclipse Ni light microscope (Nikon) fitted  
465 with a C11440 digital camera (Hamamatsu). Immunofluorescence analysis was performed  
466 with *P. falciparum* *pv5-3xHA:loxP* parasites that were fixed in 4% formaldehyde using rat anti-  
467 HA (1:500; Sigma Aldrich) and rabbit anti-SERA5 (1:500) (54) primary antibodies in  
468 combination with appropriate fluorophore-coupled secondary antibodies (1:1,000; Thermo  
469 Fisher Scientific). Stainings with Lysosensor Blue DND-167 (Thermo Fisher Scientific),  
470 BODIPY 581/591 C11 (Image-iT Lipid Peroxidation Kit, Thermo Fisher Scientific) and  
471 CellROX Green (Thermo Fisher Scientific) were performed according to the manufacturer's  
472 instructions.

473 **Scanning electron microscopy.** Hz was isolated from nycodenz (*P. berghei*) or percoll (*P.*  
474 *falciparum*) gradient-purified infected red blood cells. Cells were lysed in water at room  
475 temperature for 20 minutes, followed by a 10-minute centrifugation step at 17,000 x *g*. The  
476 pellet was resuspended in 2% SDS in water and centrifuged as above. Three more washing  
477 steps with 2% SDS were then followed by three washing steps with distilled water, before the  
478 crystals were resuspended and transferred onto round glass cover slips (12 mm), where they  
479 were dried. Cover slips were mounted on SEM specimen stubs, sputter-coated, and then  
480 imaged on a LEO 1430 (Zeiss) or on a Quanta FEG 250 scanning electron microscope  
481 (Thermo Fisher Scientific).

482 **Transmission electron microscopy.** Infected erythrocytes were initially fixed in 2.5%  
483 glutaraldehyde, embedded in beads of 2% agarose, fixed and contrasted with 1% osmium  
484 tetroxide, and further contrasted *en bloc* using 0.5% uranyl acetate. Following dehydration in  
485 a graded series of ethanol and propylene oxide, beads were embedded in epoxy resin and  
486 cured at 60°C for at least 24 hours. 60 nm sections were made with a Reichert Ultracut S  
487 ultramicrotome (Leica) using a diamond knife. Sections were retrieved on copper hexagonal  
488 mesh grids, and stained with 2% uranyl acetate and Reynold's lead citrate before imaging on  
489 an EM 900 transmission electron microscope (Zeiss) equipped with a wide-angle slow-scan  
490 2K CCD camera (Tröndle Restlichtverstärkersysteme).

491 **Electron diffraction.** Purified Hz was added to glow-discharged Lacey carbon films on 400  
492 mesh copper grids which were then transferred to a Vitrobot Mark IV plunge freezer (Thermo  
493 Fisher Scientific) with 100% humidity at 7°C. The grids were blotted for 3 seconds with blot  
494 force 1 and plunge frozen in liquid ethane cooled by liquid nitrogen. Electron diffraction data  
495 were collected on a Talos cryo-electron microscope (Thermo Fisher Scientific) operated at  
496 200 keV equipped with a hybrid pixel Timepix detector (512 x 512 pixels, 55 x 55 µm pixel  
497 size; Amsterdam Scientific Instruments). Still and rotation (70°) datasets were collected with  
498 a beam size of 2 µm. The recording time varied between 15 and 100 seconds. To determine  
499 the resolution, a powder pattern of an aluminum diffraction standard was recorded. Since the  
500 Hz crystals had a strong tendency to stick together, we measured diffraction data of crystal  
501 clusters. For each individual data set, we determined the location of the central electron beam  
502 and shifted the patterns to make the beams coincide. Since crystal clustering prevented  
503 indexing of the diffraction data, the radial median and maximum intensities were determined  
504 as a function of resolution. Hereafter, the WT and *pv5::5'hsp101* datasets were averaged and  
505 normalized to the local background median intensity at 0.18 Å<sup>-1</sup>.

506 **Subcellular fractionation and immunoblotting.** *P. falciparum* parasites were released from  
507 erythrocytes by treatment with 0.15% saponin in PBS. Murine erythrocytes infected with  
508 the *pv5-tag-GFP<sup>PV</sup>* or *exp2-mCherry P. berghei* lines (64) were purified on a nycodenz  
509 gradient and lysed hypotonically for 1 hour on ice in 10 mM TRIS-HCl, pH 7.5. *P. berghei*  
510 lysates were spun 50 minutes at 100,000 × *g*. Membrane pellets were resuspended in 0.1 M  
511 Na<sub>2</sub>CO<sub>3</sub> in PBS or in 1% Triton X-100 in PBS, respectively, and spun 50 minutes at  
512 100,000 × *g*. Proteins were separated on SDS-polyacrylamide and transferred onto a  
513 nitrocellulose membrane. Western blotting was performed using rat anti-mCherry (1:5,000;  
514 ChromoTek), chicken anti-GFP (1:5,000; Abcam), rat anti-HA (1:1,000, Sigma Aldrich), rat  
515 anti-*Pf*BiP (1:1,000) (62) and rabbit anti-human-haemoglobin-α primary antibodies (1:1,000,  
516 Abcam) followed by chemiluminescence detection with horseradish peroxidase-coupled  
517 secondary antibodies (1:10,000; Sigma Aldrich, or 1:5,000, Jackson ImmunoResearch).

518 **Quantitative haemozoin analysis.** Hz was visualized and quantified by microscopy of  
519 methanol-fixed infected red blood cells. Hz from *P. berghei* was analysed by reflection contrast  
520 polarized light microscopy using a Leica DMR widefield microscope equipped with a ProgRes  
521 MF camera (Jenoptik) and the POL filter set 513813 (Leica). Hz from *P. falciparum* was  
522 analysed by transmitted polarized light (488 nm) microscopy using an LSM 710 confocal  
523 microscope (Zeiss) equipped with a crossed polarizer in the condenser. Cellular granularity  
524 was approximated by quantification of side scattered light using an LSRFortessa flow  
525 cytometer (BD Biosciences). Hz crystal dimensions were analysed using FIJI. Due to the

526 variation in the *P. berghei* mutant's crystal width, individual crystals were divided into nine  
527 evenly spaced segments along the dominant axis. The width of each segment was determined  
528 and the values were averaged. For the *pv5::5'hsp101* parent crystal and the crystal outgrowth,  
529 transects were drawn through the central axis of either structure and their shared angle was  
530 determined. Hz movement within the FV of *pv5-3xHA:loxP* parasites was imaged live 36 hours  
531 following treatment with DMSO or rapamycin, respectively.

532 REFERENCES

- 533 1. A. G. Maier, K. Matuschewski, M. Zhang, M. Rug, *Plasmodium falciparum*. *Trends*  
534 *Parasitol* **35**, 481-482 (2019).
- 535 2. P. A. Sigala, D. E. Goldberg, The peculiarities and paradoxes of *Plasmodium* heme  
536 metabolism. *Annu Rev Microbiol* **68**, 259-278 (2014).
- 537 3. V. L. Lew, T. Tiffert, H. Ginsburg, Excess hemoglobin digestion and the osmotic  
538 stability of *Plasmodium falciparum*-infected red blood cells. *Blood* **101**, 4189-4194  
539 (2003).
- 540 4. D. E. Goldberg, A. F. Slater, A. Cerami, G. B. Henderson, Hemoglobin degradation in  
541 the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle.  
542 *Proc Natl Acad Sci U S A* **87**, 2931-2935 (1990).
- 543 5. C. Slomianny, G. Prensier, P. Charet, [Comparative ultrastructural study of the process  
544 of hemoglobin degradation by *P. berghei* (Vincke and Lips, 1948) as a function of the  
545 state of maturity of the host cell]. *J Protozool* **32**, 1-5 (1985).
- 546 6. A. R. Dluzewski *et al.*, Formation of the food vacuole in *Plasmodium falciparum*: a  
547 potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1(19)). *PLoS*  
548 *One* **3**, e3085 (2008).
- 549 7. S. Kumar, U. Bandyopadhyay, Free heme toxicity and its detoxification systems in  
550 human. *Toxicol Lett* **157**, 175-188 (2005).
- 551 8. S. Pagola, P. W. Stephens, D. S. Bohle, A. D. Kosar, S. K. Madsen, The structure of  
552 malaria pigment beta-haematin. *Nature* **404**, 307-310 (2000).
- 553 9. A. F. Slater *et al.*, An iron-carboxylate bond links the heme units of malaria pigment.  
554 *Proc Natl Acad Sci U S A* **88**, 325-329 (1991).
- 555 10. I. Weissbuch, L. Leiserowitz, Interplay between malaria, crystalline hemozoin  
556 formation, and antimalarial drug action and design. *Chem Rev* **108**, 4899-4914 (2008).
- 557 11. M. Olivier, K. Van Den Ham, M. T. Shio, F. A. Kassa, S. Fougeray, Malarial pigment  
558 hemozoin and the innate inflammatory response. *Front Immunol* **5**, 25 (2014).
- 559 12. T. J. Egan, W. W. Mavuso, K. K. Ncokazi, The mechanism of beta-hematin formation  
560 in acetate solution. Parallels between hemozoin formation and biomineralization  
561 processes. *Biochemistry* **40**, 204-213 (2001).
- 562 13. T. J. Egan, D. C. Ross, P. A. Adams, Quinoline anti-malarial drugs inhibit spontaneous  
563 formation of beta-haematin (malaria pigment). *FEBS Lett* **352**, 54-57 (1994).
- 564 14. M. M. Chen, L. Shi, D. J. Sullivan, Jr., *Haemoproteus* and *Schistosoma* synthesize  
565 heme polymers similar to *Plasmodium* hemozoin and beta-hematin. *Mol Biochem*  
566 *Parasitol* **113**, 1-8 (2001).
- 567 15. A. Dom, R. Stoffel, H. Matile, A. Bubendorf, R. G. Ridley, Malarial haemozoin/beta-  
568 haematin supports haem polymerization in the absence of protein. *Nature* **374**, 269-  
569 271 (1995).
- 570 16. D. Jani *et al.*, HDP – a novel heme detoxification protein from the malaria parasite.  
571 *PLoS Pathog* **4**, e1000053 (2008).
- 572 17. D. J. Sullivan, Jr., I. Y. Gluzman, D. E. Goldberg, *Plasmodium* hemozoin formation  
573 mediated by histidine-rich proteins. *Science* **271**, 219-222 (1996).
- 574 18. A. F. Slater, A. Cerami, Inhibition by chloroquine of a novel haem polymerase enzyme  
575 activity in malaria trophozoites. *Nature* **355**, 167-169 (1992).

- 576 19. A. N. Hoang, R. D. Sandlin, A. Omar, T. J. Egan, D. W. Wright, The neutral lipid  
577 composition present in the digestive vacuole of *Plasmodium falciparum* concentrates  
578 heme and mediates beta-hematin formation with an unusually low activation energy.  
579 *Biochemistry* **49**, 10107-10116 (2010).
- 580 20. M. A. Ambele, T. J. Egan, Neutral lipids associated with haemozoin mediate efficient  
581 and rapid beta-haematin formation at physiological pH, temperature and ionic  
582 composition. *Malar J* **11**, 337 (2012).
- 583 21. J. M. Pisciotta *et al.*, The role of neutral lipid nanospheres in *Plasmodium falciparum*  
584 haem crystallization. *Biochem J* **402**, 197-204 (2007).
- 585 22. D. J. Sullivan, Jr., I. Y. Gluzman, D. G. Russell, D. E. Goldberg, On the molecular  
586 mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A* **93**, 11865-  
587 11870 (1996).
- 588 23. K. N. Olafson, T. Q. Nguyen, J. D. Rimer, P. G. Vekilov, Antimalarials inhibit hematin  
589 crystallization by unique drug-surface site interactions. *Proc Natl Acad Sci U S A* **114**,  
590 7531-7536 (2017).
- 591 24. T. Herraiz, H. Guillen, D. Gonzalez-Pena, V. J. Aran, Antimalarial quinoline drugs  
592 inhibit beta-hematin and increase free hemin catalyzing peroxidative reactions and  
593 inhibition of cysteine proteases. *Sci Rep* **9**, 15398 (2019).
- 594 25. S. N. Cohen, K. O. Phifer, K. L. Yielding, Complex formation between chloroquine and  
595 ferrihaemic acid *in vitro*, and its effect on the antimalarial action of chloroquine. *Nature*  
596 **202**, 805-806 (1964).
- 597 26. U. D'Alessandro, H. Buttiens, History and importance of antimalarial drug resistance.  
598 *Trop Med Int Health* **6**, 845-848 (2001).
- 599 27. J. M. Matz, K. Matuschewski, An *in silico* down-scaling approach uncovers novel  
600 constituents of the *Plasmodium*-containing vacuole. *Sci Rep* **8**, 14055 (2018).
- 601 28. D. R. Flower, The lipocalin protein family: structure and function. *Biochem J* **318** ( Pt  
602 **1**), 1-14 (1996).
- 603 29. D. R. Flower, A. C. North, C. E. Sansom, The lipocalin protein family: structural and  
604 sequence overview. *Biochim Biophys Acta* **1482**, 9-24 (2000).
- 605 30. H. E. Bullen *et al.*, Biosynthesis, localization, and macromolecular arrangement of the  
606 *Plasmodium falciparum* translocon of exported proteins (PTEX). *J Biol Chem* **287**,  
607 7871-7884 (2012).
- 608 31. M. Morita *et al.*, PV1, a novel *Plasmodium falciparum* merozoite dense granule protein,  
609 interacts with exported protein in infected erythrocytes. *Sci Rep* **8**, 3696 (2018).
- 610 32. H. Iriko *et al.*, *Plasmodium falciparum* exported protein 1 is localized to dense granules  
611 in merozoites. *Parasitol Int* **67**, 637-639 (2018).
- 612 33. M. Lamarque *et al.*, Food vacuole proteome of the malarial parasite *Plasmodium*  
613 *falciparum*. *Proteomics Clin Appl* **2**, 1361-1374 (2008).
- 614 34. T. D. Otto *et al.*, A comprehensive evaluation of rodent malaria parasite genomes and  
615 gene expression. *BMC Biol* **12**, 86 (2014).
- 616 35. J. M. Matz *et al.*, *In vivo* function of PTEX88 in malaria parasite sequestration and  
617 virulence. *Eukaryot Cell* **14**, 528-534 (2015).
- 618 36. M. Zhang *et al.*, Uncovering the essential genes of the human malaria parasite  
619 *Plasmodium falciparum* by saturation mutagenesis. *Science* **360** (2018).
- 620 37. Y. L. Hong, Y. Z. Yang, S. R. Meshnick, The interaction of artemisinin with malarial  
621 hemozoin. *Mol Biochem Parasitol* **63**, 121-128 (1994).



- 622 38. R. K. Haynes *et al.*, Artemisinin antimalarials do not inhibit hemozoin formation.  
623 *Antimicrob Agents Chemother* **47**, 1175 (2003).
- 624 39. W. Asawamahasakda, I. Ittarat, C. C. Chang, P. McElroy, S. R. Meshnick, Effects of  
625 antimalarials and protease inhibitors on plasmodial hemozoin production. *Mol*  
626 *Biochem Parasitol* **67**, 183-191 (1994).
- 627 40. A. V. Pandey, B. L. Tekwani, R. L. Singh, V. S. Chauhan, Artemisinin, an endoperoxide  
628 antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in  
629 malarial parasite. *J Biol Chem* **274**, 19383-19388 (1999).
- 630 41. N. Basilico, E. Pagani, D. Monti, P. Olliario, D. Taramelli, A microtitre-based method  
631 for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs.  
632 *J Antimicrob Chemother* **42**, 55-60 (1998).
- 633 42. T. F. de Koning-Ward *et al.*, A newly discovered protein export machine in malaria  
634 parasites. *Nature* **459**, 945-949 (2009).
- 635 43. J. M. Matz, C. Goosmann, K. Matuschewski, T. W. A. Kooij, An unusual prohibitin  
636 regulates malaria parasite mitochondrial membrane potential. *Cell Rep* **23**, 756-767  
637 (2018).
- 638 44. X. Y. Liu, "Heterogeneous nucleation and crystal network formation" in Crystal growth  
639 of technologically important electronic materials, K. Byrappa, T. Ohachi, H. Klapper,  
640 R. Fornari, Eds. (Allied Publishers PVT. Limited, New Delhi, 2003), pp. 118-135.
- 641 45. R. Huber *et al.*, Molecular structure of the bilin binding protein (BBP) from *Pieris*  
642 *brassicae* after refinement at 2.0 Å resolution. *J Mol Biol* **198**, 499-513 (1987).
- 643 46. R. Huber *et al.*, Crystallization, crystal structure analysis and preliminary molecular  
644 model of the bilin binding protein from the insect *Pieris brassicae*. *J Mol Biol* **195**, 423-  
645 434 (1987).
- 646 47. H. M. Holden, W. R. Rypniewski, J. H. Law, I. Rayment, The molecular structure of  
647 insecticyanin from the tobacco hornworm *Manduca sexta* L. at 2.6 Å resolution. *EMBO*  
648 *J* **6**, 1565-1570 (1987).
- 649 48. A. Waterhouse *et al.*, SWISS-MODEL: homology modelling of protein structures and  
650 complexes. *Nucleic Acids Res* **46**, W296-W303 (2018).
- 651 49. A. Schiefner, L. Chatwell, D. A. Breustedt, A. Skerra, Structural and biochemical  
652 analyses reveal a monomeric state of the bacterial lipocalin Blc. *Acta Crystallogr D Biol*  
653 *Crystallogr* **66**, 1308-1315 (2010).
- 654 50. J. Yang, Y. Zhang, Protein structure and function prediction using I-TASSER. *Curr*  
655 *Protoc Bioinformatics* **52**, 5 8 1-5 8 15 (2015).
- 656 51. V. Campanacci, R. E. Bishop, S. Blangy, M. Tegoni, C. Cambillau, The membrane  
657 bound bacterial lipocalin Blc is a functional dimer with binding preference for  
658 lysophospholipids. *FEBS Lett* **580**, 4877-4883 (2006).
- 659 52. J. M. Matz, K. Matuschewski, T. W. Kooij, Two putative protein export regulators  
660 promote *Plasmodium* blood stage development *in vivo*. *Mol Biochem Parasitol* **191**,  
661 44-52 (2013).
- 662 53. C. J. Janse *et al.*, High efficiency transfection of *Plasmodium berghei* facilitates novel  
663 selection procedures. *Mol Biochem Parasitol* **145**, 60-70 (2006).
- 664 54. R. Stallmach *et al.*, *Plasmodium falciparum* SERA5 plays a non-enzymatic role in the  
665 malarial asexual blood-stage lifecycle. *Mol Microbiol* **96**, 368-387 (2015).
- 666 55. J. M. Matz, T. W. Kooij, Towards genome-wide experimental genetics in the *in vivo*  
667 malaria model parasite *Plasmodium berghei*. *Pathog Glob Health* **109**, 46-60 (2015).



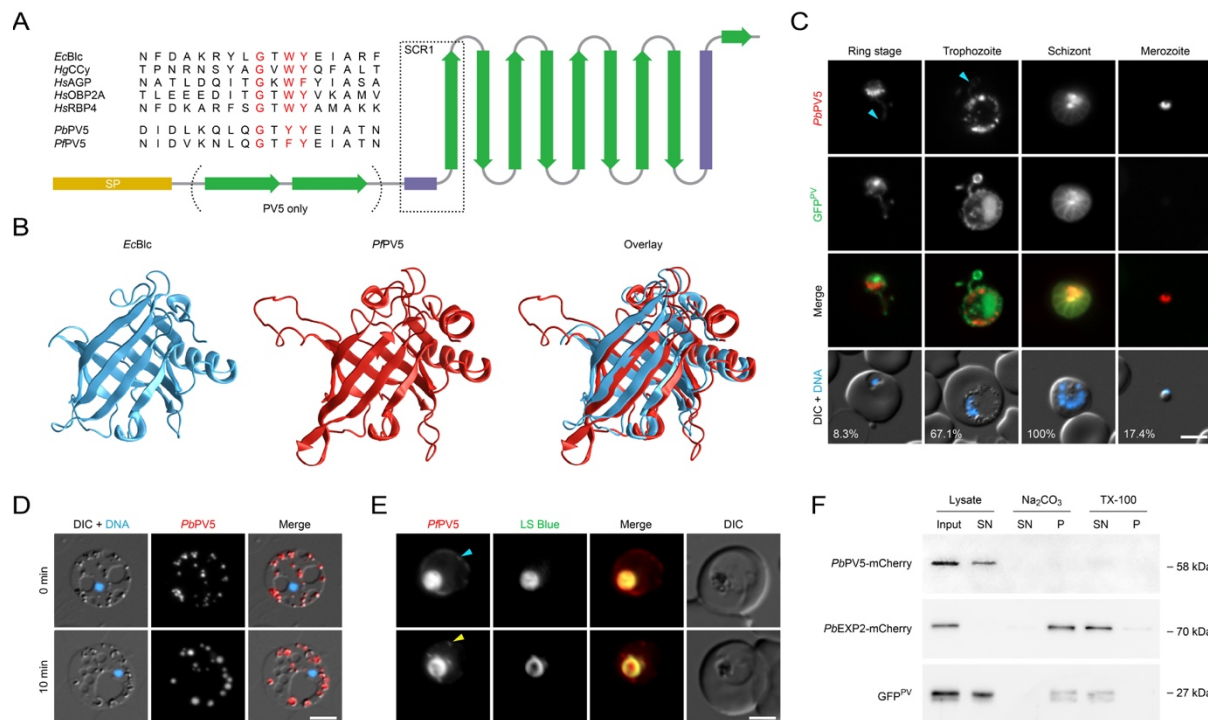
- 668 56. T. W. Kooij, M. M. Rauch, K. Matuschewski, Expansion of experimental genetics  
669 approaches for *Plasmodium berghei* with versatile transfection vectors. *Mol Biochem*  
670 *Parasitol* **185**, 19-26 (2012).
- 671 57. C. R. Collins *et al.*, Robust inducible Cre recombinase activity in the human malaria  
672 parasite *Plasmodium falciparum* enables efficient gene deletion within a single asexual  
673 erythrocytic growth cycle. *Mol Microbiol* **88**, 687-701 (2013).
- 674 58. M. L. Jones *et al.*, A versatile strategy for rapid conditional genome engineering using  
675 *loxP* sites in a small synthetic intron in *Plasmodium falciparum*. *Sci Rep* **6**, 21800  
676 (2016).
- 677 59. E. Knuepfer, M. Napiorkowska, C. van Ooij, A. A. Holder, Generating conditional gene  
678 knockouts in *Plasmodium* – a toolkit to produce stable DiCre recombinase-expressing  
679 parasite lines using CRISPR/Cas9. *Sci Rep* **7**, 3881 (2017).
- 680 60. J. A. Thomas *et al.*, Development and application of a simple plaque assay for the  
681 human malaria parasite *Plasmodium falciparum*. *PLoS One* **11**, e0157873 (2016).
- 682 61. J. M. Matz *et al.*, The *Plasmodium berghei* translocon of exported proteins reveals  
683 spatiotemporal dynamics of tubular extensions. *Sci Rep* **5**, 12532 (2015).
- 684 62. E. Knuepfer *et al.*, RON12, a novel *Plasmodium*-specific rhopty neck protein important  
685 for parasite proliferation. *Cell Microbiol* **16**, 657-672 (2014).
- 686 63. R. Buller, M. L. Peterson, Ö. Almarsson, L. Leiserowitz, Quinoline binding site on  
687 malaria pigment crystal: a rational pathway for antimalaria drug design. *Cryst Growth*  
688 *Des* **2**, 553-562 (2002).
- 689 64. J. Friesen *et al.*, Natural immunization against malaria: causal prophylaxis with  
690 antibiotics. *Sci Transl Med* **2**, 40ra49 (2010).

691 **AUTHOR CONTRIBUTIONS**

692 JMM conceived the study and performed all experiments requiring genetic manipulation and  
693 cultivation of *P. berghei* and *P. falciparum* parasites. JMM, BD, AB, PM, TS and LC contributed  
694 to Hz analysis by SEM and TEM. TBB, EvG and JPA performed and analysed electron  
695 diffraction experiments. KM and MJB contributed to data interpretation. The manuscript was  
696 written by JMM with input from TBB, EvG, JPA, KM and MJB.

697 **ACKNOWLEDGEMENTS**

698 We thank Volker Brinkmann and Christian Goosmann (Max Planck Institute for Infection  
699 Biology, Berlin) as well as Kurt Anderson (The Francis Crick Institute, London) for assistance  
700 with polarization microscopy. We also thank Darren Flower (Aston University, Birmingham)  
701 and Leslie Leiserowitz (Weizmann Institute of Science, Rehovot) for fruitful discussion. This  
702 work was supported by a stipend from the Deutsche Forschungsgemeinschaft (DFG, project  
703 number 419345764 to JMM) and in part by funding to JMM, LC, AB and MJB from the Francis  
704 Crick Institute, which receives its core funding from Cancer Research UK (FC001043), the UK  
705 Medical Research Council (FC001043), and the Wellcome Trust (FC001043). JMM, BD, PM,  
706 TS and KM were also supported by the Humboldt University and by the Alliance Berlin  
707 Canberra “Crossing Boundaries: Molecular Interactions in Malaria”, which is co-funded by a  
708 grant from the DFG for the International Research Training Group (IRTG) 2290 and the  
709 Australian National University. We further acknowledge funding from the Swiss National  
710 Science Foundation (project 31003A\_17002 to TBB and project 200021\_165669 to TBB and  
711 JPA). We thank the Center for Cellular Imaging and NanoAnalytics for support and use of the  
712 cryo-electron microscope.



713

714 **Fig. 1.** The *Plasmodium* lipocalin PV5 is trafficked to the parasite's digestive compartments.

715 (A) PV5 is a lipocalin family member. Secondary structure of *Plasmodium* PV5. Yellow,  
 716 signal peptide (SP); green,  $\beta$ -strands; purple,  $\alpha$ -helices. Note the two amino-terminal  $\beta$ -  
 717 strands specific to PV5. Alignments of the structurally conserved region 1 (SCR1) from  
 718 different lipocalin family members and PV5 are shown in the upper left corner. Signature  
 719 residues are highlighted in red. *EcBlc*, *Escherichia coli* bacterial lipocalin; *HgCCy*, *Homarus*  
 720 *gammarus* (European lobster) crustacyanin; *HsAGP*, *Homo sapiens*  $\alpha_1$ -acid glycoprotein;  
 721 *HsOBP2A*, *H. sapiens* odorant-binding protein 2A; *HsRBP4*, *H. sapiens* retinol-binding protein  
 722 4; *Pb/PfPV5*, PV5 from *P. berghei* and *P. falciparum*.

723 (B) Structure homology modelling predicts a lipocalin fold for *PfPV5*. Shown are the  
 724 experimentally validated structure of *EcBlc* (blue, left, residues 27-175, PDB ID: 3MBT), the  
 725 derived model of *PfPV5* (red, centre, residues 35-214) using *EcBlc* as a homology template,  
 726 and an overlay (right). Modelling was performed with SWISS-MODEL and supported by I-  
 727 TASSER. Amino acid sequence identity is 20%, similarity calculated from BLOSUM62  
 728 substitution matrix is 0.3.

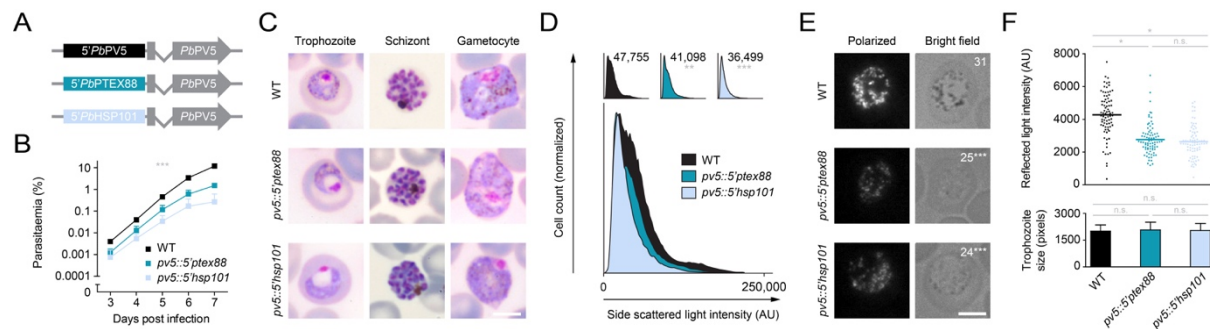
729 (C) Dual protein localization of PV5 to extensions of the PV and to intraparasitic structures  
 730 in *P. berghei*. Transgenic parasites expressing the PV marker GFP<sup>PV</sup> and the endogenous  
 731 *PbPV5* gene fused to mCherry-3xMyc were imaged live (27). Shown are the mCherry (red,  
 732 first row) and GFP channels (green, second row), a merge of both signals (third row) and a  
 733 merge of differential interference contrast images (DIC) and Hoechst 33342 nuclear stain  
 734 (blue, fourth row). Cyan arrowheads, *PbPV5* in PV tubules. Numbers represent normalized

735 mCherry intensity values obtained by quantitative live fluorescence microscopy. N=44  
736 parasites.

737 (D) Intraparasitic PV5 localizes to Hz-containing DVs in *P. berghei*. Parasites were  
738 incubated in 1-2  $\mu$ l under a large coverslip (22 x 40 mm) for several minutes, leading to lysis  
739 of the host erythrocyte and the PV, and to mechanical expansion of the parasite (top). Shown  
740 are a merge of DIC and Hoechst 33342 nuclear stain (blue, first row), the signal of tagged  
741 *PbPV5* (red, second row), as well as a merge of all three channels (third row). Swelling of  
742 *PbPV5*-containing DVs was observed ten minutes later (bottom). Note the even distribution of  
743 *PbPV5* throughout the swollen DVs.

744 (E) PV5 localizes to the central FV, intraparasitic vesicles and to the PV in *P. falciparum*.  
745 Transgenic parasites expressing the endogenous *PfPV5* gene fused to mCherry were imaged  
746 live in the presence of Lysosensor Blue DND-167 (LS Blue). Shown are the signals of mCherry  
747 (red, first row), LS Blue (green, second row), a merge of both signals (third row), and DIC  
748 images (fourth row). Cyan arrowhead, *PfPV5* in PV (top). Yellow arrowhead, *PfPV5* in small  
749 intraparasitic vesicles (bottom). Bars, 5  $\mu$ m.

750 (F) PV5 is a soluble protein. Subcellular fractionation was performed using the *PbPV5*-  
751 tagged *P. berghei* line, which also expresses the soluble marker GFP<sup>PV</sup>, and a *P. berghei* line  
752 expressing the transmembrane protein *PbEXP2* fused to mCherry-3xMyc (64). Cell lysates  
753 were centrifuged and resultant membrane pellets were subjected to solubilization with Na<sub>2</sub>CO<sub>3</sub>  
754 and Triton X-100 (TX-100). Input, supernatant (SN) and pellet fractions (P) were analysed by  
755 Western blot using anti-mCherry and anti-GFP primary antibodies.



756

757 **Fig. 2.** Deregulated expression of *PV5* impacts haemozoin formation in *Plasmodium berghei*.

758 (A) Schematic representation of the genotypes of WT (top) and transgenic *pv5::5'ptex88*  
 759 (middle) and *pv5::5'hsp101* parasites (bottom). In the mutants, the endogenous *PbPV5*  
 760 promoter (black) was exchanged for the promoter of *PbPTEX88* (dark blue) or *PbHSP101*  
 761 (light blue), respectively.

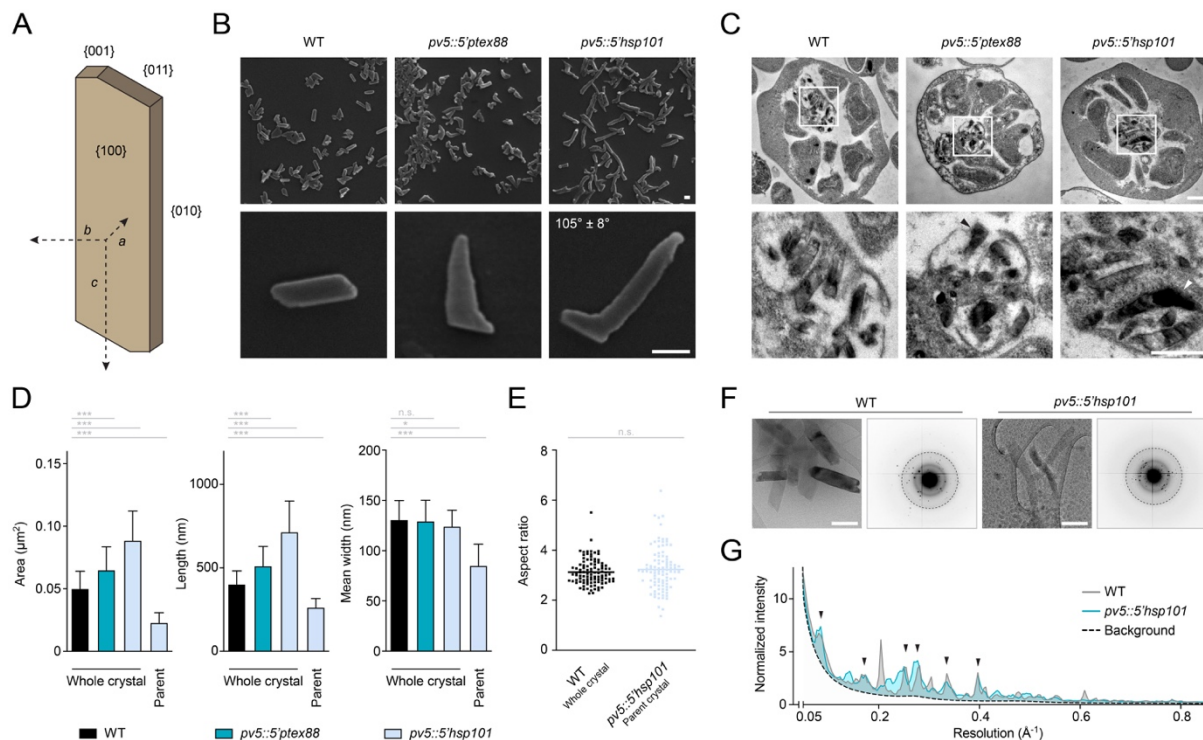
762 (B) Reduced parasite proliferation upon *PbPV5* promoter swapping. Asexual blood stage  
 763 development was analysed using the intravital competition assay (55). Average parasite  
 764 multiplication rates were 11.4 (WT), 9.1 (*pv5::5'ptex88*) and 6.2 (*pv5::5'hsp101*). Shown are  
 765 mean values +/- SD. \*\*\*, P<0.001; Two-way ANOVA. N=3 independent infections.

766 (C) Morphology of trophozoite, schizont and gametocyte stages in the WT, *pv5::5'ptex88*  
 767 and *pv5::5'hsp101* lines as observed by Giemsa staining. Note the lack of prominent dark  
 768 pigment granules in mutant trophozoites and gametocytes as well as the dilation of the FV in  
 769 mutant trophozoites. Bar, 5  $\mu$ m.

770 (D) *PbPV5* promoter swap mutants are less granular. Infected blood was subjected to flow  
 771 cytometry and the intensity of the side scattered light was determined. Shown are individual  
 772 histograms including the mean side scatter intensity values (top) as well as a merge of WT,  
 773 *pv5::5'ptex88* and *pv5::5'hsp101* histograms (bottom). Significances are shown for the  
 774 comparison of the mutants with WT. n.s., non-significant; \*\*, P<0.01; \*\*\*, P<0.001; One-way  
 775 ANOVA and Tukey's multiple comparison test. N=6 independent infections.

776 (E, F) *PbPV5* is required for efficient Hz formation. (E) Trophozoites were visualized by  
 777 polarization microscopy (left) and bright field imaging (right). Numbers indicate the mean  
 778 quantity of bright puncta in polarization images. Significances are shown for the comparison  
 779 of the mutants with WT. Bar, 5  $\mu$ m. (F) Quantitative polarization microscopy. Depicted are  
 780 individual and mean intensity values of reflected polarized light in methanol-fixed WT,  
 781 *pv5::5'ptex88* and *pv5::5'hsp101* trophozoites (bars, upper graph). Only trophozoites of similar  
 782 size were analysed (lower graph). Depicted are mean values +/- SD. n.s., non-significant; \*,  
 783 P<0.05; \*\*\*, P<0.001; One-way ANOVA and Tukey's multiple comparison test. N=80  
 784 trophozoites from 4 independent infections.





785

786 **Fig. 3. PV5 determines haemozoin morphology in *Plasmodium berghei*.**

787 (A) Hz crystal architecture. In *Plasmodium* WT parasites, Hz assembles as triclinic high  
788 aspect ratio parallelograms (63). Characteristic crystal axes and faces are indicated.

789 (B) Scanning electron micrographs of Hz purified from WT, *pv5::5'ptex88* and  
790 *pv5::5'hsp101* mixed blood stage parasites. The angle between the regularly shaped  
791 *pv5::5'hsp101* parent crystal and the outgrowth is indicated. N=130 crystals. Bars, 100 nm.

792 (C) Crystal morphology *in situ*. Shown are TEM images of WT, *pv5::5'ptex88* and  
793 *pv5::5'hsp101* schizonts (top) as well as their residual body at higher magnification (bottom).  
794 Abnormal crystal shapes in the mutants are indicated by arrowheads. Bars, 500 nm.

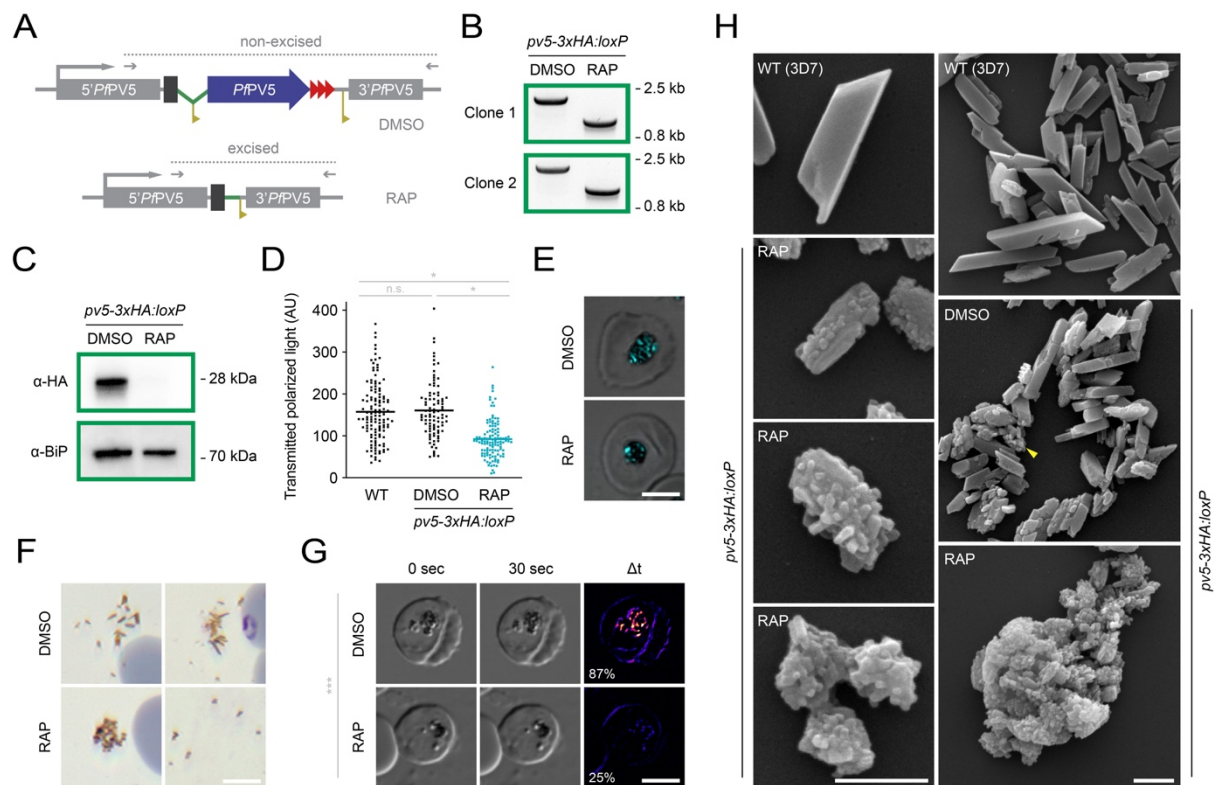
795 (D) Hyperactive Hz growth is unidirectional. Shown are the dimensions of whole individual  
796 Hz crystals extracted from WT, *pv5::5'ptex88* and *pv5::5'hsp101* parasites including the area  
797 exposed to the electron beam (left) as well as the length (centre) and mean width of the  
798 crystals (right). The theoretical dimensions of the *pv5::5'hsp101* parent crystal were  
799 interpolated and are depicted as well. Shown are mean values +/- SD. n.s., non-significant; \*,  
800 P<0.05; \*\*\*, P<0.001; One-way ANOVA and Tukey's multiple comparison test. N=100  
801 crystals.

802 (E) Normal aspect ratio of *pv5::5'hsp101* parent crystals. Shown are the aspect ratios of  
803 whole Hz crystals from WT parasites and of the parent crystal from *pv5::5'hsp101*-generated

804 Hz. Depicted are individual and mean values (bars). n.s., non-significant; Student's *t*-test.  
805 N=100 crystals.

806 (F, G) Unaltered crystalline order in Hz of *pv5::5'hsp101* parasites. (F) Depicted are TEM  
807 images (left) of Hz purified from WT and *pv5::5'hsp101* parasites as well as their  
808 corresponding electron diffraction patterns (right) showing comparable resolution of the Bragg  
809 peaks. Dashed circles demark a resolution of  $0.5 \text{ \AA}^{-1}$ . Bars, 200 nm. (G) Plot of the radial  
810 maximum diffracted intensity as a function of resolution. Data were normalized to the average  
811 median intensity at  $0.18 \text{ \AA}^{-1}$ , in order to correct for differences in diffracted volume. Arrowheads  
812 denote overlapping peaks. N=10 (WT) and 18 (*pv5::5'hsp101*) diffraction data sets.





813

814 **Fig. 4.** Absence of PV5 causes haemozoin branching in *Plasmodium falciparum*.

815 (A) Schematic representation of DiCre-mediated *PfPV5* excision. The endogenous locus  
 816 was modified to introduce *loxP* sites (yellow) flanking the majority of the coding sequence of  
 817 the 3xHA (red)-tagged *PfPV5* (blue). The artificial intron is indicated in green. See also Fig.  
 818 S3A. Treatment with rapamycin (RAP) induces Cre recombinase-mediated excision of the  
 819 *loxP*-flanked sequence which results in truncation of *PfPV5* leaving behind only the sequence  
 820 encoding the protein's signal peptide. Excision-sensitive primer combinations are indicated by  
 821 arrows and expected diagnostic PCR fragments by dotted lines.

822 (B) Diagnostic PCR of the modified *PfPV5* locus following treatment with dimethyl  
 823 sulfoxide (DMSO) or RAP, respectively, using the primer combinations depicted in A. Results  
 824 are shown from two independent *pv5-3xHA:loxP* clones.

825 (C) Loss of *PfPV5* protein. Western blot analysis of parasite extracts following treatment  
 826 with DMSO or RAP, respectively, using anti-HA and anti-*PfBiP* primary antibodies.

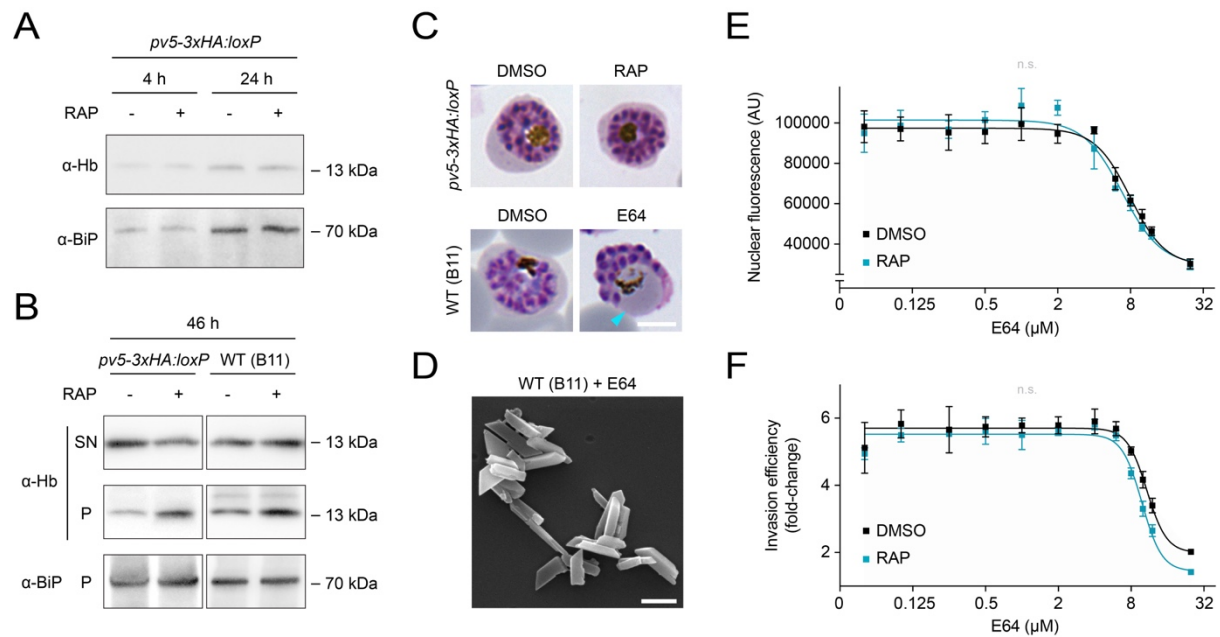
827 (D, E) *PfPV5* is required for efficient Hz formation. WT and *pv5-3xHA:loxP* parasites  
 828 were treated with DMSO or RAP, respectively, and visualized by polarization microscopy 36 hours  
 829 after invasion. (D) Quantification of the polarized light intensity. Depicted are values from  
 830 individual parasites as well as the mean intensity values (bars). n.s., non-significant; \*,  $P < 0.05$ ;  
 831 One-way ANOVA and Tukey's multiple comparison test.  $N \geq 93$  parasites from 3 independent  
 832 experiments. (E) Exemplary images of DMSO- and RAP-treated *pv5-3xHA:loxP* parasites.

833 Shown is a merge of polarized light (cyan) and DIC. Bar, 5  $\mu\text{m}$ .

834 (F) Abnormal Hz morphology in the absence of *PfPV5*. Hz released from residual bodies  
835 during parasite egress was imaged in Giemsa-stained thin culture smears of DMSO and RAP-  
836 treated *pv5-3xHA:loxP* parasites. Note the spreading of elongated Hz crystals in DMSO-  
837 treated cultures and the clumping of granular Hz upon RAP treatment (left). Only in very few  
838 instances did the crystals detach from one another in RAP-treated cultures (right). Bar, 5  $\mu\text{m}$ .

839 (G) Reduced Hz movement in the FV upon loss of *PfPV5*. Shown are DIC images of live  
840 DMSO and RAP-treated *pv5-3xHA:loxP* parasites 36 hours after invasion. Parasites were  
841 imaged twice at an interval of 30 seconds (left and centre) and the difference of both images  
842 was visualized with pixel-by-pixel intensity subtraction (right). Note the absence of Hz  
843 movement in the RAP-treated parasite (see also Movie S1). The percentage of parasites with  
844 moving Hz is indicated. \*\*\*,  $P < 0.001$ ; Student's *t*-test.  $N = 4$  independent experiments with  $> 300$   
845 parasites each. Bar, 5  $\mu\text{m}$ .

846 (H) Scanning electron micrographs of Hz purified from WT (3D7) and from DMSO or RAP-  
847 treated *pv5-3xHA:loxP* schizonts. Bars, 500 nm.



848

849 **Fig. 5.** *PfpV5* regulates haem sequestration independently from haemoglobin processing.

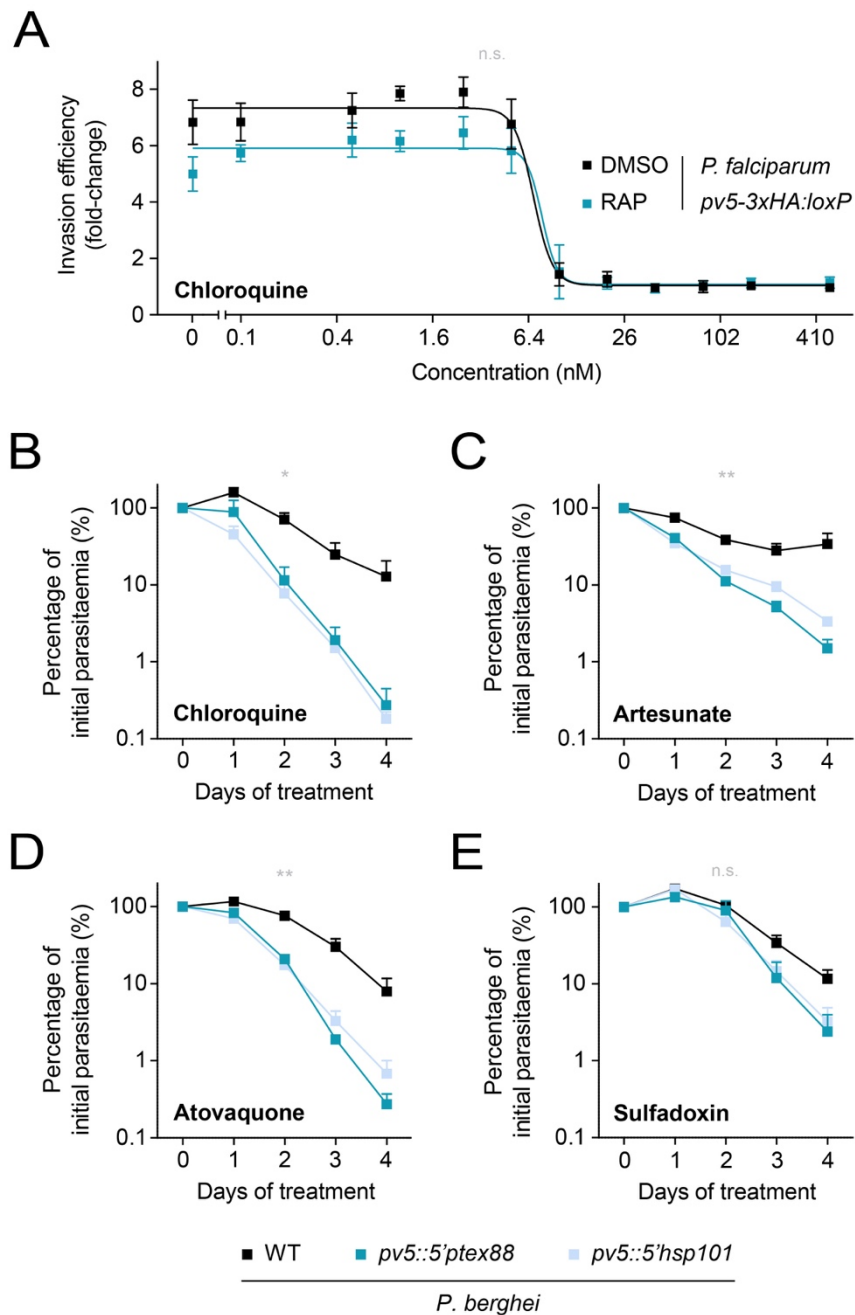
850 (A, B) Normal uptake and digestion of host cell haemoglobin by *PfpV5*-deficient parasites.

851 (A) Western blot analysis of induced and non-induced *pv5-3xHA:loxP* parasites released from  
 852 their host cells by saponin lysis 4 and 24 hours after invasion. (B) 46 hours after invasion,  
 853 induced and non-induced *pv5-3xHA:loxP* or WT (B11) schizonts were released by saponin  
 854 treatment. Resultant supernatants (SN) and schizont pellets were isolated. Blots were probed  
 855 with antibodies directed against human haemoglobin  $\alpha$  (Hb) and *Pf*BiP. Note an increase in  
 856 intraparasitic haemoglobin upon rapamycin treatment (RAP) in both *pv5-3xHA:loxP* and WT  
 857 (B11) schizonts.

858 (C) *PfpV5*-deficient schizonts exhibit no vacuolar bloating. Shown are *pv5-3xHA:loxP*  
 859 parasites treated from the ring stage onward with dimethyl sulfoxide (DMSO) or RAP (top) and  
 860 *Plasmodium falciparum* WT parasites treated from 24 hours post invasion onward with DMSO  
 861 or 21.7  $\mu$ M E64 (bottom). Cyan arrowhead, bloated food vacuole. Bar, 5  $\mu$ m.

862 (D) Inhibition of haemoglobin catabolism does not result in abnormal Hz morphology.  
 863 Shown is a scanning electron micrograph of Hz crystals isolated from the E64-treated *P.*  
 864 *falciparum* WT parasites shown in C. Bar, 500 nm.

865 (E, F) *PfpV5*-deficient parasites display unaltered sensitivity towards E64. DMSO and  
 866 RAP-treated *pv5-3xHA:loxP* parasites were grown in various concentrations of E64 from the  
 867 ring stage onward. (E) Nuclear SYBR Green fluorescence 44 hours after invasion. (F) Invasion  
 868 efficiency under static conditions following a 36-hour incubation in the presence of E64 and  
 869 subsequent inhibitor washout. Mean values  $\pm$  SD are shown. n.s., non-significant; fitting of  
 870 IC<sub>50</sub> values following non-linear regression, N=3.



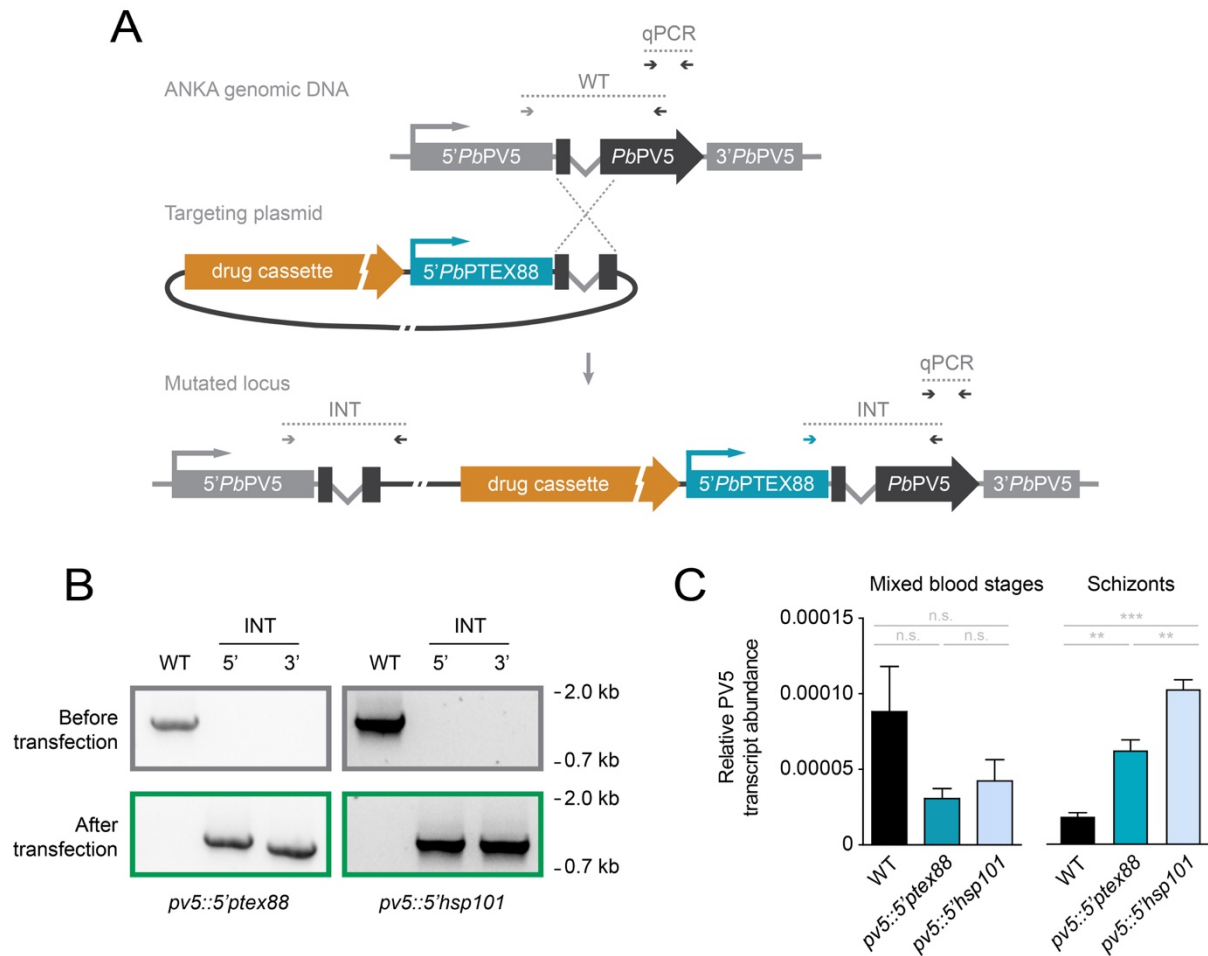
871

872 **Fig. 6.** Targeting PV5 function results in parasite hypersensitivity towards antimalarial drugs  
873 *in vivo*.

874 (A) PV5-deficient *P. falciparum* parasites retain normal sensitivity towards chloroquine.  
875 Shown is a dose-response analysis of DMSO- and RAP-treated *pv5-3xHA:loxP* parasites.  
876 Static ring stage cultures were treated with varying chloroquine concentrations and the  
877 transition into the following intraerythrocytic cycle was quantified. Depicted are mean values  
878 +/- SD. n.s., non-significant; fitting of IC<sub>50</sub> values following non-linear regression, N=3.

879 (B-E) Enhanced drug susceptibility of the PbPV5 mutants *in vivo*. 5 x 10<sup>6</sup> mCherry-  
880 fluorescent *P. berghei* WT parasites were injected into SWISS mice together with 5 x 10<sup>6</sup> GFP-

881 fluorescent *pv5::5'ptex88* or *pv5::5'hsp101* parasites, respectively. From day three onward,  
882 mice were treated with curative doses of (A) chloroquine (288 mg/l in drinking water, *ad*  
883 *libitum*), (B) artesunate (50 mg/kg body weight, i.p.), (C) atovaquone (1.44 mg/kg body weight,  
884 i.p.) or (D) sulfadoxin (1.4 g/l in drinking water, *ad libitum*) and the respective parasitaemias  
885 were determined daily by flow cytometry of peripheral blood (50). Values are normalized to  
886 the parasitaemias on day 0 of treatment. Shown are mean values +/- SEM. n.s., non-  
887 significant; \*, P<0.05; \*\*, P<0.01; Two-way ANOVA. N=3 independent infections.



888

889 **Fig. S1.** Generation and validation of *PbPV5* promoter swap mutants.

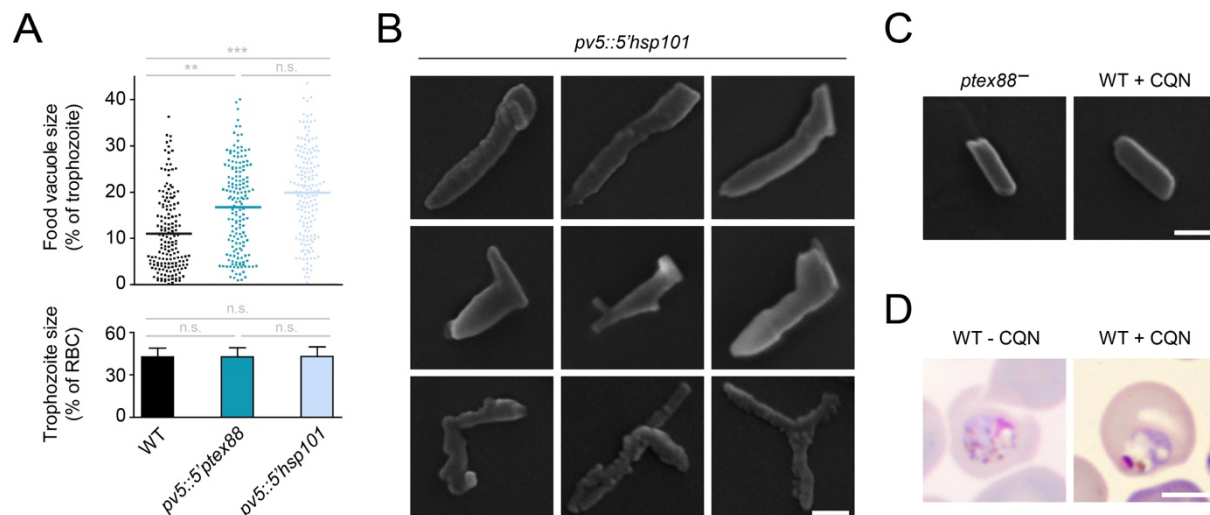
890 (A) Genetic strategy to exchange the endogenous promoter (light grey) of *PbPV5* (dark  
 891 grey) using single homologous recombination. The endogenous *PbPV5* locus was targeted  
 892 with an insertion plasmid containing the promoter sequence of *PbPTEX88* (shown, blue) or  
 893 *PbHSP101* (not shown) fused to the amino-terminal sequence of *PbPV5* as well as the drug-  
 894 selectable hDHFR-yFcu cassette (orange). Successful insertion yields parasites expressing  
 895 full length *PbPV5* from a heterologous promoter and a non-functional amino-terminal fragment  
 896 from the endogenous promoter, encoding the *PbPV5* signal peptide. Primer combinations for  
 897 wild-type (WT) and integration-specific reactions (INT) as well as for quantitative real-time  
 898 PCR (qPCR) are indicated by arrows and expected amplicons by dotted lines.

899 (B) Diagnostic PCR of the WT locus (top) and of the drug-selected and isolated *PbPV5*  
 900 mutants (bottom) using the primer combinations depicted in A.

901 (C) Dynamic changes in *PbPV5* transcription upon promoter swap. Mixed blood stages  
 902 and mature segmented schizonts were purified from mouse blood or from *in vitro* culture,  
 903 respectively, and subjected to qPCR using primers targeting a carboxy-terminal portion of  
 904 *PbPV5*, as depicted in A. *PbPV5* transcript abundance was normalized to *Pb18S* rRNA.



905 Shown are mean values +/- SEM. n.s., non-significant; \*\*, P<0.01; \*\*\*, P<0.001; One-way  
906 ANOVA and Tukey's multiple comparison test. N=6 for mixed blood stages and 3 for schizonts.



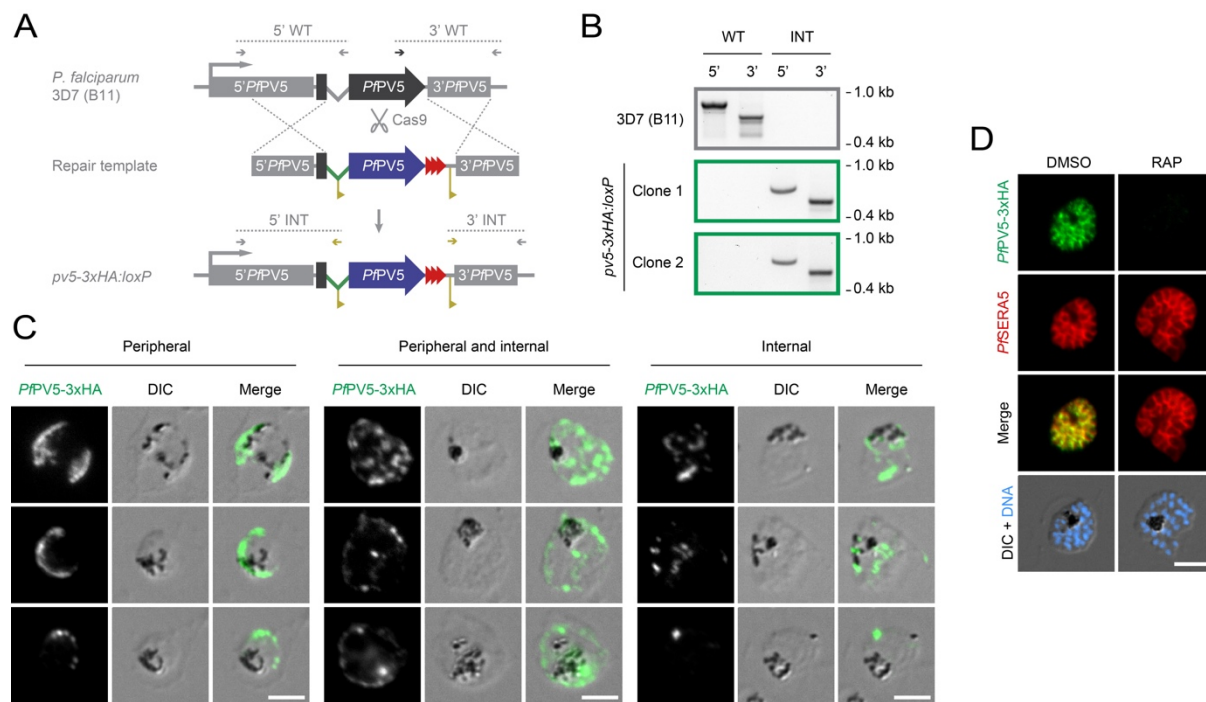
907

908 **Fig. S2.** Vacuolar dilation and defective haemozoin formation in the *PbPV5* mutants.

909 (A) Quantification of vacuolar dilation. The translucent area observed in Giemsa-stained  
910 trophozoites corresponding to the FV was measured microscopically and expressed as the  
911 percentage of the entire trophozoite area (upper graph). Depicted are individual and mean  
912 values (bars). Only trophozoites of identical size were analysed (lower graph). Shown are  
913 mean values +/- SD. n.s., non-significant; \*\*, P<0.01; \*\*\*, P<0.001; One-way ANOVA and  
914 Tukey's multiple comparison test. N=165 trophozoites from 5 independent infections.

915 (B) A selection of Hz crystals generated by *pv5::5'hsp101* parasites as visualized by  
916 scanning electron microscopy. Bar, 100 nm.

917 (C, D) Hz morphology is not affected by slow parasite growth or mortality. (C) Crystals  
918 were extracted from slow-growing *PTEX88* knockout parasites (left) and from WT parasites  
919 treated with curative doses of chloroquine (CQN, 288 mg/l in drinking water, *ad libitum*) (right)  
920 and were visualized by scanning electron microscopy. Bar, 100 nm. (D) Morphology of  
921 untreated (left) and CQN-treated WT parasites (right) as shown by Giemsa staining. Note the  
922 pigment clumping and vesiculation in the dying parasite. Bar, 5 μm.



923

924 **Fig. S3.** Generation and validation of conditional *PfPV5* knockout parasites.

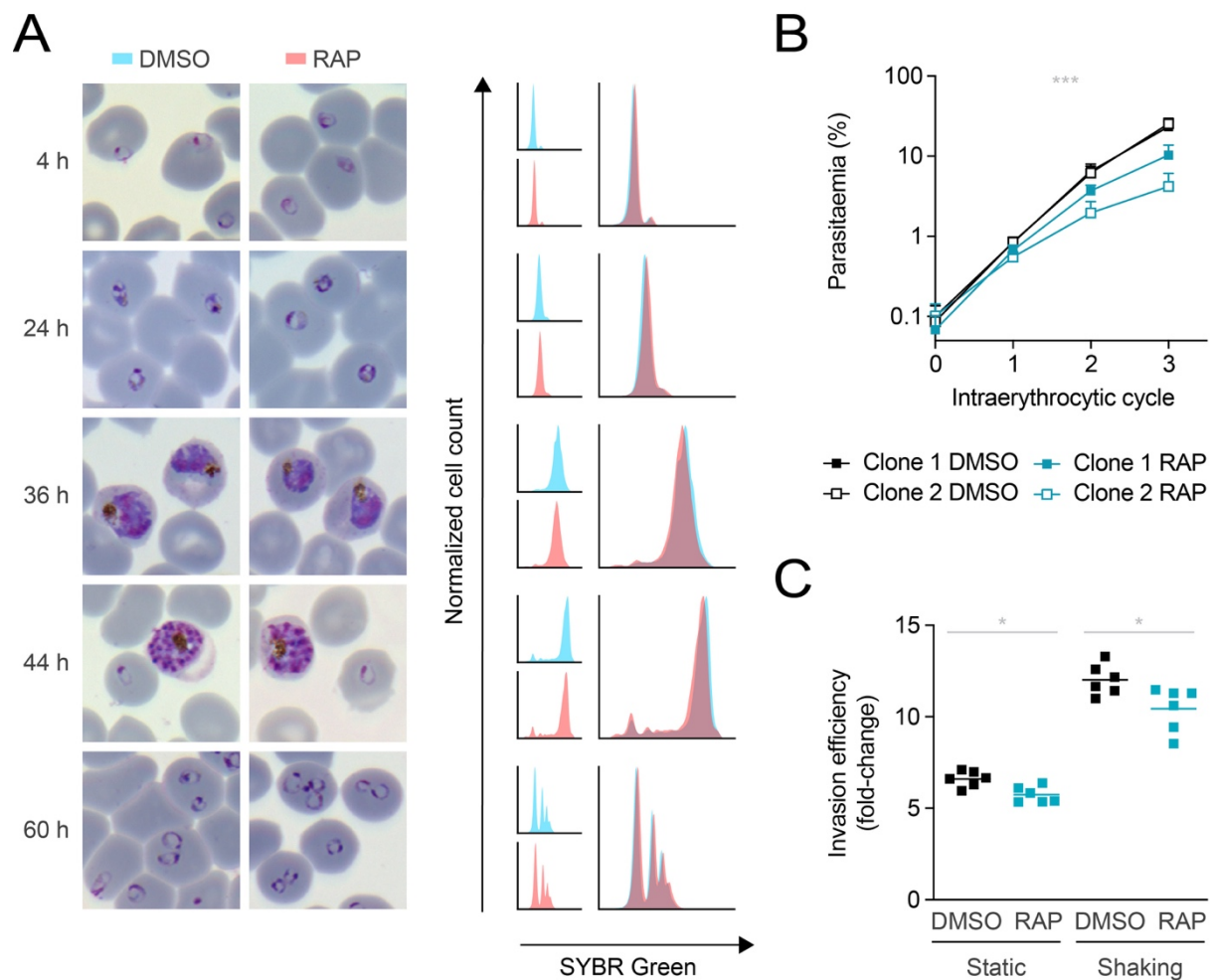
925 (A) Genetic strategy for the generation of a conditional *PfPV5* knockout line. The  
 926 endogenous *PfPV5* locus (dark grey) was targeted by Cas9-mediated double strand cleavage  
 927 and repaired by homologous recombination with a synthetic template containing 5' and 3'  
 928 homology arms (light grey), a 3xHA-tagged (red) re-codonised version of *PfPV5* (blue) and  
 929 *loxP* sequences (yellow) within the artificial intron (green) and behind the stop codon. Wild-  
 930 type (WT) and integration-specific primer combinations (INT) are indicated by arrows and  
 931 expected amplicons by dotted lines. Note that a second conditional knockout line was  
 932 generated expressing *PfPV5* fused to mCherry (Fig. 1E).

933 (B) Diagnostic PCR of the recipient *P. falciparum* 3D7 (B11) line (top) and of two isolated  
 934 *pv5-3xHA:loxP* clones (bottom) using the primer combinations depicted in A.

935 (C) Dual localization of 3xHA-tagged *PfPV5*. Immunofluorescence analysis was  
 936 performed using anti-HA primary antibodies. Depicted are exemplary *pv5-3xHA:loxP*  
 937 trophozoites and young schizonts demonstrating localization of 3xHA-tagged *PfPV5*  
 938 exclusively to the parasitophorous vacuole (PV, left), to the PV and internal parasite structures  
 939 (centre) or to intraparasitic structures only (right). Shown are the signal of tagged *PfPV5*  
 940 (green, left columns), differential interference contrast images (DIC, centre columns) and a  
 941 merge (right columns).

942 (D) Loss of *PfPV5* protein. Immunofluorescence analysis of *pv5-3xHA:loxP* schizonts was  
 943 performed with primary antibodies directed against HA and the PV protein *PfSERA5*. Shown  
 944 are the individual signals of 3xHA-tagged *PfPV5* (green, first row) and *PfSERA5* (red, second

945 row), a merge of both signals (third row) as well as a merge of DIC with Hoechst 33342 nuclear  
946 stain (blue, fourth row) following treatment with DMSO (left) or RAP (right), respectively. Bars,  
947 5  $\mu\text{m}$ .



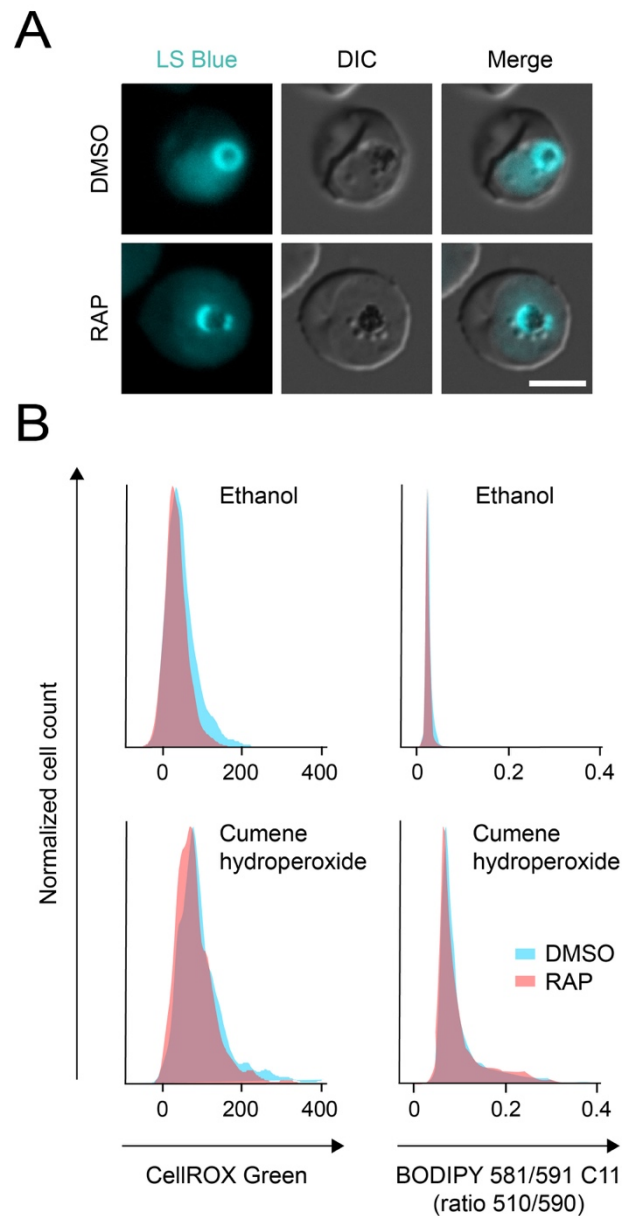
948

949 **Fig. S4.** Impaired fitness of *in vitro* cultivated *Plasmodium falciparum* in the absence of PV5.

950 (A) *PfPV5*-deficient parasites mature normally *in vitro*. Tightly synchronized *pv5-*  
 951 *3xHA:loxP* ring stages were treated with dimethyl sulfoxide (DMSO, blue) or rapamycin (RAP,  
 952 red) and visualized by Giemsa staining 4, 24, 36, 44 and 60 hours later (left). In parallel,  
 953 nuclear SYBR Green fluorescence was quantified by flow cytometry. Individual and merged  
 954 histograms are depicted (right). Results are representative of two independent experiments.

955 (B) Asexual parasite proliferation is impaired upon loss of *PfPV5*. Shown are growth  
 956 curves of two independent *pv5-3xHA:loxP* clonal lines upon treatment with DMSO or RAP,  
 957 respectively. Averaged parasite multiplication rates are 7.6 (DMSO) and 4.6 (RAP). Shown  
 958 are mean values +/- SD. \*\*\*,  $P < 0.001$ ; Two-way ANOVA.  $N = 6$  independent infections.

959 (C) Impaired schizont to ring stage transition in the absence of *PfPV5*. Schizonts from  
 960 DMSO- and RAP-treated *pv5-3xHA:loxP* cultures were added to fresh erythrocytes and  
 961 incubated under static or shaking conditions for 24 hours. Shown is the fold-change in  
 962 parasitaemia, depicted as individual and mean values (bars). \*,  $P < 0.05$ ; paired *t*-test.  $N = 6$   
 963 independent infections.



964

965 **Fig. S5.** Absence of *PfPV5* does not cause dissipation of the vacuolar pH gradient nor an  
966 increase in oxidative stress.

967 (A) *PfPV5*-deficient parasites maintain an intact and acidic FV. Synchronized *pv5-*  
968 *3xHA:loxP* parasites were treated with dimethyl sulfoxide (DMSO) or rapamycin (RAP) from  
969 the ring stage onward and were stained with LysoSensor Blue DND-167 (LS Blue) 36 hours  
970 later. Shown are the LS Blue channel (cyan, left), differential interference contrast images  
971 (DIC, centre) and a merge (right). Bar, 5  $\mu$ m.

972 (B) No increased oxidative stress in the absence of *PfPV5*. Synchronized *pv5-3xHA:loxP*  
973 parasites were treated with DMSO (blue) or RAP (red) from the ring stage onward, stained  
974 with the oxidative stress sensor CellROX Green (left) or with the ratiometric lipid peroxidation  
975 dye BODIPY 581/591 C11 (right) 36 hours later and analysed by flow cytometry. In addition,



976 parasites had been treated with the oxidative stress-inducing agent cumene hydroperoxide  
977 (bottom) or with ethanol as the solvent control (top). Shown are the histograms of CellROX  
978 Green fluorescence intensity or of the 510/590 nm fluorescence ratio of BODIPY 581/591 C11.  
979 Results are representative of two independent experiments.

980

981 **Movie S1.** Absence of *PfPV5* ablates haemozoin movement within the food vacuole of  
982 *Plasmodium falciparum*.  
983 Shown are differential interference contrast recordings of dimethyl sulfoxide (DMSO, left) and  
984 rapamycin-treated (RAP, right) *pv5-3xHA:loxP* parasites 36 hours following invasion. The  
985 video contains 120 frames shown at a 4x acceleration. Elapsed time is indicated in the upper  
986 right corner. Bar, 5  $\mu\text{m}$ .

987 **Table S1. Haemozoin crystal morphometry.**

	WT	<i>pv5-3xHA:loxP</i>		WT vs. DMSO	WT vs. RAP	DMSO vs. RAP
		DMSO	RAP			
Area ( $\mu\text{m}^2$ )	0.164 ( $\pm 0.088$ )	0.107 ( $\pm 0.052$ )	0.102 ( $\pm 0.039$ )	***	***	n.s.
Aspect ratio	3.673 ( $\pm 1.047$ )	2.781 ( $\pm 1.061$ )	1.604 ( $\pm 0.337$ )	***	***	***
Branching (%)	0	27.5	96.1			

988 n.s., non-significant; \*\*\*,  $P < 0.001$ ; One-way ANOVA and Tukey's multiple comparison test.  
989  $N > 300$  crystals.

990 **Table S2. Primer sequences.**

Primer Name	Primer Sequence (restriction sites)	WT (bp) <sup>a</sup>	INT (bp) <sup>b</sup>	EX (bp) <sup>c</sup>	Use <sup>d</sup>	Target	Reference
NT-PbPV5-F-BamHI	agtttggatccaaaatgaaattttatagcattttgcaatg	707			TV	NT PbPV5	This study
NT-PbPV5-R-SacII	agtattccgggtttaaataacatttctgattttttctcc				TV	NT PbPV5	This study
5'-PbPTEX88-F-BamHI	aaatagcatcctttttgtgaaataagttgttggtg	1,496			TV	5' PbPTEX88	This study
5'-PbPTEX88-R-BamHI	atatagcatcctttttgtgaaataagttgttggtg				TV	5' PbPTEX88	This study
5'-PbHSP101-F-BamHI	ttaaaaggatccaaaataatacaaatgcgtgtggc	1,489			TV	5' PbHSP101	This study
5'-PbHSP101-R-BamHI	ttttcaggatccatttatagaaatagatataattttatcttcattc				TV	5' PbHSP101	This study
mCherry-F-AatII	aatttagactcatgaaggtagcaagggcg	735			TV	mCherry	This study
mCherry-R-AatII	aatttagactcctgtacagctcgtccatg				TV	mCherry	This study
T7	taatacgactcactataggg		1,206		GT	T7	-
5'-PbPV5-F	gtgggtcgtattgtatttttaattagg	1,440			GT	5' PbPV5	This study
CT-PbPV5-R1	gatggatcataaccagcaacg				GT	CT PbPV5	This study
5'-PbPTEX88-F	agtcgaagatataattgaaaagcc		1,091		GT	5' PbPTEX88	This study
5'-PbHSP101-F	tgcaactacattttattaccgcc		1,177		GT	5' PbHSP101	This study
CT-PbPV5-F	taaacccgtgatgaaaacactactgttg	261			qPCR	CT PbPV5	This study
CT-PbPV5-R2	ggatcataaccagcaacgtaaagagc				qPCR	CT PbPV5	This study
Pb18S-F	aagcattaataaagcgaatacatccttcc	134			qPCR	Pb18S-rRNA	(64)
Pb18S-R	ggagattggtttgacggttatgtg				qPCR	Pb18S-rRNA	(64)
NT-PfPV5-F	attgattgtattatcattccag	24			gRNA	NT PfPV5	This study
NT-PfPV5-R1	aaacctggaatgataataacaat				gRNA	NT PfPV5	This study
5'-PfPV5-F	aatgcggggaggagagaaccc	836	730	2,006 / 1,260	GT	5' PfPV5	This study
NT-PfPV5-R2	acaactccatcctatcaaaattaaag				GT	NT PfPV5	This study
CT-PfPV5-F	catgatcattatgtctaaatagaaacc	670			GT	CT PfPV5	This study
3'-PfPV5-R	atgtgaaaaaacttacaactatataccc		561	2,006 / 1,260	GT	3' PfPV5	This study
LoxP-F	taactcgtatagcatacattatagc				GT	loxP	This study
LoxP-R	aactcgtataatgatgctatacg				GT	loxP	This study

991 <sup>a</sup> Sizes of Wild-type-specific PCR products or mCherry.

992 <sup>b</sup> Sizes of integration-specific PCR products.

993 <sup>c</sup> Sizes of excision-diagnostic PCR products (non-excised / excised).

994 <sup>d</sup> Primers used for construction of Transfection Vectors (TV), for GenoTyping (GT), quantitative real-time PCR

995 (qPCR), or for guide RNAs (gRNA). Used primer combinations are indicated in Figures 3A and Figures S1A and

996 S3A.