A lipocalin mediates unidirectional heme biominalization in malaria parasites

Joachim M. Matz1,2,10, Benjamin Drepperb, Thorsten B. Blum3, Eric van Genderenc, Alana Burrelld, Peer Martinb, Thomas Stach,5 Lucy M. Collinsond, Jan Pieter Abrahams2,6,11, Kai Matuschewskib, and Michael J. Blackmana,9

1Malaria Biochemistry Laboratory, The Francis Crick Institute, NW1 1AT London, United Kingdom; 2Department of Molecular Parasitology, Institute of Biology, Humboldt University, 10115 Berlin, Germany; 3Laboratory of Nanoscale Biology, Division of Biology and Chemistry, Paul Scherrer Institute, 5232 Villigen, Switzerland; 4Electron Microscopy Science Technology Platform, The Francis Crick Institute, NW1 1AT London, United Kingdom; 5Center for Cellular Imaging and NanoAnalytics, Biozentrum, University of Basel, 4051 Basel, Switzerland; 6Institute of Biology, Leiden University, 2311 EZ Leiden, The Netherlands; and 7Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, WC1E 7HT London, United Kingdom

Edited by Daniel E. Goldberg, Washington University in St. Louis, St. Louis, MO, and accepted by Editorial Board Member Stephen M. Beverley May 21, 2020 (received for review January 22, 2020)

During blood-stage development, malaria parasites are challenged with the detoxification of enormous amounts of heme released during the proteolytic catabolism of erythrocytic hemoglobin. They tackle this problem by sequestering heme into bioinert crystals called hemozoin. The mechanisms underlying this bioninalization process remain enigmatic. Here, we demonstrate that both rodent and human malaria parasite species secrete and internalize a lipocalin-like protein, PV5, to control heme crystallization. Transcriptional deregulation of PV5 in the rodent parasite Plasmodium berghei results in inordinate elongation of hemozoin crystals, while conditional PV5 inactivation in the human malaria agent Plasmodium falciparum causes excessive multidirectional crystal branching. Although hemoglobin processing remains unaffected, PV5-deficient parasites generate less hemozoin. Electron diffraction analysis indicates that despite the distinct changes in crystal morphology, neither the crystalline order nor unit cell of hemozoin are affected by impaired PV5 function. Deregulation of PV5 expression renders P. berghei hypersensitive to the antimalarial drugs artesunate, chloroquine, and atovaquone, resulting in accelerated parasite clearance following drug treatment in vivo. Together, our findings demonstrate the Plasmodium-tailed role of a lipocalin family member in hemozoin formation and underscore the role of heme in parasite survival. It is thus crucial for understanding of the mechanisms underlying heme sequestration to provide valuable insights for future drug development efforts.

Significance

During blood-stage development, the malaria parasite replicates inside erythrocytes of the vertebrate host, where it engulfs and digests most of the available hemoglobin. This results in the build-up of cytotoxic free heme, which eventually leads to the build-up of cytotoxic free heme (22–25). The chloroquine-resistant P. falciparum strain Dd2 is hypersensitive to the antimalarial drugs artesunate, chloroquine, and atovaquone, resulting in accelerated parasite clearance following drug treatment in vivo. Together, our findings demonstrate the Plasmodium-tailed role of a lipocalin family member in hemozoin formation and underscore the role of heme in parasite survival. It is thus crucial for understanding of the mechanisms underlying heme sequestration to provide valuable insights for future drug development efforts.

Author contributions: J.M.M. designed research; J.M.M., B.D., T.B.B., E.v.G., A.B., P.M., T.S., and L.M.C. performed research; J.M.M., B.D., T.B.B., E.v.G., J.P.A., K.M., and M.J.B. analyzed data; and J.M.M., P.M., K.M., and M.J.B. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. D.E.G. is a guest editor invited by the Editorial Board.

This open access article is distributed under Creative Commons Attribution License 4.0 (CC BY).

*To whom correspondence may be addressed. Email: joachim.matz@crick.ac.uk.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2001153117/-/DCSupplemental.

First published June 29, 2020.
future drug development efforts to gain a better understanding of the mechanisms underlying this unique biomineralization event.

In this report, we demonstrate that a parasite-encoded lipocalin called PV5 is crucial for physiological heme biomineralization in *Plasmodium*.

**Results**

**Malaria Parasites Encode a Lipocalin-Like Protein, PV5.** Employing a genome-wide in silico down-scaling approach, we previously identified an essential *P. berghei* PV protein, *PbPV5* (PBANKA_0826700), which has orthologs in all other *Plasmodium* species, including *P. falciparum* (*Pf*) (PF3D7_0925900) (27). Inspection of the PV5 amino acid sequence revealed a striking similarity to members of the functionally diverse lipocalin family, barrel-shaped proteins capable of binding various hydrophobic ligands and protein interaction partners (28). The signature lipocalin fold comprises a short amino-terminal 3_10 helix followed by eight consecutive barrel-forming β-strands, an α-helix, and one more β-strand (Fig. 1A) (29). In addition, PV5 harbors two predicted preceding amino-terminal β-strands specific to *Plasmodium*, as well as a signal peptide. Multiple sequence alignments with lipocalins from phylogenetically distant organisms showed the presence of a highly conserved glycine and two aromatic residues within the structurally conserved region 1 (SCR1) of PV5, a hallmark of the extended calycin superfamily (Fig. 1A) (29).

A transgene bearing structural homology to the bacterial outer-membrane lipocalin Blc from *Escherichia coli* was predicted to share the highest similarity with PV5. Homology modeling guided by the known *E. coli* Blc structure suggests that PV5 shares the overall architecture of the lipocalin family, including the characteristic β-barrel (Fig. 1B). Together, the sequence signatures and predicted structural features support membership of *Plasmodium* PV5 in the calycin protein superfAMILY.

**PV5 Is Trafficked to the Parasite Digestive Compartments.** We first investigated the spatiotemporal expression of *PbPV5* during asexual blood-stage development. Live fluorescence microscopy of transgenic *P. berghei* parasites expressing mCherry-3xMyc-tagged *PbPV5* confirmed that the protein localizes to tubular PV extensions during ring and trophozoite stages and surrounds individual daughter merozoites in segmented schizonts (Fig. 1C) (27). In addition, a substantial fraction of the protein was restricted to the parasite cytoplasm. This was particularly prominent in schizonts, where intraparasitic *PbPV5* appeared to localize to the HZ-containing residual body (Fig. 1C). In merozoites, mCherry fluorescence was concentrated in a punctate intraparasitic region, perhaps signifying storage of *PbPV5* in the dense granules, as has been demonstrated for several other important PV5 proteins (30–32), but this fraction was minimal as compared to the protein contained in the residual body. Quantification of the mCherry-fluorescence intensity in live parasites indicated that *PbPV5* is much more abundant in mature parasite stages than in rings and merozoites, suggesting substantial levels of de novo synthesis throughout parasite maturation (Fig. 1C). In trophozoites, the intraparasitic fraction of *PbPV5* was associated with spherical structures at the parasite periphery (Fig. 1C) and microscopic examination of mechanistically expanded free parasites revealed that these were the HZ-containing DVs (Fig. 1D).

To test whether this localization is conserved across different *Plasmodium* species, we generated and imaged transgenic *P. falciparum* parasites expressing mCherry-tagged *PbPV5* (Fig. 1E and *Appendix*, Fig. S1). In younger parasite stages, fluorescence was restricted to one or more foci in the parasite periphery. With parasite maturation, most of the fluorescent signal overlapped with the HZ crystals and with the signal of the acidotropic dye Lysosensor blue DND-167, which accumulates in the acidic FV (Fig. 1E and *Appendix*, Fig. S1D). This is in good agreement with the reported detection of *PbPV5* in the proteme of purified *P. falciparum* FVs (33). In addition, we frequently observed staining of the PV and of small foci in the parasite cytoplasm (Fig. 1E and *Appendix*, Fig. S1D). Some trophozoites also showed staining of a perinuclear region corresponding to the endoplasmic reticulum (*Appendix*, Fig. S1D). As in *P. berghei*, faint fluorescent foci were observed associated with the budding merozoites in mature *P. falciparum* schizonts (*Appendix*, Fig. S1D).

Western blot analysis indicated the presence of full-length *PbPV5-mCherry* in the transgenic *P. falciparum* parasites (*Appendix*, Fig. S1C). However, the predominant fraction of mCherry appeared to have been cleaved off *PbPV5*, presumably by the FV-resident proteases. This cleavage was not observed in the tagged *P. berghei* line, possibly due to differences in the spacer regions linking *PbPV5* to the fluorescent protein. Subcellular fractionation and Western blot analysis revealed that full-length *PbPV5-mCherry* is freely soluble (Fig. 1F). Together, our findings suggest that in both *Plasmodium* species, PV5 is trafficked to the PV via the secretory pathway and then internalized through endocytosis of host cell cytoplasm to eventually accumulate in the matrix of the parasite’s digestive compartments.

**Transcriptional Deregulation of *PbPV5* Impairs Asexual Parasite Propagation In Vivo.** Our previous attempts to disrupt the genomic *PbPV5* locus resulted only in atypical integration of the targeting construct without perturbing the endogenous gene, which is indicative of essential functions during the asexual blood-stage cycle in vivo (27). As an alternative genetic strategy to analyze *PbPV5* function, we sought to deregulate *PbPV5* expression by employing a promoter swap approach (Fig. 2A). Toward this aim, we generated parasites expressing the endogenous *PbPV5* gene from the promoters of *Plasmodium* translocon of exported proteins 88 (*PTEX88*) or heat shock protein 101 (*HSP101*, respectively (*Appendix*, Fig. S2A and B). Quantitative real-time PCR analysis of the mutants indicated that the knockdown efficiency was ~60% in asynchronous blood stages (*Appendix*, Fig. S2C). Impaired growth prevented quantification of knockdown levels in synchronized ex vivo early blood stages. Strikingly, in the schizont stage, the mutants exhibited significantly elevated *PbPV5* transcript levels, corresponding to ~3.4 (*p* = 5.96 *ptex88*) and ~5.6-fold (*p* = 5.96 *hsp101*) more than in WT schizonts (*Appendix*, Fig. S2C), showing that the promoter swap strategy was successful in deregulatin the translational expression of *PbPV5* throughout the asexual replication cycle.

To investigate whether altered *PbPV5* transcription results in reduced parasite fitness, we examined asexual propagation of the mutants in vivo. Growth of the promoter swap mutants was significantly impaired, with the *p* = 5.96 *ptex88* parasites growing at 80% and *p* = 5.96 *hsp101* parasites at only 54% of the WT growth rate (Fig. 2B). These results underscore the importance of correct *PbPV5* expression during asexual replication of the parasite in vivo.

***PbPV5* Mutants Form Less HZ.** Inspection of Giemsa-stained thin blood films revealed striking morphological differences between WT parasites and the promoter swap mutants. In trophozoite stages, the FV of the mutants was significantly swollen, visible as a large bright area within the parasite cytoplasm close to the nucleus (Fig. 2C). Microscopic quantification revealed this area to be 1.5-fold (*p* = 5.96 *ptex88*) or 1.8-fold (*p* = 5.96 *hsp101*) larger than in WT parasites (*Appendix*, Fig. S3A). Comparison of the light intensities in the swollen FVs and the host cell cytoplasm provided no indication for an accumulation of native hemoglobin within the *PbPV5* mutants (*Appendix*, Fig. S3 B and C). The vacuolar swelling was transient, as mature schizonts did not exhibit comparable abnormalities (Fig. 2C).

Another striking phenomenon was the low visibility of dark granular material in mutant trophozoites, as compared to WT (Fig. 2C). This lack of granularity was most noticeable in the *p* = 5.96 *hsp101* mutant and became particularly apparent during

Matz et al.
the gametocyte stages, where pigment granules are usually very prominent. To validate this finding, we subjected mixed blood-stage parasites to flow cytometry and measured the intensity of the side scattered light, a commonly used proxy for cellular granularity (SI Appendix, Fig. S3D). In agreement with our microscopic analysis, the PbPV5 mutants displayed reduced side scattering and the phenotype was again more severe in the pv5:5′:hsp101 mutant.

Because we suspected a Hz formation defect in the mutants, we fixed intraerythrocytic parasites with methanol and subjected them to polarization microscopy, which exploits the birefringent properties of Hz to specifically visualize the crystals. This approach revealed a weaker signal for the mutants, which correlated with reduced visibility of dark pigment in brightfield (Fig. 2D). Enumeration of individual bright entities showed the presence of fewer Hz-containing structures within the mutants (~80% of WT) (Fig. 2D). Quantification of the polarized light intensity also indicated that pv5:5′:pepx88 parasites form only 64% and the pv5:5′:hsp101 61% of the Hz generated in WT parasites (Fig. 2E). Together, these observations show that perturbation of PbPV5 expression results in reduced heme biomineralization in vivo.

Protracted Hz Extension upon Deregulation of PbPV5 Expression. We next aimed to examine how lower levels of Hz correlate with crystal size. To do this, we isolated Hz from mixed blood-stage parasites and examined the material by scanning electron microscopy (SEM). This confirmed the characteristic high aspect

---

**Fig. 1.** The *Plasmodium* lipocalin PV5 is trafficked to the parasite digestive compartments. (A) PV5 is a lipocalin family member. Secondary structure of *Plasmodium* PV5. Yellow, signal peptide (SP); green, β-strands; purple, helices. Note the two amino-terminal β-strands specific to PV5. Alignments of the SCR1 from different lipocalin family members and PV5 are shown in the upper left corner. Signature residues are highlighted in red. Ecblic, *Escherichia coli* bacterial lipocalin; NcGc, *Homo gammarus* (European lobster) crustacyanin; HsAGP, *Homo sapiens* α-1-acid glycoprotein; HsOBP2A, *H. sapiens* odorant-binding protein 2A; HsRBP4, *H. sapiens* retinol-binding protein 4; PbPV5, PV5 from *P. berghei* and *P. falciparum*. (B) Structure homology modeling predicts a lipocalin fold for PV5. Shown are the experimentally validated structure of Ecblic (blue, Left, residues 27 to 175, PDB ID 3MBT), the derived model of PV5 (red, Center, residues 35 to 214) using Ecblic as a homology template, and an overlay (Right). Modeling was performed with SWISS-MODEL and supported by I-TASSER. Amino acid sequence identity is 20%, similarity calculated from BLOSUM62 substitution matrix is 0.3. (C) Dual protein localization of PV5 to extensions of the PV and to intraparasitic structures in *P. berghei*. Transgenic parasites expressing the PV marker GFP*PV* and the endogenous PbPV5 gene fused to mCherry-3xMyc were imaged live (27). Shown are the mCherry (red, first row) and GFP channels (green, second row), a merge of both signals (third row) and a merge of differential interference contrast images (DIC), and Hoechst 33342 nuclear stain (blue, fourth row). Cyan arrowheads, PbPV5 in PV tubules. Numbers represent normalized mCherry intensity values obtained by quantitative live fluorescence microscopy, n = 44 parasites. (D) Intraparasitic PV5 localizes to Hz-containing DVs in *P. berghei*. Parasites (1 to 2 μL) were incubated under a coverslip (22 × 40 mm) for several minutes, leading to lysis of the host erythrocyte and the PV, and to mechanical expansion of the parasite (Upper). Shown are a merge of DIC and Hoechst 33342 nuclear stain (blue, first column), the signal of tagged PbPV5 (red, second column), as well as a merge of all three channels (third column). Swelling of PbPV5-containing DVs was observed 10 min later (Lower). Note the even distribution of PbPV5 throughout the swollen DVs. (E) PV5 localizes to the central PV, intraparasitic vesicles and to the PV in *P. falciparum*. Transgenic parasites expressing the endogenous PV5 gene fused to mCherry were imaged live in the presence of Lysosensor blue DND-167 (LS Blue). Shown are the signals of mCherry (red, first column), LS Blue (green, second column), a merge of both signals (third column), and DIC images (fourth column). Cyan arrowhead, PV5 in PV (Upper). Yellow arrowhead, PV5 in small intraparasitic vesicles (Lower). (Scale bars, 5 μm.) (F) PV5 is a soluble protein. Subcellular fractionation was performed using the PbPV5-tagged *P. berghei* line, which also expresses the soluble marker GFP*PV*, and a *P. berghei* line expressing the transmembrane protein PbEXP2 fused to mCherry-3xMyc (62). Cell lysates were centrifuged and resulting membrane pellets were subjected to solubilization with Na$_2$CO$_3$ and Triton X-100 (TX-100). Input, supernatant (SN), and pellet fractions (P) were analyzed by Western blot using anti-mCherry and anti-GFP primary antibodies.
Deregulated expression of PV5 impacts Hz formation in *Plasmodium berghei*. (A) Schematic representation of the genotypes of WT (Top), and transgenic *pv5::5ptex88* (Middle) and *pv5::5'hsp101* parasites (Bottom). In the mutants, the endogenous PbPV5 promoter (black) was exchanged for the promoter of *PbPTEX88* (dark blue) or *PbHSP101* (light blue), respectively. (B) Reduced parasite proliferation upon PbPV5 promoter swapping. Asexual blood-stage development was analyzed using the intravitral competition assay (53). Average parasite multiplication rates were 11.4 (WT), 9.1 (*pv5::5ptex88*), and 6.2 (*pv5::5'hsp101*). Shown are mean values ± SD. **P < 0.001; two-way ANOVA. n = 3 independent infections. (C) Morphology of trophozoite, schizont, and gametocyte stages in the WT, *pv5::5ptex88* and *pv5::5'hsp101* lines as observed by Giemsa staining. Note the lack of prominent dark pigment granules in mutant trophozoites and gametocytes as well as the dilation of the FV in mutant trophozoites. (Scale bar, 5 μm.) (D and E) PbPV5 is required for efficient Hz formation. (D) Trophozoites were visualized by polarization microscopy (Left) and bright field imaging (Right). Numbers indicate the mean quantity of bright puncta in polarization images. Significance values are shown for the comparison of the mutants with WT. (Scale bar, 5 μm.) (E) Quantitative polarization microscopy. Depicted are individual and mean intensity values of reflected polarized light in methanol-fixed WT, *pv5::5ptex88* and *pv5::5'hsp101* trophozoites (bars, upper graph). Only trophozoites of similar size were analyzed (lower graph). Depicted are mean values ± SD. n.s., nonsignificant; **P < 0.001; one-way ANOVA and Tukey’s multiple comparison test. (F) Hz crystal architecture. In WT *Plasmodium* parasites Hz assembles as triclinic high aspect ratio parallelepipeds (64). Characteristic crystal axes and faces are indicated. (G) SEM images of Hz purified from WT, *pv5::5ptex88* and *pv5::5'hsp101* mixed blood-stage parasites. The angle between the regularly shaped *pv5::5'hsp101* parent crystal and the outgrowth is indicated. n = 130 crystals. (Scale bars, 100 nm.) (H) Crystal morphology in situ. Shown are TEM images of WT, *pv5::5ptex88* and *pv5::5'hsp101* schizonts (Upper) as well as their residual body at higher magnification (Lower). Abnormal crystal shapes in the mutants are indicated by arrowheads. (Scale bars, 500 nm.) (I) Hyperactive Hz growth is unidirectional. Shown are the dimensions of whole individual Hz crystals extracted from WT, *pv5::5ptex88*, and *pv5::5'hsp101* parasites, including the area exposed to the electron beam (Left) as well as the length (Center) and mean width of the crystals (Right). The theoretical dimensions of the *pv5::5'hsp101* parent crystals were interpolated and are depicted as well. Shown are mean values ± SD. n.s., nonsignificant; **P < 0.005; ***P < 0.001; one-way ANOVA and Tukey’s multiple comparison test. n = 100 crystals. (J) Normal aspect ratio of *pv5::5'hsp101* parent crystals. Shown are the aspect ratios of whole Hz crystals from WT parasites and of the parent crystal from *pv5::5'hsp101*-generated Hz. Depicted are individual and mean values (bars), n.s., nonsignificant; Student’s t test. n = 100 crystals. (K and L) Unaltered crystalline order in Hz of *pv5::5'hsp101* parasites. (K) Depicted are TEM images (Left) of Hz purified from WT and *pv5::5'hsp101* parasites as well as their corresponding electron diffraction patterns (Right) showing comparable resolution of the Bragg peaks. Dashed circles demark a resolution of 0.5 Å⁻¹. (Scale bars, 200 nm.) (L) Plot of the radial maximum diffracted intensity as a function of resolution. Data were normalized to the average median intensity at 0.18 Å⁻¹ in order to correct for differences in diffracted volume. Arrowheads denote overlapping peaks. n = 10 (WT) and 18 (*pv5::5'hsp101*) diffract dataset.

Matz et al

PNAS | July 14, 2020 | vol. 117 | no. 28 | 16549
ratio parallelogram morphology of WT Hz (Fig. 2 F and G). Strikingly, this was not the case for crystals isolated from the promoter swap mutants. Hz from both transgenic parasite strains exhibited highly irregular shapes and rough edges and showed only few of the distinctive Hz crystal vertices (Fig. 2G). Crystals from pv5::5 ptx88 parasites most often had a pointed and canine tooth-like appearance, extending from a single straight crystal face. These abnormalities were even more pronounced in the pv5::5 hsp101 mutant, where in most cases there was a region of normal crystal morphology with two or three straight edges, corresponding to the (100), (011), and (001) faces. From this regular parent crystal emerged an enormous outgrowth that usually surpassed the dimensions of the parent crystal (Fig. 2G). This outgrowth consistently grew at an obtuse angle of ∼105° in relation to the dominant c axis of the parent crystal, although accurate determination of the angle was complicated by the slightly bent and irregular shape of the outgrowth, which might be attributed to the space restrictions encountered in the P. berghei DVs. The outgrowth’s angle was not reflected in the physiological morphology of Hz (Fig. 2 F and G) and at least two faces of the regular parent crystal appeared to be involved. Indeed, in most cases the outgrowth emerged from sites where the (100) and (011) faces meet and always grew along a plane corresponding to one of the original crystal faces (Fig. 2G). Other crystal formations were also observed, albeit at lower frequency, namely with some with multiple crystal branches and some with very rough surfaces (SI Appendix, Fig. S3E). We observed similar crystal abnormalities in situ by transmission electron microscopy (TEM) of purified schizonts (Fig. 2H).

Despite the lower overall levels of Hz formed and the abnormal crystal morphology, we found that the mutants formed larger Hz crystals, as indicated by the area exposed to the SEM electron beam (Fig. 2I). The pv5::5 hsp101 mutant formed the largest crystals, which were ∼180% of WT size, while the pv5::5 ptx88 crystals were at 130%. Importantly, the mutant Hz crystals displayed greater dimensions only in length but not in width because of the unidirectional expansion of the outgrowth (Fig. 2I). Examination of the parent crystals from pv5::5 hsp101 parasites showed that these were roughly half the size of whole WT crystals (Fig. 2I). The aspect ratios of WT crystals and the pv5::5 hsp101 parent crystals were identical, together indicating that a period of normal crystallization during early pv5::5 hsp101 parasite development is followed by irregular crystal extension later on (Fig. 2J). Hz morphology was unaffected in an unrelated slow-growing mutant (34) and in chloroquine-treated WT parasites, suggesting that Hz crystal dysmorphism, as observed in the PfPV5 mutants, is not a phenomenon generally associated with poor parasite growth or mortality (SI Appendix, Fig. S3 F and G).

To determine whether the crystalline order was affected by deregulation of PfPV5, we obtained electron diffraction patterns from WT- and pv5::5 hsp101-derived Hz (Fig. 2K) and analyzed the maximum diffracted intensities in concentric bins as a function of resolution. There was no difference in the drop-off of diffracted intensity between WT and mutant and the peak positions of the maxima corresponded (Fig. 2L). Differences in the magnitude of individual peaks can be attributed to preferential orientation, especially of the pv5::5 hsp101-derived crystals, which most often come to lie at their (100) faces. Together, these data indicate no differences in crystalline order or unit cell upon functional impairment of PfPV5. We conclude that altered crystal morphology is not caused by alternative nucleation into a different hematin polymorph. Thus, deregulation of PfPV5 leads to the formation of ordered elongated Hz crystals with a highly variable and abnormal architecture.

**Loss of PV5 Causes Hz Branching in P. falciparum.** To investigate the consequences of PV5 disruption, we generated a conditional PV5 knockout line of the human pathogen P. falciparum, allowing rapamycin (RAP)-induced DiCre-mediated excision of the PfPV5 gene (Fig. 3A and SI Appendix, Figs. S1A and S4A). Correct modification of the locus was indicated by diagnostic PCR and by the successful tagging of PfPV5 with a 3xHA tag, as demonstrated by Western blot and immunofluorescence analysis (Fig. 3 B and C and SI Appendix, Fig. S3 B–D). The 3xHA-tagged PfPV5 localized to the PV and to intraparasitic vesicular structures (SI Appendix, Fig. S4B). Surprisingly, no signal was detected in the FV. However, global inhibition of the parasite’s cytochrome b oxidase with Eth6 restored localization of 3xHA-tagged PfPV5 to the FV and significantly increased the amount of tagged protein, together suggesting that the 3xHA tag is proteolytically cleaved upon FV delivery (SI Appendix, Fig. S4 C and D).

Treatment of pv5::3xHA:loxP parasites with RAP led to efficient gene excision and complete loss of PfPV5 protein expression during the same intraerythrocytic cycle (Fig. 3 B and C and SI Appendix, Fig. S4E). This did not detectably affect parasite maturation but did result in a modest merozoite invasion defect upon rupture of the PfPV5-null schizonts, reducing parasite replication (SI Appendix, Fig. S5). Extended monitoring of the RAP-treated parasites indicated an estimated fitness cost of ∼40% (SI Appendix, Fig. S5B). This is in good agreement with a proposed mutagenesis index score of 0.22 from a genome-wide piggyBac insertion screen (35). Accordingly, PfPV5, although not essential under standard P. falciparum culture conditions, is required for optimal parasite propagation in vitro.

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

The dramatic abnormalities in Hz crystal morphology resulting from ablation of PfPV5 were even more evident by SEM analysis. While the regular morphologies of WT crystals, including the brick-like morphology, individual Hz units from RAP-treated pv5::3xHA:loxP parasites appeared smaller and more globular (Fig. 3H and SI Appendix, Table S1). The surfaces of these Hz units were covered in scales and stubby crystal buds. Individual crystals of comparable bud-like dimensions were not observed, indicating a branching rather than an aggregation phenomenon. In some instances, a lower number of crystal buds allowed the visualization of an ordered Hz core, suggesting that branching is initiated from a regular parent crystal (Fig. 3H). We detected several morphological intermediates between slightly scaled Hz, highly branched crystal units, and fused congregations (Fig. 3H). We frequently observed enormous aggregates of spherical proportions, mirroring the shape of the central FV (Fig. 3H). This suggested that hyperactive crystal branching in the absence of PfPV5 caused individual studded Hz units to stick together and subsequently merge during Hz growth, which might explain the absence of motion in the parasite FV (Fig. 3G). NonRAP-treated pv5::3xHA:loxP control parasites mainly formed regular Hz crystals; however, 27.5% of the crystals exhibited a modest degree of branching (Fig. 3H and SI Appendix, Table S1). Furthermore, crystal size and aspect ratio were reduced in comparison to WT parasites, suggesting a moderate functional impairment of 3xHA-tagged PfPV5 (Fig. 3H and SI Appendix, Table S1). Our data demonstrate that PV5 is critical for the efficient sequestration of hemoglobin and for the ordered expansion of Hz crystals in P. falciparum.
Efficient Hemoglobin Processing in the Absence of *PfPV5*. To exclude an indirect effect mediated by defective hemoglobin catabolism, we examined the hemoglobin content of saponin-released parasites. *PfPV5*-deficient ring stages and trophozoites contained normal quantities of internalized hemoglobin (Fig. 4A). Only mature segmented *pv5-3xHA:loxP* schizonts exhibited slightly elevated concentrations of residual hemoglobin upon induction (Fig. 4B). However, this was also observed in WT schizonts upon RAP treatment, suggesting a minor compound-specific effect.

In WT parasites, inhibition of the vacuolar cysteine proteases with E64 caused significant bloating of the FV, because of an accumulation of undigested hemoglobin (Fig. 4C). A comparable bloating phenotype was not observed in untreated *PfPV5*-null parasites, which retained normal FV morphology. We also noted that, unlike *PfPV5* deletion, E64 treatment produced no changes in the architecture of Hz (Fig. 4D), and *PfPV5*-deficient parasites retained normal E64 sensitivity (Fig. 4E and F). Furthermore, saponin treatment released normal amounts of hemoglobin from schizont-infected erythrocytes in the absence of *PfPV5*, indicating unaltered hemoglobin ingestion (Fig. 4B).

Combined, these findings suggest that inhibition of hemoglobin catabolism does not directly translate into altered Hz morphology and that *PfPV5* is not involved in the overall consumption of host cell cytoplasm.

*PfPV5* Expression Influences Antimalarial Drug Sensitivity In Vivo. In the light of our evidence implicating *PV5* in Hz formation, we next tested whether parasites with affected *PV5* function display altered sensitivity toward chloroquine, a 4-aminoquinoline which is thought to inhibit heme biominalization in *Plasmodium*...
survival, respectively), followed by atovaquone (3.5% and 8.6%)
hypersensitivity toward chloroquine (2.1% and 1.4% of WT
fourth day of drug treatment suggested the greatest degree of
reduced parasite multiplication is unlikely to cause drug hyper-
sensitivity. Collectively, these results suggest that interference
with PV5 activity toward drug-mediated insult during in vivo blood infection.

Fig. 4. PPV5 regulates heme sequestration independently from hemoglobin processing. (A and B) Normal uptake and digestion of host cell hemoglobin by PPV5-deficient parasites. (A) Western blot analysis of induced and noninduced pv5-3xHA:loxP parasites released from their host cells by saponin lysis 4 and 24 h after invasion. (B) Forty-six hours after invasion, induced and noninduced pv5-3xHA:loxP or WT (B11) schizonts were released by saponin treatment. Resultant supernatants (SN) and schizont pellets were isolated. Blots were probed with antibodies directed against human hemoglobin a (Hb) and PbiP. Note an increase in intraparasitic hemoglobin upon RAP treatment in both pv5-3xHA:loxP and WT (B11) schizonts. (C) PPV5-deficient schizonts exhibit no vacuolar bloating. Shown are pv5-3xHA:loxP parasites treated from the ring stage onward with DMSO or RAP (Upper) and P. falciparum WT parasites treated from 24 h postinvasion onward with DMSO or 21.7 μM E64 (Lower). Cyan arrowhead, bloated food vacuole. (Scale bar, 5 μm.) (D) Inhibition of hemoglobin catabolism does not result in abnormal Hz morphology. Shown is an SEM image of Hz crystals isolated from the E64-treated P. falciparum WT parasites shown in C. (Scale bar, 500 nm.) (E and F) PPV5-deficient parasites display unaltered sensitivity toward E64. DMSO- and RAP-treated pv5-3xHA:loxP parasites were grown in various concentrations of E64 from the ring stage onward. (E) Nuclear SYBR Green fluorescence 44 h after invasion. (F) Parasite multiplication under static conditions following a 36-h incubation in the presence of E64 and subsequent inhibitor washout. Mean values ± SD are shown. n.s., nonsignificant; fitting of IC50 values following nonlinear regression, n = 3.

Discussion
In this work, we have demonstrated that Hz formation in malaria parasites involves a secreted calycin family member called PV5. While transcriptional deregulation of PV5 in P. berghei resulted in protracted Hz elongation along a preexisting crystal plane, multidirectional branching was observed in the complete absence of PV5 in P. falciparum. Species differences aside, the disparity between those two phenotypes can be explained by the unique transcriptional dynamics observed in the P. berghei mutants. Here, the hyperactive crystal elongation that follows an initial phase of normal Hz growth coincides with a substantial increase in PfPV5 transcript abundance during late parasite development. In their physiological context, PTEX88 and HSP101 are coexpressed as members of the same protein complex, with HSP101 being more abundant than PTEX88 (42, 43), as supported by our qPCR analysis. Thus, the increased phenotypic severity in the pv5::5 hsp101 mutant over the pv5::5 pte88 mutant indicates that the deficiencies in Hz formation can be attributed to late overexpression of PV5. Together with the chaotic Hz crystal branching observed in PV5-deficient P. falciparum parasites, this leads us to propose that PV5 acts as a facilitator of unidirectional Hz extension.

The highly polymorphic appearance of Hz in the absence of PV5 is reminiscent of the variable crystal morphology following β-hematin formation in vitro (12). While the exact biophysical mechanisms that govern PV5-mediated Hz morphogenesis remain to be delineated, our results support a model of conventional crystallization within the aqueous milieu of the parasite’s
The molecular functions of PV5 might also involve protein-protein interactions, as lipocalins from various organisms share the tendency to oligomerize and form complexes with different proteins and membrane receptors (28, 29).

The crystal branching characterizing PV5-deficient P. falciparum parasites could also be elicited by nonheme impurities adsorbing onto the crystal surface, where they would generate novel nucleation sites (44). In this alternative functional model, PV5 could act to bind these impurities to create a vacuolar environment permissive for proper biominalization. PV5-deficient parasites maintain an intact FV with a transvacuolar proton gradient as indicated by staining with LysoSensor blue DND-167 (SI Appendix, Fig. S7A). In the absence of indications for FV membrane damage, differences in leakage of impurities from the parasite cytoplasm appear unlikely. However, we cannot rule out the possibility that specific ions or organic compounds are more abundant in the vacuolar matrix of PV5-deficient parasites.

The striking localization pattern of PV5 is suggestive of initial secretion into the PV followed by endocytic uptake. Since we ruled out an involvement of PV5 in hemoglobin ingestion and catabolism, the transient vacuolar swelling in the P. berghei mutants likely reflects a secondary effect mediated by the grave deficiencies in heme sequestration. This is supported by a previous report demonstrating that defective hemoglobin catabolism causes chloroquine resistance in P. berghei (48), a phenotype that is in stark contrast to our own observations.

Our previous experiments had indicated that PbPV5 is essential for asexual blood-stage development in P. berghei (27), and the deregulation of PbPV5 transcription in the mutants described here indeed led to a striking fitness cost during in vivo infection. In contrast, complete ablation of PVPV5 in P. falciparum only resulted in a moderate fitness loss in vitro. The apparent dispensability of PVPV5 is therefore puzzling, but can be resolved by the notion that in vitro culture does not necessarily reflect all adversities encountered during host infection. For example, Hz-mediated stiffening of the residual body in the absence of PV5 might hinder the passage of infected cells through capillaries or through the interendothelial slits of the spleen, a scenario that would result in parasite elimination only during in vivo infection. Similarly, we observed enhanced drug susceptibility only in the P. berghei mutants. Thus, it appears plausible that any imbalance in heme biominalization in the absence of PV5 is compensated for under optimal culture conditions, revealing its adverse effects only during in vivo infection. We thus propose that the molecular mechanism by which PV5 regulates Hz formation might involve host factors that are not encountered in a cell culture setting.

The observation of enhanced drug susceptibility in the PbPV5 mutants is in agreement with the notion that aberrant heme biominalization chemo-sensitizes parasites to partner drugs. Although oxidative stress and lipid peroxidation remained unchanged upon deletion of PVPV5 (SI Appendix, Fig. S7B), the reduced Hz formation efficiency in the PV5 mutants suggests elevated heme concentrations within the parasite mediating the fitness loss and drug hypersensitivity.

In summary, we provide conclusive evidence for a parasite factor mediating Hz formation in vivo. Since this Plasmodium-encoded member of the calycin superfamily also governs parasite viability and susceptibility to drug-mediated insult during blood infection, our observations reinforce Hz formation as an excellent pathway for therapeutic intervention. Further investigation of the parasite heme detoxification machinery in vivo, as exemplified here for PV5, will significantly improve our understanding of this unique biominalization process and holds great promise for the development of novel malaria intervention strategies.

Materials & Methods

Structure Homology Modeling. Structure homology modeling was performed using the SWISS-MODEL server (49). PVPV5 (residues 35 to 214) was aligned to the experimentally validated structure of EcBlt (residues 27 to 175, PDB ID 2CSV).

Fig. 5. Targeting PV5 function results in parasite hypersensitivity toward antimalarial drugs in vivo. Enhanced drug susceptibility of the PdPV5 mutants in vivo. The 5 × 10⁶ mCherry-fluorescent P. berghei WT parasites were injected into SWISS mice together with 5 × 10⁶ GFP-fluorescent pv5::5 ptex88 or pv5::5 hsp101 parasites, respectively. From day 3 onward, mice were treated with curative doses of (A) chloroquine (288 mg/L in drinking water, ad libitum), (B) artesunate (50 mg/kg body weight, i.p.), (C) atovaquone (1.44 mg/kg body weight, i.p.) or (D) sulfadoxine (1.4 g/L in drinking water, ad libitum). Subsequent parasitemia values were determined daily by flow cytometry of peripheral blood (41). Values are normalized in each case to the parasitemia on day 0 of treatment. Shown are mean values ± SEM. n.s., nonsignificant; *P < 0.05; **P < 0.01; Two-way ANOVA. n = 3 independent experiments.
code 3MBT) (50), resulting in a global model quality estimate value of 0.39 and a qualitative model energy analysis value of −5.48. Modeling was confirmed with I-TASSER (51), which also identified Blc (PDB ID code 2ACO) as the most closely related structural analog of PPVPs with a template modeling score of 0.75. Due to a lack of sequence similarity, the structure of the extended PPVPs amino terminus was not modeled.

**P. berghei** Cultivation. *P. berghei* parasites were propagated in SWISS mice under constant drug pressure with pyrimethamine (70 mg/L in drinking water, ingested ad libitum; MP Biomedicals) to avoid homology-induced reversion of the promoter swap mutants to the original WT genotype. This was routinely checked by diagnostic PCR of genomic DNA as shown in *SI Appendix*, Fig. S2 A and B. Drug pressure was withdrawn 5 d prior to experimentation to avoid secondary effects of pyrimethamine treatment. Pyrimethamine-resistant *Berred* WT parasites (53) were treated accordingly. All infection experiments were carried out in strict accordance with the German "Tierschutzgesetz in der Fassung vom 22. Juli 2009" and the Directive 2010/63/EU of the European Parliament and Council “On the protection of animals used for scientific purposes.” The protocol was approved by the ethics committee of the Berlin state authority (“Landesamt für Gesundheit und Soziales Berlin,” permit no. G0294/15).

*P. berghei* growth was determined with the previously described intravitreal challenge assay (53). In short, 500 mCherry-fluorescent Berred WT and *P. berghei* Nycodenz gradient centrifugation (54). per day injected intraperitoneally; Sigma Aldrich), or sulfadoxine (1.4 g/L in injected intraperitoneally; GlaxoSmithKline), artesunate (50 mg/kg body weight injected intraperitoneally; Sigma Aldrich), atovaquone (1.44 mg/kg body weight per day in-jected intraperitoneally; Sigma-Aldrich), and various concentrations of E64 or equivalent volumes of DMSO from the early ring stage onward. Growth assays were performed as described previously (55) and parasitaemia as well as DNA content were measured by flow cytometry using the nuclear dye SYBR Green (1:10,000; Thermo Fisher Scientific). Staining with 24 h after inoculation and the fold-change was calculated. For drug- and 106 WT and 5 × 106 parasites were imaged live using an AxioImager Z2 epifluorescence microscope equipped with an AxioCam MR3 camera (Zeiss). For mechanical parasite expansion, 1 to 2 μL of infected blood was incubated under a 22 × 40-mm coverslip for several minutes until erythrocyte lysis became apparent. *P. falciparum* parasitoids were imaged on an Eclipse Ni light microscope (Nikon) fitted with a C1440 digital camera (Hamamatsu). Immunofluorescence analysis was performed with *P. falciparum* pv5-tag-GFP parasites that were fixed in 4% formaldehyde using rat anti-HA (1:500; Sigma Aldrich) and rabbit anti-SEAR5 (1:500) (55) primary antibodies in combination with appropriate fluorescein-conjugated secondary antibodies (1:1,000; Thermo Fisher Scientific). Scanning Electron Microscopy. H2 was isolated from Nycodenz (*P. berghei*) or Percoll (*P. falciparum*) infected enrich drug blood cells. Cells were lysed in water at room temperature for 20 min, followed by a 10-min centrifugation step at 17,000 × g. The pellet was resuspended in 2% SDS in water and centrifuged as before. Three more washing steps with 2% SDS were then followed by three washes with 2% SDS in water. Four washing steps with water were performed, and the pellets were resuspended and transferred onto round glass coverslips (12 mm), where they were dried. Coverslips were mounted on SEM specimen stubs, sputter-coated, and then imaged on a LEO 1430 (Zeiss) or on a Quanta FEG 250 SEM (Thermo Fisher Scientific).

**P. falciparum** Cultivation. *P. falciparum* parasites were propagated in type A human red blood cells at 90% NaCl, 5% CO2, and 5% O2 at 37 °C in RPMI 1640 (Thermo Fisher Scientific) supplemented with 2 mM L-glutamine. Parasites were routinely synchronized using a combination of Percoll gradient centrifugation and sorbitol lysis and were treated with 100 nM RAP, various concentrations of E64 or equivalent volumes of DMSO from the early ring stage onward. Growth assays were performed as described previously (55) and parasitaemia as well as DNA content were measured by flow cytometry using the nuclear dye SYBR Green (1:10,000; Thermo Fisher Scientific). For invasion assays, mature schizonts were incu-bated at 2% initial parasitaemia under static or shaking (120 rpm) conditions, as confirmed by flow cytometry. Parasitaemia was measured again 24 h after inoculation and the fold-change was calculated. For drug- and inhibitor-response analyses, *psv*-3xHA:loxP/rap ring-stage cultures at 0.5 to 2% parasitaemia were treated with varying concentrations of chloroquine, WR143189, atovaquone, or E64. Parasitaemia for drug RAP or E64 in a 96-well format. Parasitaemia was determined 3 d later and the fold-change was calculated. E64 was washed out after 36 h to allow for reinvahion. In addition, nuclear SYBR Green fluorescence was determined by flow cytometry following 2K CCD camera (Tröndle Rüdelsrichtverstärkersystem). Transmission Electron Microscopy. Infected erythrocytes were fixed in 2.5% glutaraldehyde, embedded in beads of 2% agarose, treated with 1% osmium tetroxide, and further contrasted en bloc using 0.5% uranyl acetate. Following dehydration in a graded series of ethanol and propylene oxide, beads were embedded in epoxy resin and cured at 60 °C for at least 24 h. The 60-nm sections were produced with a Reichert Ultracut S ultramicrotome (Leica) using a diamond knife. Sections were retrieved on copper hexagonal mesh grids, and stained with 2% uranyl acetate and Reynolds’s lead citrate before imaging on an EM 900 TEM (Zeiss) equipped with a wide-angle slow-scan 2K CCD camera (Tröndle Rüdelsrichtverstärkersystem).

Electron Diffraction. Purified Hz was added to glow-discharged Lacey carbon films on 400-mesh copper grids, which were then transferred to a Vitrobot Mark IV plunge freezer (Thermo Fisher Scientific) with 100% humidity at 7 °C. The grids were blotted for 3 s with blot force 1 and plunged frozen in liquid ethane cooled by liquid nitrogen. Electron diffraction data were collected on a Talos cryoelectron microscope (Thermo Fisher Scientific) operated at 200 keV equipped with a hybrid pixel Temipex detector (512 × 512 pixels, 55 × 55 μm pixel size; Amsterdam Scientific Instruments). Still and rotation (70°) datasets were collected with a beam size of 2 μm. The recording time varied between 15 and 100 s. To determine the resolution, a powder pattern of an aluminum diffraction standard was recorded. Since the Hz crystals had a strong tendency to stick together, we measured diffraction data of crystal clusters. For each individual dataset, we determined the location of the central electron beam and shifted the patterns to make the beams coincide. Since crystal cluster preventing indexing of the diffraction data, the radial median and maximum intensities were determined as a function of resolution. Hereafter, the WT and *psv*-5:5 hap101 datasets were averaged and normalized to the local background median intensity at 0.18 Å−1.

**Subcellular Fractionation and Immunoblotting.** *P. falciparum* parasites were released from erythrocytes by treatment with 0.15% saponin in PBS. Murine erythrocytes infected with the *psv*-tag-GFP+ or *exp2-mCherry* *P. berghei* lines (62) were purified on a Nycodenz gradient and lyzed hypotonically for 1 h on ice in 10 μL Tris-HCl, pH 7.5. *P. berghei* lysates were centrifuged for 50 min at 100,000 × g. Membrane pellets were resuspended in 0.1 M Na2CO3 in PBS or in 1% Triton X-100 in PBS, respectively, and centrifuged for 50 min at 100,000 × g. Proteins were separated on SDS-polyacrylamide and...
Quantitative Hz Analysis. Hz was visualized and quantified microscopically in methods to fixed infected red blood cells. Hz from P. berghei was analyzed by reflection contrast polarized light microscopy using a Leica DMR widefield microscope equipped with a ProgRes MF camera (Jenoptik) and the POL filter set 513813 (Leica). Hz from P. falciparum was analyzed by transmitted polarized light (488 nm) microscopy using an LSM 710 confocal microscope (Zeiss) equipped with a crossed polarizer in the condenser. Cellular granularity was approximated by quantification of side scattered light using an LSRFortessa flow cytometer (BD Biosciences). Hz cell dimensions were analyzed using Fiji. Due to the variation in the P. berghei mutant's crystal width, individual crystals were divided into nine evenly spaced segments along the dominant axis. The width of each segment was determined and the values were averaged. For the psV5:5′hsps101 parent crystal and the crystal outgrowth, transects were drawn through the central axis of either structure and their shared angle was determined. Hz movement within the FV of psV5-3HAloxP parasites was imaged live 36 h following treatment with DMSO or RAP.

Data Availability. All data are presented in the main text or are available in the SI Appendix. Generated plasmodium and parasite lines are available from the corresponding author.

ACKNOWLEDGMENTS. We thank Volker Brinkmann and Christian Gooss- mann (Max Planck Institute for Infection Biology, Berlin) as well as Kurt Anderson (Francis Crick Institute, London) for assistance with polarization microscopy; Darren Flower (Aston University, Birmingham) and Leslie Leiserson (Weizmann Institute of Science, Rehovot) for fruitful discussion; and the Center for Cellular Imaging and NanoAnalytics for support and use of the electron microscope. This work was supported by a stipend from the Deutsche Forschungsgemeinschaft (DFG) to J.M.M. (19345764) and in part by funding to J.M.M., L.M.C., A.B., and M.J.B. from the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001043), the UK Medical Research Council (FC001043), and the Wellcome Trust (FC001043). J.M.M., B.D., P.M., T.S., and K.M. were also supported by the Humboldt University and by the Alliance Berlin Canberra “Crossing Boundaries: Molecular Interactions in Malaria,” which is funded by a grant from the DFG for the International Research Training Group 2290 and the Australian National University. We further acknowledge funding from the Swiss National Science Foundation Project 31003A_17002 (to T.B.B.) and Project 200021_165669 (to T.B.B. and J.P.A.).
45. R. Huber et al., Molecular structure of the bilin binding protein (BBP) from Pieris brassicae after refinement at 2.0 Å resolution. J. Mol. Biol. 195, 499–513 (1987).

46. R. Huber et al., Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect Pieris brassicae. J. Mol. Biol. 195, 423–434 (1987).

47. H. M. Holden, W. R. Rypniewski, J. H. Law, I. Rayment, The molecular structure of insecticyanin from the tobacco hornworm Manduca sexta L. at 2.6 Å resolution. EMBO J. 6, 1565–1570 (1987).

48. J. W. Lin et al., Replication of Plasmodium in reticulocytes can occur without hemozoin formation, resulting in chloroquine resistance. J. Exp. Med. 212, 893–903 (2015).

49. A. Waterhouse et al., SWISS-MODEL: Homology modelling of protein structures and complexes. Nucleic Acids Res. 46, W296–W303 (2018).

50. A. Schiefner, L. Chatwell, D. A. Breustedt, A. Skerra, Structural and biochemical analyses reveal a monomeric state of the bacterial lipocalin Blc. Acta Crystallogr. D Biol. Crystallogr. 66, 1308–1315 (2010).

51. J. Yang, Y. Zhang, Protein structure and function prediction using I-TASSER. Curr. Protoc. Bioinformatics 52, S.8.1–S.8.15 (2015).

52. V. Campanacci, R. E. Bishop, S. Blangy, M. Tegoni, C. Cambillau, The membrane bound bacterial lipocalin Blc is a functional dimer with binding preference for lysophospholipids. FEBS Lett. 580, 4877–4883 (2006).

53. J. M. Matz, K. Matuschewski, T. W. Kooij, Two putative protein export regulators promote Plasmodium blood stage development in vivo. Mol. Biochem. Parasitol. 191, 44–52 (2013).

54. C. J. Janse et al., High efficiency transfection of Plasmodium berghei facilitates novel selection procedures. Mol. Biochem. Parasitol. 145, 60–70 (2006).

55. R. Stallmach et al., Plasmodium falciparum SERAS plays a non-enzymatic role in the malarial asexual blood-stage lifecycle. Mol. Microbiol. 96, 365–387 (2015).

56. J. M. Matz, T. W. Kooij, Towards genome-wide experimental genetics in the in vivo malaria model parasite Plasmodium berghei. Pathog. Glob. Health 109, 46–60 (2015).

57. T. W. Kooij, M. M. Rauch, K. Matuschewski, Expansion of experimental genetics approaches for Plasmodium berghei with versatile transfection vectors. Mol. Biochem. Parasitol. 185, 19–26 (2012).

58. C. R. Collins et al., Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. Mol. Microbiol. 88, 687–701 (2013).

59. M. L. Jones et al., A versatile strategy for rapid conditional genome engineering using loxp sites in a small synthetic intron in Plasmodium falciparum. Sci. Rep. 6, 21800 (2016).

60. E. Knuepfer, M. Napiorkovska, C. van Onsij, A. A. Holder, Generating conditional gene knockouts in Plasmodium—A toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. Sci. Rep. 7, 3881 (2017).

61. J. A. Thomas et al., Development and application of a simple plaque assay for the human malaria parasite Plasmodium falciparum. PLoS One 11, e0157873 (2016).

62. J. M. Matz et al., The Plasmodium berghei translocon of exported proteins reveals spatiotemporal dynamics of tubular extensions. Sci. Rep. 5, 12532 (2015).

63. E. Knuepfer et al., RON12, a novel Plasmodium-specific rhoptry neck protein important for parasite proliferation. Cell. Microbiol. 16, 657–672 (2014).

64. R. Buller, M. L. Peterson, Ö. Almarsson, L. Leiserowitz, Quinoline binding site on malaria pigment crystal: A rational pathway for antimalaria drug design. Cryst. Growth Des. 2, 553–562 (2002).