Maternally supplied S-acyl-transferase is required for crystalloid organelle formation and transmission of the malaria parasite

Short Title: Palmitoylation and *Plasmodium* organelle formation

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Abstract

Transmission of the malaria parasite from the mammalian host to the mosquito vector requires the formation of adequately adapted parasite forms and stage-specific organelles. Here we show that formation of the crystalloid—a unique and short-lived organelle of the *Plasmodium* ookinete and oocyst stage required for sporogony—is dependent on the precisely timed expression of the S-acyl-transferase DHHC10. DHHC10, translationally repressed in female *P. berghei* gametocytes, is activated translationally during ookinete formation, where the protein is essential for the formation of the crystalloid, the correct targeting of crystalloid-resident protein LAP2 and malaria parasite transmission.

Keywords

sporogony; DHHC; zygote; translational repression; DOZI

Significance statement

The malaria parasite requires the formation of a stage-specific, enigmatic organelle—the crystalloid—for mosquito invasion. Perhaps acting as storage for proteins and lipids, the crystalloid is generated during ookinete formation and dissolved during sporogony in the oocyst. We show here that *Plasmodium berghei* expresses an S-acyl-transferase DHHC10 that localises to the crystalloid. Parasites depleted of *dhhc10* produce ookinetes that successfully establish oocysts but fail to produce sporozoites; knock-out parasites are characterised by the absence of the crystalloid and the mis-localization of a crystalloid-resident protein.

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Introduction

The malaria parasite is capable of infecting both vertebrate host and mosquito vector. Following a mosquito blood meal, sexual precursor cells rapidly differentiate into mature gametes. In the mosquito midgut they mate to form a zygote that develops further into the motile ookinete. After crossing the midgut epithelium and establishing a sessile oocyst it gives rise to thousands of sporozoites capable of infecting a subsequent mammalian host (1). Sharing key organelles like the nucleus, ER, Golgi and mitochondria with other eukaryotes this parasite has evolved specialised, stage-specific structures that are necessary for developmental progression during parasite transmission. These include for example osmiophilic bodies (secretory vesicles) that release protein factors capable of lysing the parasitophorous vacuole and erythrocyte membranes thus producing free gametes (2); the formation in the ookinete of a gliding motility motor anchored to the inner membrane complex enabling the ookinete to migrate across the mosquito midgut epithelium and establish an oocyst (3); sporozoite formation in the oocyst finally requires the presence of a stage-specific organelle, the crystalloid, a multivesicular structure assembled in the ookinete and putative reservoir of proteins and lipids used during sporogony. Although discovered more than 40 years ago the formation and function of this enigmatic organelle remains largely unknown (4-9); four LCCL proteins have been shown to reside within (9) and maintain the stability (8, 9) of these organelles essential for sporogony (10).

The morphological changes taking place during zygote to ookinete development and the generation of thousands of sporozoites inside a single oocyst require extensive protein translation and membrane biogenesis to support the formation of organelles and plasma membrane surrounding each new parasite. One third of the proteins identified in the oocyst and oocyst-derived (midgut) sporozoites of the human parasite *P. falciparum* are putatively membrane-bound (11). Targeting of such proteins to organelles, and perhaps formation of certain organelles *per se*, requires appropriate sorting signals, and transmembrane (TM) domains to keep these factors in place; post-translational modifications like lipidation can increase the affinity of a modified protein for membranes and alter its subcellular localization. Only palmitoylation—the addition of a C-16 long chain fatty acid to a cysteine residue—is reversible and thus able to dynamically influence protein-protein interactions, function, and gene expression (12-17). Catalysed by TM-spanning enzymes called palmitoyl-S-acyl-transferases (DHHC-PATs, PATs) this post-translational modification is evolutionarily conserved; 25 PATs are known in human and seven in *Saccharomyces cerevisiae*, twelve are annotated in the human malaria parasite *P. falciparum*, and eleven in the rodent malaria parasite *P. berghei.* In asexual blood stages of *P. falciparum* several hundred proteins have been found to be palmitoylated and addition of the inhibitor 2-bromopalmitate (2-BMP) to *P. falciparum*

in vitro cultures caused developmental as well as red blood cell invasion defects in schizonts, the latter probably through the destabilisation of gliding motility motor components (15). In *P. berghei*, the inhibitor causes severe ookinete formation defects (18). Despite known localisation data for several *Plasmodium* PATs (14, 19, 20), the specific functions of individual S-acyl-transferases for life cycle progression are almost completely unknown; only recently have we identified DHHC2 to be required for ookinete morphogenesis, specifically zygote elongation, and a likely essential role for blood stage parasite development (18). Here we provide conclusive genetic evidence for the essential role of the stage-specific S-acyl-transferase for mosquito infection by *P. berghei*: *dhhc10* is maternally provided as a translationally repressed mRNP to the developing ookinete by the female gametocyte; in the ookinete the protein controls formation of the crystalloid, and ultimately guarantees the success of sporozoite formation and transmission to a subsequent host.

Results

DHHC10 is required for mosquito infection. Palmitoylation is crucial for *P. falciparum* schizogony and *P. berghei* ookinete formation (15, 18). Here we investigated the role of a maternally provided, translationally repressed S-acyl-transferase for developmental progression in the mosquito vector. Following a mosquito blood meal, mating of males and females produces the round zygote that develops into a motile, banana-shaped ookinete. This transformation process relies heavily on translationally repressed (stored) mRNAs provided by the female gametocyte (21-23). Of eleven annotated *P. berghei* DHHC-PATs (17, 24) we had identified *dhhc10* together with *dhhc2* and *dhhc3* to be upregulated in gametocytes (**Fig. 1A**) (25); while *dhhc2* (18) and *dhhc3* expression was also evident in asexual blood stage forms, only *dhhc10* was exclusively transcribed in sexual stages (**Fig. 1B**) suggesting a role for the protein during transmission of gametocytes to the mosquito vector. DHHC10 is 275 amino acids in length and has four distinct transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/), a feature it shares with the syntenic orthologs from *P. falciparum* and *P. vivax*, two major malaria parasite species that infect humans (**Fig. 1C; SI Appendix, Fig. S1**). Using standard methods of *P. berghei* genetic modification (26) we generated two independent *dhhc10* null mutants, Δ*dhhc10*-a and Δ*dhhc10*-b (**SI Appendix, Table S1, Fig. S2**). Although asexual growth and schizont formation in *P. falciparum* requires palmitoylation (15), our *dhhc10* null mutants showed normal asexual growth and gametocyte production rates (see **SI Appendix, Table S2** for key developmental data for both independent knock-out clones). This could indicate that DHHC10 exerts a non-essential role during schizont formation (asexual development) and gametocytogenesis

(intraerythrocytic sexual development), but given its transcriptional pattern, supports that DHHC10-driven palmitoylation does not take place.

To identify a possible role for DHHC10 during developmental progression and transmission (ookinete formation, establishment of oocysts, or sporogony) we allowed *Anopheles stephensi* mosquitoes to feed on mice infected with Δ*dhhc10* parasites. Two independent null mutant clones (Δ*dhhc10-a and* Δ*dhhc10-b*) produced oocyst numbers that were similar to those upon infection with wildtype (WT) parasites (**Fig. 1D; SI Appendix, Table S2**) and were thus not compromised in their ability to form infection-competent, motile ookinetes and establish oocysts that resisted elimination by the host's immune response. Although similar in size to wildtype oocysts, mutants displayed an 'empty' appearance and showed no signs of sporulation (**Fig. 1E**), the process during which a single oocyst gives rise to thousands of motile sporozoites. These parasites lacked appreciable expression of circumsporozoite protein (CSP), the major sporozoite surface protein and a key developmental marker of sporogony. Strongly expressed in wildtype oocysts of the same age, immunofluorescence assays (**Fig. 1F**) and Western analyses (**Fig. 1G**) of protein extracts from pooled infected midguts showed minimal expression of CSP in Δ*dhhc10* parasites although labelling of the same cells with the DNA-specific dye Hoechst-33342 showed no defect in DNA replication (**Fig. 1F**). Mutants failed entirely to colonize the mosquito salivary glands (**Fig. 1H; SI Appendix, Table S2**) and were consequently unable to transmit the infection to naïve mice (**Fig. 1I; SI Appendix, Table S2**).

***dhhc10* mRNA is maternally supplied, translated during ookinete formation and targeted to crystalloids.** The ability of Δ*dhhc10* mutants to generate normal numbers of oocysts while failing to develop further into sporozoites was unexpected in view of the transcription profile identified by RT-PCR; mRNA was clearly detected in gametocytes (**Fig. 1A, B**). In order to characterize DHHC10 protein expression, timing and localisation in detail, we generated a parasite expressing a GFP-tagged version of the endogenous protein (**Fig. 2A; SI Appendix, Fig. S3**). This mutant showed normal progression throughout the life cycle, including ookinete, oocyst and sporozoite production and transmission to mice via mosquito bite (**SI Appendix, Table S2**). Thus, GFP-tagging of DHHC10 did not affect protein function or parasite viability. As expected from the RT-PCR results we found no protein expression in asexual stage parasites; in addition DHHC10::GFP was not detected in gametocytes until translated in the ookinete (**Fig. 2B; SI Appendix, Fig. S4**), a behaviour consistent with translational regulation during transmission of *P. berghei* found for many genes (21-23). Storage of this mRNA in cytoplasmic messenger ribonucleoproteins (mRNPs) for translation in the ookinete was confirmed in an RNA immunoprecipitation experiment; *dhhc10* co-purified with both translational repressors

DOZI (a homolog of yeast DHH1p) and CITH (a homolog of yeast Scd6p) (**Fig. 2C**) similar to two well-characterised translationally repressed mRNAs encoding the ookinete surface proteins P25 and P28 (21-23). Failure to store translationally repressed mRNAs in the absence of DOZI and CITH affects exclusively female but not male fertility (21-23). Highly downregulated in *dozi* null mutants (22) it is likely that *dhhc10* mRNA is transcribed and provided to developing zygotes solely by females. To test this, we crossed Δ*dhhc10* parasites with the Δ*p47* parasite line, a mutant producing fertile males but sterile females (27). The resulting ookinetes lacked crystalloids, while ookinetes from the control experiment—crossing Δ*p47* males with fertile females from line Δ*p48/45* (28)—did not (**SI Appendix, Fig. S5**). Fertilisation rates in both crosses were similar (**SI Appendix, Fig. S5**), while no fertilisation took place in Δ*p47* nor Δ*p48/45* parasites. The Δ*dhhc10*/Δ*p47* cross does not rescue the crystalloid formation defect of Δ*dhhc10* ookinetes (**SI Appendix, Fig. S5**), confirming that the wildtype dhhc10 allele of the male Δ*p47* gene does not allow for proper crystalloid biogenesis. DHHC10 expression in ookinetes results thus from translationally repressed, DOZI and CITH-associated *dhhc10* mRNA provided by the female gamete.

In ookinetes DHHC10::GFP highlighted distinct cytoplasmic foci (**Fig. 2B; SI Appendix, S4**) known for members of the LAP/CCp protein family that are typical for the ookinete/oocyst-specific crystalloid organelle (8, 29, 30). Believed to act as a reservoir for proteins needed for sporozoite development within the oocyst, these organelles are generated in ookinetes but dissolved in early and absent in late oocysts (6, 9). Focal localisation of DHHC10::GFP in one or two spots was observed in approximately 75% of ookinetes (n=60); the remaining 25% showed a somewhat more diffuse GFP signal throughout the ookinete cytoplasm; dim and diffuse fluorescence was also present in ookinetes that presented clear DHHC10::GFP foci (**SI Appendix, Fig. S4**). To corroborate targeting of DHHC10 to crystalloid bodies, we transfected *dhhc10*::*gfp* parasites with a plasmid construct encoding a C-terminally mCherry-tagged version of LAP3 (**SI Appendix, Fig. S6**). This plasmid contains *lap3* upstream regulatory regions allowing for episomal expression of LAP3::mCherry under its endogenous promoter. LAP3 has previously been localised to the ookinete crystalloids by both live fluorescence imaging and immuno-electron microscopy (30). DHHC10 perfectly co-localised with LAP3 in these experiments, unambiguously identifying this PAT in the ookinete crystalloid (**Fig. 2D**). In the differential interference contrast (DIC) image the crystalloid is seen surrounded by haemozoin, the remnant of haemoglobin digestion from the intraerythrocytic female gametocyte and also known as malaria pigment (**Fig. 2D**).

Crystalloids are formed by microtubule-dependent transport and assembly of endoplasmic reticulum-derived vesicles (9). Concomitantly, LAP proteins such as LAP3 are shuttled to

common assembly points and incorporated into mature crystalloids. We took advantage of the *dhhc10*::*gfp*;*lap3*::*mCherry* line to closely follow DHHC10 translation and trafficking in relation to LAP3. DHHC10::GFP was detected as early as 3 hours (h) post-fertilisation; both DHHC10 and LAP3 showed showed signs of protein concentration by 9 h post-fertilisation; at 12 h, more than 50% of retorts displayed focal accumulation of both proteins and by the end of ookinete development (24 h), GFP and mCherry signals co-localised in the crystalloid in more than 90% of mature ookinetes (**SI Appendix, Fig. S7**). No significant differences in both the timing of expression and subcellular localisation were thus found between the two proteins.

DHHC10 is required for crystalloid formation. *In vivo* Δ*dhhc10* mutants produced wildtype numbers of oocysts, *in vitro* they showed normal ookinete formation rates (**SI Appendix, Table S2**). Compared to wildtype ookinetes (**Fig. 3A**), mutant ookinetes displayed a normal shape and efficiently invaded mosquito midguts but lacked haemozoin clusters (**Fig. 3B**) that typically surround and highlight crystalloids in wildtype *P. berghei* ookinetes (4, 31); they were dispersed throughout the cytoplasm in live and fixed, Giemsa-stained cells (**Fig. 3B**). To unequivocally confirm the absence of this organelle we compared wildtype and Δ*dhhc10* mutants by transmission electron microscopy. In 50% of wildtype ookinetes (6 out of 12) we were able to visualise the crystalloid, appearing as a highly ordered (almost crystalline) structure surrounded by malaria pigment (**Fig. 3C, D**). The crystalloid is located close to the nucleus, often posterior to the apical complex which harbours the micronemes (**Fig. 3E**). Micronemes are secretory vesicles that provide adhesive proteins needed for gliding motility and invasion of the midgut epithelium. The apical complex contains the gliding motility motor (3) which is anchored to the IMC or inner membrane complex, between the plasma membrane (PM) and the subpellicular microtubules (MT) (**Fig. 3E**). Micronemes are clearly visible in the knock-out mutant, and the organisation of the inner membrane complex and the subpellicular network appears normal (**Fig. 3F**) explaining the wildtype invasion behaviour in the *in vivo* transmission experiments (**Fig. 1D; SI Appendix, Table S2**). On the contrary, in all 30 Δ*dhhc10* ookinetes examined we failed to localize characteristic crystalloids and found haemozoin scattered throughout the cytoplasm (**Fig. 3G**).

To identify the consequences of crystalloid absence in Δ*dhhc10* ookinetes for crystalloid-resident proteins we tagged LAP2 with GFP in the wildtype and Δ*dhhc10* null mutant genetic backgrounds (30). *P. berghei* LAP2 is a member of the LCCL multigene family of proteins expressed in female gametocytes with a typically scattered staining pattern in the wildtype background; during ookinete formation it is concentrated in the crystalloid (30)(**Fig. 4A**). The resulting Δ*dhhc10*;*lap2*::*gfp* parasites (**SI Appendix, Fig. S8**) also presented LAP2::GFP-positive

female gametocytes with protein dispersed throughout the cytoplasm as shown previously (30). While in wildtype ookinetes LAP2::GFP is targeted to the crystalloids, the absence of DHHC10 left LAP2::GFP protein scattered throughout the cell (**Fig. 4B**).

To assess how the lack of DHHC10 affects crystalloid formation during zygote to ookinete transformation, we imaged Δ*dhhc10*;*lap2*::*gfp* parasites in a time-course experiment. More than 50 parasites were imaged at each time point and in all developmental stages, GFP signal showed exclusively a diffuse cytoplasmic location with no evidence for LAP2::GFP clustering, or haemozoin crystals seen by microscopic analysis of live or fixed, Giemsa-stained parasites. Instead, small haemozoin crystals remained scattered throughout the cytoplasm (**SI Appendix, Fig. S9**). These results strongly support the conclusion that in the absence of DHHC10 crystalloid formation is abrogated. As a result, crystalloid-resident proteins remain localised in the cytoplasm. However, the absence of crystalloid assembly could also be a consequence of mis-trafficking of non-palmitoylated LAP2 itself. Whether LAPs are palmitoylated is, however, unknown.

Finally, to unequivocally attribute the lack of crystalloid formation and protein targeting to this organelle to the absence of DHHC10, we complemented the Δ*dhhc10*-a *parasite line with dhhc10*::*gfp* (**SI Appendix, Fig. S10**). In the clonal line Δ*dhhc10*;*dhhc10*::*gfp* we readily observed the presence of DHHC10::GFP in bright fluorescent spots in mature ookinetes (**SI Appendix, Fig. S11**), characteristic of crystalloids as shown in *dhhc10*::*gfp* ookinetes (**Fig. 2B; SI Appendix, Fig. S4**). Clustered haemozoin crystals were visible under DIC imaging, indicating the normal formation of crystalloids. These results show that the introduction of a wildtype copy of *dhhc10* into the genome of Δ*dhhc10* parasites rescued crystalloid formation.

Discussion

Our studies highlight the essential nature of a protein S-acyltransferase for malaria parasite life cycle progression in the mosquito vector. We show that DHHC10 is essential for sporozoite formation, a prerequisite for malaria transmission to the subsequent mammalian host. Although transcribed and translationally repressed in the gametocyte, DHHC10 plays no immediate role in the development of gametes or ookinetes. Gametes are fertile, form zygotes, and develop into ookinetes that infect the mosquito midgut epithelium in wildtype numbers. Instead, DHHC10 is essential for the formation of crystalloids, transient organelles of the ookinete and oocyst that exert their function in the oocyst. In the absence of DHHC10, crystalloids could not be detected at light or electron microscopy levels resulting in an

aberrant localisation of the crystalloid-resident protein LAP2. Mutants ultimately failed to produce sporozoites. Our work confirms that crystalloids are dispensable for ookinete development and initial colonization of the mosquito midgut, but essential for the development of sporozoites in oocysts and thus parasite transmission between host and vector.

Formed in ookinetes, the crystalloid persists up to young oocysts (6). It has been suggested that the organelle acts as a protein (4, 32) or lipoprotein-energy reservoir (7) that is used during development of the oocyst and formation of sporozoites (5, 6). The crystalloid appears as a prominent honeycomb-patterned structure believed to consist of individual vesicles that during sporogony traffic or provide cargo to their destination. That this organelle is likely membranous in nature is highlighted by the localization of the 4-transmembrane domain protein DHHC10::GFP to this structure. How the regular shape of the crystalloid is established or maintained, is unclear; perhaps palmitoylation of crystalloid-resident proteins helps organise, maintain and tether individual vesicles together in this typical arrangement. As illustrated by the Δ*dhhc10;lap2::gfp* parasites, the crystalloid-resident protein LAP2 is not degraded in the mutant. The protein either fails to be trafficked to and concentrated in the now absent crystalloid, or requires itself DHHC10-mediated palmitoylation to promote crystalloid formation. Therefore, proper expression and packaging of such components is important for sporozoite development. Lack of crystalloid formation has also been reported for null mutants of the crystalloid-resident LCCL protein PbSR (LAP1), although the block in sporogony is not absolute (8), and more recently also for parasites lacking LAP3 (9). Gene deletion mutants of additional LCCL/LAP protein family members (10, 33, 34), plasmepsin VI (35) and rhomboid 3 (36) (the two latter may be *bona fide* crystalloid-residents) present similar phenotypes with respect to oocyst differentiation. In *P. berghei*, haemozoin-containing vacuoles accumulate around the crystalloid's edges (4, 31). In the DHHC10 knock-out, malaria pigment remained scattered throughout the cytoplasm. Whether this is biologically relevant is unknown.

The targets of protein palmitoylation in the early mosquito stages of the malaria parasite remain to be identified, although inhibition of palmitoylation and deletion of *P. berghei dhhc2* severely affect zygote elongation (18). The published rodent *P. berghei* (37, 38) and avian *P. gallinaceum* (39) malaria zygote and ookinete proteomes comprehend 277 out of 1368 (20%) *P. falciparum* orthologs found to be palmitoylated in *P. falciparum* schizonts; a comparison of oocyst and oocyst-derived sporozoite (ODS) proteome data from *P. falciparum* (11) with the palmitome of *P. falciparum* blood stage schizonts (15) showed that 54/134 proteins detected in 7-8 days old oocysts were targets of palmitoylation in blood

stages, while of the 453 proteins detected in 13-14 days old ODS, 176 were present in the schizont palmitome. However, it remains unclear whether the enzymatic activity of DHHC10 is required for the phenotype observed here. Palmitoylation is clearly important for protein stability and trafficking (40) but enzymatic activity-independent function of the yeast palmitoyl-transferase Swf1p is also reported (41). With poorly defined palmitoylation motifs (42) proteomic strategies (15, 43) will be required to identify the true extent of protein palmitoylation in these stages, allow their effect on parasite morphogenesis to be identified, and determine whether crystalloid-formation and maintenance necessitates palmitoylation of crystalloid-resident proteins such as the LAPs. The identification of a clear phenotype linked to DHHC10 will help define enzyme-substrate pairs.

Our results serve as a starting point for further experiments to identify targets that require palmitoylation for proper function and formation of the *Plasmodium* crystalloid. Affecting the transmission from the mosquito vector to the mammalian host, the development of parasite-specific DHHC-PAT inhibitors will certainly be of interest for novel intervention strategies. The transcription, yet translational repression, of an mRNA (*dhhc10*) in the blood stage gametocyte, whose protein product directs the generation and maintenance of an organelle, the crystalloid, which *per se* is redundant for ookinete formation, morphology and midgut invasion but plays an essential role in the developmental progression during the next life cycle stage (oocyst sporogony) to guarantee successful transmission, is an astonishing evolutionary development.

Materials and Methods

Experimental animals. Female Balb/c ByJ and OF-1 (6–8 weeks-old bred at Charles River, France) mice were used. All animal experiments in this study were carried out in accordance with the European Guideline 86/609/EEC and followed the FELASA (Federation of European Laboratory Animal Science Associations) guidelines and recommendations concerning laboratory animal welfare;experiments performed at the Leiden University Medical Center (LUMC, Leiden, The Netherlands) were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 10099; 12042; 12120);experiments performed at the Instituto de Medicina Molecular (IMM, Lisbon, Portugal) were approved by the IMM Animal Ethics Committee (under authorisation AEC_2010_018_GM_Rdt_General_IMM) and the Portuguese authorities (Direção Geral de Alimentação e Veterinária), and were in compliance with the Portuguese Law (Portaria 1005/92);experiments in Heidelberg were approved by the Regierungspräsidium Karlsruhe.

Reference *P. berghei* ANKA lines and generation of mutant parasite lines; reverse Transcriptase-PCR (RT-PCR); *in vivo* multiplication rate of asexual blood stages; gametocyte production and ookinete formation assays; oocyst and sporozoite production, and transmission experiments are described in SI Appendix, Materials and Methods.

Live imaging and immunofluorescence assays (IFAs) of blood stages, ookinetes and oocysts. Live imaging of transgenic parasites expressing GFP-tagged DHHC10, GFP-tagged LAP2 and LAP3::mCherry was done by collecting tail blood samples from infected mice and samples from ookinete cultures at different time points post gametocyte activation and staining with 1 µg/mL of Hoechst 33342/PBS. To detect DHHC10::GFP expression in blood stages by IFA, mouse RBCs infected with *dhhc10*::*gfp* parasites were stained with rabbit polyclonal anti-GFP (Abcam®, #ab6556; 1:500) as primary antibody. As secondary antibody, goat anti-rabbit IgG-Alexa Fluor®488 (Jackson ImmunoResearch Laboratories, Inc., #111-545-003; 1:500) was used. To detect CSP expression in Fluo-frmg and Δ*dhhc10*-a oocysts, parasites were stained with 3D11 mouse anti-PbCSP (44) (10 µg/mL) as primary antibody and goat anti-mouse IgG-Cy™3 (Jackson ImmunoResearch Laboratories, Inc., #115-166-003; 1:400) as secondary antibody. In all IFAs, samples were fixed with 4% PFA/PBS for 10 min at RT, permeabilised with 0.1-0.5% TritonX-100/PBS and blocked for 1h at RT with 1-3% BSA/PBS. All antibody incubations were done in blocking solution for 1h at RT and 1-5 µg/mL of Hoechst-33342/PBS was used to stain nuclei. Images were taken with a Leica DM5000B, Leica DM IRBE or Zeiss Axiovert 200M fluorescence microscope and processed using ImageJ 1.47n software (imagej.nih.gov/ij).

Transmission electron microscopy of ookinetes is described in SI Appendix, Materials and Methods.

Western analysis of CSP expression in Δ*dhhc10* oocysts is described in SI Appendix, Materials and Methods.

Genetic crosses, fertilisation rates and crystalloid body formation. The fertility of wildtype and mutant gamete populations was analysed by standard *in vitro* fertilisation and ookinete maturation assays (see above). The fertilisation rate is defined as the percentage of female gametes that develop into zygotes or ookinetes and is determined by counting female gametes and zygotes/ookinetes in Giemsa-stained blood smears 16-18 h after *in vitro* induction of gamete formation. Fertility of individual sexes (macro- and microgametes) was determined by *in vitro* cross-fertilisation studies (22, 23, 45) in which gametes are cross-fertilised with gametes of parasite lines that produce only fertile male (Δ*p47*; clone 270cl1;

(27)) or only fertile female (Δ*p48/45*; clone 137cl7; (28)) gametes. Crystalloid formation was scored on the same Giemsa-stained smears used for fertilisation rate determination.

Statistical methods. Statistical analyses of oocyst and sporozoite numbers were performed using Mann-Whitney test as part of Prism software package 5 (GraphPad Software).

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. DHHC10 is essential for transmission to the mosquito vector. (A) RNA sequencing data from blood and mosquito stages for all 11 *P. berghei dhhc* genes reveal *dhhc2, dhhc3* and *dhhc10* to be upregulated in gametocytes (25). Error bars correspond to SEM values. FPKM: Fragments Per Kilobase of transcript per Million mapped reads. **(B)** RT-PCR of *dhhc3* and *dhhc10* confirm the gametocyte-specific nature of *dhhc10* while *dhhc3* is also transcribed in asexual stage parasites. RT+: RT positive reaction; RT-: RT negative reaction. **(C)** Schematic representation of rodent and human malaria DHHC10 with 4 transmembrane (TM) domains; the signature DHHC motif is located between TM domains 2 and 3; drawn to scale. **(D)** Δ*dhhc10*-a parasites develop wildtype (WT) oocyst numbers on days 12 to 13 p.i. Absolute numbers of oocysts/midgut from 5 independent experiments are presented for both WT (*n*=70) and Δ*dhhc10*-a (*n*=48) parasites. Mean ± SEM values are shown; p-value for Mann-Whitney test. **(E)** Δ*dhhc10*-a oocysts appear normal in size at day 14 p.i., but lack signs of sporulation and remain empty (arrowheads) while WT oocysts have already formed sporozoites (arrows). Scale bar = 20 µm. **(F)** Immunofluorescence of oocyst-infected midguts at day 14 p.i. reveals strongly reduced expression of the developmental marker CSP in Δ*dhhc10*-a parasites; DNA staining (Hoechst-33342) is comparable. ↑CSP indicates long exposure. Scale bar = 20 µm. **(G)** Western blot analyses of oocyst-infected midguts at day 13 p.i. confirms low CSP expression in Δ*dhhc10*-a mutants. HSP70 serves as loading control. ↑CSP indicates overexposure. **(H)** Δ*dhhc10*-a parasites do not colonize the mosquito salivary glands. WT (5 independent experiments, *n*=15); Δ*dhhc10*-a (4 independent experiments, *n*=10). Mean ± SEM values are shown. **(I)** Mice bitten by Δ*dhhc10*-a-infected mosquitoes fail to develop blood stage infections. Mean parasitaemias ± SEM from 3 independent experiments are shown.

Fig. 2. *dhhc10* is translationally repressed in gametocytes and translated in ookinetes, where it localises to the crystalloid bodies. (A) Only *dhhc10*::*gfp* mRNA is transcribed in *dhhc10*::*gfp* parasites. *p28* serves as control gene in the RT-PCR. **(B)** DHHC10::GFP is only translated in ookinetes. DNA stained with Hoechst-33342. Scale bar = 5 µm. **(C)** RT-PCR analyses of DOZI::GFP and CITH::GFP gametocyte immunoprecipitation (IP) eluates showing that *dhhc10* like *p25* and *p28* mRNAs are bound by both translational repressors. Translated mRNAs (*dozi, cith* and *alba-3*) are not enriched in the IP-GFP fractions. Input: total gametocyte mRNA; IP-GFP: IP with anti-GFP antibody; IP-c-myc: IP with anti-c-myc antibody; IP beads: no antibody used for IP. **(D)** DHHC10::GFP co-localises with the crystalloid-resident protein LAP3 (here

tagged with mCherry). Second image is a higher magnification of the crystalloid body as seen by DIC microscopy. N: nucleus stained with Hoechst-33342. Scale bar = 5 µm.

Fig. 3. DHHC10 is required for crystalloid formation. (A) WT ookinetes with crystalloids (+) under DIC microscopy (live) and after Giemsa staining (fixed). Scale bar = 5 µm. **(B)** Δ*dhhc10*-a ookinetes show no evidence of crystalloid formation. Scale bar = 5 µm. **(C)** Longitudinal transmission electron microscopy (TEM) section of a WT ookinete. N: nucleus; C: crystalloid surrounded by haemozoin (+). Scale bar = 1 µm. **(D)** TEM of WT crystalloid (C) reveals ordered, honeycomb arrangement surrounded by haemozoin (+). Scale bar = 1 µm. **(E)** Apical complex of a WT ookinete with micronemes (∗) and typical arrangement of subpellicular microtubules (MT), inner membrane complex (IMC) and plasma membrane (PM). Scale bar = 1 µm. **(F)** Apical complex of Δ*dhhc10*-b ookinetes showing that micronemes (∗), subpellicular microtubules (MT), inner membrane complex (IMC) and plasma membrane (PM) arrangement is not affected. Scale bar = 1 µm. **(G)** Longitudinal TEM section of a Δ*dhhc10*-b ookinete highlights the nucleus (N) but does not reveal a distinct crystalloid. Haemozoin (+) is scattered throughout the cytoplasm. Scale bar = 1 µm.

Fig. 4. Absence of DHHC10 disturbs localisation of crystalloid-resident protein LAP2. Live imaging of *lap2::gfp* **(A)** and Δ*dhhc10;lap2::gfp* **(B)** female gametocytes shows LAP2::GFP expression throughout the gametocyte cytoplasm in both strains. Only in WT ookinetes is LAP2::GFP trafficked to crystalloids, while in Δ*dhhc10;lap2::gfp* parasites the protein remains distributed throughout the ookinete cytoplasm. DNA stained with Hoechst-33342. Scale bars = 5 µm.