Published in final edited form as:

Cell Microbiol. 2016 November ; 18(11): 1625–1641. doi:10.1111/cmi.12601.

Palmitoyl transferases have critical roles in the development of mosquito and liver stages of *Plasmodium*

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Summary

As the *Plasmodium* parasite transitions between mammalian and mosquito host, it has to adjust quickly to new environments. Palmitoylation, a reversible and dynamic lipid post-translational modification, plays a central role in regulating this process and has been implicated with functions for parasite morphology, motility and host cell invasion. While proteins associated with the gliding motility machinery have been described to be palmitoylated, no palmitoyl transferase responsible for regulating gliding motility has previously been identified. Here, we characterize two palmitoyl transferases with gene tagging and gene deletion approaches. We identify DHHC3, a palmitoyl transferase, as a mediator of ookinete development, with a crucial role for gliding motility in ookinetes and sporozoites, and we co-localize the protein with a marker for the inner membrane complex in the ookinete stage. Ookinetes and sporozoites lacking DHHC3 are impaired in gliding motility and exhibit a strong phenotype *in vivo*; with ookinetes being significantly less infectious to their mosquito host and sporozoites being non-infectious to mice. Importantly, genetic complementation of the DHHC3-ko parasite completely restored virulence. We generated parasites lacking both DHHC3, as well as the palmitoyl transferase DHHC9, and found an enhanced phenotype for these double knockout parasites, allowing insights into the functional overlap and compensational nature of the large family of PbDHHCs. These findings contribute to our understanding of the organization and mechanism of the gliding motility machinery, which as is becoming increasingly clear, is mediated by palmitoylation.

Introduction

Malaria is a vector-borne disease caused by eukaryotic pathogens of the genus *Plasmodium* and continues to be a leading cause of childhood mortality in sub-Saharan Africa with an estimated 584 000 deaths annually (WHO, 2013). *Plasmodium* parasites cycle between the mosquito and mammalian host; transmission of the parasite to the mosquito occurs when a female *Anopheles* mosquito takes a blood meal on an infected mammalian host and ingests

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Supporting information

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gametocytes, the sexual stage of the parasite. Following fertilization, zygotes mature to motile ookinetes, which traverse the mosquito midgut and form oocysts. These oocysts subsequently produce sporozoites, which upon release traverse the mosquito hemolymph to reach the salivary glands. During a subsequent blood meal, motile sporozoites are then injected into the skin of the mammalian host and migrate through the dermis to enter blood vessels and travel through the circulation to establish infection in the liver, which eventually initiates blood stage infection.

This complex life cycle is regulated at multiple points by post-translational modifications, which allow rapid modulations of protein function (Doerig *et al.*, 2015). One relatively understudied post-translational modification is the covalent attachment of palmitic acid, a saturated C¹⁶-chain, to a cysteine residue via a thioester bond, and this modification is referred to as palmitoylation (Linder and Deschenes, 2007). Unlike prenylation and myristoylation, S-acylation is reversible, and similar to phosphorylation or ubiquitination, it allows dynamic regulation of protein function by regulating protein localization between and within membranes, influencing protein–protein interactions or affecting protein stability (Linder and Deschenes, 2007). Global biochemical approaches, which characterized the palmitoylated proteome, discovered ~50 palmitoylated proteins in yeast (Roth *et al.*, 2006), ~600 proteins in plants (Hemsley *et al.*, 2013), several hundreds in mammals (Martin and Cravatt, 2009) and 401 palmitoylated proteins in the related apicomplexan *Toxoplasma gondii* (Foe *et al.*, 2015; Caballero *et al.*, 2016). Palmitoylation in *Plasmodium* has only recently been described, and a proteomic analysis of the palmitome of the blood stage of *Plasmodium falciparum* identified more than 400 putative palmitoylated proteins with roles in cytoadherence, host cell invasion, organelle biogenesis and protein export (Jones *et al.*, 2012).

Palmitoyl transferases are the enzymes catalysing the transfer of palmitate from palmitoyl-CoA to the thiol group of a cysteine residue on a protein substrate. The catalytic domain, which harbours a conserved DHHC (Asp-His-His-Cys) motif, required for protein palmitoylation, is typically located cytosolically between two transmembrane domains (Linder and Deschenes, 2007). *Saccharomyces cerevisiae*, where DHHC enzymes were first discovered, has 7 DHHCs (Roth *et al.*, 2006); in *Arabidopsis*, the DHHC family encompasses 24 genes (Hemsley *et al.*, 2013); and in mammals, 23 DHHCs have been identified (Fukata *et al.*, 2004). A recent analysis of the repertoire of DHHC enzymes in the apicomplexan parasites *T. gondii*, *P. falciparum* and *Plasmodium berghei* revealed that these parasites possess large families of putative DHHCs, with 18, 12 and 11 genes respectively (Frénal *et al.*, 2013). A comprehensive study of essentiality and subcellular localization of DHHCs in *T. gondii* tachyzoites and *P. berghei* blood stage parasites has been carried out (Frénal *et al.*, 2013), and while functional redundancy of DHHCs in other eukaryotes were reported (Roth *et al.*, 2006), some apicomplexan DHHCs were found critical for parasite survival (Frénal *et al.*, 2013). In *T. gondii*, five DHHCs were essential for parasite survival, and 16 DHHCs were found to be expressed and trafficked to different subcellular localizations within the tachyzoite (Frénal *et al.*, 2013).

In *P. berghei* blood stage parasites, the majority of DHHCs could be successfully disrupted without phenotype (Frénal *et al.*, 2013), and only five were expressed in different subcellular

compartments of blood stage schizonts (Frénal *et al.*, 2013). It is likely that the remaining six DHHCs are functional in other life cycle stages, thus allowing the parasite to rapidly adapt to the different environments of its mammalian and insect host. However, DHHCs had not been investigated in the mosquito or liver stages of *Plasmodium* until recently, when DHHC2 was shown to be essential for both the *P. berghei* blood stage and ookinete stage: DHHC2 was found refractory to gene deletion in the blood stage, and a promoter swap mutant demonstrated an important function in ookinete morphogenesis (Santos *et al.*, 2015).

The genes of two *P. berghei* DHHCs, DHHC3 (PBANKA_092730) and DHHC9 (PBANKA_093210), have previously been deleted in *P. berghei* blood stages, where the proteins were localized to the inner membrane complex (IMC) (Frénal *et al.*, 2013). In a subsequent study of *P. falciparum* blood stages, by Wetzel and co-workers (Wetzel *et al.*, 2014), mCherry-tagged PfDHHC3 and PfDHHC9, overexpressed from the PfAMA1 promoter, did not localize to the IMC. However, the same study was able to confirm an IMC localization for PfDHHC1 (Wetzel *et al.*, 2014), an additional DHHC that was localized to the IMC in *T. gondii* (TgDHHC14, (Beck *et al.*, 2013; Frénal *et al.*, 2013)) and *P. berghei* (Frénal *et al.*, 2014).

Reasoning that these DHHCs may play a more important role at other life cycle stages, using gene knock out and C-terminal epitope tagging approaches, we investigated the localization and function of DHHC3 and DHHC9 in the mosquito and pre-erythrocytic stages of the parasite. The data demonstrate that both palmitoyl transferases have critical functions in these life cycle stages, and therefore critical functions in transmission. This suggests that unlike model eukaryotes, *Plasmodium* may use its repertoire of DHHCs in different ways during different stages of development, raising the possibility that these enzymes may have stage-specific targets.

Results

Localization of DHHC3 and DHHC9 in ookinetes, oocysts, sporozoites and liver stages

Given that DHHC3 and DHHC9 had previously been successfully deleted in the blood stage of *P. berghei* (Frénal *et al.*, 2013), we hypothesized that they might be expressed and have a primary function in the mosquito or liver stages of the parasite. To investigate the spatiotemporal expression pattern of DHHC3 and DHHC9, we cloned by limiting dilution, lines in which the endogenous genes were fused to a C-terminal triple HA epitope tag (Frénal *et al.*, 2013) (Fig. S1) and used these to infect *Anopheles stephensi* mosquitoes. Immunofluorescence microscopy of ookinetes showed that DHHC3-HA localized to the periphery of ookinetes and the staining overlapped with that of the IMC marker myosin tail interacting protein (MTIP, (Bergman *et al.*, 2003)) (Fig. 1A), mirroring the location of DHHC3 in *P. berghei* blood stage parasites (Frénal *et al.*, 2013). DHHC3-HA is also expressed in oocyst sporozoites, resulting in strong labelling of oocysts (Fig. 1B). Interestingly, in oocyst sporozoites, DHHC3-HA localizes close to the nucleus, and punctate staining throughout the sporozoite was observed (Fig. 1C); however, DHHC3-HA was not detected in salivary gland sporozoites (Fig. 1D). We did not observe expression of DHHC3-HA in early liver stages in the mammalian host cell *in vitro* and observed only weak staining

in late liver stages 65 h after infection, localizing to the individual hepatic merozoites (Fig. 2A).

Generally, the level of DHHC9-HA expression detectable by immunofluorescence was low in sporozoites; however, similar to DHHC3-HA, it was detectable in oocyst sporozoites but not in salivary gland sporozoites (Fig. 1C and D). In liver stages, it was not observed until 48 h after invasion of the mammalian host cell, where it appeared to localize with dividing hepatic merozoites, a staining pattern that persisted into mature, 65 h liver stage parasites. Interestingly, at 48 h, DHHC9-HA was also localized to a discrete streak in the periphery of the parasitophorous vacuole, which showed partial overlap with staining for uis4 (up-regulated in infective sporozoites gene 4) (Mueller *et al.*, 2005). This distinct DHHC9-HA streak was observed in all infected hepatocytes at this time point in liver stage development but was absent from parasites at 65 h after infection (Fig. 2B). We did not observe this staining pattern with the DHHC3-HA parasites, suggesting that the two enzymes have different functionalities at this stage. Given that both DHHC3 and DHHC9 are both expressed in mosquito and liver stages, we set out to assess their functionality during these life cycle stages using a gene knock out approach.

Roles for DHHC3 and DHHC9 during the development of *P. berghei* in the mosquito

To investigate whether DHHC3 and DHHC9 are critical for the development of mosquito and liver stages of *P. berghei* parasites, we used DHHC3-ko and DHHC9-ko parasites (Fig. S2). Clonal parasites, obtained by limiting dilution, were used to infect *A. stephensi* mosquitoes, and parasite development from the oocyst to the salivary gland sporozoite was followed. The number of DHHC3-ko oocysts in infected mosquitoes was 10-fold lower than in mosquitoes infected with wild-type parasites (Fig. 3A). The number of DHHC3-ko sporozoites found in the hemolymph was fourfold reduced, and the number of DHHC3-ko salivary gland sporozoites was 25-fold decreased compared with mosquitoes infected with wild-type parasites (Fig. 3B). This phenotype was in keeping with the expression of DHHC3-HA in ookinetes and oocyst sporozoites, which suggested a possible function at this stage of the life cycle. To demonstrate that these phenotypes were a direct result of the absence of DHHC3, we used the same 3' HA tagging vector from the *PlasmoGEM* resource (Gomes *et al.*, 2015) with which the tagged line had been generated, to reintroduce the DHHC3 gene into the DHHC3 locus (Fig. S3). Oocyst production in the resulting complemented parasite clones was restored, and normal numbers of hemolymph and salivary gland sporozoites were generated (Fig. 3).

In contrast to the DHHC3-ko parasite, the DHHC9-ko parasite generates normal numbers of oocysts, as well as hemolymph and salivary gland sporozoites (Fig. 3). Given the high level of redundancy between DHHCs (Frénal *et al.*, 2013), we wanted to determine whether DHHC9 was partly compensating for the lack of DHHC3 in the DHHC3-ko. To test this, we generated DHHC3/9 double knockout parasites. Dual mutants lacking both DHHC3 and DHHC9 (Fig. S2), were found to produce 30-fold fewer oocysts (Fig. 3A) and generate only very few salivary gland sporozoites (Fig. 3B). The exacerbated phenotype of the double knockout parasite suggests a degree of functional overlap between DHHC3 and DHHC9, such that they might be able to catalyze palmitoylation of some common substrates.

However, there is clearly limited redundancy with the remaining DHHCs, as very few oocysts are produced in the double knockout parasite line.

Zygote to ookinete transformation is blocked in palmitoyl transferase mutants

The decrease in oocyst numbers in the palmitoyl transferase mutants could result from impairment in fertilization or ookinete to oocyst development. Given the fact that DHHC3-HA is expressed in the ookinete stage, we first investigated the functions for DHHC3 and DHHC9 at this stage. Following ingestion of *Plasmodium* parasites to the mosquito vector, gametes mate to form a round zygote, which over the following 16–19 h develops into a banana-shaped, motile ookinete. Immunofluorescence microscopy of mutant ookinetes grown *in vitro* was performed (Fig. 4), revealing normal ookinete morphology and subcellular localization of MTIP in DHHC3-ko, complemented DHHC3-ko and DHHC9-ko parasites (Fig. 4A). However, while in wild-type, 85% of all MTIP-positive parasites (asexual parasites were not counted) develop into mature ookinetes, DHHC3/9-ko ookinete cultures were dominated by aberrant, shorter ookinete forms, and less than 1% of normal mature ookinetes were present (Fig. 4B). Approximately 36% of double knockout parasites never develop into ookinetes and instead appear stuck in what resembles parasites in stage II at the beginning of differentiation, which is not seen in wild-type cultures at 16–19 h after fertilization. This stage has previously been observed in other mutant parasites (Moon *et al.*, 2009) and may represent parasites that initiated but failed to complete morphogenesis. Of the remaining DHHC3/9-ko, a large percentage (48%) develop into ookinetes; however, these are shorter than normal (Fig. 4B). Apart from the abnormal shape, mutant ookinetes appeared to have a normal MTIP localization, suggesting that the overall IMC structure is intact (Fig. 4B). While the individual loss of DHHC3 and DHHC9 genes does not affect ookinete maturation, the phenotype of the double mutant suggests functional redundancy of the two genes at this stage of the parasite and likely accounts for the greater reduction of oocyst numbers seen with this mutant.

Gliding motility in palmitoyl transferase mutant ookinetes

While abnormal development explains the 30-fold reduction in oocyst numbers of the DHHC3/9-ko, the DHHC3-ko mutant appeared not to have a morphological defect, yet still exhibited a 10-fold reduction in oocyst numbers. To investigate the viability of DHHC3-ko ookinetes, we performed gliding motility assays. Wild-type and DHHC3-ko ookinetes cultured *in vitro* were imaged in Matrigel with time-lapse brightfield microscopy over 10 min, and motile ookinetes were tracked using ImageJ software (Fig. 5A). Comparable with previous reports, wild-type ookinetes in Matrigel moved at an average speed of $6.9 \pm 2.0 \mu\text{m min}^{-1}$ (Moon *et al.*, 2009; Volkmann *et al.*, 2012; Kan *et al.*, 2014) (Fig. 5B). DHHC3-ko ookinetes were gliding with a slightly reduced average speed of $5.13 \pm 2.36 \mu\text{m min}^{-1}$ (Fig. 5B), a defect that was entirely restored in the complemented parasite line. The gliding phenotype of the DHHC3-ko is consistent with the localization of the DHHC3-HA to the IMC at this parasite stage (Fig. 1A) and suggests that DHHC3 has substrates that are part of the gliding machinery. Of note, DHHC9-ko ookinetes did not show a gliding impairment (Fig. 5); however, the small number of morphologically mature DHHC3/9 double knockout ookinetes moved with a much reduced speed of $2.07 \pm 1.37 \mu\text{m min}^{-1}$ compared with

DHHC3-ko ookinetes (Fig. 5B), consistent with the more dramatic decrease in oocyst numbers of the double knockout compared with the DHHC3-ko single deletion mutant.

We then investigated the impact of slower gliding on the area explored by the moving ookinete by quantifying their mean displacement over the 10-min time frame of the movie. Both the DHHC3-ko and the double knockout had decreased mean displacement (Fig. 5C), significantly reducing the area explored by these mutants (Beltman *et al.*, 2009; Kan *et al.*, 2014). Compared with wild-type, the complemented DHHC3 line and the DHHC9-ko, which disseminate approximately 30 μm from their origin in the observed 10min interval, migration of DHHC3-ko ookinetes is reduced by half to 16 μm . The displacement of DHHC3/9-ko ookinetes is reduced fivefold to 6 μm , and thus, this mutant exhibits an even greater reduction in dispersal than DHHC3-ko ookinetes (Fig. 5C). The decrease in gliding speed and displacement may directly hinder mosquito midgut epithelium traversal and could thus explain the decreased oocyst numbers in both the DHHC3-ko and the double knockout.

Gliding motility in palmitoylase transferase mutant sporozoites

Given the ookinete gliding phenotype of the DHHC3-ko and DHHC3/9-ko parasites, we sought to examine gliding of mutant sporozoites. Although sporozoites move 10-times faster than ookinetes, the underlying mechanics of their motility are the same. The fast, robust gliding of sporozoites is crucial for their migration to the mammalian liver and ultimately for their infectivity (Douglas *et al.*, 2015; Hopp *et al.*, 2015). To begin, we used a trail assay in which sporozoites are incubated on glass coverslips for 1 h. The trails that they leave behind as they move can be visualized and quantified by detection of circumsporozoite protein (CSP) present in the shed material (Fig. 6A). Approximately 80% of wild-type hemolymph and 90% of wild-type salivary gland sporozoites were found to be motile, although hemolymph sporozoites only glide for short intervals and few are capable of leaving large numbers of contiguous, overlapping circular trails compared with salivary gland sporozoites (Fig. 6A and B). While DHHC3-ko sporozoites collected from mosquito hemolymph are capable of the same short spurts of gliding as wild-type hemolymph sporozoites, gliding motility of salivary gland DHHC3-ko sporozoites is severely impaired, with only 10% of sporozoites producing trails (Fig. 6A and B). Gliding motility is completely restored in complemented DHHC3-ko sporozoites (Fig. 6A and B), again suggesting a role for DHHC3 in the regulation of the motor complex function.

To further investigate the gliding defects of DHHC3-ko salivary gland sporozoites, we performed live imaging experiments of mutant and wild-type sporozoites as they moved on glass slides *in vitro* (Fig. 6C–E). The overall number of motile sporozoites was reduced in the mutant to 34% compared with 61% in the wild-type (Fig. 6C). Additionally, mutant sporozoites were unable to continuously glide and frequently detached from the glass slide. This was quantified by manual tracking of the motile sporozoites using ImageJ software. In the observed time frame of the 2 min videos, wild-type sporozoites continuously glide for an average of 160 μm , while DHHC3-ko sporozoites only glide for an average of 97 μm before detaching (Fig. 6D). During these shorter gliding intervals, mutant sporozoites were able to glide with a speed comparable with that of wild-type sporozoites (Fig. 6E). These data

suggest that continuous gliding motility and possibly adhesion of salivary gland sporozoites may be affected in this palmitoyl transferase mutant.

Similar to what was observed in DHHC9-ko ookinetes, DHHC9-ko hemolymph and salivary gland sporozoites glide normally compared with wild-type sporozoites (Fig. 6A and B). By contrast, sporozoites of the DHHC3/DHHC9 double knockout behave as the DHHC3-ko parasites, suggesting that redundancies between the two DHHCs in the ookinete stage do not exist in the sporozoite stage. Sporozoite morphology was assessed by staining of surface CSP on wild-type and mutant sporozoites and collected from mosquito midguts, hemolymph and salivary glands, and a normal localization of CSP on the surface of all sporozoite populations was found with no obvious differences in shape between mutants and wild-type sporozoites (Fig. S4). To investigate how the gliding defects translate to the establishment of an infection *in vivo*, we subsequently performed sporozoite infectivity studies.

***In vivo* infectivity of palmitoyl transferase mutant sporozoites**

To determine whether the observed gliding motility defects would have an impact on infectivity, we inoculated mutant and wild-type sporozoites intravenously into mice and determined the time to blood stage infection, termed as the prepatent period, by Giemsa-stained blood smears. Wild-type sporozoites from hemolymph or salivary glands are similarly infective to rodents when inoculated intravenously (Sato *et al.*, 2014) and 10 000 hemolymph or 5000 salivary gland sporozoites result in infection with a prepatent period of 3 days. However, mice injected with 5000 or 10000 hemolymph sporozoites of the DHHC3-ko mutant failed to develop blood stage parasitemia (Table 1). Thus, while hemolymph DHHC3-ko sporozoites have similar motility to wild-type parasites in *in vitro* gliding assays, they are unable to cause an infection *in vivo*. Salivary gland sporozoites were found to be equally impaired in initiating infection: mice inoculated with 5000 DHHC3-ko sporozoites failed to develop blood stage parasitemia and upon injection with 10000 sporozoites, only one out of three mice developed a blood stage infection, with a delay of 3 days compared with wild-type (Table 1). Importantly, while this analysis revealed a vital point in the life cycle requiring DHHC3, complementation of the DHHC3-ko restored normal infectivity.

Mutant sporozoites that cannot initiate a blood stage infection may be impaired in one of many different steps: they may not localize to the liver, invade hepatocytes, develop to exoerythrocytic stages or exit the liver. To look at the DHHC3-ko infection phenotype more closely, we inoculated mutant and wild-type sporozoites intravenously and harvested livers for quantification of liver parasite burden. As shown in Fig. 7A, only very few DHHC3-ko sporozoites develop into exoerythrocytic stages *in vivo*. Because invasion of hepatocytes and development to exoerythrocytic stages *in vitro* was only mildly reduced (Fig. 7B and C), these data suggest that hemolymph, as well as salivary gland DHHC3-ko sporozoites, have exacerbated impairments *in vivo*.

By contrast, DHHC9-ko sporozoites infect mice with a normal prepatent period after intravenous injection, and even 500 DHHC9-ko sporozoites result in blood stage infection with a prepatent period of 3 days (Table 1). Because mosquitoes inject sporozoites into the dermis (Sidjanski and Vanderberg, 1997), we assessed whether DHHC9-ko sporozoites are

able to infect mice after intradermal inoculation. Intradermal injection did not significantly alter sporozoite infectivity (Table 1), and the normal prepatency period and furthermore the normal DHHC9-ko liver stage development in cultured HepG2 cells *in vitro* (Fig. S5) suggest that DHHC9 is functionally redundant or not required for *in vivo* sporozoite exit from the dermis and liver stage development.

Discussion

Previous studies suggested a high level of redundancy between the DHHCs. In yeast, all seven DHHCs were individually nonessential (Roth *et al.*, 2006), and in the protozoan parasite *Trypanosoma brucei*, all 12 DHHCs could be targeted by RNA interference knockdown without phenotype (Emmer *et al.*, 2011). However, in the plant *Arabidopsis thaliana*, DHHCs were found not to act completely redundantly, because deletion of two DHHCs resulted in visible phenotypes (Schiefelbein *et al.*, 1993). In the blood stages of *P. berghei*, the majority of DHHCs, apart from DHHC2, DHHC4 and DHHC8, could be successfully disrupted (Frénal *et al.*, 2013; Santos *et al.*, 2015), and it was suggested that the parasite DHHCs might have stage-specific functions, a speculation that was supported by evidence of stage-specific expression in *P. falciparum* (López-Barragán *et al.*, 2011). Other than the recent identification of PbDHHC2 as a palmitoyl transferase involved in mediating ookinete development (Santos *et al.*, 2015), nothing is known about the DHHCs involved in the transition from ookinete to sporozoite stage, through to liver stage development. The present data strengthen the hypothesis of stage-specific functions for DHHCs and illustrate that while there is functional redundancy in some stages, this is not true in other stages.

Epitope tagging of the endogenous loci of DHHC3 and DHHC9 revealed stage-specific regulation, and while DHHC3 expression was primarily present in ookinetes and oocyst sporozoites, weak DHHC9 expression was seen in oocyst sporozoites and in liver stage parasites from 48 h onwards. Strong DHHC9 expression was only detected in the liver stage and spatio-temporally confined to a thin compartment at the edge of the parasite 48 h after hepatocyte invasion. Given this localization, we hypothesized that DHHC3 and DHHC9 have functions in the mosquito and liver stages, and we characterized parasites individually disrupted for DHHC3 and DHHC9, as well as a double DHHC3/9-ko strain. Interestingly, as we describe in the succeeding discussion, DHHC3-ko salivary gland sporozoites had a strong phenotype, yet we did not detect expression of this palmitoyl transferase in salivary gland sporozoites. One possibility is that the amounts expressed are below the detection limit of indirect immunofluorescence, and another possibility is that its activity in oocyst sporozoites sets the stage for its role downstream.

Disruption of the DHHC3 locus had a striking impact on ookinete and sporozoite gliding speed and behaviour. All invasive stages of apicomplexan parasites, which includes ookinetes and sporozoites, exhibit gliding motility that is driven through an internal actin-myosin motor associated with the IMC (Soldati *et al.*, 2004; Frénal *et al.*, 2013; Caballero *et al.*, 2016). Palmitoylation had previously been established as a key post-translational modification for proteins associated with the IMC, and many motor complex-related proteins involved in motility and invasion have been shown to be palmitoylated in *T. gondii*, as well as the blood stages of *P. falciparum* (Rees-Channer *et al.*, 2006; Jones *et al.*, 2012;

Volkman *et al.*, 2012; Foe *et al.*, 2015). It has been shown that treatment of *T. gondii* with the broad-spectrum protein palmitoylation inhibitor 2-bromopalmitate (2-BP) alters gliding motility of tachyzoites (Alonso *et al.*, 2012), and inhibition of a thioesterase, the enzyme that is responsible for reversing palmitoylation, was found to result in increased motility and invasive capacity in tachyzoites (Child *et al.*, 2013). Our data are consistent with previous findings in *P. berghei*, which showed that addition of 2-BP blocks ookinete development *in vitro* (Santos *et al.*, 2015) and the DHHC3-ko/DHHC9-ko mutant is reminiscent of a mutant parasite deficient in PbDHHC2 (Santos *et al.*, 2015), which resulted in a developmental block at the zygote stage, implicating palmitoylation in ookinete development.

Here, we demonstrate a central role for the palmitoyl transferase DHHC3 in the regulation of development and motility of ookinetes and additional functions of DHHC3 for sporozoite motility and infectivity, thus further implicating palmitoylation in the regulation of parasite motility and the invasive capacity of these motile stages (Fig. 8). Palmitoylation, as a reversible modification, can fine-tune protein function and allow a protein to associate with specific membranes, thus affecting localization or protein–protein interactions. The actin–myosin motor is linked to transmembrane proteins with adhesive functions, and a controlled turnover of adhesion sites is necessary for continuous movement (Münter *et al.*, 2009). It is tempting to speculate that dynamic palmitate cycling may be involved in regulating the concerted action of the motor complex, and adhesive surface proteins and a slower turnover of the motor complex machinery could result in the observed decreased gliding speed of the palmitoyl transferase mutant ookinetes. The motility deficits of the DHHC3-ko ookinetes could account for their impairment to develop into oocysts by making them more prone to an attack by the mosquito's immune system as they attempt to cross the midgut epithelium. A similar link between ookinete gliding speed and oocyst numbers has previously been observed in a mutant parasite lacking the calcium-dependent protein kinase 3 (CDPK3), which produces morphologically normal ookinetes that exhibit a gliding defect and a similarly reduced transmission to the mosquito (Siden-Kiamos *et al.*, 2006). It will therefore be interesting to test whether PbCDPK3 is palmitoylated in ookinetes, particularly as the related CDPK1, which has been proposed to have functions regulating parasite motility (Kato *et al.*, 2008), as well as the *T. gondii* orthologue TgCDPK3, have been found to be palmitoylated in multiple studies (Jones *et al.*, 2012; Foe *et al.*, 2015; Caballero *et al.*, 2016).

While gliding motility of the hemolymph DHHC3-ko sporozoites was normal, they were not able to cause an infection after intravenous inoculation in mice, suggesting an additional dysregulation at this stage. Interestingly, DHHC3-ko salivary gland sporozoites, also non-infectious, exhibited a strong gliding impairment with a higher rate of detachment. Sporozoite adhesion is an active process dependent on actin dynamics, as well as membrane proteins that engage with the environment (Hegge *et al.*, 2010). Compared with salivary gland sporozoites, oocyst and hemolymph sporozoites have previously been shown to have reduced the capacity to attach (Hegge *et al.*, 2010), and it was suggested that an endogenous developmental programme leads to maturation of the sporozoite, resulting in a higher capacity to adhere (Hegge *et al.*, 2010). We hypothesize that this maturation process, which leads to infectivity and full gliding motility, as exhibited by wild-type salivary gland sporozoites, is affected in the DHHC3-ko mutant, thus explaining the normal gliding motility of hemolymph sporozoites, which does not require a high capacity for

attachment (Hegge *et al.*, 2010). It is possible that surface proteins involved with roles in substrate adhesion during salivary gland sporozoite gliding are affected in the DHHC3-ko parasite.

Contrasting with the DHHC3-ko, our phenotypic characterization of the DHHC9-ko strain indicates that DHHC9, which has previously been described as non-essential in blood stages (Frénal *et al.*, 2013), is also functionally redundant for *P. berghei* transmission. Despite the intriguing localization of DHHC9 to the periphery of the liver stage parasite, which in terms of both timing and localization was remarkably similar to the staining described for the IMC protein IMC1h-HA (Volkman *et al.*, 2012) and is the first localization of a *Plasmodium* DHHC in the liver stage of the parasite, DHHC9 is dispensable and likely fully functionally compensated by other DHHCs at this stage. However, while DHHC9 was found to be individually nonessential and no expression of DHHC9-HA was detected in the ookinete stage, the more dramatic phenotype of the double DHHC3 and DHHC9 knockout suggests partial compensation by DHHC9 in the DHHC3-ko. This indicates some functional redundancy of the two DHHCs in the ookinete, which in turn suggests overlap in the substrate specificities for these two DHHCs.

The present data indicate a strong role for palmitoylation in structure and function of the motor complex in the ookinete and sporozoite stages. Future work has to address the targets of DHHC3 and DHHC9, and comparative palmitoyl-proteomic approaches using strains deficient for one or multiple DHHCs will identify DHHC targets and may reveal both proteins relying on specific DHHC proteins, as well as overlapping substrate specificities, and will allow us to speculate more on the mechanism(s) by which DHHCs function. Over 20 proteins are known to localize to the IMC or its vicinity, and the majority of these has been found to be palmitoylated in *P. falciparum* (Jones *et al.*, 2012). And given the localization of DHHC3-HA, as well as the gliding motility and attachment phenotype in the DHHC3-ko parasite, proteins associated with the gliding machinery, as well as proteins involved in secretion of surface proteins and substrate adhesion, are possible targets for DHHC3. The present data suggest that DHHCs or their substrates may expand the list of potential drug targets, and identification of DHHC cognate substrates may provide us with novel targets for pharmacological interventions.

Experimental procedures

Ethics statement

All animal work was conducted in accordance with the recommendations by the Johns Hopkins University Animal Care and Use Committee (ACUC), under the ACUC-approved protocols #M011H467 and #M014H363. All efforts were made to minimize suffering.

Generation and genotyping of clonal DHHC-ko and DHHC-3xHA parasites

Plasmodium berghei ANKA DHHC3-ko (RMgm-888), DHHC9-ko (RMgm-892), DHHC3-3xHA (RMgm-897) and DHHC9-3xHA (RMgm-903) parasites from a systematic knock out screen of apicomplexan DHHC genes (Frénal *et al.*, 2013) were cloned by limiting dilution, and genomic DNA of clonal parasite lines was subjected to PCR to

confirm correct integration of the targeting cassette (Figs S1 and S2; see Table S1 for primer sequences). All experiments were performed with two clones of HA-tagged or ko parasites, apart from the DHHC9-ko, for which only one clone was available (Figs S1 and S2).

Genetic complementation of DHHC3-ko parasites

Negative drug selection with 5-FC in drinking water at 1 mgmr⁻¹ (Orr *et al.*, 2012) was applied to the DHHC3-ko clone A1, to select for parasites that excised the selection marker cassette, consisting of a fusion gene of two selectable markers: *hdhfr* (human dihydrofolate reductase; positive SM) and *yfcu* (yeast cytosine deaminase and uridyl phosphoribosyl transferase; negative SM), through homologous recombination in the 450 bp of *pbdhfr*-terminator sequence flanking the *hdhfr::yfcu* (Orr *et al.*, 2012). The resulting drug selection marker-free transfected line (Fig. S2) was cloned by limiting dilution in mice, and absence of the *hdhfr::yfcu* selection cassette was verified by PCR with primers 4698 and 4699 (see Table S1 for primer sequences). The homologous recombination event was confirmed by PCR with primers CassF and CassR, which anneal in areas flanking the *pbdhfr*-terminator sequences (Fig. S3; see Table S1 for primer sequences), and a 0.6 kbp band was expected after homologous recombination. Further cloning by limiting dilution resulted in a pure population, clone B2, which was sensitive to pyrimethamine. To re-introduce a copy of the 3×HA-tagged DHHC3 into the disrupted locus, we used standard procedures (Janse *et al.*, 2006) to transfect with the DHHC3-3×HA epitope tagging construct *PbGEM*-094114, available from the *PlasmoGEM* resource (Gomes *et al.*, 2015). The *PlasmoGEM* project (<http://plasmogem.sanger.ac.uk/>) generates genetic modification vectors with long homology arms through a process of phage recombineering from a library of *P. berghei* ANKA genomic inserts in a pJAZZ linear vector system (PMID: 20040575; PMID: 22020067). The transfected, complemented DHHC3-ko parasite line was cloned by limiting dilution, and PCRs on clones B5 and C3 were carried out to confirm presence of the DHHC3 gene with primers QCR3-F and QCR3-R and presence of the *hdhfr::yfcu* selection cassette with primers CassF and CassR. Integration at the 5'-end of the DHHC3 locus was confirmed via PCR with primers IntF and IntR (Fig. S3; see Table S1 for primer sequences).

Generation of DHHC3/9-ko parasites

The drug selection marker-free DHHC3-ko line clone B2, obtained after negative selection with 5-FC selection as described previously (Fig. S2), was transfected with a KO construct for the DHHC9 locus (*PbGEM*-121226), which was linearized through a *NotI* restriction digest as described previously (Fréna *et al.*, 2013). And after pyrimethamine-resistant parasites were recovered, PCRs on genomic DNA were carried out to verify correct integration at the 3'-end of the DHHC9 locus, with primers GT-9/GW2-9. Presence of the 5'-integration at the DHHC3 locus was verified via PCR with primers IntF and IntR. Absence of both the DHHC9 and DHHC3 open reading frames was confirmed by PCRs with primer pairs QCR9-F/QCR9-R and QCR3-F/QCR3-R respectively (Fig. S2; see Table S1 for primer sequences). All experiments were performed with two clones of the DHHC3/9-ko parasite (Fig. S2).

Ookinete cultures

Phenyl hydrazine-treated mice were used to obtain blood with high gametocytemia, and 3–4 days post-infection, exflagellation was quantified as previously described (Brochet *et al.*, 2014) by adding 1.5 μ l of mouse blood to 1 μ l of 200 units/ml heparin to 100 μ l exflagellation medium (RPMI1640 with 25 mM HEPES, 2 mM glutamine, supplemented with 20% FCS and 100 μ M xanthurenic acid). After 10–15 min of incubation at RT, the number of exflagellating microgametocytes was counted in a haemocytometer. If $1\text{--}6 \times 10^4$ exflagellation events per μ l blood were observed, ookinete cultures were set up in exflagellation medium and incubated at 19°C for 18–20 h as described previously (Brochet *et al.*, 2014).

Mosquito infection

Plasmodium berghei ANKA parasites were maintained in outbred female Swiss Webster mice and infections monitored on Giemsa-stained blood films. *A. stephensi* mosquitoes were reared in the Johns Hopkins insectary using standard procedures and fed on infected mice. On day 10 and 13 after infective blood meal, mosquitoes were dissected, and the midguts were observed for oocyst counts using an upright Nikon E600 (Nikon Instruments, Inc., Melville, NY, USA) microscope with a phase contrast PlanApo 10 $\times$ objective. For salivary gland sporozoite numbers, salivary glands were harvested on day 18–20, and organs from 15 to 20 mosquitoes were pooled, and released sporozoites were counted using a haemocytometer. For hemolymph sporozoites, thorax and abdomen of 15–20 mosquitoes were perfused with DMEM on day 18 post-feeding, as described previously (Hillyer *et al.*, 2007), and sporozoites were counted on a haemocytometer.

Parasite development in hepatocyte cultures

Human hepatoma cells (HepG2 ATCC HB 8065) were cultured in glass eight-chambered Lab-Tek wells in DMEM (supplemented with 10% FCS, 0.1 mM glutamine and 5mg/ml penicillin/streptomycin) at 37°C and 5% CO₂, as previously described (Sinnis *et al.*, 2013). For development of parasite EEFs, 5×10^4 sporozoites per well were added and incubated for 24, 48 or 65 h at 37°C and 5% CO₂. Wells were washed, fixed in 4% paraformaldehyde/PBS and permeabilized in methanol over night at –20°C and blocked with 1% BSA/PBS for 1 h at RT before incubation with primary and secondary antibodies.

Immunofluorescence assays

For immunofluorescence assays (IFAs), midgut, hemolymph and salivary gland sporozoites were centrifuged onto coverslips in 24 well plates at 800 g for 3 min, and for ookinete IFAs, thin blood smears of ookinete cultures were air-dried. All samples were fixed with 4% paraformaldehyde/PBS permeabilized with 0.1% Triton X–100/PBS and blocked with 3% BSA/PBS. Primary and secondary antibodies were diluted in 1% BSA/PBS, and antibodies included mouse monoclonal CSP antibody (clone 3D11), used at 1 μ gml^{–1}; polyclonal rabbit anti-MTIP (kindly provided by Stefan Kappe, Seattle Biomedical Research Institute) (Bergman *et al.*, 2003), diluted 1:400; mouse monoclonal anti-HA (clone 16B12) (BioLegend, San Diego, CA, USA), diluted 1:200; affinity purified rabbit anti-HA (Sigma-Aldrich Corporation, St. Louis, MO, USA), diluted 1:200; and polyclonal rabbit anti-uis4

diluted 1:1000. Secondary detection was with Alexa-Fluor 488 goat anti-mouse IgG (Molecular Probes, A11029, Eugene, OR, USA), Alexa-Fluor 594 goat anti-rabbit IgG (Molecular Probes, A11037). All samples were preserved in Prolong Gold mounting medium containing DAPI (LifeTechnologies, Gaithersburg, MD, USA) and imaged using a LSM700 laser scanning confocal microscope (Zeiss AxioObserver, Carl Zeiss AG, Oberkochen, Germany) with a 63×/1.4 PlanApo oil objective, and images were acquired using ZEN software (Carl Zeiss AG, Oberkochen, Germany).

Ookinete Matrigel motility assay

Ookinete cultures were mixed on ice with an equal volume of Matrigel (BD Bioscience, San Diego, CA, USA), and 50 µl were dropped onto a slide, covered with a coverslip and sealed with nail polish. Parasites were visualized using a 40× objective, resulting in an imaging area of 206 × 165 µm. To allow the Matrigel to solidify, slides were incubated for 15 min at RT, following which time-lapse videos were captured at 0.2 Hz over a total time of 4 min using epifluorescence microscopy on an upright Nikon E600 microscope with a 40× objective, and images were acquired with a digital camera DsRi1 Nikon (Nikon Instruments, Inc., Melville, NY, USA) and NIS Elements software (Nikon Instruments, Inc., Melville, NY, USA). The resulting two-dimensional dataset was processed and manually tracked using Fiji software with the manual tracking plugin. Results of at least two independent ookinete cultures are shown.

Manual tracking and data analysis

Average speed—Average gliding speed was calculated as the sum of distances between track locations, divided by the track duration. This measure is highly sensitive to the frame rate with which the video was acquired (Kan *et al.*, 2014), and only datasets acquired at the same interval times were compared.

Mean displacement—The mean displacement was calculated for parasites that exhibited motility, and tracks spanning less than the total video time of 10 min were discarded.

Sporozoite gliding motility assays—Sporozoite gliding motility was assayed as previously described (Ejigiri *et al.*, 2012). Round coverslips were coated in 24-well plates with 10 µg µl⁻¹ mAb 3D11 (Yoshida *et al.*, 1980) in PBS overnight at 4°C. A total of 10⁵ salivary gland sporozoites in 2% BSA in DMEM were added to each well, centrifuged onto the coverslip for 3 min at 800 × g and incubated for 1 h at 37°C. Wells were fixed in 4% paraformaldehyde/PBS, blocked over night in 3% BSA/PBS and stained with biotinylated mAb 3D11 for 1 h at RT, followed by detection with streptavidin-conjugated to Alexa-Fluor-488 (Molecular Probes, S11223) diluted 1:100 in 1% BSA/PBS for 1 h at RT. Samples were preserved in Prolong Gold mounting medium (LifeTechnologies, Gaithersburg, MD, USA), and the number of trails associated with sporozoites was counted using fluorescence microscopy on an upright Nikon E600 and 40× objective. For live sporozoite gliding assays, 5 µl of salivary gland sporozoites in 2% BSA in DMEM were dropped on a microscopy slide and covered with a coverslip. To allow sporozoites to settle, slides were incubated for 10 min at 37°C, following which time-lapse videos were captured at 1 Hz over a total time of 2 min using epifluorescence microscopy on an upright Nikon E600 microscope with a 40×

objective, and images were acquired with a digital camera (DsRi1 Nikon) and NIS Elements software (Nikon). Sporozoites were manually tracked using Fiji software with the manual tracking plugin.

Assay for sporozoite infectivity in vivo—Swiss/Webster mice were inoculated i.v. with 4500 salivary gland sporozoites. After 38.5 h, livers were harvested, total RNA was isolated and infection was quantified using reverse transcription followed by real-time PCR using primers that recognize *P. berghei*-specific sequences within the 18S rRNA as outlined previously (Bruña-Romero *et al.*, 2001).

Sporozoite infectivity as determined by prepatent period—Four- to six-week-old Swiss Webster mice were infected with a total of 500, 5000 or 10 000 hemolymph or salivary gland sporozoites, either through intravenous or intradermal injection. For intradermal inoculation, mice were lightly anaesthetized by intraperitoneal injection of ketamine/xylazine (35–100 µg ketamine/g body weight) and maintained at 37°C on a slide warmer. Sporozoites were injected into the ear pinna in a total volume of 0.2 µl DMEM with a Flexifill microsyringe (World Precision Instruments, Sarasota, FL, USA). The onset of blood stage infection was determined by daily observation of Giemsa-stained blood smears, beginning on day 3 after inoculation.

Statistical analysis—Statistical analysis of scatter dot plots was analysed using unpaired student's t-test, and statistical significance between samples is indicated with asterisks as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns, nonsignificant ($P > 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Abai Tripathi, Godfree Mlambo and Chris Kizito, the team of the insectary and parasitology core facilities at the Johns Hopkins Malaria Research Institute for their outstanding work. We thank Stefan H. I. Kappe (Center for Infectious Disease Research, Seattle) for his generous gift of the MTIP antibody. This work was supported by the National Institutes of Health (R01 Grant No. A1056840) (P. S., A. E. B. and C. S. H.) and a Johns Hopkins Malaria Research Institute fellowship (C. S. H.). Work at the Sanger Institute was funded by the Wellcome Trust (Grant No. WT098051).

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Fig. 1.

Localization of DHHC3-HA and DHHC9-HA in ookinetes as well as oocyst and salivary gland sporozoites.

A. DHHC3-HA and WT ookinetes labelled with anti-HA (green) and IMC-marker MTIP (magenta) and DAPI (blue).

B. Expression of DHHC3-HA (green) in developing DHHC3-HA oocysts 10 days after the infectious blood meal. DNA was stained with DAPI (blue).

C and D. DHHC3-HA, DHHC9-HA and WT oocyst sporozoites and salivary gland sporozoites were isolated 14 and 18 days, respectively, after the infectious blood meal. Sporozoites were stained for HA (green) and MTIP (magenta); DNA was stained with DAPI (blue). Scale bars 20 μ m.

Fig. 2.

Localization of DHHC3-HA and DHHC9-HA in liver stages.

A. DHHC3-HA or B. DHHC9-HA sporozoites were added to HepG2 cell monolayers, and liver stage development was assayed at 24, 48 and 65 h after infection, when monolayers were fixed and stained for HA (green) to localize the palmitoyl transferase and uis4 (up-regulated in infective sporozoites gene 4, (Mueller *et al.*, 2005)) (magenta) to visualize the parasitophorous membrane. Parasite and host cell DNA was stained with DAPI (blue). Scale bars 10 μm.

Fig. 3. Roles for DHHC3 and DHHC9 during *Plasmodium berghei* transmission to mosquitoes. A. Oocyst formation of WT and mutant parasites. Midguts from *Anopheles stephensi* mosquitoes 10 and 13 days after the infectious blood meal were dissected and oocysts counted. Results of *n* independent feeding experiments are shown: WT (grey) *n* = 5, DHHC3-ko (red) *n* = 4, complemented DHHC3-ko (blue) *n* = 2, DHHC9-ko (purple) *n* = 2 and DHHC3/9-ko (orange) *n* = 2. Horizontal lines represent mean values. B. Number of hemolymph and salivary gland sporozoites of WT and mutant parasites. For collection of hemolymph, mosquitoes were dissected 18 days after blood meal, and salivary glands were harvested 18–20 days after blood meal.

Fig. 4.

Palmitoylation is required for normal ookinete maturation. Ookinete *in vitro* cultures were stained for the IMC-marker MTIP (green) and DAPI (blue).

A. Normal subcellular localization of MTIP in permeabilized WT and DHHC3-ko, complemented DHHC3-ko and DHHC9-ko ookinetes. Scale bars 5 μ m.

B. DHHC3/9-ko ookinetes were stained for MTIP (green) and DAPI (blue) to illustrate aberrant ookinete forms (a high number of stage II-like forms and shorter than normal ookinetes) present after 16–19 h of *in vitro* culture. Results of two biological replicates are shown. Scale bars 5 μ m.

Fig. 5.

DHHC3-ko ookinetes are less motile in Matrigel.

A. Ookinetes cultured *in vitro* were imaged in Matrigel with time-lapse brightfield microscopy over a total time of 10 min. Ookinetes were tracked using ImageJ software and colored tracks were superimposed to the first timepoint. Scale bars 50 μm .

B. Average ookinete gliding speed with horizontal bars representing mean values. Compared to WT, complemented DHHC3-ko and the DHHC9-ko lines, DHHC3-ko parasites exhibit significantly decreased average gliding speed. Double DHHC3/9-ko ookinetes have an impairment in gliding speed, which is significantly decreased compared to the DHHC3-ko. Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple-comparison test.

C. Mutant DHHC3-ko and DHHC3/9-ko ookinetes display decreased mean displacement. Data in panels B.–C. originate from two WT, four DHHC3-ko, two complemented DHHC3-ko, one DHHC9-ko and one DHHC3/9-ko biological replicates and a total number of 166 WT, 175 DHHC3-ko, 205 complemented DHHC3-ko, 16 DHHC9-ko and 16 DHHC3/9-ko ookinetes were tracked for analysis.

Fig. 6.

Gliding motility of DHHC3-ko, complemented DHHC3-ko, DHHC9-ko and DHHC3/9-ko sporozoites.

A. To characterize sporozoite gliding motility, WT and mutant sporozoites were allowed to glide for 1 h at 37°C on coated glass coverslips and trails were visualized by detection of shed surface protein CSP. Panels show representative fields of view; scale bars 25 μm .

B. The number of circular trails associated with sporozoites was counted. Total number of hemolymph sporozoites counted was: WT ($n = 601$), DHHC3-ko ($n = 329$), complemented DHHC3-ko ($n = 249$), DHHC9-ko ($n = 186$), DHHC3/9-ko ($n = 262$) and total number of salivary gland sporozoites counted was: WT ($n = 214$), DHHC3-ko ($n = 100$), complemented DHHC3-ko ($n = 249$), DHHC9-ko ($n = 100$) and DHHC3/9-ko ($n = 213$) sporozoites counted. Scale bars 30 μm .

C.–E. Live gliding motility of WT and DHHC3-ko salivary gland sporozoites *in vitro* on glass slides. Two-minute videos were acquired, and results of three biological replicates are shown.

C. Sporozoite motility patterns were manually counted and shown is the percentage of attached/non-motile, non-attached/floating and motile sporozoites. Sporozoites were manually tracked using ImageJ software.

D. Length of tracks during continuous gliding and

E. average gliding speed of motile sporozoites are displayed. Bars represent mean values.

Fig. 7. DHHC3-ko sporozoites are impaired for liver infection *in vivo* and *in vitro*. **A.** *In vivo* infectivity: mice were inoculated intravenously with WT or DHHC3-ko salivary gland sporozoites. Two days later, total liver RNA was extracted and infection was measured by quantifying the copy number of *Plasmodium berghei* 18S rRNA through quantitative PCR. **B.** *In vitro* infectivity: WT or DHHC3-ko sporozoites were harvested from mosquito hemolymph or salivary glands and added to HepG2 cells for 1 h, after which time cells were fixed and stained with a double-staining assay that distinguishes intracellular from

extracellular sporozoites. The mean and standard deviation of two biological replicates are shown.

C. Immunofluorescence of DHHC3-ko liver stages at the indicated time points of development. Parasites were stained for parasite proteins CSP (green) and uis4 (magenta) to visualize the parasitophorous membrane. Parasite and host cell DNA was stained with DAPI (blue). Scale bars 10 μ m.

Fig. 8.

Summary of DHHC3 and DHHC9 localization and essentiality across the mosquito and liver stages of *Plasmodium berghei*. Schematic representation of DHHC3-HA and DHHC9-HA expression (grey boxes) across the mosquito and liver stages, with DHHC3-HA being expressed in ookinetes and oocyst sporozoites, and DHHC9-HA being spatio-temporally restricted to expression in the liver stage. Crosses represent the impact of the DHHC3 and DHHC9 deletions, with DHHC3-ko (green) being impaired at the ookinete and salivary gland sporozoite stages and the DHHC3/9-ko (pink) being severely impaired at the ookinete stage.

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Table 1
In vivo infectivity of palmitoyl transferase mutants as determined by prepatent period.

Sporozoite origin	Route of inoculation	Parasite line	No. of sporozoites injected	No. of positive mice/No. of infected mice	Prepatent period (days)
Hemolymph	i.v.	WT	10 000	8/8	3.1
	i.v.	DHHC3-ko	5000	0/5	–
	i.v.	DHHC3-ko	10 000	0/5	–
Salivary gland	i.v.	WT	500	5/5	3
	i.v.	WT	5000	8/8	3.1
	i.v.	DHHC3-ko	5000	0/5	–
	i.v.	DHHC3-ko	10 000	1/3	6
	i.v.	Complemented DHHC3-ko	5000	5/5	3.4
	i.v.	DHHC9-ko	500	5/5	3.2
	i.v.	DHHC9-ko	5000	5/5	3
	i.d.	WT	5000	5/5	3.4
	i.d.	DHHC9-ko	5000	5/5	3.2