Copper-transporting ATPase is important for malaria parasite fertility

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Summary

Homeostasis of the trace element copper is essential to all eukaryotic life. Copper serves as a cofactor in metalloenzymes and catalyses electron transfer reactions as well as the generation of potentially toxic reactive oxygen species. Here, we describe the functional characterization of an evolutionarily highly conserved, predicted copper-transporting P-type ATPase (CuTP) in the murine malaria model parasite Plasmodium berghei. Live imaging of a parasite line expressing a fluorescently tagged CuTP demonstrated that CuTP is predominantly located in vesicular bodies of the parasite. A P. berghei loss-of-function mutant line was readily obtained and showed no apparent defect in in vivo blood stage growth. Parasite transmission through the mosquito vector was severely affected, but not entirely abolished. We show that male and female gametocytes are abundant in cutp− parasites, but activation of male microgametes and exflagellation were strongly impaired. This specific defect could be mimicked by addition of the copper chelator neocuproine to wild-type gametocytes. A cross-fertilization assay demonstrated that female fertility was also severely abrogated. In conclusion, we provide experimental genetic and pharmacological evidence that a healthy copper homeostasis is critical to malaria parasite fertility of both genders of gametoocyte and, hence, to transmission to the mosquito vector.

Introduction

Copper is a trace element essential to all eukaryotic life and functions as a cofactor in many key enzymes, such as mitochondrial cytochrome c oxidase and superoxide dismutase. Its properties as a transition metal enable the electron transfer by metalloenzymes and yet make it toxic to cells (Festa and Thiele, 2011; Hodgkinson and Petris, 2012; Samanovic et al., 2012). Consequently, the redox property of copper can catalyse the production of hydroxyl radicals under aerobic conditions through the Fenton reaction leading to oxidative damage to proteins, DNA, lipids, etc. (Jomova and Valko, 2011). Therefore, copper homeostasis needs to be tightly controlled and cells express a range of copper-specific transporting and sequestering proteins (Lutsenko, 2010; Argüello et al., 2012).

Plasmodium species are unicellular ancient eukaryotic parasites that are the sole cause of malaria. These obligate intracellular parasites harbour at least three genes, which are predicted to be involved in copper transport and the maintenance of a healthy copper homeostasis: two proteins harbouring a putative Ctr copper transporter domain (Pfam04145; PBANKA_102150 and PBANKA_130290) (Martin et al., 2009; Choveaux et al., 2012) and one copper-transporting P-type ATPase (CuTP; PBANKA_041650) (Rasoloson et al., 2004). The sources of copper for parasites during blood stage development are unknown. The relatively abundant red blood cell derived copper superoxide dismutase taken up along with haemoglobin in the parasite food vacuole was suggested as one possible source (Rasoloson et al., 2004). Alternatively, one or both of the putative Ctr copper transport proteins could be involved in import. Localization of the P. falciparum copper transport protein (PF3D7_143900) to the erythrocyte and parasite plasma membranes could support a role in either import or export (Choveaux et al., 2012).

The effect of intracellular depletion of Cu+ can be tested with the cell-permeable copper chelator neocuproine (2,9-dimethyl-1,10-phenanthroline; CID 65237) (Smith and McCurdy, 1952), which is highly specific for Cu+. In a previous study, addition of neocuproine to cultured blood stage Plasmodium falciparum, the parasite responsible for the majority of malaria-related deaths, was reported to inhibit ring-to-trophozoite transition but did not affect infectivity of schizonts (Rasoloson et al., 2004). The
identity of copper containing enzymes and their importance for blood stage development remains unknown, particularly since Plasmodium parasites lack an orthologue of Cu/Zn superoxide dismutase.

The P. falciparum CuTP was localized to both parasite and host cell membrane and it was suggested that it mediates Cu⁺ efflux in order to minimize toxic effects of excess Cu⁺ (Rasoloson et al., 2004). There are two CuTP homologues in human and mouse genomes, ATP7A and B, and mutations therein are linked to Menkes and Wilson disease respectively (La Fontaine et al., 2010). Both transporters usually reside at the trans-Golgi network where they facilitate the biosynthesis of cuproenzymes. In addition, they can detoxify by exporting excess intracellular Cu⁺ (La Fontaine and Mercer, 2007; Hasan and Lutsenko, 2012).

Here, we have used the murine malaria model Plasmodium berghei to investigate the in vivo role(s) of this evolutionarily highly conserved transport protein during the malaria parasite life cycle. We demonstrate that PbCuTP is dispensable for blood stage development and important only for male and female fertility. Localization of the fluorescently tagged protein to vesicular bodies suggests that such bodies might serve as copper storage organelles throughout the Plasmodium life cycle.

Results

Apicomplexan parasites encode evolutionary conserved CuTP proteins

We identified genes encoding CuTP in all queried Plasmodium species and some related apicomplexan parasites, e.g. Cryptosporidium parvum and Toxoplasma gondii (Table S1). All proteins contained at least one predicted metal-ion scavenging motif (MxCxxC) at the amino-terminal end and at least three pairs of predicted transmembrane domains. In addition, the intramembranous ‘CPC’ and ‘MxxSS’ motifs required for the selective translocation of Cu⁺ ions across membranes (Argüello, 2003) are conserved in all apicomplexan CuTP sequences placing them into the family of intracellular P₁B₁-type ATPases (Fig. S1). Phylogenetic analysis of selected CuTP protein sequences from a variety of model organisms and species with a biological or evolutionary link to malaria parasites confirmed an ancestry reflecting the known evolutionary relationships (Fig. S2). Together, consistent presence of CuTP in all parasitic and free-living eukaryotes indicates important functions.

CuTP is expressed in all Plasmodium life cycle stages and localizes to vesicle-like structures

To examine the temporal and spatial expression of CuTP, we generated a transgenic parasite line where the endogenous CuTP was carboxy-terminally fused to a red fluorescent mCherry-triple c-myc hybrid protein (Fig. S3A). This was achieved by tailored double cross-over homologous recombination, generating stable recombinant parasites. Transfection of WT parasites, followed by flow-cytometric isolation of recombinant parasites yielded an isogenic parasite line, termed cutp::tag (Fig. S3B). Live imaging of cutp::tag parasites revealed abundant expression of PbCuTP in asexual and sexual blood stages, ookinetes, and sporozoites (Fig. 1A). Intriguingly, in all intra- and extracellular parasite stages the tagged CuTP consistently localized to large vesicle-like structures inside the parasite cytoplasm (Fig. 1B). This was most striking in ookinetes, where CuTP localizes to multiple vesicle-like structures of unknown origin. In addition, staining with an anti-mCherry antibody on fixed liver stage parasites 48 h after infection revealed intracellular expression of CuTP similar to the other parasite life cycle stages (Fig. 1C). Western blot analysis demonstrated integrity of the fusion protein and excluded potentially aberrant localization of a processed carboxy-terminal fluorescent tag, at least in mixed blood stages (Fig. S3C). We conclude that CuTP is present throughout the entire Plasmodium life cycle and localizes to intraparasitic structures that might represent storage vesicles.

Localization of Toxoplasma gondii CuTP to intraparasitic structures

We initially hypothesized that CuTP might localize to acidocalcisomes, acidic organelles rich in calcium and phosphorus that can be found in Plasmodium species as well as T. gondii (Miranda et al., 2008; Moreno and Docampo, 2009). Since an antibody against the T. gondii plant-like vacuolar proton pyrophosphatase (VP1) that localizes to acidocalcisomes and a plant-like vacuole (Miranda et al., 2010) was not cross-reactive with P. berghei blood stage parasites (data not shown), we generated a stable recombinant T. gondii line expressing endogenous CuTP (TGGT1_020170) fused in-frame with a 2xMyc tag using single cross-over homologous recombination (Fig. S4).

Double immunofluorescence of intracellular Tgcutp::myc parasites with the anti-TgVP1 and anti-Myc antibodies revealed that TgCuTP localizes to intraparasitic structures juxtaposed to the acidocalcisomes and/or plant-like vacuole (Fig. 2). This spatial distribution closely reflects our findings in P. berghei, indicative of potential functional similarities of the CuTPs in the two apicomplexan parasites. Although we could not provide definitive assignment of the parasite organelle, we did not detect CuTPs at the parasite plasma membranes, in good agreement with their phylogenetic placement as intracellular P₁B₁-type ATPases.
Targeted deletion of CuTP does not affect asexual blood stage growth

We next wanted to study the in vivo role(s) of CuTP and generated a targeting vector, termed pCuTP-KO, to ablate CuTP in P. berghei (Fig. 3A). Successful generation of cutp::tag parasites already indicated that the gene locus is amenable to genetic manipulation. Employing a similar gene targeting strategy, we performed two independent transfections followed by flow-cytometric isolation of recombinant parasites. To our surprise, we readily obtained cutp− parasites lines in both cases (Fig. 3A–C), indicating that loss of CuTP is compatible with asexual blood stage growth.

In order to directly compare in vivo blood stage growth, we infected mice (n = 3) by intravenous injection of 1000
infected erythrocytes of either cutp− or WT parasites and monitored parasitemia side-by-side over the course of one week (Fig. 3D). We observed no difference in the exponential growth phase, and both Plasmodium lines resulted in similar high parasite burden.

We next synchronized P. berghei asexual parasites by high dose intravenous injection of culture-enriched schizonts. Blood was collected 2 h later for a highly synchronized in vitro parasite culture. Flow-cytometric analysis of WT and cutp− parasites cultured for 20 h suggested a reduction of multi-nucleated schizonts in cutp− parasites when compared with WT parasites in two independent experiments (Fig. 3E). However, this mild arrest did apparently not affect exponential propagation of asexual blood stages in vivo (Fig. 3D). Together, successful generation of two independent cutp− parasite lines establishes a dispensable role of the copper-transporting ATPase during asexual blood infection.

Additive inhibition by the cell-permeable copper chelator neocuproine

The observed reduction of in vitro-cultured schizonts in cutp− parasites encouraged us to test whether depletion of intracellular copper could, at least partially, phenocopy genetic ablation of CuTP. We tested the effect of a broad range of neocuproine concentrations on schizont formation in an in vitro culture assay (Fig. S5). Though schizont formation in cutp− parasites was typically reduced to 35–65% of WT at any given concentration, WT and cutp− parasites were affected to a similar extent by increasing neocuproine concentrations. Negligible schizont formation was reached in WT and cutp− parasites at 500 nM neocuproine.

Severe impairment of natural transmission in cutp− parasites

Since CuTP is dispensable for asexual blood stages, we next explored whether it plays an important role during life cycle progression. To this end, we first performed a natural transmission experiment (Fig. 4A). Anopheles stephensi mosquitoes were infected by feeding on cutp− and WT-infected mice. On day 17, when sporozoites have colonized salivary glands, these mosquitoes were used to infect naïve mice. Monitoring of blood stage parasitemia revealed a severe defect in cutp−-infected mice (Fig. 4A). Mice infected with cutp− sporozoites stayed either parasite-free or became blood-film positive with a 2-day delay on average, indicating a severe defect during Plasmodium life cycle progression.

We next dissected infected Anopheles mosquitoes and determined the oocyst burden (Fig. 4B and C). While WT-infected mosquitoes contained large numbers of oocysts, cutp−-infected mosquitoes displayed very few oocysts. Quantification of oocyst numbers in repeated feeding experiments with the two isogenic cutp− parasite lines confirmed this finding (Fig. 4B). In order to test whether this phenotype is stable, we subjected cutp− parasites selected by the first transmission experiment to a second transmission cycle. Again, oocyst numbers were dramatically reduced, indicating that loss of CuTP function results in a robust phenotype that cannot be swiftly adapted to. As expected, quantification of sporozoites in infected mosquitoes revealed low numbers (Fig. 4D), in good agreement with the severe defect in the transmission experiment. We conclude that the main defect in cutp− parasites is efficient colonization of the insect vector, the definitive host of the Plasmodium life cycle.

Male exflagellation is significantly reduced in cutp− parasites

For the effective colonization of the anopheline mosquito midgut and subsequent successful transmission to a new vertebrate host, malaria parasites need to go through a round of sexual reproduction (Kooij and Matuschewski, 2007). This complex process involves the activation of dormant gametocytes in the mosquito midgut. Upon emer-
gence from the red blood cell highly motile male microgametes aim to fertilize a female macrogamete.

To establish whether a fertility defect might be the cause of the low cutp− oocyst numbers, we quantified the number of exflagellating male gametocytes (Fig. 5A). Exflagellation activities of both cutp− lines were reduced to 10–15% of the activity of WT parasites. In contrast, cutp::tag male gametocytes showed exflagellation levels within the WT range (Fig. 5A). This finding demonstrated that the fluorescently tagged CuTP is functional and the observed targeting to intracellular vesicles (Fig. 1A and B) is physiologically relevant.

Of note, no differences between WT and cutp− lines in either gametocyte production or ratios of male and female gametocytes (Fig. 5B) were detected. Immunofluorescent microscopic analysis of activated male cutp− gametes revealed the presence of healthy and degenerated males (Fig. 5C). Together, our findings establish that the trans-
mission defect in the absence of CuTP can be, at least partially, attributed to impaired microgamete activation and/or formation.

Inhibition of Cu⁺ impairs exflagellation of wild-type gametes

To further substantiate the link between male fertility and copper, we investigated the effects of copper ion addition and chelation on exflagellation in an in vitro assay. Chelation of intracellular Cu⁺ with neocuproine in WT parasites resulted in a substantial reduction of male exflagellation, mimicking the defect observed in cutp⁻ parasites (Fig. 6). In contrast, we were not able to restore the exflagellation deficit of the cutp⁻ parasites or neocuproine-treated WT parasites by addition of extracellular Cu⁺ or Cu²⁺ (data not shown).

Ablation of CuTP impairs female fertility

So far, we determined a ∼ 10-fold reduction in microgamete formation, yet observed a ∼ 100-fold reduced oocyst burden in cutp⁻ parasites. To confirm a male fertility defect and test female fertility, we performed a cross-fertilization assay and quantified the resulting ookinetes, a functional read-out for gamete fertility (van Dijk et al., 2001; 2010). As published previously, no ookinetes were detectable in cultures containing p48/45⁻ or p47⁻ parasites alone, whereas in co-cultures p47⁻ male microgametes were able to productively fertilize p48/45⁻ females and ookinetes were readily formed (Fig. 7). Next, we tested ookinete formation in cultures containing cutp⁻ parasites alone. Ookinetes were observed only infrequently, approximately 2% of WT oocyst formation (Fig. 7). This prominent reduction correlates with the very low oocyst formation (Fig. 4B), and apparently exceeds reduced exflagellation rates (Fig. 5A).

Strikingly, co-culturing cutp⁻ parasites with fertile p47⁻ males did not result in higher ookinete numbers (Fig. 7). A likely interpretation of this result is that healthy male gametes in p47⁻ were unable to compensate for the reduced exflagellation activity of cutp⁻ parasites, indicative of an additional female fertility defect. Indeed, co-cultures of cutp⁻ and fertile p48/45⁻ female parasites yielded approximately 10% ookinetes (Fig. 7), strongly indicating a partial rescue of female fertility defects in cutp⁻ females by addition of p48/45⁻ parasites. Together, our cross-fertilization studies establish that Plasmodium CuTP is critical only for fertility of male and female gametes.

Discussion

Plasmodium berghei CuTP functions in parasite fertility

In this study, we establish an important function for the putative Plasmodium Cu⁺-transporting P_{1B1}-type ATPase in parasite fertility. Thus far, very little is known about possible links between fertility and copper homeostasis in any eukaryotic organism. A first indication for a potential
Fig. 5. Male microgamete formation is defective in cutp− parasites.
A. Quantification of exflagellation events in vitro (**P < 0.01; ns, non-significant; Kruskal–Wallis test followed by Dunn’s post-test).
B. In vivo gametocyte conversion rate (left) and ratio between female and male gametocytes (right) did not differ significantly (ns, non-significant; Kruskal–Wallis test followed by Dunn’s post-test).
C. Immunofluorescence analysis of activated male gametes. Parasites were fixed 14 min after activation and stained with anti-heat shock protein 70 antibody (cytoplasm). Male gametes were identified by staining with anti-SET antibody and Hoechst for DNA. The presence or absence of DNA replication, DNA segregation, formation of flagella, and the formation of blebs were used to monitor the health status of the male gametes and to define six categories as described to the left of the exemplary immunofluorescence images. Proportions of healthy (shades of green) and abnormal (shades of red) gametocytes were scored for WT (n = 30) and cutp− (n = 56) parasites according to these categories that are represent in the pie charts by the colour coding as it appears to the right of the images. Bars, 5 μm.

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correlation between copper levels and spermatozoal motility was noted in a study that compared highly and less motile human sperm cells (Battersby and Chandler, 1977). More recently, a redundant copper transporter was shown to play an important role in male fertility in *Drosophila melanogaster* (Steiger et al., 2010). In male mice, Atp7a mutants displayed abnormal testes morphology and decreased male gamete quantity and quality, including motility and membrane integrity (Kowal et al., 2010). Together with our findings, a universal link between copper homeostasis and male fertility emerges.

Thus far, copper transporters have not yet been implicated in female fertility. Malaria parasites lack sex-specific chromosomes. Instead, sex-specific gene expression is regulated by transcriptional and translational control mechanisms (Kooij and Matuschewski, 2007). Our analysis of temporal and spatial CuTP expression revealed a remarkably uniform expression and localization to intracellular vesicles. Therefore, we consider regulation of *Plasmodium CuTP* expression of minor, if any, importance. However, loss of CuTP function results in specific defects in male and female fertility *in vitro* and, as a consequence, in severe impairment of colonization of the definitive invertebrate host *in vivo*. While this unexpected finding offers new opportunities for transmission blocking strategies, additional experimentation is warranted to elucidate the roles of Cu²⁺ and, most likely, one or more critical cuproenzymes in gamete fertility. It is tempting to hypothesize that copper homeostasis might also play previously unrecognized roles in female fertility of other eukaryotes.

**Copper export or storage?**

At present, it is unknown, whether *Plasmodium* CuTP mediates copper export or intracellular redistribution. A previous study proposed a role for *P. falciparum* CuTP in copper export and localization of the protein to the erythrocyte plasma membrane (Rasoloson et al., 2004). A second *P. falciparum* copper transport protein (PF3D7_1439000) thought to mediate copper transport has also been localized to the plasma membrane of ring-stage infected red blood cells (Choveaux et al., 2012). Accordingly, the observed phenotype of CuTP loss-of-function parasites might be explained by a defect in copper export, leading to toxic accumulation and excessive generation of reactive oxygen species. Our data demonstrate a predominant localization at rounded vesicular bodies inside the cytoplasm of all life cycle stages as well as an intraparasitic localization in *T. gondii*. This localization is

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**Fig. 6.** Reduction of male microgamete exflagellation in the presence of the cell-permeable Cu⁺-chelator neocuproine. Shown are the numbers of exflagellating WT and cutp⁻ male gametocytes in the presence or absence of 1 μM neocuproine (*P < 0.05; non-parametric, two-tailed Mann–Whitney’s test).

**Fig. 7.** Cutp⁻ parasites display reduced female fertility. Cross-fertilization assay to determine fertility of female and male cutp⁻ gametes. The fertilization partners are p48/45⁻ (pink), which produces only healthy female gametes and p47⁻ (blue), which produces only healthy males. As positive and negative controls, a p47⁻ × p48/45⁻ cross-fertilization and homologous ookinete cultures were included. Ookinete cultures of cutp⁻ parasites (black) yielded only 2% of the reference culture. A similar low level of ookinetes resulting from cross-fertilization with healthy male gametes (p47⁻) suggests a defect in female fertility of cutp⁻ parasites. Partial rescue in cross-fertilization with healthy females (p48/45⁻) corroborates male gamete deficiency of cutp⁻ parasites. Results were obtained from three independent experiments (repeated measures analysis of variance with Bonferroni’s post-test; **P < 0.01; ns, non-significant).
more consistent with an important role in intracellular copper redistribution or storage, most likely for biosynthesis of cuproenzymes. We cannot entirely rule out residual PbCuTP targeting to the parasite vacuolar and plasma membranes or the erythrocyte plasma membrane (Fig. 1). However, our data do not support such a localization. Normal exflagellation rates of the cutp::tag parasites suggest that the mCherry-3xMyc tagged protein is functional and is therefore expected to localize correctly. In fact, alternative interpretations of the single published immunofluorescence image, obtained with a polyclonal PCuP-ATPase antiserum, are possible. We note a strong vesicular-like pattern, reminiscent of the localization of the endogenously tagged protein, which was not addressed by the authors (Rasoloson et al., 2004). In conclusion, we favour a central role of CuTP in intracellular copper homeostasis and, possibly, export via vesicles, comparable to ATP7 in higher eukaryotes (La Fontaine and Mercer, 2007; Hasan and Lutsenko, 2012).

What is the CuTP-labelled vesicle-like compartment?

In the absence of validated subcellular markers or antibodies to perform colocalization studies and assign a definitive identity to the PbCuTP-labelled vesicle-like structures, we note that in T. gondii CuTP associates with the acidocalcisomes and/or the plant-like vacuole (Miranda et al., 2010). Copper has not yet been unequivocally reported in these organelles, in marked contrast to other essential transition metals, such as zinc and iron. Intriguingly, X-ray microanalysis of acidocalcisomes showed a significant copper peak, which was, however, attributed to the copper grids used (Scott et al., 1997; Ruiz et al., 2010). Nevertheless, presence of copper in acidocalcisomes could not be ruled out either.

Alternatively, the vesicles may be part of a trans-Golgi network where PbCuTP could assist the biosynthesis of cuproenzymes, similar to mammalian ATP7A and B at low or basal copper concentrations (La Fontaine and Mercer, 2007; Hasan and Lutsenko, 2012). It is tempting to speculate, that some as yet unidentified cuproenzyme is critical to malaria parasite fertility thus leading to the observed phenotype. At high copper levels, ATP7A and B relocate and sequester excess copper in vesicles, which eventually fuse to the plasma membrane (Hasan and Lutsenko, 2012). In asexual blood stage, we observed that the CuTP-labelled vesicles were often at the parasite periphery, which could be an indication for a second function in copper detoxification, thus linking the observation that P. falciparum-infected erythrocytes contain lower copper levels than uninfected ones (Rasoloson et al., 2004) with the intraparasitic localization of CuTP.

Finally, the CuTP-labelled structures may represent novel as yet unidentified (storage) organelles. The distinct patterns of the CuTP-labelled vesicle-like structures in different phases of the Plasmodium life cycle ranging from a singular, small juxta-plasma membrane spot in ring stages to several large vesicles in ookinetes, together with a similar intraparasitic stain in the related parasite T. gondii, clearly merits further studies to resolve the cellular function(s) of this compartment.

Taken together, our data provide strong evidence for a link between copper homeostasis and malaria parasite fertility. Although malaria parasites have a highly specialized, obligate intracellular life style, our experimental genetics findings illustrate that a Plasmodium protein with the key signatures of a Cu⁺-transporting P₁B₁-type ATPase is particularly important for male and female fertility.

Experimental procedures

Experimental animals

This study was 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 number G0469/09). C57BL/6 mice were used for sporozoite infections. All other parasite infections were conducted with NMRI mice.

Generation and imaging of Tgcup::myc parasites

For the generation of Tgcup::myc parasites, we used single cross-over homologous recombination. Briefly, T. gondii RH Δku80 parasites (Huynh and Carruthers, 2009) were cultivated on human foreskin fibroblasts (HFF) and free tachyzoites transfected with ~30 μg linearized pTgCuTP-myc. Recombinant parasites were selected with 25 μg ml⁻¹ mycophenolic acid and 40 μg ml⁻¹ xanthine as described previously (Donald et al., 1996). Approximately one week later, HFF colonized coverslips were infected with either untransfected or transfected parasites.

For colocalization studies of Tgcup::myc parasites, infected HFF cells were fixed two days after invasion with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton-X in PBS, blocked with 3% bovine serum albumin in PBS, and incubated with monoclonal mouse anti-myc antibodies (Santa Cruz Biotechnology, 1:1000 dilution) and rabbit anti-plant-like vacuolar proton pyrophosphatase (VP1) antibodies (1:4000 dilution) (Miranda et al., 2010) in 3% BSA/PBS for 70 h at 4°C. Bound antibodies were detected using donkey anti-mouse Alexa Fluor 546 and goat anti-rabbit fluorescein conjugated antibodies respectively (1:1000 dilution; Invitrogen) in 3% BSA/PBS. DNA was visualized by Hoechst 33342 (Invitrogen).

Generation of cutp⁻ and cutp::tag parasites

We used the advanced gene replacement strategy (Janse et al., 2006; Kooij et al., 2012) to generate parasites with their CuTP deleted or tagged at the carboxy-terminus with a fluo-
rescent protein-epitope tag. Details of the molecular cloning strategy to generate the two resulting plasmids, termed pCuTP-KO and pCuTP-tag, can be found in the Supplemental Experimental Procedures and Figs 2 and S3. The plasmids were verified by commercial Sanger sequencing. For transfection into wild-type *P. berghei* strain ANKA (WT) parasites, vectors were linearized with Sall and Scal. Isogenic mutant parasite lines were isolated by flow cytometry 7–8 days after transfection (Kenthirapalan et al., 2012). Correct integration of the transfection vectors in the mutant parasite lines and absence of contaminating WT parasites was confirmed by diagnostic PCR (see Supplemental Experimental Procedures for details). The cutp* line used for the majority of the experiments was analysed by Southern blot using the PCR DIG Probe Synthesis kit and the DIG Luminescent Detection kit (Roche), according to the manufacturer’s protocol. The 5′ probe was amplified using primers CuTP-F2-Sacl and CuTP-R1-EcoRI (see Table S2 for all primer sequences) and annealed to EcoRV restriction-digested gDNA, resulting in bands of 9.1 kb (WT) and 4.8 kb (cutp*). The 3′ probe was amplified using primers CuTP-F5-AvrII and CuTP-R4-KpnI and annealed to NdeI restriction-digested gDNA resulting in bands of 3.2 kb (WT) and 7.0 kb (cutp*).

Phenotypic analysis of the mutant parasite lines was done in direct comparison with two GFP-expressing parasite lines; BG/6 (Kooij et al., 2012) was used for all asexual and sexual blood stage development and ookinete cultures; GFPcon (Janse et al., 2006) was used for mosquito infections and liver stage development. As both lines display WT behaviour and life cycle progression, both are referred to as WT parasites.

**Analysis of Plasmodium life cycle progression**

To compare blood stage development of cutp* and WT parasites, 1000 infected erythrocytes were injected intravenously into naive recipient NMRI mice. The progress of the infection was monitored by daily microscopic examination of Giemsa-stained thin blood smears. Gametocyte conversion rates, male:female gametocyte ratios, and exflagellation rates were determined at day 3 after intravenous infection of 10⁷ blood stage parasites into naïve NMRI mice. 5 µl tail blood was mixed with 125 µl RPMI (complemented with 50 mM xanthurenic acid), and an aliquot transferred into a Neubauer chamber for incubation at 20°C. Exflagellation centres were determined by microscopic observation at 400× magnification for 6 min, starting 12 min after incubation. For ookinete *in vitro* cultures, blood from infected mice at day 3 after infection with 10⁷ blood stage parasites was used. 1 ml infected blood obtained via cardiac puncture was immediately added to ookinete medium (RPMI 1640 with L-glutamine and 25 mM Hepes supplemented with 100 mM sodium bicarbonate, 125 mM l- penicillin/streptomycin, 10% fetal calf serum, and 50 µM xanthurenic acid, pH 8.0) and incubated for 16–20 h at 20°C. Ookinetes were purified with anti-P28 antibody-coated magnetic beads, and the number determined by microscopic observation (400×). *Anopheles stephensi* mosquitoes were raised at 28°C and 75% humidity under a 14 h light/10 h dark cycle. Mosquito stage development was analysed using standard techniques (Vanderberg, 1975). Briefly, to determine infectivity and the number of midgut sporozoites, mosquitoes were dissected on day 14 after feeding. The number of salivary gland sporozoites was determined on day 17. To determine the sporozoite infectivity to mice, naïve recipient C57BL/6 mice were infected by exposure to 10–12 infected mosquitoes. Patency was determined by microscopic examination of daily Giemsa-stained thin blood smears. Liver stages of cutp*::tag parasites were cultured *in vitro* and analysed as described (Hausig et al., 2011). At 48 h after infection, cultured hepatoma cells were fixed and incubated with monoclonal mouse anti- *P. berghei* heat shock protein 70 (1:300 dilution) (Tsuij et al., 1994) and rat anti-mCherry (1:500 dilution; Chromotek) antibodies. Bound antibodies were detected using goat anti-mouse Alexa Fluor 488 and anti-rat IgG Alexa Fluor 546 conjugated antibodies respectively (1:2000 dilution; Invitrogen). Nuclei of live and fixed parasites were visualized with the DNA-dye Hoechst 33342 (Invitrogen).

**Plasmodium berghei exflagellation assay**

*In vitro* exflagellation was analysed as described (Raabe et al., 2009). Briefly, mice were infected with 10⁵ WT or cutp*::tag-infected blood stage parasites. Three days later mice were bled by heart puncture and aliquots of 50 µl infected blood were transferred immediately to 500 µl of preheated normal or supplemented gametocyte maintenance buffer (GM: 4 mM NaHCO₃, 20 mM glucose, 137 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM HEPES, pH 7.24–7.29, 0.1% BSA). 1 µM neocuproine, 1 µM CuCl₂ (for Cu²⁺), or 1 µM CuCl₂ (incubated for 30 min with 1 mM ascorbic acid in order to obtain Cu⁺ ions) were tested. Following incubation at 37°C for 1 h, the infected red blood cells were collected by a short spin and resuspended in 1 ml RPMI (prewarmed at 20°C and complemented with 50 µM xanthurenic acid). 10 µl was immediately transferred to a Neubauer chamber and incubated at 20°C. Exflagellation centres were quantified by microscopic observation at 400× magnification from 12–18 min after activation.

**Image acquisition**

All images were recorded on a Leica DMR or Zeiss AxioObserver Z1 epifluorescence microscope and processed minimally with ImageJ.

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Supporting information

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