Identification of Vital and Dispensable Sulfur Utilization Factors in the *Plasmodium* Apicoplast

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Abstract

Iron-sulfur [Fe-S] clusters are ubiquitous and critical cofactors in diverse biochemical processes. They are assembled by distinct [Fe-S] cluster biosynthesis pathways, typically in organelles of endosymbiotic origin. Apicomplexan parasites, including *Plasmodium*, the causative agent of malaria, harbor two separate [Fe-S] cluster biosynthesis pathways in their mitochondrion and apicoplast. In this study, we systematically targeted the five nuclear-encoded sulfur utilization factors (SUF) of the apicoplast ([Fe-S] cluster biosynthesis pathway) by experimental genetics in the murine malaria model parasite *Plasmodium berghei*. We show that four SUFs, namely SUFC, D, E, and S are refractory to targeted gene deletion, validating them as potential targets for antimalarial drug development. We achieved targeted deletion of SUFA, which encodes a potential [Fe-S] transfer protein, indicative of a dispensable role during asexual blood stage growth in *vivo*. Furthermore, no abnormalities were observed during *Plasmodium* life cycle progression in the insect and mammalian hosts.

In *Plasmodium*, a genus of eukaryotic, single cell parasites that are the causative agents of malaria, components of the SUF, ISC, and GIA systems are required for biogenesis in distinct cellular compartments, namely the ISC system in the mitochondrion and the SUF system in plastids. The mitochondrial ISC proteins were found to supply [Fe-S] clusters to the mitochondrial [Fe-S] proteins and to [Fe-S] proteins in the cytosol [13], where the cytosolic iron-sulfur protein assembly (CIA) machinery is responsible for the maturation of cytosolic [Fe-S] proteins [14].

In spite of the differences between bacteria and eukaryotes, the basic principles of [Fe-S] clusters biogenesis seem to be conserved. First, the [Fe-S] cluster is assembled *de novo* onto a scaffold protein. For this step, sulfur is mobilized from cysteine by a cysteine desulfurase (SufS, IscS, NisS) [15]. The iron source is mostly unknown. Second, the [Fe-S] cluster is transferred from the scaffold protein to a target apoprotein and assembled into the polypeptide chain. The most common [Fe-S] clusters are rhombic [2Fe-2S] or cubic [4Fe-4S]. More complex structures have also been described, some of which include additional heavy metals [16].

The SUF system in *E. coli*, for instance, consists of six genes organized in the *sufABCDEF* operon [11]. SufE acts as cysteine desulfurase that provides the sulfur for the [Fe-S] cluster and SufE has been shown to interact with SufS to enhance its activity up to 50-fold [17,18]. SufB, C, and D form a functional complex serving as a [Fe-S] scaffold [19,20] and also enhancing SufS function [18]. SufC was shown to contain ATPase activity in *Erwinia chrysanthemi* [21] and the crystal structure of *E. coli* SufC confirmed it to be an ABC-type ATPase [22]. SufA interacts with the SufBCD complex to accept [Fe-S] clusters formed *de novo* [19].

In *Plasmodium*, a genus of eukaryotic, single cell parasites that are the causative agents of malaria, components of the SUF, ISC, and GIA system have been identified by bioinformatic analyses [23–25]. Like in other eukaryotes, the ISC system is predicted to localize to the mitochondrion and the SUF system to the apicoplast, a vestigial, non-photosynthetic plastid of red algal
origin [24]. In *Plasmodium*, all components of the SUF system are nuclear-encoded, except for SUFB, which is encoded in the small circular apicoplast genome [26].

A biochemical study focusing on the *P. falciparum* plastid SUF system provided evidence that SUFC localizes to the apicoplast [27]. In bacteria and plants, SUFC interacts with SUFB [21,28,29], which was confirmed for the corresponding *P. falciparum* proteins [27]. In *Arabidopsis thaliana*, SUFB and SUFC both display ATPase activity [29], whereas the bacterial SUFB seems to lack this activity. ATPase activity of recombinant PSUFB and PSI SUFC proteins provided supporting evidence for the evolutionary conservation of the plastid SUF system between plants and apicomplexan parasites [27]. PSI SUFE and PSUFS have also been assigned to the apicoplast, while PSI SUFS was shown to functionally complement *E. coli* deficient in *SufS* [30].

Data-mining and bioinformatic analysis of potential *Plasmodium* [Fe-S] cluster-containing proteins revealed at least 31 candidates, seven of which are predicted to localize to the apicoplast (Table 1). These proteins are involved in diverse pathways, such as mevalonate-independent isoprenoid biosynthesis, lipoic acid metabolism, and biogenesis of [Fe-S] clusters itself. Because some proteins are components of essential biosynthesis pathways, most notably the DOXP pathway of isoprenoid biosynthesis [31], plastid [Fe-S] cluster assembly is likely essential for parasite survival.

We previously reported that a *Plasmodium*-specific component of the apicoplast [Fe-S] cluster biosynthesis pathway, nitrogen fixation factor U (NifU)-like domain containing protein (NFUapi), can be deleted in the murine malaria model parasite *P. berghei* and plays an auxiliary role in liver stage merozoite formation [32]. Here, we present a systematic experimental genetics analysis of the *P. berghei* apicoplast SUF system. We show that four of five nuclear-encoded *P. berghei* SUF genes are refractory to gene deletion and, hence, can be considered likely essential for blood stage proliferation. Endogenous tagging confirmed accessibility of the loci to targeted gene modification and the resulting fluorescent fusion proteins showed co-localizations with an apicoplast resident protein.

**Results**

**The Principal Components of the *Plasmodium berghei* SUF Pathway are Refractory to Targeted Deletion in vivo**

We first wanted to investigate whether the SUF genes of the *Plasmodium* apicoplast [Fe-S] cluster biosynthesis pathway were susceptible or refractory to targeted gene deletion. For this systematic genetic characterization, we employed the murine malaria model system *P. berghei* that permits in vivo selection of recombinant parasites. We attempted to generate loss-of-function mutants of the five nuclear-encoded *P. berghei* SUF genes refractory to gene deletion and, hence, can be considered likely essential for blood stage proliferation. Endogenous tagging confirmed accessibility of the loci to targeted gene modification and the resulting fluorescent fusion proteins showed co-localizations with an apicoplast resident protein.

SUF is Dispensable for *Plasmodium* life Cycle Progression

Repeated successful deletion of *SUFA* (PBANKA_123740) during our transfection experiments already indicated non-vital roles of the target gene for erythrocytic parasite propagation. In order to determine potential in vivo roles during the parasite life cycle, we selected two isogenic *sufA* populations. Genotyping, including Southern blot analysis (Fig. 1D), confirmed the homogenous presence of *sufA* parasites only.

To mimic a natural infection, we propagated the two selected *sufA* populations through the mosquito vector, female *Anopheles stephensi*, and isolated sporozoites from infected salivary glands. Intravenous injection of 10,000 wild type (WT) or *sufA* sporozoites were performed to infect and monitor transmission to C57BL/6 mice (Fig. 2). This analysis showed the typical pre-patent period, which is the time until first detection of blood stage parasites in peripheral blood following infection with sporozoites and which includes the liver stage development, of three days in all animals tested. Moreover, during the following days, *sufA* and WT-infected animals displayed similar development of parasitemias. All parasites first replicated exponentially before entering a plateau phase, once the parasitemia was close to 1%. The exposure of three naive C57BL/6 mice to bites of five infected mosquitoes also resulted in successful natural transmission in two mice. In conclusion, this analysis demonstrated that *SUFA* does not play important roles in establishment and propagation of an erythrocytic infection and, hence, is not a valid target for rational drug design.

Successful *sufA* sporozoite isolation and infection of mice were already indicative of unaltered life cycle progression in the absence of *SUFA*. To exclude modest defects, we systematically assessed parasite development in the mosquito vector and during pre-erythrocytic growth (Fig. 3). Transmission to mosquitoes and sporogony were indistinguishable from WT parasites, as exemplified by similar infectivity to mosquitoes (Fig. 3A) and normal sporozoite numbers in salivary glands, the final target organ in the mosquito vector (Fig. 3B). Also when we quantified intrahepatic parasite stages in cultured hepatoma cells (Fig. 3C) and merozoites from culture supernatant, representing emerging merozoites (Fig. 3D), we could not distinguish *sufA* from WT parasites. In both parasite lines, merozoites are released into the supernatant between 48 and 72 h after infection, leading to a remarkable drop in parasite numbers in hepatoma cells seen at 72 h after infection.
Table 1. Confirmed and predicted [Fe-S] cluster-containing proteins in *Plasmodium*.

<table>
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<th></th>
<th><em>P. berghei</em></th>
<th><em>P. falciparum</em></th>
<th>Annotation</th>
<th>PlasmoAP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ApicoAP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PlasMit&lt;sup&gt;b&lt;/sup&gt;</th>
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*Note: Gene IDs of the *P. berghei* and *P. falciparum* orthologs and the predicted localizations of the proteins were retrieved from PlasmoDB (http://PlasmoDB.org) or identified by similarity searching using *A. thaliana* [Fe-S] cluster proteins as query sequences [41].

Aputative targeting of the *P. falciparum* [Fe-S] cluster-containing proteins to the apicoplast or mitochondrion was predicted using 20 different algorithms. PlasmoAP (51) indicates the likelihood of the presence of the required signal peptide followed by the likelihood of an apicoplast localization ("–" = unlikely, "0" = undecided, "+" = likely, "++" = very likely). ApicoAP (52) is a different algorithm that can identify apicoplast proteins in multiple Apicomplexa ("No SP" = no signal peptide, "No ATP" = signal peptide but no transit peptide, "ATP" = apicoplast targeted protein). PlasMit (53) predicts the likelihood of the mitochondrial localization for *P. falciparum* proteins ("non-mito" (99%), possibly (91%), and "mito" (99%). MitoProtII (54) gives a probability score for the likelihood of mitochondrial localization but is not optimized for Plasmodium sequences.

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Together, our analysis demonstrates that absence of *SUF4* is compatible with effective host switch, parasite stage conversion, and population expansion throughout the entire *Plasmodium* life cycle.

**Apicomplexa Localization of *Plasmodium* SUF Proteins**

In order to gain independent confirmation that the four essential *SUF* loci are susceptible to genetic manipulation, we targeted *SUF3, D, E, and S* along with *SUF4* by double homologous/ends-out recombination introducing a carboxy-terminal in-frame fusion with the combined mCherry-3xMyc tag.
(Fig. 4A), using the same strategy we previously employed to tag NFUapi [32]. Upon successful recombination, selection of recombinant parasites by the antifolate pyrimethamine, and WT-free isolation by flow cytometry, we readily obtained all five desired recombinant parasites, i.e. sufA::tag, sufC::tag, sufD::tag, sufE::tag, and sufS::tag, after the first transfection attempt (Fig. 4B).

In addition to demonstrating accessibility of the loci to genetic modification, the parasite populations provided templates for the verification of the respective diagnostic PCRs of the 3’ integration of the unsuccessful gene deletion attempts (Fig. 1B), which use the same homologous sequence for integration.

These parasites also provided an opportunity to verify the predicted apicoplast targeting by fluorescence microscopy (Fig. 5 and 6). As expected, all five fusion proteins displayed a punctate staining in live P. berghei blood stage trophozoites, reminiscent of the apicoplast (Fig. 5A). The localization of PbSUF::tag fully
supports the previous finding of a punctate pattern in P. falciparum-infected erythrocytes observed with an anti-SUFC serum [27]. We confirmed the apparent apicoplast localization in fixed blood stage parasites by staining these with anti-mCherry antibodies and an anti-serum recognizing acyl carrier protein (ACP), an apicoplast signature protein (Fig. 5B). Using the same antibodies, we obtained supporting evidence for an apicoplast localization of three SUF fusion proteins, SUFD::tag, SUFE::tag, and SUFS::tag, in developing liver stage parasites (Fig. 6A). As expected in the case of an apicoplast localization, the characteristic, SUFD, E, and S-positive branched structures were destroyed following treatment with azithromycin (Fig. 6B).

We note that all tagged SUF proteins displayed similar localization, yet differed in intensity in the live blood stage parasites, with SUFC::tag yielding the most prominent signal, while SUFA::tag and SUFD::tag were only detectable after prolonged exposure times. Transcription data available online (http://PlasmoDB.org) confirm generally low transcript levels, particularly for SUFA and SUFD, with SUFE being most abundantly expressed [37]. Though we could not confirm this in live blood stage parasite, SUFE::tag signals were the most prominent during liver stage development. This initial observation indicates that a detailed biochemical study to investigate the stoichiometry and order of the apicoplast [Fe-S] cluster biogenesis pathway is warranted.

**Discussion**

Our data provide the first genetics evidence that four of five SUF proteins are refractory to targeted gene deletion and, hence,
most likely vital for blood stage development. We provide additional experimental support for this notion by successful fluorescent tagging through a complementation strategy. Based on the important role(s) of SUFC for parasite growth, we hypothesize that, by analogy, its partner protein SUFB, encoded in the apicoplast genome and inaccessible to experimental genetics, likely exerts essential functions as well.

Two aspects provide further confidence that the central components of the *Plasmodium* apicoplast SUF system, namely SUFC, SUFD, SUFE, and SUFS, are critical for survival during blood stage growth. First, we used the most recent and efficient techniques available for experimental genetics in *P. berghei* [34,35] that have previously enabled the challenging generation of a slow-growing recombinant line lacking a putative protein export regulator [38]. Second, we successfully deleted only two components of the apicoplast [Fe-S] cluster biogenesis pathway, namely SUFA, described herein, and NFUapi [32], both of which are estimated to have carrier rather than assembly functions in *E. coli* [19,39]. This finding combined with the absence of a plastid-like SUF system in humans provides a rationale for further studies towards the development of antimalarial drugs targeting the *Plasmodium* SUF pathway.

[Fe-S] cluster biogenesis has been well studied in bacteria and yeast, and to a lesser extent in other eukaryotes [16,24,40–42]. However, very little functional data are yet available for apicomplexan parasites. The dispensable function of *PbSUFA* could be compatible with a role as a transfer protein as suggested by studies in *E. coli* [19] rather than that of a scaffold protein. Similarly, we discussed a potential function of *PbNFUapi* as a transfer protein [32]. Together, our data suggest that the putative transfer proteins in the apicoplast [Fe-S] biosynthesis pathway are not essential for parasite survival. Alternatively, both proteins might perform at least partially redundant functions, a possibility that might be further tested through the generation of recombinant parasite lines lacking both *NFUapi* and *SUFA*.

The corresponding SUF [Fe-S] cluster biogenesis pathway in the related apicomplexan parasite, *Toxoplasma gondii*, which causes toxoplasmosis, is likely also localized to the apicoplast, despite the uniform absence of a signal peptide and apicoplast-targeting sequences in the SUF proteins [24]. It remains elusive how this alternative targeting to the apicoplast might have evolved and if there are specific reasons why all of the SUF components in *T. gondii* are targeted through this alternative pathway. Apparently, other apicomplexan parasites, such as *Babesia bovis* and *Theileria annulata*, target these components through a classical signal peptide and apicoplast-targeting sequence-dependent import pathway, although these parasites appear to encode a reduced set of proteins, namely *SUFE* and *SUFS* and, in the case of *T. annulata*, also *NFUapi* [25,32].

In *E. coli*, deficiencies in the SUF pathway do not result in phenotypes under normal growth conditions. However, when cultured in low iron or increased oxidative stress conditions, bacteria demonstrate marked growth problems [21,43,44]. As all our analyses were performed under optimized *in vivo* and cell culture conditions, we cannot exclude a phenotype of *suFA* under suboptimal growth conditions, e.g. in malnourished mice.

*Plasmodium* is an obligate intracellular eukaryotic pathogen and apparently cannot compensate for the loss of the apicoplast SUF pathway. Recent data suggest that a functional non-mevalonate isoprenoid biosynthesis (DOXP) pathway is the major vital role of the apicoplast in *P. falciparum parasites in vitro* [31]. Presence of [Fe-S] clusters in the penultimate and ultimate enzymes, ISPG and

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**Figure 4. Control transfections of *Plasmodium berghei* SUF genes and generation of fluorescently tagged SUF parasite lines.** (A) Replacement strategy to generate stable parasite lines that express the endogenous SUF proteins fused to an mCherry-3xMyc tag (red). The respective ANKA strain wild type (WT) SUF loci were targeted with replacement plasmids (pSUF-Tag) containing carboxy terminal (CT) and downstream 3' regions (dark gray bars), a high expressing GFP cassette (green), and the hDHFR-yFcu drug-selectable cassette (blue). Integration-specific (CT INT and 3'INT) and wild type-specific (CT WT and 3' WT) primer combinations (Table S1) and expected fragments are indicated by arrows and dotted lines, respectively. (B) PCR-based genotyping of the *suf::tag* parasites to verify successful fusion of the respective SUF genes with the mCherry-3xMyc tag and WT-free isolation of the recombinant *suf::tag* parasites. Note that the 3' WT- and integration-specific PCRs are identical to those designed for targeted gene deletion (Figure 1).

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ISPH, provide a plausible explanation for the observed refractoriness of the SUF assembly machinery to targeted gene deletion. To test if the DOXP pathway of isoprenoid biosynthesis is the sole reason for the essentiality of [Fe-S] cluster biogenesis in the apicoplast, one could attempt to delete *P. falciparum* SUF pathway components under isopentenyl pyrophosphate supplementation [31].

In conclusion, our study identified the four key components of the *Plasmodium* apicoplast [Fe-S] biosynthetic pathway and revealed that *SUFA* is dispensable for efficient progression through the *Plasmodium* life cycle.

Materials and Methods

Ethics Statement

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).

Experimental Animals, Parasites, and Cell Lines

Female NMRI and C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). C57BL/6 mice...
were used for sporozoite infections. All other parasite infections were conducted with NMRI mice. Experimental genetics were all performed in P. berghei strain ANKA (WT), as control lines GFPcon [33] or Berred [38] parasites were used. In vitro liver stage parasite development was analyzed using cultured HuH7 hepatoma cells.

**Generation of SUF Targeting and Tagging Plasmids**

For targeted gene deletion of the P. berghei SUF genes, fragments of the upstream 5' and downstream 3' flanking regions (FR) were amplified from gDNA using gene-specific primers. PCR fragments were cloned into the P. berghei adaptable transfection vector (pBAT-SIL6) [34], which contains drug-selectable and high-expressing GFP cassettes. First, the 3' FR homologous sequences were cloned following restriction digestion of vector and insert with HindIII and KpnI. Then, the 5' FR homologous sequences digested with SacII and EcoRV were cloned into SacII and PvuII linearized vector, thus removing the mCherry-3xMyc tag from the original vector. The resulting plasmids were linearized with SalI. To provide transfection controls and confirm the apicoplast localization of SUF proteins, mCherry-3xMyc tagged parasite lines were generated. For this purpose, the carboxy-terminal parts of the SUF genes were PCR amplified using gene-specific primers. After restriction digestion, the respective fragments were cloned into the SacII and HpaI digested pBAT-SIL6 vector already containing the 3' FR sequence of the respective SUF genes, thus fusing the SUF carboxy-terminal sequence in frame with the mCherry-3xMyc tag sequence. The resulting plasmids were linearized withSalI. All primers are listed in Table S1.

**Parasite Transfection, Selection and Genotyping of Recombinant Parasites**

For targeted gene deletions and carboxy-terminal tagging, 10^5 to 10^6 purified P. berghei schizonts were transfected with digested plasmids using the Amaxa Nucleofector system as described [33]. Transfected parasites were subsequently injected into naïve NMRI mice selected by oral pyrimethamine (70 μg/ml) in the drinking water. Genotyping of drug-resistant parasites was performed by diagnostic PCR using gDNA as template and integration-specific primers. Two isogenic sufA– parasite lines from two independent transfection experiments were generated by flow cytometry-assisted isolation as described [35]. The genotype of the two selected sufA– parasite populations was confirmed by Southern blot analysis using the PCR DIG Probe Synthesis kit and the DIG Luminescent Detection kit (Roche), according to the manufacturer’s protocol. For amplification of the hybridization probe, gene-specific primers TV-5'SUFA-F and TV-5'SUFA-R were used. The hybridization probe was annealed to EcoRI restriction-digested gDNA, resulting in bands of 4.5 kb (WT) and 9.8 kb (sufA–). All primers are listed in Table S1.

**Plasmodium Life Cycle Progression**

Gametocyte differentiation and exflagellation of microgametes were examined prior to mosquito feeding. Anopheles stephensi mosquitoes were raised under a 14 h light/10 h dark cycle, 75% humidity and at 28°C (non-infected) or 20°C (infected), respectively. Sporozoite populations were isolated and analyzed as described previously [45]. Mosquito infectivity, i.e. the percentage of mosquitoes with midgut oocysts, was assessed through dissection.
of mosquito midguts at day 10 after feeding. All parasite strains that were used express GFP in all life cycle stages, allowing the determination of the number of midguts containing GFP-positive oocysts using a fluorescence binocular. Salivary gland-associated sporozoites were quantified at days 17–21. To determine sporozoite infectivity, sporozoites were liberated from salivary glands and injected intravenously into young, naïve C57BL/6 mice (10,000 sporozoites/inoculation). Patency was determined by daily examination of Giemsa-stained thin blood smears.

*P. berghei* in vitro liver stages were cultured and analyzed using standard techniques [46]. In brief, 30,000 hepatoma cells were seeded per well in 12-well chamber slides (Nalge Nunc International) and inoculated with freshly-dissected 10,000 sporozoites 24 h later. Thereafter, standard procedures for culturing infected hepatoma cells were followed [47]. Merosomes were harvested from the cell culture supernatant and counted in a Neubauer chamber 72 h after inoculation.

**Fluorescence Microscopy**

For confirmation of expression and determination of the subcellular localization of tagged SUF proteins, live and fixed *suf*::tag blood stage parasites were imaged using Leica DMR epifluorescence microscope. Infected erythrocytes were fixed using a previously published protocol with minor modifications [48]. 5 μL of tail blood from an infected mouse was mixed with 125 μL of RPMI1640 and 15 μL of cell suspension was allowed to settle 5 min onto poly-L-lysine coated cover slips. Coverslips were mounted with Fluoromount-G (Southern Biotech). Total numbers of parasites were counted using a Leica DM2500 epifluorescence microscope. Images were recorded using a Zeiss AxioObserver Z1 epifluorescence microscope. All images were processed minimally with ImageJ (http://rsb.info.nih.gov/ij/). Following subtraction of background fluorescence levels of non-infected cells within the same recording, minimum and maximum intensities of the specific signals were optimized to use the full dynamic range of the look-up-tables. No gamma adjustments were applied.

**Supporting Information**

**Table S1** Primer sequences.

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**Author Contributions**

Conceived and designed the experiments: JMH KM TWAK. Performed the experiments: JMH TWAK. Analyzed the data: JMH KM TWAK. Wrote the paper: JMH KM TWAK.

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