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. Fusion of a fluorescent tag to the endogenous P. berghei SUFs demonstrated that all loci were accessible to genetic modification and that all five tagged SUFs localize to the apicoplast. Together, our experimental genetics analysis identifies the key components of the SUF [Fe-S] cluster biosynthesis pathway in the apicoplast of a malarial parasite and shows that absence of SUFC, D, E, or S is incompatible with Plasmodium blood infection in vivo.

Introduction

Iron-sulfur [Fe-S] clusters are small inorganic cofactors that are present in most organisms. Proteins containing [Fe-S] clusters are involved in numerous biological processes, ranging from mitochondrial oxidative phosphorylation [1] and photosynthesis [2] to DNA replication [3], DNA repair [4], ribosome biogenesis [5], and regulation of gene expression [6]. Accordingly, the list of [Fe-S] cluster-containing proteins is continuously expanding. Although early in vitro studies suggested a spontaneous assembly [7], [Fe-S] clusters are not formed spontaneously in living cells but rather assembled through distinct [Fe-S] biosynthesis pathways.

Bacteria harbor the sulfur utilization factor (SUF) and iron-sulfur cluster (ISC) systems for assembly of [Fe-S] clusters. In Escherichia coli, the ISC system is thought to mediate housekeeping functions, whereas the SUF system was shown to be especially important under stress conditions such as iron starvation [8,9]. However, deletion of individual operons is not lethal [10,11].

In eukaryotes, [Fe-S] biogenesis machineries are thought to have evolved from their bacterial counterparts that had been acquired by endosymbiosis [12]. Different [Fe-S] cluster assembly 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], whereas 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, NifS) [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 sufABCDE operon [11]. SufS 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 CIA 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...
Here, we present a systematic experimental genetics analysis of the encoded P. berghei SUF can be deleted in the murine malaria model parasite fixation factor U (NifU)-like domain containing protein (NFUapi), the apicoplast [Fe-S] cluster biosynthesis pathway, nitrogen evolution conservation of the plastid SUF system between plants and apicoplast [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 (NiF)-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 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.

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 isoegenic 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 supernatants, 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.
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.

Apicomplexan Localization of Plasmodium SUF Proteins

In order to gain independent confirmation that the four essential SUF loci are susceptible to genetic manipulation, we targeted SUFC, SUFD, SUFE, and SUFS along with SUFA by double homologous/ends-out recombination introducing a carboxy-terminal 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 P8SUFC::tag fully

Figure 1. Systematic gene targeting of Plasmodium berghei SUF genes. (A) Replacement strategy to delete the five nuclear-encoded PbSUF genes. The respective ANKA strain wild type (WT) SUF loci were targeted with replacement plasmids (pKO) containing upstream 5’ and downstream 3’ regions (dark gray bars) flanking the open reading frames (light gray arrow), a high-expressing GFP cassette (green), and the hDHFR-yFcu drug-selectable cassette (blue). Integration-specific (5’INT and 3’INT) and wild type-specific (5’WT and 3’WT) primer combinations (Table S1) are indicated by arrows; expected PCR fragments by dotted lines. The probe used for Southern blot analysis of the two isogenic sufA– parasites lines corresponds to the 5’ integration sequence and hybridized to EcoRI (E) restriction-digested gDNA; expected fragments and their sizes are indicated by gray dashed lines. (B) Representative diagnostic PCR results of the SUF loci of WT ANKA (pre transfection) and drug-selected (post transfection) parasites are shown. For SUFA, diagnostic PCR of isogenic gene deletion parasites confirms successful integration and absence of WT parasite contamination. (C) Overview of all transfection experiments summarizing the number of times no pyrimethamine-resistant parasites were selected (black), selection of pyrimethamine-resistant parasites was achieved (red), integration-specific PCR demonstrated targeted deletion of the SUF gene (orange), and isolation of WT-free, isogenic recombinant parasites (green). (D) Southern blot analysis of two isogenic sufA– parasite lines reveals the expected size shifts.

doi:10.1371/journal.pone.0089718.g001
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 [36]. 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 P. falciparum 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 PlsNFUapi 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 vivo [31]. Presence of [Fe-S] clusters in the penultimate and ultimate enzymes, ISPG and

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).
ISPH, provide a plausible explanation for the observed refracto-

riness 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 \(SUF\) 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 with SalI. All primers are listed in Table S1.

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

For targeted gene deletions and carboxy-terminal tagging, 10^6 to 10^7 purified *P. berghei* schizonts were transfected with digested plasmids using the Amaxa Nucleofector system as described [33]. Transfected parasites were subsequently injected into naive 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). Pathency 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 8-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 polystyrene-coated cover slips. Cover slips were transferred to a 24-well plate containing 500 μl of 4% EM-grade paraformaldehyde and 0.0075% EM-grade glutaraldehyde in microtubes stabilizing buffer (MTSB, 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM MgCl₂ pH 6.9), fixed for 20 min and washed with PBS. Cells were permeabilized for 10 min with 0.1% Triton X-100 in PBS and blocked 3 h with 10% foetal calf serum (FCS) in PBS. The samples were incubated with rat anti-mCherry antibodies (1:1,000 dilution, Chromotek) and rabbit anti-P. berghei ACP peptide antisera (1:300 dilution; [49]) in 10% FCS in PBS overnight at 4°C. Bound antibodies were detected using goat anti-rabbit/rat/mouse IgG Alexa Fluor 488/546 conjugated antibodies (1:3,000 dilution, Invitrogen). Nuclei were visualized with DNA-dyes Hoechst 33342 (Invitrogen) and DRAQ5 (Axxora; both 1:1,000 dilution). Coverslips were mounted with Fluormount-G (Southern Biotech). Total numbers of parasites were counted using a Zeiss AxioObserver Z1 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.

(DOCX)

Acknowledgments

We would like to acknowledge the assistance of the FCCF at the Deutsches Rheuma-Forschungszentrum (Berlin).

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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