HELOISA BERTI GABRIEL

Caracterização funcional de farnesil difosfato sintase/geranilgeranil difosfato sintase (FPPS/GGPPS) e 1,4-dihidroxi-2-naftoato preniltransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K em *Plasmodium falciparum*

Tese apresentada ao Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

São Paulo
2015

HELOISA BERTI GABRIEL

Caracterização funcional de farnesil difosfato sintase/geranilgeranil difosfato sintase (FPPS/GGPPS) e 1,4-dihidroxi-2-naftoato preniltransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K em *Plasmodium falciparum*

Tese apresentada ao Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

Área de concentração: Biologia da Relação Patógeno-Hospedeiro

Orientador: Prof. Dr. Alejandro Miguel Katzin
Coorientador: Dr. Mauro Ferreira de Azevedo

Versão Original

São Paulo
2015

DADOS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
Serviço de Biblioteca e Informação Biomédica do
Instituto de Ciências Biomédicas da Universidade de São Paulo

© reprodução total

Gabriel, Heloisa Berti.

Caracterização funcional de farnesil difosfato sintase/geranylgeranyl difosfato sintase (FPPS/GGPPS) e 1,4-dihidroxi-2-naftoato preniltransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K em *Plasmodium falciparum* / Heloisa Berti Gabriel. -- São Paulo, 2015.

Orientador: Prof. Dr. Alejandro Miguel Katzin.

Tese (Doutorado) – Universidade de São Paulo. Instituto de Ciências Biomédicas. Departamento de Parasitologia. Área de concentração: Biologia da Relação Patógeno-Hospedeiro. Linha de pesquisa: Protozoologia de parasitas.

Versão do título para o inglês: Functional characterization of farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase (FPPS/GGPPS) and 1,4-dihydroxy-2-naphthoate prenyltransferase (MenA) respectively involved in the isoprenoid pathway and vitamin K in *Plasmodium falciparum*.

1. *Plasmodium* 2. Malaria 3. Isoprenóides 4. Enzimas I. Katzin, Prof. Dr. Alejandro Miguel II. Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro III. Título.

ICB/SBIB0128/2015

UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Candidato(a): Heloisa Berti Gabriel.

Título da Tese: Caracterização funcional de farnesil difosfato sintase/geranylgeranyl difosfato sintase (FPPS/GGPPS) e 1,4-dihidroxi-2-naftoato prenilttransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K em *Plasmodium falciparum*.

Orientador(a): Prof. Dr. Alejandro Miguel Katzin.

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a/...../....., considerou

() Aprovado(a)

() Reprovado(a)

Examinador(a): Assinatura:
 Nome:
 Instituição:

Examinador(a): Assinatura:
 Nome:
 Instituição:

Examinador(a): Assinatura:
 Nome:
 Instituição:

Examinador(a): Assinatura:
 Nome:
 Instituição:

Presidente: Assinatura:
 Nome:
 Instituição:

UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Cidade Universitária "Armando de Salles Oliveira"
Av. Prof. Lineu Prestes, 2415 – CEP. 05508-000 São Paulo, SP – Brasil
Telefone : (55) (11) 3091-7733 - telefax : (55) (11) 3091-8405
e-mail: cep@icb.usp.br

Comissão de Ética em Pesquisa

CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB Nº 469/11 referente ao projeto intitulado: "*Caracterização funcional de farnesil pirofosfato sintase (FPPs) e 1,4-dihidroxi-2-naftoato preniltransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K durante o desenvolvimento intra-eritrocítico de Plasmodium falciparum*" sob a responsabilidade de **Heloisa Berti Gabriel**, foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSh - COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº196 de 1996.

São Paulo, 17 de agosto de 2011.

PROF. DR. WOTHAN TAVARES DE LIMA
Coordenador da CEUA - ICB/USP

PROF. DR. PAOLO M.A. ZANOTTO
Coordenador da CEPsh - ICB/USP

*Aos meus pais Antônio e
Maria Jandira, por todo
apoio e carinho...*

AGRADECIMENTOS

Agradeço a todas as pessoas que direta ou indiretamente colaboraram comigo ao longo desse trabalho, em especial:

A Alejandro, pela oportunidade, ensinamentos, apoio, paciência e confiança depositada todos esses anos.

Ao Mauro, Emília e Gerhard por todos os ensinamentos, discussões, idéias e imensa colaboração durante todo o desenvolvimento do projeto.

Aos meus colegas de laboratório por toda a colaboração, em especial ao Rodrigo, Fabiana e Alexandre pela sua imensa amizade, incentivo e apoio sempre que necessário, e a Márcia pelo auxílio em partes do projeto.

Ao grupo do Professor Gerhard e Tânia por todo apoio, colaboração e paciência em ceder o espaço, sempre que necessário, em especial o Wolfgang, Fernanda, Rose, André e Alexandre.

Ao Professor Dean Crick da Universidade do Estado do Colorado e seu grupo, pela confiança, ensinamentos e apoio durante um período de desenvolvimento de parte do projeto.

Aos Professores João M. P. Alves e Giuseppe Palmisano pelos ensinamentos e auxílio em partes do desenvolvimento do projeto.

Ao laboratório do Professor Cláudio Marinho e Cefap pelos auxílios com os experimentos de fluorescência.

A todo o pessoal do Departamento de Parasitologia: alunos, técnicos e professores, pela convivência, amizade e muitas vezes pelos auxílios e discussões.

Aos meus grandes amigos do departamento e os de fora do departamento, pelo apoio, carinho, paciência e incentivo.

Ao meu amigo, colega e companheiro Wesley, por todas as discussões, auxílios e apoio sempre que necessário.

Aos meus pais e familiares, pelo amor e apoio que nunca me faltaram.

*“A ciência ainda não nos
provou se a loucura é ou não
o mais sublime da
inteligência.”*
Edgar Allan Poe

Este trabalho contou com o apoio financeiro do Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundo de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

PREFÁCIO

“Esta tese foi elaborada de acordo com as normas da GPG/ICB, relativas a outras formas de elaboração de tese de doutoramento, que permitem a inclusão dos anexos cujos resultados já foram publicados ou submetidos em periódicos internacionais indexados em língua inglesa. Permite ainda, que detalhes metodológicos e resultados sejam aqueles contidos nos artigos anexados no corpo da tese.”

Anexos que compõem o corpo desta tese:

- Anexo I** - Single-target high-throughput transcription analysis reveal high levels of alternative splicing present in the FPPS/GGPPS from *Plasmodium falciparum*. (submitted)
- Anexo II** - Squalenstatin is an inhibitor of carotenoid biosynthesis in *P. falciparum*.
- Anexo III** - Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*.
- Anexo IV** - Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite *Plasmodium falciparum*.

RESUMO

Gabriel, HB. Caracterização funcional de farnesil difosfato sintase/geranilgeranil difosfato sintase (FPPS/GGPPS) e 1,4-dihidroxi-2-naftoato preniltransferase (MenA) envolvidas respectivamente na via de isoprenóides e da vitamina K em *Plasmodium falciparum*. [Tese (Doutorado em Parasitologia)]. São Paulo. – Instituto de Ciências Biomédicas, Universidade de São Paulo, 2015.

A malária é uma das principais e a mais disseminada das parasitoses humanas, se constituindo em um grave problema de saúde mundial, especialmente nos países africanos. Muitos esforços têm sido mobilizados nos últimos anos com o intuito de se desenvolver novas formas de tratamento e novos quimioterápicos contra a doença. A falta de uma vacina eficaz e o problema da resistência aos fármacos tem contribuído para o adiamento da solução do controle desta infecção. A busca de novos alvos biológicos tem se concentrado, em parte, na pesquisa e compreensão de vias metabólicas, sendo uma das abordagens a caracterização e localização de enzimas dessas vias, bem como a demonstração de terem uma função essencial no ciclo do parasita. Em *P. falciparum*, identificamos a biossíntese das duas formas da vitamina K (filoquinona e menaquinona), provenientes das vias do chiquimato e da via MEP, assim como a biossíntese de ubiquinona (Ub₇₋₈), que também é produto da biossíntese de isoprenóides no parasita. Seguindo os estudos na via MEP, foram caracterizadas duas importantes enzimas bifuncionais, a farnesil difosfato sintase/geranilgeranil difosfato sintase (FPPS/GGPPS) capaz de formar farnesil difosfato e geranilgeranil difosfato, essenciais para a biossíntese de diversos produtos secundários, e octaprenil pirofosfato sintase/fitoeno sintase (OPP/PSY) responsável pela biossíntese da cadeia isoprênica que se liga ao anel da via de ubiquinona, como também forma o primeiro caroteno na via de carotenóides. Este projeto tem como principal objetivo caracterizar o gene MenA da biossíntese de MQ, determinar a localização de FPPS/GGPPS em *P. falciparum* e investigar a importância de OPP/PSY e de FPPS/GGPPS no ciclo intraeritrocítico de *P. falciparum*. Os resultados gerados poderão ajudar a compreender mais sobre os mecanismos de sobrevivência de *P. falciparum*, potencializando assim, novos alvos para drogas antimaláricas.

Palavras-chave: *Plasmodium*. Malária. Isoprenóides. Transfecção. Enzimas. Menaquinona.

ABSTRACT

Gabriel, HB. Functional characterization of farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase (FPPS/GGPPS) and 1,4-dihydroxy-2-naphthoate prenyltransferase (MenA) respectively involved in the isoprenoid pathway and vitamin K in *Plasmodium falciparum*. [Thesis (PhD, Parasitology)] São Paulo. - Instituto de Ciências Biomédicas, Universidade de São Paulo, 2015.

Malaria is one of the main and the most widespread human parasites, constituting into a serious health problem worldwide, especially in African countries. Many efforts have been deployed in recent years in order to develop new treatments and new chemotherapeutic agents against the disease. The lack of an effective vaccine and the problem of drug resistance have contributed to the delay of the control solution of this infection. The search for new biological targets has focused in part on the research and understanding of metabolic pathways, one of the approaches to characterize enzymes and localization of these pathways, as well as the demonstration of having a key role in the parasite cycle. In *P. falciparum*, we identified the biosynthesis of the two forms of vitamin K (phylloquinone and menaquinone), from the shikimate and MEP pathways and as well as ubiquinone biosynthesis (Ub7-8), which is also a product of isoprenoid biosynthesis in the parasite. Following the studies towards in the MEP pathway, we characterized two important bifunctional enzyme, farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase (FPPS/GGPPS) able to form farnesyl diphosphate and geranylgeranyl diphosphate, essential for the biosynthesis of many secondary products and octaprenyl pyrophosphate synthase/phytoene synthase (OPP/PSY) responsible for the biosynthesis of isoprenic side chains attached to the benzoquinone ring of ubiquinones, but also forms the first carotene in the carotenoid pathway. This project aims to characterize the MenA gene from the MQ biosynthesis, determine the localization of FPPS/GGPPS in *P. falciparum* and investigate the importance of OPP/PSY and FPPS/GGPPS in intra-erythrocytic cycle of *P. falciparum*. The results generated will help understand more about *P. falciparum* survival mechanisms, enhancing thus new targets for antimalarial drugs.

Keywords: *Plasmodium*. Malaria. Isoprenoids. Transfection. Enzymes. Menaquinone.

LISTA DE FIGURAS

Figura 1- Casos confirmados de malária no mundo em 2013..	20
Figura 2- Ciclo de vida do parasita da malária.....	22
Figura 3- Via de biossíntese de retinóides, vitamina K e carotenóides.	26
Figura 4- Via de biossíntese de carotenoides em <i>P. falciparum</i>	28
Figura 5- Representação dos intermediários da via de biossíntese da MQ.	30
Figura 6- Detecção da proteína FPPS/GGPPS por Western blot na linhagem FPPS/GGPPS-HA.	51
Figura 7- Detecção da proteína FPPS/GGPPS por Western blot na linhagem FPPS/GGPPS-HA-DD24.	51
Figura 8- Esquema de integração de pFPPS/GGPPS-HA, pFPPS/GGPPS-HA-DD24 e pFPPS/GGPPS-GFP-HA no locus genômico.....	52
Figura 9- Expressão de FPPS/GGPPS-HA durante o ciclo intraeritrocítico de <i>P. falciparum</i> .	53
Figura 10- Localização de FPPS/GGPPS.....	56
Figura 11- Alinhamento de FPPS/GGPPS e respectivas isoformas.....	57
Figura 12- Digestão das isoformas que deletam o exon 7 com a enzima TaqI.	58
Figura 13- Cobertura geral das sequências a partir do RNA-seq.	59
Figura 14- Isoformas viáveis.....	61
Figura 15- PCR e RT-PCR para confirmação de algumas isoformas.	63
Figura 16- Expressão e purificação das proteínas codificadas pelos genes PF3D7_0607500 e PF3D7_0202700 (OPP/PSY).	66
Figura 17- Expressão de pOPP/PSY-HA-int durante o ciclo intraeritrocítico de <i>P. falciparum</i>	67
Figura 18- Super Expressão de OPP/PSY em <i>P. falciparum</i>	69
Figura 19- Curva de crescimento por 96 horas comparando-se as linhagens OPP/PSY-HA-int, OPP/PSY-HA-epi e a selvagem 3D7.	70
Figura 20- Efeito da super expressão de OPP/PSY na biossíntese de carotenoides e GGPP.	71
Figura 21- LC-MS após a extração de lipídeos para análise de MQ-8 e DMQ-8..	73
Figura 22- Perfil radioativo, DPM (desintegração por minuto), das amostras após reação enzimática para a enzima codificada pelo gene MenA..	75
Figura 23- Perfil radioativo, DPM (desintegração por minuto), e TLC das amostras após reação enzimática para FPPS.....	76

Figura 24- Evidência bioquímica da biossíntese da Demetilmenaquinona-4 em <i>P. falciparum</i>	78
Figura 25- Perfil de HPLC após atividade para MenA utilizando a proteína recombinante OPP/PSY..	79

LISTA DE TABELAS

Tabela 1- Oligonucleotídeos utilizados.....	48
Tabela 2- Eventos de retenção de introns de FPPS/GGPPS durante o ciclo intraeritrocítico de <i>P. falciparum</i>	63

LISTA DE ABREVIATURAS E SIGLAS

CQ	cloroquina
IPP	isopentenil difosfato
DMAPP	dimetilalil difosfato
DOXP	1-deoxi-D-xilulose 5-fosfato
MEP	2C-metil-D-eritritol-4-fosfato
FPPS	farnesil difosfato sintase
GGPPS	geranilgeranil difosfato sintase
GPP	geranil difosfato
FPP	farnesil difosfato
GGPP	geranilgeranil difosfato
PSY	fitoeno sintase
OPPS	octaprenil difosfato sintase
OPP	octaprenil difosfato
pABA	p-aminobenzoato
EPSP sintase	5-enolpiruvil-chiquimato-3-fosfato-sintase
DAHPS sintase	3-deoxi-D-arabino-heptolusonato-7-fosfato-sintase
PhQ	filoquinona
MQ	menaquinona
DMQ	demetilmenaquinona
Hepes	ácido N-(2-hidroxietil) piperazina-N'-2-etanossulfónico
SDS	dodecil sulfato de sódio
EDTA	ácido etilenodiamino tetra-acético
DTT	ditiotreitól
PBS	<i>phosphate Buffer saline</i>
BSA	albumina sérica bovina
NMD	mutação nonsense
HA	hemaglutinina
DD	domínio de desestabilização
GFP	<i>green fluorescent protein</i>
PCR	<i>polymerase chain reaction</i>
ACP	<i>acyl carrier protein</i>
RT-PCR	<i>Reverse transcription polymerase chain reaction</i>
Ct	<i>cycle threshold</i>
IPTG	isopropil- β -D-1-tiogalactopiranosídeo
ESI-MS/MS	<i>electrospray ionization tandem mass spectrometry</i>
HPLC	<i>high-performance liquid chromatography</i>
TLC	<i>thin-layer chromatography</i>
LC-MS	<i>liquid chromatography-mass spectrometry</i>
DHNA	1,4-dihidroxi-2-ácido naftóico
FARM	<i>first Asp-rich motif</i>
SARM	<i>second Asp-rich motif</i>
CDL	<i>chain length determination</i>
MSP	proteína de superfície do merozoíta
EXP	proteína exportada
GC-MS	<i>gas chromatography-mass spectrometry</i>

SUMÁRIO

1 INTRODUÇÃO.....	18
1.1 Malária.....	19
1.1.1 Generalidades.....	19
1.1.2 O parasita e seu ciclo de vida	20
1.1.3 Os problemas enfrentados no combate a doença.....	22
1.2 Vias Metabólicas	24
1.2.1 Via 2C-metil-D-eritritol-4-fosfato (MEP)	24
1.2.2 Via do Chiquimato.....	28
1.3 Genética Reversa	30
1.4 Processamento do RNA – splicing alternativo	32
2 JUSTIFICATIVAS E OBJETIVOS	34
3 MATERIAIS E MÉTODOS.....	36
3.1 Digestão, purificação e clonagem dos fragmentos de DNA.....	37
3.2 Cultura de <i>Plasmodium falciparum</i>	38
3.3 Sincronização dos estágios intraeritrocitários	38
3.4 Transfecção em <i>P. falciparum</i> e seleção dos parasitas.....	38
3.5 Integração dos plasmídeos nos cromossomos dos parasitas.....	39
3.6 Detecção da proteína por Western blot.....	39
3.7 Clonagem dos parasitas por diluição limitante.....	40
3.8 Extração de DNA genômico de <i>P. falciparum</i>	40
3.9 PCR (<i>Polymerase chain reaction</i>).....	41
3.10 Imunofluorescência	41
3.11 Extração do RNA e síntese da dupla fita (cDNA) dos estágios intraeritrocitários de <i>P. falciparum</i>	42
3.12 RT-PCR (<i>Reverse transcription polymerase chain reaction</i>)	42
3.13 Sequenciamento de nova geração.....	43
3.14 Análises dos dados do RNA-seq	43
3.15 Preparação das proteínas para espectrometria de massa	43
3.16 Espectrometria de massa	44
3.17 Clonagem de PF3D7_0607500 e PF3D7_0202700 (OPP/PSY).....	44

3.18 Expressão das proteínas codificadas por PF3D7_0607500 e PF3D7_0202700 (OPP/PSY)	44
3.19 Marcação metabólica e extração de DMQ-4 e Carotenoides	45
3.20 RP-HPLC (<i>Reverse phase- High-performance liquid chromatography</i>)	45
3.21 Extração de Lipídeos e TLC (<i>Thin-layer chromatography</i>)	46
3.22 Preparação de membrana	46
3.23 Atividade enzimática para MenA	47
3.24 Atividade enzimática para FPPS	47
3.25 Desfosforilação enzimática	47
4 RESULTADOS E DISCUSSÃO	49
4.1 Geração das linhagens transgênicas com os plasmídeos integrados	50
4.2 Detecção da integração por PCR	51
4.3 Perfil de expressão de FPPS/GGPPS	53
4.4 Clonagem por diluição limitante de pFPPS/GGPPS-HA-DD24	53
4.5 Localização de FPPS/GGPPS	55
4.6 Identificação de possíveis isoformas da enzima FPPS/GGPPS durante o ciclo intraeritrocítico	56
4.7 Expressão de PF3D7_0607500 e OPP/PSY	65
4.8 Perfil de expressão de OPP/PSY	66
4.9 Super expressão de OPP/PSY	67
4.10 Caracterização de MenA	71
5 CONCLUSÕES	81
REFERÊNCIAS	83
ANEXOS	104
I- Single-target high-throughput transcription analysis reveal high levels of alternative splicing present in the FPPS/GGPPS from <i>Plasmodium falciparum</i> . (submitted)	105
II- Squalastatin is an inhibitor of carotenoid biosynthesis in <i>P. falciparum</i> .	171
III- Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from <i>Plasmodium falciparum</i> .	181
IV- Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite <i>Plasmodium falciparum</i> .	197

1.1 Malária

1.1.1 Generalidades

A malária é considerada um dos principais flagelos da humanidade desde a antiguidade. É uma doença também conhecida como paludismo, impaludismo, maleita, febre terçã ou quartã. Sua origem é uma questão ainda discutida nos dias atuais, suas teorias seriam que tivesse se desenvolvido a partir de adaptação de Coccídios do epitélio intestinal para tecidos de órgãos internos e células sanguíneas, ou através de uma transferência lateral de parasitos de outros vertebrados (1).

A malária humana teve origem provavelmente no continente Africano e acompanhou as migrações dos seres humanos pelas regiões do mediterrâneo, Índia e Sudeste Asiático. No século XVIII recebeu o nome italiano de “*mal aria*”, que significa mal ar ou ar insalubre, quando Lancisi considerou que a doença seria causada pelas emanções e miasmas provenientes dos pântanos (2). No Novo Mundo a chegada da malária continua a ser motivo de especulações (3). Estima-se que no início do século XVII, uma das espécies do parasita, o *Plasmodium vivax*, teria sido transportado do sudeste Asiático para as colônias americanas em Jamestown e Virginia, enquanto o *Plasmodium falciparum* teria sido introduzido na América Central por meio de escravos africanos infectados (3). Análises de DNA mitocondrial em espécies do parasita na Ásia, América do Sul e Papua Nova Guiné mostraram que essas populações são mais antigas (50.000 a 100.000 anos atrás) que o evento de migração dos africanos, sugerindo que, o parasita tenha migrado da África antes da expansão ocorrida neste continente, talvez durante o Pleistoceno (4).

De acordo com as últimas estimativas, 198 milhões de casos de malária ocorreram globalmente em 2013 com 584 mil mortes, sendo que 90 % desse total foi atribuído a região Africana, com cerca de 78% de mortes em crianças menores de 5 anos. Atualmente 3,2 bilhões de pessoas em áreas de risco podem estar infectados com malária e desenvolver a doença, sendo que 1,2 bilhão estão presentes em áreas de alto risco (> 1 em 1000 chances de contrair malária em um ano) (**Figura1**), representando um decréscimo na incidência de casos de malária e taxas de mortalidade de 30% e 47%, respectivamente, desde o ano 2000 (5). No Brasil, a área endêmica é conhecida como Amazônia Legal, composta pelos estados do Acre, Amapá, Amazonas, Maranhão, Mata Grosso, Mato Grosso do Sul, Pará, Rondônia e Tocantins. Em 2013 foram mais de 177 mil casos confirmados da doença (5).

Figura 1- Casos confirmados de malária no mundo em 2013. As cores representam o número de casos registrados da doença a cada 1000 habitantes, como indicado (5). O branco indica regiões onde não houve casos registrados e o cinza onde não se aplica a estatística.

1.1.2 O parasita e seu ciclo de vida

O agente etiológico da malária é um protozoário do gênero *Plasmodium* pertencente à família **Plasmodiidae** e ao filo Apicomplexa. Laveran foi o responsável pela descoberta do plasmódio, ao observá-los nos eritrócitos humanos e foi o primeiro a descrevê-lo, em 1880 (6). A doença é transmitida pela fêmea do mosquito do gênero *Anopheles*, hospedeiro em que ocorre a fase sexuada do ciclo biológico do parasita. Apenas as fêmeas são hematófagas e são as responsáveis pela transmissão do parasita. Esse gênero inclui aproximadamente 400 espécies no mundo, mas apenas 60 são capazes de transmitir o parasita em condições naturais (2). Há quase 100 espécies de plasmódios, 22 das quais infectam macacos e 50 são parasitas de aves ou répteis (6). As espécies de *Plasmodium* que infectam o homem são cinco: *P. falciparum* descrita por Welch em 1897, causa a maioria dos casos de morbidade e mortalidade; *P. vivax* descrita por Grassi e Feletti em 1890; *P. ovale* descrita por Strphens em 1922, com distribuição limitada ao continente Africano; *P. malariae* descrita por Laveran em 1881 (6); e por último *P. knowlesi* isolado pela primeira vez na Índia no início de 1930 por Campbell e Napier como parasita de macacos, posteriormente Knowlesi e Das Gupta assemelharam sua morfologia ao do *P. malariae*. No entanto, o primeiro caso de uma infecção natural num ser humano só foi relatado 34 anos mais tarde (7).

O ciclo biológico dos parasitas humanos do gênero *Plasmodium* é muito semelhante entre as espécies, apresentando duas fases distintas (**Figura 2**), uma com reprodução sexuada

(esporogonia) no hospedeiro definitivo invertebrado e a outra fase com reprodução assexuada (esquizogonia) no hospedeiro vertebrado (6).

O ciclo sexuado se inicia com a liberação de formas sexuais do parasita na luz do intestino do hospedeiro invertebrado através da picada por fêmeas dos anofelinos em indivíduos infectados por *Plasmodium*. Os gametócitos masculinos e femininos se diferenciam em micro e macrogametas, respectivamente, pelo processo de exflagelação. Os gametas se unem formando uma célula-ovo ou zigoto, que se diferencia em oocineto, uma forma ameboide que se locomove e penetra na lâmina basal intestinal, originando o oocisto. No interior do oocisto ocorrem muitas divisões nucleares, a primeira meiótica e as subsequentes mitóticas, dando origem a milhares de esporozoítos. Essas formas infectantes migram para as glândulas salivares do mosquito, sendo liberados durante um novo repasto sanguíneo em outro hospedeiro vertebrado dando início a fase assexuada. Nesta fase os esporozoítos liberados, após a picada do anofelino, alcançam o fígado do hospedeiro vertebrado e invadem os hepatócitos originando a forma arredondada denominada criptozoíta que crescem e realizam a esquizogonia pré-eritrocitária. Ocorrem variações no tempo do ciclo nos hepatócitos para cada espécie do parasita, sendo em média 6 dias para *P. falciparum*, 10 dias para *P. vivax* e 15 dias para *P. ovale* e *P. malariae*. Os merozoítas da esquizogonia pré-eritrocitária são então liberados pelo rompimento dos hepatócitos e os que sobrevivem à fagocitose e destruição pelas células de Kupffer invadem as hemácias iniciando o ciclo intra-eritrocitário.(6)

No ciclo intra-eritrocitário o parasita se diferencia em três fases distintas: trofozoíto jovem ou anel, trofozoíto maduro e esquizonte. A duração do ciclo intra-eritrocitário do parasita se distingue entre as espécies de *Plasmodium*. *P. knowlesi* tem o ciclo mais curto entre as espécies que infectam os humanos, com aproximadamente 24 h, já para *P. falciparum*, *P. vivax* e *P. ovale* o ciclo é de aproximadamente 48 h, enquanto que para *P. malariae* a duração do ciclo chega a 72 h (7). Ao final do ciclo intra-eritrocitário novos merozoítas se originam durante a esquizogonia, sendo então liberados na corrente sanguínea, através do rompimento do esquizonte, podendo invadir novas hemácias, iniciando um novo ciclo intra-eritrocitário. Alguns trofozoítos diferenciam-se em formas sexuadas, os gametócitos, que são os responsáveis pela infecção do hospedeiro invertebrado, completando o ciclo.

No sangue, o ciclo esquizogônico repete-se em ciclos bastante regulares e característicos para cada espécie, preferencialmente nos capilares profundos das vísceras. No caso de *P. falciparum*, as formas esquizogônicas raramente são encontradas no sangue

periférico devido à sua capacidade de citoaderência em células endoteliais, favorecendo assim a retenção dessas formas nas paredes dos vasos profundos o que pode ocasionar as formas graves da infecção (6).

Figura 2- Ciclo de vida do parasita da malária. Durante a alimentação sanguínea, a fêmea do mosquito *Anopheles* inocula os esporozoítas no hospedeiro humano (1). Os esporozoítas infectam as células do fígado (2), formando os esquizontes (3). A ruptura dos hepatócitos leva à liberação dos merozoítas (4). Após a replicação no fígado, ciclo pré-eritrocitário (A), os parasitas sofrem multiplicação assexuada nos eritrócitos (B). Os merozoítas infectam as hemácias (5). O estágio de anel diferencia-se em trofozoíto e esquizonte (6). Alguns parasitas diferenciam-se em gametócitos (7), os quais são ingeridos pelo mosquito (8) em um novo repasto sanguíneo. No mosquito a multiplicação é conhecida como ciclo esporogônio (C). No estômago do mosquito, ocorre a geração dos zigotos (9). Os oocinetos (10) invadem a parede do estômago se desenvolvendo em oocisto (11). No oocisto são produzidos os esporozoítas (12) que liberados migram até a glândula salivar do mosquito. Modificado de www.cdc.gov

1.1.3 Os problemas enfrentados no combate a doença

Os danos econômicos atribuídos à malária classificam esta doença como uma das causas da pobreza no mundo, acarretando problemas sócio-econômicos e contribuindo para o menor desenvolvimento dos países afetados. A falta de uma vacina eficaz, o problema da resistência aos fármacos e a falta de investimento na procura e aplicação de novos compostos, contribuem para o adiamento da solução do controle desta endemia (8).

No Brasil, mudanças socioeconômicas ocorridas principalmente a partir da década de 50, levaram a migrações internas no país em especial para a região norte. Alterações no meio

ambiente (9) como assentamentos, construções de usinas hidroelétricas, extrativismos vegetal e mineral, desorganização espacial, concentração de pessoas em condições sanitárias inadequadas, entre outras, foram determinantes para a disseminação da malária no país, considerando também condições ecológicas, geográficas, econômicas, sociais e culturais.

Nos últimos tempos observou-se o surgimento de parasitas cada vez mais resistentes a drogas e vetores aos inseticidas (8). A reprodução sexuada do parasita e a utilização inadequada de antimaláricos, nos últimos cinquenta anos, tem prestado uma enorme pressão sobre a seleção natural dos parasitas da malária humana, aumentando o número de cepas resistentes. Esses fatores colaboram para o aumento do número de casos de morbidade e mortalidade ocasionados pela doença.

Durante grande parte do século XX o tratamento antimalárico indicado era a droga cloroquina (CQ), um medicamento caracterizado por sua rápida ação, segurança e baixo custo (10). A cloroquina inibe a degradação da hemoglobina pelo parasita através da formação de um complexo com o heme ou ferriprotoporfirina IX (Fe(III) PPIX), impedindo a polimerização deste (11). Cerca de 80% da hemoglobina é degradada, para a alimentação do parasita, por enzimas proteolíticas, em peptídeos que, posteriormente, serão degradados a aminoácidos. Com essa degradação da hemoglobina forma-se um resíduo livre denominado heme ou ferriprotoporfirina IX (Fe(III) PPIX) tóxico ao parasita, sendo portanto polimerizado, formando um composto inerte, insolúvel e não tóxico ao parasita, o pigmento malárico hemozoína (12).

A resistência às drogas é um dos grandes obstáculos encontrados no combate a doença. Em 1967 já era observada, em um programa de erradicação da doença, a resistência à cloroquina na maioria das regiões onde o *P. falciparum* era endêmico (13), além da resistência de outros antimaláricos habitualmente utilizados hoje em dia como sulfadoxina, pirimetamina e mefloquina. A resistência à cloroquina surgiu lentamente, mas já no início de 1990 foram encontrados parasitas resistentes em praticamente todas as regiões endêmicas da doença, no mundo (10). Recentemente foi descrito resistência do *P. falciparum* a artemisinina em cinco países do Sudeste Asiático e, provavelmente, na América do Sul (5). A vantagem particular de artemisinina sobre outras drogas antimaláricas é que ela mata parasitas em circulação no estágio anel e assim acelera a resposta terapêutica (5). Porém, a previsão é de que parasitas resistentes a artemisinina se espalhem na África, onde a incidência da malária ocasionada por *P. falciparum* é muito maior. No século passado essa dispersão aconteceu com parasitas resistentes à cloroquina e posteriormente a pirimetamina, contribuindo para a morte de milhões de crianças africanas (14).

Os eventos genéticos que conferem resistência às drogas antimaláricas são espontâneos e raros, independentes da droga utilizada. Eles são mutações ou alterações no número de cópias de genes relacionados com os alvos (15). A descoberta de regiões de baixa variação ou de pressões seletivas identificou numerosos genes que sofreram evolução adaptativa recente. Notavelmente, alguns dos exemplos mais marcantes de pressões seletivas estão em genes envolvidos na evolução da resistência aos medicamentos antimaláricos. Mutações nos genes *pfprt*, *dhfr* e *dhps* causam resistência à CQ, pirimetamina e sulfadoxina (16).

O desafio atual se baseia em melhorar as técnicas de combate à doença, aprimorando o acesso às drogas apropriadas e suas combinações, fornecendo medicamentos com baixo custo, vigilância crescente a fim de orientar o uso adequado e mais atenção para estratégias alternativas de prevenção, como uso de mosquiteiros tratados com inseticidas, por exemplo.

Os alvos visados no desenvolvimento de novas terapias para o tratamento da malária abrangem tanto funções celulares, tais como detoxificação do heme ou ferriprotoporfirina IX (Fe(III)PPIX) (17), e o metabolismo do folato, já explorados para drogas estabelecidas como antimaláricos, assim como também vias metabólicas, tais como síntese de ácidos graxos, e biossíntese de isoprenóides (18).

1.2 Vias Metabólicas

1.2.1 Via 2C-metil-D-eritritol-4-fosfato (MEP)

O *Plasmodium* é um protozoário que pertence ao grupo dos apicomplexas, parasitas intracelulares obrigatórios que são responsáveis por uma série de doenças graves que afetam uma ampla gama de hospedeiros animais, incluindo os seres humanos. Relatado pela primeira vez em *Toxoplasma* e *Plasmodium*, o parasita apresenta uma organela denominada apicoplasto, possivelmente resultante de uma endossimbiose secundária envolvendo uma alga vermelha (19).

Essa organela reteve algumas vias biossintéticas como a biossíntese de ácidos graxos e de isoprenóides. Neste último caso, em parasitas da malária, especialmente a espécie mais virulenta, *P. falciparum*, uma série de novas enzimas "*plantlike*", relacionadas a essa via metabólica, foram recentemente descobertas (20). Essas vias são excelentes alvos para a produção de novas drogas uma vez que não são compartilhadas com o hospedeiro humano.

No curso da evolução, o apicoplasto perdeu sua função fotossintética, e especulações demonstraram sua importância na formação de componentes essenciais incorporados na membrana do vacúolo parasitóforo (21). Recentemente foi demonstrado que a biossíntese de

isoprenóides não é apenas essencial para o parasita, mas, de fato, a única função do apicoplasto durante o crescimento em fase sanguínea (22) e formas sexuais (23).

Todos os isoprenóides derivam de um precursor comum, o isopentenil difosfato (IPP) e seu isômero dimetilalil difosfato (DMAPP) (**Figura 3**). Por várias décadas, a via do Mevalonato, presente em animais e plantas, foi considerada a única via de síntese para as unidades isoprênicas da biossíntese de isoprenóides. A existência de uma segunda via para a biossíntese de unidades isoprênicas foi descoberta em 1988 por Flesch e Rohmer quando estudavam a biossíntese de hopanóides (esteróides triterpênicos pentacíclicos) em bactérias (24). Originalmente chamada de via de Rohmer ou via independente do mevalonato, teve seu nome modificado após a identificação do primeiro passo da via (via do piruvato/gliceraldeído-3-fosfato GAP) ou o primeiro intermediário, 1-deoxi-D-xilulose 5-fosfato (via DOXP). Entretanto, o nome mais aceito é via do 2C-metil-D-eritritol-4-fosfato (MEP) (25), uma vez que este composto é o primeiro precursor exclusivo da via.

A identificação e caracterização do farnesil pirofosfato (FPP) em *P. falciparum* (26), assim como a presença de dolicois (27), e proteínas covalentemente modificadas por isoprenóides (28) foram as primeiras evidências para o estudo da biossíntese de isoprenóides em *Plasmodium*. Na última década, houve uma ampla caracterização de produtos da biossíntese de isoprenóides no parasita (27-33) resultantes da via alternativa 2C-metil-D-eritritol-4-fosfato (MEP) (25) (**Figura 3**). O passo essencial e importante no metabolismo da biossíntese de todos os isoprenóides é o alongamento da cadeia isoprênica por enzimas denominadas prenilttransferases. Estas enzimas são classificadas de acordo com o comprimento da cadeia do produto final e a estereoquímica da dupla ligação formada por condensações, sendo FPPS (farnesil difosfato sintase) e GGPPS (geranilgeranil difosfato sintase) as prenilttransferases mais estudadas (34). FPPS catalisa a condensação de IPP com DMAPP e geranil difosfato (GPP) para formar o isoprenóide composto de 15 carbonos, o farnesil difosfato (FPP). O FPP é o substrato que catalisa o primeiro passo para a biossíntese de ubiquinona, carotenóides, dolicois e isoprenilação de proteínas. O FPP também pode ser condensado com uma molécula adicional de IPP pela enzima GGPPS para formar o isoprenóide de 20 carbonos, o geranilgeranil difosfato (GGPP), também essencial na isoprenilação de proteínas (**Figura 3**) (35).

Os genes que codificam FPPS e GGPPS já foram identificados e caracterizados em várias espécies como *Saccharomyces cerevisiae*, *Trypanosoma cruzi*, *T. brucei*, *Toxoplasma gondii*, *Plasmodium vivax* (35-39) e recentemente em *P. falciparum* (34). O genoma humano contém genes para as duas enzimas distintas responsáveis pela síntese de GGPP e FPP (40,

41). Em *T. cruzi* e *P. vivax*, a FPPS ou a GGPPS está presente, respectivamente (37, 39), porém, há indícios da GGPPS de *P. vivax* ser uma enzima bifuncional (39). Em *T. brucei* experimentos utilizando a técnica de interferência no RNA (RNAi) silenciando o gene, mostraram que FPPS é essencial para a sobrevivência do parasita, assim como parece ser em muitos outros organismos (38). A enzima FPPS em *P. falciparum* bem como em *T. gondii* mostrou ser bifuncional (FPPS/GGPPS), sendo capaz de formar os compostos isoprênicos: farnesil difosfato e geranylgeranyl difosfato (34, 35).

Figura 3- Via de biossíntese de retinóides, vitamina K e carotenóides. Os retinóides (retinal, retinol e ácido retinóico), as vitaminas K (filoquinona e menaquinona) e carotenóides são produtos finais da via dos isoprenóides. A formação das unidades isoprênicas pode ocorrer por meio da via do Mevalonato (animais) ou de ambas (bactérias e plantas). O anel aromático de ambas as formas de vitamina K é proveniente da via do Chiquimato. DMAPP (dimetilalil difosfato), DOXP (1-deoxi-D-xilulose 5-fosfato), FPP (farnesil pirofosfato), GAP (gliceraldeído-3-fosfato), GPP (geranyl difosfato), GGPP (geranylgeranyl difosfato), IPP (isopentenil difosfato) e MEP (metil-eritritol-fosfato).

Muitos tipos de isoprenóides, provenientes da via MEP, como por exemplo, carotenóides e ubiquinonas (biossintetizados por *P. falciparum*) (32, 42), são componentes essenciais da maquinaria celular de muitos organismos, participando de uma variedade de processos biológicos. Entre estas vias bioquímicas, a biossíntese de carotenóides é um alvo atrativo para estudos, uma vez que são essenciais em algas, plantas superiores, bactérias e fungos, mas ausentes em mamíferos, e os seus produtos estão envolvidos em várias funções metabólicas importantes (43). Todos os carotenóides possuem uma estrutura poliisoprênica (**Figura 4**), uma cadeia longa de ligações duplas conjugadas, e uma simetria bilateral em torno da ligação dupla central (43). Diferentes carotenóides são derivadas essencialmente por modificações na estrutura de base, tal como a ciclização dos grupos terminais e por introdução das funções de oxigênio, que confere suas cores características e propriedades antioxidantes (44). Em *P. falciparum* foi demonstrado a biossíntese de carotenóides em fases intraeritrocitárias, sugerindo que podem desempenhar um papel no desenvolvimento do parasita e, por conseguinte, ser um alvo para drogas antimaláricas (42). A biossíntese de carotenos começa com a condensação de duas moléculas de geranylgeranyl difosfato (GGPP) para formar fitoeno, o primeiro caroteno da via (43). Esta reação é catalisada pela enzima fitoeno sintase (PSY) (42), sendo essa uma enzima bifuncional, uma vez que exerce a atividade também de octaprenil difosfato sintase (OPPS) (33). OPPS pertence a uma família de preniltransferase que catalisa as reações de condensação de FPP com cinco moléculas de IPP para produzir um C40 OPP (octaprenil difosfato) (33). São responsáveis pela biossíntese de cadeias laterais ligadas a ubiquinonas em *Escherichia coli* (45, 46) e *P. falciparum* (33), neste último representou a primeira caracterização de uma preniltransferase em um parasita da malária (34). Essa enzima plasmodial é um exemplo de uma enzima carotenogênica com uma linha contínua de evolução de arquea para bactérias (cianobactérias) e plantas (47, 48) contendo duas atividades.

Figura 4- Via de biossíntese de carotenoides em *P. falciparum*. Através da via MEP, a biossíntese de carotenóides se inicia com a condensação de duas moléculas de geranilgeranil difosfato (GGPP) pela enzima fitoeno sintase (PSY) para formar o fitoeno, o C40, esqueleto inicial (49). **Anexo 2**

1.2.2 Via do Chiquimato

Além da via MEP, foi descrita a via de biossíntese do chiquimato (**Figura 3**) em *P. falciparum*, melhor estudada em algas, plantas superiores, bactérias e fungos. A via do chiquimato (50) tem como produtos finais os aminoácidos aromáticos: fenilalanina, tirosina e triptofano, e como intermediário o corismato(51).

São sete reações até a formação do corismato, iniciando com a condensação de fosfoenolpiruvato e eritrose 4-fosfato. Os intermediários da via biossíntese do chiquimato são pontos de ramificação para diversas vias. As sete enzimas da via do Chiquimato foram originalmente descobertas em bactérias, principalmente *Escherichia coli* e *Salmonella typhimurium* (51).

Em *P. falciparum* a via foi descoberta como parte da biossíntese de folatos onde, o p-aminobenzoato (pABA), um intermediário da via essencial para a produção de ácido fólico, foi descrito como essencial para a sobrevivência do parasita, sendo capaz de reverter a ação

de inibição ocasionada pelo herbicida glifosato, inibidor da enzima 5-enolpiruvil chiquimato-3-fosfato-sintase (EPSP sintase) (50, 52). Posteriormente as enzimas chiquimato quinase, 3-deoxi-D-arabino-heptolusonato-7-fosfato-sintase (DAHP sintase) e chiquimato desidratase, foram descritas no parasita (50).

Por ser uma via metabólica encontrada em plantas e microorganismos como *Mycobacterium tuberculosis* e *P. falciparum*, e ausente em mamíferos, considera-se um alvo importante para o desenvolvimento de herbicidas, vacinas e outras drogas.

Além dos aminoácidos aromáticos, o corismato, produto final da via do Chiquimato, pode dar origem a diversos outros compostos aromáticos, como tetraidrofolato, ubiquinonas, vitaminas E e as duas formas de vitamina K (filoquinona - PhQ, menaquinona - MQ) (**Figura 3**) (51).

Na via do chiquimato, o anel aromático da vitamina K é sintetizado a partir do corismato, onde reações enzimáticas subsequentes conduzem à formação do anel naftoquinona, ou 1,4-dihidroxi-2-naftoil-CoA, por meio de enzimas codificadas pelos genes MenF, MenD, MenC, MenE, MenB. Este anel é isoprenilado por uma molécula de geranilgeranil difosfato (proveniente da via MEP) com a ação da enzima codificada pelo gene MenA (**Figura 5**) e então metilado pela enzima codificada pelo gene MenG (53). A biossíntese de menaquinona nos estágios intraeritrocitários de *P. falciparum* foi inicialmente caracterizada por meio de marcações metabólicas com precursor direto [1-(n)-³H] geranilgeranil pirofosfato e confirmado por ESI-MS/MS (*Electrospray ionization tandem mass spectrometry*) (54). Observou-se uma ação de inibição no crescimento dos parasitas e posteriormente na biossíntese de menaquinona proporcionada pela droga Ro 48-8071 (derivada de amino alquoxi difenilmetano, inibidor da enzima 1,4-dihidroxi-2-naftoato preniltransferase - MenA) (**Figura 5**). Essa droga foi utilizada inicialmente por Dhiman e colaboradores (2009) para inibir a biossíntese de menaquinona em *Mycobacterium tuberculosis* (55).

Figura 5- Representação dos intermediários da via de biossíntese da MQ. Respectivas enzimas, mostrando o local de inibição da droga Ro 48 – 8071.

1.3 Genética Reversa

Um estágio crítico no avanço de novas terapias é desenvolver um meio para compreender aspectos específicos da biologia do parasita, como vias metabólicas ou de sinalização e outros mecanismos fundamentais da célula (56). Como *Plasmodium* não possui maquinaria enzimática necessária para RNAi (57), a identificação da função de genes importantes tem tradicionalmente utilizado abordagens clássicas de genética reversa, a qual consiste em se modificar de forma controlada regiões específicas do genoma de um organismo e posteriormente analisar o fenótipo resultante.

O genoma de *P. falciparum* é quase duas vezes o tamanho do genoma da levedura *Schizosaccharomyces pombe*. O genoma haplóide desse parasita tem pouco mais de 23 milhões de pares de base (Mb), uma composição global de AT de 80,6% e contém cerca de 90% em introns e regiões intergênicas. A análise comparativa do genoma de *P. falciparum* com outros eucariotas, que possuem o genoma completo disponível, revelou que, em termos de conteúdo global, é ligeiramente mais semelhante ao de *Arabidopsis thaliana* do que outros

organismos estudados (58), provavelmente devido à presença, no genoma nuclear, de genes derivados de plastídeos. Embora, a afinidade aparente de *Plasmodium* e *Arabidopsis*, pode não refletir a verdadeira história filogenética da linhagem de *P. falciparum*.

O parasita da malária utiliza sua variabilidade genética para se defender do sistema imunológico do hospedeiro e da ação de drogas utilizadas para o tratamento (59). Apesar do genoma completo do clone 3D7 ter sido sequenciado em 2002, (60) auxiliando assim em vários estudos com cepas resistentes, existem seções do genoma que permanecem não identificadas (61). Sendo assim, informação sobre a variação genética responsável por fenótipos como resistência e virulência ainda é pouco elucidada (59).

Uma das técnicas mais importantes, e muito utilizada para a análise funcional de genes de um organismo e suas respectivas proteínas, é a transfecção de parasitas. Existem basicamente dois tipos de transfecção, a transiente e a estável. A transfecção estável é utilizada para criar uma linhagem de células permanentemente transformada e é amplamente utilizada para estudos funcionais dos produtos de genes específicos. Basicamente consiste em introduzir sequências específicas de DNA ou RNA exógenos capazes de recrutar as maquinarias de transcrição, tradução e/ou replicação de células hospedeiras eucariontes. Transformações genéticas estáveis em protozoários *tripanossomatídeos* e em *T. gondii* (Apicomplexa) são realizadas desde o início da década de 90 (62, 63). Transfecção estável e integração homóloga através de recombinação simples em *P. falciparum* foi obtida inicialmente por Wu e colaboradores em 1996 (64) através da construção de plasmídeos com o gene *dhfr-ts* de *T. gondii* (*Tgdhfr-ts*) modificado para conferir resistência à pirimetamina. A partir de então, esses trabalhos permitiram vários estudos como a localização sub-celular de proteínas através da transfecção de genes em fusão com genes que codificam proteínas fluorescentes ou epítomos conhecidos (65, 66), a regulação da expressão (67) e a super expressão de genes (49, 68) para determinação de fenótipo.

Em *P. falciparum*, já foi observado que a expressão de proteínas em níveis ou momentos diferentes do original pode alterar sua localização (69). A localização de proteínas utilizando-se vetores de transfecção onde ela será expressa em fusão com uma proteína fluorescente ou um epítomo, será confiável somente após a sua integração no locus genômico permitindo a manutenção da expressão (70).

Os recentes avanços em sistemas de expressão gênica induzíveis também passaram a permitir o nocaute condicional e a expressão regulada de proteínas de interesse, abrindo caminho para a investigação da função de genes essenciais para a sobrevivência do parasita (56, 71-73). O atrativo destes sistemas para investigar a função de proteínas é a sua

capacidade de inibir especificamente uma proteína de forma regulada e reversível, através da redução dos níveis de expressão pelo nocaute condicional, ou a regulação da expressão de uma variante negativa dominante, onde uma proteína modificada ou parte dela é expressa a fim de se inibir a proteína alvo (74). Já a super expressão de genes, além de complementar estudos que identificam funções de proteínas e suas interações (75, 76), pode ser uma estratégia utilizada para confirmar a função de proteínas ainda não caracterizadas, como também para determinar a especificidade de inibidores (49, 77).

1.4 Processamento do RNA – splicing alternativo

Compreender o perfil de transcrição dos genes, processamento de RNA (tal como splicing), modificações pós-traducionais e mecanismos enzimáticos, são essenciais para se conhecer melhor a fisiologia do parasita.

Com o aumento dos dados de sequenciamento do DNA provenientes dos projetos genomas de *Plasmodium* spp, surgiram novos estudos de transcriptoma em escalas muito maiores (78, 79). Já nos primeiros estudos foi possível observar a evolução na regulação transcricional desse parasita, com a maioria dos genes transcritos durante o ciclo intraeritrocítico (78).

Em todas as células vivas, a expressão da informação genética envolve transcrição de moléculas de DNA em RNA e a posterior tradução destas para proteínas. Em muitos casos o transcrito primário não é idêntico ao funcional, pois o RNA maduro passa anteriormente por um processamento, a fim de estabilizar e garantir uma maior eficiência na síntese protéica, que geralmente inclui, remoção de fragmentos, adição de nucleotídeos ao término da cadeia de RNA e alterações de bases e unidades de ribose (80). A comparação de mRNA com sequências genômicas no final dos anos 1970 demonstrou que, antes da exportação para o citosol, sequências virais são removidas no processamento do RNA (81), esse processo ficou conhecido como splicing, identificado em quase todos os mamíferos (82). Este fenômeno explica como o RNA nuclear mais longo que o RNA citoplasmático pode ter a mesma terminação e uma calda de poli (A), considerando a diferença de tamanho (83). No processo de splicing apenas uma pequena fração das sequências dos transcritos primários são unidas entre si e exportadas como exons para o citosol, formando assim o mRNA maduro. A maioria das sequências intervenientes (introns) permanece no núcleo, onde são posteriormente degradadas (83). Um grande complexo macromolecular, spliceossoma, reconhece esses exons e remove as sequências de introns enquanto o pré-mRNA é sintetizado pela RNA polimerase II no núcleo (82). A grande maioria dos pré-mRNAs contém exons que podem ser

alternativamente incluídos no mRNA maduro ou removidos a partir dele, esse processo é chamado de splicing alternativo (82).

A análise de transcriptoma mostrou que splicing alternativo desempenha um papel importante gerando um grande número de mRNA e proteínas isoformas (82, 84). Estimou-se que mais de 50% de todos os genes humanos sofrem, em algum momento, o processamento por splicing alternativo, possivelmente apresentando diferentes isoformas com funções importantes (83). Essas mudanças, na estrutura primária da proteína, podem alterar as propriedades de ligação da mesma, interferindo em suas funções, atividade enzimática, estabilidade ou mesmo na localização intracelular (85).

Recentemente, observou-se que as ocorrências de eventos de splicing alternativo de *P. falciparum* não são tão raros como estudos anteriores tinham relatado (85). Embora o genoma do parasita tenha apresentado 7406 introns previstos, eventos de splicing alternativo que podem afetar a função da proteína foram observados em apenas alguns genes (85). O processamento alternativo pode resultar na modulação dos níveis de expressão do mRNA transcrito sujeitando este a deterioração mediada por mutações nonsense (NMD), através da adição de códons de terminação (stop códon), ou alteração da estrutura do produto do gene por mutação, com eliminação ou inserção de aminoácidos na proteína (84). Recentemente, estudos tendo como base sequenciamento de nova geração do transcriptoma e epigenoma de *P. falciparum*, demonstraram a ocorrência desses eventos em fases sexuada e assexuada do parasita, incluindo amostras de isolados de pacientes (86-89). Alguns desses estudos identificaram um grande número de junções adicionais de splicing (intron-exon), transcrições antisense e eventos de splicing alternativo não incluídos no genoma inicial do parasita. Estes estudos têm melhorado bastante a anotação do genoma e ajudado a compreender os mecanismos de regulação de genes e a diversidade de proteínas.

2 JUSTIFICATIVAS E OBJETIVOS

A malária é uma das principais parasitoses humanas e um dos grandes problemas de saúde mundial. Dos principais obstáculos enfrentados no controle e combate a doença, destacam-se o surgimento de parasitas cada vez mais resistentes a drogas comumente utilizadas e vetores aos inseticidas. Sendo assim, é de extrema importância focar no desenvolvimento de estratégias que objetivem tratamentos eficazes, como possíveis vacinas e novas drogas antimaláricas.

O conhecimento sobre a bioquímica, biologia e fisiologia dos parasitas abre novas alternativas no estudo contra a malária. Os conhecimentos adquiridos até o presente momento sobre aspectos gerais de *P. falciparum* estimulam a investigação de vias metabólicas, novos compostos antimaláricos, como também a especulação do papel desempenhado por produtos e enzimas dessas vias no ciclo do parasita.

A via MEP, onde vários produtos já foram caracterizados pelo nosso grupo em *P. falciparum*, assim como a via do Chiquimato, apresentam-se como alvos quimioterápicos por serem vias não compartilhadas com o hospedeiro humano. A identificação de produtos destas vias metabólicas do parasita, distintas do hospedeiro, abre caminho para novas frentes de desenvolvimento de antimaláricos, no entanto, é necessário identificar também, genes que codificam as enzimas e caracterizá-los, bem como determinar seu papel desempenhado durante o ciclo intraeritrocítico de *P. falciparum*, conhecendo melhor assim, mecanismos de sobrevivência e adaptação utilizados pelo parasita.

Baseados nesses argumentos foram propostos os seguintes objetivos iniciais:

- Determinar se o gene PF3D7_0607500 ou PF3D7_0202700 (OPP/PSY) codificam uma enzima com atividade compatível com MenA.
- Investigar a importância de ambas as enzimas bifuncionais: FPPS/GGPPS e OPP/PSY, no ciclo intraeritrocítico de *P. falciparum*, através de perfil de expressão, localização e regulação da expressão.

3 MATERIAIS E MÉTODOS

3.1 Digestão, purificação e clonagem dos fragmentos de DNA

Os vetores e insertos de DNA foram digeridos com as enzimas de restrição por 2 horas num volume de 20 a 50 µl na temperatura indicada pelo fabricante de cada enzima. Esses fragmentos de DNA foram separados através de eletroforese em gel de agarose de 0,8 a 1,5% preparado em tampão TAE 1X (40 mM Tris, 1 mM EDTA; 20 mM ácido acético), também usado para a eletroforese. Os fragmentos, com o tamanho esperado, foram cortados do gel, purificados com o kit “Quick Gel Extraction Kit” (Invitrogen, São Paulo, SP, Brasil) e ressuspensos em 20 µl de água.

As reações de ligação foram realizadas durante 1 hora, à temperatura ambiente, e com quantidades e proporções variáveis de vetor e inserto. A enzima T4 DNA ligase (Invitrogen) foi utilizada com o tampão fornecido pelo fabricante na concentração 1X, no volume final de 5 a 10 µl. De 1 a 5 µl do produto da reação acima foram utilizados para transformar bactérias *E. coli* da cepa DH10B eletro ou químico-competentes.

A sequência de DNA que codifica a região C-terminal de FPPS/GGPPS (nucleotídeos 706-1129 da região codificante (CDS) do gene PF3D7_1128400) foi amplificada, a partir de DNA genômico de *P. falciparum* da cepa 3D7, com os oligonucleotídeos: F-FPPS/GGPPS-BglII e R-FPPS/GGPPS-PstI (**Tabela 1**), inicialmente clonada em um vetor de clonagem (pGEM-T easy) e posteriormente digerido com as enzimas BglII/PstI. O gene FPPS/GGPPS foi clonado no vetor pTEX150-HA/Stre3 (90), que contém o epítipo da proteína hemaglutinina (HA), digerido com as mesmas enzimas. Obtivemos ao final o vetor de integração pFPPS/GGPPS-HA (34) (**Anexo 3**).

A sequência de DNA correspondente ao epítipo HA repetido 3 vezes (HA) em fusão com o domínio de desestabilização mutante 24 (DD24) foi amplificada por PCR a partir de DNA do plasmídeo pEF-Luc-GFP-HA-DD24 (56) (**Anexo 4**), digerida com *PstI/NcoI* e clonada em pTEX150-HA-Str (90) gerando pTEX150-HA-DD24 (Azevedo et al., dados não publicados). O vetor pTEX150-HA-DD24 foi digerido com *PstI/NcoI* para a obtenção do fragmento HA-DD24, que foi re-ligado ao plasmídeo pFPPS/GGPPS-HA (34) (**Anexo 3**) digerido com as mesmas enzimas gerando o vetor pFPPS/GGPPS-HA-DD24.

A sequência correspondente à proteína fluorescente verde - GFP (*green fluorescent protein*) em fusão com HA e com DD24 foi retirada de pEF-Luc-GFP-HA-DD24 (56) (**Anexo 4**) pela digestão com as enzimas *PstI/SpeI*, e clonado em pTEX150-HA-DD24 digerido com *PstI/SpeI* gerando pTEX150-GFP-HA-DD24 (Azevedo et al., dados não publicados). Este plasmídeo foi digerido com *NheI/SpeI* e religado, removendo DD24 e gerando pTEX150-GFP-HA. A sequência alvo para integração no locus endógeno do gene que codifica

FPPS/GGPPS foi retirada de pFPPS/GGPPS-HA (34) digerido com *Bgl*III/*Pst*I e clonada em pTEX150-GFP-HA, gerando o vetor de integração pFPPS/GGPPS-GFP-HA (**Anexo 1**).

A sequência de DNA que codifica a enzima OPP/PSY de *P. falciparum*, foi amplificada a partir de cDNA do parasita com os oligonucleotídeos F-PSY-Xho e R-PSY-Mlu (**Tabela 1**), clonados em vetor de clonagem (pGEM-T easy) e posteriormente clonados no vetor pRM2-GFP-HA (**Anexo 2**), gerando o vetor de expressão pRM2-PSY-HA (49) (**Anexo 2**).

O mapa de restrição dos plasmídeos foi analisado por extenso, o que consistiu na digestão com pelo menos três combinações de enzimas de restrição e a autenticidade foi confirmada por sequenciamento de DNA. Foram realizadas maxi-preparações de cada plasmídeo de forma a se obter a quantidade necessária de DNA para transfectar os parasitas (100 µg).

3.2 Cultura de *Plasmodium falciparum*

A cepa 3D7 bem como os transfectantes de *P. falciparum* foram cultivados de acordo com o método de Trager e Jensen (91). Os parasitas foram cultivados em garrafas de cultivo em meio RPMI-1640 suplementado com 25 mM de Hepes (ácido N-(2-hidroxietil) piperazina-N'-2-etanossulfônico), 21 mM de bicarbonato de sódio, 300 mM de hipoxantina, 11 mM de glicose, 40 g/ml de gentamicina e 0,5% (v/v) de Albu-max I. Eritrócitos foram adicionados à cultura obtendo um hematócrito de 5%. As garrafas foram mantidas em estufa a 37°C com trocas diárias de meio e injeção de uma mistura gasosa composta por 5,05% CO₂, 4,93% O₂ e 90,2% N₂. O controle da parasitemia foi realizado com a verificação microscópica diária de esfregaços corados com Giemsa.

3.3 Sincronização dos estágios intraeritrocitários

As culturas com mais de 10% de parasitemia no estágio anel jovem foram centrifugados, retirado o sobrenadante, adicionada a solução Sorbitol na proporção 1:25 (v/v, precipitado: solução Sorbitol 5% a 37 °C). Após incubar a 37 °C por 5 minutos, os parasitas foram centrifugados a 800 x g por 10 minutos. O precipitado, que corresponde ao concentrado de parasitas no estágio anel, foi introduzido novamente à cultura (92).

3.4 Transfecção em *P. falciparum* e seleção dos parasitas

Foi utilizada a metodologia descrita por Wu e colaboradores (93) nas condições de eletroporação estabelecidas por Fidock e Wellens (94). O volume de 600 µl de hemácias

humanas não parasitadas foi previamente lavado em cytomix (120 mM KCl; 0,15 mM CaCl₂; 10 mM K₂HPO₄/KH₂PO₄ pH 7,6; 25 mM Hepes pH 7,6; 2 mM EDTA (ácido etilenodiamino tetra-acético); 3 mM MgCl₂) e depois misturado a 100 µg dos plasmídeos circulares purificados por colunas de maxi prep, ressuspensos no volume de 400µl do mesmo tampão. Essa mistura foi eletroporada usando-se cubetas de 0,2 cm (Biorad, São Paulo, SP, Brasil) nas condições de eletroporação 0,31 kv e 960 µf e os eritrócitos eletroporados foram adicionados a aproximadamente 10⁷ parasitas da linhagem 3D7.

A cultura de parasitas foi mantida por 2 dias quando a pressão seletiva foi iniciada com a droga WR99210 na concentração de 2,5 nM. Após o início da pressão seletiva, o meio de cultura foi trocado diariamente nos primeiros 5 dias e depois em dias alternados até a detecção dos parasitas.

3.5 Integração dos plasmídeos nos cromossomos dos parasitas

Os parasitas transfectados que sobreviveram à seleção por WR99210 e, portanto possuem os plasmídeos de interesse na forma episomal, foram submetidos no mínimo a três ciclos de retirada da droga WR99210. A droga WR é retirada do meio de cultura por 21 dias e, após esses 21 dias, é reintroduzida ao meio por cerca de 7 dias para seleção de eventos de integração por recombinação simples. Nesse intervalo de 7 dias de reintrodução da droga, a integração no locus desejado é detectada por PCR. Se a integração não for detectada, nesse intervalo, inicia-se outro ciclo com retirada da droga.

No caso das linhagens que precisam ser utilizadas para análises quantitativas e comparativas de fenótipo, como por exemplo expressão gênica, crescimento da população de parasitas em cultura, resistência a drogas, entre outros, assim que a integração no locus desejado é detectada, os parasitas são clonados por diluição limitante.

3.6 Detecção da proteína por Western blot

As hemácias parasitadas são lisadas com saponina 0,1%, a hemoglobina e as proteínas solúveis presentes no eritrócito e no vacúolo parasitóforo removidas através de lavagem com PBS e o extrato protéico são extraídos do pellet de parasitas com 50mM Tris-HCl pH 6,8, 10% Glicerol, 2mM EDTA, 2% SDS (dodecil sulfato de sódio), 144 mM 2- Mercaptoetanol e 0,008% Bromofenol Blue na presença de 2% de inibidores de protease (Protease Inhibitor Cocktails – Sigma-Aldrich, São Paulo, SP, Brasil) e 50mM de DTT (ditiotreitól), para a proteína OPP/PSY utilizamos o tampão 2D: 7M urea, 2M tiourea, 2% ASB-14 (amidosulfobetaina-14) (65). Posteriormente as proteínas são separadas através de gel de

poliacrilamida SDS-PAGE. O gel é então transferido para uma membrana de nitrocelulose usando um electroblotter semi-seco (Trans-Blot, Bio-Rad) (95) e posteriormente a membrana é bloqueada com 1% de caseína em PBS por 1 hora. Posteriormente as membranas são então incubadas com um anticorpo monoclonal α -HA (1:500 diluição; Sigma) ou com anticorpos controles como α -PTEX150 (1:1000) – proteína de transporte situada na membrana do vacúolo parasitóforo expressa constitutivamente durante o ciclo intraeritrocítico de *P. falciparum* (90) ou α -MSP2 (1:500) – glicoproteína ancorada a membrana plasmática do merozoíto, expressa somente durante a esquizogonia (96), por 1 hora a temperatura ambiente ou 14 horas a 4°C. Ao final as membranas são lavadas com PBS/tween20 (0,05% tween20 em PBS), incubadas com um anticorpo secundário anti-mouse IgG ou anti-rabbit IgG marcados com peroxidase diluído em PBS/1% caseína por 1 hora, lavadas como previamente descrito, incubadas por cerca de 2 min com kit de detecção de ECL (*enhanced Chemiluminescence detection*) e então utilizadas para expor filmes de raio-x ou o sinal detectado através de câmera CCD de alta sensibilidade (Biorad) (**Anexo 2 e 3**).

3.7 Clonagem dos parasitas por diluição limitante

Os parasitas são cultivados em placas de 96 poços na diluição de aproximadamente 1/3 de parasita por poço. Estes são mantidos em um hematócrito reduzido de 2% num volume final de 100 μ l. Durante os 10 dias iniciais, os parasitas são mantidos em meio sem a presença da droga WR, a fim de aumentar a chance de selecionar clones com o plasmídeo integrado. Plasmídeos na forma episomal costumam ser perdidos em uma população reduzida de parasitas. O meio de cultura é trocado a cada 4 dias e, após 15 dias iniciamos a detecção de parasitas através da observação da mudança de cor do meio de cultura nos poços positivos. A presença de parasitas é identificada por citometria de fluxo utilizando brometo de etídio como marcador e a confirmação é feita por esfregaço e coloração com Giemsa.

3.8 Extração de DNA genômico de *P. falciparum*

Uma alíquota de 200 μ l de hemácias infectadas com uma parasitemia de 5-10% de esquizontes da linhagem 3D7, pFFPS/GGPPS-HA, pFFPS/GGPPS-GFP-HA e pFFPS/GGPPS-HA-DD24 foram lisadas com saponina e lavadas em PBS. Os parasitas foram ressuspendidos em 300 μ l de solução de extração (10 mM Tris-HCl ; 1 mM EDTA pH 8,0; 100 mM NaCl; 0,5% SDS; 200 μ g/ μ l proteinase K) e incubados durante a noite ou 4 horas a 37°C. Foram então adicionados 200 μ l de fenol equilibrado em TE (Tris-EDTA), sendo o tubo mantido em agitação branda por 10 minutos e depois centrifugado por 10 minutos a 12.000 x

g. A fase aquosa foi transferida para outro tubo e a este foram adicionados 200 µl de solução fenol-clorofórmio (1:1, v/v). Nova agitação e centrifugação foram realizadas como anteriormente e a fase aquosa foi recuperada. Foram adicionados 200 µl de clorofórmio/álcool isoamílico (24:1, v/v) e o tubo foi agitado e centrifugado como anteriormente. A fase aquosa foi transferida para outro tubo e a este foram adicionados 20 µl de acetato de sódio 3 M pH 5,2 e 500 µl de etanol 100%. O tubo foi invertido 4 vezes e armazenado a -20 °C por pelo menos 2 horas, quando foi centrifugado a 12.000 x g por 10 minutos a 4 °C. O líquido foi removido e o DNA foi lavado com 500 µl de etanol 70% e centrifugado nas mesmas condições. O etanol 70% foi removido e o DNA foi seco a 37 °C e ressuspensionado em 50 µl de TE.

3.9 PCR (*Polymerase chain reaction*)

As reações de PCR foram realizadas com as instruções do manual da enzima Taq polimerase (Invitrogen). Para experimentos de detecção da integração no locus genômico, utilizamos o seguinte programa para os oligos 1 e 3 (F-FPPS/GGPPS-BamHI; R-HA-Nhe, **Tabela 1**): 94 °C por 5 minutos; 25 ciclos de 94 °C por 30 segundos; temperatura de anelamento de 44 °C por 30 segundos; 72 °C por 1 minuto e meio; seguido de uma extensão final a 72 °C por 10 minutos. Para os oligos 1 e 2 (F-FPPS/GGPPS-BamHI; R-FPPS/GGPPS-PstI, **Tabela 1**) utilizamos o seguinte programa: 95 °C durante 5 minutos; 35 ciclos de 95 °C durante 40 segundos; temperatura de anelamento de 59 °C durante 40 segundos; 72 °C durante 90 segundos; seguido por uma extensão final de 72 °C durante 10 minutos (**Anexo 3**).

A fim de realizarmos o sequenciamento de nova geração para o gene que codifica a enzima FPPS/GGPPS, utilizamos oligonucleotídeos (F-FPPS/GGPPS-BamHI; R-FPPS/GGPPS-PstI – **Tabela 1**) que amplificam o transcrito que codifica a enzima FPPS/GGPPS com o programa descrito acima (**Anexo 1**).

Para experimentos de confirmação de eventos de splicing alternativo, os oligonucleotídeos (F-iso5; R-iso5 - F-iso7; R-iso7 - F-iso10; R-iso10 - **Tabela 1**) que amplificam regiões para a detecção de um evento específico de splicing alternativo, utilizamos o seguinte programa: 95 °C durante 5 minutos; 35 ciclos de 95 °C por 40 segundos; temperatura de anelamento de 59 °C para F-iso5; R-iso5; 57 °C para F-iso7; R-iso7 e 59 °C para F-iso10; R-iso10 por 40 segundos; 72 °C durante 90 segundos; seguido por uma extensão final de 72 °C durante 10 minutos (**Anexo 1**).

3.10 Imunofluorescência

Para a análise de imunofluorescência seguimos o protocolo descrito por Furtado e colaboradores (97) com modificações. Eritrócitos infectados (5-10% de parasitemia, hematócrito de 10%) foram fixadas com 3,7% formaldeído/PBS (*Phosphate Buffer saline*) durante 3 horas, bloqueados e permeabilizados com 0,1% de BSA (albumina sérica bovina), 0,005% de PBS-saponina durante 25 min, duas vezes, incubado com o anticorpo primário α -HA (diluição 1:1000, Sigma) ou α -ACP (*acyl carrier protein* - diluição 1: 200) diluídos em 0,1% BSA, 0,001% de saponina/PBS durante 1 hora a 37 °C, e finalmente incubado com o anticorpo secundário Alexa Fluor (®) 488 conjugado também diluído em 0,1% BSA, 0,001% de saponina/PBS durante 45 min a 37 °C. Depois da lavagem em PBS, o material foi seco ao ar sobre lâminas de Imunofluorescência, preparados com o Vectashield e, em seguida, analisadas por microscopia de fluorescência (Zeiss, LSM710) (**Anexo 1**).

Para a análise com parasitas vivos que expressam GFP, utilizamos Mitotracker, (Molecular Probes, São Paulo, SP, Brasil - diluição 1: 10000) durante 30 min a 37 ° C e posteriormente visualizados por microscopia de fluorescência (Zeiss LSM710) ou microscopia confocal (LSM 780 NLO-) (**Anexo 1**).

3.11 Extração do RNA e síntese da dupla fita (cDNA) dos estágios intraeritrocíticos de *P. falciparum*.

Os RNAs totais dos estágios intraeritrocíticos de *P. falciparum* (Anel, Trofozoíta jovem – 24 horas, Trofozoíta maduro – 35 horas, Esquizonte) como também dos isolados S20 e Cand (**Anexo 1**) e as linhagens pRM2-PSY-HA e pPSY/OPP-HA (**Anexo 2**) foram extraídos utilizando Trizol (Invitrogen), precipitados com isopropanol e posteriormente lavados com etanol 70%, ressuspensos em água e estocados a -80 °C. As concentrações de RNA foram estimadas a partir da medida de absorbância a 260 nm, utilizando o espectrofotômetro *Nanodrop* 1000 (Thermo Scientific, São Paulo, SP, Brasil). Uma unidade de absorbância a 260 nm corresponde aproximadamente a 40 µg/ml de RNA. Os cDNAs foram sintetizados a partir de 4 µg de RNA total, os quais foram tratados 3 vezes com DNase (Fermentas, São Paulo, SP, Brasil) e, posteriormente submetidos a transcrição reversa utilizando a enzima *MuLV-Reverse transcriptase* (Fermentas) (98).

3.12 RT-PCR (*Reverse transcription polymerase chain reaction*)

Foram desenhados pares de oligonucleotídeos para confirmação de um evento de splicing alternativo onde ocorre deleção específica de um exon (controle – F-iso; R-iso e F-

iso7; R-iso7 - **Tabela 1**) (**Anexo 1**), como também oligos que amplificam o transcrito que codifica a enzima OPP/PSY (F-PSY; R-PSY - **Tabela 1**) para detecção e quantificação de super expressão da enzima (**Anexo 2**). Todos os oligonucleotídeos que foram utilizados amplificaram com o mesmo desempenho em relação ao gene de controle interno (± 1 Ct), *seril-tRNA sintetase* (PF07_0073 (29)), após realização de curva padrão. Os ensaios de PCR quantitativo foram realizados utilizando o *mix SYBR Green* (Thermo Scientific, São Paulo, SP, Brasil) na máquina de PCR modelo StepOne™ Real-Time PCR (Applied Biosystems, São Paulo, SP, Brasil). Todas as reações foram realizadas em triplicatas (valores de *Ct* - *cycle threshold*) permitindo não mais do que 0,5 unidades de desvio entre os valores individuais de *Ct*. As amostras com *Cts* mais de 35 foram descartadas. Para todas as reações foram analisadas as curvas de dissociação dos oligonucleotídeos, onde cada par de oligonucleotídeos apresenta uma temperatura específica, caracterizando a especificidade dos produtos obtidos. O ΔCt para cada par de oligonucleotídeos foi determinado individualmente subtraindo-se o valor de *Ct* medido para o gene alvo menos o valor de *Ct* do controle *seril-tRNA sintetase*. A expressão relativa de mRNA foi então obtida pela formula $2^{-\Delta Ct}$ (99).

3.13 Sequenciamento de nova geração

Transcritos amplificados de FPPS/GGPPS foram sequenciados usando a plataforma GS FLX 454 (realizado por Macrogen Inc., Seoul, Coréia do Sul). Para cada um dos quatro estágios do ciclo intraeritrocítico do parasita, uma biblioteca foi construída utilizando-se: 25 ng / μ l de parasitas em estágio anel, 75 ng / μ L de trofozoitas jovens – 24 horas, 45 ng / μ l de trofozoíto maduro – 35 horas e 65 ng / μ l de esquizontes de produtos amplificados com a expectativa de geração de cerca de 50.000 leituras por ponto (**Anexo 1**).

3.14 Análises dos dados do RNA-seq

As análises do sequenciamento de nova geração foram realizadas em colaboração com o Prof. Dr. João Marcelo Pereira Alves e os detalhes se encontram no **Anexo 1**.

3.15 Preparação das proteínas para espectrometria de massa

Parasitas de uma cultura assíncrona de *P. falciparum* foram recuperados e tratados com 0,15% de saponina em meio RPMI para lise das hemácias. As proteínas foram extraídas com tampão: 0,05 M de Tris-HCl pH 6,8, 10% glicerol, 2mM EDTA, 2% SDS, 0,05% de azul de bromofenol e 50 mM de ditiotreitol (65) e posterior separação por SDS-PAGE. Foram cortadas bandas em intervalos de 1kDa na região de 30 a 50 kDa de peso molecular.

3.16 Espectrometria de massa

Os experimentos e análises das proteínas por espectrometria de massa foram realizados em colaboração com o Dr. Giuseppe Palmisano e os detalhes se encontram no **Anexo 1**

3.17 Clonagem de PF3D7_0607500 e PF3D7_0202700 (OPP/PSY)

Amplificamos o fragmento do gene PF3D7_0202700 a partir do DNA genômico de *P. falciparum* com os oligonucleotídeos: F-Fito-Xho e R-PFB0130w-Mlu (**Tabela 1**), clonamos em um vetor de clonagem, pGEM-T easy e posteriormente subclonamos em um vetor de expressão, pRSETC (cauda de poli-histidina - his-tag). Quanto ao gene PF3D7_0607500, obtivemos um gene sintético (GenScript USA Inc., Piscataway, USA) em um vetor de clonagem para posteriormente subclonarmos também no vetor de expressão pRSETC. Os clones bem como o vetor pRSETC foram digeridos com as enzimas PstI/NcoI. As reações de ligação foram realizadas durante 1 hora, à temperatura ambiente, e com quantidades e proporções variáveis de vetor e inserto. A enzima T4 DNA ligase (Invitrogen) foi utilizada com o tampão fornecido pelo fabricante na concentração 1X, num volume final de 5 a 10 µl. De 1 a 5 µl do produto da reação acima foram utilizados para transformar bactérias *E. coli* da cepa DH10B eletro ou químico-competentes. Os clones foram analisados por PCR e/ou restrição e tiveram sua autenticidade confirmada por sequenciamento de DNA.

3.18 Expressão das proteínas codificadas por PF3D7_0607500 e PF3D7_0202700 (OPP/PSY)

Após a obtenção dos clones, purificamos as proteínas através de uma coluna de níquel sepharose por cromatografia de afinidade através da afinidade da cauda de poli-histidina, fusionada na proteína, por níquel. Os clones em vetor pRSETC foram utilizados para transformar bactérias *E. coli* da cepa BL-21Hil e, uma colônia foi lançada em pré-inóculo à 37 °C “overnight” e posteriormente diluída em um inóculo no volume final de 100-200 ml cultivadas até atingir a fase log (OD₆₀₀ de 0,6-1,0). A indução ocorreu “overnight” com 300 mM de IPTG (isopropil-β-D-1-tiogalactopiranosídeo). Após centrifugação a 3000 x g por 30 min a 4 °C o pellet foi ressuspenso em 10ml de PBS/Triton 100X e incubado com lisozima a - 20 °C por 3 horas. Após a lise bacteriana através de um disruptor de células coletamos o sobrenadante por centrifugação a 3000 x g a 4 °C por 15 minutos e assim purificamos a proteína através da coluna de níquel sepharose por 1 hora e eluímos a mesma com 500 mM de

imidazol em 500 mM de NaCl em PBS. Ao separarmos as proteínas através de gel de poliacrilamida SDS-PAGE obtivemos nossa proteína recombinante com o tamanho esperado de 63kDa. Para OPP/PSY, ao final confirmamos as atividades de OPPS e PSY através de metodologias já padronizadas em nosso laboratório.

3.19 Marcação metabólica e extração de DMQ-4 e Carotenóides

Culturas de *P. falciparum* com pelo menos 20% de parasitemia e sincronizadas no estágio trofozoíta jovem foram marcadas com [$1\text{-}^3\text{H}$] GGPP (0.75 $\mu\text{Ci/ml}$ - GE Healthcare, São Paulo, SP, Brasil - atividade específica 15 Ci/mmol) ou [$1\text{-(n)}\text{-}^3\text{H}$] FPP (0.75 $\mu\text{Ci/ml}$ - GE Healthcare, São Paulo, SP, Brasil - atividade específica 23 Ci/mmol) durante 16 horas. Subsequentemente, hemácias infectadas com parasitas na fase esquizonte foram concentradas com colunas magnéticas.

O padrão de DMQ-4 (Demetilmenaquinona – 4) foi sintetizado em colaboração com Dr. Guillermo Labadie da Faculdade de Bioquímica e Ciências Farmacêuticas da Universidade Nacional de Rosário. Parasitas foram homogeneizados em uma solução de 3 ml gelado de 0,2 M HClO_4 em metanol que proporciona o rompimento das hemácias, em seguida foi adicionado éter de petróleo, misturado em um *vórtex* e então centrifugado (2000 x g, 10 min) (100). O sobrenadante foi seco em pressão de nitrogênio e posteriormente analisado por sistema de HPLC (*High-performance liquid chromatography*) previamente padronizado.

Para extração de carotenoides usamos quatro vezes o volume do pellet de acetona gelada e posterior centrifugação a 8000 x g durante 5 min. O sobrenadante foi seco em pressão de nitrogênio e posteriormente analisado por sistema de HPLC previamente padronizado.

3.20 RP-HPLC (*Reverse phase- High-performance liquid chromatography*)

Para análise de DMQ-4 utilizamos uma coluna de Phenomenex Luna C18 (250 mm x 4,6 milímetros x 5 mm) (Phenomenex, CA, EUA), acoplada com uma pré-coluna C18 (Phenomenex, CA, EUA), um detector Gilson UV 152 / UV-VIS ou um *Diode Array detector* (DAD) Gilson 170 e um coletor de frações FC203B. O software utilizado para o processamento de dados foi o Sistema de Software 3.0 UniPoint LC TM. O sistema consistiu de um gradiente linear, com a fase móvel sendo o solvente A metanol 70% e solvente B etanol 100% começando com 60/40% (A/B) respectivamente e chegando em 40 minutos a 5/95% (A/B). As condições iniciais foram retornadas em 45 minutos em um fluxo de 0.8 ml/min. O comprimento de onda monitorado foi de 225 nm. A fase móvel foi filtrada em uma

membrana de PTFE (solvente A) e NYLON (solvente B) de 0,20 μm . As frações radioativas foram secas por evaporação em uma capela a temperatura ambiente e ressuspensas em líquido de cintilação. A quantificação da radioatividade (c.p.m – contagem por minuto) foi realizada no aparelho *Beckman 5000 β -radiation scintillation counter* (Beckman, CA, USA).

Para análise de carotenos o extrato de acetona foi ressuspensão em 20 μl de metil tert-butil éter (MTBE) e analisado em uma coluna YMC C30 polimérica (4.6 $\times$ 250 mm, 3 μm and/or 5 μm) (YMC Inc.) em um sistema de gradiente (101) (**Anexo 2**).

3.21 Extração de Lipídeos e TLC (*Thin-layer chromatography*)

As cultura de *E. coli* foram cultivadas até atingir a fase log (OD_{600} de 0,6-0,8). As células foram centrifugadas transferidas para tubos de vidro. Foram adicionados 6 ml de clorofórmio/metanol (2:1, v/v) e a mistura foi agitada suavemente durante 2 h. A solução bifásica resultante foi centrifugada e a camada aquosa superior foi removida. A fase orgânica, inferior, foi lavada com água: metanol: clorofórmio (48:47:3, v/v/v) e transferidas para tubos novos. O solvente foi evaporado e a amostra foi ressuspensa em clorofórmio. Lipídeos polares foram removidos da amostra por cromatografia em coluna de ácido silícico utilizando clorofórmio para eluição (55). A amostra foi ressuspensa em hexano:éter dietílico (95:5 v/v) e aplicado a placas de TLC de sílica gel, que foram desenvolvidas em hexano:éter dietílico (95:5 v/v) ou ressuspensas em etanol e posteriormente enviadas para análises em LC-MS (*liquid chromatography–mass spectrometry*) na Universidade do Estado do Colorado, HPLC Agilent Technologies 1200 HPLC acoplado a um Agilent Technologies 6210 TOF-MS (*time of flight – mass spectrometer*) (55).

3.22 Preparação de membrana

As amostras foram cultivadas até atingir a fase log (OD_{600} de 0,6-0,8). As células foram centrifugadas e ressuspensas em tampão de homogeneização contendo fosfato de potássio 50 mM (pH 7,2), 10% de glicerol, 5 mM de MgCl_2 e 5 mM DTT e rompidas por sonicação com sonda em gelo com um Sanyo Soniprep 150 (10 ciclos de 60s e 90s off). O resultante foi centrifugado a 27 000 $\times g$ durante 20 min. O sedimento foi descartado e o sobrenadante foi centrifugado a 100 000 $\times g$ durante 2 h num rotor Beckman Ti 70.1. O pellet (membranas) foi ressuspensão no tampão de homogeneização, dividido em alíquotas e congeladas a -70 $^{\circ}\text{C}$ (55). A concentração da proteína foi estimada utilizando um kit de ensaio de proteínas BCA (Pierce).

3.23 Atividade enzimática para MenA

Protocolo 1: As misturas da reação continham 500 mM DHNA (1,4-dihidroxi-2-ácido naftóico) ou MQ-1 ou MQ-3, 10 mM [^3H] farnesil difosfato ou geranilgeranil difosfato (atividade específica, 44 $\mu\text{Ci}/\text{mmol}$ - American Radiolabelled Chemicals, St. Louis, USA), 5 mM MgCl_2 , 0.1% CHAPS em 100 mM Tris-HCl (pH 8.0) e 50-100 mg de proteína membrana. Reações foram paradas pela adição de 0,1 M de ácido acético em metanol. A mistura resultante foi extraída duas vezes com hexano, o sobrenadante foi evaporado em N_2 líquido e a amostra foi ressuspensa em hexano/éter (95:5, v/v) sendo então os produtos da reação separados através de uma coluna de ácido silícico eluídos com hexano/éter (95:5, v/v), posteriormente secos em uma pressão de N_2 ressuspensos em líquido de cintilação (55). A abundância relativa foi em seguida, utilizada para calcular a parte da radioatividade total, determinada por contagem de cintilação em meio líquido, em que a amostra poderia ser atribuída a menaquinona recentemente sintetizado.

Protocolo 2: As misturas da reação continham 500 mM DHNA (1,4-dihidroxi-2-ácido naftóico), [$1\text{-}^3\text{H}$] GGPP (0.75 $\mu\text{Ci}/\text{ml}$ - GE Healthcare), 5 mM MgCl_2 , 0.1% CHAPs em 100 mM Tris-HCl (pH 8.0) e 50-100 μg de proteína recombinante. Reações foram paradas pela adição de 0,1 M de ácido acético em metanol (55). A mistura resultante foi extraída duas vezes com éter de petróleo, o sobrenadante foi evaporado e a amostra foi ressuspensa em metanol 70% e etanol 100% (60:40 v/v) sendo então o produto da reação separado por HPLC para identificação de DMQ-4.

3.24 Atividade enzimática para FPPS

As misturas da reação continham 50 μM GPP ou FPP ou GGPP, 30 μM [^{14}C] isopentenil difosfato (American Radiolabelled Chemicals), 50 mM Mops (pH 7.9), 10 mM Na_3VO_4 , 5 mM MgCl_2 , 2,5 mM DTT, 0,2% Triton X -100 e 50-100 mg de proteína membrana. Reações foram paradas pela adição de 1ml de água saturada em NaCl. A mistura resultante foi extraída duas vezes com butanol saturado em água, o supernadante foi evaporado em N_2 líquido ressuspensos em líquido de cintilação ou em tampão para desfosforilação enzimática. A abundância relativa foi utilizada para calcular a parte da radioatividade total, determinada por contagem de cintilação em meio líquido, em que a amostra poderia ser atribuída ao produto recentemente sintetizado (102).

3.25 Desfosforilação enzimática

Após a extração, o butanol foi removido sob uma corrente de N₂, e os produtos radiomarcados foram dissolvidos em 5 ml de tampão contendo 100 mM acetato de sódio (pH 4,8), 0,1% de Triton X-100, e 60% de metanol. Depois de uma breve sonicação (2min), 3mg da enzima fosfatase ácida foi adicionado à mistura e incubada a 25 °C durante a noite. Produtos desfosforilados foram extraídos três vezes com 1 ml de n-hexano e posteriormente lavados com 1 ml de água. O solvente foi evaporado sob N₂. As amostras foram dissolvidas em 200 µl de clorofórmio-metanol (2:1, v/v), e aplicadas a placas de TLC de sílica gel, que foram desenvolvidas em metanol/acetona (8:2 v/v) (102).

Tabela 1- Oligonucleotídeos utilizados.

Nome	Sequência (5' - 3')	T.A.
F-FPPS/GGPPS-BglII	AGATCTGGTATGCAAATGGGGGGTATA	59 °C
R-FPPS/GGPPS-PstI	CTGCAGCAGCGCCTGTAAACAAAATGTC	59 °C
F-FPPS/GGPPS-BamHI	CCGGATCCATGGAGAACGAGCAGAATAAC	57 °C
F-iso5	GTAGCAGATGATATTATGGATAAGG	55 °C
R-iso5	AAAGTGGCTTCTCTAAAGGATG	57 °C
F-iso7	CATCCTTTAGAGAAGCCACTTTAA	57 °C
R-iso7	AATCATCATGGACCACAAACAATA	58 °C
F-iso10	CACAACCTGAAAAAGAGGACAT	58 °C
R-iso10	CAAGACATATTCTATACTCTAATATTTTCATC	56 °C
F-isso	TTCATTCTTTTTACCTATTGTTTG	53 °C
R-isso	CCAGGGCCACGTTAATTT	57 °C
R-HA-Nhe	GCTAGCAGCGGCATAATCTGG	48 °C
F-PSY-Xho	CTCGAGATGGTTCACCTAAGTAAAAGAAATAATATT	57 °C
R-PSY-Mlu	ACGCGTTTTGACGTTTCTTGATAACACGTTTAAG	60 °C
F-PSY	TGGTACGGGTTACCAAAAAT	59 °C
R-PSY	CATTTTGAGTGCTTCTTCAACA	56 °C
F-Fito-Xho	CTCGAGATGGTTCACCTAAGTAAAAGAAATAATATT	57 °C
R-PFB0130w-Mlu	ACGCGTTTTGACGTTTCTTGATAACACGTTTAAG	60 °C

4 RESULTADOS E DISCUSSÃO

4.1 Geração das linhagens transgênicas com os plasmídeos integrados

Transfectamos os parasitas de *P. falciparum*, da cepa 3D7, com plasmídeos vetores pFPPS/GPPS-HA, pFPPS/GPPS-GFP-HA e pFPPS/GPPS-HA-DD24. Este domínio (DD) possui um sítio para um ligante (Shld-1) e é estruturado para ser instável na sua ausência, portanto, a proteína de fusão de interesse é degradada. Esta abordagem se torna interessante uma vez que a expressão da proteína de interesse pode ser dose-dependente do ligante e reversível. Este processo é revertido com a adição do composto Shld-1 que estabiliza a molécula, permitindo que dessa forma se regule o nível de expressão da proteína de interesse. Este sistema já foi estabelecido para diferentes tipos de organismo (103, 104), incluindo *P. falciparum* (70, 72, 73, 105). Assim que os parasitas são detectados após a transfecção, garantindo o sucesso desta, todo o processo de integração dos plasmídeos no genoma do parasita é realizado na presença de Shld-1, para garantir que a proteína seja expressa uma vez que ocorra a integração.

Obtivemos sucesso na transfecção com os três plasmídeos, obtendo assim três linhagens transfectadas na forma episomal.

Em nossos plasmídeos, o gene que codifica a proteína de fusão, nesse caso a FPPS/GGPPS, não tem promotor, impedindo assim que a proteína seja expressa antes da integração. Uma vez integrado no genoma somente a proteína de fusão é expressa em níveis similares aos da endógena, (106) cujo gene se torna truncado, sem promotor e, portanto, não é expresso.

Os parasitas transfectados que sobreviveram à seleção por WR99210 e, portanto, possuem os plasmídeos de interesse na forma episomal, foram submetidos a ciclos de retirada da droga e reintrodução da mesma para seleção de eventos de integração. Nos intervalos dos ciclos, a integração no locus desejado foi detectada por PCR e a expressão da proteína em questão fusionada com os “tags” de interesse foi detectada por Western blot.

Através de Western blot com anticorpo α -HA observamos a expressão da proteína correspondente a FPPS/GGPPS com tamanho esperado de 44 kDa fusionada a HA (~47kDa) na linhagem agora então nomeada de FPPS/GGPPS-HA, no final do segundo ciclo, mostrando assim que, possivelmente ocorreu a integração no locus desejado, pois os parasitas estão expressando a proteína de interesse (FPPS/GGPPS) fusionada com o tag HA como esperado (**Figura 6**).

Figura 6- Detecção da proteína FPPS/GGPPS por Western blot na linhagem FPPS/GGPPS-HA. As proteínas são extraídas e separadas por SDS-PAGE; o gel é transferido para uma membrana que é bloqueada e incubada com o anticorpo de interesse. A) Detecção da proteína de interesse na linhagem pFPPS/GGPPS-HA. B) Controle demonstrando a especificidade do anticorpo HA; linhagem 3D7 incubada com anticorpo α-HA e α-pTEX150. α-HA (anticorpo contra o epítipo da proteína hemaglutinina (HA) presente no plasmídio em fusão com a proteína de interesse –FPPS/GGPPS), α-pTEX150 (anticorpo contra pTEX150, proteína expressa constitutivamente durante o ciclo intraeritrocítico do parasita (90)).

Após 5 ciclos de retirada e reintrodução da droga WR, conseguimos detectar, através de Western blot com anticorpo α-HA, a expressão da proteína correspondente a FPPS/GGPPS fusionada a HA-DD24 (~48kDa) na linhagem agora então nomeada de FPPS/GGPPS-HA-DD24 (**Figura 7**).

Figura 7- Detecção da proteína FPPS/GGPPS por Western blot na linhagem FPPS/GGPPS-HA-DD24. As proteínas são extraídas e separadas por SDS-PAGE; o gel é transferido para uma membrana que é bloqueada e incubada com o anticorpo de interesse; α-HA (anticorpo contra o epítipo da proteína hemaglutinina (HA) presente no plasmídio em fusão com a proteína de interesse –FPPS/GGPPS); FPPS/GGPPS-HA (linhagem integrada utilizada como controle positivo; 3D7 (linhagem selvagem demonstrando a especificidade do anticorpo).

4.2 Detecção da integração por PCR

Para a determinação ou confirmação por PCR do locus de integração, foram desenhados pares de oligonucleotídeos que fazem pareamento ao plasmídeo e ao DNA genômico (1-F-FPPS/GGPPS-BamHI 2- R-FPPS/GGPPS-PstI e 3- R-HA-Nhe) como

mostrado na **Figura 8A**, produzindo um produto de amplificação somente se a integração ocorrer através de um evento de simples recombinação no determinado locus. Assim, o DNA genômico das linhagens FPPS/GGPPS-HA, FPPS/GGPPS-GFP-HA, FPPS/GGPPS-HA-DD24 e 3D7 foram extraídos e as reações de PCR foram realizadas como descrito em materiais e métodos, utilizando-se os pares de oligos 1-3 e 1-2 respectivamente.

Assim, confirmamos a integração de pFPPS/GGPPS-HA no genoma de *P. falciparum* (**Figura 8B**) amplificando o fragmento de DNA com o tamanho esperado de FPPS/GGPPS (2.1kb) fusionada ao tag HA a partir do DNA genômico de *P. falciparum*. Observamos bandas sugerindo a integração de pFPPS/GGPPS-GFP-HA (2.6kb) e uma banda fraca de aproximadamente 2.2kb sugerindo a integração de pFPPS/GGPPS-HA-DD24 a partir do DNA genômico de *P. falciparum* (**Figura 8B**) e, como controle, amplificamos o fragmento de DNA que corresponde ao gene de FPPS/GGPPS endógeno da linhagem 3D7 e da linhagem FPPS/GGPPS-HA (**Figura 8C**).

Figura 8- Esquema de integração de pFPPS/GGPPS-HA, pFPPS/GGPPS-HA-DD24 e pFPPS/GGPPS-GFP-HA no locus genômico. A) esquema ilustrando o evento de integração por recombinação simples e os primers desenhados para a detecção desse evento; os números 1 e 3 indicam a região onde os primers foram desenhados para detectar a integração do gene no locus. **B)** PCR detectando a integração de pFPPS/GGPPS-HA

e a possível integração de pFPPS/GGPPS-HA-DD24 e pFPPS/GGPPS-GFP-HA no locus genômico de *P. falciparum* utilizando-se os primers 1 e 3. C) PCR controle demonstrando a especificidade do anticorpo detectando a amplificação do gene de FPPS/GGPPS endógeno nas linhagens 3D7 e FPPS/GGPPS-HA utilizando-se os primers 1 e 2.

4.3 Perfil de expressão de FPPS/GGPPS

A linhagem FPPS/GGPPS-HA foi utilizada para determinar o perfil de expressão de FPPS/GGPPS no ciclo intraeritrocítico de *P. falciparum*. Análise por Western blot, utilizando amostras de proteína extraídas de parasitas sincronizados nos 3 principais estágios (anel, trofozoíta e esquizonte) e, reveladas com o anticorpo anti HA, demonstraram que a enzima FPPS/GGPPS é expressa constitutivamente, em todos os estágios, durante o ciclo intraeritrocítico de *P. falciparum* (**Figura 9**) (34). Como controles foram utilizados anticorpos que reconhecem pTEX150 (90), expressa de forma constitutiva e MSP2 (107), expressa somente em esquizontes.

FPP e GGPP são substratos para as proteínas preniltransferases (farnesil transferase e geranilgeranil transferase), catalizando modificações pós-traducional de proteínas (108). Estudos anteriores têm demonstrado que a modificação pós-traducional das proteínas ocorre em todas as fases do ciclo intraeritrocítico de *P. falciparum*, o que sugere que essa enzima seja mesmo ativa também em todas as fases do ciclo (28, 108).

Figura 9- Expressão de FPPS/GGPPS-HA durante o ciclo intraeritrocítico de *P. falciparum*. (A) anel, (T) trofozoíta, (E) esquizonte; α-HA (anticorpo contra o epítipo da proteína hemaglutinina (HA) presente no plasmídeo em fusão com a proteína de interesse – FPPS/GGPPS), controles: α-pTEX-150 (anticorpo contra pTEX150, proteína expressa constitutivamente durante o ciclo intraeritrocítico do parasita (90)), α-MSP2 (anticorpo contra MSP2, proteína expressa somente no estágio de esquizonte (96)). **Anexo 3**

4.4 Clonagem por diluição limitante de pFPPS/GGPPS-HA-DD24

Detectamos a expressão da proteína em fusão com os tags por western blot através de um anticorpo monoclonal α-HA (**Figura 7**), no final do quinto ciclo. Sendo assim, iniciamos

a clonagem dos parasitas por diluição limitante em placas de 96 poços (detalhado em materiais e métodos), com o intuito de obtermos 100% de parasitas com a proteína FPPS/GGPPS fusionada a HA-DD24 integrada no genoma.

Essa clonagem é essencial para realizarmos os experimentos reduzindo a concentração do ligante Shld-1 a fim de obtermos nosso objetivo de controlar a expressão dessa enzima.

Foram feitas cerca de 10 placas ao longo de 6 meses de trabalho a fim de obtermos os parasitas da linhagem de interesse clonados por diluição limitante. Os parasitas que foram detectados por citometria de fluxo e confirmados por esfregaço foram ampliados em cultivo para a extração da proteína e do DNA genômico, para assim, realizarmos as análises de integração por western blot e PCR respectivamente como descrito anteriormente, porém não obtivemos nenhum resultado positivo.

Durante sua permanência em cultivo por um longo período, a linhagem FPPS/GGPPS-HA-DD24 poderia ter sofrido algum problema como: contaminação com outras linhagens ou deficiência do ligante Shld-1 no meio de cultura trocado diariamente, ocasionando assim a perda da linhagem, pois, este domínio (DD) possui um sítio para um ligante (Shld-1) e é estruturado para ser instável na ausência do ligante, ou mesmo o uso de concentrações altas do ligante que podem ser tóxicas para o parasita, interferindo em seu ciclo celular e em sua proliferação (56). A fim de certificarmos que não ocorrera nenhum problema com nossa linhagem ao longo desses meses de trabalho, extraímos a proteína e o DNA genômico para, como controle, novamente identificarmos a proteína de interesse e o gene fusionado a HA-DD24 integrado no genoma.

Porém, não conseguimos mais essa confirmação da integração do plasmídeo no genoma do parasita por PCR ou mesmo a expressão da proteína por western blot. Descongelamos uma alíquota dessa linhagem, ainda no 2º ciclo, e recomeçamos outros ciclos com introdução e retirada da droga WR a fim de obtermos novamente a linhagem integrada. Em paralelo, re-transfectamos parasitas da linhagem 3D7 com o plasmídeo pFPPS/GGPPS-HA-DD24 adicionando o ligante Shld-1 no meio cultura na concentração inicial de 0,5µM no momento em que é adicionado a droga WR, após 48 horas de transfecção, a fim de obtermos uma maior expressão da proteína quando ocorre a integração por recombinação simples.

Depois de três transfecções independentes, parasitas com o plasmídeo integrado não foram recuperados, sugerindo que a FPPS/GGPPS não pode manter a sua função fisiológica quando em fusão com um tag de 15 kDa ou seus níveis de expressão foram demasiado baixos, mesmo na presença do ligante Shld-1. Nós mostramos anteriormente (**Anexo 4**) (56) que DD24 diminui a quantidade de proteínas fusionadas de forma significativa, o que não é

totalmente revertido pela presença de Shld-1.

4.5 Localização de FPPS/GGPPS

A fim de determinar a localização de FPPS/GGPPS em parasitas vivos, uma outra linhagem transgênica foi gerada onde FPPS/GGPPS é expressa em fusão com GFP-HA, como descrito anteriormente. A análise por microscopia de fluorescência de parasitas vivos confirma a expressão ao longo do ciclo intraeritrocítico e mostra a localização de FPPS/GGPPS ao longo do citoplasma e também formando pontos, que aumentam em número em parasitas maduros a partir da fase trofozoítas para esquizonte (**Figura 10A**). Para investigar a que compartimento subcelular os pontos detectados correspondem, parasitas marcados com o marcador de mitocôndria, MitoTracker, foram analisados de forma semelhante (**Figura 10B**, painel superior). Um padrão distinto é detectado, sugerindo que FPPS/GGPPS não colocaliza com essa organela. Parasitas fixados foram analisados por imunofluorescência utilizando anticorpo contra o tag HA e contra a proteína acil-carregadora (ACP), marcadora de apicoplasto (109), mostrando que a enzima não colocaliza também com o apicoplasto (**Figura 10B**, painel inferior). Nestas organelas já foram bem caracterizadas a localização de precursores (110), produtos (22) ou enzimas (42) que participam da via dos isoprenóides em *Plasmodium falciparum*. Localização citosólica de FPPS foi demonstrada em *Leishmania major* (111), *Trypanosoma cruzi* e *Trypanosoma brucei* (112). No entanto, em *Toxoplasma gondii*, cuja enzima é também caracterizada como uma bifuncional, a localização é mitocondrial (35). Nossos resultados sugerem um direcionamento celular desta proteína, possivelmente influenciado por isoformas geradas por meio de eventos de splicing alternativo, podendo alterar a sua localização ao longo da maturação das formas assexuadas no ciclo intraeritrocítico do parasita. Em muitos organismos, tais como plantas (113), mamíferos (114) e insetos (115) mais de uma isoforma de FPPS e/ou GGPPS está presente, com diferentes padrões de localização. Em *Toxoplasma gondii* foi demonstrado a existência de duas isoformas, onde o nível de transcrição de uma é muito mais elevado do que a de outra, embora estes níveis de expressão relativa não variem entre os estágios taquizoíto e bradizoíto do parasita (35).

Figura 10- Localização de FPPS/GGPPS. Imagens de parasitas vivos (linhagem FPPS/GGPPS-GFP-HA) que expressa GFP e imunofluorescência da linhagem FPPS/GGPPS-HA visualizados por microscopia fluorescência ou confocal. **A)** Imagens de parasitas vivos, FPPS/GGPPS-GFP-HA, por microscopia de fluorescência durante o ciclo intraeritrocítico do parasita. **B)** Parte Inferior - imunofluorescência da linhagem FPPS/GGPPS-HA com anticorpos tal como indicado, visualizados por microscopia confocal. Parte Superior - Imagens de parasitas vivos, FPPS/GGPPS-GFP-HA, com marcadores, como indicado, visualizados por microscopia confocal. A1 (Estágio Anel); A2 (Anel); T1 (Trofozoíta); T2 (Trofozoíta); E1 (Esquizonte); E2 (Esquizonte); R (rosetas); M (merozoítos); DAPI (marcador de núcleo). Ampliação original para todas as imagens 1.000X. **Anexo 1**

4.6 Identificação de possíveis isoformas da enzima FPPS/GGPPS durante o ciclo intraeritrocítico

A região codificante de FPPS/GGPPS foi amplificada a partir de cDNA dos 3 estágios intraeritrocíticos de *P. falciparum* (anel, trofozoíta e esquizonte), clonada em vetor de clonagem (pGEM-T easy) e os clones foram sequenciados para a verificação de possíveis diferenças no sequenciamento que poderiam ser explicadas pelo fenômeno de splicing alternativo.

Através do sequenciamento de diversos clones dos estágios intraeritrocíticos do parasita, encontramos 2 isoformas distintas na fase trofozoíta, a primeira com a deleção do exon 7 e a segunda com a deleção do exon 10, ambas com adição de stop codons prematuros após o evento de splicing alternativo, levando assim, a formação de proteínas truncadas (**Figura 11**). Esse tipo de splicing alternativo, onde ocorre a deleção de um exon, é o padrão mais comum, ocorre em cerca de 38% dos casos em humanos (85).

Alinhamento de sequências de aminoácidos de FPPS e/ou GGPPS de diferentes organismos revelou regiões conservadas com dois motivos ricos em aspartato característicos,

um na região II chamada FARM (*first Asp-rich motif*) e o outro na região VI chamada SARM (*second Asp-rich motif*). Foi demonstrado, claramente, que o tamanho da cadeia de produtos naturais de FPPS e GGPPS são regulados, principalmente, por resíduos de aminoácidos localizados na posição 4 e 5 “upstream” a região FARM, designada assim, de região CDL (*chain length determination*) (116). A nossa primeira isoforma encontrada manualmente, onde há deleção do exon 7 e adição de stop codons prematuros, verificamos a perda da região conservada SARM (**Figura 11**), que pode resultar na alteração da compatibilidade entre enzima e substratos, perda de uma ou total função de FPPS/GGPPS, ou mesmo alteração de sua função.

Figura 11- Alinhamento de FPPS/GGPPS e respectivas isoformas. Alinhamento demonstrando a proteína primária – FPPS/GGPPS, em comparação com as duas isoformas encontradas (a primeira com deleção do exon 10 e a segunda com deleção do exon 7), os domínios conservados (FARM e SARM) estão em destaque respectivamente.

Como complemento desse resultado, descartando um possível erro de sequenciamento, realizamos uma digestão com a enzima TaqI utilizando os 2 clones encontrados que tiveram a deleção do exon 7, e 2 clones controles contendo a sequência completa de FPPS/GGPPS. Essa enzima, apresenta 5 sítios no vetor pGEM-T easy e um no exon 7 de FPPS/GGPPS, apresentando assim, um padrão de digestão diferente com a perda dessa região (**Figura 12**).

Figura 12- Digestão das isoformas que deletam o exon 7 com a enzima TaqI. Os clones foram digeridos com a enzima TaqI de acordo com o manual do fabricante (117). (PM) peso molecular; (1 e 2) clones com a sequência anotada de FPPS/GGPPS; (3 e 4) clones que apresentam deleção do exon 7.

A fim de averiguarmos a presença de variadas isoformas que podem ter um papel fundamental durante o ciclo intraeritrocítico do parasita, bem como a abundância destas na população, partimos para uma análise mais complexa, utilizando o cDNA de parasitas ao longo do ciclo intraeritrocítico para realizarmos um sequenciamento de nova geração (Plataforma 454 GS FLX).

Alguns dados de cDNA foram utilizados para análise de estrutura de transcritos e variantes, melhorando anotação do genoma e modelos de genes em *P. falciparum* (86-89). RNA-Seq permite obter milhares de sequências da mesma amostra, análises de sequências que ainda não estão determinadas, bem como, as sequências curtas disponíveis através de adaptadores, permite determinar o limite de transcrição e obter informações precisas sobre a conexão entre dois ou mais exons (118). Otto e colaboradores, 2010 (86), sugeriram que aproximadamente 90% do genoma do parasita são transcricionalmente ativos durante esta fase. Sequenciamento dos transcritos amplificados de cada uma das quatro bibliotecas rendeu uma boa cobertura, com cerca de 26.8-44.3 milhões de bases sequenciadas, distribuídos por cerca de 49.000 (anel), 81.000 (trofozoíto jovem – 24 horas), 54.000 (trofozoíto maduro – 35 horas), ou 76.000 (esquizonte) leituras. Com o corte de qualidade, somente cerca de 300-900 leituras por biblioteca (ou cerca de 1% do total, no máximo) foram descartadas devido a qualidade da leitura. Uma vez que a duração média de leitura foi ao redor de 519 e 621 bases, a janela utilizada foi entre 52-62 bases de comprimento. A cobertura global do sequenciamento, está apresentada na **Figura 13** e, devido ao comprimento médio da sequência de leitura em comparação com o tamanho total do transcrito, a cobertura de sequência é menor no ponto médio do gene, entre os exons 6 e 7, no entanto, a cobertura mínima ainda é bastante elevada, em torno de 5.000 vezes. Cobertura em regiões intrônicas (**Figura 13**) é, obviamente, quase ausente e é representado por linhas fixas em partes do gráfico onde não existem bases sequenciadas. Mesmo nesta representação grosseira, já é possível identificar alguns dos eventos de splicing alternativos que foram descritos, como a retenção parcial da extremidade do intron 2, mais evidente na fase de esquizonte, ou a retenção parcial da extremidade do intron 7, bem distinguível em todas as fases.

O programa utilizado (STAR) foi capaz de mapear entre 88% e 94% das leituras com a sequência de referência, que consistia no gene completo anotado de FPPS/GGPPS do início ao códon de terminação, e incluindo todos os introns anotados. Identificamos 329 regiões de

junções de splicing, que após a inspeção manual caiu para 98 previstas junções de alta confiança (**Arquivo adicional 3 - Anexo 1**). Todas as análises subsequentes foram realizadas utilizando estas junções de alta confiança. Essas novas junções foram classificadas como canônicas (GT ... AG) ou alternativas (qualquer outro sítio). Junções canônicas foram responsáveis por 78 leituras, enquanto as alternativas foram responsáveis por 252 de todas as junções originalmente identificadas. Após filtragem, esses números caíram para 58 e 40, respectivamente, sugerindo fortemente que a maioria das junções, de baixa confiança, foi produzida por erros de sequenciamento ou alinhamento, eliminados por inspeção manual.

Figura 13- Cobertura geral das sequências a partir do RNA-seq. Os níveis de cobertura indicam o número de leituras cobrindo cada posição ao longo do gene anotado, FPPS/GGPPS de *P. falciparum*. Cobertura ao longo de regiões intrônicas está normalmente representada por uma linha a seguir a partir da última cobertura exônica disponível, com exceção dos casos em que alguma sequência intrônica foi adicionada. Cada estágio do ciclo intraeritrocítico está representado por uma linha, de acordo com a legenda. Áreas brancas verticais representam exons anotados (numerados). **Anexo 1**

P. falciparum tem um ciclo de vida complexo com diferentes características funcionais associadas com padrões de expressão de genes específicos (78). Assim, observações nas diferenças de transcrição estágio-específico sugerem que estas novas junções de splicing estão provavelmente associadas a algumas características únicas dos estágios de desenvolvimento do parasita e, provavelmente, com a regulação da via de isoprenóides. López-Barragán e colaboradores, 2011 (89) demonstraram 201 eventos de splicing alternativo afetando 178 genes em *P. falciparum*, e, destes, 124 isoformas ocorreram de maneira estágio-específica. Nós observamos que, em um total de 98 novas junções de alta confiança preditas como citado anteriormente, 63 estão presentes apenas em um ou dois estágios do ciclo do parasita

analisados (**Arquivo adicional 3 – Anexo 1**), embora as mais abundantes, em relação a primária, ocorra de maneira constitutiva, ao longo de todo o ciclo intraeritrocítico. (**Arquivo adicional 3 – Anexo 1**). Devido a quantidade de AT no genoma do parasita, a maioria das novas junções introduzem códons de parada, sugerindo uma proteína muito mais curta como produto final, ou, provavelmente, a produção de um mRNA que será degradado, caso em que a função desses eventos de *splicing* alternativo seja puramente reguladora. No entanto, foram identificados 40 casos (**Figura 14** e Arquivos adicionais 4 e 5 do Anexo 1), onde é potencialmente possível obter um produto de proteína razoável (isto é, o comprimento da proteína final produzido é, pelo menos, metade do da proteína anotada). Dado o grande número de possibilidades combinatórias (cerca de 3.630 milhões, em nossa estimativa) para a geração de isoformas utilizando as junções de *splicing* identificadas neste trabalho, analisamos apenas as combinações de variantes que foram experimentalmente observados em nossos dados de sequenciamento. Uma vez que nenhuma leitura poderia cobrir uma transcrição completa, nós "completamos" as variantes com sequências de acordo com as junções de *splicing* da variante anotada (**Arquivo adicional 4 – Anexo 1**). Deste modo, foi possível gerar variantes preditas com sequências iniciando no exon 1 e terminando no exon 11.

A maioria, dessas variantes, apresenta perda de partes de um ou mais exons, com apenas algumas apresentando inserção de novas sequências curtas - com exceção das variantes var230, var148, e var067. Essa última é a única variante que termina de forma significativa antes do códon de parada anteriormente anotado, levando à remoção completa dos exons 8 ao 11. Uma variante (var357) não tem uma porção significativa da região N-terminal, enquanto quatro outras variantes diferem ligeiramente na região N-terminal em comparação com a isoforma anotada, o que poderia ser significativo para um provável alvo celular (embora nossas análises *in silico* tenha mostrado uma diferença significativa).

Figura 14- Isoformas viáveis. Possíveis isoformas, permitindo a tradução para uma proteína funcional. A isoforma no topo é a versão anotada. Exon são marcados pela alternância de barras pretas e azuis na parte superior, e os domínios FARM e SARM são representados por retângulos nos exons 4 e 8, respectivamente. Os códons de parada estão representados em preto. As cores diferentes ao longo das sequências nas variantes representam a diferença em relação à isoforma anotada. **Anexo 1**

Um códon de parada em fase foi observado em cinco das isoformas potencialmente viáveis (**Figura 14**) - embora no caso de var148, ocorre apenas em 38 aminoácidos antes do final da proteína. Esta observação levanta a possibilidade de um fenômeno conhecido como *stop codon readthrough* (119) nos transcritos FPPS/GGPPS em *P. falciparum*. Este fenômeno foi recentemente mostrado ser comum em organismos como *Drosophila* e outros (120, 121) e consiste na leitura de um códon de parada como um sinal para a incorporação de um aminoácido.

As variantes var75, var128 e var148 apresentam um UGA como um códon de parada em fase (**Figura 14**). Um dos mecanismos ainda estudados seria o reconhecimento do códon UGA e a então incorporação de uma selenocisteína (119). A análise do genoma de *P. falciparum* (58) mostrou características que suportam a possibilidade de *stop codon readthrough*, como a presença de sequências de inserções de selenocisteínas (SECIS) encontradas na 3'-UTR de quatro genes específicos de *Plasmodium* (122, 123), como também experimentos de suplementação com selênio que mostram um aumento no crescimento dos parasitas (124). Para a confirmação desse fenômeno nesse estudo seriam necessários mais dados experimentais adequados, analisando as proteínas produzidas.

As outras duas variantes (var024 e var235) apresentam um códon de terminação UAG, localizado entre os exons 8 e 9 (**Figura 14**). Além da incorporação da selenocisteína, como mencionado acima, outros mecanismos para *stop codon readthrough* bem menos estudados

também foram caracterizados. Particularmente os códons UAA e UAG codificam uma glutamina, tirosina ou lisina, enquanto o triptofano, cisteína e arginina foram codificados por um códon UGA (121).

Neste trabalho podemos observar também, entre essas isoformas selecionadas como viáveis, uma isoforma (var094) que exclui o domínio FARM e duas (var034 e var067) que excluem o domínio SARM (**Figura 14**), indicando que as proteínas correspondentes são transcritas na íntegra, sem essas regiões. Como citado anteriormente sobre a importância desses domínios para as atividades enzimáticas dessa enzima, uma das principais funções destas variantes geradas por eventos de splicing alternativo, poderia ser a regulação na formação dos principais precursores da via de isoprenóides: GPP, FPP ou GGPP.

Uma variante de splicing com importância significativa é a deleção de um exon, onde uma sequência que está normalmente presente no RNA maduro é removida juntamente com os seus introns que a flanqueiam, resultando em um transcrito mais curto. Neste trabalho, observou-se um número de potenciais deleções parciais ou completas de exons onde parte ou a totalidade do exon é removido (**Figura 14**). Para validar esses resultados de eventos de splicing alternativo detectados aqui por RNA-seq, selecionamos três dessas novas junções identificadas (**Figura 15A**). Desenhamos pares de oligonucleotídeos que flanqueiam os locais de splicing selecionados e amplificamos por PCR os cDNAs da mesma amostra de esquizonte utilizada para a construção da biblioteca (isolado 3D7) e de dois isolados de pacientes (S20 e Cand), para descartar a possibilidade de qualquer artefato em cultura mantida durante longos períodos. Nós confirmamos a ocorrência destes três eventos de splicing alternativo, e corroboramos a quantificação dos dados por meio de RT-PCR, indicando que a isoforma que deleta o exon 7, por exemplo, é cerca de 100 vezes menos expressa que a anotada (**Figura 15C**).

Figura 15- PCR e RT-PCR para confirmação de algumas isoformas. **A)** Os oligos foram desenhados em regiões específicas para a detecção de um evento de splicing alternativo com deleção específica de um determinado exon. **A-I)** Oligos para amplificar um produto somente se o exon 5 é deletado. **A-II)** Oligos para amplificar um produto somente se o exon 7 é deletado. **A-III)** Oligos para amplificar um produto somente se o exon 10 é deletado. **B)** Esquema de oligonucleotídeos designados para detectar um evento de splicing alternativo onde ocorre deleção do exon 7, a mesma estratégia foi utilizada para todos os oligos mostrados na figura 15A. **C)** RT-PCR. Comparação da expressão entre a isoforma que deleta o exon 7 com o transcrito primário. Os níveis de transcrição do gene que codifica FPPS/GGPPS no isolado 3D7 foram normalizados pelo gene de controle interno K1. A significância estatística foi determinada por *one-way* ANOVA. Todas as diferenças entre o controle e a variante foram significativas ($p < 0,05$). 3D7 (tipo selvagem); S20 (isolado de um paciente); Cand (isolado de um paciente); gDNA (DNA genômico de 3D7); - (Controlo negativo); Controle (transcrito para a proteína primária); - exon7 (isoforma que deleta o exon 7); a e b (F-iso e R-iso); c e d (F-iso7 e R-iso7); TJ (trofozoíto jovem – 24 horas); TM (trofozoíto maduro – 35 horas). **Anexo 1**

Um outro caso de variante de splicing seria a retenção de um intron, é o caso em que uma parte da sequência, que é normalmente removida do transcrito primário, está incluída no RNA maduro. Nós investigamos a presença de retenção de introns em FPPS/GGPPS em todas as quatro fases analisadas neste estudo. A **Tabela 2** apresenta o número de eventos de retenção de introns putativos observados em nosso gene de interesse. Os casos onde ocorreram retenção dos introns 1, 2, ou 4 foram significativamente diferentes entre as fases do ciclo intraeritrocítico do parasita, ao passo que os introns 7, 8 e 10 apresentaram os níveis de retenção semelhantes em todas as fases (baixo). Os introns 3, 5, 6 e 9 não apresentaram envolvimento em qualquer caso de retenção.

Tabela 2- Eventos de retenção de introns de FPPS/GGPPS durante o ciclo intraeritrocítico de *P. falciparum*. O número total de leituras são apenas para referência e não foram utilizados nas análises estatísticas. Para as análise estatísticas foi utilizado o Teste exato de Fisher. Intron 1: significativo ($P < 2,2 \times 10^{-16}$); Intron 2: significativo ($P = 5,3 \times 10^{-5}$); Intron 4: significativo ($P = 3,7 \times 10^{-13}$); Intron 7: não significativo (P

= 0,149); Intron 8: não significativa (P = 0,011); Intron 10: não significativa (P = 0,041). E (Esquizonte); TJ (trofozoíta jovem – 24 horas); TM (trofozoíta maduro – 35 horas). **Anexo 1**

	Anel	E	TJ	TM	Anel nº	E nº	TJ nº	TM nº
Intron 1	31	155	43	20	30648	35261	48270	24389
Intron 2	14	22	4	4	28029	31344	40393	22250
Intron 3								
Intron 4	31	18	21	61	27000	33069	38097	22862
Intron 5								
Intron 6								
Intron 7	1	5	0	1	11593	23046	18994	17064
Intron 8	6	4	0	3	15300	28808	24926	21584
Intron 9								
Intron 10	0	1	5	0	18122	32245	30220	24091
Total reads	48944	76087	80727	54352				

Determinadas fases do parasita pareceram ser mais intensamente envolvidas em acontecimentos de retenção de introns. A presença de retenção do intron 1, por exemplo, foi significativamente diferente comparando-se esquizonte com as outras formas do parasita. Para o intron 4, a retenção em trofozoíta maduro é significativamente diferente das demais formas, já o intron 2, por outro lado, a retenção em trofozoíta jovem é significativamente diferente comparando-se com esquizonte e anel, mas não com trofozoíta maduro (estatísticas na tabela 2 do anexo 1). O fato da retenção de introns parecer ser um evento que ocorre de maneira estágio-específico sugere que estes acontecimentos podem ter importância reguladora. Trabalhos anteriores relataram que casos de retenção de intron em *P. falciparum*, ocorrem em menos de 50% dos transcritos analisados (87, 125). Nossos dados suportam estas conclusões, mostrando que esta é uma das categorias mais raras de splicing alternativo nesses organismos.

No caso da variante que retém o intron 1, embora o significado dessa sequência extra não seja completamente claro neste momento, é muito interessante notar que uma pesquisa de sinal de direcionamento com o programa TargetP, indicou um nível muito elevado (0,974) de *score* indicando para "outro" local (ou seja, não correspondendo com mitocôndria, nem apicoplasto ou secretor), enquanto a isoforma anotada apresenta um nível ligeiramente inferior, de 0,881. Outras investigações experimentais desta possível variante poderia

correlaciona-lá ao padrão de localização de FPPS/GGPPS apresentado nas formas maduras do ciclo.

A fim de investigar se qualquer uma das isoformas presentes no parasita foi traduzida em proteína, analisamos extratos de proteína do parasita por espectrometria de massa. De um total de 17 amostras a partir do gel SDS-PAGE, com as proteínas variando de tamanho entre 30 a 50 kDa, obteve-se um total de 1383 peptídios de alta confiança. Em nossos resultados, encontramos proteínas já bem estudadas neste parasita, como MSP2 – Proteína de superfície do merozoita-2 (107) ou EXP-2 – Proteína exportada-2 (126). Encontramos também uma proteína SR1, um fator de splicing de pré-mRNA envolvida na regulação do splicing alternativo em *Plasmodium falciparum* (127).

Significativamente, como um controle interno, encontramos, na banda do gel de tamanho aproximado de 41 kDa, três peptídeos de confiança da versão anotada da proteína FPPS/GGPPS. Em conjunto, estas observações mostram que os nossos dados refletem corretamente o proteoma esperado de *P. falciparum*, por outro lado, não conseguimos detectar qualquer mapeamento de peptídios para as formas previstas de variantes decorrentes de eventos de splicing alternativo aqui descobertos. Tendo em conta os limites de sensibilidade da metodologia utilizada, e tendo em conta que a isoforma anotada é quase sempre 1000 vezes mais abundante do que qualquer uma das variantes do transcriptoma, é provável que métodos diferentes, mais sensíveis, devem ser utilizados de modo a detectar quaisquer proteínas derivadas de potenciais isoformas do gene FPPS/GGPPS em *P. falciparum*.

4.7 Expressão de PF3D7_0607500 e OPP/PSY

As regiões codificantes de PF3D7_0607500 e PF3D7_0202700 (OPP/PSY) foram amplificadas a partir de DNA genômico de *P. falciparum*, clonadas em vetor de clonagem (pUC 57 e pGEM-T easy respectivamente) e posteriormente subclonadas em vetores de expressão em bactéria (pRSETC -cauda de poli-histidina - his-tag). Ambas as proteínas foram expressas e purificadas. Ao separarmos as proteínas através de gel de poliacrilamida SDS-PAGE, obtivemos nossas proteínas recombinante com o tamanho esperado de aproximadamente 55kDa para PF3D7_0607500 e 60kDa para OPP/PSY (**Figura 16**).

Figura 16- Expressão e purificação das proteínas codificadas pelos genes PF3D7_0607500 e PF3D7_0202700 (OPP/PSY). O produto de DNA amplificado foi clonado em vetor de clonagem (pUC 57 e pGEM-T easy repectivamente) e posteriormente em vetor de expressão (pRSETC), a proteína foi purificada por coluna de níquel sepharose. A) Purificação da proteína codificada pelo gene PFF0370w. As colunas representam diferentes eluições com concentrações variando de 300mM, 100mM e 500mM de imidazol repectivamente. B) Purificação da proteína codificada pelo gene PFB0130w (OPP/PSY). As colunas representam diferentes eluições com concentrações de imidazol variando em 500 mM, 300mM e 100mM respectivamente.

4.8 Perfil de expressão de OPP/PSY

A fim de obter uma prova genética que a biossíntese de OPP/PSY seria essencial para o parasita, procurou-se gerar uma linhagem transgênica em que a expressão da enzima bifuncional OPP/PSY poderia ser regulada. Para este fim, parasitas 3D7 foram transfectadas com o vetor pOPP/PSY-HA-DD24 e cultivados na presença de Shld-1, a mesma estratégia utilizada para FPPS/GGPPS descrita anteriormente. Depois de três transfecções independentes, parasitas com o plasmídeo integrado não foram recuperados (**Anexo 2**) assim como FPPS/GGPPS, sugerindo que OPP/PSY não pode manter a sua função fisiológica quando em fusão com um taq de 15 kDa ou seus níveis de expressão foram demasiados baixos, mesmo na presença de Shld- 1. Esse resultado demonstra que OPP/PSY desempenha um papel importante e possivelmente essencial durante o ciclo intra-eritrocítico e não pode desempenhar completamente a sua função fisiológica quando em fusão com grandes taqs na sua região C-terminal. PSY de plantas estão normalmente associadas com as membranas e com outras enzimas, e, por conseguinte, uma região C-terminal pode ser de extrema importância para algumas dessas interações acontecerem (128).

Para observar se o locus genômico do gene que codifica OPP/PSY seria susceptível a integração de plasmídeos, foi gerada uma linha transgênica semelhante, em que a enzima se encontra em fusão com um taq menor, o HA de 3 kDa. Parasitas com o plasmídeo integrado foram clonados por diluição limitante gerando a linhagem clonal de pOPP/PSY-HA-int, em que a integração foi confirmada por PCR (**Anexo 2**).

Análise por Western blot, utilizando amostras de proteína extraídas de parasitas

sincronizados nos 3 principais estágios (anel, trofozoíta e esquizonte) e, reveladas com o anticorpo anti HA, demonstraram que a enzima OPP/PSY é expressa em todos os estágios, durante o ciclo intraeritrocítico de *P. falciparum*, com pico na fase esquizonte (**Figura 17**) (49). Como controle, foram utilizados anticorpos que reconhecem pTEX150 (90), expressa de forma constitutiva e MSP2 (107), expressa somente em esquizontes. Embora OPP/PSY seja expressa constitutivamente, os seus produtos são, principalmente, detectados em parasitas em fase madura (33, 42), sugerindo que a sua atividade pode ser limitada pela disponibilidade de alguns dos precursores.

Figura 17- Expressão de pOPP/PSY-HA-int durante o ciclo intraeritrocítico de *P. falciparum*. (A) anel, (T) trofozoíta, (E) esquizonte; α-HA (anticorpo contra o epítipo da proteína hemaglutinina (HA) presente no plasmídeo em fusão com a proteína de interesse – OPP/PSY), controles: α-pTEX-150 (anticorpo contra pTEX150, proteína expressa constitutivamente durante o ciclo intraeritrocítico do parasita (90)), α-MSP2 (anticorpo contra MSP2, proteína expressa somente no estágio de esquizonte (96)). **Anexo 2**

4.9 Super expressão de OPP/PSY

Como citado anteriormente, foi sugerido que a biossíntese de carotenóides, em fases intraeritrocitárias de *P. falciparum*, pode desempenhar um papel no desenvolvimento do parasita e/ou a replicação e, por conseguinte, ser um alvo de drogas (42). Nós demonstramos que a droga esqualestatina, um ácido carboxílico com a estrutura molecular $C_{35}H_{43}O_{14}Na_3$ (2,8-dioxabicyclo [3.2.1] - octano-3 núcleo do ácido, 4,5-tricarboxílico) descoberta na busca de metabólitos de fungos filamentosos como inibidores de esqualeno-sintase, a enzima responsável pelo primeiro passo na biossíntese de esterol (129-132), tem efeito inibitório na biossíntese de fitoeno em *P. falciparum* (49) (**Anexo 2**). A fim de tentar demonstrar que a biossíntese de fitoeno é essencial para o parasita e que a enzima PSY é o principal alvo da esqualestatina no parasita, tentamos super expressar OPP/PSY usando um vetor pEF (56), a partir do qual o gene estaria sob o controle do promotor constitutivo *ef1-α* (133, 134).

Esperava-se que a OPP/PSY fosse super expressa nos parasitas transfectados uma vez que os plasmídeos são geralmente mantidos na forma episomal em múltiplas cópias. No entanto, a transfecção utilizando esse plasmídeo não foi capaz de gerar parasitas transfectados, sugerindo que a super expressão constitutiva pode ser tóxica. Os efeitos tóxicos da super expressão de PSY foram relatados tanto em bactérias quanto em plantas, sendo a principal causa o esgotamento do substrato GGPP, induzindo um decréscimo de outros produtos da via dos isoprenóides (135, 136). Na linhagem super expressa, OPP também é abundante e GGPP é um dos seus produtos intermediários e, portanto, outros precursores da via de isoprenóides tais como FPP, GPP, IPP ou DMAPP poderiam estar esgotados. Uma vez que estes metabólitos são principalmente biossintetizados em parasitas maduros (30 h após a infecção), parasitas na fase inicial poderiam ser ainda mais sensíveis a super expressão constitutiva. Para atenuar qualquer possível efeito tóxico da super expressão de OPP/PSY, o promotor *efl-α* foi substituído pelo promotor específico de esquizonte MSP2 (107) e o plasmídeo resultante pRM2-OPP/PSY-HA foi transfectado com sucesso em parasitas da cepa 3D7, gerando a linha transgênica OPP/PSY-HA-epi (**Figura 18A**). Para comparar a expressão de OPP/PSY nas linhagens transgênicas (OPP/PSY-HA-int e OPP/PSY-HA-epi) e selvagem 3D7, aplicamos PCR em tempo real analisando parasitas em estágio esquizontes, devido ao promotor específico utilizando na linhagem OPP/PSY-HA-epi. Enquanto a expressão na linhagem integrada foi semelhante a 3D7, como esperado, a linhagem super expressa, OPP/PSY-HA-epi, apresentou uma expressão cerca de 5 vezes superior (**Figura 18B**).

Figura 18- Super Expressão de OPP/PSY em *P. falciparum*. **A)** Representação esquemática do vetor utilizado para super expressar OPP/PSY em *P. falciparum*. **B)** Expressão de OPP/PSY da linhagem integrada (OPP/PSY-HA-int) em comparação com a linhagem super expressora, que possui o plasmídeo epissomal (OPP/PSY-HA-epi) analisados por PCR em tempo real. Os níveis de transcrição do gene que codifica OPP/PSY foram normalizados pelo gene de controle interno K1 e são representados em relação ao 3D7. A significância estatística foi determinada por *One-way* ANOVA, valor de *p* indicado. **Anexo 2**

A falha para super expressar constitutivamente OPP/PSY sugere que um excesso desta enzima poderia ser tóxico para o parasita. Para investigar se a linhagem OPP/PSY-HA-epi poderia ter o crescimento dos parasitas reduzido se comparada com a selvagem 3D7, uma curva de crescimento de 96 horas comparando-se todas as linhagens foi realizada (**Figura 19**). Parasitas que super expressam OPP/PSY cresceram mais lentamente do que a selvagem e a linhagem integrada (**Figura 19**). Especulamos que a linhagem OPP/PSY-HA-epi possa ser instável e, eventualmente, perder o plasmídeo ou a expressão do gene ao longo do tempo devido à toxicidade da super expressão da enzima, por isso, todos os experimentos foram realizados com parasitas recém-transfectados, não mais que 4 semanas estabelecidos em cultivo.

Figura 19- Curva de crescimento por 96 horas comparando-se as linhagens OPP/PSY-HA-int, OPP/PSY-HA-epi e a selvagem 3D7. Super expressão de OPP/PSY afeta negativamente o crescimento do parasita. Parasitemia foi determinada pelo exame microscópico de esfregaços corados com Giemsa. Os pontos referem-se às médias de experimentos realizados em triplicatas e as barras de erro informam o desvio padrão. **Anexo 2**

Investigamos também, através de marcação metabólica, se os produtos de OPP e PSY iriam ser biossintetizados em uma taxa mais elevada na linhagem transgênica super expressora (OPP/PSY-HA-epi) em comparação com a selvagem 3D7 (**Figura 20**). A biossíntese do produto direto de PSY, fitoeno, e do produto subsequente, fitoflueno, foram aumentadas, $57,3 \pm 3\%$ e $47 \pm 5\%$ respectivamente (**Figura 20A**). Em contraste, a biossíntese de β -caroteno, um produto mais distante na via de carotenóides, não foi afetada. A biossíntese de GGPP, o substrato de PSY, e um dos produtos intermédios da OPP, foi aumentada $46,2 \pm 5\%$ (**Figura 20B**).

Figura 20- Efeito da super expressão de OPP/PSY na biossíntese de carotenoides e GGPP. A) Biossíntese de fitoeno, fitoflueno e β - caroteno na linhagem super expressa OPP/PSY-HA-epi em comparação com a selvagem 3D7 . Carotenos foram extraídos a partir de amostras de esquizontes marcados metabolicamente com [1- (n) -3H] GGPP e analisados por HPLC. Os picos radioativos correspondentes ao tempo de retenção de fitoeno, fitoflueno e β - caroteno foram representados graficamente. **B)** Biossíntese de GGPP na linhagem super expressa OPP/PSY-HA-epi em comparação com a selvagem 3D7. GGPP foi extraído a partir de amostras de esquizontes marcados metabolicamente com [1- (n) -3H] FPP e analisados por HPLC. O pico radioativo correspondente ao tempo de retenção de GGPP foi representado graficamente. A significância estatística foi determinada por *One-way* ANOVA. *p*-valores indicados; ns indica associações não significativas ($p > 0,05$).

Anexo 2

A correlação entre a super expressão de OPP/PSY e o aumento da biossíntese de seus produtos sugeriu que esses parasitas poderiam tolerar uma inibição da atividade da enzima em níveis mais elevados do que os parasitas que expressam OPP/PSY em níveis normais. Para testar esta hipótese, o efeito inibitório de esqualestatina no crescimento de *P. falciparum* foi avaliado comparando-se as três linhagens aqui descritas (experimentos executados por Márcia Ferreira). Especificamente, enquanto que a linhagem transfectada com o plasmídeo integrado (OPP/PSY-HA-int) era tão sensível a esqualestatina quanto a selvagem 3D7, a IC₅₀ de esqualestatina na linhagem super expressa (OPP/PSY-HA-epi) foi cerca de 5,5 vezes mais elevada (**Anexo 2**). Esse fenótipo de resistência apresentado pelos parasitas que super expressam OPP/PSY, sugere que PSY é o principal alvo desta droga nos parasitas, e que fitoeno pode ser essencial durante o ciclo intraeritrocitária do parasita.

É incerto se fitoeno é o único carotenóide necessário para o desenvolvimento do parasita ou se os seus produtos derivados podem também desempenhar um papel importante. Uma maneira para investigar esta questão seria inibir as reações envolvidas na biossíntese dos derivados de carotenóides. No entanto, os genes que codificam estas enzimas não foram identificados em *Plasmodium* e, por conseguinte, ainda não é possível utilizar abordagem de genética reversa ou testar a ação de inibidores.

4.10 Caracterização de MenA

Foram realizadas análises *in silico* no banco de dados *PlasmoDB* (www.plasmodb.org) para busca de sequências/genes relacionados(as)/envolvidos(as) na via de biossíntese de vitamina K. Essas análises foram feitas pelo técnico do Departamento de parasitologia do Instituto de Ciências Biomédicas da USP, Márcio M. Yamamoto. Foi possível encontrar sequências similares aos genes MenA (PF3D7_0607500 ou PF3D7_0202700), comparado ao gene MenA de *Arabidopsis thaliana*, apesar da baixa similaridade encontrada (~10%). É sabido que o genoma de *P. falciparum* é mais parecido com o de uma planta, ou seja,

Arabidopsis thaliana, do que com outros organismos não apicomplexos como *E.coli* ou *Saccharomyces cerevisiae* (137).

A fim de averiguarmos se um dos nossos genes candidatos possuía atividade de MenA, em colaboração com o Dr. Dean C. Crick da Universidade do Estado do Colorado, utilizamos um mutante de *E. coli* com deleção desse gene de interesse para experimentos de complementação. A proteína codificada por PF3D7_0607500 possui em sua estrutura, 6 domínios transmembranas, portanto, sua purificação após a expressão poderia conferir uma conformidade diferente e alterar assim sua atividade.

Nossos genes de interesse, subclonados no vetor de expressão (pRSETC-PF3D7_0607500, pRSETC-PF3D7_0202700), foram usados para transformar bactérias *E. coli* com a deleção do gene MenA (Mt-3, Mt-5 respectivamente). Utilizamos como controle positivo *E. coli* selvagem (WT *E. coli*), e como controle negativo somente o mutante sem inserção dos plasmídeos de interesse (MenA Mt). Como o mutante de *E. coli* utilizado, tem deleção específica do gene MenA, ele não é capaz de sintetizar tanto Menaquinona como Demetilmenaquinona. Após crescimento das células até atingir a fase log, extraímos lipídeos a fim de averiguarmos a formação dos produtos finais (MQ-8, DMQ-8) inicialmente através de Thin layer chromatography (TLC) e posteriormente por Liquid chromatography–mass spectrometry (LC-MS) (**Figura 21**).

Figura 21- LC-MS após a extração de lipídeos para análise de MQ-8 e DMQ-8. Os lipídeos foram extraídos com clorofórmio e separados através de cromatografia em coluna de ácido silícico. **A)** TIC (total ion current) das 4 amostras analisadas. **B)** TIC de WT *E. coli* demonstrando o tempo em que ocorre a formação de MQ-8 e DMQ-8, bem como mass spectrometry (Ms) desses padrões. **C)** TIC de MenA MT mostrando a não formação dos produtos. **D)** TIC das amostras MT-3 e MT-5 mostrando a não formação dos produtos. WT *E. coli* (wild type – *E. coli* selvagem), MenA Mt (mutante de *E. coli* com deleção do gene MenA), Mt-3 (plasmídeo pRSETC-PF3D7_0607500 expresso em MenA Mt), Mt-5 (plasmídeo pRSETC-PF3D7_0202700 expresso em MenA Mt).

Na TLC não foi possível visualizar a formação de MQ-4 (menaquinona de 4 unidades isoprênicas), MQ-8 (menaquinonade 8 unidades isoprênicas) ou DMQ-8 (demetilmenaquinona -8) no mutante e nos plasmídeos expressos no mutante (dados não apresentados). Foram utilizados, na corrida, os padrões correspondentes, bem como foi possível à visualização de MQ-8 e DMQ-8 na linhagem selvagem.

Nas análises de LC-MS (**Figura 21**), observamos as formações dos produtos MQ-8 (MS = 703.5461) e DMQ-8 (MS = 717.532) na linhagem selvagem (**Figura 21B**) e não na

mutante (**Figura 21C**), como esperado. Porém, não observamos a formação dos produtos em nenhum dos nossos plasmídeos de interesse expressos no mutante (**Figura 21D**). Provavelmente devido a pouca sensibilidade do método utilizado para preparação da amostra. Além disso, nesses experimentos, analisamos, principalmente, a formação de Menaquinona de 8 unidades isoprênicas, pois é o produto biossintetizado por *E. Coli* (138) (organismo com mutação específica em MenA utilizado para experimentos de complementação), embora em *P. falciparum* caracterizamos como produto final a MQ-4, de 4 unidades isoprênicas (54). Utilizamos também, o padrão de MQ-4 como controle, mas também não foi possível observar a formação desse produto em nossas amostras.

A fim de confirmarmos a expressão dos plasmídeos de interesse na linhagem mutante com deleção de MenA, realizamos um western blot, com anticorpo anti His-tag, epítipo presente no vetor de expressão pRSETC para purificação, onde esses plasmídeos foram subclonados. Porém, não identificamos nenhuma banda nesse experimento (dados não apresentados). Sendo assim, mudamos a estratégia para subclonar nosso gene de interesse no vetor pVV16, derivado do pMV261(139) que carrega um gene marcador de resistência à higromicina. Esse vetor já foi utilizado com sucesso no laboratório para experimentos de complementação.

Os plasmídeos pRSETC- PF3D7_0607500, pRSETC-PF3D7_0202700 bem como o vetor pVV16 foram digeridos com PST1/BstB1. Obtivemos ao final os plasmídeos pVV16-PF3D7_0607500 (pVV16-3) e pVV16- PF3D7_0202700 (pVV16-5).

Os Plasmídeos pVV16- PF3D7_0607500 e pVV16- PF3D7_0202700 foram usados para transformar bactérias *E. coli* com a deleção do gene MenA (pVV16-3, pVV16-5 respectivamente). Posteriormente realizamos uma extração de membrana e em sequência a reação enzimática (**Protocolo 1**), utilizando como substratos 1,4-dihidroxi-2-ácido naftóico e [³H] farnesil difosfato ou geranilgeranil difosfato (55) (**Figura 22**). Como também uma nova extração de lipídeos para análise por LC-MS.

Figura 22- Perfil radioativo, DPM (desintegração por minuto), das amostras após reação enzimática para a enzima codificada pelo gene MenA. Após a preparação de membrana a reação foi iniciada com os substratos 500 mM DHNA, 10 mM [^3H] farnesil difosfato (American Radiolabelled Chemicals), os produtos foram extraídos com hexano e separados através de uma coluna de ácido silícico. Controles positivos (*Mycobacterium smegmatis* – *M. Seg.*; *E. coli* selvagem – WT *E. coli*; MenA de *Mycobacterium tuberculosis* expressa em *E.coli* mutante para o gene MenA – TB MenA/Mt); Controle negativo (MenA Mt – Mutante para MenA); pVV16-3 (pVV16- PF3D7_0607500 expresso em MenA Mt); pVV16-5 (pVV16- PF3D7_0202700 expresso em MenA Mt).

Não obtivemos, novamente, nenhum resultado satisfatório nas análises de LC-MS (dados não apresentados). Na reação enzimática utilizando FPP (**Figura 22**) ou GGPP (dados não apresentados) como substratos, não conseguimos observar nenhum pico radioativo correspondente a formação de MQ-3, MQ-4, DMQ-3 ou DMQ-4 em nossas amostras (pVV16-3 ou pVV16-5) enquanto que nos controles positivos utilizados, observamos picos correspondentes (*Mycobacterium smegmatis*; *E. coli* selvagem; MenA de *Mycobacterium tuberculosis* expressa em *E.coli* mutante para o gene MenA).

Como controle da preparação do extrato de membrana utilizado na reação, realizamos uma atividade enzimática, onde observamos a atividade de uma farnesil difosfato sintase (FPPS), utilizando como substratos GPP (geranil difosfato) e [^{14}C] IPP (isopentenil difosfato) (102) (**Figura 23**).

Figura 23- Perfil radioativo, DPM (desintegração por minuto), e TLC das amostras após reação enzimática para FPPS. Após a preparação de membrana a reação foi iniciada com os substratos 50 μ M GPP e 30 μ M [14 C] Isopentenil Difosfato (American Radiolabelled Chemicals), os produtos foram extraídos com butanol saturado em água. **A)** Perfil radioativo após atividade enzimática. Grcc1 (controle positivo – poliprenil - proteína recombinante); WT (*Wild type* E. coli); MenA Mt (Mutante para MenA); pVV16-3 (pVV16- PF3D7_0607500 expresso em MenA Mt); pVV16-5 (pVV16- PF3D7_0202700 expresso em MenA Mt). **B)** TLC após atividade enzimática. Após a desforilação enzimática, as amostras foram ressupensas em clorofórmio/metanol (2:1 v/v) e aplicadas em placas de TLC de sílica gel, que foram desenvolvidas em metanol/acetona (8:2 v/v). 1 (Controle); 2 (*Wild type* E. coli); 3 (MenA Mt); 4 (pVV16- PF3D7_0607500 expresso em MenA Mt); 5 (pVV16- PF3D7_0202700 expresso em MenA Mt). FOH (Farnesol); GGOH (geranilgeraniol); p25 (prenil de 25 carbonos); p30 (prenil de 30 carbonos); p35 (prenil de 35 carbonos); p40 (prenil de 40 carbonos); p45 (prenil de 45 carbonos).

Demonstramos assim, como um controle da preparação dos extratos de membrana, a atividade de outra enzima não relacionada à biossíntese de Menaquinona, (102). Essa enzima (FPPS) é capaz de sintetizar farnesil pirofosfato (FPP - 15 carbonos) catalizando a reação de isopentenil pirofosfato (IPP - 5 carbonos) com geranil pirofosfato (GPP - 10 carbonos) (102). Podemos observar na TLC a formação do produto direto da reação, farnesol, em todas as amostras utilizadas (**Figura 23B**), porém, em nosso gene PF3D7_0607500, observamos um pico radioativo (**Figura 23A**) como também a formação de produtos maiores de 40 e 45 carbonos (**Fig. 23B linha 4**).

O gene, PF3D7_0202700 (**Fig. 23B linha 5**), codifica a enzima bifuncional OPP/PSY, citada anteriormente (33, 42). OPPS pertence a uma família de prenil-transferases que catalisa a reação de condensação de FPP (farnesil pirofosfato) com cinco moléculas de IPP para produzir um produto de 40 carbonos o OPP (octaprenil pirofosfato) (33). Essas enzimas são

responsáveis pela biossíntese de cadeias laterais ligadas a ubiquinonas em *Escherichia coli* (45, 46). Portanto, esperávamos que esses produtos fossem identificados nesse gene (pVV16-PF3D7_0202700 - linha 5), nessas condições, apesar de usarmos GPP (10 carbonos) não FPP (15 carbonos) como substrato.

Esse resultado nos leva a especulações futuras sobre outras possíveis atividades de PF3D7_0607500 a serem estudadas em *P. falciparum*.

Nakagawa e colaboradores, 2010 (140), demonstraram que em humanos, MQ-4 é obtida pela clivagem e substituição da cadeia lateral de filoquinona por uma nova cadeia de GGPP, processo realizado por uma enzima prenil transferase homóloga a MenA de *E. coli*. Foi demonstrado que essa conversão também pode ser realizada utilizando-se menadiona (3 unidades isoprênicas) ou mesmo a própria MQ-4.

Como em *P. falciparum* também caracterizamos a biossíntese de filoquinona (dados ainda não publicados) e até o momento ainda não possuímos nenhuma enzima caracterizada de ambas as vias de vitamina K, fomos verificar se poderia ocorrer a mesma obtenção de MQ-4 em *P. falciparum* assim como em humanos. Para isso, realizamos o mesmo ensaio enzimático para MenA, como descrito nos resultados anteriores, mas utilizando agora menadiona (MQ-3) como substrato no lugar do anel, 1,4-dihidroxi-2-ácido naftóico, que provém da via do chiquimato. Porém, não obtivemos nenhum pico radioativo em nossas amostras comparadas com os controles Wt *E. coli* (*Wide Type E. coli*, controle positivo) e MenA Mt (mutante para o gene MenA, controle negativo) (dados não apresentados).

Ainda seguindo a mesma linha de raciocínio e a fim de confirmar a presença da via ativa de biossíntese de menaquinona em *P. falciparum*, iniciamos uma colaboração com o grupo do Dr. Guillermo Labadie da Faculdade de Bioquímica e Ciências Farmacêuticas da Universidade Nacional de Rosário que sintetizou e nos cedeu o composto Demetilmenaquinona-4, produto direto da atividade de MenA anterior a metilação pela enzima MenG (**Figura 5**). Através de experimentos com marcação metabólica e análise por HPLC, podemos caracterizar um pico radioativo no tempo de retenção de DMQ-4 (**Figura 24**), confirmando então que o parasita possui a via ativa para a biossíntese de menaquinona, sendo o único organismo até o momento a possuir as duas formas de vitamina K.

Figura 24- Evidência bioquímica da biossíntese da Demetilmenaquinona-4 em *P. falciparum*. Perfil de análise por RP-HPLC (coluna C18) de extratos de esquizonte metabolicamente marcadas com [1- (n) -3H] GGPP. TM Software Unipoint (Gilson Inc.) foi utilizado como o sistema operacional e analítico. As amostras foram co-injectados com os padrões descritos nos respectivos picos. A confirmação se obteve depois de 3 experimentos realizados.

Como agora possuímos o produto direto da reação de MenA já padronizado por metodologia de HPLC e a proteína recombinante OPP/PSY, realizamos a reação enzimática para MenA (**Protocolo 2**) e analisamos a presença do produto direto da reação, DMQ-4 (**Figura 25**).

Figura 25- Perfil de HPLC após atividade para MenA utilizando a proteína recombinante OPP/PSY. Utilizamos os substratos 500 mM DHNA e [1-(n)-³H] geranilgeranil pirofosfato. A reação foi mantida por 1 hora à 37°C e ao final submetida à extração de DMQ-4 com éter de petróleo e então purificado por *RP-HPLC*. As frações foram coletadas em intervalos de 0,8 ml/min. Demonstração da presença de frações radioativas coincidente ao padrão de DMQ-4 – fração 35; MQ-4 – fração 41; UQ-8 – fração 20.

Identificamos, por HPLC, um pico radioativo que corresponde ao mesmo tempo de retenção do padrão Demetilmenaquinona-4. Porém nas análises por GC-MS (*Gas chromatography–mass spectrometry*), não foi possível obter essa confirmação (dados não apresentados). Portanto, especulamos que provavelmente esse perfil obtido por HPLC seja inespecífico, pois é possível observar outros picos radioativos não identificados, mesmo no controle negativo.

Durante a realização desses experimentos, o grupo da Dra Maria Belen Cassera em Virginia Tech, tiveram indícios da presença do composto fualosina em formas assexuais de *P. falciparum* (dados ainda não publicados). Fualosina é um nucleosídeo incomum que consiste de inosina, sendo uma via alternativa recente de biossíntese de menaquinona que atua em bactérias, incluindo alguns patógenos, como a *Helicobacter pylori*, *Campylobacter jejuni*, *Chlamydia trachomatis*, e *Leptospira borgpetersenii* (141-143). MqnA catalisa a reação de condensação de corismato, inosina, e uma unidade de C2, provavelmente derivado de Fosfato fosfoenolpiruvato, para formar fualosina. Então hipoxantina é liberada para dehipoxanthinilfualosina (DHFL), uma dehipoxantina derivada de fualosina, por fualosina hidrolase (MqnB). A enzima codificada pelo gene MqnC cicliza DHFL, seguido pela

clivagem de um C3 para uma unidade de 1,4-dihidroxi-6-naftoato por MqnD. Nas duas últimas etapas, ortólogos de MenA e MenG catalisam a prenilação e metilação respectivamente para formar menaquinona (144). Assim, provavelmente, todos os resultados negativos obtidos até o momento tenham ocorrido devido ao substrato utilizado, pois na via de Fualosina o grupo carboxila se encontra na posição 6 do anel de naftoquinona e não na posição 2 como no substrato da via clássica (**Figura 5**). Acreditamos que o parasita possua a via alternativa de Fualosina, sendo uma linha de pesquisa de extrema importância para ser investigada no parasita.

5 CONCLUSÕES

No presente trabalho objetivou-se compreender melhor a fisiologia do parasita, visando à caracterização de enzimas presente na via de isoprenóides bem como determinar o papel desempenhado, para a sobrevivência do parasita, de enzimas já caracterizadas, abrindo caminho assim, para a seleção de novos alvos e drogas antimaláricas. A partir dos resultados obtidos pode-se concluir que:

5.1. FPPS/GGPPS é expressa constitutivamente durante o ciclo intraeritrocítico do parasita.

5.2. FPPS/GGPPS não pode manter a sua função fisiológica quando em fusão com HA-DD24, pois seus níveis de expressão provavelmente são demasiado baixos.

5.3. FPPS/GGPPS está localizada ao longo do citoplasma em formas assexuais de *P. falciparum*, formando pontos, que aumentam em número em parasitas maduros a partir da fase trofozoíta para esquizonte, sugerindo a presença de transportadores ou isoformas dessa proteína.

5.4. FPPS/GGPPS apresenta uma variabilidade inédita de eventos de *splicing* alternativo em *P. falciparum*, mostrando uma grande variabilidade de isoformas, sugerindo que, a complexidade proteômica neste organismo pode ser muito maior do que o estimado no genoma, relativamente pequeno, anotado.

5.5. OPP/PSY é expressa constitutivamente durante o ciclo intraeritrocítico do parasita.

5.6. OPP/PSY não pode manter a sua função fisiológica quando em fusão com um tag de pelo menos 15 kDa.

5.7. A super expressão de OPP/PSY é tóxica para o parasita, mesmo utilizando um promotor estágio-específico.

5.8. Através da abordagem de genética reversa, demonstramos que, fitoeno sintase é, aparentemente, o principal alvo da droga esqualestatina, sugerindo que, a biossíntese de carotenóides poderia ser um alvo para o desenvolvimento de novos antimaláricos.

5.9. A proteína codificada pelo gene PF3D7_0607500 apresentou uma atividade enzimática de poliprenil sintase, nos levando a especulações futuras sobre essa possível atividade desse gene a ser estudada em *P. falciparum*.

5.10. *P. falciparum* possui a via ativa para a biossíntese de menaquinona.

5.11. OPP/PSY e PF3D7_0607500 não apresentaram atividade para a enzima codificada pelo gene MenA com os substratos que foram utilizados até o momento.

REFERÊNCIAS*

1. Sherman IW. A brief history of malaria and discovery of the parasite's life cycle. In: _____. Malaria: Parasite biology, Pathogenesis and Protection. Washington, DC: ASM; 1998. p. 3-10.
2. Bruce-Chwatt L. Essential Malariology. London: William Heinemann Medical Books; 1980. 354 p.
3. Toler S. The plasmodial apicoplast was retained under evolutionary selective pressure to assuage blood stage oxidative stress. *Med Hypotheses*. 2005;65(4):683-90.
4. Joy DA, Feng X, Mu J, Furuya T, Chotivanich K, Krettli AU, et al. Early origin and recent expansion of *Plasmodium falciparum*. *Science*. 2003;300(5617):318-21.
5. WHO. World Malaria Report. Switzerland: World Health Organization; 2014. 242 p.
6. Rey L. Parasitologia. 3 ed. Rio de Janeiro: Guanabara Koogan; 2001. 731 p.
7. Singh B, Daneshvar C. Human infections and detection of *Plasmodium knowlesi*. *Clin Microbiol Rev*. 2013;26(2):165-84.

* De acordo com: International Committee of Medical Journal Editors. Uniform requirements for manuscripts submitted to Biomedical Journal: sample references. Available from: <http://www.icmje.org> [2007 May 22].

8. Trigg PI, Kondrachine AV. Commentary: malaria control in the 1990s. *Bull World Health Organ.* 1998;76:11-6.
9. Phillips RS. Current status of malaria and potential for control. *Clin Microbiol Rev.* 2001;14(1):208-26.
10. Petersen I, Gabryszewski SJ, Johnston GL, Dhingra SK, Ecker A, Lewis RE, et al. Balancing drug resistance and growth rates via compensatory mutations in the *Plasmodium falciparum* chloroquine resistance transporter. *Mol Microbiol.* 2015; 97(2):381-95.
11. Fitch CD. Ferriprotoporphyrin IX, phospholipids, and the antimalarial actions of quinoline drugs. *Life sciences.* 2004;74(16):1957-72.
12. Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG. Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. *Nature.* 1995;374(6519):269-71.
13. Trigg PI, Kondrachine AV. Commentary: malaria control in the 1990s. *Bull World Health Organ.* 1998;76(1):11-6.
14. White LJ, Flegg JA, Phyo AP, Wiladpai-Ngern JH, Bethell D, Plowe C, et al. Defining the In Vivo Phenotype of Artemisinin-Resistant *Falciparum* Malaria: A Modelling Approach. *PLoS Med.* 2015;12(4):e1001823.
15. White NJ. Antimalarial drug resistance. *J Clin Invest.* 2004;113:1084-92.

16. Kim Y, Schneider, K. A. Evolution of Drug Resistance in Malaria Parasite Populations. *Nature Education Knowledge*. 2013;4(8):6.
17. Ginsburg H, Ward AS, Bray PG. An integrated model of chloroquine action. *Parasitol Today*. 1999;15(9):357-60.
18. Macreadie I, Ginsburg H, Sirawaraporn W, Tilley L. Antimalarial drug development and new targets. *Parasitol Today*. 2000;16:438-44.
19. Fast NM, Kissinger JC, Roos DS, Keeling PJ. Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol Biol Evol*. 2001;18(3):418-26.
20. Luo S, Marchesini N, Moreno SN, Docampo R. A plant-like vacuolar H(+)-pyrophosphatase in *Plasmodium falciparum*. *FEBS Lett*. 1999;460(2):217-20.
21. Wilson RJ. Progress with parasite plastids. *J Mol Biol*. 2002;319(2):257-74.
22. Yeh E, DeRisi JL. Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. *PLoS biology*. 2011;9(8):e1001138.
23. Wiley JD, Merino EF, Krai PM, McLean KJ, Tripathi AK, Vega-Rodriguez J, et al. Isoprenoid precursor biosynthesis is the essential metabolic role of the apicoplast during gametocytogenesis in *Plasmodium falciparum*. *Eukaryotic cell*. 2015;14(2):128-39.

24. Flesch G, Rohmer M. Prokaryotic hopanoids: the biosynthesis of the bacteriohopane skeleton. Formation of isoprenic units from two distinct acetate pools and a novel type of carbon/carbon linkage between a triterpene and D-ribose. *Eur J Biochem.* 1988;175(2):405-11.
25. Eisenreich W, Bacher A, Arigoni D, Rohdich F. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell Mol Life Sci.* 2004;61(12):1401-26.
26. Mbaya B, Rigomier D, Edorh GG, Karst F, Schrevel J. Isoprenoid metabolism in *Plasmodium falciparum* during the intraerythrocytic phase of malaria. *Biochem Biophys Res Commun.* 1990;173(3):849-54.
27. Couto AS, Kimura EA, Peres VJ, Uhrig ML, Katzin AM. Active isoprenoid pathway in the intra-erythrocytic stages of *Plasmodium falciparum*: presence of dolichols of 11 and 12 isoprene units. *Biochem J.* 1999;341 (Pt 3):629-37.
28. Moura IC, Wunderlich G, Uhrig ML, Couto AS, Peres VJ, Katzin AM, et al. Limonene arrests parasite development and inhibits isoprenylation of proteins in *Plasmodium falciparum*. *Antimicrob Agents Chemother.* 2001;45(9):2553-8.
29. de Macedo CS, Uhrig ML, Kimura EA, Katzin AM. Characterization of the isoprenoid chain of coenzyme Q in *Plasmodium falciparum*. *FEMS Microbiol Lett.* 2002;207(1):13-20.
30. Cassera MB, Merino EF, Peres VJ, Kimura EA, Wunderlich G, Katzin AM. Effect of fosmidomycin on metabolic and transcript profiles of the methylerythritol phosphate pathway in *Plasmodium falciparum*. *Mem Inst Oswaldo Cruz.* 2007;102(3):377-83.

31. Rodrigues Goulart H, Kimura EA, Peres VJ, Couto AS, Aquino Duarte FA, Katzin AM. Terpenes arrest parasite development and inhibit biosynthesis of isoprenoids in *Plasmodium falciparum*. *Antimicrob Agents Chemother*. 2004;48(7):2502-9.
32. Cassera MB, Gozzo FC, D'Alexandri FL, Merino EF, del Portillo HA, Peres VJ, et al. The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem*. 2004;279(50):51749-59.
33. Tonhosolo R, D'Alexandri FL, Genta FA, Wunderlich G, Gozzo FC, Eberlin MN, et al. Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*. *Biochem J*. 2005;392(Pt 1):117-26.
34. Jordao FM, Gabriel HB, Alves JM, Angeli CB, Bifano TD, Breda A, et al. Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*. *Malar J*. 2013;12:184.
35. Ling Y, Li ZH, Miranda K, Oldfield E, Moreno SN. The farnesyl-diphosphate/geranylgeranyl-diphosphate synthase of *Toxoplasma gondii* is a bifunctional enzyme and a molecular target of bisphosphonates. *J Biol Chem*. 2007;282(42):30804-16.
36. Anderson MS, Yarger JG, Burck CL, Poulter CD. Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *J Biol Chem*. 1989;264(32):19176-84.
37. Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R. Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. *J Biol Chem*. 2001;276(36):33930-7.

38. Montalvetti A, Fernandez A, Sanders JM, Ghosh S, Van Brussel E, Oldfield E, et al. Farnesyl pyrophosphate synthase is an essential enzyme in *Trypanosoma brucei*. In vitro RNA interference and in vivo inhibition studies. *J Biol Chem*. 2003;278(19):17075-83.
39. Artz JD, Wernimont AK, Dunford JE, Schapira M, Dong A, Zhao Y, et al. Molecular characterization of a novel geranylgeranyl pyrophosphate synthase from *Plasmodium* parasites. *J Biol Chem*. 2011;286(5):3315-22.
40. Kavanagh KL, Dunford JE, Bunkoczi G, Russell RG, Oppermann U. The crystal structure of human geranylgeranyl pyrophosphate synthase reveals a novel hexameric arrangement and inhibitory product binding. *J Biol Chem*. 2006;281(31):22004-12.
41. Ding VD, Sheares BT, Bergstrom JD, Ponpipom MM, Perez LB, Poulter CD. Purification and characterization of recombinant human farnesyl diphosphate synthase expressed in *Escherichia coli*. *Biochem J*. 1991;275 (Pt 1):61-5.
42. Tonhosolo R, D'Alexandri FL, de Rosso VV, Gazarini ML, Matsumura MY, Peres VJ, et al. Carotenoid biosynthesis in intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem*. 2009;284(15):9974-85.
43. Mijts BN, Schmidt-Dannert C. Engineering of secondary metabolite pathways. *Current opinion in biotechnology*. 2003;14(6):597-602.
44. Stahl W, Sies H. Antioxidant activity of carotenoids. *Molecular aspects of medicine*. 2003;24(6):345-51.

45. Okada K, Suzuki K, Kamiya Y, Zhu X, Fujisaki S, Nishimura Y, et al. Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochimica et biophysica acta*. 1996;1302(3):217-23.
46. Okada K, Kamiya Y, Zhu X, Suzuki K, Tanaka K, Nakagawa T, et al. Cloning of the *sdsA* gene encoding solanesyl diphosphate synthase from *Rhodobacter capsulatus* and its functional expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *J Bacteriol*. 1997;179(19):5992-8.
47. Sandmann G. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chembiochem : a European journal of chemical biology*. 2002;3(7):629-35.
48. Sato S, Clough B, Coates L, Wilson RJ. Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*. *Protist*. 2004;155(1):117-25.
49. Gabriel HB, Silva MF, Kimura EA, Wunderlich G, Katzin AM, Azevedo MF. Squalenstatin Is an Inhibitor of Carotenoid Biosynthesis in *Plasmodium falciparum*. *Antimicrob Agents Chemother*. 2015;59(6):3180-8.
50. McConkey GA. Targeting the shikimate pathway in the malaria parasite *Plasmodium falciparum*. *Antimicrob Agents Chemother*. 1999;43(1):175-7.
51. Herrmann KM, Weaver LM. The Shikimate Pathway. *Annu Rev Plant Physiol Plant Mol Biol*. 1999;50:473-503.

52. McConkey GA, Ittarat I, Meshnick SR, McCutchan TF. Auxotrophs of *Plasmodium falciparum* dependent on p-aminobenzoic acid for growth. *Proc Natl Acad Sci U S A*. 1994;91(10):4244-8.
53. Johnson TW, Shen G, Zybailov B, Kolling D, Reategui R, Beauparlant S, et al. Recruitment of a foreign quinone into the A(1) site of photosystem I. I. Genetic and physiological characterization of phylloquinone biosynthetic pathway mutants in *Synechocystis* sp. pcc 6803. *J Biol Chem*. 2000;275(12):8523-30.
54. Tonhosolo R, Gabriel HB, Matsumura MY, Cabral FJ, Yamamoto MM, D'Alexandri FL, et al. Intraerythrocytic stages of *Plasmodium falciparum* biosynthesize menaquinone. *FEBS Lett*. 2010;584(23):4761-8.
55. Dhiman RK, Mahapatra S, Slayden RA, Boyne ME, Lenaerts A, Hinshaw JC, et al. Menaquinone synthesis is critical for maintaining mycobacterial viability during exponential growth and recovery from non-replicating persistence. *Mol Microbiol*. 2009;72(1):85-97.
56. de Azevedo MF, Gilson PR, Gabriel HB, Simoes RF, Angrisano F, Baum J, et al. Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite *Plasmodium falciparum*. *PLoS One*. 2012;7(7):e40981.
57. Baum J, Papenfuss AT, Mair GR, Janse CJ, Vlachou D, Waters AP, et al. Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. *Nucleic Acids Res*. 2009;37(11):3788-98.
58. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*. 2002;419(6906):498-511.

59. Volkman SK, Sabeti PC, DeCaprio D, Neafsey DE, Schaffner SF, Milner DA, Jr., et al. A genome-wide map of diversity in *Plasmodium falciparum*. *Nat Genet.* 2007;39(1):113-9.
60. Gardner MJ, Shallom SJ, Carlton JM, Salzberg SL, Nene V, Shoaibi A, et al. Sequence of *Plasmodium falciparum* chromosomes 2, 10, 11 and 14. *Nature.* 2002;419(6906):531-4.
61. Liao Q, Shen J, Liu J, Sun X, Zhao G, Chang Y, et al. Genome-wide identification and functional annotation of *Plasmodium falciparum* long noncoding RNAs from RNA-seq data. *Parasitology research.* 2014;113(4):1269-81.
62. Clayton CE. Genetic manipulation of kinetoplastida. *Parasitol Today.* 1999;15(9):372-8.
63. Soete M, Hettman C, Soldati D. The importance of reverse genetics in determining gene function in apicomplexan parasites. *Parasitology.* 1999;118 Suppl:S53-61.
64. Wu Y, Kirkman LA, Wellems TE. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *Proc Natl Acad Sci U S A.* 1996;93(3):1130-4.
65. Bullen HE, Tonkin CJ, O'Donnell RA, Tham WH, Papenfuss AT, Gould S, et al. A novel family of Apicomplexan glideosome-associated proteins with an inner membrane-anchoring role. *J Biol Chem.* 2009;284(37):25353-63.

66. Thavayogarajah T, Gangopadhyay P, Rahlfs S, Becker K, Lingelbach K, Przyborski JM, et al. Alternative Protein Secretion in the Malaria Parasite *Plasmodium falciparum*. PLoS One. 2015;10(4):e0125191.
67. Crabb BS, Triglia T, Waterkeyn JG, Cowman AF. Stable transgene expression in *Plasmodium falciparum*. Mol Biochem Parasitol. 1997;90(1):131-44.
68. Waller KL, Muhle RA, Ursos LM, Horrocks P, Verdier-Pinard D, Sidhu AB, et al. Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. J Biol Chem. 2003;278(35):33593-601.
69. Rug M, Wickham ME, Foley M, Cowman AF, Tilley L. Correct promoter control is needed for trafficking of the ring-infected erythrocyte surface antigen to the host cytosol in transfected malaria parasites. Infect Immun. 2004;72(10):6095-105.
70. Azevedo MF, Sanders PR, Krejany E, Nie CQ, Fu P, Bach LA, et al. Inhibition of *Plasmodium falciparum* CDPK1 by conditional expression of its J-domain demonstrates a key role in schizont development. Biochem J. 2013;452(3):433-41.
71. de Koning-Ward TF, Gilson PR. Keeping it simple: an easy method for manipulating the expression levels of malaria proteins. Trends Parasitol. 2009;25(1):4-7.
72. Russo I, Oksman A, Vaupel B, Goldberg DE. A calpain unique to alveolates is essential in *Plasmodium falciparum* and its knockdown reveals an involvement in pre-S-phase development. Proc Natl Acad Sci U S A. 2009;106(5):1554-9.

73. Dvorin JD, Martyn DC, Patel SD, Grimley JS, Collins CR, Hopp CS, et al. A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science*. 2010;328(5980):910-2.
74. Chu BW, Banaszynski LA, Chen LC, Wandless TJ. Recent progress with FKBP-derived destabilizing domains. *Bioorg Med Chem Lett*. 2008;18(22):5941-4.
75. Josling GA, Petter M, Oehring SC, Gupta AP, Dietz O, Wilson DW, et al. A *Plasmodium Falciparum* Bromodomain Protein Regulates Invasion Gene Expression. *Cell host & microbe*. 2015;17(6):741-51.
76. Komaki-Yasuda K, Okuwaki M, Nagata K, Kawazu S, Kano S. Identification of a novel and unique transcription factor in the intraerythrocytic stage of *Plasmodium falciparum*. *PLoS One*. 2013;8(9):e74701.
77. Gardiner DL, Trenholme KR, Skinner-Adams TS, Stack CM, Dalton JP. Overexpression of leucyl aminopeptidase in *Plasmodium falciparum* parasites. Target for the antimalarial activity of bestatin. *J Biol Chem*. 2006;281(3):1741-5.
78. Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS biology*. 2003;1(1):E5.
79. Le Roch KG, Zhou Y, Blair PL, Grainger M, Moch JK, Haynes JD, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science*. 2003;301(5639):1503-8.
80. Perry RP. Processing of RNA. *Annual review of biochemistry*. 1976;45:605-29.

81. Chow LT, Gelinas RE, Broker TR, Roberts RJ. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell*. 1977;12(1):1-8.
82. Kelemen O, Convertini P, Zhang Z, Wen Y, Shen M, Falaleeva M, et al. Function of alternative splicing. *Gene*. 2013;514(1):1-30.
83. Sharp PA. The discovery of split genes and RNA splicing. *Trends in biochemical sciences*. 2005;30(6):279-81.
84. Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, et al. Function of alternative splicing. *Gene*. 2005;344:1-20.
85. Iriko H, Jin L, Kaneko O, Takeo S, Han ET, Tachibana M, et al. A small-scale systematic analysis of alternative splicing in *Plasmodium falciparum*. *Parasitol Int*. 2009;58(2):196-9.
86. Otto TD, Wilinski D, Assefa S, Keane TM, Sarry LR, Bohme U, et al. New insights into the blood-stage transcriptome of *Plasmodium falciparum* using RNA-Seq. *Mol Microbiol*. 2010;76(1):12-24.
87. Sorber K, Dimon MT, DeRisi JL. RNA-Seq analysis of splicing in *Plasmodium falciparum* uncovers new splice junctions, alternative splicing and splicing of antisense transcripts. *Nucleic acids research*. 2011;39(9):3820-35.
88. Bartfai R, Hoeijmakers WA, Salcedo-Amaya AM, Smits AH, Janssen-Megens E, Kaan A, et al. H2A.Z demarcates intergenic regions of the *plasmodium falciparum* epigenome that are dynamically marked by H3K9ac and H3K4me3. *PLoS pathogens*. 2010;6(12):e1001223.

89. Lopez-Barragan MJ, Lemieux J, Quinones M, Williamson KC, Molina-Cruz A, Cui K, et al. Directional gene expression and antisense transcripts in sexual and asexual stages of *Plasmodium falciparum*. *BMC genomics*. 2011;12:587.
90. de Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, Papenfuss AT, et al. A newly discovered protein export machine in malaria parasites. *Nature*. 2009;459(7249):945-9.
91. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science*. 1976;193(4254):673-5.
92. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol*. 1979;65(3):418-20.
93. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. Transfection of *Plasmodium falciparum* within human red blood cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(4):973-7.
94. Fidock DA, Wellems TE. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(20):10931-6.
95. Hawthorne PL, Trenholme KR, Skinner-Adams TS, Spielmann T, Fischer K, Dixon MW, et al. A novel *Plasmodium falciparum* ring stage protein, REX, is located in Maurer's clefts. *Mol Biochem Parasitol*. 2004;136(2):181-9.

96. Gerold P, Schofield L, Blackman MJ, Holder AA, Schwarz RT. Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. *Mol Biochem Parasitol*. 1996;75(2):131-43.
97. Furtado GC, Moura IC, Pudles J, Alvarez JM, D'Imperio Lima MR. *Plasmodium chabaudi chabaudi*: a monoclonal antibody raised against soluble antigens present in the plasma of infected mice recognizes a 250-kDa schizont glycoprotein that is secreted during schizogony. *Exp Parasitol*. 1999;91(1):97-100.
98. Golnitz U, Albrecht L, Wunderlich G. Var transcription profiling of *Plasmodium falciparum* 3D7: assignment of cytoadherent phenotypes to dominant transcripts. *Malar J*. 2008;7:14.
99. Shestopalov AI, Bogachev AV, Murtazina RA, Viryasov MB, Skulachev VP. Aeration-dependent changes in composition of the quinone pool in *Escherichia coli*. Evidence of post-transcriptional regulation of the quinone biosynthesis. *FEBS Lett*. 1997;404(2-3):272-4.
100. Bekker M, Kramer G, Hartog AF, Wagner MJ, de Koster CG, Hellingwerf KJ, et al. Changes in the redox state and composition of the quinone pool of *Escherichia coli* during aerobic batch-culture growth. *Microbiology*. 2007;153(Pt 6):1974-80.
101. de Rosso VV, Mercadante AZ. Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *J Agric Food Chem*. 2007;55(13):5062-72.
102. Dhiman RK, Schulbach MC, Mahapatra S, Baulard AR, Vissa V, Brennan PJ, et al. Identification of a novel class of omega,E,E-farnesyl diphosphate synthase from *Mycobacterium tuberculosis*. *Journal of lipid research*. 2004;45(6):1140-7.

103. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell*. 2006;126(5):995-1004.
104. Herm-Gotz A, Agop-Nersesian C, Munter S, Grimley JS, Wandless TJ, Frischknecht F, et al. Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nat Methods*. 2007;4(12):1003-5.
105. Armstrong CM, Goldberg DE. An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*. *Nat Methods*. 2007;4(12):1007-9.
106. Alexeeva S, Hellingwerf KJ, Teixeira de Mattos MJ. Requirement of ArcA for redox regulation in *Escherichia coli* under microaerobic but not anaerobic or aerobic conditions. *J Bacteriol*. 2003;185(1):204-9.
107. Wickham ME, Thompson JK, Cowman AF. Characterisation of the merozoite surface protein-2 promoter using stable and transient transfection in *Plasmodium falciparum*. *Mol Biochem Parasitol*. 2003;129(2):147-56.
108. Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, et al. Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J Biol Chem*. 2002;277(44):42066-73.
109. Waller RF, Reed MB, Cowman AF, McFadden GI. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. *EMBO J*. 2000;19(8):1794-802.
110. Wiesner J, Jomaa H. Isoprenoid biosynthesis of the apicoplast as drug target. *Current drug targets*. 2007;8(1):3-13.

111. Ortiz-Gomez A, Jimenez C, Estevez AM, Carrero-Lerida J, Ruiz-Perez LM, Gonzalez-Pacanowska D. Farnesyl diphosphate synthase is a cytosolic enzyme in *Leishmania major* promastigotes and its overexpression confers resistance to risidronate. *Eukaryotic cell*. 2006;5(7):1057-64.
112. Ferella M, Li ZH, Andersson B, Docampo R. Farnesyl diphosphate synthase localizes to the cytoplasm of *Trypanosoma cruzi* and *T. brucei*. *Exp Parasitol*. 2008;119(2):308-12.
113. Cunillera N, Boronat A, Ferrer A. The *Arabidopsis thaliana* FPS1 gene generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate synthase isoform. *J Biol Chem*. 1997;272(24):15381-8.
114. Martin D, Piulachs MD, Cunillera N, Ferrer A, Belles X. Mitochondrial targeting of farnesyl diphosphate synthase is a widespread phenomenon in eukaryotes. *Biochim Biophys Acta*. 2007;1773(3):419-26.
115. Taban AH, Tittiger C, Blomquist GJ, Welch WH. Isolation and characterization of farnesyl diphosphate synthase from the cotton boll weevil, *Anthonomus grandis*. *Archives of insect biochemistry and physiology*. 2009;71(2):88-104.
116. Wang K, Ohnuma S. Chain-length determination mechanism of isoprenyl diphosphate synthases and implications for molecular evolution. *Trends in biochemical sciences*. 1999;24(11):445-51.
117. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997;25(17):3389-402.

118. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics*. 2009;10(1):57-63.
119. Bertram G, Innes S, Minella O, Richardson J, Stansfield I. Endless possibilities: translation termination and stop codon recognition. *Microbiology*. 2001;147(Pt 2):255-69.
120. Jungreis I, Lin MF, Spokony R, Chan CS, Negre N, Victorsen A, et al. Evidence of abundant stop codon readthrough in *Drosophila* and other metazoa. *Genome research*. 2011;21(12):2096-113.
121. Blanchet S, Cornu D, Argentini M, Namy O. New insights into the incorporation of natural suppressor tRNAs at stop codons in *Saccharomyces cerevisiae*. *Nucleic acids research*. 2014;42(15):10061-72.
122. Lobanov AV, Delgado C, Rahlfs S, Novoselov SV, Kryukov GV, Gromer S, et al. The *Plasmodium* selenoproteome. *Nucleic acids research*. 2006;34(2):496-505.
123. Mourier T, Pain A, Barrell B, Griffiths-Jones S. A selenocysteine tRNA and SECIS element in *Plasmodium falciparum*. *Rna*. 2005;11(2):119-22.
124. Gamain B, Arnaud J, Favier A, Camus D, Dive D, Slomianny C. Increase in glutathione peroxidase activity in malaria parasite after selenium supplementation. *Free radical biology & medicine*. 1996;21(4):559-65.
125. Lu F, Jiang H, Ding J, Mu J, Valenzuela JG, Ribeiro JM, et al. cDNA sequences reveal considerable gene prediction inaccuracy in the *Plasmodium falciparum* genome. *BMC genomics*. 2007;8:255.

126. Fischer K, Marti T, Rick B, Johnson D, Benting J, Baumeister S, et al. Characterization and cloning of the gene encoding the vacuolar membrane protein EXP-2 from *Plasmodium falciparum*. *Mol Biochem Parasitol*. 1998;92(1):47-57.

127. Eshar S, Allemand E, Sebag A, Glaser F, Muchardt C, Mandel-Gutfreund Y, et al. A novel *Plasmodium falciparum* SR protein is an alternative splicing factor required for the parasites' proliferation in human erythrocytes. *Nucleic Acids Res*. 2012;40(19):9903-16.

128. Cunningham FX, Gantt E. Genes and Enzymes of Carotenoid Biosynthesis in Plants. *Annu Rev Plant Physiol Plant Mol Biol*. 1998;49:557-83.

129. Bergstrom JD, Kurtz MM, Rew DJ, Amend AM, Karkas JD, Bostedor RG, et al. Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. *Proc Natl Acad Sci U S A*. 1993;90(1):80-4.

130. Baxter A, Fitzgerald BJ, Hutson JL, McCarthy AD, Motteram JM, Ross BC, et al. Squalstatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol in vivo. *J Biol Chem*. 1992;267(17):11705-8.

131. Sidebottom PJ, Highcock RM, Lane SJ, Procopiou PA, Watson NS. The squalostatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. II. Structure elucidation. *The Journal of antibiotics*. 1992;45(5):648-58.

132. Dawson MJ, Farthing JE, Marshall PS, Middleton RF, O'Neill MJ, Shuttleworth A, et al. The squalostatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. *The Journal of antibiotics*. 1992;45(5):639-47.

133. de Koning-Ward TF, Speranca MA, Waters AP, Janse CJ. Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter. *Mol Biochem Parasitol*. 1999;100(1):141-6.
134. Fernandez-Becerra C, de Azevedo MF, Yamamoto MM, del Portillo HA. *Plasmodium falciparum*: new vector with bi-directional promoter activity to stably express transgenes. *Exp Parasitol*. 2003;103(1-2):88-91.
135. Neudert U, Martinez-Ferez IM, Fraser PD, Sandmann G. Expression of an active phytoene synthase from *Erwinia uredovora* and biochemical properties of the enzyme. *Biochim Biophys Acta*. 1998;1392(1):51-8.
136. Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, et al. Constitutive Expression of a Fruit Phytoene Synthase Gene in Transgenic Tomatoes Causes Dwarfism by Redirecting Metabolites from the Gibberellin Pathway. *Plant Journal*. 1995;8(5):693-701.
137. Bastien O, Lespinats S, Roy S, Metayer K, Fertil B, Codani JJ, et al. Analysis of the compositional biases in *Plasmodium falciparum* genome and proteome using *Arabidopsis thaliana* as a reference. *Gene*. 2004;336(2):163-73.
138. Cox GB, Gibson F. The role of shikimic acid in the biosynthesis of vitamin K2. *Biochem J*. 1966;100(1):1-6.
139. Stover CK, de la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, et al. New use of BCG for recombinant vaccines. *Nature*. 1991;351(6326):456-60.

140. Nakagawa K, Hirota Y, Sawada N, Yuge N, Watanabe M, Uchino Y, et al. Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme. *Nature*. 2010;468(7320):117-21.

141. Hiratsuka T, Furihata K, Ishikawa J, Yamashita H, Itoh N, Seto H, et al. An alternative menaquinone biosynthetic pathway operating in microorganisms. *Science*. 2008;321(5896):1670-3.

142. Arakawa C, Kuratsu M, Furihata K, Hiratsuka T, Itoh N, Seto H, et al. Diversity of the early step of the futasosine pathway. *Antimicrob Agents Chemother*. 2011;55(2):913-6.

143. Barta ML, Thomas K, Yuan H, Lovell S, Battaile KP, Schramm VL, et al. Structural and biochemical characterization of *Chlamydia trachomatis* hypothetical protein CT263 supports that menaquinone synthesis occurs through the futasosine pathway. *J Biol Chem*. 2014;289(46):32214-29.

144. Hiratsuka T, Itoh N, Seto H, Dairi T. Enzymatic properties of futasosine hydrolase, an enzyme essential to a newly identified menaquinone biosynthetic pathway. *Biosci Biotechnol Biochem*. 2009;73(5):1137-41.

I- Single-target high-throughput transcription analysis reveal high levels of alternative splicing present in the FPPS/GGPPS from *Plasmodium falciparum*. (submitted)

1 **Single-target high-throughput transcription analysis reveal high levels of**
2 **alternative splicing present in the FPPS/GGPPS from *Plasmodium falciparum***

3

4 Heloisa B. Gabriel¹, heloisaberti@usp.br

5 Mauro F. de Azevedo¹, maurousp@gmail.com

6 Giuseppe Palmisano¹, palmisano.gp@usp.br

7 Gerhard Wunderlich¹, gwunder@usp.br

8 Emília A. Kimura¹, eakimura@usp.br

9 Alejandro M. Katzin¹, amkatzin@icb.usp.br

10 João M. P. Alves^{1*}, jotajj@usp.br

11

12 Author affiliations:

13 1 – Department of Parasitology, Institute of Biomedical Sciences, University of São
14 Paulo, São Paulo, Brazil.

15

16 * Corresponding author

17

18 **Abstract**

19 Malaria is a tropical disease with significant morbidity and mortality. A better
20 understanding of the metabolism of its most important etiological agent, *Plasmodium*
21 *falciparum*, is paramount to the development of better treatment and other mitigation
22 measures. Farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase (FPPS/GGPPS)
23 is a key enzyme in the synthesis of isoprenic chains present in many essential structures. In *P.*
24 *falciparum*, as well as a handful of other organisms, FPPS/GGPPS has been shown to be a
25 bifunctional enzyme. By genetic tagging and microscopy, we observed a changing localization
26 of FPPS/GGPPS in blood stage parasites. Given the great importance of alternative splicing and
27 other transcriptional phenomena in gene regulation and the generation of protein diversity,
28 we have investigated the processing of the FPPS/GGPPS transcript in *P. falciparum* by high-
29 throughput sequencing methods in four time-points along the intraerythrocytic cycle of *P.*
30 *falciparum*. We have identified levels of transcript diversity an order of magnitude higher than
31 previously observed in this organism, as well as a few stage-specific splicing events. Our data
32 suggest that alternative splicing in *P. falciparum* is an important feature for gene regulation
33 and the generation of protein diversity, and that the FPPS/GGPPS gene plays a crucial role in
34 isoprenoid biosynthesis.

35

36 **Keywords:** *Plasmodium falciparum*; malaria; isoprenoid pathway; alternative splicing

37

38 **Introduction**

39 Malaria is one of the most important infectious diseases in the world, causing about
40 200 million clinical cases and nearly 600 thousand deaths every year. The causative agent of
41 malaria is a protozoan of the genus *Plasmodium*, transmitted by the female *Anopheles*
42 mosquito host, in which the sexual phase of the parasite's life cycle takes place [1]. Of the five
43 *Plasmodium* species that infect humans, *P. falciparum* is responsible for the vast majority of

44 severe forms of the disease, including deaths. The increasing resistance of this parasite to
45 virtually all current drugs, such as artemisinin and its derivatives in five South-East Asian
46 countries and probably in South America [2], calls for combined therapy using drugs to which
47 the parasites have not yet developed resistance, as well as for identifying new drug targets [3].
48 Therefore, the exact knowledge about parasite metabolic pathways is crucial for a better
49 understanding of the parasite's physiology and consequently the development of new
50 chemotherapeutics.

51 An important target for the development of new antimalarial drugs is isoprenoid
52 biosynthesis (Figure 1), which occurs via the 2-C-methyl-D-erythritol-4-phosphate (MEP)
53 pathway [4-8] in *P. falciparum*, some plants, and most bacteria [9, 10]. In contrast, most animal
54 cells, certain eubacteria, archaea, and fungi synthesize isoprenoid precursors through the
55 mevalonate pathway [11]. Many types of isoprenoids biosynthesized by *P. falciparum*, such as
56 carotenoids, menaquinone and α -tocopherol [8, 12-14], are essential components of the
57 cellular machinery of many organisms, participating in a variety of biological processes. All
58 isoprenoids are derived from a common precursor, isopentenyl diphosphate (IPP), and its
59 isomer dimethylallyl diphosphate (DMAPP). Farnesyl diphosphate synthase (FPPS), which
60 belongs to a family of enzymes classified as prenyltransferases, catalyzes the consecutive
61 head-to-tail condensation of IPP with DMAPP to form geranyl diphosphate (GPP). Then, a
62 second condensation between GPP and IPP forms farnesyl diphosphate (FPP). In *P. falciparum*,
63 this enzyme is bifunctional (PF3D7_1128400), having geranylgeranyl diphosphate synthase
64 (GGPS) activity able to condense FPP with a further molecule of IPP to form the 20-carbon
65 isoprenoid geranylgeranyl diphosphate (GGPP) (Figure 1)[15]. FPP and GGPP function as
66 substrates for the first reaction of several branched pathways leading to the synthesis of
67 compounds such as ubiquinone, dolichol, menaquinone, carotenoids, and prenylated proteins.
68 FPPS and GGPPS are the most studied prenyltransferases and have been described in various
69 organisms from all three domains, Eukarya, Bacteria, and Archaea [16]. In protist parasites,

70 the FPPS and/or GGPPS gene from *Trypanosoma cruzi* [17], *Trypanosoma brucei* [18],
71 *Plasmodium vivax* [19], and *Toxoplasma gondii* were identified [20]. In this last case, the
72 enzyme has been described as a bifunctional enzyme, and this is also true for *P. falciparum*. In
73 the case of malaria parasites, especially the most virulent species, *P. falciparum*, a number of
74 new “plant-like” enzymes participating in the isoprenoid pathway were recently discovered.
75 Some of these enzymes are associated with the apicoplast [21], , an organelle acquired by a
76 secondary symbiosis [22] and absent in mammalian cells. This obviously turns all structures
77 contained therein prime targets for chemotherapeutic intervention.

78 Transcriptome analysis has shown that alternative splicing plays an important role
79 generating a large number of mRNA and protein isoforms [23, 24]. Recently, it was observed
80 that the occurrence of alternative splicing events in *P. falciparum* is not as rare as previous
81 studies had reported [25]. Although 7,406 introns have been predicted in its genome,
82 alternative splicing that might affect protein function has been observed only for a few genes
83 [25]. Alternative splicing can result in modulation of transcript expression levels by subjecting
84 mRNAs to nonsense-mediated decay (NMD) by stop codon addition, or in alteration of the
85 structure of the gene product by changing, deleting or inserting amino acids in the protein,
86 therefore influencing their intracellular localization and modifying their enzymatic activity
87 and/or protein stability[23].

88 Studies have reported the occurrence of several isoforms of the FPPS and/or GGPPS,
89 through alternative splicing events, in some organisms [26]. *Martin et. al.* [27] showed that
90 the FPPS gene undergoes alternative splicing in mammals leading to two different transcripts:
91 a transcript encoding an isoform containing an extension of 66 amino acids with an N-terminal
92 peptide that targets the mitochondria, while the smaller transcript is directed to the cytosol. In
93 *P. falciparum*, antisense transcripts corresponding to FPPS/GGPPS have been recently
94 demonstrated in sexual forms of the parasite [28].

95 In next generation sequencing efforts of the transcriptome and epigenome of *P.*
96 *falciparum* sexual and asexual stages, including samples from clinical isolates [28-31], a large
97 number of additional intron-exon splicing junctions missed by the initial genome annotation
98 have been reported. Also, antisense transcripts and alternative splicing events were
99 encountered and provided improved EST coverage and genome annotation.

100 In this study, during analysis of the localization of the protein FPPS/GGPPS, we
101 observed different patterns of localization along the intra-erythrocytic cycle of the parasite,
102 which led to the hypothesis that alternative splicing might be contributing to these differences.
103 Thus, we have used the 454 sequencing platform for deep mRNA sequencing of the
104 FPPS/GGPPS gene exclusively, using material from four time points of intraerythrocytic stages
105 representing rings (R), early trophozoites (ET), late trophozoites (LT), and schizonts (S). We
106 have detected high levels of alternative splicing of the FPPS/GPPS transcript, including possibly
107 stage-specific, alternatively spliced isoforms. Our data suggest that this gene might play a
108 crucial role in the survival of the parasite, given alternative splicing's potential as an important
109 strategy in the parasite for gene regulation and the generation of protein diversity.

110

111 **Results and discussion**

112 Farnesyl pyrophosphate synthase/geranylgeranyl pyrophosphate synthase
113 (FPPS/GGPPS) is a major elongation bifunctional enzyme of the isoprenoid pathway which
114 belongs to the prenyltransferases family. It is a branch-point enzyme responsible for
115 elongation of the chain isoprene. Changes in FPPS/GGPPS activity could alter the flux of
116 isoprenoids towards various branches of this pathway and, hence, play a crucial role in the
117 regulation of isoprenoid metabolism [26]. IPP and DMAPP are required to synthesize
118 isoprenoid products, and *Yet and DeRisi* 2011 [32] demonstrated that the isoprenoid precursor
119 biosynthesis is not only essential for the parasite but in fact the sole essential function of the
120 apicoplast during blood-stage growth.

121

122 *Protein localization*

123 Using a parasite transgenic line where FPPS/GGPPS is expressed in fusion with a HA
124 tag, we have previously demonstrated that the enzyme is present throughout the asexual
125 stages in the intra-erythrocytic cycle [15]. In order to determine its localization in live
126 parasites, another transgenic line was generated where FPPS/GGPPS is expressed in fusion
127 with GFP-HA (data not shown). Analysis by fluorescence microscopy of live parasites confirms
128 expression along the intra-erythrocytic cycle and shows FPPS/GGPPS localization throughout
129 the cytoplasm and also forming spots, which increase in number as parasites mature from
130 trophozoite to schizont stages (Figure 2A). To investigate to which subcellular compartment
131 the detected spots correspond, parasites labeled with the mitochondrial marker MitoTracker
132 were similarly analyzed (Figure 2B, upper panel). A distinct pattern is detected, suggesting
133 FPPS/GGPPS does not colocalize with the mitochondria. Fixed parasites were analyzed by
134 immunofluorescence using antibodies against the HA tag and the apicoplast marker acyl
135 carrier protein (ACP) [33], showing that the enzyme does also not colocalize to the apicoplast
136 (Figure 2B, bottom panel). These organelles are the location of the precursors [34], products
137 [32] or enzymes [12] already described in the isoprenoid pathway in *Plasmodium falciparum*.
138 Cytosolic localization of FPPS was demonstrated in *Leishmania major* [35], *Trypanosoma cruzi*
139 and *Trypanosoma brucei* [36]. However, in *Toxoplasma gondii*, whose enzyme is also
140 characterized as bifunctional, FPPS is located in the mitochondria [20]. Our results suggest a
141 differential cellular targeting of this protein, possibly influenced by isoforms generated
142 through alternative splicing events. In many organisms, such as plants [37], mammals [27] and
143 insects [38] more than one isoform for FPPS and/or GGPPS is present, with different
144 localization patterns. In *Toxoplasma gondii* the presence of two isoforms has been
145 demonstrated. Interestingly, the transcription level of one is much higher than that of the

146 other isoform, but these relative expression levels do not vary between tachyzoite and
147 bradyzoite stages of the parasite [20].

148

149 *Transcript sequencing and mapping*

150 Gene regulation through alternative splicing is more versatile than regulation through
151 promoter activity [23]. Some full-length cDNA and EST data have been used for analysis of
152 transcript structure and variants, improved genome annotation and gene models in *P.*
153 *falciparum* [28-31]. RNA-Seq provides the advantage of capturing an entire transcriptome at
154 great depth, enabling detection of low copy number transcripts and variants [30]. We
155 demonstrate for the first time, to our best knowledge, a specific RNA-Seq analysis, for just one
156 gene (FPPS/GGPPS) from four timepoints in the intraerythrocytic cycle of the parasite. *Otto et.*
157 *al.* 2010 [29] suggest that roughly 90% of the genome of this parasite is transcriptionally active
158 during this stage. Sequencing of the amplified transcripts from each of the four libraries
159 yielded good coverage, with about 26.8 to 44.3 million bases sequenced, distributed
160 throughout about 49,000 (ring), 81,000 (early trophozoite), 54,000 (late trophozoite), or
161 76,000 (schizont) reads. After quality trimming, only between 300 to 900 reads per library (or
162 about 1% of the total, at the most) were discarded due to having regions of average quality
163 lower than 20 or trimmed read shorter than 30 bases. Since the average read length was
164 between 519 and 621 bases, the window used by the trimmer (sickle) was typically about 52 to
165 62 bases in length. Overall sequence coverage is shown in Additional file 2 and, as expected
166 due to the average read sequence length compared to the full transcript size, it is clear that
167 sequence coverage is lowest in the mid-point of the gene, between exons 6 and 7.
168 Nonetheless, minimum coverage is still quite high (around 5,000-fold). Coverage at intronic
169 regions (gray bands) is obviously mostly absent and is represented by flat lines in parts of the
170 graph where there are no sequenced bases (Add. file 2). Even in this coarse representation it is
171 already possible to identify some of the alternative splicing events described below, like the

172 partial retention of the end of intron 2, most evident in the schizont stage, or the partial
173 retention of the end of intron 7, well distinguishable in all stages.

174 The aligner (STAR) was able to map between 88% and 94% of the trimmed reads to the
175 reference sequence, which consisted of the full length FPPS/GGPPS gene from start to stop
176 codon, and including all annotated introns. Using STAR's output files, isoform_threader was
177 able to identify 329 putative splice junction regions, which after manual inspection dropped to
178 98 high confidence predictions (Additional file 3). All subsequent analyses were performed
179 using these filtered junctions. Intron donor and acceptor splicing sites were classified as
180 canonical (GT...AG) or alternative (anything else). Canonical junctions accounted for 78 while
181 alternative ones accounted for 252 of all junctions originally seen; after filtering, these
182 numbers dropped to 58 and 40, respectively, strongly suggesting that most low confidence
183 alternative splicing junctions originally seen were produced by sequencing and/or alignment
184 errors eliminated by manual curation.

185

186 *Stage-specific junctions*

187 *P. falciparum* has a complex life cycle with different functional characteristics
188 associated with specific gene expression patterns [39]. Thus, the observation of stage-specific
189 transcriptional differences suggests that these new splice junctions are likely associated with
190 some unique features of the parasite's developmental stages, and probably with isoprenoid
191 pathway regulation. López-Barragán *et al.* 2011 [28] demonstrated 201 alternative splicing
192 events affecting 178 genes in *P. falciparum*, and, of these, 124 isoforms occurred in a stage-
193 specific manner. Figure 3 shows the different splice junctions, and their relative quantities (see
194 also Additional file 3), found for each of the life-cycle stages. Our results observed a total of 98
195 high confidence predictions splice junction regions for FPPS/GGPPS, and among these, 63 are
196 only present in one or two stages of the parasite cycle analyzed (Additional file 3). The most
197 abundant novel junctions appear in all four life cycle stages analyzed, such as junctions 179 or

198 199, which delete exon 7 or parts thereof, respectively, or 238 and 248, which delete exon 10
199 or part of exon 11, respectively. Many other junctions, on the other hand, are distributed in a
200 stage-specific way, such as junction 2 (deletes part of exon 1), which is absent in the ring stage,
201 or junction 17, which deletes exon 2 (absent in early trophozoite and schizont), or junctions 76
202 and 77, exclusively seen in late trophozoite, and which delete about half of exon 3 or that plus
203 the whole of exon 4, respectively. We can also observe isoforms present only in the schizont
204 stage, such as 216, which deletes a part of exon 8 and the whole of exon 9. Apart from a few
205 splice junction combinations (see below), most of the new junctions introduced stop codons a
206 few residues thereafter, suggesting either a much shorter final protein product or, more likely,
207 the production of an mRNA that will be subjected to nonsense-mediated decay and not
208 generate any protein product – in which case the function of these alternative splicing events
209 might be purely regulatory.

210

211 *Viable isoforms*

212 Most of the novel splice junctions identified in this work seem to, by themselves and
213 absent any other variant or compensating transcriptional or translational phenomenon, lead to
214 frame-shifts and therefore stop codons that would terminate translation soon after the splice
215 junction – a possibility exacerbated by *P. falciparum*'s very high AT-content. However, we
216 identified 40 cases (Figure 4 and Additional files 4 and 5) where it is potentially possible to get
217 a reasonable protein product (i.e., the length of the final protein produced is at least half of
218 that of the annotated protein). Given the huge number of combinatorial possibilities (about
219 3.63 billion, in our estimate) for generating isoforms using the splice junctions identified in this
220 work, we have only analyzed variant combinations that have been experimentally seen in our
221 sequencing data. Since no single 454 read can cover a complete transcript, and most cover at
222 the best half of the predicted complete transcript, we have “padded” variant isoforms with
223 sequence processed according to the splice junctions of the annotated variant (Additional file

224 4). In that way, it was possible to generate predicted variants that present sequences starting
225 from exon 1 and ending as close as possible to exon 11, regardless of the direction in which
226 they were originally sequenced. The exact splice junction composition of each variant
227 analyzed, as well as the count of each variant in each of the four life-cycle stages analyzed
228 here, can be found in Additional file 5.

229 As can be seen in Figure 4, most variants display deletion in parts of one or more
230 exons, with only a few presenting the insertion of short new sequences – with the exception of
231 variants var230, var148, and var067. The last one, incidentally, is the only variant that
232 terminates significantly before the previously annotated stop codon, leading to the complete
233 removal of exons 8 through 11. One variant (var357) lacks a significant portion of the N-
234 terminal region, and is also the only one without an obvious candidate for starting codon. Four
235 other variants present slightly different N-terminal regions compared to the annotated
236 isoform, which could potentially have significance for protein targeting in the cell (although
237 our searches using *in silico* cellular targeting predictors did not show a significant difference
238 between the scores of these variants).

239

240 *Stop codon readthrough*

241 One in-frame stop codon was observed in each of five of the potentially viable
242 isoforms – although in the case of var148, it occurs just 38 amino acids before the end of the
243 protein. This observation raises the possibility of in-frame stop codon readthrough [40] in the
244 FPPS/GGPPS transcripts in *P. falciparum*. This phenomenon, recently shown to be common in
245 organisms like *Drosophila* and others [41, 42] and shown to be strictly regulated [43], consists
246 of the reading of a stop codon as a signal for the incorporation of an amino acid instead.

247 Variants var75, var128, and var148 present UGA as the in-frame stop codon. As
248 previously seen, at first mainly in viruses but later also in cellular organisms from all domains
249 of life, one of the mechanisms of in-frame stop codon readthrough is recoding, from a UGA

250 stop codon to selenocysteine [40]. The analysis of the *P. falciparum* genome [44] has shown
251 features that support the possibility of stop codon readthrough. This would lead to the
252 incorporation of selenocysteine to proteins in this parasite, which is supported by the presence
253 of a selenocysteinyl-tRNA, proteins necessary for the selenocysteine insertion machinery, and
254 also conserved putative selenocysteine-insertion sequences (SECIS) found in the 3'-UTR of four
255 *Plasmodium*-specific genes [45, 46]. Considering that *P. falciparum* presents very limited
256 redundancy in its tRNA gene repertoire [44], it seems significant that a selenocysteinyl-tRNA
257 has been retained in the genome. It has also been observed experimentally that
258 supplementation of the culture medium with selenium increases the parasite growth rate [47].
259 Together, these observations strongly suggest the importance of selenoproteins for the
260 parasite, and thus the need for in-frame UGA stop codon recoding. Whether this phenomenon
261 is actually happening in the novel FPPS/GGPPS transcripts characterized here is uncertain until
262 confirmation based on further, more suitable experimental data analyzing the proteins
263 produced.

264 The other two variants (var024 and var235) presenting an in-frame stop codon have a
265 UAG, located exactly between exons 8 and 9. In the case of var024 there is an insertion,
266 exclusive to this variant, of nine amino acids (IIIIFFFL) immediately before the in-frame stop
267 codon. Besides the above-mentioned selenocysteine incorporation, other amino acids have
268 been observed replacing an in-frame stop codon, employing other, less well-characterized
269 mechanisms. Particularly, UAA and UAG codons have been detected as coding for glutamine,
270 tyrosine, or lysine, whereas tryptophan, cysteine, and arginine were coded for by a UGA codon
271 [42]. Thus, if readthrough is indeed happening in the newly described isoforms, it might be
272 occurring a different number of ways. Again, more experimental data focusing on the actual
273 proteins will help clarify these questions.

274

275 *Functional domains*

276 Amino acid sequence alignment of FPPS from different organisms revealed conserved
277 regions I to VII with two characteristic aspartate rich motifs, one in region II called FARM (first
278 Asp-rich motif) and in region VI called SARM (second Asp-rich motif) [15]. A hydrophilic side
279 chain at the fifth amino acid upstream of the FARM region plays a crucial role in the
280 production of both GGPP and FPP [48]. This region determines the final products of all *E*-
281 isoprenyl diphosphate synthases and can thus be designated as the chain-length
282 determination (CLD) region [48]. It has been shown that the presence of a cysteine at the fifth
283 position is essential for the FPPS/GGPPS bifunctionality in *T. gondii* [20] for example, and a
284 bulky phenylalanine at this same position in the methanobacterial version of this enzyme also
285 leads to the production of GGPP and FPP [49]. Narita *et al* [50] showed that in *B.*
286 *stearothermophilus* FPP synthase could be converted to GPP synthase by the substitution of
287 phenylalanine by serine at the fourth position before the FARM. In *P. falciparum*, the FARM
288 occurs in the middle of exon 4, while the SARM occurs very close to the beginning of exon 8.

289 In this work we can observe one isoform (var094) that deletes the FARM and two
290 (var034 and var067) that deletes the SARM (Figure 4) indicating that the corresponding protein
291 are transcribed in their entirety without these regions, and with probable loss of FPPS and/or
292 GGPPS function. Thus one of the major functions of these variants generated by alternative
293 splicing events may be the regulation in the formation of the main precursors of the isoprenoid
294 pathway: GPP, FPP or GGPP.

295

296 *Exon-skipping, partial deletion, or new intron creation*

297 A splicing variant with significant importance is exon-skipping, where an exonic
298 sequence that is usually present in the mature transcript is removed together with its flanking
299 introns, resulting in a shorter transcript. In this work, we have observed a number of potential
300 partial exon deletions or complete exon-skipping events – where part of or the whole of the
301 exon gets removed, respectively (Additional file 3 and Figure 4). We have also seen three cases

302 of new intron creation, where a sequence internal to a previously annotated exon gets
303 removed from the transcript; one of these events involved exon 2, and the other two involved
304 exon 6.

305 To validate the intron-exon junctions and alternatively spliced events detected here by
306 RNA-seq, we randomly selected three of the splicing junctions identified (Additional file 6). We
307 designed primers flanking the newly described splice sites and PCR amplified cDNA from the
308 same schizont RNA samples used for library construction (isolate 3D7) and two field isolates
309 from patients, to discard the possibility of any artifacts from strains kept in culture for
310 extended periods. We confirm the occurrence of these three alternative splicing events, and
311 corroborate the data quantification by RT-PCR, indicating that the isoform(s) lacking exon 7,
312 for example, are about 100 times less expressed than those presenting it (Additional file 7).

313

314 *Intron retention*

315 Intron retention is the splicing variant event where a piece of sequence that is usually
316 removed from the primary transcript is included in the mature transcript. We have
317 investigated the presence of intron retention in FPPS/GGPPS from all four stages present in
318 this study. To try to minimize the number of false positives due to the possible sequencing of
319 unprocessed transcripts, we have only considered those events occurring in reads that
320 presented at least one other intron removed from the transcript. Table 1 presents the number
321 of putative intron retention events observed in our FPPS/GGPPS reads. Retention events of
322 introns 1, 2, or 4 were significantly different between stages of the intraerythrocytic cycle of
323 the parasite, while introns 7, 8, and 10 presented similar (low) levels of retention in all stages.
324 Introns 3, 5, 6, and 9 were not involved in any retention event.

325 Certain life-cycle stages seemed to be more intensely involved in different retention
326 events (Table 2). Thus, the schizont retention count was significantly different from counts
327 from other stages for intron 1 (but the other three stages had similar counts to each other), for

example. For intron 4, the retention count for late trophozoite was the outlier. For intron 2, on the other hand, the early trophozoite retention count was significantly different from both ring and schizont counts, but not from the late trophozoite count. It is not clear why certain introns, especially those closer to the start of the transcript, are much more retained than others (Table 1). However, the fact that intron retention seems to be stage-specific suggests that these events may have regulatory importance. Previous work reported intron retention cases in *P. falciparum* which occurs in less than 50% of the analyzed transcripts [30, 51]. Our data support these conclusions, showing that this is among the rarest categories of alternative splicing in these organisms, and again emphasizes the importance of the new splice junctions in the regulation and development of the parasite.

The only intron retention event that could eventually lead to a functional protein is the one involving intron 1, which introduces an in-frame stop codon (UAA) about halfway through the translated intron sequence. As mentioned above for other kinds of splicing variants, this could be another case of in-frame stop codon-readthrough leading to extra proteins in the organism. Although the significance of this extra sequence is not completely clear at present, it is very interesting to note that a search for targeting signal with TargetP indicates a very high (0.974) NN score for “other” location (i.e. not mitochondria, chloroplasts, nor secretory), while the annotated isoform presents a slightly lower score of 0.881. Further experimental investigation of this possible variant could show whether it is contributing to the concentration of FPPS/GGPPS in the spots seen in the cytoplasm described above.

348

349 *Antisense transcripts*

P. falciparum variability in transcription of the FPPS/GGPPS enzyme had been previously demonstrated in sexual stages by López-Barragán *et al* 2011 [28], in the antisense transcript category. Antisense transcripts are noncoding RNAs that may act predominantly as regulators of sense gene expression [28]. Genes with antisense transcription have an increased

354 number of annotated isoforms compared to genes without antisense transcription, as
355 described in several organisms, such as amphibians, fish, insects, birds, nematodes, and
356 mammals [52]. In the RNA masking model, an antisense RNA may mask a splice site on the
357 sense pre-mRNA sequence, leading to an alternative splicing event [28]. Due to the nature of
358 the method we used, which uses primers to PCR-amplify only one gene in particular, the
359 description of the likely occurring antisense transcripts was not possible in this work.

360

361 *Proteomic investigation*

362 In order to investigate whether any of the isoforms present in the parasite got
363 translated into protein, we have analyzed parasite protein extracts by mass spectrometry.
364 From a total of 17 samples from the SDS-PAGE, with proteins in the size range of 30 to 50 kDa,
365 we obtained a total of 1,383 high confidence peptides. In our results, we have found proteins
366 already well-studied in this parasite, such as MSP2 - merozoite surface protein-2 [53] or EXP-2 -
367 Exported protein-2, a vacuolar membrane protein exposed into the vacuolar space [54]. We
368 also found SR1 protein, a pre-mRNA splicing factor involved in regulation of alternative splicing
369 in *Plasmodium falciparum* [55].

370 More significantly, as an internal control, is the fact that we have found, in the 41 kDa
371 gel band, three high confidence peptides from the annotated version of the FPPS/GGPPS
372 protein. Taken together, these observations show that our data correctly reflects the expected
373 proteome of *P. falciparum*.

374 On the other hand, we have failed to detect any peptide mapping to variant protein
375 forms predicted from the alternatively spliced transcripts uncovered here. Given the sensitivity
376 limits of the proteomic methodology employed here, and taking into account that the
377 annotated isoform is almost always more than 1,000 times more abundant than any of the
378 variants in the transcriptome, it is likely that a different, more sensitive method must be used

379 in order to detect any proteins potentially derived from variant isoforms of the FPPS/GGPPS
380 gene in *P. falciparum*.

381

382 **Conclusions**

383 FPPS/GGPPS is an essential enzyme, responsible for the regulation of a whole pathway
384 that is extremely important for the survival of the parasite, as previously mentioned [15, 32]
385 Alternative splicing events in *P. falciparum*, as seen in many other organisms, are important
386 mechanisms that both work in increasing protein diversity and possible functionalities beyond
387 what could be afforded by the organism's gene complement by itself. Such variation could also
388 contribute to differences in protein localization throughout the cell, as well as provide
389 significant post-transcriptional gene regulation. In this work, we have examined in detail the
390 transcriptional products for the FPPS/GGPPS gene, uncovering unprecedented variability in
391 splicing of this *P. falciparum* gene. This suggests that proteomic complexity in this organism
392 could be much higher than estimated by looking at the relatively small annotated genome, and
393 more in line with what seems necessary for a complex life-cycle like the one displayed by
394 malaria parasites.

395

396 **Methods**

397 *P. falciparum* culture

398 Cultures of *P. falciparum* clone 3D7 were grown as described [56], except that human
399 serum was replaced with Albumax I (0.5%, Invitrogen/Life Technologies). Parasite
400 development and multiplication were monitored by microscopic evaluation of Giemsa-stained
401 thin smears.

402

403 *Plasmid construction*

404 The integration vector pFPPS/GGPPS-HA used herein is described elsewhere [57]. The
405 HA tag of this vector was replaced with the sequence encoding a GFP-HA fusion, retrieved
406 from pEF-Luc-GFP-HA-DD24 [58], generating pFPPS/GGPPS-GFP-HA.

407

408 *Parasite transfection*

409 Parasites were transfected as previously described [59], using the electroporation
410 conditions established elsewhere [60]. Briefly, *P. falciparum* 3D7 was cultured in 4%
411 hematocrit in RPMI-HEPES supplemented with 0.5% Albumax 1 (Invitrogen). Ring stage
412 parasites at 5–8% parasitemia were transfected with 150 µg of pFPPS/GGPPS-GFP-HA and
413 submitted to drug pressure with 2.5 nM WR99210 starting two days later. Parasites were
414 cultivated in standard conditions until parasites re-appeared and normal growth was re-
415 established. The integration at the genomic FPPS/GGPPS locus was selected by intermittent
416 exposure and retrieval of WR99210 and checked by PCR under standard conditions using
417 oligonucleotides inside and outside the integrated locus. Parasites transfected with
418 pFPPS/GGPPS-HA are described in *Jordão et al* [57].

419

420 *Immunofluorescence*

421 For immunofluorescence analysis, we have followed the protocol described by *Furtado*
422 *et al.* [61] with modifications. Infected erythrocytes (5-10% parasitemia in 10% hematocrit)
423 were fixed with 3.7% formaldehyde / PBS for 3 hours, permeabilized with 0.1% BSA, 0.005%
424 PBS-saponin for 25 min twice and incubated with α-HA primary antibody (1: 100 dilution,
425 Sigma), and α-ACP primary antibody (1: 200 dilution) diluted in 0.1% BSA, 0.001% saponin /
426 PBS for 1 hour at 37 ° C and finally incubated with their secondary antibody Alexa Fluor (®) 488
427 diluted in 0.1% BSA, 0.001% saponin / PBS for 45 min at 37 ° C. After washing, the material was
428 dried in air for immunofluorescence slides prepared with Vectashield and then analyzed by
429 confocal microscopy (LSM 780-NLO).

430 For analysis with live parasites expressing GFP, we have utilized Mitotracker (1:10000
431 dilution, Molecular Probes) for 30 min at 37 ° C and then visualized by fluorescence microscopy
432 (Zeiss LSM710) or confocal microscopy (LSM 780-NLO).

433

434 *cDNA preparation; PCR and RT-PCR*

435 The total RNA of intraerythrocytic stages of *P. falciparum* (ring – at 10 hours, early
436 trophozoite - at 24 hours, late trophozoite – at 35 hours, and schizont – at 45 hours) was
437 extracted using TRIZOL LS (Invitrogen) following manufacturer instructions. The washed RNA
438 pellet was briefly dried at room temperature and dissolved in water and stored at –80°C until
439 use. About 4 µg of total RNA were used for cDNA synthesis. Briefly, total RNA was treated 3
440 times with DNase I (Fermentas) prior to synthesis to prevent genomic DNA contamination.
441 Treated RNA was reverse transcribed using MuLV-Revert Aid reverse transcriptase (Fermentas)
442 and random hexamer primers following the manufacturer's instructions. Oligonucleotides (F-
443 FPPS/GGPPS-BamHI; R-FPPS/GGPPS-PstI – additional file 1) that amplify the transcript that
444 encodes the FPPS enzyme were designed using the Primer3 server (<http://frodo.wi.mit.edu/>).
445 PCR reactions were performed with Taq polymerase enzyme (Invitrogen) using the following
446 program: 95 ° C for 5 minutes; 35 cycles of 95 °C for 40 seconds; annealing temperature of 58
447 °C for 40 seconds; 72 °C for 90 seconds; followed by a final extension at 72 °C for 10 minutes.

448 For confirmation experiments, oligonucleotides (F-iso5; R-iso5 - F-iso7; R-iso7 - F-iso10;
449 R-iso10 – additional file 1) that target regions specific for detection of a particular alternative
450 splicing event were designed using Primer3. PCR reactions were performed with Taq
451 polymerase enzyme (Invitrogen) using the following program: 95 °C for 5 minutes; 35 cycles of
452 95 °C for 40 seconds; annealing temperatures of: 59 °C for F-iso5; R-iso5; 57 °C for F-iso7; R-
453 iso7 and 59 °C for F-iso10; R-iso10 for 40 seconds; 72 °C for 90 seconds; followed by a final
454 extension at 72 °C for 10 minutes.

455 For RT-PCR experiments, the oligonucleotides (control – F-iso; R-iso and F-iso7; R-iso7
456 – additional file 1) amplified with the same performance regarding the internal control gene ($\pm$
457 1 Ct), seryl-tRNA synthetase (PF3D7_0717700[62]). Quantitative PCR assays were performed
458 using the SYBR Green Mix (Thermo Scientific), using the PCR template in a StepOne Real-Time
459 PCR machine (Applied Biosystems). All reactions were performed in triplicate permitting not
460 more than 0.5 units deviation between individual Ct values. Samples with Cts over 35 were
461 considered as unamplified targets. Dissociation curves of amplicons were analyzed for all
462 reactions, wherein each amplicon has a specific temperature characterizing the specificity of
463 the obtained products. The relative transcript abundance for each pair of primers was
464 determined independently by subtracting the Ct value measured for each sample from the Ct
465 value of the internal control target using the formula $2^{-\Delta Ct}$ [63].

466

467 *High-throughput sequencing*

468 Amplified FPPS/GGPPS transcripts were sequenced to high depth using the 454 GS FLX
469 platform (performed by Macrogen Inc., South Korea), with expected read lengths of about
470 600-800 bases on average. For each of the four intra-erythrocytic time-points, one sequence
471 library was constructed using 25 ng/ μ l for ring, 75 ng/ μ l for early trophozoite, 45 ng/ μ l for late
472 trophozoite and 65 ng/ μ l for schizonts of the amplification product. The sequencing was
473 designed to generate an average of 50,000 reads per library.

474

475 *Transcript sequence analysis*

476 Resulting transcript sequences were quality trimmed by sickle [64] using a minimum
477 average quality of 20 and minimum trimmed read length of 30 bases. Trimmed reads were
478 mapped to the full FPPS/GGPPS gene (locus tag PF11_0295, GeneID:810842) using STAR [65],
479 with visualization and coverage analysis performed in Tablet [66]. Graphs of sequence
480 coverage and splice junction positioning and abundance along the gene were generated using

481 R <http://www.r-project.org/>). STAR results pertaining to intron boundaries were analyzed
482 using software developed specifically for this work (isoform_threader, available at
483 <http://isoformthreader.sourceforge.com/>), which uses the SJ (splice junction) and SAM files
484 generated by STAR, plus the original FASTQ files containing the raw transcript sequencing
485 reads, and i) generates a report file detailing how many reads support each splice junction
486 seen (both those found in the SJ file and those not, due to not being one of the three known
487 junction sequence combinations); ii) identifies all combinations of splice junctions and the
488 reads supporting them; and iii) separates reads to different files according to which splice
489 junction combination they support. The identification of the potential intron boundaries are
490 identified from the N operations in the SAM file's CIGAR strings.

491 To quantitatively characterize events of intron retention, all FPPS/GGPPS intronic
492 sequences (as defined in the annotated isoform) were searched by BLASTN against the full
493 gene sequence (low complexity filter turned off, 95% minimum identity threshold, 1E-30
494 maximum E-value threshold), and only instances where the whole intron is contained within
495 the read were scored as positive. Also, a minimum of 30 (25 in the case of the first and last
496 exons) bases aligning in the flanking exon were required to avoid false positives from bad
497 alignments, and a read supporting intron retention must have at least one other intron
498 removed, to minimize the chance of false positives due to unprocessed transcripts being
499 sequenced.

500 All statistical analyses of splice junction or intron retention event counts were
501 performed in R by using Fisher's exact test with Bonferroni-corrected threshold P-values (using
502 0.05 as the family-wide error rate).

503 Potential cellular targeting signals were investigated using TargetP 1.1 [67], with
504 organism group set to "plant".

505

506 *SDS-PAGE separation and in gel-digestion*

Asynchronous cultures of *P. falciparum* were recovered and treated with 0.15% saponin in RPMI media to release hemoglobin from the red blood cells. Proteins were extracted with buffer: 0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2 mM EDTA, 2% SDS, 0.05% bromophenol blue, 50 mM dithiothreitol [68] for separation by SDS-PAGE. Seventeen bands differing by about 1kDa were cut from the gel, ranging from 30 to 50 kDa.

Excised gel bands were in-gel digested with trypsin as previously described [69]. Briefly, gel bands were minced and destained using 100mM Ammonium bicarbonate/Acetonitrile (1:1, vol/vol), followed by neat Acetonitrile to shrink the gel. Disulfide bonds were reduced by 10mM DTT at 56°C for 30min and alkylated with 40mM iodoacetamide for 30min at room temperature in the dark. Proteins were digested with sequencing grade trypsin (Promega) overnight at 37°C. Tryptic activity was quenched by TFA acidification. Peptides were extracted by acetonitrile/water and desalted and concentrated on ZipTip C18-microcolumns (Millipore) according to manufacturer instructions.

Mass spectrometry analysis

Peptides were separated by nano-LC-MS/MS on an Acclaim® PepMap100 15 cm x 75 µm packed with C18 material (3 µm; 100Å, Thermo Fisher) using an Easy-LC nano-HPLC (Thermo Fisher). The HPLC gradient was 0–35% solvent B (A = 0.1% formic acid; B = Acetonitrile, 0.1% formic acid) in a total of 70 mins run at a flow of 250 nL/min. Mass spectrometric analysis was performed using an LTQ Orbitrap Velos ETD (Thermo Scientific, Bremen, Germany). An MS scan (400–2000 m/z) was recorded in the Orbitrap mass analyser at a resolution of 60,000 at 400 m/z for a target of 10^6 ions followed by data-dependent collision-induced dissociation (CID) MS/MS analysis of the top twenty most intense ions with charge state ≥ 2 . The following parameters: activation time = 10 ms, normalized energy = 35, Q-activation = 0.25, dynamic exclusion = enabled with repeat count 1, exclusion duration = 30 s and, intensity threshold = 10,000, target ions = 10^4 .

533

534 *Data Analysis*

535 Raw files were analyzed using Proteome Discoverer v1.4 (Thermo Scientific). MS/MS
536 spectra were converted to .mgf files and searched against the UniProtKB *Plasmodium* database
537 (March 2015) using Sequest HT search engine. Database searches were performed with the
538 following fixed parameters: precursor mass tolerance 10 ppm; MS/MS mass tolerance 0.6 Da
539 and full trypsin cleavage with two possible missed cleavages. Fixed modifications: cysteine
540 carbamidomethylation. Variable modifications included: methionine oxidation. Shared peptide
541 sequences were reported as protein grouped accessions. False discovery rates were obtained
542 using Percolator [70] selecting identification with a q-value equal or less than 0.01. A minimum
543 of two peptides was chosen to identify a protein.

544

545 **Competing interests:**

546 The authors declare that they have no competing interests.

547

548 **Authors' contributions:**

549 JMPA, AMK, MFA designed the study; JMPA, HBG, GP performed experiments; JMPA,
550 GP, HBG, EAK analysed data; JMPA, HBG, EAK, GW, MFA wrote the manuscript. All authors
551 revised and approved the manuscript.

552

553 **Acknowledgements:**

554 This work was supported by grants from CNPq and FAPESP (AMK, grant #2014/23417-
555 7; JMPA, grant #2013/14622-3, Sao Paulo Research Foundation, FAPESP). The authors would
556 like to thank S. Wendel (Blood bank at the Srio Libans Hospital) for providing the
557 erythrocytes, Lucile Maria Floeter-Winter for suggestions, the confocal core facility and the
558 proteomic facility BIOMASS at CEFAP-USP for performing the confocal microscopy analyses

559 and the MS analyses, respectively. HBG is the recipient of a post-graduate fellowship, grant
560 #2010/19518-1, Sao Paulo Research Foundation (FAPESP).

561

562 **References**

563

- 564 1. Singh B, Daneshvar C: **Human infections and detection of Plasmodium**
565 **knowlesi**. *Clin Microbiol Rev* 2013, **26**(2):165-184.
- 566 2. WHO: **World Malaria Report**. In. Switzerland: World Health Organization;
567 2014.
- 568 3. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI: **The global distribution of**
569 **clinical episodes of Plasmodium falciparum malaria**. *Nature* 2005, **434**(7030):214-217.
- 570 4. Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, Allen CM:
571 **Protein farnesyltransferase and protein prenylation in Plasmodium falciparum**. *J Biol Chem*
572 2002, **277**(44):42066-42073.
- 573 5. Borrmann S, Issifou S, Esser G, Adegnika AA, Ramharter M, Matsiegui PB,
574 Oyakhirome S, Mawili-Mboumba DP, Missinou MA, Kun JF *et al*: **Fosmidomycin-clindamycin**
575 **for the treatment of Plasmodium falciparum malaria**. *The Journal of infectious diseases* 2004,
576 **190**(9):1534-1540.
- 577 6. Moura IC, Wunderlich G, Uhrig ML, Couto AS, Peres VJ, Katzin AM, Kimura EA:
578 **Limonene arrests parasite development and inhibits isoprenylation of proteins in**
579 **Plasmodium falciparum**. *Antimicrob Agents Chemother* 2001, **45**(9):2553-2558.
- 580 7. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M,
581 Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK *et al*: **Inhibitors of the nonmevalonate**
582 **pathway of isoprenoid biosynthesis as antimalarial drugs**. *Science* 1999, **285**(5433):1573-
583 1576.

- 584 8. Cassera MB, Gozzo FC, D'Alexandri FL, Merino EF, del Portillo HA, Peres VJ,
585 Almeida IC, Eberlin MN, Wunderlich G, Wiesner J *et al*: **The methylerythritol phosphate**
586 **pathway is functionally active in all intraerythrocytic stages of Plasmodium falciparum.** *J Biol*
587 *Chem* 2004, **279**(50):51749-51759.
- 588 9. Flesch G, Rohmer M: **Prokaryotic hopanoids: the biosynthesis of the**
589 **bacteriohopane skeleton. Formation of isoprenic units from two distinct acetate pools and a**
590 **novel type of carbon/carbon linkage between a triterpene and D-ribose.** *Eur J Biochem* 1988,
591 **175**(2):405-411.
- 592 10. Eisenreich W, Bacher A, Arigoni D, Rohdich F: **Biosynthesis of isoprenoids via**
593 **the non-mevalonate pathway.** *Cell Mol Life Sci* 2004, **61**(12):1401-1426.
- 594 11. Goldstein JL, Brown MS: **Regulation of the mevalonate pathway.** *Nature* 1990,
595 **343**(6257):425-430.
- 596 12. Tonhosolo R, D'Alexandri FL, de Rosso VV, Gazarini ML, Matsumura MY, Peres
597 VJ, Merino EF, Carlton JM, Wunderlich G, Mercadante AZ *et al*: **Carotenoid biosynthesis in**
598 **intraerythrocytic stages of Plasmodium falciparum.** *J Biol Chem* 2009, **284**(15):9974-9985.
- 599 13. Tonhosolo R, Gabriel HB, Matsumura MY, Cabral FJ, Yamamoto MM,
600 D'Alexandri FL, Sussmann RA, Belmonte R, Peres VJ, Crick DC *et al*: **Intraerythrocytic stages of**
601 **Plasmodium falciparum biosynthesize menaquinone.** *FEBS Lett* 2010, **584**(23):4761-4768.
- 602 14. Sussmann RA, Angeli CB, Peres VJ, Kimura EA, Katzin AM: **Intraerythrocytic**
603 **stages of Plasmodium falciparum biosynthesize vitamin E.** *FEBS Lett* 2011, **585**(24):3985-
604 3991.
- 605 15. Jordao FM, Gabriel HB, Alves JM, Angeli CB, Bifano TD, Breda A, de Azevedo
606 MF, Basso LA, Wunderlich G, Kimura EA *et al*: **Cloning and characterization of bifunctional**
607 **enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from Plasmodium**
608 **falciparum.** *Malar J* 2013, **12**:184.

- 609 16. Wang KC, Ohnuma S: **Isoprenyl diphosphate synthases**. *Biochim Biophys Acta*
610 2000, **1529**(1-3):33-48.
- 611 17. Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R:
612 **Bisphosphonates are potent inhibitors of Trypanosoma cruzi farnesyl pyrophosphate**
613 **synthase**. *J Biol Chem* 2001, **276**(36):33930-33937.
- 614 18. Montalvetti A, Fernandez A, Sanders JM, Ghosh S, Van Brussel E, Oldfield E,
615 Docampo R: **Farnesyl pyrophosphate synthase is an essential enzyme in Trypanosoma brucei.**
616 **In vitro RNA interference and in vivo inhibition studies**. *J Biol Chem* 2003, **278**(19):17075-
617 17083.
- 618 19. Artz JD, Wernimont AK, Dunford JE, Schapira M, Dong A, Zhao Y, Lew J, Russell
619 RG, Ebetino FH, Oppermann U *et al*: **Molecular characterization of a novel geranylgeranyl**
620 **pyrophosphate synthase from Plasmodium parasites**. *J Biol Chem* 2011, **286**(5):3315-3322.
- 621 20. Ling Y, Li ZH, Miranda K, Oldfield E, Moreno SN: **The farnesyl-**
622 **diphosphate/geranylgeranyl-diphosphate synthase of Toxoplasma gondii is a bifunctional**
623 **enzyme and a molecular target of bisphosphonates**. *J Biol Chem* 2007, **282**(42):30804-30816.
- 624 21. Luo S, Marchesini N, Moreno SN, Docampo R: **A plant-like vacuolar H(+)-**
625 **pyrophosphatase in Plasmodium falciparum**. *FEBS Lett* 1999, **460**(2):217-220.
- 626 22. Janouskovec J, Horak A, Obornik M, Lukes J, Keeling PJ: **A common red algal**
627 **origin of the apicomplexan, dinoflagellate, and heterokont plastids**. *Proceedings of the*
628 *National Academy of Sciences of the United States of America* 2010, **107**(24):10949-10954.
- 629 23. Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, Thanaraj TA, Soreq H:
630 **Function of alternative splicing**. *Gene* 2005, **344**:1-20.
- 631 24. Kelemen O, Convertini P, Zhang Z, Wen Y, Shen M, Falaleeva M, Stamm S:
632 **Function of alternative splicing**. *Gene* 2013, **514**(1):1-30.

633 25. Iriko H, Jin L, Kaneko O, Takeo S, Han ET, Tachibana M, Otsuki H, Torii M,
634 Tsuboi T: **A small-scale systematic analysis of alternative splicing in *Plasmodium falciparum*.**
635 *Parasitol Int* 2009, **58**(2):196-199.

636 26. Dhar MK, Koul A, Kaul S: **Farnesyl pyrophosphate synthase: a key enzyme in**
637 **isoprenoid biosynthetic pathway and potential molecular target for drug development.** *New*
638 *biotechnology* 2013, **30**(2):114-123.

639 27. Martin D, Piulachs MD, Cunillera N, Ferrer A, Belles X: **Mitochondrial targeting**
640 **of farnesyl diphosphate synthase is a widespread phenomenon in eukaryotes.** *Biochim*
641 *Biophys Acta* 2007, **1773**(3):419-426.

642 28. Lopez-Barragan MJ, Lemieux J, Quinones M, Williamson KC, Molina-Cruz A, Cui
643 K, Barillas-Mury C, Zhao K, Su XZ: **Directional gene expression and antisense transcripts in**
644 **sexual and asexual stages of *Plasmodium falciparum*.** *BMC genomics* 2011, **12**:587.

645 29. Otto TD, Wilinski D, Assefa S, Keane TM, Sarry LR, Bohme U, Lemieux J, Barrell
646 B, Pain A, Berriman M *et al*: **New insights into the blood-stage transcriptome of *Plasmodium***
647 ***falciparum* using RNA-Seq.** *Mol Microbiol* 2010, **76**(1):12-24.

648 30. Sorber K, Dimon MT, DeRisi JL: **RNA-Seq analysis of splicing in *Plasmodium***
649 ***falciparum* uncovers new splice junctions, alternative splicing and splicing of antisense**
650 **transcripts.** *Nucleic acids research* 2011, **39**(9):3820-3835.

651 31. Bartfai R, Hoeijmakers WA, Salcedo-Amaya AM, Smits AH, Janssen-Megens E,
652 Kaan A, Treeck M, Gilberger TW, Francoijs KJ, Stunnenberg HG: **H2A.Z demarcates intergenic**
653 **regions of the plasmodium falciparum epigenome that are dynamically marked by H3K9ac**
654 **and H3K4me3.** *PLoS pathogens* 2010, **6**(12):e1001223.

655 32. Yeh E, DeRisi JL: **Chemical rescue of malaria parasites lacking an apicoplast**
656 **defines organelle function in blood-stage *Plasmodium falciparum*.** *PLoS biology* 2011,
657 **9**(8):e1001138.

658 33. Waller RF, Reed MB, Cowman AF, McFadden GI: **Protein trafficking to the**
659 **plastid of Plasmodium falciparum is via the secretory pathway.** *EMBO J* 2000, **19**(8):1794-
660 1802.

661 34. Wiesner J, Jomaa H: **Isoprenoid biosynthesis of the apicoplast as drug target.**
662 *Current drug targets* 2007, **8**(1):3-13.

663 35. Ortiz-Gomez A, Jimenez C, Estevez AM, Carrero-Lerida J, Ruiz-Perez LM,
664 Gonzalez-Pacanowska D: **Farnesyl diphosphate synthase is a cytosolic enzyme in Leishmania**
665 **major promastigotes and its overexpression confers resistance to risedronate.** *Eukaryotic cell*
666 2006, **5**(7):1057-1064.

667 36. Ferella M, Li ZH, Andersson B, Docampo R: **Farnesyl diphosphate synthase**
668 **localizes to the cytoplasm of Trypanosoma cruzi and T. brucei.** *Exp Parasitol* 2008, **119**(2):308-
669 312.

670 37. Cunillera N, Boronat A, Ferrer A: **The Arabidopsis thaliana FPS1 gene**
671 **generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate synthase**
672 **isoform.** *J Biol Chem* 1997, **272**(24):15381-15388.

673 38. Taban AH, Tittiger C, Blomquist GJ, Welch WH: **Isolation and characterization**
674 **of farnesyl diphosphate synthase from the cotton boll weevil, Anthonomus grandis.** *Archives*
675 *of insect biochemistry and physiology* 2009, **71**(2):88-104.

676 39. Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL: **The transcriptome**
677 **of the intraerythrocytic developmental cycle of Plasmodium falciparum.** *PLoS Biol* 2003,
678 **1**(1):E5.

679 40. Bertram G, Innes S, Minella O, Richardson J, Stansfield I: **Endless possibilities:**
680 **translation termination and stop codon recognition.** *Microbiology* 2001, **147**(Pt 2):255-269.

681 41. Jungreis I, Lin MF, Spokony R, Chan CS, Negre N, Victorsen A, White KP, Kellis
682 M: **Evidence of abundant stop codon readthrough in Drosophila and other metazoa.** *Genome*
683 *research* 2011, **21**(12):2096-2113.

684 42. Blanchet S, Cornu D, Argentini M, Namy O: **New insights into the**
685 **incorporation of natural suppressor tRNAs at stop codons in *Saccharomyces cerevisiae*.**
686 *Nucleic acids research* 2014, **42**(15):10061-10072.

687 43. Dunn JG, Foo CK, Belletier NG, Gavis ER, Weissman JS: **Ribosome profiling**
688 **reveals pervasive and regulated stop codon readthrough in *Drosophila melanogaster*.** *eLife*
689 2013, **2**:e01179.

690 44. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain
691 A, Nelson KE, Bowman S *et al*: **Genome sequence of the human malaria parasite *Plasmodium***
692 ***falciparum*.** *Nature* 2002, **419**(6906):498-511.

693 45. Lobanov AV, Delgado C, Rahlfs S, Novoselov SV, Kryukov GV, Gromer S, Hatfield
694 DL, Becker K, Gladyshev VN: **The *Plasmodium* selenoproteome.** *Nucleic acids research* 2006,
695 **34**(2):496-505.

696 46. Mourier T, Pain A, Barrell B, Griffiths-Jones S: **A selenocysteine tRNA and SECIS**
697 **element in *Plasmodium falciparum*.** *Rna* 2005, **11**(2):119-122.

698 47. Gamain B, Arnaud J, Favier A, Camus D, Dive D, Slomianny C: **Increase in**
699 **glutathione peroxidase activity in malaria parasite after selenium supplementation.** *Free*
700 *radical biology & medicine* 1996, **21**(4):559-565.

701 48. Wang K, Ohnuma S: **Chain-length determination mechanism of isoprenyl**
702 **diphosphate synthases and implications for molecular evolution.** *Trends in biochemical*
703 *sciences* 1999, **24**(11):445-451.

704 49. Chen A, Poulter CD: **Purification and characterization of farnesyl**
705 **diphosphate/geranylgeranyl diphosphate synthase. A thermostable bifunctional enzyme**
706 **from *Methanobacterium thermoautotrophicum*.** *J Biol Chem* 1993, **268**(15):11002-11007.

707 50. Narita K, Ohnuma S, Nishino T: **Protein design of geranyl diphosphate**
708 **synthase. Structural features that define the product specificities of prenyltransferases.**
709 *Journal of biochemistry* 1999, **126**(3):566-571.

710 51. Lu F, Jiang H, Ding J, Mu J, Valenzuela JG, Ribeiro JM, Su XZ: **cDNA sequences**
711 **reveal considerable gene prediction inaccuracy in the Plasmodium falciparum genome.** *BMC*
712 *genomics* 2007, **8**:255.

713 52. Morrissy AS, Griffith M, Marra MA: **Extensive relationship between antisense**
714 **transcription and alternative splicing in the human genome.** *Genome research* 2011,
715 **21(8):1203-1212.**

716 53. Wickham ME, Thompson JK, Cowman AF: **Characterisation of the merozoite**
717 **surface protein-2 promoter using stable and transient transfection in Plasmodium**
718 **falciparum.** *Mol Biochem Parasitol* 2003, **129(2):147-156.**

719 54. Fischer K, Marti T, Rick B, Johnson D, Benting J, Baumeister S, Helmbrecht C,
720 Lanzer M, Lingelbach K: **Characterization and cloning of the gene encoding the vacuolar**
721 **membrane protein EXP-2 from Plasmodium falciparum.** *Mol Biochem Parasitol* 1998,
722 **92(1):47-57.**

723 55. Eshar S, Allemand E, Sebag A, Glaser F, Muchardt C, Mandel-Gutfreund Y, Karni
724 R, Dzikowski R: **A novel Plasmodium falciparum SR protein is an alternative splicing factor**
725 **required for the parasites' proliferation in human erythrocytes.** *Nucleic Acids Res* 2012,
726 **40(19):9903-9916.**

727 56. Trager W, Jensen JB: **Human malaria parasites in continuous culture.** *Science*
728 1976, **193(4254):673-675.**

729 57. Jordao FM, Gabriel HB, Alves JM, Angeli CB, Bifano TD, Breda A, de Azevedo
730 MF, Basso LA, Wunderlich G, Kimura EA *et al*: **Cloning and characterization of bifunctional**
731 **enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from Plasmodium**
732 **falciparum.** *Malaria journal* 2013, **12(1):184.**

733 58. de Azevedo MF, Gilson PR, Gabriel HB, Simoes RF, Angrisano F, Baum J, Crabb
734 BS, Wunderlich G: **Systematic analysis of FKBP inducible degradation domain tagging**

735 **strategies for the human malaria parasite *Plasmodium falciparum*. *PLoS One* 2012,**
736 **7(7):e40981.**

737 59. Wu Y, Sifri CD, Lei HH, Su XZ, Wellem TE: **Transfection of *Plasmodium***
738 ***falciparum* within human red blood cells. *Proceedings of the National Academy of Sciences of***
739 ***the United States of America* 1995, **92**(4):973-977.**

740 60. Fidock DA, Wellem TE: **Transformation with human dihydrofolate reductase**
741 **renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of**
742 **proguanil. *Proceedings of the National Academy of Sciences of the United States of America***
743 **1997, **94**(20):10931-10936.**

744 61. Furtado GC, Moura IC, Pudles J, Alvarez JM, D'Imperio Lima MR: ***Plasmodium***
745 ***chabaudi chabaudi*: a monoclonal antibody raised against soluble antigens present in the**
746 **plasma of infected mice recognizes a 250-kDa schizont glycoprotein that is secreted during**
747 **schizogony. *Exp Parasitol* 1999, **91**(1):97-100.**

748 62. de Macedo CS, Uhrig ML, Kimura EA, Katzin AM: **Characterization of the**
749 **isoprenoid chain of coenzyme Q in *Plasmodium falciparum*. *FEMS Microbiol Lett* 2002,**
750 **207(1):13-20.**

751 63. Shestopalov AI, Bogachev AV, Murtazina RA, Viryasov MB, Skulachev VP:
752 **Aeration-dependent changes in composition of the quinone pool in *Escherichia coli*. Evidence**
753 **of post-transcriptional regulation of the quinone biosynthesis. *FEBS Lett* 1997, **404**(2-3):272-**
754 **274.**

755 64. Joshi NA. FJ: **Sickle: A sliding-window, adaptive, quality-based trimming tool**
756 **for FastQ files (Version 1.33) [Software]. In., vol. 2015, 1.33 edn.**
757 **<https://github.com/najoshi/sickle>; 2011.**

758 65. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson
759 M, Gingeras TR: **STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013, **29**(1):15-21.**

- 760 66. Milne I, Bayer M, Cardle L, Shaw P, Stephen G, Wright F, Marshall D: **Tablet--**
 761 **next generation sequence assembly visualization.** *Bioinformatics* 2010, **26**(3):401-402.
- 762 67. Emanuelsson O, Brunak S, von Heijne G, Nielsen H: **Locating proteins in the**
 763 **cell using TargetP, SignalP and related tools.** *Nature protocols* 2007, **2**(4):953-971.
- 764 68. Bullen HE, Tonkin CJ, O'Donnell RA, Tham WH, Papenfuss AT, Gould S, Cowman
 765 AF, Crabb BS, Gilson PR: **A novel family of Apicomplexan glideosome-associated proteins with**
 766 **an inner membrane-anchoring role.** *J Biol Chem* 2009, **284**(37):25353-25363.
- 767 69. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M: **In-gel digestion for mass**
 768 **spectrometric characterization of proteins and proteomes.** *Nature protocols* 2006, **1**(6):2856-
 769 2860.
- 770 70. Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ: **Semi-supervised**
 771 **learning for peptide identification from shotgun proteomics datasets.** *Nat Methods* 2007,
 772 **4**(11):923-925.

773

774 **FIGURE LEGENDS**

775

776 **Figure 1. Schematic diagram of the isoprenoid biosynthesis in *P. falciparum*.**

777 Downstream of the MEP pathway, all isoprenoids are derived from a common precursor, IPP,
 778 and its isomer DMAPP. FPPS/GGPPS catalyzes the consecutive condensation of IPP with
 779 DMAPP to form GPP. Then, a second condensation between GPP and IPP forms FPP. In *P.*
 780 *falciparum* and condense FPP with a further molecule of IPP to form GGPP.

781

782 **Figure2. FPPS/GGPPS localization.** Images of live parasites (FPPS/GGPPS-HA-GFP

783 strain) expressing GFP and immunofluorescence of the FPPS/GGPPS-HA strain visualized by
 784 fluorescence or confocal microscopy. **A)** Images of live parasites FPPS/GGPPS-HA-GFP by
 785 fluorescence microscopy during the intra-erythrocytic cycle of the parasite. **B)** Bottom -

786 immunofluorescence of the FPPS/GGPPS-HA strain with antibody as indicated, visualized by
787 confocal microscopy. Upper- Images of live parasites FPPS/GGPPS-HA-GFP with markers as
788 indicated visualized by confocal microscopy. ET1 (early trophozoites); ET2 (early trophozoites);
789 LT (late trophozoites); LT (late trophozoites) S1 (schizont); S2 (schizont); R (rosettes); M
790 (Merozoites); DAPI (nucleus marker). Original magnification for all images 1.000x.

791

792 **Figure 3. Splice junctions from FPPS/GGPPS and their relative quantities during the**
793 **intra-erythrocytic cycle.** A) Ring; B) Early trophozoite – 24 hours; C) Late trophozoite – 35
794 hours; D) Schizont. Green line – annotated junction; blue line – other GT-AG junctions; red line
795 – other junctions (not GT-AG); white bars – annotated exons, numbered; pink bars – FARM and
796 SARM domains.

797

798 **Figure 4. Viable isoforms.** Isoforms possibly allowing the translation to a functional
799 protein. The top isoform is the annotated version. Exon positions are marked by alternating
800 black and blue bars on the top, and the FARM and SARM are represented by rectangles in
801 exons 4 and 8, respectively. The five in-frame stop codons, as well as the final stop in the
802 predicted proteins, are represented in black. Different colors along sequence segments in the
803 variants denote difference compared to the annotated isoform.

804

805 **ADDITIONAL FILES**

806 **Additional file 1**

807 **Title:** Oligonucleotides utilized.

808 **Description:** Oligonucleotides used for RNA-seq experiments, plasmids for transfection
809 and validation experiments by PCR and RT-PCR.

810

811 **Additional file 2**

812 **Title:** Overall sequence coverage from RNA-seq.

813 **Description:** Coverage levels indicate number of reads covering each position along
814 the annotated *P. falciparum* FPPS/GGPPS gene. Coverage along intronic regions is usually
815 represented by a line following from the last available exonic coverage, with the exception of
816 cases where some intronic sequence was added to an exon (see main text). Each life-cycle
817 stage is represented by a line, according to the legend. Vertical white areas represent
818 annotated exons (numbered).

819

820 **Additional file 3**

821 **Title:** High confidence splice junctions

822 **Description:** Splice junction identifiers, coordinates, and junction types are
823 represented, as well as number of reads where each junction has been observed in each of the
824 four life-cycle stages studied here. Notes indicate predicted effect each alternative junction
825 might have on an eventual protein product.

826

827 **Additional file 4**

828 **Title:** Viable isoform splice junction composition

829 **Description:** Alignment version of Figure 4, representing the amino acid composition
830 of each predicted viable isoform found. Boldface type distinguishes positions differing
831 between a variant and the annotated isoform, and highlights help visualize in-frame stop
832 codons (red) and length differences (yellow). Alternating black and blue bars above the
833 alignment blocks represent the annotated exon positions along the FPPS/GGPPS, identified by
834 numbers immediately above.

835

836 **Additional file 5**

837 **Title:** Splice junction combinations detected and their quantifications during the intra-
838 erythrocytic cycle

839 **Description:** Named variants represent the viable isoforms presented in Figure 4 and
840 Additional file 4.

841

842 **Additional file 6**

843 **Title:** Reverse-transcriptase PCR essays for confirming isoforms in the isolated from
844 patients and wild type strains

845 **Description:** Primers were designed to target regions specific for detecting an
846 alternative splicing event occurring only if deletion of a particular exon occurred (as an
847 example, see sketch in Additional file 7 part A). **A)** Primers for amplifying a product only if exon
848 5 is deleted. **B)** Primers for amplifying a product only if exon 7 is deleted. **C)** Primers for
849 amplifying a product only if exon 10 is deleted. Isolates: 3D7 (wild type); S20 (isolated from a
850 patient); Cand (isolated from a patient); gDNA (genomic DNA from 3D7); - (negative control).

851

852 **Additional file 7**

853 **Title:** qPCR comparison of expression between isoforms lacking exon 7 or generating
854 the intact primary protein

855 **Description:** **A)** Oligonucleotide design for detecting an alternative splicing event. **B)**
856 Transcript levels of the gene encoding FPPS/GGPPS in isolate 3D7 were normalized by the
857 control gene K1. Statistical significance was determined by one-way ANOVA. All differences
858 between control and variant isoforms were significant ($p < 0.05$). Control (transcript for the
859 primary protein); - exon7 (isoform lacking exon 7); a and b (F-iso and R-iso); c and d (F-iso7 and
860 R-iso7); ET (early trophozoite); LT (late trophozoite).

Table 1 – FPPS/GGPPS intron retention events in four *P. falciparum* stages

Total read numbers are for reference only and were not used in the statistical analyses.

Statistical analysis (two-sided Fisher exact test, $\alpha = 0.05 / 6 = 0.008$) of differential number of retention events: Intron 1: significant ($P < 2.2 \times 10^{-16}$); Intron 2: significant ($P = 5.3 \times 10^{-5}$); Intron 4: significant ($P = 3.7 \times 10^{-13}$); Intron 7: not significant ($P = 0.149$); Intron 8: not significant ($P = 0.011$); Intron 10: not significant ($P = 0.041$).

	Ring	Schizont	Early tropho	Late tropho	Ring no	Schizont no	ET no	LT no
Intron 1	31	155	43	20	30648	35261	48270	24389
Intron 2	14	22	4	4	28029	31344	40393	22250
Intron 3								
Intron 4	31	18	21	61	27000	33069	38097	22862
Intron 5								
Intron 6								
Intron 7	1	5	0	1	11593	23046	18994	17064
Intron 8	6	4	0	3	15300	28808	24926	21584
Intron 9								
Intron 10	0	1	5	0	18122	32245	30220	24091
Total reads	48944	76087	80727	54352				

Table2. Intron retention comparison between intra-erythrocytic forms.

Only the three introns whose retention numbers were statistically significant in the global analysis are included. All pairwise comparisons of life cycle stage retention numbers were performed with a level of significance $\alpha = 0.05 / 18 = 0.0028$.

	Intron 1	Intron 2	Intron 4
Ring x Schizont	< 2.2 x 10⁻¹⁶	0.4042	0.01377
Ring x Early tropho	0.6336	0.002709	0.01062
Ring x Late tropho	0.4846	0.09386	9.12 x 10⁻⁵
Schizont x Early tropho	< 2.2 x 10⁻¹⁶	2.813 x 10⁻⁵	1
Schizont x Late tropho	< 2.2 x 10⁻¹⁶	0.008283	8.441 x 10⁻¹¹
Early x Late tropho	0.8939	0.4664	1.691 x 10⁻¹¹
Note: numbers in bold and red typeface indicate significant difference (Fisher's exact test P-value below 0.0028)			

Single-target high-throughput transcription analysis reveal high levels of alternative splicing present in the FPPS/GGPPS from *Plasmodium falciparum*

Heloisa B. Gabriel, Mauro F. de Azevedo, Giuseppe Palmisano, Gerhard Wunderlich, Emília

A. Kimura, Alejandro M. Katzin, João M. P. Alves

ADDITIONAL FILES

Additional file 1

Title: Oligonucleotides utilized.

Description: Oligonucleotides used for RNA-seq experiments, plasmids for transfection and validation experiments by PCR and RT-PCR.

Name	Sequence (5' - 3')	T.A.
F-FPPS/GGPPS-BglII	AGATCTGGTATGCAAATGGGGGGTATA	59°C
R-FPPS/GGPPS-PstI	CTGCAGCAGCGCCTGTAAACAAAATGTC	59°C
F-FPPS/GGPPS-BamHI	CCGGATCCATGGAGAACGAGCAGAATAAC	57°C
F-iso5	GTAGCAGATGATATTATGGATAAGG	55°C
R-iso5	AAAGTGGCTTCTCTAAAGGATG	57°C
F-iso7	CATCCTTTAGAGAAGCCACTTTAA	57°C
R-iso7	AATCATCATGGACCACAAACAATA	58°C
F-iso10	CACAACCTGAAAAAGAGGACAT	58°C
R-iso10	CAAGACATATTCTATACTCTAATATTTTCATC	56°C
F-iso	TTCATTCTTTTACCTATTGTTTG	53°C
R-iso	CCAGGGCCACGTTAATT	57°C

Additional file 2

Title: Overall sequence coverage from RNA-seq.

Description: Coverage levels indicate number of reads covering each position along the annotated *P. falciparum* FPPS/GGPPS gene. Coverage along intronic regions is usually represented by a line following from the last available exonic coverage, with the exception of cases where some intronic sequence was added to an exon (see main text). Each life-cycle stage is represented by a line, according to the legend. Vertical white areas represent annotated exons (numbered).

Additional file 3

Title: High confidence splice junctions

Description: Splice junction identifiers, coordinates, and junction types are represented, as well as number of reads where each junction has been observed in each of the four life-cycle stages studied here. Notes indicate predicted effect each alternative junction might have on an eventual protein product.

Junction	Start	End	Ring	ET	LT	Schizont	Type	Notes
2	38	165	0	4	8	1		deletes about 1/3 of the end of exon 1
5	56	401	0	1	0	8		deletes 2 bases at the end of exon 1 plus the whole of exon 2
10	58	131	0	1	0	0	GT: TG	
11	58	154	0	2	2	2	GT: TG	
12	58	165	19045	32788	23630	32627		
14	58	176	50	108	60	119		deletes 11 bases at the start of exon 2
15	58	182	0	9	1	0		
17	58	401	1	0	7	0		deletes exon 2
19	61	165	1	4	0	3	AG: AG	
20	62	165	5	11	15	8		
25	71	165	0	0	0	1		
30	103	401	0	0	2	0		
32	193	253	1	0	0	0	AT: CT	creates a new intron inside exon 2, removing about 1/2 of its sequence
33	214	401	0	0	0	1		
34	222	689	0	0	0	1	AG: GC	
35	266	401	0	1	0	0	TT: AG	
44	283	401	207	300	25	76	GG: AG	"stage-specific error" (4 instead of 5 G)
47	284	649	3	3	19	0		
48	284	685	0	0	1	0		deletes exon 3 and part of exon 4
52	284	389	0	0	0	3	GT: TT	
53	284	400	7575	7914	276	204	GT: TA	"stage-specific error" (6 instead of 5 G)
54	284	401	8917	17169	21215	28506		
55	284	417	6	5	16	4		deletes part of exon 3
56	284	428	4	1	22	4		deletes part of exon 3
58	285	410	0	0	22	0	TA: AA	
63	293	401	18	12	20	43		adds 9 bases to the end of exon 2
67	293	459	0	0	12	21	AG: TA	
69	322	401	1	3	6	0		
73	338	809	0	0	1	0		adds about 1/2 of intron 2 to exon 2, deletes exons 3 and 4
75	405	649	0	0	2	0		
76	445	649	0	0	4	0		deletes about 1/2 of exon 3
77	445	809	0	0	2	0		deletes about 1/2 of exon 3 plus the whole of exon 4
78	474	646	0	0	0	2	AA: TT	
81	491	649	0	0	6	0	GC: AG	
83	493	654	1	3	0	2	TT: GG	
84	495	654	0	0	0	1	GT: GG	
92	500	652	0	5	0	0	GG: AT	
94	501	649	16451	24863	21934	29546		
95	501	650	0	1	0	0	GT: GC	
96	501	661	7	14	40	26		deletes 12 bases at the start of exon 4
97	501	685	1	8	1	1		deletes about 1/4 at the start of exon 4

98	501	688	0	0	1	2		deletes about ¼ at the start of exon 4
103	734	809	15	20	45	19		deletes 4 bases at the end of exon 4
104	734	1024	0	0	6	0		deletes end of exon 4 plus the whole of exon 5
109	736	822	0	0	1	0	TG:AA	
111	738	808	2	0	192	94	?	"stage-specific error" (3 instead of 2 G), GT:TA or TA:AG
112	738	809	15281	22611	21705	29828		
114	738	823	41	67	117	76		deletes about 1/5 of the start of exon 5
116	738	837	0	5	3	3		deletes about 2/5 of the start of exon 5
117	738	1024	17	9	30	27		deletes exon 5
118	738	1084	1	0	0	7		deletes exon 5 plus about ¼ of the start of exon 6
119	738	1107	2	0	0	0		
121	740	810	25	23	0	32	AT:GG	
125	742	809	24	21	29	27		adds 4 bases at the end of exon 4
126	742	812	75	151	11	130		"stage-specific error" (5 instead of 4 T at start of exon 5)
130	745	809	0	0	0	3	TT:AG	adds 7 bases at the end of exon 4
131	748	844	0	0	1	0	TG:GG	
133	864	1024	0	9	0	17		deletes about ¼ of exon 5 at the end
134	880	1023	0	1	5	4	AA:CA	
137	881	1024	76	213	3511	5960	AG:AG	"stage-specific error" (2 instead of 3 A)
138	882	1014	0	0	1	0	GT:TT	
139	882	1024	14773	20621	20253	26043		
140	882	1032	1	0	0	0	GT:TG	
141	882	1069	1	0	0	1	GT:TT	deletes about 1/6 of exon 6
142	882	1084	2	1	4	3		deletes about 1/5 of exon 6
143	883	1015	0	0	0	1	TT:TA	
145	887	1028	0	1	0	0	AT:TA	
146	892	1216	5	1	0	0	AC:AA	
152	1107	1232	3	0	11	14		creates a new intron inside exon 6, removing about ½ of its sequence
153	1114	1241	0	0	0	6	AA:AA	creates a new intron inside exon 6, removing about ½ of its sequence
154	1120	1618	0	1	0	0	TA:TA	
161	1201	1221	0	1	0	0	AT:GT	
163	1233	1400	2	0	2	0		deletes about ¼ of exon 6
166	1266	1384	1	0	0	0	TA:GA	
171	1290	1400	1	0	0	0		
173	1294	1400	1572	2098	46	58		
176	1296	1400	1	8	15	24	?	dubious alignment, GT:GG or GG:AG
178	1297	1400	1734	2618	11191	9709		
179	1297	1602	39	21	34	19		deletes exon 7
199	1448	1602	46	72	48	79		deletes about ½ of exon 7
205	1487	1602	11050	17976	14462	19195		
207	1487	1621	0	0	0	1		
212	1654	1813	3	5	1	7		deletes about ½ of exon 8

216	1654	2087	0	0	0	4		deletes about ½ of exon 8 plus the whole of exon 9
223	1702	1783	0	1	0	0	GT:TT	
224	1702	1810	1	6	1	1	GT:TG	
225	1702	1813	14658	23699	18392	24215		
227	1702	1844	1	2	0	1		
228	1702	2087	0	0	19	0		
231	1715	1839	0	2	0	0	AA:GA	
233	1891	1960	1	0	0	0		
236	1975	2087	17118	28281	20495	27086		
237	1975	2089	4	16	3	10		
238	1975	2237	100	58	56	15		deletes exon 10
239	1995	2087	1	3	0	2		adds 20 bases to the end of exon 9
242	2112	2237	0	0	0	2	AG:AG	
247	2114	2237	17361	28805	20705	27453		
248	2114	2243	91	97	65	112		deletes 6 bases from exon 11

Legend

ET	Early trophozoite
LT	Late trophozoite
Start	start coordinate for the putative intron involved in the current splice junction
End	end coordinate for the putative intron involved in the current splice junction
Ring	number of reads corroborating the current splice junction, as reported by STAR, in the ring life cycle stage
Early trophozoite	same as above, but for the early trophozoite stage
Late trophozoite	same as above, but for the late trophozoite stage
Schizont	same as above, but for the schizont stage
Type	type of junction, classified by splice donor and acceptor sequences; when absent, it is the canonical pair (GT:AG); ? when undetermined
Notes	any pertinent notes on the effect, on the final protein, of a particular splice junction variant

Other observations: Rows in **bold typeface** indicate splice junctions that were present in the annotated isoform present in GenBank
Splice junctions whose IDs are in **red background** indicate "stage-specific errors"
Splice junctions whose IDs are in **light blue background** indicate those, excluding previously annotated ones, present in at least one of the 40 newly identified isoforms (see main text)

Additional file 4

Title: Viable isoform splice junction composition

Description: Alignment version of Figure 4, representing the amino acid composition of each predicted viable isoform found. Boldface type distinguishes positions differing between a variant and the annotated isoform, and highlights help visualize in-frame stop codons (red) and length differences (yellow). Alternating black and blue bars above the alignment blocks represent the annotated exon positions along the FPPS/GGPPS, identified by numbers immediately above.

Additional file 5

Title: Splice junction combinations detected and their quantifications during the intra-erythrocytic cycle

Description: Named variants represent the viable isoforms presented in Figure 4 and Additional file 4.

Variant	Splice junctions	Ring	Early trophozoite	Late trophozoite	Schizont	Total
var006	2		1	1		2
	5				1	1
	10		1			1
	11		1		1	2
	12	2418	7596	2166	4052	16232
	14	13	54	8	24	99
	15		5			5
	19	1	1		1	3
	20		2			2
	25				1	1
	52				3	3
	53	1	2			3
	54	4	9	10	16	39
	94	6	12	1	8	27
	112		1			1
	139	2	5	2	1	10
	178	1	6	10	4	21
	205	2	3		2	7
	225	70	132	38	113	353
	231		2			2
	236	18	37	8	17	80
	238	13	25	3	5	46
	247	424	775	314	620	2133
	248	5	4	9	11	29
var009	103 137 178 205 225 236 247			2	1	3
	109 137 178 205 225 236 247			1		1
	11 54 94 112 139		1	1	1	3
	11 54 94 112 139 178			1		1
	111 137 178 205 225				1	1
	112 134 178 205 225 236 247			1	1	2
	112 137 173 205			1		1
	112 137 173 205 225			1	2	3
	112 137 173 205 225 236 247			6	10	16
	112 137 176 205 225 236 247			1	4	5
	112 137 178 205			3	3	6

	112 137 178 205 224 236 247				1	1
	112 137 178 205 225			1	10	11
	112 137 178 205 225 236			2	6	8
	112 137 178 205 225 236 242				1	1
	112 137 178 205 225 236 247			1475	1848	3323
	112 137 178 205 225 237 247				2	2
	112 137 178 205 225 239 247				1	1
	112 139	13	9	1	2	25
	112 139 153				6	6
	112 139 163 205 225 236 247			1		1
	112 139 176		1			1
	112 139 176 205		2		1	3
	112 139 178	9	11	4	1	25
	112 139 178 199 225 236 247	2	3	3	3	11
	112 139 178 205	6	11	5	10	32
	112 139 178 205 216 247				1	1
	112 139 178 205 225	15	12	12	27	66
	112 139 178 205 225 236	1		2	4	7
	112 139 178 205 225 236 247	37	56	251	342	686
	112 139 178 205 225 236 248	3		4	6	13
var148	112 139 178 205 225 238	1		1	1	3
	112 139 178 205 225 238		1			1
	112 139 178 205 228 247			1		1
	112 139 179	1				1
	112 139 179 225 236 247		1			1
	112 139 179 225 236 247	2		4		6
	112 142 178 205 225 236 247	1				1
	114 137 178 205 225 236 247			7	6	13
	117 178 205 225 236 247		1			1
	117 178 205 225 236 247	1		10	4	15
	117 179 225 236 247				5	5
	118 178 205 225 236 247				1	1
	12 32 54 94	1				1
var208	12 33 94 112 139 178				1	1
var078	12 34 112 139				1	1
	12 35 94		1			1

	12 44	10	12	3	10	35
	12 44 94	13	27	1	10	51
	12 44 94 111			1		1
	12 44 94 112	14	17	4	4	39
	12 44 94 112 137				2	2
	12 44 94 112 139	149	220	7	45	421
	12 44 94 112 139 178	14	11	4	1	30
	12 44 94 112 139 178 205	1		1	1	3
	12 44 94 117	1	1			2
	12 44 96 112 139		3	1		4
	12 47		1	1		2
	12 47 112			1		1
	12 47 112 139	3	1	12		16
var036	12 47 112 139 178		1	4		5
var038	12 48 112 139			1		1
	12 53	198	239	24	16	477
	12 53 83 112 139		1			1
	12 53 92		2			2
	12 53 92 112		3			3
	12 53 94	469	673	36	24	1202
	12 53 94 103 139	5	5			10
	12 53 94 111			1		1
	12 53 94 111 139			6		6
	12 53 94 112	518	965	4	7	1494
	12 53 94 112 133		4			4
	12 53 94 112 137	44	78		3	125
	12 53 94 112 139	6177	5778	136	136	12227
	12 53 94 112 139 163	2				2
	12 53 94 112 139 173	1	2			3
	12 53 94 112 139 178	43	9	56	6	114
	12 53 94 112 142	1				1
	12 53 94 114	11	13			24
	12 53 94 114 139	8	5			13
	12 53 94 117	5	2			7
	12 53 94 119	1				1
	12 53 94 121	6	3			9

	12 53 94 121 139	2	2		4	
	12 53 94 125		1		1	
	12 53 94 125 139	11	7	1	19	
	12 53 94 126	8	10		18	
	12 53 94 126 139	30	41		71	
	12 53 94 139	2			2	
	12 53 95		1		1	
	12 53 96		1		1	
	12 53 96 112		1		1	
	12 53 96 112 139	2	1		3	
	12 53 97		1		1	
	12 53 97 112 139	1	1		2	
	12 54	306	587	551	886	2330
	12 54 76 112 139			3		3
var093	12 54 76 112 139 178			1		1
	12 54 77 139			2		2
	12 54 78 112				1	1
var124	12 54 78 112 139				1	1
	12 54 81			1		1
	12 54 81 111			1		1
	12 54 81 111 139			4		4
	12 54 83		2		1	3
	12 54 83 111 139				1	1
	12 54 83 112 139	1				1
	12 54 84 112 139				1	1
	12 54 94	521	1297	1590	1559	4967
	12 54 94 103		4	3		7
	12 54 94 103 139	7	11	36	14	68
	12 54 94 104			6		6
	12 54 94 111			24	5	29
	12 54 94 111 139	2		124	56	182
	12 54 94 111 139 173				1	1
	12 54 94 111 139 178			13	7	20
	12 54 94 112	580	1892	589	1020	4081
	12 54 94 112 133		4		15	19
	12 54 94 112 134		1		2	3

	12 54 94 112 137	23	118	6	1032	1179
	12 54 94 112 137 152			1	1	1
	12 54 94 112 137 178		1	2	3	3
	12 54 94 112 138			1	1	1
	12 54 94 112 139	7127	12732	13680	22453	55992
	12 54 94 112 139 152			1	9	10
	12 54 94 112 139 152 178			2	3	5
var107	12 54 94 112 139 152 178 205			3		3
var060	12 54 94 112 139 173	1	1	19	10	31
var067	12 54 94 112 139 176	1	2	7	6	16
	12 54 94 112 139 178	108	78	4096	719	5001
	12 54 94 112 139 178 199			1		1
	12 54 94 112 139 178 205			1	3	4
	12 54 94 112 139 179			5		5
	12 54 94 112 140	1				1
var156	12 54 94 112 141				1	1
var151	12 54 94 112 142		1	4	2	7
	12 54 94 112 143				1	1
	12 54 94 112 145		1			1
	12 54 94 114	10	29	20	7	66
	12 54 94 114 133		1			1
	12 54 94 114 137		2			2
	12 54 94 114 139	8	17	71	55	151
	12 54 94 114 139 178	1			3	4
	12 54 94 116		2			2
	12 54 94 116 139		3	3	2	8
	12 54 94 116 139 178				1	1
	12 54 94 117	8	5	17	10	40
	12 54 94 117 178				2	2
	12 54 94 117 178 205			1		1
var128	12 54 94 117 178 205 225			1		1
var075	12 54 94 118	1			6	7
	12 54 94 119	1				1
	12 54 94 121	8	5		7	20
	12 54 94 121 137	1			4	5
	12 54 94 121 139	8	12		20	40

	12 54 94 125	2	2	2		6
	12 54 94 125 137				2	2
	12 54 94 125 139	11	10	21	22	64
	12 54 94 125 139 178			4		4
	12 54 94 126	9	13		8	30
	12 54 94 126 137	1	1		6	8
	12 54 94 126 139	24	78	3	102	207
	12 54 94 126 139 152				1	1
	12 54 94 126 139 173			1		1
	12 54 94 130 139				3	3
	12 54 94 131 139			1		1
	12 54 94 139		1			1
	12 54 96	1	1	6	4	12
	12 54 96 111 139			1	16	17
	12 54 96 112			3		3
	12 54 96 112 139	4	5	28	6	43
var147	12 54 96 112 139 178			1		1
	12 54 96 126 139		1			1
	12 54 97		2			2
	12 54 97 112		1			1
	12 54 97 112 139		1			1
var188	12 54 97 112 139 173				1	1
var189	12 54 98 112 139			1	1	2
	12 54 98 114 139				1	1
	12 55		1		1	2
	12 55 94	1	2	1	2	6
	12 55 94 111				1	1
	12 55 94 112	2				2
	12 55 94 112 139	3	2	10		15
	12 55 94 112 139 178			5		5
	12 56		1			1
	12 56 94			1	2	3
	12 56 94 111 139			12		12
	12 56 94 111 139 178			1		1
	12 56 94 112 139	3			2	5
var115	12 56 94 112 139 178	1				1

	12 56 94 114 139			8		8
	12 58 94 112 139			14		14
	12 58 94 112 139 178			8		8
	12 63	3		1	1	5
	12 63 94	4	2	1	3	10
	12 63 94 112		1		3	4
	12 63 94 112 137				5	5
var218	12 63 94 112 139	10	8	11	29	58
	12 63 94 112 139 178		1	4	1	6
	12 69 94 112	1				1
	12 69 94 112 139			6		6
	12 73 139			1		1
	12 94				5	5
	12 94 112			1		1
	121 139 178				1	1
	125 137 178 205 225 236 247			1	1	2
	126 137 178 205 225 236 247			4	6	10
var116	126 139 178 205 225		1		1	2
	133 178 205 225 236 247				2	2
	134 178 205 225 236 247			2	1	3
	137 173 205 225			1	1	2
	137 173 205 225 236 247			5	14	19
	137 176 205 225 236 247				5	5
	137 178			1		1
	137 178 199 225 236 247			2	3	5
	137 178 205	1	1	1	5	8
	137 178 205 225			4	7	11
	137 178 205 225 236			1	6	7
	137 178 205 225 236 242				1	1
	137 178 205 225 236 247	2	5	1291	1683	2981
	137 178 205 225 238				2	2
	137 178 205 228 247			1		1
var157	139 152 173 205 225	1				1
	139 152 178 205 225 236 247			1		1
	139 171 205	1				1
	139 173 205 225	2	4			6

	139 173 205 225 236	1				1
	139 173 205 225 236 247	26	33			59
	139 176 205 225 236 247		1	2	3	6
	139 178	3	2	28	1	34
	139 178 199 225 236 247	6	14	17	14	51
	139 178 205	42	45	5	5	97
var062	139 178 205 212 236 247		1			1
	139 178 205 225	11	24	18	32	85
	139 178 205 225 236	5	7	10	15	37
	139 178 205 225 236 247	418	737	794	911	2860
	139 178 205 225 236 248	9	8	4	13	34
	139 178 205 225 238	6	1	10	1	18
	139 178 205 228 247			2		2
	139 179 225 236 247	8	7	12		27
	139 179 225 238			1		1
	14 53 94 112 139				2	2
	14 53 94 112 139 178				1	1
	14 54	6	10	1	9	26
	14 54 94	8	15	3	19	45
	14 54 94 103 139			1		1
	14 54 94 111 139				4	4
	14 54 94 112		1	1		2
	14 54 94 112 139	20	24	37	43	124
var310	14 54 94 112 139 173				2	2
var311	14 54 94 112 139 178		1	9	13	23
	14 54 94 114 139	3				3
	14 54 96 112 139		1			1
	142 178 205 225 236 247				1	1
	146 173 205 225 236 247	5	1			6
	15 44 94 112 139		1			1
	15 54 94 111 139			1		1
	15 69 94 112 139		3			3
	152 178 205 225 236 247			1		1
var025	161 173 205 225 236 247		1			1
	163 205 225 236 247			1		1
var267	166 205 225 236 247	1				1

	17 94 112 139	1		6		7
	173 205 225	14	9	1	1	25
	173 205 225 236	15	20			35
	173 205 225 236 247	1503	2018	11	13	3545
	173 205 225 237 247	1				1
	173 205 225 239 247		2			2
	176 199 225 236 247			1	2	3
	176 205 225		1			1
	176 205 225 236 247		1	2	2	5
	178 199 225		1			1
	178 199 225 236 247	12	13	10	19	54
	178 205	23	18	2	20	63
	178 205 212 236 247	3	1	1		5
	178 205 216 247				1	1
var235	178 205 224 236 247		1			1
	178 205 225	62	45	21	68	196
	178 205 225 236	21	14	7	21	63
	178 205 225 236 247	768	1390	2062	2492	6712
	178 205 225 236 248	6	14	7	10	37
	178 205 225 238	29	9	9		47
	178 205 227 236 247	1				1
	178 205 228 247			4		4
	178 207 225 236 247				1	1
	179 225	2	3			5
	179 225 236 247	25	10	11	12	58
	19 53 94 112		1			1
	19 53 94 112 139		1			1
	19 54 94				1	1
	19 54 94 112 137		1		1	2
	199 225			6		6
	199 225 236		1			1
	199 225 236 247	26	39	8	36	109
	2 44		1			1
	2 54 94 112		1			1
var001	2 54 94 112 139		1	7	1	9
	20 53 94	1		1		2

	20 53 94 112		1			1
	20 53 94 112 139	1	2			3
	20 54			2		2
	20 54 94			1		1
	20 54 94 112		1			1
	20 54 94 112 137				1	1
	20 54 94 112 139	3	5	8	7	23
	20 54 94 114 139			3		3
	205 216 247				2	2
	205 224 236 247	1	4	1		6
	205 225	28	65	26	52	171
	205 225 236	10	26	16	24	76
	205 225 236 247	7874	13227	7484	10042	38627
	205 225 236 248	8	10	10	11	39
	205 225 237 247	2	7		5	14
	205 225 238	18	9	13	3	43
	205 225 239 247	1				1
	205 227 236 247		1			1
	205 228 247			1		1
	205 236 247			1	2	3
	212 236 247		3		7	10
var024	223 236 247		1			1
	224 236 247		1			1
	225 236	14	16	17	37	84
	225 236 247	3453	5519	3801	4807	17580
	225 236 248	44	28	21	34	127
	225 237 247		5	1	1	7
	225 238	32	12	19	3	66
	227 236 247		1		1	2
	228 247			5		5
	236 247	2694	4763	2247	3121	12825
	236 248	15	31	7	25	78
var230	237 247	1	4	2	2	9
var339	239 247		1		1	2
var357	30 94 112 139			2		2
	44 94 112	1		1		2

	44 94 112 139				2	2
	44 94 112 139 178	1		1		2
	44 94 112 139 178 199		1			1
	44 94 112 139 178 205	1			1	2
	5 94				1	1
	5 94 112 139		1		1	2
var071	5 94 112 139 178 205				4	4
	53 94	3	4	1	2	10
	53 94 112		4			4
	53 94 112 139	10	27	2	5	44
	53 94 112 139 178			1		1
	53 94 126 139	2	1			3
	54 94	15	20	4	23	62
	54 94 103 139	3				3
	54 94 112	3	9	9	5	26
	54 94 112 137				3	3
	54 94 112 139	34	82	86	128	330
	54 94 112 139 178	1	1	14	12	28
	54 94 112 139 178 205	3	4	11	17	35
	54 94 112 139 178 205 225		1		1	2
	54 94 112 139 179			1		1
	63 94 112 137				1	1
	63 94 112 139	1				1
	67 94 112 137 178 205 225 236 247			12	21	33
var021	75 112 139			2		2
	94 103 137				1	1
	94 103 137 178 205 225 236 247			3	3	6
	94 111 139			2	1	3
	94 112		1	2		3
	94 112 134 178 205 225 236 247			2		2
	94 112 137	2	4		42	48
	94 112 137 176 205 225 236 247				1	1
	94 112 137 176 205 225 236 247			2		2
	94 112 137 178 205	2		1	1	4
	94 112 137 178 205 225			1		1
	94 112 137 178 205 225 236 247			600	1046	1646

	94 112 137 178 205 225 236 248				2	2
	94 112 139	190	292	192	427	1101
	94 112 139 152	1				1
	94 112 139 152 178 205 225	1				1
var034	94 112 139 154 225 236 247		1			1
	94 112 139 178	13	7	27	10	57
var045	94 112 139 178 199 225 236 247				2	2
	94 112 139 178 205	11	4	8	10	33
	94 112 139 178 205 225	15	17	9	19	60
	94 112 139 178 205 225 236 247			17	20	37
var169	94 112 139 178 205 225 236 248			1		1
	94 112 139 178 205 228 247			5		5
	94 112 139 179	1				1
	94 112 141	1				1
	94 114 137 178 205 225 236 247			1	3	4
	94 114 139			5	1	6
	94 114 139 178 205			1		1
	94 114 139 178 205 225			1		1
	94 117	2				2
	94 117 178 205 225 236 247			1		1
	94 117 179 225 236 247				2	2
	94 121 139		1			1
	94 125 139			1	1	2
	94 126 137		1			1
	94 126 137 178 205 225 236 247			2	4	6
	94 126 139	1	4		2	7
	94 126 139 178 205 225 236 247			1		1
	94 139 178			5		5
var017	97 112 139 178 205 225 236 247			1		1

Variant	variant name, only present for those variants displayed in Figures X and Suppl. Figure Y
Splice junctions	splice junctions (as named in Additional file 3) present in each variant
Ring	number of reads in the ring life-cycle stage that display a particular combination of splice junctions
Early trophozoite	same as above, but for early trophozoite
Late trophozoite	same as above, but for late trophozoite

Schizont same as above, but for **schizont**

Other observations: Splice junction combinations in **bold typeface** are those containing only splice junctions present in the annotated isoform of the gene, present in GenBank

Additional file 6

Title: Reverse-transcriptase PCR essays for confirming isoforms in the isolated from patients and wild type strains

Description: Primers were designed to target regions specific for detecting an alternative splicing event occurring only if deletion of a particular exon occurred (as an example, see sketch in Additional file 7 part A). **A)** Primers for amplifying a product only if exon 5 is deleted. **B)** Primers for amplifying a product only if exon 7 is deleted. **C)** Primers for amplifying a product only if exon 10 is deleted. Isolates: 3D7 (wild type); S20 (isolated from a patient); Cand (isolated from a patient); gDNA (genomic DNA from 3D7); - (negative control).

Additional file 7

Title: qPCR comparison of expression between isoforms lacking exon 7 or generating the intact primary protein

Description: **A)** Oligonucleotide design for detecting an alternative splicing event. **B)**

Transcript levels of the gene encoding FPPS/GGPPS in isolate 3D7 were normalized by the control gene K1. Statistical significance was determined by one-way ANOVA. All differences between control and variant isoforms were significant ($p < 0.05$). Control (transcript for the primary protein); - exon7 (isoform lacking exon 7); a and b (F-iso and R-iso); c and d (F-iso7 and R-iso7); ET (early trophozoite); LT (late trophozoite).

II- Squalestatin is an inhibitor of carotenoid biosynthesis in *P. falciparum*.

Squalestatin Is an Inhibitor of Carotenoid Biosynthesis in *Plasmodium falciparum*

Heloisa B. Gabriel, Marcia F. Silva, Emília A. Kimura, Gerhard Wunderlich, Alejandro M. Katzin, Mauro F. Azevedo

Department of Parasitology, Institute of Biomedical Science, University of Sao Paulo, Sao Paulo, Brazil

The increasing resistance of malaria parasites to almost all available drugs calls for the characterization of novel targets and the identification of new compounds. Carotenoids are polyisoprenoids from plants, algae, and some bacteria, and they are biosynthesized by *Plasmodium falciparum* but not by mammalian cells. Biochemical and reverse genetics approaches were applied to demonstrate that phytoene synthase (PSY) is a key enzyme for carotenoid biosynthesis in *P. falciparum* and is essential for intraerythrocytic growth. The known PSY inhibitor squalestatin reduces biosynthesis of phytoene and kills parasites during the intraerythrocytic cycle. PSY-overexpressing parasites showed increased biosynthesis of phytoene and its derived product phytofluene and presented a squalestatin-resistant phenotype, suggesting that this enzyme is the primary target of action of this drug in the parasite.

Malaria is one of the most important infectious diseases in the world, causing about 200 million clinical cases and over 600,000 deaths every year. Lethal forms of the disease are caused mostly by *Plasmodium falciparum*, and there is an increasing resistance of this parasite to virtually all current drugs, including artemisinin, in five southeast Asian countries and probably in South America (1). This calls for the identification of new therapeutic targets and the development of new drugs (2). The causative agent of malaria is a protozoan of the genus *Plasmodium*, transmitted by the female *Anopheles* mosquito host, in which occurs the sexual phase of the parasite's life cycle. Five species of *Plasmodium* infect humans, and *P. falciparum* causes most cases of morbidity and mortality (3). Given the genetic flexibility and the resulting development of resistance to almost every drug, a comprehensive understanding of plasmodial metabolic pathways is essential for the development of new chemotherapeutic strategies. An important target for the development of new antimalarial drugs is isoprenoid biosynthesis (Fig. 1), which occurs via the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) (4–8) in *P. falciparum*, some plants, and bacteria (9, 10). In contrast, most animal cells, certain eubacteria, archaea, and fungi synthesize isoprenoid precursors through the mevalonate pathway (11). In the case of malaria parasites, especially the most virulent species, *P. falciparum*, a series of new plantlike enzymes from this metabolic pathway was discovered recently. Some of these enzymes are associated with the apicoplast (12), whereas the nature of the others and the pathways they are involved in remain elusive to current bioinformatics approaches.

Many isoprenoids from the MEP pathway, such as carotenoids and ubiquinones (biosynthesized by *P. falciparum*) (8, 13), are essential components of the cellular machinery of many organisms, participating in a variety of biological processes. All carotenoids possess a polyisoprenoid structure (Fig. 1), a long conjugated chain of double bonds, and an almost bilateral symmetry around the central double bond. Among these biochemical pathways, carotenoid biosynthesis is an attractive target for investigation, because it is essential in algae, higher plants, bacteria, and fungi, and it is absent from most animals, including mammals (14). Carotenoid biosynthesis in intraerythrocytic stages of *P. falciparum* has been demonstrated, suggesting it plays a role in par-

asite development and/or replication; therefore, it could be a drug target (13). Carotenoid biosynthesis starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene, the initial C₄₀ carotenoid skeleton (14). This reaction is catalyzed by the enzyme phytoene synthase (PSY). The gene encoding PSY in *P. falciparum* has been identified (PlasmoDB accession no. PF3D7_0202700) and its product characterized (13). It appears to be a bifunctional enzyme, since it exerts octaprenyl pyrophosphate synthase (OPP) activity, which is involved in the elongation of the isoprenic chain, which then is attached to the benzoquinone ring that comes from the shikimate pathway (15). The plasmodial enzyme is an example of a carotenogenic enzyme with a continuous line of evolution from archaea to bacteria (via cyanobacteria) and plants (16, 17) containing two activities.

Inhibiting *Plasmodium* PSY and, therefore, the first step in carotenoid biosynthesis could help to reveal the role of these isoprenoid compounds in the parasite intraerythrocytic cycle, demonstrating whether this metabolic pathway could be a drug target. The compound zaragozic acid, also known as squalestatin, is a carboxylic acid, with the molecular structure C₃₅H₄₃O₁₄Na₃ (2,8-dioxabicyclo-[3.2.1]-octan-3 core acid, 4,5-tricarboxylic), that was discovered by screening metabolites of filamentous fungi for inhibitors of squalene synthase, the enzyme responsible for the first step of sterol biosynthesis (18–21). Detailed analysis disclosed that it acts as a competitive inhibitor of squalene synthase by mimicking the farnesyl-PP substrate or the stable intermediate pres-

Received 8 October 2014 Returned for modification 3 December 2014

Accepted 9 March 2015

Accepted manuscript posted online 16 March 2015

Citation Gabriel HB, Silva MF, Kimura EA, Wunderlich G, Katzin AM, Azevedo MF. 2015. Squalestatin is an inhibitor of carotenoid biosynthesis in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 59:3180–3188.
doi:10.1128/AAC.04500-14.

Address correspondence to Alejandro M. Katzin, amkatzin@icb.usp.br, or Mauro F. Azevedo, maurousp@gmail.com.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.04500-14

FIG 1 Carotenoid biosynthesis pathway in *P. falciparum*. Downstream of the MEP pathway, the carotenoid biosynthesis starts with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) by phytoene synthase enzyme (PSY) to form phytoene, the initial C₄₀ carotenoid skeleton.

qualene-PP with its bicyclic, highly acidic core (22). Neudert et al. showed that squalestatin also inhibits PSY from the enterobacterium *Erwinia uredovora* (23). The inhibition of phytoene synthase by an inhibitor of squalene synthase presumably can be explained by similar catalytic mechanisms proposed for both enzymes during conversion of two molecules of GGPP or farnesyl pyrophosphate ammonium salt (FPP), respectively (23, 24).

Recent data showed that squalestatin has an inhibitory effect on *P. falciparum* *in vitro* growth and a synergistic effect when combined with other drugs (M. F. da Silva, A. Y. Saito, V. J. Peres, A. C. Oliveira, and A. M. Katzin, submitted for publication), suggesting PSY activity is the target, since *Plasmodium* does not have squalene synthase or synthesize sterols (25). In this study, we ap-

plied biochemical and reverse genetics approaches to demonstrate that PSY is the main target of squalestatin in *P. falciparum* and that the first carotenoid, phytoene, is essential for parasite development during the intraerythrocytic cycle.

MATERIALS AND METHODS

Reagents. [1-(*n*)-³H]geranylgeranyl pyrophosphate triammonium salt, [1-(*n*)-³H]GGPP (16.5 Ci/mmol), and [1-(*n*)-³H]farnesyl pyrophosphate triammonium salt, [1-(*n*)-³H]FPP (23 Ci/mmol), were obtained from GE Healthcare. Life Technologies supplied Albumax I. All solvents used were high-performance liquid chromatography (HPLC) grade or better. Sigma-Aldrich provided isopentenyl pyrophosphate (IPP), FPP, GGPP, all-*trans*-lutein, all-*trans*-β-carotene, squalestatin, and all other biochemical

TABLE 1 Oligonucleotides utilized in PCR amplification

Name	Sequence ^a
F-int-PSY-Sma	<u>CCCGGGTTTCAAATCAAATAAACTCACGTC</u>
R-PSY-Mlu	<u>ACGCGTTTTGACGTTTCTTGATAACACGTTTAAG</u>
F-PSY-Xho	<u>CTCGAGATGGTTCACCTAAGTAAAAAGAAATAATATT</u>
F-PSY	TGGTACGGGTTACCAAAAAT
R-PSY	CATTTTGAGTGCTTCTTCAACA

^a Underlining denotes restriction sites.

reagents. Carotenoid standards were donated by DSM Nutritional Products (Basel, Switzerland).

Plasmid construction. The sequences for the oligonucleotides used are described in Table 1. The expression vector pRM2-GFP-HA-DD24 (26) was digested with NheI and SpeI and religated to delete the DD24 sequence, generating pRM2-GFP-HA. The *P. falciparum* genomic DNA (gDNA) sequence that encodes the bifunctional enzyme OPP/PSY (PlasmoDB no. PF3D7_0202700), nucleotides 687 to 1614, was PCR amplified with oligonucleotides F-int-PSY-Sma and R-PSY-Mlu, digested with SmaI and MluI, and cloned in pRM2-GFP-HA via the same sites, replacing the MSP2 promoter and the green fluorescent protein (GFP) gene and generating the integration vector pPSY/OPP-HA. DD24 was retrieved from pRM2-GFP-HA-DD24 digested with MluI and NotI and cloned via the same sites in pPSY/OPP-HA to generate the integration vector pPSY/OPP-HA-DD24.

The coding sequence of the *P. falciparum* OPP/PSY gene was PCR amplified from cDNA with oligonucleotides F-PSY-Xho and R-PSY-Mlu, digested with XhoI and MluI, and cloned in the same sites of pRM2-GFP-HA, replacing GFP and generating *Plasmodium* expression vector pRM2-PSY-HA.

***P. falciparum* culture.** Cultures of *P. falciparum* clone 3D7 were grown as described previously (27), except that human serum was replaced with Albumax I (0.5%; Invitrogen/Life Technologies). Parasite multiplication was monitored by microscopic evaluation of Giemsa-stained thin smears. Schizont stages were purified with magnetic columns (magnetically activated cell sorting [MACS] separation columns; CS; Miltenyi Biotec) (28). Column preequilibration, washing, and elution all were carried out at room temperature with RPMI 1640 (Sigma-Aldrich). For schizont purification, the culture was centrifuged ($2,000 \times g$ for 5 min) and the pellet resuspended in RPMI 1640 (1:10, vol/vol), and 10 ml of the 10% suspension of erythrocytes was applied to a CS column assembled in a magnetic unit, where only schizonts are retained. After washing the column with 50 ml of RPMI 1640, the column was removed from the magnetic field and its contents eluted with 50 ml of RPMI 1640, and the schizont-stage parasites were centrifuged at $2,000 \times g$ for 5 min at room temperature. The supernatant was discarded, and the pellet of parasites was stored in liquid N₂ for subsequent analysis.

Parasite transfection. Parasites were transfected as previously described (29), using the electroporation conditions established elsewhere (30). Briefly, *P. falciparum* 3D7 was cultured in 4% hematocrit in RPMI-HEPES supplemented with 0.5% Albumax I (Invitrogen). Ring-stage parasites at 5 to 8% parasitemia were transfected with 150 µg of plasmid DNA. The culture media were changed on the second day, and parasites were subjected to drug pressure with 2.5 nM WR99210 (stable) on the third day. Transfected parasites were cultured under standard conditions until parasites reappeared and normal growth was reestablished. When necessary, the integration at the genomic OPP/PSY locus was selected by intermittent exposure and retrieval of WR99210 and detected by PCR.

cDNA preparation and qRT-PCR. RNA was extracted using TRIzol LS (Invitrogen) by following instructions of the manufacturer. The washed RNA pellet was dried briefly at room temperature, dissolved in water, and stored at -80°C until use. About 5 µg of total RNA was used for cDNA synthesis. Briefly, total RNA was treated 3 times with DNase I (Fermentas) prior to synthesis to prevent genomic DNA contamination. The treated RNA was reverse transcribed using MuLV-Revert Aid reverse

transcriptase (Fermentas) and random hexamer primers by following the manufacturers' instructions. Oligonucleotides that amplify the transcript that encodes the enzyme OPP/PSY were designed using Primer3 (F-PSY; R-PSY) (<http://frodo.wi.mit.edu/>). The internal control transcript used for calibration throughout the experiments was locus seryl t-RNA transferase (PlasmoDB no. PF3D7_0717700), previously shown as a reliable control (31), since its transcript level does not vary greatly during the intraerythrocytic cycle. Thus, the relative mRNA expression was obtained using the formula $2^{-\Delta CT}$. All experiments were performed in duplicate.

Inhibition tests with zaragozic acid (squalestatin). Squalestatin was dissolved in water, resulting in 10 mM stock solutions (23). We applied the method proposed by Desjardins et al. and Moneriz et al. (32, 33) to determine the 50% inhibitory concentrations after 48 h (IC₅₀s). Briefly, synchronous ring-stage parasite cultures (5% hematocrit and 1% parasitemia) were exposed to increasing drug concentrations, and parasitemia was determined by Giemsa-stained smears after 2 days. All tests were performed in triplicates from three independent experiments. The IC₅₀s for growth inhibition were calculated by nonlinear regression in GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

For isoprenoid biosynthesis inhibition, schizont-stage parasites were treated with squalestatin at the IC₅₀ for 48 h, and parasites were labeled with the metabolic precursors during the last 16 h.

Metabolic labeling and carotenoid extraction. Synchronous cultures of *P. falciparum* in the trophozoite stage were labeled with [³H]GGPP (0.75 µCi/ml) or [1-(*n*)-³H]FPP (0.75 µCi/ml) diluted in complete RPMI 1640 medium for 16 h. Subsequently, infected red blood cells with parasites in the schizont stage were concentrated with magnetic columns. Extraction was performed by mixing the cell pellet with four volumes of ice-cold acetone, which was centrifuged at $8,000 \times g$ for 5 min. The supernatant was collected and the extraction repeated 3 times. The pooled acetone phases were dried under a nitrogen stream and stored in liquid nitrogen (34).

RP-HPLC. For reverse-phase HPLC (RP-HPLC), the acetone extracts were resuspended in 20 µl of methyl tertiary-butyl ether and then analyzed using a YMC C₃₀ polymeric column (4.6 by 250 mm, 3 µm and/or 5 µm) (YMC Inc.) in a gradient system (13). A carotenoid extract mixture in methyl tertiary-butyl ether was coinjected and served as a standard. We carried out the analyses in an HPLC-photodiode array (PDA) equipped with a model 600 quaternary solvent delivery system (Waters, Milford, MA) and an on-line degasser, a Rheodyne injection valve with a 20-µl loop, and an external oven coupled to the model 996 PDA detector (Waters). We used Millennium Waters software for data acquisition and processing. The PDA was set at 450, 346, and 288 nm for the analysis of carotenoids (34).

Western blot analyses. Synchronous cultures of transfected parasites were recovered in each stage. Ring, trophozoite, and schizont stages were treated with 0.15% saponin in RPMI media to release hemoglobin from the red blood cells. Proteins were extracted with 2D buffer (7 M urea, 2 M thiourea, 2% ASB-14) (35) for separation by SDS-PAGE. The gel then was transferred to nitrocellulose membrane (Amersham) for 1 h using a Trans-Blot semidry electroblotter (Bio-Rad) (36). After blocking with 1% casein in phosphate-buffered saline (PBS), the membranes were incubated with an α-hemagglutinin (HA) monoclonal antibody (1:500 dilution; Sigma-Aldrich) or with the control antibody α-PTEX150 (1:1,000) (37) or α-MSP2 (1:500) (38), diluted in blocking solution for 1 h at room temperature or 14 h at 4°C , washed in PBS, and incubated with an anti-mouse IgG-labeled, peroxidase-conjugated secondary antibody diluted in blocking solution. After PBS washing, blots were incubated with an ECL (enhanced chemiluminescence) detection kit according to the instructions of the manufacturer, and the signal was detected with the IQ350 apparatus (GE Healthcare).

RESULTS

Effect of squalestatin on *P. falciparum* carotenoid biosynthesis. To determine the effect of squalestatin on carotenoid biosynthesis, parasites in schizont stage were cultured at the IC₅₀ of the drug

FIG 2 Effect of squalenstatin on carotenoid biosynthesis. Parasites were treated with 5 μ M squalenstatin for 48 h, and carotenoid biosynthesis was determined by HPLC. The bars represent the relationship between squalenstatin-treated parasites and controls (means $\pm$ standard deviations; $n = 3$ experiments). Significant differences comparing control parasites and parasites treated with squalenstatin were determined by one-way analysis of variance (ANOVA). Significant P values ($P < 0.05$) are shown, and ns denotes nonsignificant P values ($P > 0.05$).

(5 μ M) for 48 h and metabolically labeled with the carotenoid precursor [3 H]GGPP during the last 16 h. Equal quantities of extracts from schizont-stage parasites, treated and untreated, were analyzed by RP-HPLC, which revealed that biosynthesis of the first carotenoid phytoene had significantly diminished by $43.6\% \pm 5\%$ in parasites treated with drug compared to the levels in untreated parasites (Fig. 2). Biosynthesis of the other carotenoids phytofluene and β -carotene were not significantly affected, suggesting specific inhibition of PSY rather than a general toxic effect of the drug.

OPP/PSY is essential and constitutively expressed in the intraerythrocytic cycle. In order to obtain genetic proof that the biosynthesis of phytoene actually is essential for *P. falciparum*, we attempted to generate a transgenic parasite line where expression of the bifunctional enzyme OPP/PSY could be regulated (Fig. 3). To this end, 3D7 parasites were transfected with the vector pOPP/PSY-HA-DD24 and cultured in the presence of Shld-1. After the expected integration into the genomic locus (Fig. 3A), OPP/PSY should be expressed in fusion at its C terminus with a triple repeat of the HA epitope, which is used for detection by Western blotting, and the destabilization domain (DD24). Expression would be dependent on the ligand Shld-1, permitting the regulation of OPP/PSY levels. After three independent transfections, parasites

FIG 3 OPP/PSY is constitutively expressed and essential during the intraerythrocytic cycle. (A) Outline of the HA and HA-DD24 targeting vectors and their predicted integration into the wild-type OPP/PSY genomic locus. (B) Diagnostic PCR of integration using genomic DNA from the wild-type 3D7 strain and the transfected lines and oligonucleotides specific to the integrated lines (3 + 2) or common to all lines (1 + 4). Binding sites for the oligonucleotides used are shown in panel A. (C) Expression levels of OPP/PSY during the three intraerythrocytic stages of OPP/PSY-HA-int parasites were determined by Western blotting using α -HA antibody. Antibodies for a constitutively expressed protein (pTEX150) and a schizont-expressed protein (MSP2) were used as synchronization controls. R, ring; T, trophozoite; S, schizont.

FIG 4 OPP/PSY overexpression in *P. falciparum*. (A) Schematic diagram of the vector used to overexpress OPP/PSY in *P. falciparum*. (B) OPP/PSY expression in the integrated HA-tagged line OPP/PSY-HA-int and in transfected parasites where the overexpression vector is episomally maintained (OPP/PSY-HA-epi) was accessed by real-time PCR. The transcript levels of the gene encoding OPP/PSY were normalized by the control gene K1 and are represented relative to levels for 3D7. Statistical significance was determined by one-way ANOVA, and the *P* value is indicated.

with the plasmid integrated were never recovered (Fig. 3B), suggesting that OPP/PSY does not retain its physiological function when in fusion with a 15-kDa tag or that its expression levels were too low even in the presence of Shld-1.

To confirm that the OPP/PSY genomic locus was not refractory to DNA integration, we generated a similar transgenic line, except that after integration the bifunctional enzyme should be expressed at endogenous levels in fusion with the significantly smaller 3-kDa HA tag (Fig. 3A). Parasites with the plasmid integrated were detected by PCR (data not shown) and cloned by limiting dilution, generating the clonal line OPP/PSY-HA-int, in which the integration was confirmed by PCR (Fig. 3B).

After plasmid integration, the gene encoding the fusion OPP/PSY-HA is supposed to be under the control of the endogenous promoter and only one copy expressed, making this line suitable to determine the expression profile of OPP/PSY. Protein samples were extracted from OPP/PSY-HA-int parasites synchronized in the three main stages (ring, trophozoite, and schizont) and detected with a monoclonal antibody against HA (Fig. 3C). As controls of parasite synchronization, antibodies that recognize the constitutively expressed protein pTEX150 (37) and the schizont-specific protein MSP2 (38) were used. The results indicate that OPP/PSY is constitutively expressed in all stages during the asexual intraerythrocytic cycle of *P. falciparum*, with its levels peaking at schizont stage. OPP/PSY-HA-int growth was similar to that of 3D7 parasites, and expression was stable after several months in culture, suggesting that neither the 3-kDa HA tag nor the inte-

FIG 5 OPP/PSY overexpression negatively affects parasite growth. Growth of wild-type 3D7 parasites and the HA-tagged lines, either integrated (OPP/PSY-HA-int) or episomal (OPP/PSY-HA-epi). Parasitemia was determined by microscopic examination of smears stained with Giemsa. The points refer to the averages from triplicate experiments, and the error bars show the standard deviations.

grated plasmid affected the function of this enzyme (data not shown).

OPP/PSY overexpression. To gather further proof that phytoene is essential and that PSY is the main target of squalastatin in the parasites, we attempted to overexpress OPP/PSY using a pEF-based vector (26), from which the gene would be under the control of the constitutive *efl-α* promoter (39, 40). It was expected that OPP/PSY would be overexpressed in the transfected parasites, since plasmids usually are maintained as concatemeric episomes of multiple copies. However, we were never able to generate transfected parasites, suggesting constitutive overexpression is toxic (data not shown). To attenuate any possible toxic effect of OPP/PSY overexpression, the *efl-α* promoter was replaced with the schizont-specific MSP2 promoter (38), and the resulting plasmid pRM2-OPP/PSY-HA was successfully transfected in 3D7 parasites, generating the transgenic line OPP/PSY-HA-epi (Fig. 4A). In order to compare OPP/PSY expression in the transgenic lines OPP/PSY-HA-epi and OPP/PSY-HA-int to that in wild-type 3D7 parasites, real-time PCR was applied. Although OPP/PSY is constitutively expressed, MSP2 is a stage-specific promoter; therefore, transcript levels were analyzed in schizont-stage parasites. While expression in the integrated line is similar to that of 3D7, as expected, it is about 5-fold higher in OPP/PSY-HA-epi parasites (Fig. 4B).

The failure to constitutively overexpress OPP/PSY suggested that an excess of this enzyme was toxic for the parasite. To investigate whether the OPP/PSY-HA-epi line had reduced fitness, parasite growth was compared to that of wild-type 3D7 and the integrated line (OPP/PSY-HA-int) for 96 h during the erythrocytic cycle. OPP/PSY-overexpressing parasites grew at a rate slower than those of both the wild-type 3D7 strain and the transfected line with the plasmid integrated (Fig. 5). We expected that the OPP/PSY-HA-epi line was unstable and would lose the plasmid or the transgene expression over time due to toxic OPP/PSY overexpression. In order to avoid that, the following experiments always were performed with freshly transfected parasites no more than 4 weeks after the reestablishment of growth.

Effect of OPP/PSY overexpression on carotenoid biosynthesis. To determine whether the products of OPP and PSY would be

FIG 6 Effect of OPP/PSY overexpression on carotenoid and GGPP biosynthesis. (A) Phytoene, phytofluene, and β -carotene biosynthesis in OPP/PSY-HA-epi strain compared to those of 3D7. Carotenoids were extracted from [1-(*n*- 3 H)]GGPP-labeled schizonts and analyzed by HPLC. The radioactive peaks corresponding to the retention time of phytoene, phytofluene, and β -carotene were plotted. (B) GGPP was extracted from [1-(*n*- 3 H)]FPP-labeled schizonts of OPP/PSY-HA-epi and 3D7 wild-type parasites and analyzed by HPLC. The radioactive peaks corresponding to the retention time of GGPP were plotted. Statistical significance was determined by one-way ANOVA. *P* values of significant results (*P* < 0.05) are shown, and ns denotes nonsignificant associations (*P* > 0.05).

biosynthesized at a higher rate in the overexpressing transgenic line, OPP/PSY-HA-epi and 3D7 parasites were metabolically labeled and the metabolites analyzed by RP-HPLC (Fig. 6A). Biosynthesis of the direct product of PSY, phytoene, and of the product of the subsequent reaction, phytofluene, was increased $57.3\% \pm 3\%$ and $47\% \pm 5\%$, respectively. In contrast, biosynthesis of β -carotene, a downstream product in the carotenoid pathway, was not affected. Biosynthesis of GGPP, the substrate of PSY, and one of the intermediate products of OPP was increased $46.2\% \pm 5\%$ (Fig. 6B).

OPP/PSY-overexpressing parasites are more resistant to squalestatin. The correlation between OPP/PSY overexpression and increased biosynthesis of its products suggested these parasites can tolerate enzyme activity inhibition at higher levels than parasites expressing OPP/PSY at standard levels. To test that hypothesis, the inhibitory effect of squalestatin on *P. falciparum* growth was assessed by culturing the wild-type (3D7), the integrated (OPP/PSY-HA-int), and the overexpressing (OPP/PSY-HA-epi) lines in the absence or in the presence of increasing concentrations of the drug (Fig. 7). Parasite growth was inhibited in a dose-dependent manner and was correlated with OPP/PSY expression. Specifically, while the line transfected with the integrated plasmid was as sensitive to squalestatin as the wild-type parasites, the squalestatin IC_{50} in the overexpressing line was about 5.5-fold higher. This not only demonstrates that the coding DNA sequences from the plasmid, such as the human *dhfr* gene or the HA tag *per se*, are not able to confer a resistant phenotype but also that the overexpression of OPP/PSY was responsible for the increase in the observed IC_{50} .

DISCUSSION

The discovery of new antimalarials is a key aspect for the success of malaria control. High-throughput drug screening studies allow the identification of new potential hits, but those are expensive and time-consuming until new compounds can be produced in large scale and applied for malaria treatment. In contrast, the identification of parasite pathways that already can be targeted by

FIG 7 Parasites overexpressing OPP/PSY are more resistant to squalestatin. (A) Parasites of the wild-type 3D7 strain and the transgenic lines OPP-PSY-HA-int and OPP-PSY-HA-epi were cultured for 2 days in the presence of various concentrations of squalestatin. Growth is represented relative to that of control parasites cultured in the presence of the solvent control. (B) The IC_{50} s represent the means and error bars (95% confidence intervals) from 3 experiments: 3D7, 5.2 μ M (3.0 to 8.8 μ M); OPP/PSY-HA-int, 5.3 μ M (3.5 to 7.8 μ M); OPP/PSY-HA-epi, 28.7 μ M (27.1 to 30.4 μ M).

known licensed drugs, either commercially available or in the pipeline for mass production, might eventually speed up the process.

Squalastatin is a potential cholesterol-lowering drug and is expected to produce fewer side effects, since it inhibits the first specific step in sterol biosynthesis. In contrast, the standard treatment based on statins inhibits the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, from which the product mevalonate is a precursor of several other metabolites. Reduction of serum cholesterol in rodents has been achieved successfully with squalastatin, suggesting it can be used *in vivo* in mammals (19). Further studies are required to determine its safety and efficacy for humans, as well as large-scale production optimizations. As for use as an antimalarial, *in vivo* tests also are required to determine its potency against *Plasmodium* infection.

In this report, we applied reverse genetic strategies in order to investigate the importance of *P. falciparum* OPP/PSY during the intraerythrocytic cycle. The possibility of altering the parasite genome only when OPP/PSY would be expressed in fusion with a 3-kDa HA tag indicated the genomic locus is amenable to knock-in experiments. However, the failure to generate transgenic lines where OPP/PSY would be expressed in fusion with the 15-kDa HA-DD24 tag suggested the protein levels expressed are insufficient, even in the presence of the stabilizing ligand Shld-1, or that its physiological function is lost when in fusion with the larger tag. In a previous study, it was shown that DD24 decreases the amount of fused proteins to a significant extent, which is not fully reversed by the presence of Shld-1 (26). We also attempted to generate a transgenic line expressing OPP/PSY in fusion with a 30-kDa GFP-HA tag, which should not affect its expression, but also failed (data not shown). Taken together, these experiments suggest OPP/PSY plays an important and possibly essential role during the intraerythrocytic cycle, and it cannot fully perform its physiological function when in fusion with large tags at its C-terminal region. PSY from plants usually is associated with membranes and with other enzymes; therefore, a reasonably free C-terminal region may be required for some of those interactions to happen (41).

Once the likely essential role for this bifunctional enzyme had been proposed from the knock-in experiments, gene overexpression and treatment with squalastatin were carried out to investigate the importance of its PSY activity and, consequently, of carotenoid biosynthesis. The squalastatin-mediated inhibition of phytoene biosynthesis, but not of other carotenoids, and the resistance phenotype presented by the overexpressing parasites suggest PSY is the main target of this drug in the parasites, and that phytoene is essential during the intraerythrocytic cycle.

It is uncertain whether phytoene is the only carotenoid required for parasite development or if its derived products also play important roles. A way to investigate this issue would be to inhibit the reactions involved in the biosynthesis of the derived carotenoids. However, the genes encoding these enzymes have not been identified in *Plasmodium*; therefore, it is not yet possible to target them either by reverse genetics or to test inhibitors *in vitro*.

Rather than a general toxic effect, squalastatin affected mainly parasites at the schizont stage before segmentation (data not shown). Although OPP/PSY is constitutively expressed, its products are detected mostly in mature-stage parasites (13, 15), suggesting its activity is limited by the availability of some of the precursors, which could explain the failure to generate transgenic

parasites where OPP/PSY would be constitutively overexpressed. Toxic effects of PSY overexpression have been reported both in bacteria and plants, which was caused mainly by the depletion of its substrate, GGPP, inducing a decrease of other products of the isoprenoid pathway (23, 42). In the overexpressing parasite line, OPP also is overexpressed, and GGPP is one of its intermediate products; therefore, other isoprenoids precursors, such as FPP, GPP, IPP, or DMAPP (dimethylallyl pyrophosphate), could be the ones being depleted. Since these metabolites are biosynthesized mostly in mature parasites (30 h postinfection), early-stage parasites could be even more sensitive to their consumption caused by a constitutive overexpression.

The resistance to squalastatin in parasites overexpressing OPP/PSY under the control of the MSP2 schizont-specific promoter also points to a specific function during this part of the life cycle which needs to be further investigated. However, since the overexpression is limited to schizont-stage parasites, it is not possible to investigate if there is a function for this enzyme in ring- and/or trophozoite-stage parasites. It is possible that carotenoids act as antioxidants during hemoglobin digestion or even as precursors of signaling molecules. Parasites live in a prooxidant environment containing oxygen and iron, which are the key players for the formation of reactive oxygen species. As a consequence, *P. falciparum* is heavily dependent on efficient antioxidant systems (43–46).

An indication that carotenoids are involved in the parasite antioxidant system has been demonstrated previously (13). During studies to determine the free radical scavenger capacity in terms of the electron donor mechanism and the deactivation of singlet oxygen, phytoene and phytofluene presented a higher antioxidant capacity than expected considering the small number of conjugated double bonds (47). Other probable functions essential for the parasite may be taken into account, such as coordination of plastid and nuclear gene expression (48) and/or membrane structure modification (49).

In conclusion, we demonstrated through biochemical and reverse genetics approaches that phytoene synthase is essential for the parasite and apparently the main target of squalastatin. The data presented here suggest that carotenoid biosynthesis is a target for the development of new antimalarials and squalastatin, or that derived compounds can be further exploited as potential antiparasitic drugs.

ACKNOWLEDGMENTS

This work was supported by grants from CNPq and FAPESP. H.B.G. is the recipient of a postgraduate fellowship from FAPESP.

We thank S. Wendel (Blood Bank at the Srio Libans Hospital) for providing the erythrocytes.

REFERENCES

1. WHO. 2013. World malaria report. Report 9789241564694. World Health Organization, Geneva, Switzerland.
2. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434:214–217. <http://dx.doi.org/10.1038/nature03342>.
3. Singh B, Daneshvar C. 2013. Human infections and detection of *Plasmodium* knowlesi. *Clin Microbiol Rev* 26:165–184. <http://dx.doi.org/10.1128/CMR.00079-12>.
4. Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, Allen CM. 2002. Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J Biol Chem* 277:42066–42073. <http://dx.doi.org/10.1074/jbc.M202860200>.

5. Borrmann S, Issifou S, Esser G, Adegnik AA, Ramharter M, Matsiegui PB, Oyakhirrome S, Mawili-Mboumba DP, Missinou MA, Kun JF, Jomaa H, Kremsner PG. 2004. Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. *J Infect Dis* 190:1534–1540. <http://dx.doi.org/10.1086/424603>.
6. Moura IC, Wunderlich G, Uhrig ML, Couto AS, Peres VJ, Katzin AM, Kimura EA. 2001. Limonene arrests parasite development and inhibits isoprenylation of proteins in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 45:2553–2558. <http://dx.doi.org/10.1128/AAC.45.9.2553-2558.2001>.
7. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E. 1999. Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* 285:1573–1576. <http://dx.doi.org/10.1126/science.285.5433.1573>.
8. Cassera MB, Gozzo FC, D'Alessandri FL, Merino EF, del Portillo HA, Peres VJ, Almeida IC, Eberlin MN, Wunderlich G, Wiesner J, Jomaa H, Kimura EA, Katzin AM. 2004. The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem* 279:51749–51759. <http://dx.doi.org/10.1074/jbc.M408360200>.
9. Flesch G, Rohmer M. 1988. Prokaryotic hopanoids: the biosynthesis of the bacteriohopane skeleton. Formation of isoprenic units from two distinct acetate pools and a novel type of carbon/carbon linkage between a triterpene and D-ribose. *Eur J Biochem* 175:405–411.
10. Eisenreich W, Bacher A, Arigoni D, Rohdich F. 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell Mol Life Sci* 61:1401–1426. <http://dx.doi.org/10.1007/s00018-004-3381-z>.
11. Goldstein JL, Brown MS. 1990. Regulation of the mevalonate pathway. *Nature* 343:425–430. <http://dx.doi.org/10.1038/343425a0>.
12. Luo S, Marchesini N, Moreno SN, Docampo R. 1999. A plant-like vacuolar H(+)-pyrophosphatase in *Plasmodium falciparum*. *FEBS Lett* 460:217–220. [http://dx.doi.org/10.1016/S0014-5793\(99\)01353-8](http://dx.doi.org/10.1016/S0014-5793(99)01353-8).
13. Tonhosolo R, D'Alessandri FL, de Rosso VV, Gazarini ML, Matsumura MY, Peres VJ, Merino EF, Carlton JM, Wunderlich G, Mercadante AZ, Kimura EA, Katzin AM. 2009. Carotenoid biosynthesis in intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem* 284:9974–9985. <http://dx.doi.org/10.1074/jbc.M807464200>.
14. Mijts BN, Schmidt-Dannert C. 2003. Engineering of secondary metabolite pathways. *Curr Opin Biotechnol* 14:597–602. <http://dx.doi.org/10.1016/j.copbio.2003.09.009>.
15. Tonhosolo R, D'Alessandri FL, Genta FA, Wunderlich G, Gozzo FC, Eberlin MN, Peres VJ, Kimura EA, Katzin AM. 2005. Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*. *Biochem J* 392:117–126. <http://dx.doi.org/10.1042/BJ20050441>.
16. Sandmann G. 2002. Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chembiochem* 3:629–635. [http://dx.doi.org/10.1002/1439-7633\(20020703\)3:7<629::AID-CBIC629>3.0.CO;2-5](http://dx.doi.org/10.1002/1439-7633(20020703)3:7<629::AID-CBIC629>3.0.CO;2-5).
17. Sato S, Clough B, Coates L, Wilson RJ. 2004. Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*. *Protist* 155:117–125. <http://dx.doi.org/10.1078/1434461000169>.
18. Bergstrom JD, Kurtz MM, Rew DJ, Amend AM, Karkas JD, Bostedor RG, Bansal VS, Dufresne C, Van Middlesworth FL, Hensens OD. 1993. Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. *Proc Natl Acad Sci U S A* 90:80–84. <http://dx.doi.org/10.1073/pnas.90.1.80>.
19. Baxter A, Fitzgerald BJ, Hutson JL, McCarthy AD, Motteram JM, Ross BC, Sapra M, Snowden MA, Watson NS, Williams RJ, Wright C. 1992. Squalostatins I, a potent inhibitor of squalene synthase, which lowers serum cholesterol in vivo. *J Biol Chem* 267:11705–11708.
20. Sidebottom PJ, Highcock RM, Lane SJ, Procopiou PA, Watson NS. 1992. The squalostatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. II. Structure elucidation. *J Antibiot* 45:648–658.
21. Dawson MJ, Farthing JE, Marshall PS, Middleton RF, O'Neill MJ, Shuttleworth A, Styli C, Tait RM, Taylor PM, Wildman HG, Buss AD, Langley D, Hayes MV. 1992. The squalostatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activity. *J Antibiot* 45:639–647.
22. Haeuptle MA, Welter M, Troxler H, Hulsmeier AJ, Imbach T, Hennot T. 2011. Improvement of dolichol-linked oligosaccharide biosynthesis by the squalene synthase inhibitor zaragozic acid. *J Biol Chem* 286:6085–6091. <http://dx.doi.org/10.1074/jbc.M110.165795>.
23. Neudert U, Martinez-Ferez IM, Fraser PD, Sandmann G. 1998. Expression of an active phytoene synthase from *Erwinia uredovora* and biochemical properties of the enzyme. *Biochim Biophys Acta* 1392:51–58. [http://dx.doi.org/10.1016/S0005-2760\(98\)00017-4](http://dx.doi.org/10.1016/S0005-2760(98)00017-4).
24. Goodwin TW. 1983. Developments in carotenoid biochemistry over 40 years. The third Morton lecture. *Biochem Soc Trans* 11:473–483.
25. Vial HJ, Philippot JR, Wallach DF. 1984. A reevaluation of the status of cholesterol in erythrocytes infected by *Plasmodium knowlesi* and *P. falciparum*. *Mol Biochem Parasitol* 13:53–65. [http://dx.doi.org/10.1016/0166-6851\(84\)90101-4](http://dx.doi.org/10.1016/0166-6851(84)90101-4).
26. de Azevedo MF, Gilson PR, Gabriel HB, Simoes RF, Angrisano F, Baum J, Crabb BS, Wunderlich G. 2012. Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite *Plasmodium falciparum*. *PLoS One* 7:e40981. <http://dx.doi.org/10.1371/journal.pone.0040981>.
27. Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. *Science* 193:673–675. <http://dx.doi.org/10.1126/science.781840>.
28. Trang DT, Huy NT, Kariu T, Tajima K, Kamei K. 2004. One-step concentration of malarial parasite-infected red blood cells and removal of contaminating white blood cells. *Malar J* 3:7. <http://dx.doi.org/10.1186/1475-2875-3-7>.
29. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE. 1995. Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci U S A* 92:973–977. <http://dx.doi.org/10.1073/pnas.92.4.973>.
30. Fidock DA, Wellems TE. 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci U S A* 94:10931–10936. <http://dx.doi.org/10.1073/pnas.94.20.10931>.
31. Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, Hviid L, Theander TG. 2003. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol* 49:179–191. <http://dx.doi.org/10.1046/j.1365-2958.2003.03570.x>.
32. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* 16:710–718. <http://dx.doi.org/10.1128/AAC.16.6.710>.
33. Moneriz C, Marin-Garcia P, Bautista JM, Diez A, Puyet A. 2009. Haemoglobin interference and increased sensitivity of fluorimetric assays for quantification of low-parasitaemia *Plasmodium* infected erythrocytes. *Malar J* 8:279. <http://dx.doi.org/10.1186/1475-2875-8-279>.
34. de Rosso VV, Mercadante AZ. 2007. Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *J Agric Food Chem* 55:5062–5072. <http://dx.doi.org/10.1021/jf0705421>.
35. Bullen HE, Tonkin CJ, O'Donnell RA, Tham WH, Papenfuss AT, Gould S, Cowman AF, Crabb BS, Gilson PR. 2009. A novel family of Apicomplexan glideosome-associated proteins with an inner membrane-anchoring role. *J Biol Chem* 284:25353–25363. <http://dx.doi.org/10.1074/jbc.M109.036772>.
36. Hawthorne PL, Trenholme KR, Skinner-Adams TS, Spielmann T, Fischer K, Dixon MW, Ortega MR, Anderson KL, Kemp DJ, Gardiner DL. 2004. A novel *Plasmodium falciparum* ring stage protein, REX, is located in Maurer's clefts. *Mol Biochem Parasitol* 136:181–189. <http://dx.doi.org/10.1016/j.molbiopara.2004.03.013>.
37. de Koning-Ward TF, Gilson PR. 2009. Keeping it simple: an easy method for manipulating the expression levels of malaria proteins. *Trends Parasitol* 25:4–7. <http://dx.doi.org/10.1016/j.pt.2008.09.009>.
38. Wickham ME, Thompson JK, Cowman AF. 2003. Characterisation of the merozoite surface protein-2 promoter using stable and transient transfection in *Plasmodium falciparum*. *Mol Biochem Parasitol* 129:147–156. [http://dx.doi.org/10.1016/S0166-6851\(03\)00118-X](http://dx.doi.org/10.1016/S0166-6851(03)00118-X).
39. de Koning-Ward TF, Speranca MA, Waters AP, Janse CJ. 1999. Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter. *Mol Biochem Parasitol* 100:141–146. [http://dx.doi.org/10.1016/S0166-6851\(99\)00042-0](http://dx.doi.org/10.1016/S0166-6851(99)00042-0).
40. Fernandez-Becerra C, de Azevedo MF, Yamamoto MM, del Portillo HA. 2003. *Plasmodium falciparum*: new vector with bi-directional promoter activity to stably express transgenes. *Exp Parasitol* 103:88–91. [http://dx.doi.org/10.1016/S0014-4894\(03\)00065-1](http://dx.doi.org/10.1016/S0014-4894(03)00065-1).
41. Cunningham FX, Gantt E. 1998. Genes and enzymes of carotenoid bio-

- synthesis in plants. *Annu Rev Plant Physiol Plant Mol Biol* 49:557–583. <http://dx.doi.org/10.1146/annurev.arplant.49.1.557>.
42. Fray RG, Wallace A, Fraser PD, Valero D, Hedden P, Bramley PM, Grierson D. 1995. Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J* 8:693–701. <http://dx.doi.org/10.1046/j.1365-313X.1995.08050693.x>.
43. Sies H. 1993. Strategies of antioxidant defense. *Eur J Biochem* 215:213–219. <http://dx.doi.org/10.1111/j.1432-1033.1993.tb18025.x>.
44. Sies H. 1997. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 82:291–295. <http://dx.doi.org/10.1113/expphysiol.1997.sp004024>.
45. Muller S, Liebau E, Walter RD, Krauth-Siegel RL. 2003. Thiol-based redox metabolism of protozoan parasites. *Trends Parasitol* 19:320–328. [http://dx.doi.org/10.1016/S1471-4922\(03\)00141-7](http://dx.doi.org/10.1016/S1471-4922(03)00141-7).
46. Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. 2004. Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. *Int J Parasitol* 34:163–189. <http://dx.doi.org/10.1016/j.ijpara.2003.09.011>.
47. Martinez A, Stinco CM, Melendez-Martinez AJ. 2014. Free radical scavenging properties of phytofluene and phytoene isomers as compared to lycopene: a combined experimental and theoretical study. *J Phys Chem B* 118:9819–9825. <http://dx.doi.org/10.1021/jp503227j>.
48. Gray JC, Sullivan JA, Wang JH, Jerome CA, MacLean D. 2003. Coordination of plastid and nuclear gene expression. *Philos Trans R Soc Lond B Biol Sci* 358:135–144. <http://dx.doi.org/10.1098/rstb.2002.1180>.
49. Gruszecki WI, Strzalka K. 2005. Carotenoids as modulators of lipid membrane physical properties. *Biochim Biophys Acta* 1740:108–115. <http://dx.doi.org/10.1016/j.bbadis.2004.11.015>.

III- Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*.

RESEARCH

Open Access

Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*

Fabiana M Jordão¹, Heloisa B Gabriel¹, João MP Alves¹, Claudia B Angeli¹, Thaís D Bifano¹, Ardala Breda², Mauro F de Azevedo³, Luiz A Basso², Gerhard Wunderlich¹, Emilia A Kimura¹ and Alejandro M Katzin^{1*}

Abstract

Background: Isoprenoids are the most diverse and abundant group of natural products. In *Plasmodium falciparum*, isoprenoid synthesis proceeds through the methyl erythritol diphosphate pathway and the products are further metabolized by farnesyl diphosphate synthase (FPPS), turning this enzyme into a key branch point of the isoprenoid synthesis. Changes in FPPS activity could alter the flux of isoprenoid compounds downstream of FPPS and, hence, play a central role in the regulation of a number of essential functions in *Plasmodium* parasites.

Methods: The isolation and cloning of gene PF3D7_18400 was done by amplification from cDNA from mixed stage parasites of *P. falciparum*. After sequencing, the fragment was subcloned in pGEX2T for recombinant protein expression. To verify if the PF3D7_1128400 gene encodes a functional rPfFPPS protein, its catalytic activity was assessed using the substrate [4-¹⁴C] isopentenyl diphosphate and three different allylic substrates: dimethylallyl diphosphate, geranyl diphosphate or farnesyl diphosphate. The reaction products were identified by thin layer chromatography and reverse phase high-performance liquid chromatography. To confirm the product spectrum formed of rPfFPPS, isoprenic compounds were also identified by mass spectrometry. Apparent kinetic constants K_M and V_{max} for each substrate were determined by Michaelis–Menten; also, inhibition assays were performed using risedronate.

Results: The expressed protein of *P. falciparum* FPPS (rPfFPPS) catalyzes the synthesis of farnesyl diphosphate, as well as geranylgeranyl diphosphate, being therefore a bifunctional FPPS/geranylgeranyl diphosphate synthase (GGPPS) enzyme. The apparent K_M values for the substrates dimethylallyl diphosphate, geranyl diphosphate and farnesyl diphosphate were, respectively, $68 \pm 5 \mu\text{M}$, $7.8 \pm 1.3 \mu\text{M}$ and $2.06 \pm 0.4 \mu\text{M}$. The protein is expressed constitutively in all intra-erythrocytic stages of *P. falciparum*, demonstrated by using transgenic parasites with a haemagglutinin-tagged version of FPPS. Also, the present data demonstrate that the recombinant protein is inhibited by risedronate.

Conclusions: The rPfFPPS is a bifunctional FPPS/GGPPS enzyme and the structure of products FOH and GGOH were confirmed mass spectrometry. Plasmodial FPPS represents a potential target for the rational design of chemotherapeutic agents to treat malaria.

Keywords: *Plasmodium falciparum*, Malaria, Isoprenoids, Farnesyl diphosphate, Farnesyl diphosphate synthase, Geranylgeranyl diphosphate, Geranylgeranyl diphosphate synthase

* Correspondence: amkatzin@icb.usp.br

¹Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Lineu Prestes 1374, CEP 05508-000, São Paulo, SP, Brazil
Full list of author information is available at the end of the article

Background

Malaria is a leading cause of morbidity and mortality in tropical regions. In 2010, there were an estimated 216 million episodes of malaria of which approximately 81%, or 174 million cases, occurred in the African continent. On a worldwide scale, 655,000 individuals died of malaria, most of them in sub-Saharan Africa [1]. Of the five parasite species that infect humans, *Plasmodium falciparum* is responsible for the vast majority of severe forms of, and deaths from, the disease. Recent observations alert that the parasite is becoming resistant to virtually all drugs currently used in the treatment of the disease. Efforts to tackle this problem are based on combined therapy using drugs to which the parasites have not yet developed resistance, as well as identifying new drug targets [2].

Plasmodium falciparum parasites harbour an unusual plastid organelle called the apicoplast that has an essential role for their survival since isoprenoid precursors are synthesized there [3]. Deletion of this organelle by concomitant supplementation with isopentenyl diphosphate (IPP) proved that this is the only essential function of the apicoplast during blood stage growth [4]. Isoprenoids are very diverse and constitute an abundantly present group of natural products. Synthesis of isoprenoids is intrinsic to all organisms and leads to a vast array of metabolites with diverse functions. Despite their structural and functional variety, all isoprenoids derive from a common precursor, isopentenyl diphosphate, and its isomer, dimethylallyl

diphosphate (DMAPP). Farnesyl diphosphate synthase (FPPS), which belongs to a family of enzymes classified as prenyltransferases, catalyzes the consecutive head-to-tail condensation of IPP with DMAPP to form geranyl diphosphate (GPP), and then a second condensation between GPP and IPP to form farnesyl diphosphate (FPP). FPP serves as a substrate for the first reaction of several branched pathways leading to the synthesis of compounds such as ubiquinone, dolichol, menaquinone, and prenylated proteins. FPP can also be condensed with an additional IPP by geranylgeranyl diphosphate synthase (GGPPS) to form geranylgeranyl diphosphate (GGPP), which is also employed in protein prenylation and is related to carotenoid biosynthesis (Figure 1).

The essential and major biosynthetic step in the metabolism of all isoprenoid is the elongation of isoprene units by prenyltransferases. These enzymes, which subsequently mediate alkylation of IPP by allylic diphosphate, are classified according to the chain length of the final product and stereochemistry of the double bond formed by condensations. FPPS and GGPPS are the most studied prenyltransferases and have been described in various organisms of all three kingdoms, Eukarya, Bacteria, and Archaea [5]. In protist parasites, the FPPS gene was cloned from *Trypanosoma cruzi* [6], *Trypanosoma brucei* [7] and *Toxoplasma gondii* [8]. Recently, a GGPPS from *Plasmodium vivax* was described [9]. However, the first characterization

of a prenyltransferase in a malaria parasite was the characterization of the octaprenyl diphosphate synthase (OPPS) that catalyzes the condensation of FPP with IPP to produce octaprenyl diphosphate [10].

Human FPPS has been found to be a target for nitrogen-containing bisphosphonate (N-BP) drugs [11]. Based on “growth-rescue” and enzyme inhibition experiments, human GGPPS was shown to be a major target for the lipophilic analogues zolendronate and risedronate [12]. These reports have generated considerable interest in FPPS as a promising target for new anti-malarial drug development. Jordão *et al.* suggested the possible mechanism of action for risedronate in *P. falciparum* by inhibition of FPPS [13]. In the causative agent of sleeping sickness, *T. brucei*, the inhibition of FPPS showed that this enzyme is essential for parasite survival [7]. Considering that FPPS is a key enzyme of the biosynthesis of compounds already characterized in the parasite, such as dolichols, farnesylated proteins, and other final isoprenoid products [14], it is essential to characterize the FPPS from *P. falciparum* in order to establish an appropriate strategy for the development of specific inhibitors.

This work describes the cloning, expression and characterization of recombinant *P. falciparum* FPPS (rPfFPPS), with catalytic activity for DMAPP, GPP, and FPP as substrates, yielding FPP and GGPP as final products. Apparent kinetic parameters for the recombinant enzyme are presented, as well as IC_{50} and apparent K_i values for risedronate inhibition of rPfFPPS enzyme activity. Constitutive protein expression is also described.

Methods

Plasmodium falciparum culture

Cultures of *P. falciparum* clone 3D7 were grown as described [15], replacing human serum with Albumax I (0.5%, Invitrogen/Life Technologies) [16]. Parasite development and multiplication were monitored by microscopic evaluation of Giemsa-stained thin smears. Schizont stages were purified with magnetic columns (MACS Separation Columns “CS”, Miltenyi Biotec) [17]. Column pre-equilibration, washing and elution were all carried out at room temperature with RPMI-1640 (Sigma-Aldrich). For schizont purification, the culture was centrifuged ($2,000 \times g$ for 5 min), the pellet resuspended in RPMI-1640 (1:10; v/v), 10 ml of the 10% suspension of erythrocytes were applied to a CS column assembled in a magnetic unit, where only schizonts are retained. After washing the column with 50 ml of RPMI-1640, the column was removed from the magnetic field and its contents eluted with 50 ml of RPMI-1640 and the schizont stage parasites were centrifuged at $2,000 \times g$ for 5 min at room temperature. The supernatant was discarded, and the

pellet of parasites was stored in liquid N_2 for subsequent analysis.

Isolation and cloning of gene PF3D7_1128400

A 1,131 bp fragment of the PfFPPS gene (PlasmoDB ID PF3D7_1128400) was amplified from cDNA from mixed stage parasites using primers (Invitrogen/Life technologies) PfFPPS1 (5'-CCGGATCCATGGAGAACGAGCAGAATAAC-3') and PfFPPS2 (5'-CGGAATTCTCAAGCGCCTGTAAACAAAATGTC-3') and the amplicon cloned in pGEM T easy (Promega). After sequencing, the fragment containing the complete ORF was subcloned in pGEX2T for recombinant protein expression using the introduced BamHI and EcoRI sites.

Expression and Purification of rPfFPPS from *Escherichia coli*

Recombinant pGEX-2 T-FPPS expression vector was used to transform *Escherichia coli* BL21(DE3+) pLys RIL cells. Bacterial clones were grown in LB medium containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37°C in Luria Broth (Hi-media) until an OD_{600} of 0.6. At this time point, the expression of rPfFPPS was induced with 0.2 µM isopropyl β-D-thiogalactoside at 24°C overnight. Cells were pelleted by centrifugation and resuspended in lysis buffer PBS/0.1% Triton X-100 pH 7.2 (v/v), 0.05 mg/ml lysozyme and 0.2 mM PMSF. Lysis was completed by sonication (five pulses of 30 s at 40 W, at 4°C). Recombinant proteins were then purified using glutathione sepharose beads (GE Healthcare), following the manufacturer's instructions. Proteins were checked for purity by SDS-PAGE [18] and quantified by the Bradford method [19].

Enzymatic activity assay

The catalytic activity of rPfFPPS was assayed by measuring the conversion of $[4-^{14}C]$ IPP (56.6 mCi/mmol, Perkin Elmer Life Sciences) to $[^{14}C]$ products, by two different protocols: **Protocol I** - The method described by Ling *et al.* [8] was used with some modifications. Briefly, the assay mixtures contained 10 mM HEPES buffer pH 7.4, 2 mM $MgCl_2$, 2 mM dithiothreitol, 100 µM $[4-^{14}C]$ IPP, an allylic substrate (100 µM DMAPP, 30 µM GPP, or 15 µM FPP), and 500–1,000 ng of recombinant protein in a total volume of 100 µl. The reaction was carried out at 37°C for 30 min and stopped by addition of 10 µl of 6 M HCl. The reaction mixture was neutralized by addition of 15 µl of 6 M NaOH. The alcoholic products were then extracted twice with 500 µl hexane and analysed by reverse phase thin layer chromatography (RP-TLC). All non-radioactive substrates and chemicals were from Sigma-Aldrich. **Protocol II** - rPfFPPS activity was measured by a modification of the method described by Chang *et al.* [20]. Final assay concentrations were 50 mM Tris-HCl buffer pH 7.5, 2 mM

MgCl₂, 5 mM iodoacetamide, and 500–1,000 ng of recombinant protein. The concentrations of allylic substrate, DMAPP, GPP and FPP were the same as described above. The final reaction volume was 100 µl. After pre-incubation at 37°C for 10 min, the reaction was started by adding 50 µM [4-¹⁴C]IPP. The mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of distilled H₂O and NaCl-saturated water. The diphosphate products were then extracted twice with 500 µl of 1-butanol saturated with NaCl-saturated water and analysed by reverse phase high-performance liquid chromatography (RP- HPLC). Enzyme activity measurements using [1-(n)-³H]FPP (15 Ci/mmol, Amersham Biosciences) and IPP as substrates were also carried out.

Identification of reaction products of rPfFPPS

The alcoholic products obtained by **Protocol I** were analysed by TLC on reverse phase Silica Gel 60 plates (Merck) with acetone:H₂O (6:1; v/v) [8]. The position of the standard prenol alcohol was visualized using iodine vapour. Radioactivity was visualized by autoradiography in a Storm phospho-imager. The diphosphorylated products that were formed following **Protocol II** were identified by RP-HPLC and analysed on a Phenomenex Luna C₁₈ column (250 mm × 4.6 mm × 5 µm) (Phenomenex) coupled with a C₁₈ pre-column (Phenomenex), a UV Gilson 152/UV variable UV/visible detector at 214 nm and an FC203B fraction collector. The software used for data processing was the UniPoint LC™ 3.0 Software System. The gradient elution system used was: solvent A, 25 mM NH₄HCO₃, pH 8.0; solvent B, 100% (v/v) acetonitrile. A linear gradient was run from 0% to 100% B over a period of 40 min, after which 100% B was then pumped through for an additional 5 min. Fractions were collected in 1 ml/min intervals [21]. The resulting fractions were dried, resuspended in 500 µl of liquid scintillation mixture (PerkinElmer Life Sciences) and monitored with a Beckman 5000 β-radiation scintillation counter (Beckman).

ESI-MS/MS investigation of the products geraniol (GOH), farnesol (FOH) and geranylgeraniol (GGOH)

Identification of product formation by using **Protocol I** with non-radioactive substrates (IPP/DMAPP) in the presence of rPfFPPS were carried out by electrospray ionization tandem mass spectrometry (ESI-MS/MS) using a ion trap mass spectrometer, model LCQ™ Duo (Thermo Scientific) coupled to a nano-HPLC system (Ultimate, Dionex). After stopping the reaction, products were extracted with hexane, dried in a vacuum centrifuge, and resuspended in 40 µl of 50% acetonitrile/0.2% formic acid. The sample was injected (10 µl) in the nano-probe of the spectrometer by an autosampler (Ultimate, Dionex) at a flow rate of 2 µl/min and

analysed in the positive mode, using the following parameters: spray voltage 1.8 kV, capillary voltage 38 V, and capillary temperature 180°C. For ESI-MS/MS, relative collision energy of 30% (1.5 eV) was applied.

Partial purification of native PfFPPS

The partial purification of native PfFPPS was performed only with schizont stage parasites purified by magnetic column separation, as described above. Partial protein purification was carried out according to Tonhosolo *et al.* [10]. **Protocol II** was used to assay the enzymatic reaction and the diphosphate products were analysed by RP-HPLC, as described above.

rPfFPPS kinetic assays

For determination of apparent kinetic constants, concentration of the first substrate DMAPP (0–150 µM), GPP (0–50 µM) or FPP (0–50 µM) was varied in the presence of a fixed concentration of [4-¹⁴C]IPP (50 µM). Enzyme activity measurements were also carried out varying the concentration of [4-¹⁴C]IPP (0–80 µM) in the presence of a fixed concentration of either DMAPP (100 µM), GPP (40 µM) or FPP (50 µM). The catalytic activity of rPfFPPS was assayed by measuring the conversion of [4-¹⁴C]IPP to [¹⁴C] products, as described in **Protocol I**. Reaction products were extracted with hexane and quantified by liquid scintillation counting. Apparent kinetic constants, K_M and V_{max} , for each substrate were derived from fitting the data to Michaelis-Menten (MM, Equation 1), using SigmaPlot 10, from Systat Software. All experiments were performed in triplicate.

$$v = \frac{V_{max} [S]}{K_M + [S]} \quad (1)$$

rPfFPPS inhibition assays

Inhibition assays were performed in presence of a fixed concentration (30 µM) of one allylic substrate (GPP or FPP) and fixed concentration (30 µM) of [4-¹⁴C]IPP, with varying concentrations of risedronate (0.005 – 1,000 µM). Each assay contained 500 ng of rPfFPPS in a final volume of 100 µl. The catalytic activity of rPfFPPS was measured by the conversion of [4-¹⁴C]IPP into [¹⁴C] products, as described in **Protocol I**.

The concentration of risedronate required to reduce the fractional enzyme activity to half of its initial value in the absence of inhibitor (IC_{50}) was obtained from fitting the data to Equation (2) for partial inhibition [22], in which y is the fractional activity of the enzyme in the presence of inhibitor at concentration [I]; $y_{(max)}$ is the maximum value of y observed at [I] = 0; and y_{min} is the minimum limiting value of y at high

inhibitor concentrations. Data analysis was performed using SigmaPlot 10 (Systat Software). Relationship between IC_{50} and risedronate apparent dissociation constant (K_i) in each assay was derived according to Cheng's and Prusoff's relationship, Equation (3), for competitive inhibitors [23,24], in which $[S]$ and K_M are, respectively, the concentration of the substrate for which risedronate is a competitive inhibitor, and this substrate MM constant. All experiments were performed in triplicate.

$$y = \frac{y_{\max} - y_{\min}}{1 + \frac{[I]}{IC_{50}}} + y_{\min} \quad (2)$$

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}} \quad (3)$$

Plasmid construction

The plasmid pTEX150-HA/Stre3 [25] containing the epitope of heamagglutinin (HA) was digested with BglII/PstI to release the gene pTEX150. The genomic DNA sequence encoding the C-terminal fragment of FPPS was PCR amplified with the oligonucleotides 5'-AGATC-TGGTATGCAAATGGGGGGTATA and 5'-CTGCAG-CAGCGCCTGTAAACAAAATGTC, cloned in pGEM T-easy (Promega) and verified by sequencing. A recombinant clone was digested with BglII/PstI and ligated into the pTEX150 depleted vector pTEX150-HA/Stre3 generating the plasmid pFPPs-HA.

Parasite transfection and characterization of transfectants

Parasites were transfected as previously described [26], using the electroporation conditions established elsewhere [27]. Briefly, *P. falciparum* 3D7 was cultured in 4% haematocrit in RPMI HEPES supplemented with 0.5% Albumax I. 2×10^7 ring stage parasites at 5-8% parasitaemia were transfected with 150 µg of plasmid. Transfected parasites were submitted to drug pressure with 2.5 nM WR99210 starting on the third day of culture. Parasites were cultivated in standard conditions until parasites re-appeared and normal growth was re-established. The integration at the genomic FPPS locus was forced by intermittent exposure and retrieval of WR99210. Genomic gDNA was purified using standard protocols [28]. The integration at the genomic locus was checked by PCR under standard conditions using oligonucleotides inside and outside the integrated locus. The details of plasmid construction and the integration are presented on Additional file 1.

Western blot analyses

Synchronous cultures of transfected *P. falciparum* were recovered in each stage. Ring, trophozoite or schizont

stages were treated with 0.15% saponin in RPMI media to release haemoglobin from the red blood cells. Proteins were extracted with buffer: 0.05 M Tris-HCl, pH 6.8, 10% glycerol, 2 mM EDTA, 2% SDS, 0.05% bromophenol blue, 50 mM dithiothreitol [29] for separation by gel SDS-PAGE. The gel was then transferred to nitrocellulose membrane (Amersham) for 1 h using a Trans-Blot semidry electroblotter (BioRad) [30]. After blocking, membranes were incubated with an α -HA monoclonal antibody (1:500 dilution; Sigma-Aldrich) or antibody controls α -PTEX150 (1:1,000) [25] or α -MSP2 (1:500) [31] for 1 h at room temperature or 14 h at 4°C. After this, the membranes were incubated with an anti-mouse IgG labelled secondary antibody with peroxidase and were visualized on radiographic film using the ECL enhanced chemiluminescence detection kit according to the instructions of the manufacturer (GE Healthcare).

Sequence analysis of the chain length determination region

For sequence selection, similarity searches were done using PfFPPS as query against the full NCBI nr protein database, using a maximum E-value cut-off of 10^{-10} . Sequences with 0.5 to 1.5 times the length of the *P. falciparum*'s protein and those identical to others were removed. The final set of protein sequences was aligned using muscle 3.8.31 [32] and analysed with WebLogo 2.8.2 [33] and in-house developed scripts for amino acid composition analysis. Based on sequence conservation, 9 alignment columns either side of the First Aspartate-Rich Motif (FARM) were analysed and only sequences containing the canonical DDxxD motif were kept. Accession numbers and CLD region for all sequences used can be found in Additional file 2.

Ethical statement

This study was approved by the Ethical Committee of the Institute of Biomedical Science of University of São Paulo, Brazil (CEUA 140.09).

Results

Expression and purification of recombinant protein

The *P. falciparum* gene PF3D7_1128400 was formerly annotated as an FPPS and is currently described as a GGPPS according to plasmoDB. Using this sequence as template, primers were designed to amplify PF3D7_1128400 from total cDNA by PCR. The full length protein was expressed as a GST fusion protein in *E. coli* BL21(DE3) pLys RIL cells and purified the protein by affinity chromatography as described in the Methods section. The protein homogeneity was inferred by SDS-PAGE followed by Coomassie Blue staining, showing that the purified GST-PfFPPS (rPfFPPS) protein has an apparent molecular mass of ~70 kDa,

(sum of 26 kDa GST and 44 kDa PfFPPS) (Additional file 3).

Catalytic activity of rPfFPPS

To verify if the PF3D7_1128400 gene encodes a functional rPfFPPS protein, its catalytic activity was assessed using the substrate $[4\text{-}^{14}\text{C}]\text{IPP}$ and three different allylic substrates DMAPP, GPP or FPP under the conditions described above. The reaction products were identified by TLC and RP-HPLC.

The products formed following **Protocol I** were extracted with hexane, and the respective alcohols were submitted to TLC analysis. With the substrates $[4\text{-}^{14}\text{C}]\text{IPP}$ and DMAPP, bands with R_f values corresponding to GOH, FOH, and GGOH were observed. Bands with similar R_f to FOH and GGOH were detected when $[4\text{-}^{14}\text{C}]\text{IPP}$ and GPP were used as substrates, whereas FPP and $[4\text{-}^{14}\text{C}]\text{IPP}$ yielded only a band with an R_f coincident with GGOH (Figure 2).

Figure 2 Analyses by TLC of products synthesized by rPfFPPS.

The activity of rPfFPPS was measured by a ^{14}C radioactivity assay, utilizing $[4\text{-}^{14}\text{C}]\text{IPP}$ and DMAPP, GPP, or FPP as allylic substrate. The enzymatic reactions (**Protocol I**) and TLC were performed as described in the Methods section. Lane 1, $[4\text{-}^{14}\text{C}]\text{IPP}$ + DMAPP as substrates; lane 3, $[4\text{-}^{14}\text{C}]\text{IPP}$ + GPP as substrates; lane 5, $[4\text{-}^{14}\text{C}]\text{IPP}$ + FPP as substrates; lanes 2, 4 and 6 control reactions without enzymes for reactions showed on lanes 1, 3 and 5 respectively. Products labelled with $[4\text{-}^{14}\text{C}]\text{IPP}$ were visualized by a Bioscan System 200 Imaging Scanner. The positions of geraniol (GOH), farnesol (FOH) and geranylgeraniol (GGOH) standards are indicated on the left. The main products detected were FOH and GGOH, indicating that this were the major enzyme products. Ori: origin SF: front.

When the enzymatic reaction was carried without any enzyme, no products were observed (Figure 2, lanes 2, 4 and 6). When the enzymatic reaction was carried out with purified GST only, no products were observed.

The diphosphorylated products formed following **Protocol II** were extracted with butanol-saturated water and analysed by RP-HPLC. rPfFPPS with allylic substrates $[4\text{-}^{14}\text{C}]\text{IPP}$ and DMAPP was able to catalyze the synthesis of GPP, FPP, and GGPP (Figure 3A), whereas the reaction incubated with $[4\text{-}^{14}\text{C}]\text{IPP}$ and GPP as substrates led to the biosynthesis of FPP and GGPP (Figure 3B). When $[4\text{-}^{14}\text{C}]\text{IPP}$ and FPP were used as substrates, only GGPP synthesis was observed (Figure 3C). Similar results were obtained when the substrates $[1\text{-}(n)\text{-}^3\text{H}]\text{FPP}$ and IPP were incubated with the rPfFPPS (Figure 3D). When the reaction was carried without enzyme, no products were observed. This indicates that major products of the reactions catalyzed by the enzyme rPfFPPS are FPP and GGPP, with a minor production of GPP, showing both FPPS and GGPPS activity using two different protocols.

Identification of rPfFPPS products by ESI-MS/MS

In order to further confirm the product spectrum formed of rPfFPPS, isoprenic compounds were also identified by mass spectrometry. **Protocol I** was used for measurements of enzyme activity with non-radioactive substrates IPP/DMAPP, and investigated the structures of compounds formed in the presence of rPfFPPS by ESI-MS/MS (Figure 4). Figure 4A, C and E present the MS/MS spectra of standards GOH, FOH, and GGOH respectively. The fragmentation patterns of the precursor ions at m/z 137, corresponding to dehydration of GOH $[\text{M}-\text{H}_2\text{O}]^+$, at m/z 205; corresponding to the dehydration of FOH $[\text{M}-\text{H}_2\text{O}]^+$; and at m/z 273, corresponding to the dehydration of GGOH $[\text{M}-\text{H}_2\text{O}]^+$, were compared between standards and samples. The dissociation of the precursor ion at m/z 137 (GOH) revealed the presence of major ions at m/z 81, 94, and 108, while the dissociation of the precursor ion at m/z 205 (FOH) resulted in the major ion products at m/z 121, 134, 148, and 162. GGOH precursor ion at m/z 273 revealed the product major ions at m/z 149, 163, 189 and 217. The molecular identity was confirmed by comparing the ESI-MS/MS spectrum of the ions at m/z 137, m/z 205, and m/z 273 produced by rPfFPPS (Figure 4B, D and F) with the ESI-MS/MS spectrum of the standards (Figure 4A, C and E), revealing the same dissociation profile. Taken together, these results underscore that the rPfFPPS is able to catalyze reactions that lead to GOH, FOH, and GGOH formation.

Characterization of PfFPPS activity in parasite extracts by HPLC

In order to verify if naturally occurring PfFPPS contained in *P. falciparum* extracts exert similar activities as detected with rPfFPPS, these extracts were used instead of recombinant protein. The reaction was performed with $[4-^{14}\text{C}]$ IPP and DMAPP, GPP or FPP as substrate in accordance with **Protocol II**. The products were analysed by RP-HPLC. Incubation of extracts in the presence of $[4-^{14}\text{C}]$ IPP and DMAPP led to formation of GPP, FPP, and GGPP. Likewise, incubation of $[4-^{14}\text{C}]$ IPP and GPP as substrates yielded FPP and GGPP as products. Finally, only GGPP was observed when extracts were incubated with $[4-^{14}\text{C}]$ IPP and FPP (Figure 5). The extracts of parasites exhibited

both FPPS and GGPPS activity and these activities were similar to those of the rPfFPPS protein.

Apparent kinetic parameters of rPfFPPS and IC_{50} of risedronate

Apparent kinetic constants of the recombinant enzyme were determined using varied concentrations of $[4-^{14}\text{C}]$ IPP, DMAPP, GPP, and FPP as substrates (Additional file 4). The parameters were determined as described in the Methods section, by measuring the radioactivity in the hexane fraction. K_M and V_{max} values for each substrate are given in Table 1. Risedronate inhibitory activity against rPfFPPS, by specifically inhibiting the condensation of IPP with an allylic substrate was assayed as described in the

Methods section. Risedronate inhibition was evaluated using FPP/IPP and GPP/IPP as substrates (Additional file 5), yielding, respectively, IC_{50} values of $1.3 \pm 0.2 \mu M$ and of $10 \pm 1 \mu M$. Apparent K_i values, assuming risedronate competitive inhibition towards FPP and GPP, are equal to $0.08 \mu M$ and $1.96 \mu M$ respectively.

Analysis of rPfFPPS expression during the intra-erythrocytic cycle by Western blot

Extracts of parasite line that had the FPPS/GGPPS enzyme tagged with the HA epitope were analysed for the presence of pFPPS-HA. Samples of protein were extracted from parasites synchronized in three main stages (ring, trophozoite, and schizont) and detected with a monoclonal antibody against HA. The results indicate that the enzyme FPPS is constitutively expressed in all stages during the asexual intra-erythrocytic cycle of *P. falciparum* (Figure 6). As a control of the parasite synchronization, antibodies that recognize the constitutively expressed protein pTEX150 in three stages [25], and MSP2 [31], which is expressed only in schizont stages, were used.

CLD region sequence analysis

The CLD regions of 452 sequences containing the canonical DDxxD FARM motif were analysed by creating a sequence logo showing relative amino acid frequencies (Additional files 2 and 6). There is clear predominance

of aromatic amino acids (F and Y, although never W) in positions 4 and 5 N-terminal to the FARM (henceforth called P4 and P5). The cysteine in P5 with F or Y in P4, as found in *Toxoplasma's* bifunctional FPPS/GGPPS, is very rare, occurring in only 6 sequences (1.33% of the total), of various taxonomic affiliations. The *P. falciparum* sequence (S in P5, aromatic in P4) is slightly more common, appearing in 14 sequences (3.10%). *Theileria* spp. and all plasmodia but *P. vivax* contain SF at those positions; other organisms of diverse taxonomic lineages present this same sequence arrangement. In contrast, the other two biochemically characterized bifunctional FPPS/GGPPS enzymes present FF (*Zea mays*) or FS (*M. thermoautotrophicum*) at these positions, with the former found in 174 (65.71%) of all sequences and the later present in only 22 (4.87%). Other positions in the sequence logo have also shown high levels of conservation (Additional file 2), most markedly positions 2–4 (most frequently LQA), 7–8 (LV), 12–13 (IM), 16 (S), 18–21 (TRRG), and 23 (P).

Discussion

FPPS is a key enzyme in the metabolism of virtually all isoprenoids and it interconnects the 5-carbon moiety isoprenoid synthesis with the mid- or long-chained compound synthesis (Figure 1). In this study, the gene PfFPPS as encoding a bifunctional FPPS/GGPPS enzyme and its *in vitro* inhibition by risedronate were characterized.

Figure 5 (See legend on next page.)

(See figure on previous page.)

Figure 5 RP-HPLC analysis of radiolabeled products biosynthesized by the native PfFPPS in 5×10^{10} semipurified *Plasmodium falciparum* schizont stages. Extracts of parasites were obtained with methodology described by Tonhosolo et al. [10]. The reaction of [4- 14 C]IPP with three allylic substrates: DMAPP, GPP, or FPP were realized. Arrows indicate the elution positions of isoprenoid standards. The retention times of GPP, FPP and GGPP were identified by co-injection of commercial standards. (A) DMPP substrate, (B) GPP substrate, and (C) FPP substrate.

In many organisms, the prenyltransferases that catalyze chain elongation are highly selective for the chain length of their products. The human genome contains genes for two distinct monofunctional enzymes for GGPP and FPP synthesis [34,35]. In the protozoans *T. cruzi* and *P. vivax*, either FPPS or GGPPS is present, respectively [6,9]. On the other hand, Artz et al. discuss the possibility that GGPPS of *P. vivax* could be a bifunctional enzyme [9].

rPfFPPS expressed as a GST-fusion protein was used to characterize its functional activity and to determine the apparent kinetic parameters. Interestingly, the removal of the GST tag from rPfFPPS resulted in almost complete activity loss. An active form of GGPPS from *Thermus thermophilus* and *Sulfolobus acidocaldarius* was also overexpressed in *E. coli* cells as a GST fusion protein. Ohto et al. suggested that the presence of the GST-tag leads to thermal stability of the recombinant enzymes [36].

Previous studies have shown that many FPPS homologues can accept both DMAPP and GPP as allylic substrates [37,38]. When synthesizing FPP from DMAPP, the enzyme catalyzes two condensation reactions with IPP, releasing only trace amounts of the intermediate GPP [39], while GGPPS can accept DMAPP, GPP, and FPP as substrates [40,41]. The activity of rPfFPPS and the parasite extracts were confirmed by purification of the synthesized products by RP-HPLC. When DMAPP was used as a substrate, GPP was detected in minor amounts while FPP and GGPP were the predominant products. When the reaction was catalyzed with GPP as allylic substrate, the only products observed were FPP and GGPP. Accordingly, when FPP was used as substrate, only GGPP was observed (Figure 3). No products were detected when GGPP was used as a substrate. Hence, rPfFPPS is a bifunctional FPPS/GGPPS enzyme. Importantly, similar products were observed using a second approach where alcoholic compounds were analysed

by TLC (Figure 2). Finally, the structures of products GOH, FOH, and GGOH were confirmed by ESI-MS/MS (Figure 4).

The bifunctional property of rPfFPPS in producing GGPP as well as FPP was previously described only in three organisms: the archaeobacterium *M. thermoautotrophicum* [42], maize [43], and *T. gondii* [8]. A related enzyme was described by Artz et al. in *Cryptosporidium parvum*. Although this enzyme was annotated as an FPPS, it shows the capacity to produce GGPP and also longer polyisoprenes (up to 35 carbons) with the main products being C25 and C30-compounds with most of the substrates tested [44]. This is indicative that the enzymes from *P. falciparum* and *T. gondii* have a rather limited product spectrum compared to the *Cryptosporidium* homologue.

Amino acid sequence alignment of FPPS from different organisms revealed conserved regions I to VII with two characteristic aspartate rich motifs, one in region II called FARM (first Asp-rich motif) and in region VI called SARM (second Asp-rich motif). Wang and Ohnuma [45] clearly demonstrated that the product chain lengths of natural FPPS and GGPPS are mainly regulated by the amino acid residues located at the fourth and fifth position upstream of the FARM region. These residues are at the bottom of the active site pocket, making direct interactions with the ω -terminal region of the allylic products. For this reason, the site was designated the CLD (chain length determination) region. Usually, three possible amino acid substitutions are described for the fourth and fifth amino acid positions upstream the FARM region, and their identities determine the classification of FPPS and GGPPS: Type I FPPS present aromatic amino acids residues on both positions; Type II and Type III GGPPS present amino acid residues other than aromatic on both positions; Type II FPPS and Type I GGPPS, as well as long chain prenyl

Table 1 Apparent kinetic constants for *Plasmodium falciparum* FPPS,(rPfFPPS)

Varied substrate	Fixed substrate	K_M (μ M)	V_{max} (nmol/min/mg)
DMAPP (0–150 μ M)	IPP 50 μ M	68 $\pm$ 5	452.5 $\pm$ 16
GPP (0–50 μ M)	IPP 50 μ M	7.8 $\pm$ 1.3	341 $\pm$ 19
FPP (0–50 μM)	IPP 50 μM	2.06 $\pm$ 0.4	326.5 $\pm$ 16
IPP (0–80 μ M)	DMAPP 100 μ M	2 $\pm$ 0.3	169 $\pm$ 5.4
IPP (0–80 μ M)	GPP 40 μ M	0.81 $\pm$ 0.1	224 $\pm$ 3.4
IPP (0–50 μM)	FPP 50 μM	2.4 $\pm$ 0.3	155.6 $\pm$ 4

Concentration ranges for each varied substrate are indicated. Activity versus varied substrate concentration plots are depicted on Additional file 4.

synthases, present an aromatic amino acid residue solely at the fifth position. Upon alignment of FPPS/GGPPS from *T. gondii* and GGPPS from *P. vivax* it appears that these proteins share more features with other FPPS as already postulated by Ling *et al.* [8], and FPPS from *P. falciparum* also falls in this cluster. Accordingly, these enzymes show the apparent production of GGPP and FPP, although this is not explicitly expressed in the characterization of the *P. vivax* enzyme [9]. One may argue that a hydrophilic side chain at the fifth amino acid upstream of the FARM region plays a crucial role for the production of both GGPP and FPP. Li *et al.* [46] showed that the presence of a cysteine at the fifth position is essential for the FPPS/GGPPS bifunctionality in *T. gondii*. On the other hand, the methanobacterial version of the enzyme contains a bulky phenylalanine at this position and also produces GGPP and FPP [42], turning evident that other regions may play a role in the fine-specificity of product formation. Our analyses of the CLD from 452 putative FPPS sequences show relatively high sequence conservation of other amino acids close to the FARM, and suggest the potential for the further discovery of a number of FPPS/GGPPS bifunctionality in organisms as diverse as animals, fungi, amoeba, plants, and others. From the point of view of parasitism, it is reasonable to infer that a bifunctional enzyme would be a selective advantage. Considering the notoriously reduced genomes in parasitic organisms and the fact that no other enzyme with similarity to known short chain prenyl synthases has been identified in the currently sequenced Apicomplexa, this mutation has probably been advantageous to these parasites given the essential

nature of both FPP and GGPP as precursors of a number of compounds important for many processes of their cellular metabolism.

The results showed in TLC, HPLC, and ESI-MS/MS (Figures 2, 3 and 4) are indicative of bifunctional activity for rPfFPPS, showing catalytic activity with DMAPP, GPP, and FPP as first substrates, ultimately yielding GGPP as final product. Based on the conservation among FPPS and GGPPS enzymes, it is tempting to suggest that rPfFPPS mechanism of catalysis is bi-bi ordered, in which binding of either DMAPP, GPP, or FPP to the free enzyme is followed by IPP binding. However, other sequential or random mechanisms cannot be ruled out for the *P. falciparum* enzyme since the results here presented do not allow the determination of its kinetic mechanism. A mandatory ordered kinetic mechanism has been described for other FPPS, including the human [47], *T. cruzi* [48], *Staphylococcus aureus* and *E. coli* [49] homologues. According to such an ordered mechanism, DMAPP or GPP binds to the free enzyme, with IPP having larger binding affinity for the E:DMAPP or E:GPP binary complexes [47]. Farnesyl synthesis by these FPPS homologues is known to proceed through two subsequent steps. The reaction starts with the condensation of one molecule of DMAPP and one molecule of IPP, yielding the first product GPP. A second IPP molecule is condensed with GPP to form FPP as the final product [50]. Accordingly, *P. falciparum* bifunctional FPPS/GGPPS catalysis is a three-step, four-substrate process (Figure 1).

Data derived from activity assays of rPfFPPS were apparently hyperbolic to all tested substrate pairs (Additional file 4), suggesting that rPfFPPS follows MM kinetics. As rPfFPPS catalyzes parallel and consecutive reactions (Figure 1), the interpretation of the apparent kinetic constants for this complex enzyme system is not trivial (Table 1). The results presented here demonstrate that rPfFPPS is capable of synthesizing GPP, FPP, and GGPP from DMAPP and IPP (Figure 1, steps 1, 2 and 3); FPP and GGPP from GPP and IPP (Figure 1, steps 2 and 3); and GGPP from FPP and IPP (Figure 1, step 3). Assuming that rPfFPPS follows an ordered mechanism for substrate binding, when activity assays were carried out in the presence of DMAPP and IPP, there will be formation of GPP, followed by conversion of GPP to form FPP, which will be competitive inhibitors of the reactions catalyzed in steps 1, 2, and 3, since DMAPP, GPP, and FPP all compete for binding to the free enzyme active site (Additional file 7). On the other hand, rPfFPPS activity measurements using GPP and IPP as substrates, there will be formation of FPP, which will be competitive inhibitors of the reactions catalyzed in steps 2 and 3, since GPP and FPP compete for binding to free enzyme. In this scenario, DMAPP, GPP, and FPP will

also behave as non-competitive inhibitors towards the second substrate, IPP (Figure 1, steps 1, 2 and 3). This same issue has been described for human FPPS [47], where the authors clearly point out the difficulties of mechanistic studies modelling and interpretation.

Evaluation of the apparent kinetic constants given in Table 1 should thus be interpreted with caution. Except for the substrate pair FPP/IPP (highlighted in bold), the parameters presented for every other pair of substrates correspond to overall dissociation constants (K_M) and overall V_{max} values comprising the consecutive and parallel reactions that would be better described by modifications of the MM equation.

Similar K_M values for substrate pair IPP/FPP were reported for *Homo sapiens* GGPPS ($3 \pm 0.2 \mu\text{M}$ and $4.2 \pm 0.3 \mu\text{M}$) [34] and *P. vivax* GGPPS ($8.4 \pm 1.6 \mu\text{M}$ and $7.3 \pm 0.7 \mu\text{M}$) [9]. The *P. falciparum* substrate pair IPP/FPP also presented similar K_M values, of $2.4 \pm 0.3 \mu\text{M}$ and $2.06 \pm 0.4 \mu\text{M}$ (Table 1). The human FPPS enzyme has also been characterized, and K_M values for IPP/GPP of $0.6 \pm 0.1 \mu\text{M}$ and $0.7 \pm 0.1 \mu\text{M}$ were reported [47]. Again *P. falciparum* data for substrate pair IPP/GPP indicate similar K_M for IPP ($0.81 \pm 0.1 \mu\text{M}$) and almost ten times larger K_M value for GPP ($7.8 \pm 1.3 \mu\text{M}$). These values, however, correspond to global apparent constants for steps 2 and 3 (Figure 1).

Considering varied substrates DMAPP, GPP, and FPP, there appears to be a trend in MM constant values: $K_M(\text{FPP}) < K_M(\text{GPP}) < K_M(\text{DMAPP})$ (Table 1). Increased K_M values, without V_{max} variations, are expected for reactions catalyzed in the presence of competitive inhibitors [22], as is the case for these substrates. No such K_M variation is expected when IPP is the varied substrate as IPP is a non-competitive inhibitor with respect to FPP, GPP, and DMAPP. Non-competitive inhibitors are expected to maintain K_M values while decreasing V_{max} values [22]. These predictions appear to be borne out by the data presented in Table 1.

Nitrogen-containing bisphosphonates like risidronate are known to inhibit FPPS enzymes [11]. However, when the activities of 26 different bisphosphonates against the GGPPS protein from *P. vivax* were compared to their effect on *P. falciparum* *in vitro* growth, a poor correlation was found [51]. Risidronate is commonly used in the treatment of osteoporosis and it was shown that risidronate has a significant inhibitory effect against murine blood stage malaria [13], also inhibiting *P. vivax* GGPPS [9], and human FPPS [47]. Jordão et al. showed that risidronate presents inhibitory activity *in vitro* cultures of *P. falciparum*, with an IC_{50} of $20 \pm 1 \mu\text{M}$, also showed that risidronate inhibition is reversed by addition of FPP or GGPP to the cultures, but not by the addition of IPP [13]. These findings are in agreement with the assumed competitive risidronate inhibition

towards FPP and GPP, and non-competitive inhibition with respect to IPP.

As for the apparent kinetic constants reported in Table 1, an IC_{50} value of $10 \pm 1 \mu\text{M}$ for risidronate inhibition in the presence of GPP/IPP substrates also corresponds to a global inhibition value, in which both risidronate and FPP product could account for the inhibitory activity. When risidronate effect was evaluated in the presence of FPP/IPP as substrates, an IC_{50} value of $1.3 \pm 0.3 \mu\text{M}$ was estimated. The increased IC_{50} for the rPfFPPS/GGPPS reaction catalyzed with GPP/IPP as substrates is in agreement with the presence of an alternative substrate (FPP) as a competitive inhibitor [22]. A similar IC_{50} value was reported for the inhibition of human FPPS activity by risidronate. When GPP/IPP were used as substrates for FPPS enzyme activity measurements, in which there is no alternative substrate present in the reaction mixture, an IC_{50} value of 2.7 nM was determined. On the other hand, when DMAPP/IPP were the substrates, and reaction product GPP will also inhibit the enzyme along with risidronate, the IC_{50} value increased to 3.2 nM [47]. The larger IC_{50} values of risidronate in the presence of alternative substrates can be a consequence of some of the enzyme active sites being occupied by these substrates thereby increasing the concentration of inhibitor to achieve 50% of enzyme activity inhibition. In addition, *in vitro* inhibition assays of human FPPS also indicate that risidronate is a time dependent slow tight-binding inhibitor, with lower IC_{50} values after incubation for 30 minutes of enzyme in the presence of risidronate [47]. As described in the Methods section, rPfFPPS formation of products was evaluated only after 30 min incubation time, according to Protocol 1. This thus prevents time dependent fluctuation of the IC_{50} value for the results presented here. Nonetheless, an alternative assay may be necessary to evaluate a possible tight-binding inhibition mechanism for risidronate over rPfFPPS.

With evidence of risidronate being a competitive inhibitor towards GPP and FPP, its apparent K_i value was estimated, according to Equation (3), as being equal to $1.96 \mu\text{M}$ (GPP/IPP) and $0.082 \mu\text{M}$ (FPP/IPP). *Plasmodium vivax* GGPPS characterization studies reported an apparent K_i value of $12.4 \pm 1.7 \mu\text{M}$, when using FPP/IPP as substrates [9]; a value 151 times larger than the K_i value reported in this work. Even though true K_i values must be assigned before a more reliable comparison can be made, *P. falciparum* FPPS/GGPPS seems to be more prone to risidronate inhibition than its *P. vivax* homologue. Reasoning for this finding is rather elusive at the moment.

Gosh et al. have shown that risidronate or zoledronate were not the most potent inhibitors in *Plasmodium* spp [52]. They recently described a new generation of

bisphosphonates known as “lipophilic biphosphonates”, found to be more active against FPPS/GGPPS both *in vitro* and *in vivo* than any other currently available bisphosphonate [12]. In addition, No *et al.* demonstrated that the lipophilic analogues of risedronate and zoledronate had a stronger inhibitory activity against GGPPS from *P. vivax* and also exhibited anti-malarial activity *in vitro* and *in vivo* [53]. Although risedronate is not a potent drug against *P. falciparum*, it was showed by metabolic incorporation with [4-¹⁴C]IPP that risedronate inhibits the biosynthesis of FPP and GGPP and interferes with protein isoprenylation by inhibiting the biosynthesis of FPP and GGPP, while also interferes with the transfer of FPP to parasite proteins [13]. These findings are in agreement with the view that risedronate inhibits *in vitro* *P. falciparum* growth by inhibiting the plasmodial FPPS. Importantly, it is expected that successful inhibition of FPPS – a key enzyme between IPP/DMAPP and all longer polyisoprenoids – exerts a pleiotrophic effect on *Plasmodium* since it inhibits the function of many important parasite proteins [10,54].

The rPfFPPS is expressed constitutively in all stages during intra-erythrocytic cycle, demonstrated by using transfected parasites with pFPPS-HA (Figure 6). FPP and GGPP are substrates for prenyl:protein transferases (farnesyl transferase and geranylgeranyl transferase), catalyzing the post-translational modification of proteins [55]. Previous studies have demonstrated that post-translational modification of proteins occurs in all intra-erythrocytic stage of *P. falciparum*, suggesting that the enzyme is also active in all stages [55,56].

Conclusions

The rPfFPPS is a bifunctional enzyme, with FPPS/GGPPS activity, producing FPP and GGPP. Both FPP and GGPP occupy a central role leading to the synthesis of important classes of compounds. These two compounds were utilized for demonstrating the several isoprenoid biosynthesis pathway in *P. falciparum* [14]. Considering that: i) *P. falciparum* does not survive in the absence of the IPP produced in the apicoplast unless this precursor is supplemented [4]; ii) the FPPS/GGPPS is the only enzyme leading to the precursors for the synthesis of larger polyisoprenoids; and, iii) that FPPS/GGPPS has major structural differences compared to the human FPPS and GGPPS enzymes [43], this enzyme possibly represents an attractive drug target for the development of selective inhibitors aiming the erythrocytic stages of *P. falciparum*. The results presented here and previously published data [13] on risedronate inhibition *in vitro* and *in vivo* call for further QSAR experiments for the development of more potent bisphosphonate-based inhibitors selectivity targeting this key point of the plasmodial isoprenoid metabolism.

Additional files

Additional file 1: Schematic representation of the integration of rPfFPPS-HA in genomic locus. A) Diagram illustrating the integration event by crossing-over and primers designed to detect this event (1, 2 and 3). Numbers 1 and 3 indicate the region where the primers have been designed for detecting the integration of the gene in locus. B) PCR detecting the integration of pFPP-HA in the genomic locus of *P. falciparum* using primers 1 and 3. C) Detecting the control PCR amplification of endogenous FPPS gene in both strains (transfected and 3D7) using the primers 1 and 2. (–) –negative control; (pFPPS-HA) – transfected strain; (3D7) – wild type strain.

Additional file 2: Table of organisms, accession numbers, and CLD region sequences analyzed. *Sequences characterized as bifunctional FPP/GPPS are highlighted in gray and use bold font, *Excluded from CLD analysis (X in red) were the sequences that either did not present the canonical DDxxD FARM motif or had rare insertions (see main text).

Additional file 3: Expression of the rPfFPPS. SDS-polyacrylamide gel 12% was stained with Coomassie Brilliant Blue. Lane 1, soluble fraction from extract of *E. coli* BL21(DE3) pLys RIL/rPfFPPS; Lane 2, rPfFPPS fused with GST; lane 3, GST. Molecular size standards are indicated on the left (kDa).

Additional file 4: MM plots of the steady-state initial velocity experiments for rPfFPPS/GGPPS. R² values for each plot are: A) 0.99; B) 0.99; C) 0.98; D) 0.99; E) 0.97; F) 0.99. Experiments and data analysis were conducted as detailed under **Methods**, *rPfFPPS kinetic assays* section. Concentration ranges of each varied substrate are depicted on Table 1. Data were fitted to Equation (1).

Additional file 5: Inhibition of rPfFPPS/GGPPS activity by risedronate. A) Substrate pair FPP/IPP (R² = 0.98); B) Substrate pair GPP/IPP (R² = 0.99). rPfFPPS is expressed as its fractional activity; and risedronate concentrations were plotted on log scale. Data were fitted to Equation (2).

Additional file 6: Sequence logo analysis of the chain-length determination region. All sequences containing the canonical DDxxD FARM motif were submitted to sequence logo analysis, as described in the **Methods** section. Total height of each position reflects overall sequence conservation at that column; height of each residue in a column reflects its proportion in relation to other possible residues for that column. Colors are for clarity, with aspartate in red, aromatic amino acids in blue, serine and cysteine in cyan, and all other amino acids in black.

Additional file 7: Proposed kinetic mechanism for rPfFPPS. GGPP synthesis is proposed to follow a bi-bi ordered mechanism in an intricate system of parallel and consecutive reactions.

Abbreviations

IPP: Isopentenyl diphosphate; DMAPP: Dimethylallyl diphosphate; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; GGPP: Geranylgeranyl diphosphate; FPPS: Farnesyl diphosphate synthase; GGPPS: Geranylgeranyl diphosphate synthase; N-BP: Nitrogen-containing bisphosphonate; OPPS: Octaprenyl diphosphate synthase; rPfFPPS: *P. falciparum* FPPS; ESI-MS/MS: Electrospray ionization tandem mass spectrometry; QSAR: Quantitative structure–activity relationship models.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FMJ initiated this work and performed most of the experiments, including Figures 1, 2, 3, 4 and 5, Additional files 3 and 5 and wrote the majority of the paper; HBG and MFdA performed the experiments in Figures 6 and Additional file 1; JMPA performed computational studies (Additional files 2 and 6); CBA performed ESI-MS/MS analyses; TDB, AB and LAB contributed in enzymatic kinetic analyses and wrote these aspects of paper. GW, EAK and AMK supervised the project, analysed the data, wrote and reviewed the paper before submission. All authors read and approved the final manuscript.

Acknowledgements

FMJ is the recipient of a postgraduate fellowship from CNPq. HBG receives a postgraduate fellowship from FAPESP. We thank S Wendel (Sírío Libanes Hospital, NESTA) for providing the erythrocytes. We thank Danilo C Miguel and Carsten Wrenger for critical reading of the manuscript and Valnice J Peres for help with parasites preparation. This work was supported by grants from CNPq and FAPESP (Brazil).

Author details

¹Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Av. Lineu Prestes 1374, CEP 05508-000, São Paulo, SP, Brazil. ²Research Center for Molecular Biology and Functional, National Institute of Science and Technology on Tuberculosis, Pontifical Catholic University of Rio Grande do Sul, Rio Grande do Sul, Brazil. ³The Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia.

Received: 22 March 2013 Accepted: 29 May 2013

Published: 4 June 2013

References

- WHO: *World Malaria Report*. Geneva: World Health Organization; 2011.
- WHO: *Guidelines for the treatment of malaria*. 2nd edition. Geneva: World Health Organization; 2010.
- Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Turbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E: Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. *Science* 1999, **285**:1573–1576.
- Yeh E, DeRisi JL: Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. *PLoS Biol* 2011, **9**:e1001138.
- Wang KC, Ohnuma S: Isoprenyl diphosphate synthases. *Biochim Biophys Acta* 2000, **1529**:33–48.
- Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R: Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl pyrophosphate synthase. *J Biol Chem* 2001, **276**:33930–33937.
- Montalvetti A, Fernandez A, Sanders JM, Ghosh S, Van Brussel E, Oldfield E, Docampo R: Farnesyl pyrophosphate synthase is an essential enzyme in *Trypanosoma brucei*. In vitro RNA interference and in vivo inhibition studies. *J Biol Chem* 2003, **278**:17075–17083.
- Ling Y, Li ZH, Miranda K, Oldfield E, Moreno SN: The farnesyl-diphosphate/geranylgeranyl-diphosphate synthase of *Toxoplasma gondii* is a bifunctional enzyme and a molecular target of bisphosphonates. *J Biol Chem* 2007, **282**:30804–30816.
- Artz JD, Wernimont AK, Dunford JE, Schapira M, Dong A, Zhao Y, Lew J, Russell RG, Ebetino FH, Oppermann U, Hui R: Molecular characterization of a novel geranylgeranyl pyrophosphate synthase from *Plasmodium* parasites. *J Biol Chem* 2011, **286**:3315–3322.
- Tonhosolo R, D'Alexandri FL, Genta FA, Wunderlich G, Gozzo FC, Eberlin MN, Peres VJ, Kimura EA, Katzin AM: Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*. *Biochem J* 2005, **392**:117–126.
- Kavanagh KL, Guo K, Dunford JE, Wu X, Knapp S, Ebetino FH, Rogers MJ, Russell RG, Oppermann U: The molecular mechanism of nitrogen-containing bisphosphonates as antiosteoporosis drugs. *Proc Natl Acad Sci USA* 2006, **103**:7829–7834.
- Zhang Y, Cao R, Yin F, Hudock MP, Guo RT, Krysiak K, Mukherjee S, Gao YG, Robinson H, Song Y, No JH, Bergan K, Leon A, Cass L, Goddard A, Chang TK, Lin FY, Van Beek E, Papapoulos S, Wang AH, Kubo T, Ochi M, Mukkamala D, Oldfield E: Lipophilic bisphosphonates as dual farnesyl/geranylgeranyl diphosphate synthase inhibitors: an X-ray and NMR investigation. *J Am Chem Soc* 2009, **131**:5153–5162.
- Jordão FM, Saito AY, Miguel DC, Peres VP, Kimura EA, Katzin AM: In vitro and in vivo antiplasmodial activities of risedronate and its interference with protein prenylation in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2011, **55**:2026–2031.
- Jordão FM, Kimura EA, Katzin AM: Isoprenoid biosynthesis in the erythrocytic stages of *Plasmodium falciparum*. *Mem Inst Oswaldo Cruz* 2001, **106**(Suppl 1):134–141.
- Trager W, Jensen JB: Human malaria parasites in continuous culture. *Science* 1976, **193**:673–675.
- Kimura EA, Couto AS, Peres VJ, Casal OL, Katzin AM: N-linked glycoproteins are related to schizogony of the intraerythrocytic stage in *Plasmodium falciparum*. *J Biol Chem* 1996, **271**:14452–14461.
- Trang DT, Huy NT, Kariu T, Tajima K, Kamei K: One-step concentration of malarial parasite-infected red blood cells and removal of contaminating white blood cells. *Malar J* 2004, **3**:7.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**:680–685.
- Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**:248–254.
- Chang TH, Guo RT, Ko TP, Wang AH, Liang PH: Crystal structure of type-III geranylgeranyl pyrophosphate synthase from *Saccharomyces cerevisiae* and the mechanism of product chain length determination. *J Biol Chem* 2006, **281**:14991–15000.
- Zhang D, Poulter CD: Analysis and purification of phosphorylated isoprenoids by reversed-phase HPLC. *Anal Biochem* 1993, **213**:356–361.
- Copeland RA: Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. *Methods Biochem Anal* 2005, **46**:1–265.
- Copeland RA: Reversible Inhibitors-Dose-Response Curves of enzyme inhibition. In *Enzymes-A practical introduction to structure, mechanism and data analysis*. Edited by Robert A. New York: Copeland Wiley-VCH Inc; 2000:266–304.
- Cheng Y, Prusoff WH: Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* 1973, **22**:3099–3108.
- De Koning-Ward TF, Gilson PR: Keeping it simple: an easy method for manipulating the expression levels of malaria proteins. *Trends Parasitol* 2009, **25**:4–7.
- Wu Y, Sifri CD, Lei HH, Su XZ, Welles TE: Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci USA* 1995, **92**:973–977.
- Fidock DA, Welles TE: Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci USA* 1997, **94**:10931–10936.
- Ljungdtröm I, Schlichtherle M, Scherf A, Wahlgren M: *Methods in Malaria Research*. Virginia: Manassas; 2004.
- Bullen HE, Tonkin CJ, O'Donnell RA, Tham WH, Papenfuss AT, Gould S, Cowman AF, Crabb BS, Gilson PR: A novel family of Apicomplexan glideosome-associated proteins with an inner membrane-anchoring role. *J Biol Chem* 2009, **284**:25353–25363.
- Hawthorne PL, Trenholme KR, Skinner-Adams TS, Spielmann T, Fischer K, Dixon MW, Ortega MR, Anderson KL, Kemp DJ, Gardiner DL: A novel *Plasmodium falciparum* ring stage protein, REX, is located in Maurer's clefts. *Mol Biochem Parasitol* 2004, **136**:181–189.
- Gerold P, Schofield L, Blackman MJ, Holder AA, Schwarz RT: Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. *Mol Biochem Parasitol* 1996, **75**:131–143.
- Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004, **32**(5):1792–1797. 2004 Mar 19, PMID: 15034147.
- Crooks GE, Hon G, Chandonia JM, Brenner SE: WebLogo: a sequence logo generator. *Genome Res* 2004, **14**(6):1188–1190. 2004 Jun, PMID: 15173120.
- Kavanagh KL, Dunford JE, Bunkoczi G, Russell RG, Oppermann U: The crystal structure of human geranylgeranyl pyrophosphate synthase reveals a novel hexameric arrangement and inhibitory product binding. *J Biol Chem* 2006, **281**:22004–22012.
- Ding VD, Sheares BT, Bergstrom JD, Ponpipom MM, Perez LB, Poulter CD: Purification and characterization of recombinant human farnesyl diphosphate synthase expressed in *Escherichia coli*. *Biochem J* 1991, **275**(Pt 1):61–65.
- Ohto C, Nakane H, Hemmi H, Ohnuma S, Obata S, Nishino T: Overexpression of an archaeal geranylgeranyl diphosphate synthase in *Escherichia coli* cells. *Biosci Biotechnol Biochem* 1998, **62**:1243–1246.
- Reed BC, Rilling HC: Crystallization and partial characterization of prenyltransferase from avian liver. *Biochemistry* 1975, **14**:50–54.
- Reed BC, Rilling HC: Substrate Binding of avian liver prenyltransferase. *Biochemistry* 1976, **15**:3739–3745.

39. Ishii K, Sagami H, Ogura K: A novel prenyltransferase from *Paracoccus denitrificans*. *Biochem J* 1986, **233**:773–777.
40. Hefner J, Ketchum RE, Croteau R: Cloning and functional expression of a cDNA encoding geranylgeranyl diphosphate synthase from *Taxus canadensis* and assessment of the role of this prenyltransferase in cells induced for taxol production. *Arch Biochem Biophys* 1998, **360**:62–74.
41. Wang G, Dixon RA: Heterodimeric geranyl(geranyl)diphosphate synthase from hop (*Humulus lupulus*) and the evolution of monoterpene biosynthesis. *Proc Natl Acad Sci USA* 2009, **106**:9914–9919.
42. Chen A, Poulter CD: Purification and characterization of farnesyl diphosphate/geranylgeranyl diphosphate synthase. A thermostable bifunctional enzyme from *Methanobacterium thermoautotrophicum*. *J Biol Chem* 1993, **268**:11002–11007.
43. Cervantes-Cervantes M, Gallagher CE, Zhu C, Wurtzel ET: Maize cDNAs expressed in endosperm encode functional farnesyl diphosphate synthase with geranylgeranyl diphosphate synthase activity. *Plant Physiol* 2006, **141**:220–231.
44. Artz JD, Dunford JE, Arrowood MJ, Dong A, Chruszcz M, Kavanagh KL, Minor W, Russell RG, Ebetino FH, Oppermann U, Hui R: Targeting a uniquely nonspecific prenyl synthase with bisphosphonates to combat cryptosporidiosis. *Chem Biol* 2008, **15**:1296–1306.
45. Wang K, Ohnuma S: Chain-length determination mechanism of isoprenyl diphosphate synthases and implications for molecular evolution. *Trends Biochem Sci* 1999, **24**:445–451.
46. Li Z, Cintrón R, Koon NA, Moreno SNJ: The N-terminus and the chain length determination domain play a role in the length of the isoprenoid product of the bifunctional *Toxoplasma gondii* farnesyl diphosphate synthase. *Biochemistry* 2012, **51**:7533–7540.
47. Glickman JF, Schmid A: Farnesyl pyrophosphate synthase: real-time kinetics and inhibition by nitrogen-containing bisphosphonates in a scintillation assay. *Assay Drug Dev Technol* 2007, **5**:205–214.
48. Sigman L, Sanchez VM, Turjanski AG: Characterization of the farnesyl pyrophosphate synthase of *Trypanosoma cruzi* by homology modeling and molecular dynamics. *J Mol Graph Model* 2006, **25**:345–352.
49. Sanchez VM, Crespo A, Gutkind JS, Turjanski AG: Investigation of the catalytic mechanism of farnesyl pyrophosphate synthase by computer simulation. *J Phys Chem B* 2006, **110**:18052–18057.
50. Ashby MN, Edwards PA: Identification and regulation of a rat liver cDNA encoding farnesyl pyrophosphate synthetase. *J Biol Chem* 1989, **264**:635–640.
51. Mukkamala D, No JH, Cass LM, Chang TK, Oldfield E: Bisphosphonate inhibition of a *Plasmodium* farnesyl diphosphate synthase and a general method for predicting cell-based activity from enzyme data. *J Med Chem* 2008, **51**:7827–7833.
52. Ghosh S, Chan JM, Lea CR, Meints GA, Lewis JC, Tovian ZS, Flessner RM, Loftus TC, Bruchhaus I, Kendrick H, Croft SL, Kemp RG, Kobayashi S, Nozaki T, Oldfield E: Effects of bisphosphonates on the growth of *Entamoeba histolytica* and *Plasmodium* species in vitro and in vivo. *J Med Chem* 2004, **47**:175–187.
53. No JH, De Macedo DF, Zhang Y, Liu YL, Zhu W, Feng X, Yoo JA, Lee E, Wang K, Hui R, Freitas-Junior LH, Oldfield E: Lipophilic analogs of zoledronate and risedronate inhibit *Plasmodium* geranylgeranyl diphosphate synthase (GGPPS) and exhibit potent antimalarial activity. *Proc Natl Acad Sci USA* 2012, **109**:4058–4063.
54. Tonhosolo R, D'Alexandri FL, De Rosso VV, Gazarini ML, Matsumura MY, Peres VJ, Merino EF, Carlton JM, Wunderlich G, Mercadante AZ, Kimura EA, Katzin AM: Carotenoid biosynthesis in intraerythrocytic stages of *Plasmodium falciparum*. *J Biol Chem* 2009, **284**:9974–9985.
55. Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, Allen CM: Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. *J Biol Chem* 2002, **277**:42066–42073.
56. Moura IC, Wunderlich G, Uhrig ML, Couto AS, Peres VJ, Katzin AM, Kimura EA: Limonene arrests parasite development and inhibits isoprenylation of proteins in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2001, **45**:2553–2558.

doi:10.1186/1475-2875-12-184

Cite this article as: Jordão et al.: Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*. *Malaria Journal* 2013 **12**:184.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

IV- Systematic analysis of FKBP inducible degradation domain tagging strategies for the human malaria parasite *Plasmodium falciparum*.

Systematic Analysis of FKBP Inducible Degradation Domain Tagging Strategies for the Human Malaria Parasite *Plasmodium falciparum*

Mauro Ferreira de Azevedo^{1,2}, Paul R. Gilson^{2,3}, Heloisa B. Gabriel¹, Roseli F. Simões¹, Fiona Angrisano^{4,5}, Jacob Baum^{4,5}, Brendan S. Crabb^{2,5}, Gerhard Wunderlich^{1*}

1 Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brasil, **2** The Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia, **3** Monash University, Melbourne, Victoria, Australia, **4** The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia, **5** University of Melbourne, Victoria, Australia

Abstract

Targeted regulation of protein levels is an important tool to gain insights into the role of proteins essential to cell function and development. In recent years, a method based on mutated forms of the human FKBP12 has been established and used to great effect in various cell types to explore protein function. The mutated FKBP protein, referred to as destabilization domain (DD) tag when fused with a native protein at the N- or C-terminus targets the protein for proteosomal degradation. Regulated expression is achieved via addition of a compound, Shld-1, that stabilizes the protein and prevents degradation. A limited number of studies have used this system to provide powerful insight into protein function in the human malaria parasite *Plasmodium falciparum*. In order to better understand the DD inducible system in *P. falciparum*, we studied the effect of Shld-1 on parasite growth, demonstrating that although development is not impaired, it is delayed, requiring the appropriate controls for phenotype interpretation. We explored the quantified regulation of reporter Green Fluorescent Protein (GFP) and luciferase constructs fused to three DD variants in parasite cells either via transient or stable transfection. The regulation obtained with the original FKBP derived DD domain was compared to two triple mutants DD24 and DD29, which had been described to provide better regulation for C-terminal tagging in other cell types. When cloned to the C-terminal of reporter proteins, DD24 provided the strongest regulation allowing reporter activity to be reduced to lower levels than DD and to restore the activity of stabilised proteins to higher levels than DD29. Importantly, DD24 has not previously been applied to regulate proteins in *P. falciparum*. The possibility of regulating an exported protein was addressed by targeting the Ring-Infected Erythrocyte Surface Antigen (RESA) at its C-terminus. The tagged protein demonstrated an important modulation of its expression.

Citation: de Azevedo MF, Gilson PR, Gabriel HB, Simões RF, Angrisano F, et al. (2012) Systematic Analysis of FKBP Inducible Degradation Domain Tagging Strategies for the Human Malaria Parasite *Plasmodium falciparum*. PLoS ONE 7(7): e40981. doi:10.1371/journal.pone.0040981

Editor: Thomas J. Templeton, Weill Cornell Medical College, United States of America

Received: March 13, 2012; **Accepted:** June 15, 2012; **Published:** July 16, 2012

Copyright: © 2012 de Azevedo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), by The National Council for Scientific and Technological Development (CNPq) and by the National Health and Medical Research Council of Australia. MFA is a former postdoctoral fellow from FAPESP (Process No: 09/51026-4). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: gwunder@usp.br

Introduction

Malaria is still one of the most important parasitic diseases affecting mankind, with more than 40% of the world's population at risk of infection [1]. The deadliest form of malaria, caused by *Plasmodium falciparum*, is responsible for almost a million deaths every year, most of them children under 5 years of age (WHO report 2010). Despite global efforts towards control and elimination of the disease, parasite resistance to nearly all drugs has emerged. Realizing the goals of future control efforts will, as such, depend on our ability to quickly diagnose and treat the infected patients and to control disease transmission.

A critical stage in the advancement of novel therapies is developing a means to understand specific aspects of *Plasmodium* biology, such as metabolic or signalling pathways and other fundamental mechanisms of parasite cell or molecular processes. Since *Plasmodium* is not amenable to RNAi based methods [2], the identification of gene function has traditionally relied on classical

reverse genetics approaches [3,4,5]. Using such approaches, the specific function of a protein is determined by phenotype analysis of null mutant parasites, where the gene encoding the protein of interest is knocked out either via single crossover-mediated homologous integration [6] or by double crossover homologous integration [7]. The haploid nature of the *Plasmodium* asexual blood stage genome has only permitted mutagenesis to be conducted for non-essential *P. falciparum* proteins expressed during this stage [8,9]. These classic reverse genetic approaches have been particularly useful for studying erythrocyte invasion for the parasites which employ multiple invasion pathways using several ligands none of which by themselves are essential [9]. Genes necessary for life cycle stages other than the asexual blood stage have been successfully studied because the mutants can be made in the blood stages and then passaged through insect and liver stages to obtain phenotypes [10,11,12]. However, it is blood stage infection that produces the symptoms of malarial disease and study

of this important life cycle stage has been severely hampered by the inability of knockout approaches to functionally dissect the role of essential proteins.

In order to address the function of proteins refractory to deletion, several inducible systems have been developed where the expression of the protein of interest can be modulated and the phenotypes analysed [13]. One of the most promising systems, utilizing conditional regulation of the candidate gene/protein at the post-translational level, was established by generating mutants of the human protein FKBP12 that are unstructured and consequently targeted for degradation in the proteasome. This mutant protein, called a destabilization domain or DD is fused to the protein of interest at its N- or C-terminus, which targets the whole fusion protein for degradation. Regulation is then achieved by adding a cell-permeable ligand, Shld-1 [14], which stabilizes the unstructured protein domain preventing it from protein degradation. The system is functional in several cell types and organisms [15,16], including *P. falciparum*, where it has been used to test the function of several essential proteins [17,18,19]. A related system has also been developed using mutated versions of the *E. coli* DHFR protein, which is unstable when not bound to the drug trimethoprim [20]. Although such a system has been successfully established for *P. falciparum*, it can only be applied to parasites that already express human DHFR since wild type parasites are sensitive to TMP [21].

The attraction of inducible systems for investigating protein function is their ability to specifically inhibit a protein in a regulated and reversible manner. This can be achieved by two basic approaches: 1) conditional knockdown where the protein of interest is expressed at markedly reduced levels; or 2) the regulated expression of a dominant negative variant where modified proteins or parts of proteins are expressed that then inhibit the native target protein or its ligand. In *P. falciparum*, due to the paucity of selectable markers and the long time required to generate stable transfectant parasites, the most straightforward strategy for conditional protein regulation is to integrate a plasmid containing the DD domain into the desired locus via single or double crossover recombination, so that the protein of interest is expressed in fusion with DD at its C-terminus (with the gene remaining under the control of the endogenous promoter). Such an approach, however, may be hindered by considerable leakiness of C-terminal DD tagging that can lead to insufficient target protein degradation and knockdown. In order to improve the strength of knockdown, two new DD variants, referred to as DD24 (E31G-R71G-K105E) and DD29 (D79G-P93S-D100R), have been developed and these provide more efficient and less leaky protein degradation [22].

Conditional knockdown of a *P. falciparum* calpain [19] and a calcium dependent protein kinase [18] have been achieved by C-terminal tagging with either the original DD (L106P) or the new mutant DD29. To our knowledge, these are the only two essential proteins that have been successfully targeted using C-terminal DD tagging with many others failing to accept a C-terminal tag or failing to regulate despite successful integration of either a DD or a DD29 tag (Baum et al. and Azevedo et al., *unpublished data*). The inability of different proteins to accept a C-terminal tag or the failure of the stabilizing ligand to restore expression levels that allow parasites to grow normally likely accounts for the lack of success to achieve integrated regulation. Variation in the ability to successfully regulate those proteins that will accept a tag likely arises because the degradation seen is not sufficient or complete enough to cause a detectable phenotype (incomplete degradation). Conditional expression of proteins that produce dominant-negative phenotypes has not yet been reported for *P. falciparum*.

To gain a better understanding of the performance of the available DD tagging strategies, we have conducted a systematic approach to measure the toxicity of Shld-1 as well as the activities of the available DD variants in *P. falciparum*. The single original mutant L106P, referred to as DD, has been characterized [17,19], as well as the triple mutant DD29 [18]. The triple mutant DD24 has only been characterized in other cell types [22]. Here, using the reporter proteins luciferase (Luc) and GFP, we show that when tagged to the C-terminal, DD24 performs clearly better than the other mutants regarding protein regulation at Shld-1 concentrations that are tolerated by the parasite. In contrast, N-terminal tagging with either of the triple mutants was less efficient compared to the original DD single mutant. The possibility of using the system with secreted or exported proteins was addressed by C-terminally tagging the endogenous gene for the exported *Ring Infected Erythrocyte Surface Antigen* (RESA) with DD24. Successful regulated expression and export of RESA-DD demonstrate that proteins secreted via the endoplasmic reticulum (ER) can be regulated.

Results

Effect of the Shld-1 on Parasite Growth

The application of the DD system includes the incubation of parasite forms with the small stabilizing molecule Shld-1 which is considered non-toxic to *P. falciparum* at the concentrations used in other cell systems. However, it is possible that Shld-1 interferes subtly with *Plasmodium* cell cycle progression or proliferation leading to a misinterpretation of eventual phenotypes. *P. falciparum* conditional knockdown or knockout lines have been successfully generated [18,19] when parasites had been maintained in 0.5 μ M Shld-1. Depending on the target protein tagged, it may be desirable or necessary to increase the concentration of Shld-1. In order to quantify the toxicity of Shld-1 to the parasites and to determine whether a higher concentration would be tolerable, *P. falciparum* ring stage parasites were incubated with either 0.5 μ M or 1.0 μ M of Shld-1 for up to four days. Parasite growth was measured by flow cytometry and parasite forms were analysed by microscopy of blood smears.

Parasitemias of the cultures kept at 0.5 μ M and 1.0 μ M Shld-1 were reduced by 11% and 18% after the first reinvasion cycle (48 hours) and by 25% and 45% after the second cycle, respectively, compared to no Shld-1 controls (Fig. 1). A subtle delay in the trophozoite development could be made out over one blood stage cycle when treating with Shld-1, while the incubation for 24 h did not reveal differences between treated or untreated parasites (Fig. S3). To ascertain if *P. falciparum* could tolerate being cultured in 1.0 μ M Shld-1 for a longer period, parasites were further grown for several weeks and continued to expand, proving that despite its apparent toxicity, 1.0 μ M could be employed for long term growth (data not shown).

Vectors for Inducible Expression of Proteins in *P. falciparum*

Prior studies and our own work have demonstrated that N-terminal tagging of proteins with a Destabilization Domain (DD) tag provides a much greater efficiency of destabilization than C-terminal tagging [14,17] (Fig. S1). This observation has proven valid for a variety of cell types, including *P. falciparum*, which may explain why reversible phenotypes of only two conditional knockout lines have been reported [18,19].

To explore this concept further, a rapid read out for the effects of DD and Shld-1 on the expression of a reporter gene encoding *Photinus* luciferase (Luc) was undertaken with *P. falciparum* blood

Figure 1. Effect of Shld-1 on parasite development. *P. falciparum* 3D7 blood stage parasites were incubated with or without the indicated concentrations of Shld-1 and the parasitemia monitored for 4 days by flow cytometry.
doi:10.1371/journal.pone.0040981.g001

stage parasites. Plasmids encoding *luc* fusions were electroporated and luciferase activity was measured one day later. GFP was also incorporated into some of our Luc fusion proteins so that an option to check the expression of the fusion proteins by fluorescence microscopy or flow cytometry was available. A plasmid encoding luciferase alone and luciferase-GFP tagged with a triple haemagglutinin epitope (HA) served as positive controls for transfection efficiency and enzyme activity (pEF-Luc & pEF-Luc-GFP-HA, Fig. 2A). In addition to the originally described and functional single DD mutant (L106P) (Fig. S1), we made a Luc-GFP-HA gene fusion with the DD24 and DD29 triple mutants. The three DD containing plasmids were accordingly called pEF-Luc-GFP-HA-DD/DD24/29 (Fig. 2A).

Regulation in Transiently Transfected Parasites

The luciferase reporter plasmids were transiently transfected in *P. falciparum* and the parasites were cultured in the presence of 0, 0.5 or 1.0 μ M Shld-1 for one day after which they were harvested and their luciferase activity determined (Fig. 2B). Importantly, in the sample without Shld-1, the culture medium was adjusted to 0.1% ethanol. Also, the incubation time with Shld-1 was limited to 24 h in which no toxic inhibitory effect on parasite progression is observed (Fig. 1 and Fig. S3). In the absence of Shld-1 the presence of the GFP-HA reduced luciferase activity to 40% of the luciferase only control. The DD further reduced reporter activity to about only 21% of pEF-Luc (Fig. 2B). The reporter activity of parasites transfected with the DD24 and DD29 plasmids was only 12% and 11% respectively, and much lower than the activity detected with the original DD plasmid. This indicates that in *P. falciparum* the DD triple mutants destabilize the proteins approximately twice as efficiently as the original DD.

In the presence of 0.5 and 1.0 μ M Shld-1 the luciferase activity of pEF-Luc and pEF-Luc-GFP transfected parasites was almost the same as without the compound (Fig. 2B), excluding any nonspecific effect of Shld-1 upon luciferase activity which can be observed after longer Shld-1 incubations (Fig. 1). In contrast, cultures transfected with each of the DD vectors and kept in 0.5 μ M or 1.0 μ M Shld-1 always showed increased luciferase activity when compared to the same transfected parasites kept in the absence of the ligand. In 0.5 μ M and 1.0 μ M Shld-1, the reporter activity relative to the control was increased to a

respective 48% and 57% for the DD, 38% and 53% for DD24 and to 21% and 29% for DD29 (Fig. 2B). The culturing of DD and DD24 transfectants on 1 μ M Shld-1 elevated the luciferase activities to a level exceeding the pEF-Luc-GFP-HA control parasites. In contrast, the luciferase activity of parasites transfected with DD29 plasmid was only raised to half of the Luc-GFP-HA parasites in 1 μ M Shld-1.

Regulation in Stably Transfected Parasites

To test whether the performance of the DD mutants would be maintained in transfectants containing the luciferase and GFP genes in episomes, parasites were stably transfected with the DD reporter plasmids. Reporter expression was then induced similarly to the transient transfection experiments, except that Shld-1 was added to ring stage parasites. Luciferase reporter activity of non-induced parasites suggested DD24 is the most efficient domain in destabilizing luciferase, followed by DD29 and DD (Fig. 2C). This result is somewhat different to the transient transfection data where both DD24 and DD29 parasites destabilised luciferase to the same degree (Fig. 2B). The other difference was that in the transient data Shld-1 drug restored greater luciferase activity in DD24 compared to DD29 mutants but in the stably transfected parasites restoration was similar (Figs. 2A & B). Although luciferase activity was normalized by parasite number in the stable transfectants, small differences in parasite cell cycle synchronization could have affected reporter activity. To further ensure synchronisation was not contributing to the differences, GFP fluorescence of the stable transfectants was quantified by flow cytometry, where cells were gated on late stage parasites. DD24 still destabilized luciferase to the lowest levels, followed now by DD and DD29. The effect of Shld-1 was similar to what was detected using the luciferase assay (Fig. 2D). The results calculated as fold induction upon Shld-1 treatment are summarized in Table 1.

Despite some differences between luciferase/GFP expression in the transient versus stable transfectants, which may also have been due to plasmid copy number in the latter, the DD24 tagged line consistently showed the highest fold induction (difference between induced and non-induced state) in each test when either 0.5 μ M (3.2–4.1 x) or 1.0 μ M (4.4–5.3 x) Shld-1 was used. DD had the second best induction followed by DD29 (Fig. 2B–D, Table 1). Notably, the plasmid copy numbers as evaluated by quantitative PCR reactions using equally performing primers for a single copy gene (seryl-t-RNA synthetase, PlasmDB PF07.0073) and luciferase showed a comparable number of plasmid equivalents in the transfectants (Fig. S4). Assuming that the copy number of plasmids is not decreasing dramatically during Shld-1 removal/addition, our results indicate that the observed differences between the different DD mutants occurred as a consequence of the differing DD domain sequences and not due to transcriptional differences caused by vastly differing plasmid copy numbers in transfectants.

Performance of the DD Mutants for N-terminal Tagging

Considering the reproducibly improved regulation of protein levels achieved with DD24 when expressed at the C-terminal of the reporter proteins, the regulation of N-terminal tagging was also investigated. Since the reporter activity obtained in *P. falciparum* transient transfections is somewhat low and DD degrades proteins more efficiently when fused to its N-terminus (Fig. S1), reporter plasmids were constructed based on the pP86 vector [23], where luciferase is under the control of the HSP86 promoter, which is about ten times stronger than the EF1- α promoter (Fig. 3A).

The N-terminal DD reduced luciferase activity 10 fold, confirming the more efficient protein destabilization (Fig. 3B). Shld-1 partially prevented the degradation restoring the activity to

Figure 2. Regulation with C-terminal DD. (A) Maps of inducible expression vectors. (B) To measure expression in transiently transfected parasites ring stage parasites were transfected and the indicated Shld-1 concentrations were added the next day and maintained for 24 hours. Luciferase expression is represented relative to the positive control pEF-Luc kept without Shld-1. Results indicate the average of 4 experiments and error bars show the standard deviation. (C) Luciferase activity on stably transfected parasites. Ring stage parasites of stable transfected lines were incubated for one day with the indicated Shld-1 concentrations. The measured luciferase activity was normalized by the number of parasites and expressed as percentages to the control line 3D7 transfected with Luc-GFP kept without Shld-1. (D) GFP fluorescence of the parasites described in (C) was measured by flow cytometry and is expressed relative to the control line Luc-GFP kept without Shld-1. RLU – relative light units, FI – fold induction of reporter expression, MFI – mean fluorescence intensity.
doi:10.1371/journal.pone.0040981.g002

29% and 30% when cultures were kept on 0.5 μ M and 1.0 μ M Shld-1, respectively (3 fold induction). The triple mutant DD24 destabilized luciferase as efficiently as DD, but reporter activity

was poorly reverted by Shld-1, 18% and 16%, respectively ($\sim$ 1.5–1.6 fold induction). DD29 was an even more efficient destabiliser, reducing reporter activity even further to about 6%, but similarly

Table 1. Reporter proteins - regulation summary.

Reporter	Shld-1 induction (fold change)							
	Transient - Luc		Stable - Luc		Stable - GFP		Average	
	0.5 μ M	1.0 μ M	0.5 μ M	1.0 μ M	0.5 μ M	1.0 μ M	0.5 μ M	1.0 μ M
C-DD	2.28	2.71	2.46	2.68	3.12	3.87	2.62	3.08
C-DD24	3.16	4.41	3.75	4.37	4.11	5.34	3.67	4.70
C-DD29	1.90	2.63	2.01	2.53	1.84	2.64	1.91	2.60
N-DD	2.90	3.00	ND	ND	ND	ND	ND	ND
N-DD24	1.63	1.45	ND	ND	ND	ND	ND	ND
N-DD29	1.66	1.50	ND	ND	ND	ND	ND	ND

C- refers to Luc-GFP-HA fused to the indicated DD mutant at its C-terminus and N- to Luc fused to DD at its N-terminus. Fold change represents the means of the expression of the Shld-1 induced related to the basal (non induced) expression. See figure 2 for relative expression values.

doi:10.1371/journal.pone.0040981.t001

to DD24, reporter expression poorly recovered when Shld-1 was added, increasing to 10% and 9%, respectively.

DD System can Regulate the Expression of Exported Proteins

While proteins localized in the parasite cytoplasm have been successfully regulated by Shld-1, it was uncertain whether proteins that are secreted via the ER or exported to the red blood cell could still be destabilized. In order to investigate the efficacy of the

system to regulate an exported protein, RESA was tagged with GFP-HA-DD24 at its C-terminus (Fig. 4A). Parasites were transfected and then kept in 0.5 μ M Shld-1 and the transfected cultures were intermittently subjected to WR99210 withdrawal to select for plasmid integration. The resulting parasites were then analysed by PCR using oligonucleotides that could only amplify a product if integration had occurred (Fig. 4B). Images of fluorescence live microscopy confirmed that RESA was expressed in fusion with the tag at its expected localization, associated to the host cell membrane (Fig. 4C, Fig. S2).

We then monitored the regulation removing the Shld-1 from ring stage cultures for 2 days after which ghosts of infected RBC were fractionated and proteins extracted with SDS. Western blots probed with anti-HA antibody showed greatly reduced levels of RESA-GFP-HA-DD24 in parasites where Shld-1 had been removed (Fig. 4D). Importantly, loading controls with EXP2 (a component of the putative parasite export machinery co-purifying with IRBC membranes [24]) demonstrated the reduction of RESA-GFP-HA-DD24 was due to its DD-mediated degradation and not due to whether cultures were subjected to Shld-1 (Fig. 4D). Densitometry analysis of the bands detected in the Western blot suggests that removal of Shld-1 resulted in about 4 fold less tagged RESA protein (data not shown). Fluorescent live microscopy imaging also showed reduction in RESA-GFP-HA-DD24 expression in parasites where Shld-1 had been removed. Moreover, GFP fluorescence was more intense inside the parasite, suggesting a partial degradation prior to insertion in the endoplasmic reticulum although the resolution does not permit more specific affirmations (Fig. S2).

Discussion

The two main approaches applying inducible systems to understand gene function are i) the conditional knockdown and ii) the expression of proteins that produce a dominant negative phenotype. In the former, the proteins of interest must be stabilized during selection, such as with tolerant Shld-1 concentrations, for periods up to many months. In the second strategy, the expression of proteins or peptides, supposed to be toxic or deleterious, must be kept silent, so Shld-1 has to be added to parasites already selected for the presence of the plasmids in the form of episomes. The conditional knockouts of a protease [19] and a kinase [18] have been conducted this way, selecting parasites on 0.5 μ M Shld-1. This enabled the generation of cloned cell lines where the target proteins were regulated at sufficient levels to

Figure 3. Regulation with N-terminal DD. (A) Maps of reporter plasmids tested. (B) Reporter expression of transiently transfected parasites. Parasite transfection and Shld-1 incubation was performed as indicated in Figure 2. Luciferase activity is represented relative to pPf86 kept without Shld-1. Results are the average of 3 experiments and the error bars show the standard deviation.

doi:10.1371/journal.pone.0040981.g003

Figure 4. DD can be used to regulate exported proteins. (A) Diagram of DNA integrations into the RESA locus to produce the transgenic line. After integration in the presence of Shld-1, RESA was expressed in fusion with the GFP-HA-DD24 under the control of the native RESA promoter. (B) Integration of the plasmid in the RESA endogenous locus is confirmed by PCR. The oligonucleotides used are indicated by arrows in (A). (C) Fluorescent microscopy of live parasites labelled with DAPI showing RESA-GFP-HA-DD24 is exported to the RBC membrane. (D) Western blot analysis of RESA-GFP-HA-DD24 transfectants showing that RESA levels decrease after the removal of Shld-1. Parasites integrated in the presence of Shld-1 at ring stage were split and kept either with 0.5 μ M or no Shld-1 for 2 days when RBC ghosts were collected and protein were fractionated by SDS-PAGE. The western blot was probed with anti-HA mAb to detect RESA-GFP-HA-DD24. As the loading control, the western blot probed with polyclonal rabbit anti-Exp2 [31].

doi:10.1371/journal.pone.0040981.g004

produce inducible and reversible deleterious phenotypes providing important clues of their respective functions. According to our results, the concentration of 0.5 μ M Shld-1 is slightly toxic to the parasites, reducing their growth to about 11% per reinvasion cycle. We also showed that although parasites tolerate up to 1 μ M of Shld-1, their development is delayed and this must be taken in account when interpreting inducible phenotypes. We could not assign the Shld-1 growth defect phenotype to the inhibition of a specific intraerythrocytic stage or to RBC invasion (Fig. S3). Therefore, it remains to be shown whether unique or multiple parasite proteins are natural targets for Shld-1. A *Plasmodium* FKBP homologue is expressed throughout the life cycle and it is sensitive to the Shld-1 analogue Rapamycin [25], but whether Shld-1 targets PfFKBP is unknown.

In this study we present an in depth-analysis of the most useful system currently available to achieve controlled protein expression in *Plasmodium*. While regulation of protein levels by the DD system in *P. falciparum* has been demonstrated using a small number of

proteins, we show here the potential of the technique using transiently, stable and integrated plasmid constructs containing three different variants of the original FKBP (DD) domain. Similar to what has been characterized for NIH3T3 cells [22], data from transiently and stably transfected parasites, using either Luc or GFP as reporters, suggested that DD24, and possibly DD29, are more efficient than the original DD in the destabilization of proteins when fused to their C-terminus. Also, at Shld-1 concentrations which are tolerated by *P. falciparum* cultures, DD24 has the greatest dynamic range of the three DDs tested. While our data generally agree with what has been described for the DD mutants in other cell types, the destabilization efficiency and the dynamic range of induction observed in *P. falciparum* are quite low compared to what Chu et al. [22] measured. It was shown that in NIH3T3 cells the DD24 or DD29-tagged reporter proteins were reduced to about 5% of the control lines; however, in *P. falciparum*, the reduction was to about 10–20% depending on the control used. In the same way, induction with 1 μ M Shld-1

increased protein levels about 20 fold against 4–5 fold in *P. falciparum*. Importantly, our values were obtained in quantifications using two different reporter proteins, and both showed similar induction performance. This suggests that the regulation provided by the DD system, despite evidence for a considerable regulation, is generally lower in *P. falciparum*. Longer Shld-1 incubations were tried, but resulted in only slightly better induction and no more than 6 fold in 4 days was attained. Higher Shld-1 concentrations were tested, but they were too toxic to the parasite. It is not clear whether these differences are due to less active protein degradation in parasites compared to NIH3T3 cells.

Although DD24 seems to be the mutant with the best regulation, DD29 has been successfully applied in the CDPK5 conditional knockout [18], indicating the regulation obtained with this mutant can be sufficient to cause an inducible lethal phenotype. It is possible though, that DD24 could be successfully used to regulate proteins that cannot be targeted with DD29 because they need to be expressed at higher levels to guarantee the survival of parasites.

Tagging the proteins with DD at the N-terminus had been reported to produce more efficient protein destabilization both for the apicomplexan parasites *P. falciparum* [17] and *Toxoplasma gondii* [15] and also for other eukaryotic cell lines [14]. We investigated whether the triple mutants would allow the protein to be further degraded. While the reporter activity was equally (DD24) or more efficiently (DD29) reduced than with the classic DD, expression was poorly restored with Shld-1, suggesting the triple mutants are not suitable for N-terminal tagging, probably by rendering the reporter protein unstable.

Plasmodium parasites have unique cellular compartments where DD tagged proteins might not be as efficiently targeted for degradation as those that reside in cytosolic environments. For example, it is unclear if proteins directed to the nucleus or to the apicoplast can be efficiently DD-tagged and degraded which may depend on specific functional features of the target proteins. It is possible that proteins become non-functional despite of being normally transported to their target organelle in the presence of a stabilized DD domain. On the other hand, C-terminally tagged proteins with co-translational insertion in the endoplasmic reticulum may escape from proteasome degradation in the absence of Shld-1. Since a number of potential virulence factors are exported from the parasite, we addressed the feasibility of regulating RESA, a protein that is exported to the host cell membrane during erythrocytic stages. Removal of Shld-1 from parasites caused a clearly visible reduction in the levels of RESA present in the RBC compartment. Since the period from which Shld-1 was removed until protein samples were acquired was about one reinvasion, it seems that proteins synthesized in the absence of Shld-1 are sensitive to degradation and it is probable that the punctuate weak fluorescent signal inside the parasite was caused by GFP *en route* to degradation. It still remains to be determined what happens to tagged RESA that has already been exported and if it is sensitive to degradation. Recently, the 20S proteasome subunit was identified in human mature erythrocytes [26], turning possible that even exported destabilized proteins may be degraded by the erythrocyte proteasome.

Conclusion

In conclusion, our study demonstrates the possibilities and limits in which proteins can be modulated using the FKBP-mutants DD, DD24 and DD29 and establishes the groundwork for experimentation using this system on virtually any target in the *Plasmodium* proteome. This includes the many proteins resident in organelles or which function in pathways that lead to the secretion of proteins

to the host cell and which may prove novel targets for intervention against malaria disease.

Materials and Methods

Plasmid Construction

The plasmid pTGFP [27] was digested with *Xho* I and re-ligated to delete the transactivator and its 3'UTR. GFP was PCR amplified from the same plasmid with the oligonucleotides 5'-ctcgagctgcagaaaaatggctacacgtgca and 5'-actagtagcgtgttgtagtagttcat and cloned back in the vector digested with *Xho* I and *Spe* I, generating the plasmid pRM2-GFP. This plasmid incorporates the rep20 element for efficient segregation during mitosis [28] and has the selection and expression cassettes cloned back to back, where GFP is under the control of the MSP2 promoter. Plasmid templates for DD variants were kindly provided by Thomas Wandless (Stanford University, USA). DD combined with a PCR amplified triple haemagglutinin (HA) tag were fused by PCR using the oligonucleotides 5'-acgcgtccgtacgacgtc and 5'-actagttattccggttttagaagc and cloned in pRM2-GFP digested with *Mlu* I and *Spe* I, generating pRM2-GFP-DD. DD24 and 29 were amplified from plasmids pYFP-E31G-R71G-K105E (24) and pYFP-D79G-P93S-D100R (29), using the forward oligonucleotide 5'-gctagctgggagtgacggtggaaac and the reverse oligonucleotides 5'-actagttattccagttctagaagctccac (24) or 5'-actagttattccagtttagaagctccac (29) and cloned in pRM2-GFP-DD, generating pRM2-GFP-DD24 and pRM2-GFP-DD29. Both promoters and the Rep20 sequences of these plasmids were replaced with the bidirectional *P. berghei* EF1 α promoter, which was PCR amplified from *P. berghei* gDNA using the oligonucleotides 5'-gctctagagatcctttataaaaaattttattttatttataagca and 5'-ctcgagttttataaaaaattttattttatttataagca and cloned in pBluescript (Stratagene). The restriction sites *Spe* I and *Hind* III were destroyed by digesting the plasmid with these enzymes and then filling the ends with Klenow and re-ligating them. The promoter was then digested with *Bam* HI and *Hind* III and cloned in pRM2-GFP-DD/24/29, generating pEF-GFP-DD/24/29. Luciferase was amplified from pGL3 (Promega) with oligonucleotides 5'-ctcgaggtccatggaagacgcaaaaaaca and 5'-actagttgtctgcagccacggcgatcttccgc and cloned in pEF-GFP-DD/24/29 digested with *Xho* I and *Spe* I or *Pst* I, generating pEF-Luc and pEF-Luc-GFP-DD/24/29 respectively. The GFP-HA-DD sequence of pEF-Luc-GFP-DD was replaced with GFP-HA digesting the vector with *Pst* I and *Spe* I and the insert retrieved from pRM2-GFP-DD digested with *Pst* I and *Nhe* I, generating pEF-Luc-GFP-HA.

The DD-Luc was fused by PCR with oligonucleotides 5'-ccatgggagtgacggtggaa and 5'-gcgtcttctcagttccggttttagaagctcca (DD) and 5'-accggaactgcaggaagacgcaaaaaacataaaga and 5'-accggaactgcaggaagacgcaaaaaacataaaga (Luc), digested with *Nco* I and *Spe* I and cloned in pP86 digested with *Nco* I and *Xba* I to make pP86-DD-Luc. DD24 and DD29 were amplified by PCR with oligonucleotides 5'-ccatgggagtgacggtggaaacca and 5'-ctgcagttccagttctagaagctccaca (DD24) or 5'-ctgcagttccagtttttagaagctccacac (DD29), digested with *Nco* I and *Pst* I and cloned in pP86-DD-Luc to make pP86-DD24/29-Luc.

P. falciparum Culture and Transfection

Parasites were transfected as previously described [3], using the electroporation conditions established elsewhere [29]. Briefly, *P. falciparum* 3D7 was cultured in 4% hematocrit in RPMI-HEPES supplemented with 0.5% Albumax 1 (Invitrogen). Ring state parasites at 5–8% parasitemia were transfected with 75 μ g (transient) or 150 μ g (stable) of plasmid. The culture media were changed on the second day and parasites were harvested for

reporter assays (transient) or submitted to drug pressure with 2.5 nM WR99210 (stable) on the third day. Stably transfected parasites were cultured in standard conditions until parasites reappeared and normal growth was re-established.

Shld-1 Incubation

Shld-1 was diluted in ethanol to a stock concentration of 1 mM and stored in -20°C . Immediately prior to use, it was diluted in RPMI to the indicated concentration. For the transient transfections, parasites were split and Shld-1 added one day after the electroporation. Unless specified, parasites were kept under Shld-1 for one day.

Luciferase Assays

Parasites were saponin lysed and the pellet washed twice in PBS. After resuspension in 1 x lysis buffer (Promega), the lysed cells were mixed with luciferase assay reagent (Promega) and luciferase activity was measured in the Lumat LB 9507 luminometer (EG & G Berthold). For each experiment, the reporter activity was expressed as the percentage of the activity measured in the positive control, usually parasites transfected with similar luciferase plasmids without DD. The results are the average of at least three independent experiments for transient transfections and of a representative experiment with stable transfected parasites, done in duplicates.

Detection of Parasitemia by Flow Cytometry

Parasites were labeled with 10 $\mu\text{g}/\text{ml}$ ethidium bromide as described previously [30], washed once in PBS and analysed on a Guava cytometer (General Electric).

Supporting Information

Figure S1 Efficient Shld-1 regulation of N terminally tagged DD (original) tagged proteins in *P. falciparum*.

(A) Plasmid derived from pRM2-GFP for N terminal DD tagging of a reporter gene - here a triple HA tag fused to GFP. This plasmid contains the strong and schizont-specific msp2 promoter. B) Western blot of synchronous schizont lysates transformed with pRM2-DD-3HA-GFP with anti-GFP (1:1000, Roche) demonstrates efficient Shld-1 dependent regulation by addition of either 0.5 μM or 0.1 μM to parasite ring stages. An asterisk marks a GFP breakdown product. Note that we used a smaller Shld-1 concentration in order to avoid the delay in intraerythrocytic development observed with higher Shld-1 concentrations which

would lead to an overall decreased signal due to weaker promoter activity in earlier than schizont stages.

(TIF)

Figure S2 Fluorescent microscopy of RESA-GFP-HA-DD24 parasites cultured in the presence of Shld-1 (left) or when the ligand had been removed for 2 days (right). (TIF)

Figure S3 *Plasmodium falciparum* D10 parasites were highly synchronized by sorbitol and heparin treatment. Shld-1 (1 μM) was added to either early ring stage ($t=0$ h) or trophozoite stage parasites ($t=24$ h). Parasite development was monitored by Giemsa-stained blood smears taken every 8 hours for 48 hours. (JPG)

Figure S4 Parasites from transfectants had their genomic DNA extracted by standard methods [32] and the following oligonucleotides were employed to amplify either plasmodial seryl-t-RNA synthetase (PF07_0073, 5'-AAGTAGCAGGTCATCGTGGTT, 5'-TTCGGC-CATTCTTCCATAA) or *Photinus* luciferase (5'-CGTCGCCAGTCAAGTAACAA, 5'-TTTCTTGCGTCGAGTTTTC) by standard qPCR using Fermentas SYBR realtime PCR mix in an Eppendorf realplex² thermocycler. Equal primer performance was tested beforehand using plasmids with cloned target sequences. The differences in copy numbers were expressed as $2^{-\Delta\text{Ct}}$ values (y-axis) which indicate how many times more luciferase target molecules are in the sample in relation to the genomic control seryl-t-RNA synthetase [33]. The four indicated gDNA samples were analysed in three independent experiments using triplicates for each gDNA sample and primer pair and the standard deviation between the experiments is shown. (TIF)

Acknowledgments

The authors thank the Blood Center from Hospital Sirio-Libanês in São Paulo for the donation of human blood.

Author Contributions

Conceived and designed the experiments: MFA PRG GW. Performed the experiments: MFA HBG RFS FA. Analyzed the data: MFA PRG JB GW. Contributed reagents/materials/analysis tools: PRG FA JB BSC. Wrote the paper: MFA PRG GW.

References

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* 434: 214–217.
2. Baum J, Papenfuss AT, Mair GR, Janse CJ, Vlachou D, et al. (2009) Molecular genetics and comparative genomics reveal RNAi is not functional in malaria parasites. *Nucleic acids research* 37: 3788–3798.
3. Wu Y, Sifri CD, Lei HH, Su XZ, Wellems TE (1995) Transfection of *Plasmodium falciparum* within human red blood cells. *Proc Natl Acad Sci U S A* 92: 973–977.
4. Crabb BS, Cowman AF (1996) Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America* 93: 7289–7294.
5. Balu B, Adams JH (2007) Advancements in transfection technologies for *Plasmodium*. *Int J Parasitol* 37: 1–10.
6. Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, et al. (1997) Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell* 89: 287–296.
7. Duraisingh MT, Triglia T, Cowman AF (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *Int J Parasitol* 32: 81–89.
8. Maier AG, Rug M, O'Neill MT, Brown M, Chakravorty S, et al. (2008) Exported proteins required for virulence and rigidity of *Plasmodium falciparum*-infected human erythrocytes. *Cell* 134: 48–61.
9. Cowman AF, Crabb BS (2006) Invasion of red blood cells by malaria parasites. *Cell* 124: 755–766.
10. Ishino T, Chinzei Y, Yuda M (2005) Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Molecular microbiology* 58: 1264–1275.
11. van Dijk MR, Douradinha B, Franke-Fayard B, Heussler V, van Dooren MW, et al. (2005) Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proceedings of the National Academy of Sciences of the United States of America* 102: 12194–12199.
12. van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, et al. (2001) A central role for P48/45 in malaria parasite male gamete fertility. *Cell* 104: 153–164.
13. de Koning-Ward TF, Gilson PR (2009) Keeping it simple: an easy method for manipulating the expression levels of malaria proteins. *Trends Parasitol* 25: 4–7.
14. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ (2006) A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* 126: 995–1004.

15. Herm-Gotz A, Agop-Nersesian C, Munter S, Grimley JS, Wandless TJ, et al. (2007) Rapid control of protein level in the apicomplexan *Toxoplasma gondii*. *Nature methods* 4: 1003–1005.
16. Madeira da Silva L, Owens KL, Murta SM, Beverley SM (2009) Regulated expression of the *Leishmania major* surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins. *Proc Natl Acad Sci U S A* 106: 7583–7588.
17. Armstrong CM, Goldberg DE (2007) An FKBP destabilization domain modulates protein levels in *Plasmodium falciparum*. *Nat Methods* 4: 1007–1009.
18. Dvorin JD, Martyn DC, Patel SD, Grimley JS, Collins CR, et al. A plant-like kinase in *Plasmodium falciparum* regulates parasite egress from erythrocytes. *Science* 328: 910–912.
19. Russo I, Oksman A, Vaupel B, Goldberg DE (2009) A calpain unique to alveolates is essential in *Plasmodium falciparum* and its knockdown reveals an involvement in pre-S-phase development. *Proc Natl Acad Sci U S A* 106: 1554–1559.
20. Iwamoto M, Bjorklund T, Lundberg C, Kirik D, Wandless TJ (2010) A general chemical method to regulate protein stability in the mammalian central nervous system. *Chemistry & biology* 17: 981–988.
21. Muralidharan V, Oksman A, Iwamoto M, Wandless TJ, Goldberg DE (2011) Asparagine repeat function in a *Plasmodium falciparum* protein assessed via a regulatable fluorescent affinity tag. *Proceedings of the National Academy of Sciences of the United States of America* 108: 4411–4416.
22. Chu BW, Banaszynski LA, Chen LC, Wandless TJ (2008) Recent progress with FKBP-derived destabilizing domains. *Bioorg Med Chem Lett* 18: 5941–5944.
23. Militello KT, Wirth DF (2003) A new reporter gene for transient transfection of *Plasmodium falciparum*. *Parasitol Res* 89: 154–157.
24. Crabb BS, de Koning-Ward TF, Gilson PR (2010) Protein export in *Plasmodium* parasites: from the endoplasmic reticulum to the vacuolar export machine. *International journal for parasitology* 40: 509–513.
25. Monaghan P, Bell A (2005) A *Plasmodium falciparum* FK506-binding protein (FKBP) with peptidyl-prolyl cis-trans isomerase and chaperone activities. *Mol Biochem Parasitol* 139: 185–195.
26. Neelam S, Kakhniashvili DG, Wilkens S, Levene SD, Goodman SR (2011) Functional 20S proteasomes in mature human red blood cells. *Experimental biology and medicine* 236: 580–591.
27. Gilson PR, O'Donnell RA, Nebl T, Sanders PR, Wickham ME, et al. (2008) MSP1(19) miniproteins can serve as targets for invasion inhibitory antibodies in *Plasmodium falciparum* provided they contain the correct domains for cell surface trafficking. *Mol Microbiol* 68: 124–138.
28. O'Donnell RA, Freitas-Junior LH, Preiser PR, Williamson DH, Duraisingh M, et al. (2002) A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. *The EMBO journal* 21: 1231–1239.
29. Fidock DA, Welles TE (1997) Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci U S A* 94: 10931–10936.
30. Wilson DW, Crabb BS, Beeson JG Development of fluorescent *Plasmodium falciparum* for in vitro growth inhibition assays. *Malar J* 9: 152.
31. Bullen HE, Charmaud SC, Kalanon M, Riglar DT, Deckwadia C, et al. (2012) Biosynthesis, localisation and macromolecular arrangement of the *Plasmodium falciparum* translocon of exported proteins; PTEX. *The Journal of biological chemistry*.
32. Ljungström I, Perlmann H, Schlichtherle M, Scherf A, Wahlgren M (2004) *Methods in Malaria Research*. Manassas, VA: MR4/ATCC.
33. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods* 25: 402–408.