Protein O-fucosylation in *Plasmodium falciparum* ensures efficient infection of mosquito and vertebrate hosts

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O-glycosylation of the *Plasmodium* sporozoite surface proteins CSP and TRAP was recently identified, but the role of this modification in the parasite life cycle and its relevance to vaccine design remain unclear. Here, we identify the *Plasmodium* protein O-fucosyltransferase (POFUT2) responsible for O-glycosylating CSP and TRAP. Genetic disruption of POFUT2 in *Plasmodium falciparum* results in ookinetes that are attenuated for colonizing the mosquito midgut, an essential step in malaria transmission. Some POFUT2-deficient parasites mature into salivary gland sporozoites although they are impaired for gliding motility, cell traversal, hepatocyte invasion, and production of exoerythrocytic forms in humanized chimeric liver mice. These defects can be attributed to destabilization and incorrect trafficking of proteins bearing thrombospondin repeats (TSRs). Therefore, POFUT2 plays a similar role in malaria parasites to that in metazoans: it ensures the trafficking of *Plasmodium* TSR proteins as part of a non-canonical glycosylation-dependent endoplasmic reticulum protein quality control mechanism.
Plasmodium spp. lack many genes necessary for conventional N- and O-glycosylation, and the N-glycan-dependent protein folding quality control pathways found in most eukaryotes. This has fuelled debate about whether these protozoan parasites glycosylate their proteins. Recent advances have begun to resolve this issue. Blood stage Plasmodium falciparum parasites, which cause the most severe form of human malaria, N-glycosylate proteins with Asn-linked N-acetylgalactosamine or chitobiase. While this minimalistic N-glycan likely plays a thermodynamic role in protein folding, it remains unclear which parasite proteins are N-glycosylated and whether it occurs in other stages of the Plasmodium lifecycle. In contrast, O-glycosylation has only been detected outside of the blood stages: the P. falciparum sporozoite antigens circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) both bear an O-linked hexosyl-deoxyhexose disaccharide on their thrombospondin repeat (TSR) domains.

CSP and TRAP are essential for infection of the human host and abundant on the sporozoite surface, making them prime vaccine candidates. Indeed, the only malaria vaccine approved for date RTS.S/A01, is based on the CSP TSR domain. Simple O-glycans can enhance antigenicity and comprise part of T-cell epitopes making this parasite glycan of great relevance to the design of next-generation malaria vaccines. However, before pursuing this idea the precise chemical nature of the O-glycan in the parasite ER.

The chemical identity of the P. falciparum O-glycan is most likely the same as the O-linked β-D-glucosyl-1,3-α-L-fucose disaccharide found on metazoan TSR domains. In these systems, O-glycosylation of the cysteine-rich TSR domain occurs in the endoplasmic reticulum (ER) on correctly folded proteins at the CXX(S/T)C sequon in a stepwise manner: protein O-fucosyltransferase 2 (POFUT2) catalyzes the addition of GDP-fucose, then β-1,3-glucosyltransferase (B3GLCT) utilizes UDP-glucose to glucosylate the 3-OH of the fucose residue (Supplementary Fig. 1). This process comprises part of a non-canonical protein folding quality control mechanism. Ablation of POFUT2 or B3GLCT in mammals affects folding and trafficking of proteins with TSR domains, though the extent to which this occurs varies from protein to protein.

POFUT2 disruption in mice has an embryonic lethal phenotype while mutations in human B3GLCT cause Peters-Plus syndrome. The identification of glycosylated TSR proteins in P. falciparum suggests a similar protein quality control mechanism is present in the malaria parasite, an idea supported by the observation that heterologous expression of CSP and TRAP TSR domains in mammalian cell lines yield proteins modified with the same β-D-glucosyl-1,3-α-L-fucose disaccharide.

Here, we identify and characterize the protein O-fucosyltransferase 2 (POFUT2) conserved in all Plasmodium spp. Genetic disruption of POFUT2 in P. falciparum results in attenuation of both ookinete and sporozoite infection of their respective mosquito and vertebrate hosts. The defects are attributable to destabilization and incorrect trafficking of proteins with TSRs. This suggests that POFUT2 plays an important role in parasite transmission to mosquitoes and infection of the human host by ensuring trafficking of TSR proteins following glycosylation in the parasite ER.

**Results**

**In vitro characterization of Plasmodium POFUT2.** To investigate whether malaria parasites encode a POFUT2, a BLAST search of the Plasmodium multigenome database revealed a putative POFUT2 candidate in P. falciparum. The putative open reading frame (ORF) was amplified and sequence-confirmed by Sanger sequencing, and the recombinant ORF was overexpressed in mammalian cells. The recombinant POFUT2 was then assessed for O-fucosylation activity using recombinant CSP recombinant TRAP, and the CXX(S/T)C protein domains treated with GDP-fucose. As predicted, POFUT2 was able to fucosylate CSP and TRAP, with the addition of the expected disaccharide. These experiments demonstrate that POFUT2 is capable of O-fucosylating both CSP and TRAP in mammalian cell lines, further supporting the hypothesis that O-fucosylation is a conserved quality control mechanism in Plasmodium spp.

**Fig. 1** O-Fucosylation of TSR domains by POFUT2. a O-Fucosylation of TSR domains by GDP-fucose, as catalyzed by POFUT2, (illustration generated using 4HQ). b Deconvoluted intact ESI mass spectrum of recombinant P. falciparum TRAP TSR domain treated with GDP-fucose in the absence (blue) and presence (red) of P. vivax POFUT2. c Deconvoluted intact ESI mass spectrum of recombinant P. falciparum CSP TSR domain treated with GDP-fucose in the absence (blue) and presence (red) of P. vivax POFUT2. d Multiple sequence alignment of all TSR domain sequences from P. falciparum revealing the proteins that are likely to be O-fucosylated (red) and in what parasite stage they are expressed.
search of the *P. falciparum* genome using *Homo sapiens* POFT2 (CAC24557.1) as a search term led to the identification of PF3D7_0909200 as the putative malarial POFT2 enzyme. Highly homologous syntenic orthologs were present across the *Plasmodium* genus (Supplementary Fig. 2). The putative *P. falciparum* and *P. vivax* (PVX_098900.1) POFT2 share considerable sequence similarity with *H. sapiens* and *Caenorhabditis elegans* (NP_001255070.1) POFT2 and retain the catalytic residues of these enzymes (Supplementary Fig. 3). A homology model constructed from the *P. falciparum* POFT2 sequence has a very similar predicted structure to *P. falciparum* A homology model constructed from the catalytic residues of these enzymes (Supplementary Fig. 3). This conformational cation was docked into the model to reveal an alignment of catalytic residues and substrates that is reminiscent of a Michaelis complex and analyzed by intact electrospray ionisation mass spectrometry (ESI-MS) to reveal a mass shift of +146 for both TRAP (Fig. 1b) and CSP (Fig. 1c), indicating the addition of a single L-fucose to the proteins. This mass shift was not observed in the absence of *P. vivax* POFT2. LC-MS/MS analysis of GluC-digested samples of the O-fucosylated TRAP enabled the localization of this glycosylation to the threonine residue of the CXXT C motif (Supplementary Fig. 6). This confirmed that *Plasmodium* parasites possess a conserved syntenic POFT2 capable of O-fucosylating TSR domains on the canonical serine/threonine fucosylation sequon. 

We proceeded to inspect the sequence of every *P. falciparum* protein with a TSR domain for this O-fucosylation sequon (Fig. 1d) to and found that, in addition to CSP and TRAP, potential POFT2 substrates include: circumsporozoite- and TRAP-related protein (CTRP), expressed in oocytes in *E. coli* as well as TRAP-like protein (TLP) and thrombospondin-related sporozoite protein (TRSP) and thrombospondin-related protein 1 (TRP1) from the sporozoite stages. No blood stage proteins with a TSR domain possessed the consensus site, suggesting that O-fucosylation was not important to this stage of the parasite’s life cycle.

**Plasmodium** POFT2 localizes to the ER. To identify the subcellular location of POFT2 in *P. falciparum*, transgenic NF54 parasites were produced in which the POFT2 gene encoded triple hemagglutinin (HA) epitopes in-frame at the C-terminus such that expression was still driven by the
endogenous promoter (Supplementary Fig. 7A). Integration of the HA epitope cassette was validated by Southern blot (Supplementary Fig. 7B) and POFUT2-HA expression confirmed by immunoblot using anti-HA antibodies. POFUT2-HA migrated as a single species of circa 60 kDa in asexual parasites, consistent with an ER localization in P. falciparum (Supplementary Fig. 7D). The punctate distribution pattern within the ER suggests that POFUT2-HA localizes within sub-domains of the ER, the presence of which has been described previously. Detection of POFUT2 expression in asexual parasites is consistent with reports that GDP-fucose is biosynthesized in blood stage P. falciparum parasites, though it does not appear to be essential. It is unclear what, if any, protein(s) might be O-fucosylated by POFUT2 in the blood stage (Fig. 1d).

**Generation of POFUT2-deficient P. falciparum.** To study the function of POFUT2 in P. falciparum, isogenic NF54 parasites were generated in which the POFUT2 locus was excised by double cross over homologous recombination (Supplementary Fig. 8A). Two independent clones of ΔPOFUT2 parasites (D3 and G8) were generated by limiting dilution and validated by Southern blot analysis (Supplementary Fig. 8B). Both mutant clones developed within erythrocytes at the same rate as NF54 parasites, indicating that POFUT2 is not essential for asexual blood stage growth (Supplementary Fig. 8C), in agreement with GDP-fucose being dispensable and the absence of predicted substrates in this stage (Fig. 1d). The ΔPOFUT2 parasites were differentiated into gametocytes and no significant difference in stage V gametocytes (Fig. 1d) were observed compared to NF54, demonstrating that POFUT2 is not essential for gametocytogenesis (Supplementary Fig. 8D).

**POFUT2 facilitates P. falciparum infection of the mosquito.** To examine the function of POFUT2 in other P. falciparum lifecycle stages, mature gametocytes were fed to female Anopheles stephensi mosquitoes by membrane feeding. Parasite load and differentiation within the mosquito was determined by real-time quantitative reverse-transcription PCR (qRT-PCR) of infected midguts 27 h post-bloodmeal. Quantification of Pf18S transcripts revealed that total parasite load in the mosquitoes did not differ between NF54 and ΔPOFUT2 parasites (Fig. 2a). Pf25 transcripts, which are produced by gametes, zygotes and ookinetes and CTRP transcripts, which are expressed in ookinetes, were also statistically equal between parasite strains. This implies that POFUT2 is not essential for formation of ookinetes within the mosquito. However, the number of oocysts developing at the basal lamina of mosquito midguts was reduced for both ΔPOFUT2 clones relative to NF54 (range 63–87% reduction; P < 0.0001 using the Kruskal–Wallis one-way analysis of variance.
ANOVA)) (Fig. 2b). This indicates that POFUT2 is required for normal infection of the mosquito vector by *P. falciparum* ookinetes. The sole predicted TSR protein expressed in ookinetes is CTRP (Fig. 1d), which is essential for ookinete motility and invasion of the midgut. Our results are, therefore, consistent with perturbed glycosylation and function of CTRP, resulting in defective midgut invasion. *P. falciparum* ookinetes could not be successfully cultured in vitro to confirm this by proteomic analysis.

**POFUT2 supports *P. falciparum* sporozoite infectivity and fitness.** To investigate the function of POFUT2 in sporozoites, parasites were propagated through mosquitoes and dissected from salivary glands. Mosquitoes infected with ΔPOFUT2 parasites harboured 45–55% fewer sporozoites in their salivary glands, depending on the clone, than mosquitoes infected with the NF54 parental line (P = 0.0160 using the Kruskal–Wallis one-way ANOVA; Fig. 2c). Since ΔPOFUT2 parasites produce fewer oocysts, this result was expected. When standardizing for oocysts, the number of salivary gland sporozoites was not different (Fig. 2d), suggesting that POFUT2 function may not be critical for *P. falciparum* maturation in oocysts or salivary gland invasion. Next, we assessed whether sporozoites in the salivary glands were infectious. Cell traversal activity is required for liver infection and was measured by incubation of sporozoites with human HC-04 hepatocytes in the presence of FITC-dextran and quantifying dextran-positive cells. ΔPOFUT2 parasites were reduced for cell traversal by 30–42%, depending on the mutant clone (P < 0.0001 using the Kruskal–Wallis one-way ANOVA) (Fig. 3a). The ability for sporozoites to invade hepatocytes, which is critical for liver infection, was investigated by quantifying the

Fig. 4 POFUT2 plays a role in TSR protein trafficking in *P. falciparum*. a Immunofluorescence microscopy of NF54 and ΔPOFUT2 salivary gland sporozoites showing the localization of CSP (red), TRAP (green), and dsDNA (blue). Purple arrow, TRAP at the sporozoite membrane; white arrow, TRAP internal to parasite. Scale 5 μm. b Total sporozoite pixel intensity for PITRAP and PICSP. c Pixel intensity for PITRAP and PICSP at the sporozoite membrane only. Data in b, c is the mean ± S.E.M. from two independent experiments. In panel b, a subtle increase in total PICSP pixels was observed in ΔPOFUT2 D3 relative to NF54 (P = 0.0360) but no difference was observed at the sporozoite membrane (P = 0.1083) in panel c. P-values are for one mutant clone compared to NF54, calculated using the Mann–Whitney test.
the number of parasites inside HC-04 hepatocytes 24 h post-addition of sporozoites to cells. This revealed a strong defect in the number of intracellular ΔPOFUT2 parasites ($P = 0.0145$ using the Kruskal–Wallis one-way ANOVA) (Fig. 3b), consistent with a defect in invasion into the cells. To examine the effect of POFUT2 activity on parasite fitness in vivo, coinfection experiments were performed in which an equal inoculum of NF54 and mutant sporozoites was mixed and injected intravenously into humanized chimeric liver mice. Loss of POFUT2 function resulted in a severe fitness cost, as demonstrated by an approximate 80% reduction in parasite liver load compared to NF54 parents ($P = 0.0155$ using the paired $t$-test; Fig. 3c). Therefore, POFUT2 activity is important for liver infection by P. falciparum.

Gliding locomotion is obligatory for infectivity of sporozoites. We, therefore, assessed whether POFUT2 plays a role in gliding motility by measuring sporozoite trails on a solid substrate. Loss of POFUT2 function caused a reduction in gliding motility, reflected by an increase in non-motile sporozoites (Fig. 3d) and concomitant decrease in the number of trail circles produced by ΔPOFUT2 sporozoites that could glide (Fig. 3e) ($P < 0.0001$ using the Kruskal–Wallis test) and TRAP was commonly observed inside parasites, indicating that trafficking to the sporozoite membrane was impaired by loss of POFUT2 function ($P < 0.0001$ using the Mann–Whitney test). Furthermore, the intensity of TRAP pixels at the sporozoite membrane was also significantly reduced in ΔPOFUT2 parasites ($P < 0.0001$ using the Mann–Whitney test) and TRAP was commonly observed inside parasites, indicating that trafficking to the sporozoite membrane was impaired by loss of POFUT2 function ($P < 0.0001$ using the Mann–Whitney test). Analysis of protein expression levels by immunoblotting indicated that levels of TRAP

POFUT2 assists stabilization and trafficking of TSR proteins. Given the important function of POFUT2 in protein quality control in metazoans, we examined whether proteins were destabilized or trafficked differently following loss of POFUT2 activity. Since the two TSR proteins reported to be O-fucosylated in sporozoites are CSP and TRAP, we investigated these two proteins by immunofluorescence microscopy using antibodies directed to these proteins (Fig. 4a). While we observed no decrease in CSP pixel intensity between NF54 and ΔPOFUT2 sporozoites, the total TRAP pixel intensity was dramatically reduced in POFUT2-deficient sporozoites ($P < 0.0001$ using the Mann–Whitney test) (Fig. 4b). Furthermore, the intensity of TRAP pixels at the sporozoite membrane was also significantly reduced in ΔPOFUT2 parasites ($P < 0.0001$ using the Mann–Whitney test) and TRAP was commonly observed inside parasites, indicating that trafficking to the sporozoite membrane was impaired by loss of POFUT2 function ($P < 0.0001$ using the Mann–Whitney test) (Fig. 4c).

Fig. 5 POFUT2 stabilizes TSR proteins in P. falciparum. a Western blot analysis of 30,000 salivary gland sporozoites per lane using antibodies to PITRAP and PICSP. Anti-PiPLP1 was used as a loading control. The same blot was probed consecutively with each antibody. b Densitometry of PITRAP and PICSP levels in sporozoites measured by immunoblotting and standardized to the PiPLP1 loading control. Data are mean ± S.E.M. and pooled from three independent immunoblots. c Abundance of PITRAP and PICSP mRNA transcripts in salivary gland sporozoites relative to Pi18S, measured by qRT-PCR. No differences were observed for either ΔPOFUT2 clone compared to NF54 for PITRAP ($P = 0.5003$) and PICSP ($P = 0.3104$) mRNA. Data is the mean ± S.E.M. of four independent experiments. P-values are for both mutant clones compared to NF54, calculated using the Kruskal–Wallis one-way ANOVA.
(P = 0.0250) but not CSP (P = 0.3571) (P-values were determined using the Kruskal–Wallis one-way ANOVA) were reduced in ΔPOFUT2 sporozoites (Fig. 5a, b). To confirm that the difference was due to protein destabilization rather than decreased gene expression, qRT-PCR was performed on sporozoites. This demonstrated no significant difference in the relative abundance of PfTRAP or PfCSP messenger RNA (mRNA) transcripts between NF54 and ΔPOFUT2 sporozoites (Fig. 5c). Therefore, POFUT2 activity is required for the stabilization and trafficking of some TSR proteins in *P. falciparum*, although TRAP is more dependent than CSP on O-fucosylation for stabilization and trafficking (Fig. 5). This may be because the TSR domain of CSP possesses just two disulfides, while that of the TRAP domain contains three and is likely more susceptible to misfolding and subsequent degradation due to erroneous disulﬁde bond formation.

Discussion

The recent discovery of O-glycosylation on CSP and TRAP in *P. falciparum* sporozoites represents a very important advance in our understanding of *Plasmodium* glycobiology and complements recent work on the biosynthesis of sugar nucleotides in *P. falciparum*.

It is not clear why POFUT2 and GDP-fucose are produced in the blood stages of *P. falciparum*. While MTRAP, SPATR, and PTRAMP are expressed in asexual stages and contain TSRs, they lack the critical CXX(S/T)C O-fucosylation sequon suggesting they are not fucosylated. Nonetheless, our study indicates that POFUT2 is not essential for parasite growth in the asexual or sexual blood stages. This is supported by recent studies showing that disruption of genes involved in GDP-fucose biosynthesis, GDP-mannose 4.6-dehydratase and GDP-L-fucose synthase, had no impact on asexual or sexual development and are also expressed in mosquito stages.

Conversely, O-fucosylation is important in parasite stages that develop within the mosquito. Our analyses of infected mosquitoes suggest that O-fucosylation is not essential for *P. falciparum* differentiation into ookinete but the formation of fewer oocysts implies it is important for oocyst infection of mosquito midgut epithelial cells. An obvious role for POFUT2 in ookinete is the O-fucosylation of five of the seven TSR domains of the essential motility-associated adhesin CTRP (Fig. 1d). Given that POFUT2 proteins play a key role in protein quality control and trafficking in metazoans, it is tempting to speculate that CTRP is reliant on POFUT2 for stabilization and trafficking. A dearth of appropriate antibodies and methods for the production of large quantities of *P. falciparum* ookinete has prevented us from experimentally confirming this hypothesis, which might be better examined in a *P. berghei* system using in vitro ookinete culture.

POFUT2-deficient parasites produced oocysts, albeit less than wild type, and this provided the opportunity to study sporozoites. POFUT2 mutants produced fewer salivary gland sporozoites within the mosquito than their NF54 parents, as expected based on the formation of fewer oocysts. When the number of salivary gland sporozoites was standardized for oocysts, no defect was observed suggesting the levels of CSP, TRP1, and TRAP remaining in ΔPOFUT2 sporozoites was sufficient to allow their important functions in sporulation, oocyst egress and salivary gland invasion.

Indeed, our analysis of protein levels in sporozoites showed that TRP proteins are impacted differently by the loss of POFUT2 function, which is reminiscent of observations made in metazoans, where some proteins are impacted far more than others. We could not demonstrate statistically significantly destabilization of CSP but TRAP was substantially destabilized by loss of POFUT2 activity, though not fully degraded. Given the essential roles of CSP in so many aspects of sporozoite biology, it is plausible that its TSR domain (with only two disulphide bonds) has evolved to be more stable than the TRAP TSR domain (with its three disulphide bonds) and classical TSR topology.

Our study has also shown that POFUT2 function extends to sporozoite interactions with human hepatocytes both in vitro and in humanized mice with chimeric livers and is therefore implicated in *P. falciparum* transmission to humans. ΔPOFUT2 sporozoites were defective for cell traversal activity, invasion of human hepatocytes and for fitness of sporozoites in vivo. These phenotypes can be attributed to functional destabilization of TRAP and CSP but may also include other TSR proteins that mediate sporozoite motility and invasion of hepatocytes, such as TLP and TRSP, respectively. Our observations of reduced gliding motility of POFUT2-deficient sporozoites strongly support this hypothesis. Further study is needed to verify that these other TSR proteins are O-glycosylated and reliant on POFUT2 for correct folding and trafficking.

The discovery of O-glycosylation in *Plasmodium* parasites is an important advance in our understanding of parasite biology and the significant defects we describe illustrate the relevance of this modification to propagation of the parasite through its lifecycle. Loss of POFUT2 function abrogated protein stabilization and impaired protein trafficking, impacting on both the transmission of ookinete to mosquitoes and the infectivity of sporozoites. This demonstrates that the ER protein glycosylation process in malaria parasites confers a significant survival advantage and is important for malaria transmission. It appears unlikely that O-fucosylation is dispensable to parasites, and so incorporation of these glycans into vaccines based on CSP, TRAP, CTRP, and possibly other TSR proteins are well worth investigating. The production of such antigens should be relatively straightforward, since mammalian glycosyltransferases can recognize and modify heterologously expressed *Plasmodium* TSR domains.

Methods

POFUT2 enzyme assay. Details of recombinant protein expression are provided in the Supplementary Methods. Reactions (10 μl total volume) containing recombinant *P. falciparum* TRAP TSR (10 μM) recombinant *P. vivax* POFUT2 (10 ng), GDP-Fuc (50 μM), MgCl2 (5 mM) in buffer (20 mM Tris, 150 mM NaCl, pH 7.4) were incubated for 16 h at 37°C. Reactions included reactions without *P. vivax* POFUT2. Samples were snap-frozen and stored at -80°C until analysis by mass spectrometry, as detailed in the Supplementary Methods.

Parasite maintenance. *P. falciparum* NF54 asexual stages were maintained in human type O-positive erythrocytes (Melbourne Red Cross) in RPMI-HEPES supplemented with 10% heat-inactivated human serum (Melbourne Red Cross) at 37°C. Gametocytes for transmission to mosquitoes were generated using the “crash” method using daily media changes.

Transgenic parasites. *P. falciparum* NF54 (kindly provided by the Walter Reed Army Institute of Research) was used to generate all transgenic parasites. Details for cloning, transfection, selection, and validation of transgenic lines are provided in the Supplementary Methods.

Blood stage growth assay. Synchronized trophozoite stage parasites were added to erythrocytes to 0.2% parasitemia, 1% hematocrit. Starting parasitemia was confirmed by flow cytometry (FACSCalibur; BD) using ethidium bromide staining (1:1000 dilution in phosphate buffered saline (PBS)) and final parasitemia was

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determined 96 h later. For each line, triplicate samples of 50,000 cells were counted in each of three independent experiments. Growth was expressed as a percentage of NF54.

Mosquito infection and analysis of parasite development. Five- to seven-day old female Anopheles stephensi mosquitoes (this strain originally provided by M. Jacobs-Lorena, Johns Hopkins University) were fed on asynchronous gametocytes, diluted to 0.3–0.6 % stage V gametocytes, via water-jacketed glass membrane feeders. Mosquitoes were provided with sugar for 2 days post-bloodmeal followed by a sugar starved for 2 days post-bloodmeal for the population for blood-fed mosquitoes. Surviving mosquitoes were provided 5% glucose ad libitum via paper/cotton wicks or water wicks and sugar cubes. Oocyst numbers were obtained from midguts dissected from cold-anesthetized and ethanol-killed mosquitoes 7 days post-bloodmeal and stained with 0.1% mercuriochrome. Salivary glands were dissected from mosquitoes (day 16–20 post-bloodmeal) and proteinase K was used to obtain sporozoites used in subsequent assays. Mosquito bloodmeal bolus were isolated 27 h post-feed from mosquitoes to check for the presence of ookinetes by qRT-PCR (see Supplementary Table 1). Briefly, mosquitoes were cold anesthetized and ethanol killed. Midguts were carefully dissected and frozen immediately on dry ice. RNA was purified using TRI Reagent (Sigma) and complementary DNA (cDNA) prepared using a SensiFast cDNA synthesis kit (Bioline) according to the manufacturers’ instructions and qRT-PCR performed using a LightCycler 480 (Roche). All oligonucleotides used in this study are listed in Supplementary Table 1.

Immunofluorescence microscopy. Asexual stages were fixed in ice-cold methanol and probed with rat anti-HA (1:500; Roche 3F10) and rabbit anti-plasmodxin V (PMV) (1:1000) antibodies in 3% bovine serum albumin (BSA)/PBS. Salivary gland sporozoites were air-dried on slides, fixed in 4% paraformaldehyde and permeabilized in 0.1% triton X-100. Primary antibodies (mouse monoclonal anti-CSP (2A10); 1:2000) (rabbit anti-TRAP 1:500) were diluted in 3% BSA/PBS. Secondary antibodies were goat anti-rabbit 594 and anti-mouse or rat Alexa 488 (1:1000; Invitrogen). Micrographs were acquired on a DeltaVision Elite microscope (Applied Precision) using an Olympus 100×/1.42 PlanApoN objective equipped with a Coolsnap HQ2 CCD camera. A total range of 220 (NF54) to 840 (ΔPOFUT2) sporozoites were counted for each condition across two independent experiments.

Sporozoite expression analysis. Sporozoites were dissected from salivary glands on day 17 or 18 post-blood meal. RNA was purified using TRI Reagent (Sigma) and complementary DNA (cDNA) prepared using a SensiFast cDNA synthesis kit (Bioline) according to the manufacturers’ instructions and qRT-PCR performed using a LightCycler 480 (Roche) with oligonucleotides in Supplementary Table 1.

Immunoblotting. Proteins were separated through 4–12% Bis-Tris polyacrylamide gels (Invitrogen), transferred to nitrocellulose membrane and probed with primary antibodies: rat anti-HA (1:500; Roche 3F10), mouse monoclonal anti-CSP (2A10) 1:2000, rabbit anti-TRAP 1:500, rabbit anti-PLP1 1:200, rabbit anti-Aldolase (1:4000) followed by horse radish peroxidase-conjugated secondary antibodies (1:1000 (mouse) and 1:4000 (rabbit); Cell Signaling Technology) and chemiluminescence (Amersham). Heptocyte culturing. HC-04 hepatocytes were maintained on Isocove’s Modified Dulbecco’s Medium (IMDM), supplemented with 5% heat-inactivated fetal bovine serum and 1% non-essential amino acids. Cells were split 1:6 every 2–3 days once they reached ~90% confluence.

Cell traversal assay. Cell traversal was measured using a cell-wounding assay. HC-04 hepatocytes (5 × 104) were seeded onto all rat tail collagen-coated coverslips in 24-well plates using Dulbecco’s modified Eagle medium without glucose (Life Technologies, 11960-025), supplemented with 1 mM sodium pyruvate (Life Technologies, 11360-070) and 1 mM non-essential amino acids without l-glutamine (Sigma-Aldrich, M5550); and 1:500 dilution of Lipid Mixture 1, Chemically Defined (Sigma-Aldrich, D–288) 55, 54. Sporozoites (5 × 104) were added to the cells 12 h later and incubated for 24 h. Media was replaced after 3 h and the assay continued on for a further 21 h (to give an invasion assay of 24 h). Coverslips were fixed in 4% paraformaldehyde for 20 min at RT and then processed as described 54. Sporozoites were detected by immunofluorescence staining using mouse monoclonal antibodies against CSP (1:2000), anti-mouse Alexa–488 (1:1000), and anti-mouse Alexa 594 (1:1000). Multiple images were taken at 20× magnification (Axio observer). A minimum of 200 × 200 μm fields with approximately 10,000 HC-04 cells were counted and the percentage of cells with intracellular sporozoites was calculated from this data set. For each condition, duplicate samples were manually counted in each of two independent experiments.

Sporozoite gliding assay. Gliding assays were performed as described previously with some minor exceptions. Eight-well chambers (Nunc, Fisher Scientific 154334) were coated with CSP antibodies (1:1000 in PBS). Twenty thousand salivary gland sporozoites were seeded into each well and allowed to glide for 60 min at 37 °C in 5% CO2 in IMDM supplemented with 10% heat-inactivated human serum. Samples were fixed with 4% paraformaldehyde at 37 °C for 20 min. Primary anti-PCPS was applied followed by goat anti-mouse Alexa 488 at (both antibodies were 1:1000 in 3% BSA). Sporozoites and trails were viewed on a DeltaVision Elite microscope (Applied Precision) using an Olympus 163x/1.42 PlanApo objective equipped with a Coolsnap HQ2 CCD camera. Humanized mice production, infection, and processing. pA A inserts –SCID mice (University of Alberta) were housed in a virus- and antigen-free facility supported by the Health Sciences Laboratory Animal Services at the University of Alberta and cared for in accordance with the Canadian Council on Animal Care guidelines. All protocols involving mice were reviewed and approved by the University of Alberta Health Sciences Laboratory Animal Care Committee and the Walter and Eliza Hall Institute for Medical Research Animal Ethics Committee. pA A inserts –SCID mice at 5–14 days old (2 male, 1 female) received 106 human hepatocytes (cryopreserved human hepatocytes were obtained from BioreclamationIVT—Baltimore MD) by intrasplenic injection and engraftment was confirmed 8 weeks post- transplantation by analysis of serum human albumin. An inoculum of 4 × 107 P. falciparum NF54 sporozoites and 4.0 × 105 ΔPOFUT2 sporozoites freshly isolated from mosquito salivary glands were mixed and injected by intravenous tail injection into each of three humanized mice, as previously described. Livers were obtained 6 days post-infection from CO2-ethanized mice and individual lobes were cut as described, pooled and emulsified into a single cell suspension and flash frozen in liquid nitrogen for subsequent genomic DNA (gDNA) extraction.

Measuring exoerythrocytic development in humanized mice. To quantify parasite load in the chimeric livers, gDNA was isolated from the single cell liver suspensions and Taqman probe-based qPCRs were performed as previously described 54, 55. To specifically differentiate NF54 from ΔPOFUT2 genomes from the chimeric livers, samples of the test was employed to evaluate fitness of one mutant clone versus NF54 in each of three humanized mice. Analyses were performed using Graphpad Prism 6.

Ethics statement. All experimental protocols involving humanized mice were conducted in strict accordance with the recommendations in the National Statement on Ethical Conduct in Animal Research of the National Health and Medical Research Council and were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee (AEC2014.030). All experiments involving the use of human erythrocytes and the HC-04 human
hepatocyte cell line were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Human Research Ethics Committee (HREC 86/17 and 15/06).

**Data availability** All data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the corresponding authors upon request.

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Author contributions
S.L. generated P. falciparum strains, performed the molecular and cellular analyses with A.S.P.Y. and performed humanized mouse experiments. S.M.E., M.T.O., and C.J. helped produce sporozoites and assisted with humanized mouse experiments, A.J., J.P.L., and E.D.G.-B. cloned, expressed, purified, and characterized POFUT2 and TSR proteins. N.E.S. performed mass spectrometry analyses, N.C.M. assisted in preparing antibodies. J.A.B., M.T.O., and L.W.W. performed immunofluorescence microscopy and pixel analyses. D.N.D. and N.M.K. generated humanized mice. Experiments were designed and interpreted by E.D.G.-B. and J.A.B. All authors contributed to preparing this manuscript.

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