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The vaccine candidate Liver Stage Antigen 3 is exported during *Plasmodium falciparum* infection and required for liver-stage development

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eLife Assessment

This **useful** study provides new insights into the liver-stage antigen LSA3, its export to erythrocytes, and its role in liver-stage development. While the functional importance of LSA3 is well demonstrated, the data underlying the conclusions regarding antibody specificity, liver-stage localization, and phenotype remain **incomplete**. A key strength of the study is the use of mosquito and humanized mouse models to access life-cycle stages that are rarely studied in most laboratories.

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Abstract

Plasmodium falciparum remodels infected erythrocytes using exporting effector proteins. Parasites express the aspartyl protease plasmepsin V that processes proteins containing a PEXEL motif and the PTEX translocon to successfully export proteins. During liver-stage infection, PTEX is required for *P. falciparum* development, but which proteins are exported remain largely unclear, yet they may serve important functions and be presented by MHC-I molecules, thereby representing potential vaccine candidates. Here, we investigated liver stage antigen 3 (LSA3), an immunogenic protein of the Laverania subgenus of *Plasmodium*. We show that LSA3 possesses a PEXEL motif processed by plasmepsin V and is targeted to one or more membranes surrounding the blood-stage parasite, suggestive of the parasitophorous vacuole membrane (PVM). A subset of LSA3 also localizes in the erythrocyte, where it forms punctate structures that are not Maurer's clefts but are soluble in biochemical fractionation assays reminiscent of J-dot proteins. During infection of human hepatocytes, antibodies to LSA3 co-localize with EXP1 and EXP2 at the PVM yet these antibodies were not detected beyond this membrane. Finally, genetic disruption of *LSA3* in *P. falciparum* NF54 attenuated fitness at the liver stage, manifesting as a 40% reduction in parasite liver load by day 5 postinfection of humanized mice. The identification of LSA3 as a member of the *P. falciparum* exportome and important for liver-stage development confirms the hypothesized potential of exported proteins as promising vaccine candidates, underscoring the need for their continued discovery and biological characterization, including those expressed at the liver stage.

Introduction

Plasmodium species are protozoan protists responsible for more than 282 million cases of malaria and 610,000 deaths each year (WHO, 2025). While major progress has been made in reducing the incidence of malaria over the last decade, these advances are threatened by development of resistance to front-line antimalarials. The development of next generation therapies and vaccines require a better understanding of how these parasites survive and proliferate in the vertebrate

and mosquito hosts (Kappe *et al*, 2010). This includes mechanisms for inhabiting different host cell types, evasion of immune responses and their ability to acquire nutrients to sustain the extraordinary growth rates in the liver for onward transmission (Vaughan & Kappe, 2017). The liver-stage is currently the target of the only approved vaccines, though they offer modest efficacy (Clinical Trials Partnership, 2015; Datoo *et al*, 2021), highlighting the need for continued research.

Infections are initiated by *Plasmodium* sporozoites injected into the skin by an infected mosquito, which migrate to the liver and develop asymptomatically in hepatocytes for ~7-10 days (Frischknecht & Matuschewski, 2017). Liver merozoites subsequently egress the liver and initiate cycles of infection in red blood cells (RBC) that are responsible for clinical malaria. Both the liver and RBC stages of *Plasmodium* replicate within a unique parasitophorous vacuole membrane (PVM) and have evolved mechanisms to remodel this membrane and their host cells (Kappe *et al*., 2010). During the blood stage, this is achieved by the export of proteins to or across the PVM into the infected erythrocyte. For many exported proteins, their targeting is dependent on the *Plasmodium* export element (PEXEL) / host targeting (HT) motif (RxLxE/Q/D) (Hiller *et al*, 2004; Marti *et al*, 2004) that is recognized by the endoplasmic reticulum (ER)-resident aspartyl protease plasmepsin V (Boddey *et al*, 2010; Russo *et al*, 2010). This protease processes the PEXEL between the third and fourth residues (Boddey *et al*, 2009; Chang *et al*, 2008), licensing the matured and then N-acetylated proteins for export. A subset of PEXEL-negative proteins (PNEPs) is also exported into RBCs (Heiber *et al*, 2013; Spielmann *et al*, 2006). Export across the PVM is mediated by the *Plasmodium* translocon of exported proteins (PTEX) (Beck *et al*, 2014; de Koning-Ward *et al*, 2009; Elsworth *et al*, 2014; Ho *et al*, 2018). PEXEL cleavage by plasmepsin V can also target proteins to alternate locations such as the parasitophorous vacuole and rhoptries (Fierro *et al*, 2024; Freville *et al*, 2024).

The nucleated hepatocyte presents a vastly different cellular environment, including a complex secretory network and cell intrinsic immune programs including interferons, guanylate binding proteins (GBPs), lysosomes and autophagic proteins that can target the intracellular parasite niche; hepatocytes also use MHC presentation pathways for recruiting adaptive immune responses that can ultimately attack and clear the parasite (Cockburn *et al*, 2011; Liehl *et al*, 2014; Marques-da-Silva *et al*, 2022; Marques-da-Silva *et al*, 2025; Prado *et al*, 2015; Weiss, 1990). Whether or to what extent parasite liver-stages translocate their own proteins into or beyond the PVM to diminish hepatocyte defences remain largely unanswered. However, several lines of evidence suggest that a protein export pathway involving components characterized in blood-stages is expressed and is functionally important in liver-stage parasites, but with some important differences.

Firstly, the PTEX150 and EXP2 components of the PTEX translocon are expressed at the parasite periphery of, and are important for, *P. falciparum* growth in hepatocytes, suggesting export of proteins may occur (McConville *et al*, 2024). EXP2 serves dual roles in the PVM of RBCs, both as a protein-conducting gateway for export and as a nutrient-transporter for parasite growth (Garten *et al*, 2018); its importance during hepatocyte infection may be, by analogy, for either function. The PTEX unfoldase HSP101 is expressed in *P. falciparum* liver-stages (Zanghi *et al*, 2025) but its importance remains unknown. HSP101 is apparently absent from the liver-stage translocon in the rodent malaria parasite *P. berghei* (Kreutzfeld *et al*, 2021; Matz *et al*, 2015).

Secondly, multiple PEXEL and PNEP proteins are expressed by *P. falciparum* liver-stages (Zanghi *et al*., 2025), and in *P. berghei* LISP2 is efficiently exported beyond the PVM into the infected hepatocyte cytoplasm and nucleus (Orito *et al*, 2013); interestingly, targeting of LISP2 was limited to the PVM of *P. falciparum*-infected hepatocytes (McConville et al., 2024) yet this protein is important for late liver-stage development of this species (Zanghi *et al*., 2025). Proteomics analysis of *P. berghei* merosomes also revealed the presence of peptides arising from PEXEL cleavage by plasmepsin V including LISP2, suggesting this protease is functional in liver-stages. Finally, while only few blood-stage PEXEL proteins are trafficked to the parasitophorous vacuole (PV), the *P. falciparum* PEXEL proteins LISP2, circumsporozoite protein (CSP) and LSA3 are all trafficked to the periphery of the liver-stage parasite during hepatocyte infection (Daubersies *et al*,

2000; McConville *et al*., 2024; Zanghi *et al*., 2025) and genetic disruption of PTEX subunits reduced expression of LISP2 and CSP, suggesting a direct or indirect link between the translocon and protein trafficking and/or quality control (McConville *et al*., 2024).

Only the Laverania subgenus of *Plasmodium* possess the *LSA3* gene; in the most virulent human parasite, *P. falciparum*, LSA3 is expressed during infection of hepatocytes (Daubersies *et al*., 2000) and RBCs (Morita *et al*, 2017). LSA3 contains an N-terminal putative PEXEL motif (Maier *et al*, 2008; McConville *et al*., 2024; Morita *et al*., 2017), suggesting it may be an exported protein, yet whether the PEXEL is functional has not been definitively proven. LSA3 also possesses an N-terminal signal anchor (that would be removed following plasmepsin V cleavage of the PEXEL motif) and a putative C-terminal hydrophobic region. LSA3 is a protective antigen in immunization studies; however, despite its immunogenicity, the amino acid sequence is highly conserved across diverse *P. falciparum* geographical isolates. This is suggestive of functional importance and strong evolutionary pressure against sequence variation (Perlaza *et al*, 2001; Perlaza *et al*, 2008), although, functional characterization of *LSA3* deletion mutants have not been reported at the mosquito or liver stages of the lifecycle and genetic disruption at the blood-stage did not identify an essential phenotype (Maier *et al*., 2008). Research efforts have instead largely focused on the immunogenicity of LSA3, with its initial discovery as a pre-erythrocytic antigen involved in protection against future liver-stage infection (Daubersies *et al*., 2000).

More recently, LSA3 was identified as a novel blood-stage antigen following a screen of a wheat germ expression library of *P. falciparum* proteins with human sera from individuals living in endemic areas, with the human antibodies that bound to LSA3 reducing erythrocyte invasion by ~24% (Morita *et al*., 2017). In schizonts, LSA3 localizes to dense granules (DGs) (Morita *et al*, 2025; Morita *et al*., 2017) yet it is not known where it localizes earlier during erythrocytic infection. Recently, we genetically disrupted of *LSA3* in *P. falciparum* NF54 and characterization of this mutant showed it supports a role during completion of erythrocyte invasion (Morita *et al*., 2025).

LSA3 was historically characterised as a promising vaccine candidate due its protective effect against *P. falciparum* sporozoite challenge in both chimpanzee and Aotus monkey models (Daubersies *et al*., 2000; Perlaza *et al*., 2008). Immunofluorescence assays (IFA) of *P. falciparum* sporozoites using affinity-purified LSA3 human antibodies from protected volunteers given radiation-attenuated sporozoites (RAS) detected labelling at the sporozoite surface (Daubersies *et al*., 2000). IFA of *P. falciparum* 5-day old liver parasites in monkeys using antibodies induced by LSA3 lipopeptide injection of a chimpanzee detected labelling at the periphery of exoerythrocytic parasites, altogether suggesting LSA3 localizes at the PVM (Daubersies *et al*., 2000). However, confirming this location is challenging and requires specific PVM markers that have not been used to date, nor have LSA3-deficient parasites been characterized at the liver stage.

Here, we have undertaken a biochemical and functional study of LSA3 in *P. falciparum* across the blood, mosquito and liver stages. Using LSA3-C and LSA3-T antibodies (Morita *et al*., 2025; Morita *et al*., 2017) epitope tagging, and inhibitors, we demonstrate that LSA3 has a *bona fide* PEXEL sequence that is proteolytically processed by plasmepsin V and that LSA3 is targeted both to the PVM and into the infected erythrocyte cytoplasm where it forms punctate structures and is therefore confirmed to be a member of the malarial exportome (Sargeant *et al*, 2006). In humanized mice, we showed that antibodies to LSA3 co-localized with the PVM markers EXP1 and EXP2. However, we did not detect LSA3 beyond the PVM of infected hepatocytes, suggesting LSA3 is secreted to the PVM or may be considered exported if a domain is exposed to the hepatocyte lumen from this membrane. Genetic disruption of *LSA3* in *P. falciparum* NF54 did not prevent gametocytogenesis, transmission to or development within the mosquito; however, LSA3-deficiency caused a significant reduction of parasite fitness by day 5 of the liver stage in humanized mice, demonstrating that LSA3 is important for *P. falciparum* liver stages.

Results

LSA3 is exported to the *P. falciparum*-infected erythrocyte

LSA3, encoded by PF3D7_0220000, is conserved in all primate-infective *Plasmodium* species and contains an N-terminal hydrophobic signal anchor likely for ER entry, a PEXEL motif followed by disordered or flexible repeat regions and a predicted C-terminal hydrophobic region. AlphaFold predicts a domain (residues 908-1115) with some similarity to the substrate binding domain of the chaperone, DnaK (Daubersies *et al*., 2000; Jumper *et al*, 2021; Morita *et al*., 2017; Prieur & Druilhe, 2009) (Figure 1A). The presence of an N-terminal hydrophobic sequence and PEXEL suggested LSA3 may be a substrate for the ER-resident aspartyl protease, plasmepsin V, raising the possibility it is exported (Marti *et al*., 2004; McConville *et al*., 2024). Despite initially being discovered as a liver-stage protein (Daubersies *et al*., 2000), LSA3 is also expressed in blood stage merozoites where it localises to DGs and is required for completion of RBC invasion, but its localization in ring and trophozoite stages has not yet been characterized (Maier *et al*., 2008; Morita *et al*., 2025; Morita *et al*., 2017).

Given the possibility of being exported and the strong immunogenicity of LSA3, we functionally characterised it by generating transgenic *P. falciparum* NF54 Δ*LSA3* clones, in which the *LSA3* gene was disrupted by allelic exchange (Figure 1B). Integration of the Δ*LSA3* knockout plasmid was confirmed in independent clones by polymerase chain reaction (PCR) and clones D8 and E2 were selected for further analysis (Figure S1). Loss of LSA3 protein expression was confirmed by immunoblot with antibodies recognizing the LSA3 C-terminus (LSA3-C) for both knockout clones D8 and E2 (Figure 1C); a non-specific band at ~75 kDa was present, which was of parasite origin as it was absent in uninfected RBCs. While LSA3 has a predicted molecular weight of 175 kDa, it migrates at a larger size in SDS-PAGE (Morita *et al*., 2017). Validation that *LSA3* was successfully disrupted in NF54 was also provided using the recently generated LSA3-T antibody, which is not cross-reactive, in an accompanying manuscript (Morita *et al*., 2025).

To further validate loss of LSA3 expression, IFA of RBCs infected with either NF54 parental or Δ*LSA3* E2 parasites was performed using LSA3-C (Figure 1D). LSA3-C localized both inside the parasite and beyond the EXP2-defined PVM in punctate structures indicating it was an exported protein (Figure 1D). By contrast, in all erythrocytes infected with Δ*LSA3* parasites, the exported signal was absent, confirming LSA3 is exported and the specificity of LSA3-C, but internal puncta remained detectable, likely representing the cross-reactive species observed by immunoblot at ~75 kDa (Figure 1C, D). IFA with the recently generated LSA3-T antibody (Morita *et al*., 2025) further confirmed that LSA3 was exported into the erythrocyte in trophozoites (Figure 1D).

Interestingly, while antibodies that react with LSA3 were shown to reduce merozoite invasion of erythrocytes by ~24% in growth inhibitory assays (Morita *et al*., 2017), we were able to generate deletion mutant clones in NF54, similar to previous studies using 3D7 (Maier *et al*., 2008; Oberstaller *et al*, 2025) confirming that LSA3 is not critical here; however its genetic disruption in NF54 did slow the rate of erythrocyte invasion by merozoites and led to the presence of acollé forms, as recently reported (Morita *et al*., 2025). This demonstrates that LSA3 supports the completion of invasion by *P. falciparum* merozoites yet is not critical for blood-stage growth while, in addition to DG localization, LSA3 is expressed in trophozoites where it is exported into the infected erythrocyte and is located within punctate structures.

LSA3 does not localize to Maurer's cleft tethers

P. falciparum exports proteins into distinct punctate structures in the iRBC, including Maurer's clefts that become tethered to the erythrocyte membrane, J-dots (Kulzer *et al*, 2010) and electron-dense vesicles (EDVs) that are mobile structures within the erythrocyte cytosol. IFA with antibodies to LSA3 and membrane associated histidine rich protein 2 (MAHRP2), which localizes at Maurer's cleft tethers (Pachlatko *et al*, 2010) indicated that although antibodies to both LSA3 and

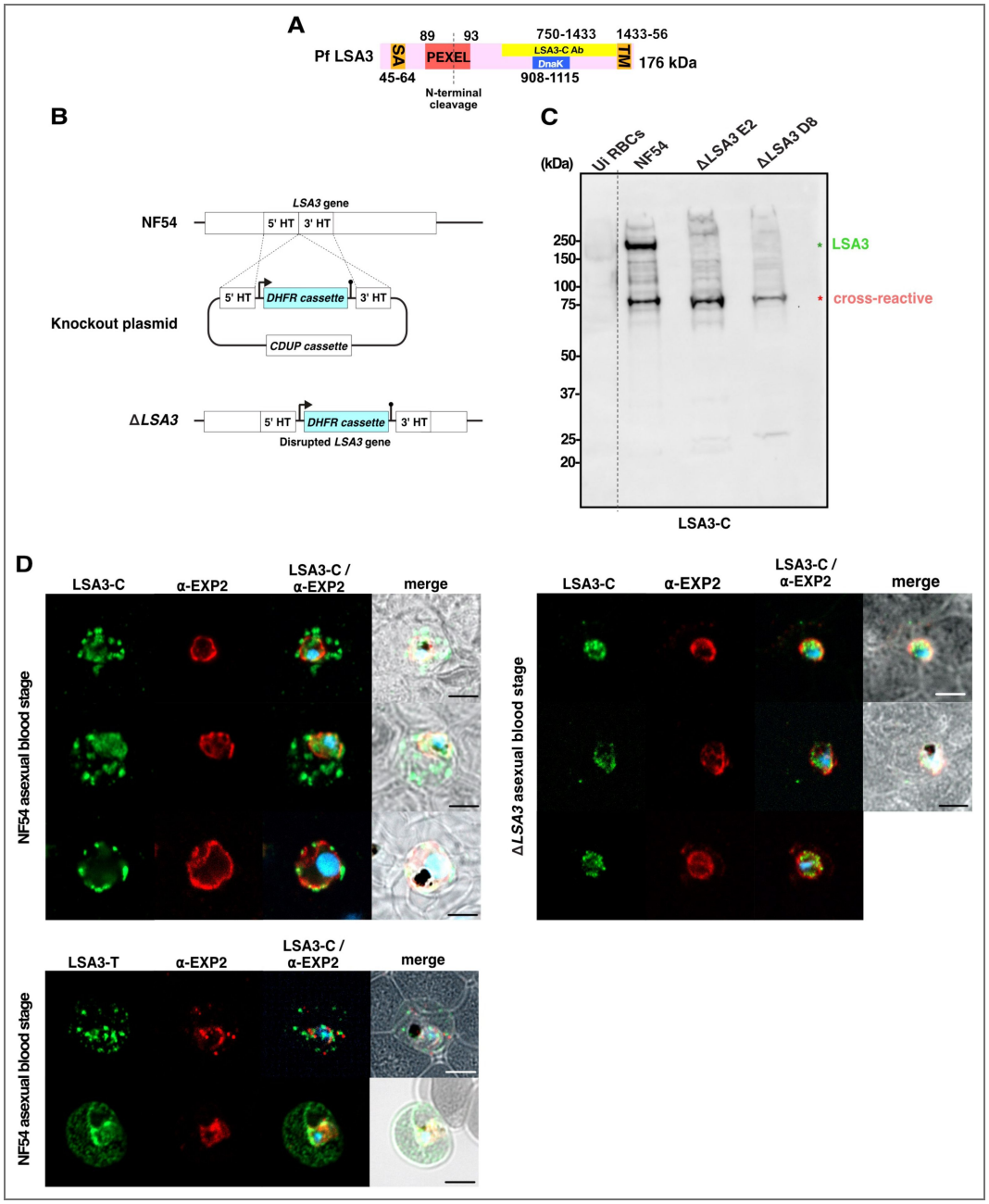

Figure 1. Genetic disruption of *LSA3* in *P. falciparum*.

A. Schematic of the 176 kDa LSA3 protein conserved across human-infective malaria species. Regions between residues 45-64 and 1433-1456 are predicted to be a hydrophobic signal anchor (SA) and transmembrane domain (TM) (orange), respectively; residues 89-93 contain a PEXEL motif (red) and residues 908-1154 have homology to the substrate binding site from the chaperone DnaK (blue). LSA3-C antibody (Ab) binds the C-terminal region (yellow). **B.** Schematic of *LSA3* genetic disruption strategy in *P. falciparum* NF54. The endogenous coding sequence of LSA3 was disrupted by allelic exchange with a selection cassette encoding a 5' promoter-dihydrofolate reductase gene (*DHFR*)-3' terminator flanked by homology targets (HT) internal to the *LSA3* gene, causing disruption of protein expression. Negative selection of the remaining knockout construct was performed with a cytosine deaminase and uracil phosphoribosyltransferase (*CDUP*) cassette by addition of 5-FC to parasite cultures. **C.** Immunoblot of uninfected human red blood cells (Ui RBCs) or parasite blood-stage lysates of NF54 (parental control) and LSA3 KO clones E2 and D8, probed with LSA3-C antibodies. **D.** IFA of NF54 parental parasites following methanol/acetone fixation and staining with LSA3-C, LSA3-T or anti-EXP2 (PVM) antibodies. IFA of Δ*LSA3* parasites using LSA3-C and anti-EXP2 antibodies confirms loss of exported LSA3 in infected erythrocytes and non-specific binding to one or more proteins inside the parasites. Representative images of n=3 experiments. Scale bars, 5 μm.

MAHRP2 exhibit punctate signals in the RBC, no colocalization occurred, suggesting LSA3 does not localize to Maurer's clefts or associated tethers (Figure 2A) but instead is located within alternate structures that were further characterized below.

LSA3 is membrane-associated at the PVM and soluble in the erythrocyte

To further understand the localization of LSA3, IFA with LSA3-C was performed with the exported protein REX3 as a control (Spielmann *et al*., 2006). This revealed distinct patterns of localisation, with LSA3-C forming aggregates and REX3 appearing diffuse throughout the cytoplasm as expected (Gruring *et al*, 2012; Schulze *et al*, 2015) (Figure 2A). Detection of REX3 required fixation with 4% paraformaldehyde (PFA) yet, under these conditions, export of LSA3 was poorly visible with LSA3-C or LSA3-T; instead, these LSA3 antibodies accumulated at the parasite periphery and co-localized with EXP2 at the PVM (Figure 2A). Therefore, the epitopes recognized by LSA3-C and LSA3-T were preserved with methanol:acetone fixation, which is not without precedent (Kulzer *et al*., 2010). As different fixatives were necessary, co-imaging of exported REX3 and LSA3 in the same infected erythrocyte was not possible.

To better understand the localization and solubility profile of LSA3, iRBCs were analyzed biochemically using a pore-forming toxin or detergents and protease digestion followed by Western blotting (Figure 2B). This approach confirmed that a soluble form of LSA3 was exported into the iRBC, as shown by release of protein into the supernatant after incubation with EQT, a pore-forming toxin from the sea anemone *Actinia equina* that selectively permeabilizes the erythrocyte membrane but not the Maurer's clefts or PVM (Jackson *et al*, 2007). Exported LSA3 in the EQT supernatant was sensitive to PK, indicating the antibody-binding domain was exposed to the iRBC cytosol; by contrast, the EQT pellet fraction of LSA3 was mostly resistant to PK, indicating this fraction was not exposed to the RBC cytosol, consistent with a location within the parasite and/or PV/PVM (Figure 2B). To ensure EQT selectively lysed the erythrocyte membrane, the exported protein REX3 was assessed as a control (Spielmann *et al*., 2006). Anti-REX3 antibodies revealed that soluble REX3 was exported (liberated into the EQT supernatant) and sensitive to PK as expected (Figure 2B). The cytoplasmic parasite protein aldolase also remained inaccessible to EQT and PK as expected, confirming integrity of the parasite membrane (Boddey *et al*., 2009). We also confirmed that the antibody-binding domain of LSA3 was not presented on the iRBC surface, as it was resistant to PK when intact iRBC were treated with this enzyme (Figure S2).

Next, the subcellular localization of LSA3 within the parasite and parasitophorous vacuole was biochemically investigated using detergents. Treatment of iRBC with the plant-derived detergent saponin, which selectively permeabilizes membranes of the RBC, Maurer's clefts and PVM, while leaving parasite membranes intact, again released exported LSA3 (the quantity released was similar to EQT alone) into the supernatant however the protein was abundant in the saponin pellet and sensitive to PK, indicating it was membrane-associated with the antibody-binding domain facing the PV lumen (Figure 2B). This indicated that LSA3 was predominantly located at the PVM or plasma membrane of *P. falciparum*-iRBCs. The REX3 control was predominantly in the saponin supernatant and sensitive to PK as expected, consistent with this protein being exported and within the PV prior to translocation into the host cell (Figure 2B). Both Aldolase and the ~75 kDa cross-reactive band recognized by LSA3-C remained inaccessible to saponin and PK, confirming the parasite membrane was intact and corroborating the IFA analysis that this cross-reactive protein was located inside the parasite.

To better understand the membrane association of LSA3, iRBC were incubated with a low concentration of the non-ionic detergent TX-100 (Gabriela *et al*, 2022), which liberated the majority of LSA3 (and REX3 as expected) into the supernatant, with both the pellet and supe fractions now accessible to PK (Figure 2B). This suggests that the majority of membrane-associated LSA3 could be peripherally rather than integrally associated with membranes with the remining protein in the pellet representing a subpopulation more tightly bound to detergent-resistant structures or incomplete solubilization due to limited detergent accessibility, which was

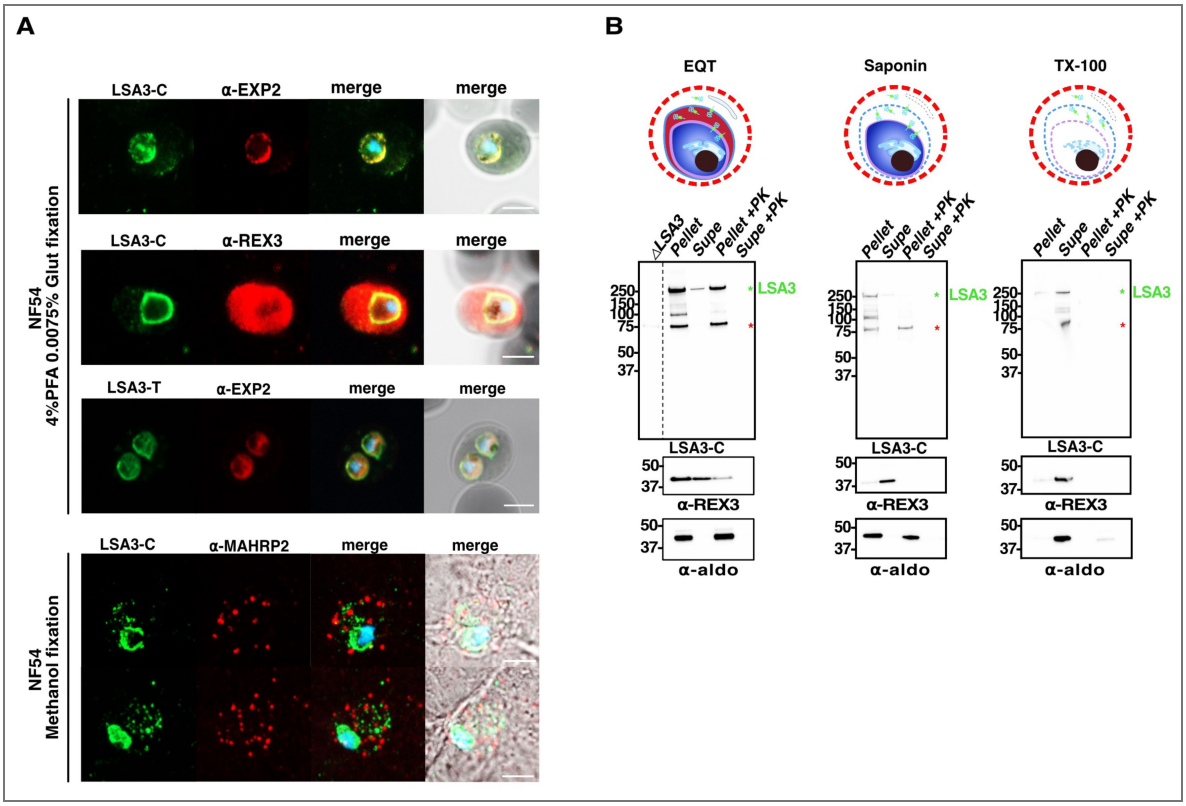

Figure 2. LSA3 is trafficked into the PVM and the erythrocyte during blood-stage development.

A. IFA of NF54 parental parasites following fixation with paraformaldehyde/glutaraldehyde (bottom) and methanol/acetone (top) and staining with anti-LSA3 (LSA3-C or LSA3-T) and antibodies to EXP2 and REX3 (top) or MAHRP2 (Maurer's cleft tethers, bottom). IFA of NF54 parasites following PFA fixation and staining with either anti-LSA3 (LSA3-C), anti-EXP2 or anti-REX3 antibodies (below). Scale bars, 5 μm. **B.** Proteinase K (PK) protection assays to localize LSA3. Erythrocytes infected with NF54 parental parasites were enriched by Percoll centrifugation and permeabilized with either equinatoxin II (EQT; selectively lyses the erythrocyte membrane), 0.03% saponin (selectively lyses the erythrocyte membrane and PVM) or 0.1% Triton-X-100 (lyses all membranes). The pellet was separated from the supernatant (supe) and both fractions were incubated with PBS containing Proteinase K (PK) at 37 °C. Protease inhibitor cocktail and PMSF was added to all samples followed by reducing sample buffer before immunoblotting with LSA3-C, anti-REX3 (exported control) or anti-aldolase (aldo; internal protein control for parasite membrane integrity) antibodies. Representative data is from n=3 experiments.

also observed for aldolase that may be bound as a complex (Figure 2B). Altogether these data suggest that LSA3 localizes peripherally to the luminal leaflet of the PVM, although we cannot exclude binding to the parasite membrane, and in soluble punctate structures in the infected erythrocyte.

The PEXEL of LSA3 is processed by plasmepsin V

The observation that LSA3 is exported during the blood-stage raised the question of molecular mechanism. Previously, we and others identified a PEXEL motif in LSA3 from *P. falciparum* that is conserved in LSA3 from *P. malariae*, *P. ovali curtsi* and *P. reichenowi* (McConville *et al*., 2024; Morita *et al*., 2017; Sargeant *et al*., 2006). To test whether the PEXEL in LSA3 was functional, *LSA3* mini-gene reporters were generated that encompassed the N-terminal 112 residues of endogenous LSA3 from *P. falciparum* with a native PEXEL sequence (RSLGE) or a mutated PEXEL RLE>A (ASAGA) fused to green fluorescent protein (GFP) (Figure 3A). This approach was chosen because native LSA3 is a large protein of 176 kDa, potentially masking minor size shifts from N-terminal processing in immunoblots. The mini genes did not encode the C-terminal sequences recognized by LSA3 antibodies and so anti-GFP antibodies were used. The constructs were transfected into *P. falciparum* 3D7 parasites and integrated into the *p230* locus by double cross-over homologous recombination to ensure that expression of other genes required for asexual parasite growth were not disrupted and expression of the reporters was driven by the *BIP* gene promoter (Figure 3A) and positive clones selected via Blasticidin-S-deaminase (Ashdown *et al*, 2020).

Transfected parasites were validated by immunoblot using anti-GFP antibodies and live microscopy of the GFP signal. mLSA3-GFP had an apparent molecular weight of ~29 kDa, consistent with proteolytic cleavage at the conserved leucine residue in the PEXEL motif by plasmepsin V and a smaller GFP core band derived from digestion of the reporter in the food vacuole, which confirmed it was secreted from the parasite (Figure 3A, B) (Boddey *et al*., 2009). In contrast, mLSA3 RLE>A-GFP had an apparent molecular weight of ~39 kDa indicating cleavage of the N-terminus was inhibited by the point mutations and therefore cleavage was PEXEL-dependent, while the GFP core band was greatly diminished confirming the protein was not secreted correctly (Figure 3B) consistent with inhibited processing and retention inside the parasite (Boddey *et al*., 2010).

LSA3 is predicted to have an N-terminal hydrophobic region between residues 45 and 64 which likely targets the protein into the ER and secretory pathway. Putative cleavage after the hydrophobic sequence by signal peptidase would result in a fragment of 32 kDa yet this was not detected by immunoblot and SignalP 3.0 did not predict processing by signal peptidase; rather, it predicted an N-terminal signal anchor. To confirm that plasmepsin V was the protease responsible for N-terminal processing, parasites expressing mLSA3-GFP were incubated with either DMSO vehicle or the inhibitor WEHI-600 (Nguyen *et al*, 2018) and processing analysed by immunoblots. The band pattern observed for mLSA3-GFP in DMSO was consistent with N-terminal processing observed in the prior blot but incubation with WEHI-600 caused a larger precursor of mLSA3-GFP, of the same size as the PEXEL mutant mLSA3 RLE>A-GFP protein (Figure 3B). Therefore, plasmepsin V processed the N-terminus of mLSA3-GFP in a PEXEL-dependent manner during erythrocytic infection, providing direct evidence that this motif in LSA3 is a *bone fide* PEXEL, in agreement with its export to the erythrocyte.

Immunoblots suggested that inhibition of PEXEL processing of mLSA3 RLE>A prevented normal secretion from the parasite, as the quantity of the 'GFP core' degradation product was reduced. Micropscopy confirmed this result, with mLSA3 RLE>A-GFP being trapped within the parasite (Figure 3C). Past studies have shown that mutation of the PEXEL results in retention in the ER, suggesting that mLSA3 RLE>A-GFP may be trapped in this organelle (Boddey *et al*., 2009). Interestingly, while mLSA3-GFP containing the native PEXEL motif was cleaved and efficiently secreted out of the ER to the periphery (parasite membrane/PV/PVM), we did not detect export beyond the PVM for this reporter by IFA. This suggested that additional sequences downstream of the PEXEL that were not included in the minigene may be required for export, or that the addition

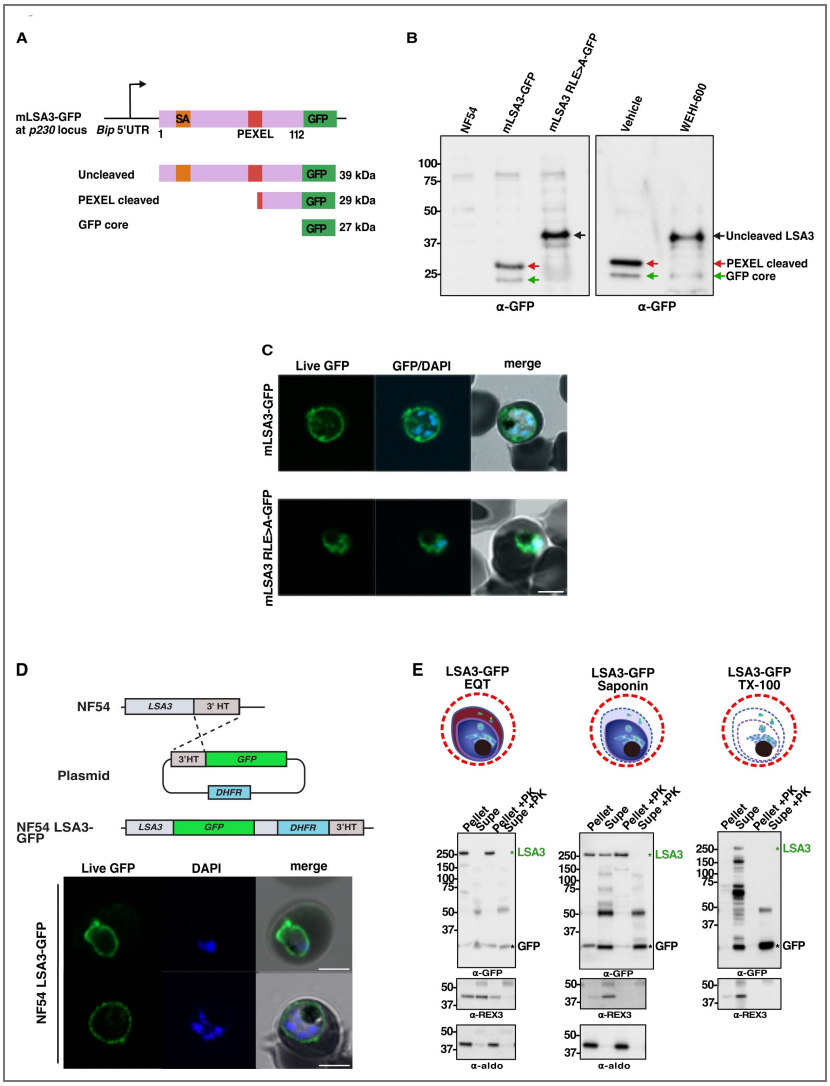

Figure 3. The PEXEL of LSA3 is processed by plasmepsin V.

A. Schematic of LSA3-GFP mini gene expressed from the *BIP* gene promoter following integration into the redundant *p230* locus. Two minigenes fused to green fluorescent protein (GFP) were generated containing endogenous LSA3 residues 1-112 encoding the N-terminal signal anchor (SA) and either a native PEXEL motif (red) or with key PEXEL residues mutated to alanine (RLE>A). Predicted molecular weights following possible putative cleavage events are depicted. **B.** Immunoblots of blood-stage lysates from parental NF54 control and transgenic NF54 expressing LSA3 minigenes probed with anti-GFP antibodies. Left blow shows accumulation of uncleaved mLSA3 RLE>A-GFP compared to mLSA3-GFP and NF54 parental controls (lanes 1-3). Right blot shows accumulation of uncleaved mLSA3-GFP in the presence of plasmepsin V inhibitor WEHI-600 for 5 hr compared to DMSO vehicle control. The uncleaved band was a similar size as uncleaved mLSA3 RLE>A-GFP in the left blot. Predicted sizes: full length minigene: 39 kDa; PEXEL processing: 29 kDa; GFP core in the food vacuole following secretion of reporter: 27 kDa. **C.** Live fluorescence imaging of transgenic NF54 blood-stage parasites expressing either mLSA3-GFP (secreted to PV; top) or mLSA3 RLE>A-GFP (retained within parasite, likely the ER; bottom). Scale bars, 5 μm. **D.** Schematic of the strategy used to endogenously tag LSA3 with GFP at the C-terminus in *P. falciparum* NF54 (top). Bottom panel shows live fluorescence imaging of LSA3-GFP expressed from the endogenous locus in transgenic NF54 parasites with the protein localized at the PV and PVM (see E). Scale bars, 5 μm. **E.** Proteinase K (PK) protection assays to localize endogenous LSA3-GFP. Erythrocytes infected with NF54 LSA3-GFP were enriched by Percoll centrifugation and permeabilized with either equinatoxin II (EQT; selectively lyses the erythrocyte and Maurer's cleft membranes), 0.03% saponin (selectively lyses the erythrocyte, Maurer's cleft, and PVM membranes) or 0.1% Triton-X-100 (lyses all membranes). The pellet was separated from the supernatant (supe) and both fractions were incubated with PBS containing Proteinase K (PK) at 37 °C. Protease inhibitor cocktail and PMSF was added to all samples followed by reducing sample buffer before immunoblotting with anti-GFP (LSA3-GFP), anti-REX3 (exported control) or anti-aldolase (aldo; internal protein control for parasite membrane integrity) antibodies. LSA3-GFP is indicated in green while GFP core in the food vacuole is indicated. Representative data is from n=2-3 experiments.

of GFP influenced the final translocation of this protein across one or more membranes, which is unusual for *PEXEL* genes but not unprecedented, for example C-terminal mCherry blocked the export of LISP2 beyond the PVM of the infected hepatocyte (Orito *et al*., 2013) and LSA3-GFP below. Nonetheless, these findings demonstrate that mLSA3-GFP was N-terminally processed by plasmepsin V during asexual blood stage development and trafficked through the secretory pathway of the parasite, both in a PEXEL-dependent manner.

Endogenous GFP tagging confirms LSA3 blood-stage expression but prevents export

While LSA3-C antibodies demonstrated expression of LSA3 in DGs (Morita *et al*., 2017) and in trophozoites (this study), we sought genetic confirmation of blood-stage expression of this liver-stage antigen. To this end, LSA3 was endogenously tagged with GFP at the C-terminus (Figure 3D) and visualised by live microscopy. Endogenous LSA3-GFP was expressed by blood-stage trophozoites and localized at the parasite periphery, suggestive of the PV / PVM. Interestingly, like the minigene mLSA3-GFP, no GFP signal was observed beyond the PVM (Figure 3D). This demonstrated by genetic epitope tagging that LSA3 is expressed during erythrocytic infection but that C-terminal fusion to GFP likely interfered with its export. Subcellular fractionation confirmed this to be the case, with LSA3-GFP present only in the pellet fraction after permeabilization of the erythrocyte membrane by EQT, and this was protected from PK digestion indicating it was not present in the erythrocyte (Figure 3E). Saponin permeabilization of the RBC membrane and PVM indicated further trafficking problems had occurred; firstly, a significant portion of LSA3-GFP was present in the saponin supernatant and could be degraded by PK, indicating it was soluble in the PV (Figure 3E). Secondly, the saponin pellet fraction of LSA3-GFP was resistant to PK, indicating the protein was located inside the parasite; based on the IFAs (Figure 3D), this was potentially at the plasma membrane with GFP facing internally (Figure 3E). Both results for LSA3-GFP were different to those observed after saponin fractionation of endogenous untagged LSA3 using LSA3-C antibodies, confirming that addition of GFP to the C-terminus affected both the trafficking and membrane association of LSA3, possibly by interfering with the C terminal hydrophobic sequence. We therefore did not study the LSA3-GFP parasite line any further. Interestingly, the GFP core of LSA3-GFP was consistently resistant to PK (Figure 3E), which agrees with the GFP fold also being resistant to proteases in the food vacuole, where the 'GFP core' species is formed following secretion from the parasite (Boddey *et al*., 2010; Boddey *et al*., 2009; Waller *et al*, 1999).

LSA3-deficiency does not inhibit gametocytogenesis or transmission

To investigate the essentiality and function of LSA3 in *P. falciparum*, we assessed the development of Δ*LSA3* mutants in which the *LSA3* gene was disrupted (Figure 1B) across the lifecycle. NF54 and Δ*LSA3* clones D8 and E2 blood-stages were differentiated to gametocytes, and no significant differences were detected in the formation or quantity of stage V gametocytes across parasite lines (Figure 4A). Gametocytes were transmitted to *An. stephensi* mosquitoes by standard membrane feeding assays (SMFA) and midgut oocyst numbers were quantified 7 days postbloodmeal: again, no significant differences were observed for oocyst burden or infection prevalence between Δ*LSA3* clones and NF54 across independent experiments (Figure 4B). We next dissected the salivary glands of *An. stephensi* mosquitoes on day 17 postbloodmeal and quantified the number of sporozoites in separate cohorts; this revealed no significant differences in sporozoite yields between parasite lines (Figure 4C). Taken together, these results showed that LSA3-deficiency did not prevent development of mature gametocytes, transmission to or development of *P. falciparum* within mosquitoes or reduce the rate of sporozoite entry into the salivary glands under the conditions used.

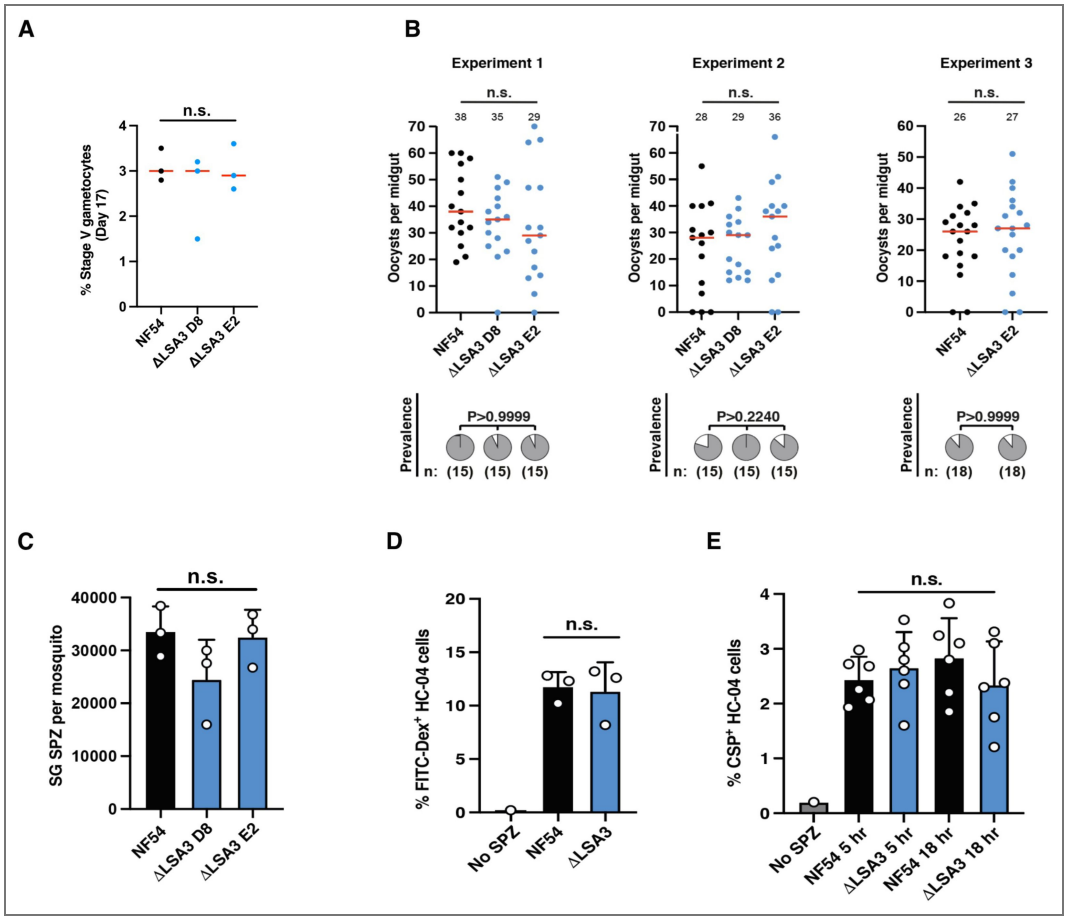

Figure 4. LSA3-deficiency does not inhibit gametocytogenesis or oocyst or sporozoite development nor sporozoite infectivity.

A. Percentage of stage V gametocytes of NF54 parental and Δ*LSA3* clones D8 and E2. Red bars indicate the mean. Data was compared by ANOVA using the Kruskal Wallis test. **B.** Oocyst counts of NF54 and Δ*LSA3* clones D8 and E2 per mosquito midgut 7 days post bloodmeal. Data include the mean (number shown and red bar) from three independent transmission experiments. Data comparing Δ*LSA3* mutant clones to NF54 parent performed by ANOVA using the Kruskal Wallis test, except experiment 3 (Mann-Whitney test). Prevalence of mosquito infection is shown below each graph, comparing NF54 to Δ*LSA3* clones by chi-square analysis. **C.** Salivary gland sporozoite (SG SPZ) counts per mosquito at 17 days postbloodmeal. Data is mean compared Δ*LSA3* mutant clones to NF54 parents by ANOVA using the Kruskal Wallis test. **D.** Cell traversal enumerated as percentage of FITC-dextran^{+} HC-04 hepatocytes after incubation with freshy dissected NF54 and Δ*LSA3* clone E2 sporozoites from mosquito salivary glands. **E.** Percentage of HC-04 cells with CSP^{+} intracellular parasites at 5 and 18 hours of incubation with NF54 parent and Δ*LSA3* clone E2 sporozoites. Data was analyzed by ANOVA using the Kruskal Wallis test. All data in the figure is mean ± SEM from n=3 (A to D) or n=6 (E) independent experiments. In all panels, n.s., not significant (P>0.05). Data is from n=3 experiments.

LSA3-deficiency does not perturb sporozoite traversal or infection of hepatocytes

Expression of LSA3 on the surface of *P. falciparum* sporozoites has been reported (Daubersies *et al*., 2000) suggesting it may be important in these forms. To investigate this, the kinetics of sporozoite traversal and infection of human hepatocytes were analysed. Firstly, cell traversal was investigated by incubating Δ*LSA3* clones or NF54 sporozoites with HC-04 human hepatocytes for 3 hours in the presence of the cell impermeant fluorescent molecule dextran-FITC. Only hepatocytes that have been traversed by sporozoites become permeable to dextran-FITC due to transient destabilization of the host cell membrane and these cells can be quantified by flow cytometry (Mota *et al*, 2001; Yang *et al*, 2017b). Both Δ*LSA3* clones and NF54 sporozoites were able to wound HC-04 hepatocytes leading to dextran-FITC uptake at similar rates (Figure 4D) indicating that LSA3 was not critical for sporozoite motility or their capacity to traverse human hepatocytes, which is a natural process of infection as sporozoites journey from the skin to blood vessels and eventually traverse hepatocytes before productively invading one in the liver.

We next investigated whether LSA3 was important for sporozoite infection of HC-04 hepatocytes using an assay that quantifies the number of intracellular parasites at different times of incubation for CSP-positive parasites by flow cytometry (Verzier *et al*, 2026). While CSP is initially located on the sporozoite surface, it is also present at the parasites periphery following human hepatocyte infection and during intracellular liver-stage development (McConville *et al*., 2024; Vaughan *et al*, 2012) and has been used to quantify productive invasion events on subsequent days (Angage *et al*, 2024; Kaushansky *et al*, 2015; Lopaticki *et al*, 2022; Verzier *et al*., 2026). We did not detect any significant difference in the number of CSP-positive Δ*LSA3* or NF54 parasites at 5 hours postinfection (hpi) or 18 hpi, indicating that LSA3 was not essential for *P. falciparum* sporozoite infection of HC-04 hepatocytes using this assay (Figure 4E).

LSA3-C co-localizes with EXP1 and EXP2 in *P. falciparum* liver-stages

To better understand the localization of LSA3 at the liver stage, IFAs were performed on *P. falciparum* NF54 liver stages on days 5 and 6 post infection of humanized mice using the PVM markers EXP1 and EXP2. The mouse liver samples investigated were from a prior study in which humanized mice were singly infected with NF54 sporozoites (McConville *et al*., 2024). On day 5 postinfection, LSA3-C antibodies were detected around, and co-localizing with, the EXP1-labelled PVM (Figure 5A), and on day 6 with EXP2 at the PVM (Figure 5B). LSA3-C antibodies also localized in puncta beyond the circular shape of the EXP1-labelled PVM in a small number of infected hepatocytes; this was apparently LSA3 derived from egressing merozoites, or material released following PV rupture since LSA3-C was closely associated with the parasite DAPI signal (Figure 5A). These results confirm that LSA3-C antibodies localize at the PVM of *P. falciparum* exoerythrocytic forms. We did not detect LSA3-C beyond the PVM under the conditions tested. Whether LSA3 is exported in hepatocytes is uncertain: it may be considered exported if part of LSA3 reaches the host cell side of the PVM but confirming this will require follow up studies. As we employed a humanized mice co-infection strategy to assess the essentiality of Δ*LSA3* versus NF54, we could not yet perform IFAs on individually infected mice deficient for LSA3 to validate the specificity of LSA3-C or LSA3-T at the liver-stage.

Genetic disruption of *LSA3* in *P. falciparum* attenuates liver-stage development

LSA3 has low sequence polymorphism across diverse geographical isolates and is a protective pre-erythrocytic antigen in chimpanzees and Aotus monkeys (Daubersies *et al*., 2000; Perlaza *et al*, 2003). To investigate its importance to parasite growth, Δ*LSA3* sporozoites were mixed in equal number with parental NF54 sporozoites and inoculated via a single intravenous (IV) injection into each of four SCID Alb-uPA humanized mice with chimeric human livers. At day 5 postinfection, livers were isolated from each mouse and half of the individual lobes were snap-frozen for sectioning and microscopy and the other half of each lobe was processed to recover genomic DNA

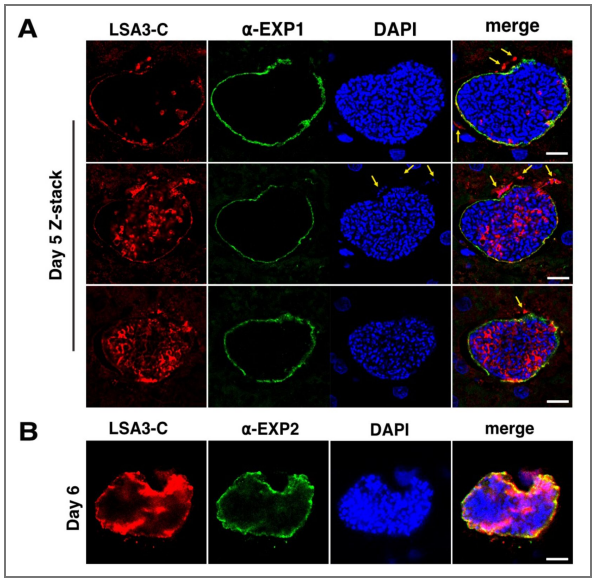

Figure 5. LSA3-C localizes to the PVM in *P. falciparum*-infected hepatocytes.

A. Liver sections from humanized mice infected with *P. falciparum* NF54 sporozoites on day 5 post infection. Each panel shown is of the same liver-stage parasite at different cross-sections of the Z-stack. Sections were stained with anti-EXP1 antibodies that label the PVM and LSA3-C antibodies. LSA3-C localized predominantly to the PVM or with egressing merozoites beyond the PVM (yellow arrows). **B.** Liver section from humanized mice infected with *P. falciparum* NF54 sporozoites on day 6 post infection stained with anti-EXP2 antibodies that label the PVM and LSA3-C antibodies. For all images, parasite and host DNA was labelled with DAPI nuclear stain; scale bars, 10 μm. Representative images are from n=1 experiment from n=2 humanized mice.

for molecular quantification of parasite liver loads by real-time polymerase chain reaction (qRT-PCR) (Foquet *et al*, 2013) using oligonucleotide pairs that amplified DNA specific to either Δ*LSA3* or NF54 from the same livers. This experimental approach allows the fitness of mutant parasites to be directly compared to parental controls in the same mice (Lopaticki *et al*, 2017; McConville *et al*., 2024; Yang *et al*, 2017a). The day 5 endpoint was chosen as this coincided with the peak of PVM localization reported previously (Daubersies *et al*., 2000) and before parasites egress from the liver on day 7. This analysis revealed a 2.3-fold reduction in the liver load for Δ*LSA3* compared to NF54 on day 5 post infection (P=0.0110) (Figure 6A). When represented as a percentage of total parasite liver load, where equal fitness of each co-infected parasite line corresponds to 50%, Δ*LSA3* averaged a 40% reduction in fitness compared to NF54 (P=0.0105) (Figure 6B). The four humanized mice had similar levels of human chimerism of approximately 20% human:80% mouse hepatocytes, as determined by qRT-PCR (Figure 6C). Together, these results show that genetic disruption of *LSA3* in *P. falciparum* caused a moderate and significant loss of fitness in each of four humanized mice, indicating that this PEXEL-containing protein is important for normal intrahepatic development of the human malaria parasite.

Discussion

Historically, LSA3 was considered a protein expressed only in pre-erythrocytic stages (Daubersies *et al*., 2000), but more recent work identified LSA3 in merozoite DGs during RBC invasion (Morita *et al*., 2025; Morita *et al*., 2017). Here, endogenous GFP tagging confirmed LSA3 expression in blood stages and motivated biochemical and functional studies across the parasite lifecycle, prompted by a previously uncharacterized PEXEL motif (Maier *et al*., 2008; McConville *et al*., 2024; Morita *et al*., 2017). LSA3 is processed by plasmepsin V (inhibited by a PEXEL-mimetic inhibitor) and is associated with the PVM, while a fraction is exported into the infected erythrocyte as soluble aggregates distinct from Maurer's clefts. Its biochemical profile resembles that of the exported chaperone HSP70-x, suggesting possible association with J-dots or other chaperone complexes (Hanssen *et al*, 2008; Kulzer *et al*, 2012; Kulzer *et al*., 2010; Petersen *et al*, 2016; Taraschi *et al*, 2003). AlphaFold predicts an LSA3 domain with similarity to the DnaK substrate binding domain (Jumper *et al*., 2021), consistent with a chaperone-like role. Limitations in fixation and antibody specificity so far precluded definitive colocalization with HSP70-x; identifying interacting partners will help clarify LSA3's role in blood-stages and may inform its liver-stage function.

LSA3's DG localization aligns with discharge during merozoite invasion, when DG contents contribute to PV and PTEX assembly and early export (Morita *et al*., 2017; Riglar *et al*, 2013). Given its DG association and recent blood-stage vaccine interest, LSA3 may act during or immediately after invasion (Morita *et al*., 2025; Morita *et al*., 2017). Trafficking of LSA3 reporter proteins required PEXEL processing by plasmepsin V, but C-terminal fluorescent fusions disrupted correct targeting, and endogenous tagging showed predominate retention at the parasite plasma membrane and a soluble PV pool, consistent with the sensitivity of pre-erythrocytic trafficking to bulky tags (Orito *et al*., 2013; Singer & Frischknecht, 2021).

Understanding of *P. falciparum* liver-stage biology remains limited, yet exported proteins expressed at this stage are plausible antigenic and functional candidates. LSA3 antibodies localized mainly to the PVM in infected hepatocytes with little evidence of export beyond the membrane. Recent reports suggest PEXEL processing can direct proteins to rhoptries or the PV in blood stages (Fierro *et al*., 2024; Freville *et al*., 2024) and the related apicomplexan *Toxoplasma*, uses an analogous TEXEL/ASP5 pathway to target to the PV or host cell compartments (Bougdour *et al*, 2014; Coffey *et al*, 2015; Hammoudi *et al*, 2015). Whether a domain of LSA3 is exported into the hepatocyte lumen from the PVM remains unresolved.

In this study, genetic disruption of *LSA3* reduced liver-stage fitness by 40%, indicating an important role for LSA3 during hepatocyte infection. This makes LSA3 the second PEXEL protein required for normal *P. falciparum* liver-stage development, after LISP2, which causes arrest later when genetically disrupted (Zanghi *et al*., 2025). The differential localization between blood and liver stages suggests stage-specific routing of PEXEL proteins: in blood stages PEXEL processing

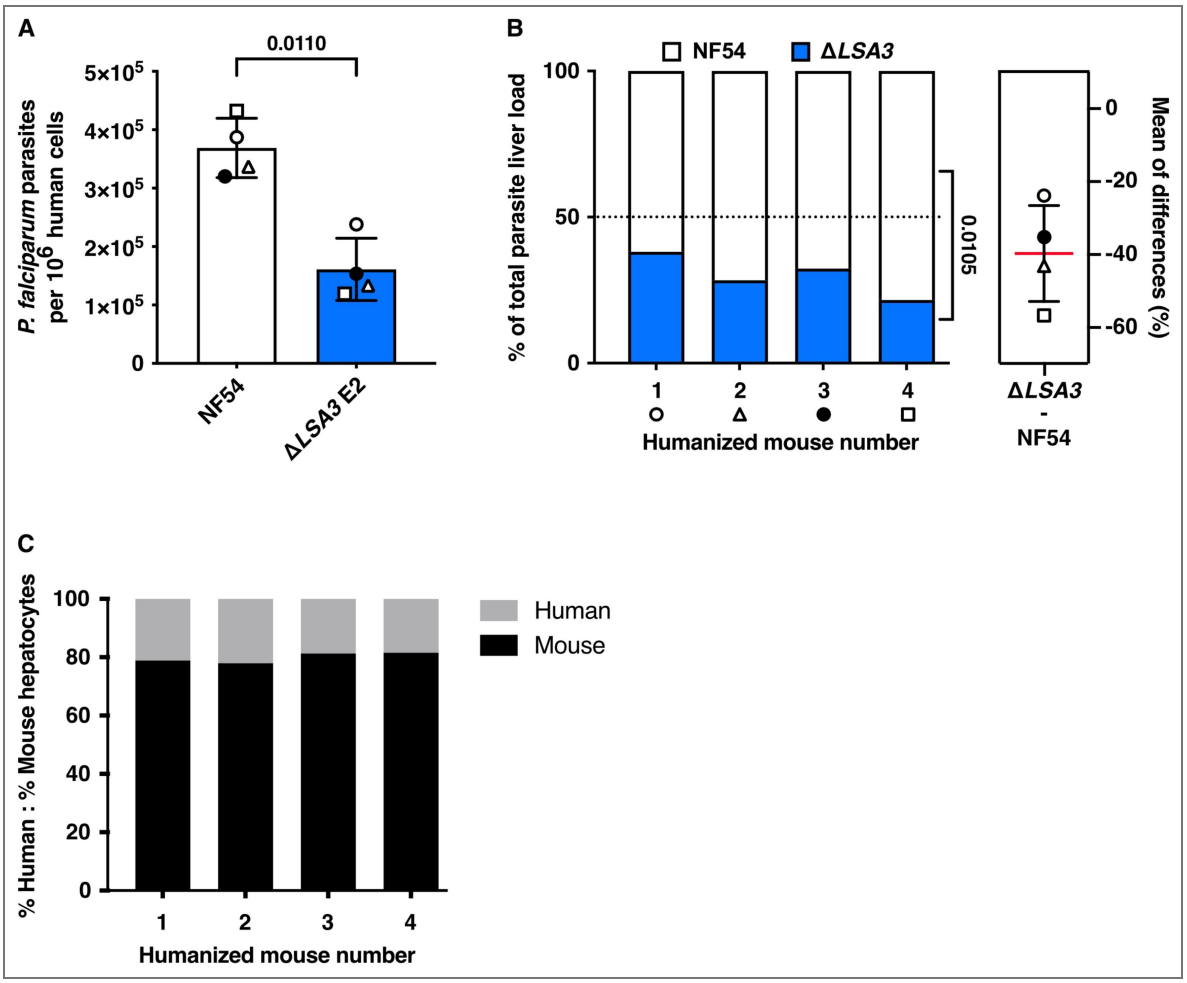

Figure 6. LSA3 is important for *P. falciparum* liver-stage development in humanized mice.

A. Quantification of *P. falciparum* genomes per million human hepatocytes on day 5 postinfection from four co-infected humanized mice with chimeric human livers, each receiving 5×10^5 Δ*LSA3* clone E2 and 5×10^5 parental NF54 sporozoites mixed for a total of 1×10^6 sporozoites via a single IV injection per mouse, by qRT-PCR. Values from each sample were normalized to a series of pretested standard curves of each amplicon and are shown as mean ± SD, statistically analysed by paired *t*-test. Each symbol corresponds to the same coinfected mouse. **B.** Percent of total parasite liver load on day 5 postinfection in each humanized mouse co-infected with equal numbers of Δ*LSA3* E2 and parental NF54 sporozoites from (A), shows reduced fitness of LSA3-deficient parasites (i.e. less than 50%). Percent differences of the mean in each mouse are shown on the right ± SD. Each symbol corresponds to the same coinfected mouse. Means were compared by paired *t*-test. **C.** Human chimerism in each humanized mouse (21%, 22%, 19% and 18%) quantified by qRT-PCR using oligonucleotides specific to human and mouse PTGER2. Data is from n=1 experiment from n=4 humanized mice.

commonly precedes export to the host cell cytosol whereas in liver stages PVM targeting is more frequent (Fougere *et al*, 2016; Ingmundson *et al*, 2012; McConville *et al*., 2024). Hence, the export pathway may influence PVM remodelling and host-pathogen interactions during liver-stage development (Liehl *et al*., 2014; Marques-da-Silva *et al*., 2025; Prado *et al*., 2015).

LSA3 is immunogenic at the liver stage (Daubersies *et al*., 2000), and more recently at the blood-stage (Morita *et al*., 2017), and its sequence conservation across geographical isolates suggests functional constraint. Structural prediction revealed similarity to a DnaK-like substrate binding domain, and a large unstructured repeat region, with different geographical isolates showing variation in the number of repeats (Prieur & Druilhe, 2009). Unstructured repeats are common features of other *Plasmodium* proteins (Orito *et al*., 2013) and disordered regions feature in exported proteins in Apicomplexa (Bougdour *et al*, 2013; Dumaine *et al*, 2021; Huang *et al*, 2022; Rodrigues *et al*, 2025; Rosenberg & Sibley, 2021).

In summary, LSA3 is a component of the *P. falciparum* exportome, required for efficient liver-stage development and capable of eliciting protective pre-erythrocytic immunity. Defining its precise functional roles at the PVM and in the host erythrocyte will help inform antigen discovery while identification of additional exported proteins trafficked to these locations may facilitate vaccine design and reveal novel drug targets.

Materials and methods

Parasite maintenance

P. falciparum NF54 (kindly provided by the Walter Reid Army Institute of Research) asexual stages were cultured in human O-positive erythrocytes (Melbourne Red Cross) at 4% haematocrit in RPMI-HEPES supplemented with 0.2% $NaHCO_3$, 7% heat-inactivated human serum (Melbourne Red Cross), and 3% AlbuMAX (ThermoFisher Scientific). Parasites were maintained at 37 °C in 94 % N_2, 5 % CO_2 and 1 % O_2. Gametocytes were cultured in RPMI-HEPES supplemented with 0.2 % $NaHCO_3$, and 10 % heat-inactivated human serum (Melbourne Red Cross). Gametocytes for transmission to mosquitoes were generated using the “crash” method (Saliba & Jacobs-Lorena, 2013) using daily media changes.

Generation of transgenic *P. falciparum*

The *LSA3* knockout clones Δ*LSA3* (E2) and (D8) were generated by amplification of the 5’ and 3’ flanks of the *LSA3* gene (PF3D7_0220000). Oligonucleotides used were: forward primer 5'-AAACCG CGGATGTAGATAAGAAATTGAATAAAC-3' and reverse primer 5'-TTGAACTAGTATTTCTTCAACACTTGG AGC-3' (5' flank); forward primer 5'-TTAGAATTCAAAAATATGGAAGAGGAGTTAATGAAG-3' and reverse primer 5'-TTACCTAGGTCTTCATCTATATCTTCATCTATATC-3' (3' flank). The flanks were ligated on either side of the human DHFR cassette in pCC1 (see Figure 1). Highly synchronous *P. falciparum* NF54 schizonts were enriched by magnet-activated cell sorting (MACS) magnetic separation columns (Miltenyi biotec) and incubated with E64 (Sigma) until parasitophorous vacuole (PV) enclosed merozoite structures (PEMS) could be visualized (Boyle *et al*, 2010). Purified *LSA3* knockout plasmid DNA (100 µg, Life Technologies) was transfected into the schizonts with an Amaxa Basic Parasite Nucleofector Kit 2 (Lonza). Parasites that had correctly integrated the knockout construct were selected by cycling on and off 4nM WR99210 and stable transfectants were cloned out by limiting dilution (Voss *et al*, 2006) and confirmed by diagnostic PCR.

Mosquito infection and analysis of parasite development

Seven-day old female *Anopheles stephensi* mosquitoes were fed on asynchronous gametocytes diluted to 0.6% stage V gametocytemia, via water jacketed glass membrane feeders as previously described (McConville *et al*., 2024). Mosquitoes were sugar starved for 48 hours following feeding to enrich for blood fed mosquitoes. Surviving mosquitoes were provided with di-ionised water via paper/cotton wicks and sugar cubes. Oocyst numbers were obtained from midguts dissected from cold-anesthetized and ethanol killed mosquitoes 7 days post feed and stained with

0.1% mercurochrome. Salivary glands were dissected from mosquitoes (day 16 to 20 post bloodmeal), crushed using pestle and then glass wool filtered to obtain sporozoites that were quantified using a hemocytometer and used in subsequent assays.

Immunofluorescence microscopy assay (IFA)

Asexual stages were fixed in 4% paraformaldehyde (PFA) PBS with 0.0075% glutaraldehyde, permeabilised with 0.01% triton X 100 or fixed and permeabilised with ice cold 90% Acetone 10% methanol and probed with primary antibodies; mouse anti-GFP (Roche; Cat # 11814460001; *RRID:AB_390913*; 1:1000), mouse anti-MAHRP2 (1:1000) (Pachlatko *et al*., 2010), mouse anti-EXP2 (1:500) (de Koning-Ward *et al*., 2009), mouse anti-REX3 (1:1000) (Spielmann *et al*., 2006), and rabbit anti-LSA3-C (1:1000) (Morita *et al*., 2017) or LSA3-T (1:1000) (Morita *et al*., 2025), in 3% normal donkey serum (NDS/ PBS). Secondary antibodies were goat anti-rabbit Alexa 594 and anti-mouse or anti-rat Alexa 488 (Thermo Fisher; Cat # A-11012, A-11029, A-11006; *RRID:AB_2534079*, *RRID:AB_2534088*, *RRID:AB_2534074*; 1:1000). For liver-stage: chimeric human livers were perfused with 1x PBS and fixed in 4% (vol/vol) PFA PBS overnight before being exchanged for sucrose and snap frozen in OCT (Invitrogen). Sections of 8 mm were cut on the cryostat apparatus HMM550. Samples were permeabilised using ice cold 10% methanol 90% acetone and blocked using 3% BSA PBS. Sections infected with NF54 parasites were then incubated with primary antibodies: rabbit anti-LSA3-C (1:1000), mouse anti-PfCSP (2A10; 1:2000) (Nicholas *et al*, 2023), rat anti-EXP1 (McConville *et al*., 2024) (1:1000), mouse anti-EXP2 (1:500), diluted in 3% BSA PBS. Secondary antibodies were the same goat anti-rabbit Alexa 594 and anti-mouse or rat Alexa 488 (1:1000). Samples were incubated with DAPI (4',6'-diamidino-2-phenylindole) at 1 mg/ml in PBS to visualize DNA and mounted in Prolong Gold mounting media (Invitrogen). For live imaging, blood stage culture was incubated with DAPI for 2 min, washed once with PBS and 4 uL blood pallet dropped onto cover slips and inverted. Images were acquired immediately. All images were acquired on the Zeiss LSM 980 microscope.

Subcellular fractionation, proteinase K treatment and immunoblots

Late-stage parasites at desired stage purified by Percoll gradient were resuspend in 40 ul PBS + 5 ul EQTII (0.5mg/ml) per 5 ul of pellet and incubate at RT for 6 min. The supernatant was collected; pellet washed in PBS and resuspend in the same volume as the supe before suspension of fractions in 4x SDS-PAGE loading buffer and stored at -20°C. For Saponin, Percoll-purified parasites were resuspended in 45 uL 0.03% saponin, incubated at RT for 3 min. Supernatant was collected, the pellet washed in PBS and resuspend in the same volume as the supe before suspension of fractions in 4x SDS-PAGE loading buffer and stored at -20°C. For Triton X-100, Percoll-purified parasites were resuspend in 45 uL 0.1% TX-100, incubated at RT for 3 min. Supernatant was collected, the pellet washed in PBS and resuspend in the same volume as the supe before suspension of fractions in 4x SDS-PAGE loading buffer and stored at -20°C (Gabriela *et al*., 2022). Proteinase K solution (20 ug/mL; Worthington) was added to samples, incubated at 37°C for 20 mins and then samples resuspended in 4x SDS-PAGE loading buffer. Proteins were separated through 4-12% Bis-Tris gels and transferred to nitrocellulose before blocking in 5% milk PBS-T and probing with mouse anti-GFP (Roche; Cat # 11814460001; *RRID:AB_390913*; 1:1000), mouse anti-REX3 (1:1000), rabbit anti-aldolase (Baum *et al*, 2006) (1:4000) followed by anti-mouse (1:1000) or anti-rabbit (1:4000) horse radish peroxidase-conjugated secondary IgG antibodies (Cell Signalling Technology; Cat # 7076S, 7074S; *RRID:AB_330924*, *RRID:AB_2099233*) and viewed by enhanced chemiluminescence (Amersham) (Jennison *et al*, 2019).

LSA3 sequence analysis and generation of the LSA3 minigene

The analysis of LSA3 from *P. falciparum* 3D7 was performed by inputting the accession Pf3D7_0220000 into PlasmoDB or AlphaFold. The first 112 amino acids of the LSA3 coding sequence were synthesised by Integrated DNA Technologies containing either the endogenous PEXEL sequence or one where the key residues, RLE, were mutated to alanines. These were then

ligated into the pKiwi003 (Ashdown *et al*., 2020) using restriction sites NheI and PstI, for integration into the *P. falciparum* 3D7 *P230* locus. The resulting plasmid was transfected into PEMs schizonts in conjunction with Cas9-U6.2-hDHFR_P230p (50 ug of each) following standard procedures (Boyle *et al*., 2010) and correct integration was selected for following addition of 4 nM WR99210 for 10 days.

Plasmepsin V cleavage inhibition assay

P. falciparum parasites at 24-34h old trophozoites were purified from uninfected erythrocytes Vario Macs magnet column (Miltenyi Biotech) separation and treated with 20 uM of the plasmepsin V inhibitor WEHI-600 (Nguyen *et al*., 2018) or DMSO vehicle for 3 h at 37°C in culture gas. Parasites were treated with 0.1% saponin to liberate contaminating hemoglobin and pellets solubilized in 4x Laemmli sample buffer before protein separation via SDS-PAGE. Proteins were separated through 4-12% Bis-Tris gels and transferred to nitrocellulose before blocking in 5% milk PBS-T and probing with mouse anti-GFP (Roche; Cat # 11814460001; *RRID:AB_390913*; 1:1000), followed by anti-mouse (1:1000) or anti-rabbit (1:4000) horse radish peroxidase-conjugated secondary IgG antibodies (Cell Signalling Technology; Cat # 7076S, 7074S; *RRID:AB_330924*, *RRID:AB_2099233*) and viewed by enhanced chemiluminescence (Amersham) (Sleebs *et al*, 2014).

Hepatocyte culturing

HC-04 hepatocytes (Sattabongkot *et al*, 2006) were maintained on Iscove's Modified Dulbecco's medium (IMDM), supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. Cells were split 1:6 every 2-3 days once they reached 90% confluency.

Cell traversal assays

Cell traversal was measured using a cell wounding assay (Lopaticki *et al*., 2022). HC-04 hepatocytes (1×10^5) were seeded onto the bottom of a 96-well plate using IMDM (Life Technologies, 11360-070). Sporozoites dissected from mosquito salivary glands into Schneider's insect medium were enumerated and placed into IMDM containing 10% human serum before addition at an MOI 0.3 to hepatocyte monolayers for 2.5 hr for cell traversal to occur in the presence of 1 mg ml-1 FITC-labelled dextran (10,000 MW, Sigma Aldrich). Cells were washed and trypsinized to obtain a single cell suspension for FACS quantification of dextran-positive and -negative cells. For each condition, triplicate samples of 10,000 cells were counted by flow cytometry in each of the three independent experiments.

Hepatocyte invasion assays

HC-04 hepatocyte invasion assays were performed as previously described (Lopaticki *et al*., 2022). Briefly, HC-04 hepatocytes (1×10^5) were seeded onto the bottom of 96-well plates using IMDM (Life Technologies, 11360-070). Sporozoites dissected from mosquito salivary glands into Schneider's insect medium were quantified and replaced in IMDM containing 10% human serum before addition at MOI 0.3 to hepatocytes for either 5 or 18 hr before being washed and trypsinized to obtain a single cell suspension and transferred to a 96 well round bottom plate. Cells were fixed and permeabilised (BD bioscience) and stained with Alexa fluor 647 conjugated mouse anti-PfCSP antibodies in 3% BSA permeabilizing solution. Cells were then washed in PBS and analysed by flow cytometry to quantify CSP^+ HC-04 cells. For each condition, triplicate samples of 10,000 cells were counted in each of the three independent experiments.

Humanized mice production and processing

$uPA^{+/+}$ SCID mice (University of Alberta) were housed in a virus and antigen free facility supported by the Health Sciences Laboratory Animal Service 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 Service Animal Welfare committee and the Walter and Eliza Hall Institute of Medical Research

Animal Ethics Committee. uPA+/+-SCID mice at 5–14 days old received 10^6 human hepatocytes (cryopreserved human hepatocytes were obtained from BioreclamationIVT—Baltimore MD) by intrasplenic injection and engraftment was confirmed 8 weeks following transplantation by analysis of serum human albumin (Mercer *et al*, 2001; Yang *et al*., 2017b). For humanized mice co-infections: 5×10^5 Δ*LSA3* clone E2 and 5×10^5 parental NF54 sporozoites (1×10^6 total) were injected IV into each of 4 mice. Livers were isolated 5 days postinfection from CO_2-ethanized mice and individual lobes were cut as described (Lopaticki *et al*., 2017), 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 (Alcoser *et al*, 2011; Lopaticki *et al*., 2017; Yang *et al*., 2017b). To specifically differentiate NF54 from Δ*LSA3* clone E2 genomes from the same mouse samples, oligonucleotides were designed as below. Human and mouse genomes were quantified using oligonucleotides specific for prostaglandin E receptor 2 (PTGER2) from each species, as described previously (Yang *et al*., 2017b). All probes were labelled 5' with the fluorophore 6-carboxy-fluorescein (FAM) and contain a double-quencher that includes an internal ZENTM quencher and a 3' Iowa Black® quencher from IDT. The following probes were used:

LSA3WT-F: TCCGCTAATTTCTACTGTTTCCTCT

LSA3WT-R: GAACAGGCAGAAGAAGAGAGC

LSA3WT: FAM/TGCAGAGAA/ZEN/TTTAGAGAA-3IABkFQ

hDHFR_F 5'-ACCTAATAGAAATA TATCAGGATCC-3'

hDHFR_R 5'-GTTTAAGATGGCCTGG GTGA-3'

ΔLSA3 (DHFR): FAM/CCGCTCAGG/ZEN/AACGAATTTAGATA-3IABkFQ

Pf18s-F: GTAATTGGAATGATAGGAATTTACAGGT

Pf18s-R: TCAACTACGAACGTTTTAACTGCAAC

18S: FAM/TGCCAGCAG/ZEN/CCGCGGTA-3IABkFQ

hPTGER2: FAM/TGCTGCTTC/ZEN/TCATTGTCTCG/3IABkFQ

mPTGER2: FAM/CCTGCTGCT/ZEN/TATCGTGGCTG/3IABkFQ

Standard curves were prepared by titration from a defined number of DNA copies for each amplicon of *P. falciparum* LSA3WT, Δ*LSA3*, 18S, and human PTGER2 and mouse PTGER2 controls. PCRs were performed on a Roche LC80 using LightCycler 480 Probe Master (Roche) (Lopaticki *et al*., 2017).

Statistics

Parasite conditions were compared between groups using an ANOVA Kruskal-Wallis test with Dunn's correction, or Mann Whitney test where indicated, whilst mosquito infection prevalence was compared using the chi-square test. Statistical analyses comparing Δ*LSA3* clone E2 to NF54 controls in co-infected mice were performed using the paired t-test and comparisons of individual mutants to control were performed using the Mann-Whitney test. Analyses were performed using Graphpad Prism 9 for macOS. $P<0.05$ was considered statistically significant.

Ethics statement

Experimental protocols involving humanized mice were conducted in 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 University of Alberta Health Sciences Animal Welfare Committee and the Walter and Eliza Hall Institute of

Medical Research Animal Ethics Committee. Experimental protocols involving the HC-04 human hepatocyte cell line were reviewed and approved by the Walter and Eliza Hall Institute of Medical Research Biosafety Committee.

Supplementary figures

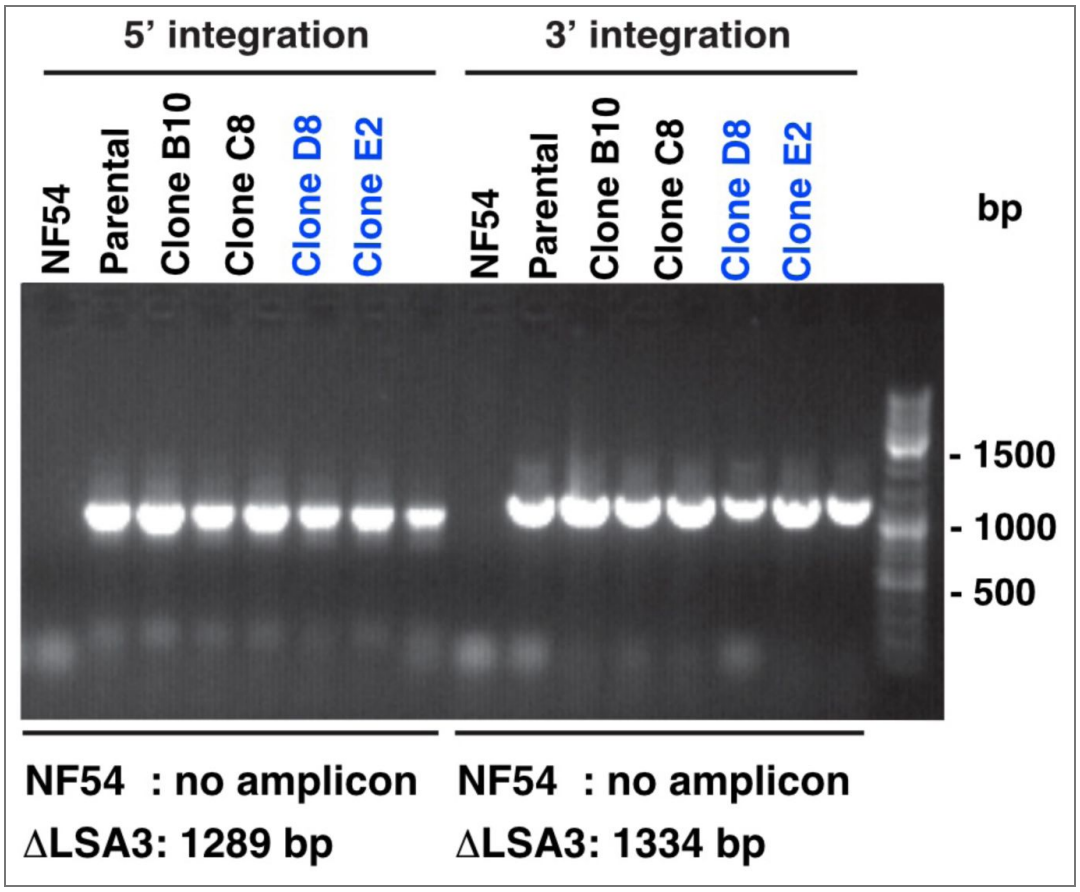

Figure S1. Genotyping *P. falciparum* NF54 and Δ*LSA3* clones. Diagnostic PCRs (MO545/aw560) confirmed correct double cross-over integration of the Δ*LSA3* knockout pCC1 construct in *P. falciparum* NF54 clones. Mutant clones D8 and E2 used in this study are shown in blue and molecular weight sizes in base-pairs (bp) are shown on the right.

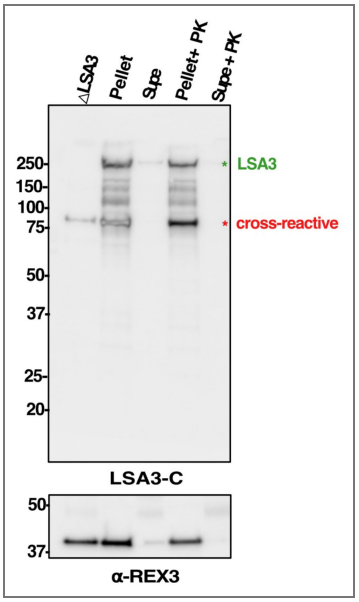

Figure S2. The LSA3-C binding domain is not localized on the infected erythrocyte surface.

Erythrocytes infected with Δ*LSA3* and NF54 parental parasites were enriched by Percoll centrifugation, with the former being included as a specificity control for the LSA3-C antibody. NF54-infected erythrocytes were incubated with PBS to maintain cells as intact. The pellet was then separated from the supernatant (supe) fraction by centrifugation, and both were incubated at 37 °C with Proteinase K (PK) in PBS. Protease inhibitor cocktail and PMSF were added to all samples followed by reducing sample buffer to stop the reactions. Samples were separated by SDS-PAGE and probed with LSA3-C or anti-REX3 (exported protein control) antibodies. The C-terminal portion of LSA3 recognized by LSA3-C antibodies was not present on the erythrocyte surface as it remained resistant to Proteinase K, like the exported REX3 control as expected. The cross-reactive internal protein recognized by LSA3-C is visible in all lanes.

Data availability

All data and materials generated in this study are available from the corresponding author upon request.

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Author contributions

Experiments were conceptualized by R.M. and J.A.B. and performed by R.M., R.W.J.S., and M.T.O. Methodology was performed by R.M., M.T.O., N.K., and J.A.B. Data was interpreted by R.M., A.F.C., and J.A.B. R.M. and J.A.B. drafted, and all authors contributed to preparing and editing, this manuscript.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The extent P. falciparum liver stage parasites export proteins into the host cell is unclear. Most blood stage exported proteins tested in liver stages were not exported. An exception is LISP2 that is exported in P. berghei but not P. falciparum liver stages. While the machinery for export is present in liver stages, efforts to demonstrate export have so far been mostly unsuccessful. Parasite proteins exported during the liver stage could be presented by MHC and thereby become the target of immune control, incentive to study liver stage export and identify proteins exported during this stage. However, particularly for P. falciparum it is very difficult to study liver stages.

This work studies LSA3 in P. falciparum blood and liver stages. The authors show that this protein is exported into the host cell in blood stages but in liver stages no or only very little export was detected. A disruption of LSA3 reduced liver stage load in a humanized mouse model, indicating this protein contributes to efficient development of the parasites in the liver.

The paper also studied the localization of LSA3 in blood stages and used a known inhibitor to show that it is processed by plasmepsin 5, a protease important for protein trafficking. The work also showed that LSA3 is not needed for passage through the mosquito.

Strengths:

The main strength of this work is the use of the humanized mouse model to study liver stages of P. falciparum, which is technically challenging and requires specialized facilities. The biochemical analysis of LSA3 localization and processing by plasmepsin 5 are thorough and mostly overcame adverse issues such as a cross-reactive antibody and negative influence of the GFP-tag on LSA3 trafficking. The mosquito stage analysis is also notable as these kinds of studies are difficult with P. falciparum. However, there was no evidence for a function of LSA3 in mosquito stages.

Weakness:

The cross-reactivity of the antibody together with the co-infection strategy prevents reliable assessment of LSA3 localization in liver stages. Despite of this it seems LSA3 is not exported in liver stages and the paper does not bring us closer to the original goal of finding an exported liver stage protein.

While the localization analysis in blood stages is well done and thorough, the advance is somewhat limited. LSA3 may be in structures like J dots, but this hypothesis was not tested. Although parasites with a disrupted LSA3 were generated, the function of this protein was not explored. However, this was now done in a separate study focussing on blood stage parasites (PMID: 41135800).

Due to the difficulty of working with humanised mice, it was not possible to refine some of the conclusions and questions remain:

The impact on liver stage development is interesting, but which phase of the liver stage is affected, and the phenotype remain largely unknown. The co-infection used (WT together with LSA3 mutant) has the advantage of a direct comparison of the mutant with the control in the same liver but complicates phenotypic analysis if the LSA3 antibody is also cross-reactive in liver stages. This issue adds a question mark to the shown localization and

precludes phenotypic comparisons. It was also not possible to determine if the cross-reactive protein is expressed at that stage. While this might have been evident from the mixed WT/mutant infection (if all cells are positive for LSA3 there is cross-reaction; if about half of the cells are negative, there isn't) but assessing this failed.

Significance:

It is important information that LSA3 contributes to efficient liver stage development. However, neither LISP2 nor LSA3 seem to be exported in P. falciparum liver stages and can't confirm the potential of vaccines with proteins exported in this stage. LSA3 is still important and may still be the target of the immune response, but based on this work, probably not due to export in liver stages.

https://doi.org/10.7554/eLife.108524.2.sa3

Reviewer #2 (Public review):

Summary:

Immunogenic Plasmodium falciparum proteins that could be targeted to prevent parasite development in the liver are of significant interest for novel anti-malarial vaccine development. In this study, McConville et al evaluate the trafficking and functional importance of LSA3, a protein expressed in the blood and liver stages and previously shown to provide protection in immunized chimpanzees. LSA3 contains a PEXEL motif but the authors have previously shown that this protein does not appear to be exported beyond the PVM in the liver stage (McConville et al PNAS 2024). However, LSA3 trafficking and functional importance have not been comprehensively evaluated across stages. In the present study, the authors find that blood-stage LSA3 undergoes PEXEL processing and a portion of the protein is exported into the erythrocyte where it localizes to punctate structures distinct from Maurer's clefts. Using a knockout mutant, LSA3 is shown to be dispensable for blood and mosquito stages but important to liver-stage development. Collectively, these results validate LSA3 as a liver-stage target and place it among several other PEXEL proteins that display differential trafficking beyond the PVM in the erythrocyte but not the hepatocyte.

Strengths:

(1) The authors present a thorough analysis of LSA3 trafficking in the blood stage. PEXEL processing by Plasmepesin 5 is clearly demonstrated through a combination of mini LSA3-GFP reporters and Plasmepsin 5 inhibitors. Importantly, an LSA3 knockout mutant is used to show that the LSA3-C anti-sera also reacts with additional, unidentified parasite proteins in the blood stage. Nonetheless, comparison between the WT and KO parasites clearly indicates that a portion of LSA3 is exported into the erythrocyte, which is further supported by protease-protection assays with fractionated iRBCs. This contrasts with the liver stage where LSA3 does not appear to traffic beyond the PVM, similar to what has been observed for other PEXEL proteins in the rodent malaria model.

(2) This study provides the first analysis of LSA3 exoerythrocytic function, showing this protein is important for liver stage development in chimeric human liver mice. Several PEXEL proteins in P. berghei have been shown to be exported into the host cell in the blood stage but do not appear to cross the PVM in the liver stage. These observations reinforce that even without detectible export into the hepatocyte, PEXEL proteins play critical roles during liver stage development.

Weaknesses:

The authors previously reported that anti-LSA3-C signal in the liver stage localizes within the parasite and at the parasite periphery but is not exported into the hepatocyte. In the present

study, it is shown that anti-LSA3-C reacts with other parasite proteins beyond LSA3 in the blood stage and this may also occur in the liver stage. However, since liver-stage IFAs were only performed on samples co-infected with both WT and ΔLSA3 parasites, non-specific anti-LSA3-C reactivity at this stage could not be determined and the localization of LSA3 in the liver stage remains somewhat unclear.

Comment on revisions:

The authors thoughtfully addressed the reviewer comments.

https://doi.org/10.7554/eLife.108524.2.sa2

Reviewer #3 (Public review):

Summary:

This manuscript provides a comprehensive characterization of the Plasmodium falciparum protein LSA3, combining biochemical, genetic, and in vivo approaches. The authors convincingly demonstrate that LSA3 is expressed during liver stage infection and that disruption of the gene leads to a modest but reproducible reduction in liver stage parasite load in humanized mice.

Strengths:

Their biochemical and cell biological analysis of blood stages provides strong evidence that LSA3 is exported to the infected erythrocyte, and the detailed analysis of its PEXEL motif processing is well executed.

Weaknesses:

The study suggests LSA3 as one of only two known P. falciparum PEXEL proteins contributing to this stage, although there is no evidence for the export beyond the vacuolar membrane. Several key conclusions, particularly regarding antibody specificity, localization in liver stage parasites, and the interpretation of the phenotypic data, are not fully supported by the current experiments.

Comments on revised version.

I appreciate the authors' efforts to revise the manuscript and to clarify several aspects of the study.

However, I remain concerned that some conclusions extend beyond the data presented. In particular, the authors acknowledge in their rebuttal letter that antibody specificity in liver stages could not be validated and that cross-reactivity cannot be excluded. Consequently, the localization data shown in Figure 5 cannot currently be considered definitive evidence for liver stage localization of LSA3 itself.

Similarly, the revised manuscript appropriately states that LSA3 was not detected beyond the PVM in liver stages and that export into the hepatocyte remains unresolved. Nevertheless, several statements continue to imply a role for liver stage protein export. At present, the possibility that a domain of LSA3 may face the host-cell side of the PVM remains speculative and is not supported by direct experimental evidence.

The liver stage fitness phenotype is convincing and supports the conclusion that LSA3 contributes to normal liver stage development. However, the current data do not establish the developmental process affected nor connect the phenotype to export beyond the PVM.

I therefore recommend that the manuscript consistently distinguish between (i) demonstrated export of LSA3 during blood stage infection and (ii) the unresolved localization and trafficking of LSA3 during liver stage infection. I would also encourage the authors to consider revising the title to better reflect the findings presented, as the current title may be interpreted as demonstrating liver stage export, which has not been shown.

https://doi.org/10.7554/eLife.108524.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The extent to which P. falciparum liver stage parasites export proteins into the host cell is unclear. Most blood-stage exported proteins tested in liver stages were not exported. An exception is LISP2, which is exported in P. berghei but not P. falciparum liver stages. While the machinery for export is present in liver stages, efforts to demonstrate export have so far been mostly unsuccessful. Parasite proteins exported during the liver stage could be presented by MHC and thereby become the target of immune control, an incentive to study liver stage export and identify proteins exported during this stage. However, particularly for P. falciparum, it is very difficult to study liver stages.*
>
> *This work studies LSA3 in P. falciparum blood and liver stages. The authors show that this protein is exported into the host cell in blood stages, but in liver stages, no or only very little export was detected. A disruption of LSA3 reduced liver stage load in a humanized mouse model, indicating this protein contributes to efficient development of the parasites in the liver.*
>
> *The paper also studies the localization of LSA3 in blood stages and uses a known inhibitor to show that it is processed by plasmepsin 5, a protease important for protein trafficking. The work also shows that LSA3 is not needed for passage through the mosquito.*
>
> *Strengths:*
>
> *The main strength of this work is the use of the humanized mouse model to study liver stages of P. falciparum, which is technically challenging and requires specialized facilities. The biochemical analysis of LSA3 localization and processing by plasmepsin 5 is thorough and mostly overcame adverse issues such as a cross-reactive antibody and the negative influence of the GFP-tag on LSA3 trafficking. The mosquito stage analysis is also notable, as these kinds of studies are difficult with P. falciparum. However, there was no evidence for a function of LSA3 in mosquito stages.*

We thank the reviewer for their perspective on the strengths of the study.

> *Weaknesses:*
>
> *The cross-reactivity of the antibody, together with the co-infection strategy, prevents reliable assessment of LSA3 localization in liver stages. Despite this, it seems LSA3 is not exported in liver stages, and the paper does not bring us closer to the original goal of finding an exported liver stage protein.*

> *While the localization analysis in blood stages is well done and thorough, the advance is somewhat limited. LSA3 may be in structures like J dots, but this hypothesis was not tested. Although parasites with a disrupted LSA3 were generated, the function of this protein was not explored. Given that a previous publication found some inhibitory effect of LSA3 antibodies on blood stage growth, a comparison of the growth of the LSA3 disruption clones with the parent would have been very welcome and easy to do. At this point, LSA3 is one more of many proteins exported in blood stages for which the function remains unclear.*
>
> *It might be possible to refine some of the conclusions. The impact on liver stage development is interesting, but which phase of the liver stage is affected, and the phenotype remains largely unknown. The co-infection (WT together with LSA3 mutant) has the advantage of a direct comparison of the mutant with the control in the same liver, but complicates phenotypic analysis if the LSA3 antibody is also cross-reactive in liver stages. This issue adds a question mark to the shown localization and precludes phenotypic comparisons. The authors write that they do not know if the cross-reactive protein is expressed at that stage. But this should be immediately evident from the mixed WT/mutant infection. If all cells are positive for LSA3, there is a cross-reaction. If about half of the cells are negative, there isn't. In the latter case, the localization shown in the paper is indeed LSA3, and morphological differences between WT and LSA3 disruption could be assessed without additional experiments.*

We thank the reviewer for their comments. While the LSA3-C antibody may cross-react with another parasite protein(s) in addition to binding LSA3 itself, we observed no strong evidence that this antibody localized beyond the liver-stage PVM, indicating that LSA3 is likely not targeted to the host cell compartment. We cannot exclude the possibility that a domain of LSA3 faces the hepatocyte lumen from this membrane and thus may be considered exported though follow-up studies are required (and are very challenging) to answer it. The phenotype of the NF54 D*LSA3* mutant generated in this study at the blood stage was underway (by an independent lab in collaboration with us) and we are happy to disclose that the outcomes were recently published (May 2026) in an accompanying manuscript (PMID: 41135800). We completely agree that independently infected humanized mice would be helpful to address further remaining questions around the localization and temporal phenotype for LSA3 essentiality, which again will require follow up studies. In the present study, we intended to address whether LSA3 is important functionally, as this had not been reported.

> *Significance:*
>
> *The conclusion from the paper that "our study presents just the second PEXEL protein so far identified as important for normal P. falciparum liver-stage development and confirms the hypothesized potential of exported proteins as malaria vaccine candidates" is partially misleading. Neither LISP2 nor LSA3 seems to be exported in P. falciparum liver stages, and we can't confirm the potential of vaccines with proteins exported in this stage. LSA3 is still important and may still be the target of the immune response, but based on this work, probably not due to export in liver stages.*

We thank the reviewer for the comment. We would like to emphasize the possibility that proteins localized at the PVM may be considered exported 'if' part or all of the protein (eg, a domain) faces the host cell lumen from the hepatocyte. We have not shown this to be the case for LSA3 or LISP2 but that possibility remains open. Nonetheless, LISP2 is exported (by *P. berghei* liver stages) and LSA3 is exported (by *P. falciparum* blood stages); both are exported proteins.

> ***Reviewer #2 (Public review):***

> *Summary:*
>
> *Immunogenic Plasmodium falciparum proteins that could be targeted to prevent parasite development in the liver are of significant interest for novel anti-malarial vaccine development. In this study, McConville et al evaluate the trafficking and functional importance of LSA3, a protein expressed in the blood and liver stages and previously shown to provide protection in immunized chimpanzees. LSA3 contains a PEXEL motif, but the authors have previously shown that this protein does not appear to be exported beyond the PVM in the liver stage (McConville et al, PNAS 2024). However, LSA3 trafficking and functional importance have not been comprehensively evaluated across stages. In the present study, the authors find that blood stage LSA3 undergoes PEXEL processing, and a portion of the protein is exported into the erythrocyte, where it localizes to punctate structures distinct from Maurer's clefts. Using a knockout mutant, LSA3 is shown to be dispensable for blood and mosquito stages but important to liver-stage development. Collectively, these results validate LSA3 as a liver-stage target and place it among several other PEXEL proteins that display differential trafficking beyond the PVM in the erythrocyte but not the hepatocyte.*
>
> *Strengths:*
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> *The authors present a thorough analysis of LSA3 trafficking in the blood stage. PEXEL processing by Plasmepsin 5 is clearly demonstrated through a combination of mini LSA3-GFP reporters and Plasmepsin 5 inhibitors. Importantly, an LSA3 knockout mutant is used to show that the LSA3-C anti-sera also react with additional, unidentified parasite proteins in the blood stage. Nonetheless, comparison between the WT and KO parasites clearly indicates that a portion of LSA3 is exported into the erythrocyte, which is further supported by protease-protection assays with fractionated iRBCs. This contrasts with the liver stage, where LSA3 does not appear to traffic beyond the PVM, similar to what has been observed for other PEXEL proteins in the rodent malaria model.*
>
> *This study provides the first direct analysis of LSA3 function by reverse genetics, showing this protein is important for liver stage development in chimeric human liver mice. Several PEXEL proteins in P. berghei have been shown to be exported into the host cell in the blood stage, but do not appear to cross the PVM in the liver stage. These observations reinforce that even without detectable export into the hepatocyte, PEXEL proteins play critical roles during liver stage development.*

We thank the reviewer for their feedback regarding the strengths of the paper.

> *Weaknesses:*
>
> *A previous study reported that anti-LSA3 antibodies inhibit blood-stage growth, suggesting a role for LSA3 during erythrocyte infection. While the authors carefully evaluate the LSA3 mutant in mosquito and liver stages, the impact on blood stage fitness is not tested. While the knockout shows LSA3 is not essential in the blood stage, its importance during erythrocyte infection remains unclear.*
>
> *The authors previously reported that anti-LSA3-C signal in the liver stage localizes within the parasite and at the parasite periphery but is not exported into the hepatocyte. In the present study, it is shown that anti-LSA3-C reacts with other parasite proteins beyond LSA3 in the blood stage, and this may also occur in the liver stage. However, since liver-stage IFAs were only performed on samples co-infected with both WT and ΔLSA3 parasites, non-specific anti-LSA3C reactivity at this stage could not be determined, and the localization of LSA3 in the liver stage remains somewhat unclear.*

We thank the reviewer for their comments. The phenotype of the NF54 D*LSA3* mutant generated in this study at the blood stage was underway (by an independent lab in collaboration with us) and we are happy to disclose that the outcomes were recently published (May 2026) in an accompanying manuscript (PMID: 41135800). While the LSA3-C antibody may cross-react with another parasite protein(s) in addition to binding LSA3 itself, we observed no strong evidence that this antibody localized beyond the liver-stage PVM, indicating that LSA3 is likely not targeted to the host cell compartment. We cannot exclude the possibility that a domain of LSA3 faces the hepatocyte lumen from this membrane and thus may be considered exported though follow-up studies are required (and are very challenging) to answer it. We completely agree that independently infected humanized mice would be helpful to address further remaining questions around the localization and temporal phenotype for LSA3 essentiality, which again will require follow up studies. In the present study, we intended to address whether LSA3 is important functionally, as this had not been reported.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
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> *This manuscript provides a comprehensive characterization of the Plasmodium falciparum protein LSA3, combining biochemical, genetic, and in vivo approaches. The authors convincingly demonstrate that LSA3 is expressed during liver stage infection and that disruption of the gene leads to a modest but reproducible reduction in liver stage parasite load in humanized mice.*
>
> *Strengths:*
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> *Their biochemical and cell biological analysis of blood stages provides strong evidence that LSA3 is exported to the infected erythrocyte, and the detailed analysis of its PEXEL motif processing is well executed.*

We thank the reviewer for their comments.

> *Weaknesses:*
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> *The study suggests LSA3 as one of only two known P. falciparum PEXEL proteins contributing to this stage, although there is no evidence for the export beyond the vacuolar membrane. Several key conclusions, particularly regarding antibody specificity, localization in liver stage parasites, and the interpretation of the phenotypic data, are not fully supported by the current experiments.*

We understand the reviewer's points. We agree that there is no evidence provided that LSA3 is targeted beyond the PVM; whether any of the protein faces the hepatocyte cytosol is unknown (and challenging to conduct) but this possibility remains plausible. LISP2- and LSA3deficient liver stages are less fit than parental controls and thus we stand by the conclusion that they are the two so far identified *P. falciparum* PEXEL proteins that are important for liver-stage development.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Line 163 says: "Altogether, this demonstrates that LSA3 is important but not critical for blood stage growth of P. falciparum": this is based on the cited Morita et al., 2017. However, previously LSA3 was considered dispensable based on a knock out in 3D7 (Maier et al., 2008; PMID: 18614010). Given that the authors generated a mutant for this work, it would be straightforward to test growth and clarify the importance of LSA3 in*

> *blood stages. If important, the analysis of the location and transport of LSA3 in blood stages would immediately become more relevant. Maybe the data for this is already in the paper: the number of stage V gams was similar between mutant and control (Figure 4A). If this was calculated from the total number of asexual starting parasitemia, it includes blood stage growth, and it can be assumed that there is no growth defect in the mutant in the blood stages. If the number of stage 5 gams was calculated from the number of committed schizonts/rings, nothing can be said about blood stage growth, and asexual blood stage growth should be tested in specific experiments.*

We thank the reviewer for raising the function of LSA3 in blood stages and agree it was an obvious omission, though for good reason - a separate, collaborative study was underway. While this eLife preprint was in revision, our accompanying manuscript on the blood stage was published, showing the characterization of our NF54 D*LSA3* mutant during blood-stage growth (PMID:41135800). The findings are now summarized and the citation included in the revised version of this preprint.

> *Manuscript line 105: "although, notably, functional characterization of lsa3 deletion mutants has not yet been reported to confirm an important function": at least in blood stages, it was reported to be dispensable, see above. The corresponding study (Maier et al., 2008, PMID: 18614010) could be cited in that context.*

The citation of PMID18614010 and 39913589 have now been added and we thank the reviewer.

> *(2) Some questions central to the conclusions of this paper remain because it was unclear whether the serum did indeed detect LSA3 in the liver or not. It would be easy to check if all cells from the WT/Mutant mix experiment show LSA3 signal (this would mean it cross-reacts) or if only about half are positive (the mutants would be negative if there is no cross-reaction). This would be important to mention for Figure 5 because, at present, it is not known that what is labeled by the LSA3-C antibody in these images is (only) LSA3.*

We thank the reviewer for this point and completely understand. We did check this via microscopy of liver sections co-infected with LSA3 mutant and control liver-stage parasites as we shared the reviewers line of enquiry. Unfortunately we could not detect parasites without LSA3 signal at the 5-day post-infection time point. This type of analysis does sound straightforward on paper but in reality is more challenging owing to several factors i) identifying sufficient individual parasites in an entire liver by microscopy can be challenging and variable from lobe to lobe and mouse to mouse, ii) the number of parasites required for a meaningful statistical analysis is increased due to coinfection of the liver (see Figure 4B as an illustration of this), iii) day 5 is a rather late liver-stage time point and so if there was a growth defect the defective parasites may be very small or sparse, iv) we cannot exclude that the LSA3 antibody may cross-react at the liver-stage, v) definitive conclusions are thus challenging and we feel require individual co-infections to be clear in the future. Nonetheless, the detailed qRT-PCR analyses identify a significant reduction in D*LSA3* parasite liver load on day 5, indicating this protein is important for the human malaria parasite's growth within human hepatocytes.

> *(3) It is also unclear which parasites were imaged in Figure 5. The text of the results states that NF54 liver stages were used, but later: "As we employed a co-infection strategy to assess the essentiality of LSA3 versus NF54 in mice, we could not perform IFAs on individually infected mice in this study to validate the specificity of LSA3-C at the liver-stage". The legend says NF54 sporozoites on day 5 post-infection were used. I suspect it was a WT/mutant mix, in which case the above applies, and in the absence of cross-reactivity, half of the cells should be LSA3-C negative. If this is not the case, the localization in the liver becomes dubious.*

We apologize for the confusion and have corrected this. In Figure 5, we utilized liver sections from NF54-infected humanized mice that were stored at -80 C from a previously published study (McConville et al, PNAS 2024). Ideally, we would validate the specificity of LSA3 antibodies at the liver-stage using liver sections containing only D*LSA3* parasites however the number of mice available was limited and the samples available to us also contained the Control line for qRT-PCR analyses (the co-infection strategy). As mentioned above, we couldn't distinguish between these two strains by IFA at the time point analysed and this precluded us unequivocally validating the LSA3-C specificity in the liver-stage; however it cannot be excluded that the signal observed at the PVM is indeed LSA3. We are currently focusing research efforts on obtaining more humanised mice to answer this.

> *Minor:*
>
> *(1) Introduction: Before the part on the PEXEL motifs, there are almost no references; please add references for all statements.*

We have added references.

> *(2) Figure 1B is unclear regarding which part of the gene was deleted. The system used would permit a complete gene deletion, but the homology flanks seem to be within LSA3. If parts of the gene are left, the 75 kDa on the western blots might be a degradation product arising from both the truncated and the full-length protein. Please clarify in the sketch exactly where the homology flanks are, with respect to the start and stop of the gene.*

The *LSA3* gene was disrupted using the flanks as shown. The DHFR selection cassette comprises its own promoter and terminator such that insertion into the coding sequence completely disrupts expression of the protein thereafter, including the C-terminus within which the LSA3-C antibody binds. The new LSA3-T antibody described in our recently published accompanying manuscript that binds more N-terminally than LSA3-C also does not label the truncated protein. The original 5' and 3' flanks used for integration of the disrupted *LSA3* allele by double cross-over recombination were then looped out into the original knockout plasmid and this was negatively selected against using exogenous 5-fluorocytidine (5-FC) via the suicide gene cassette *CDUP* (cytosine deaminase and uracil phosphoribosyl transferase that also contains a 5' promoter and 3'UTR terminating element) in the construct. These features should provide clarification and have now been indicated in the figure and legend.

> *(3) Line 161: Replace was with were.*

Corrected.

> *(4) Figure 2, 224: Why do the authors think LSA3 must be in the luminal leaflet of the PVM as opposed to the outer leaflet of the plasma membrane?*

Several pieces of evidence combined led us to this conclusion in Figure 2B. i) if LSA3 was on the outer PVM leaflet, it would be substantially degraded in the EQT Pellet + PK fraction but a substantial population remained insensitive to PK, indicating much of the total protein pool was protected by the PVM (and possibly the parasite membrane; PM), ii) yet saponin, which leaves the PM intact, allowed PK to access and almost completely degrade LSA3 (see Saponin Pellet + PK), indicating that a substantial population of LSA3-C is located inside the boundary of the PVM, and this is membrane associated as saponin did not liberate it, rather, it remained in the Saponin Pellet before PK was added, iii) the TX-100 Super fraction confirmed LSA3 is membrane associated, as more is present in the TX-100 Super than the Saponin Super fractions, iv) if LSA3 was inside the PM, the Saponin Pellet fraction should be resistant to PK (as was the case for the cross-reactive band indicated with a red asterisk) but LSA3 (green

asterisk) in the Saponin Pellet was PK sensitive. Altogether, our best conclusion from these data is that LSA3 is likely to be PVM associated with the LSA-C-binding domain facing internal to the PV, and a fraction is also exported beyond the PVM into the erythrocyte.

> *(5) Line 245: GFP core "derived from digestion of the reporter in the food vacuole, which confirmed it was secreted from the parasite". I wonder if the amount of GFP "core" really can be used as evidence for secretion, and its amount can be compared between experiments. Did the author quantify this for the full-length protein to get a proportion per sample?*

Use of GFP core to measure defects in *P. falciparum* GFP reporter secretion has been described previously (for example PMID:23387285 and 35906227). The comparison the reviewer asked for is an interesting and important question: however the control would be to compare the ratio of GFP core to uncleaved in the control lanes as well, which is not possible to do since the full-length protein is digested by plasmepsin V in the native PEXEL versions of the experiments (mLSA3-GFP in the first blot, Vehicle in the second blot) leaving no full-length protein to compare to. It stands to reason that inhibition of N-terminal processing results in less protein removal from the membrane (ER or COPII vesicle or PM) resulting in less secretion out of the parasite for retrograde transport to the food vacuole with cytostomal vacuoles (analogous to plasmepsin II). In the food vacuole, the chimeras are in normal cases digested by proteases back to the GFP core that is resistant to cleavage and evident as GFP core on the immunoblots (PMID:10775264 and 14709539 and 19055692 and 20130643).

> *(6) Figure 3 has the word plasmid in two lanes. In Figure 3E, amend the labelling of the blots.*

We apologize for the formatting error in converting the figures to PDF during the original submission and thank the reviewer for the suggestion. This has now been corrected.

> *(7) Lines 266/271/284: "live IFAs", live immunofluorescence assay. Does this mean an antibody was given to living parasites?*

The correct term is live microscopy and this has been corrected.

> *(8) Does Figure 6A fit with the data in Figure 6B? It seems 6B has a milder phenotype than 6A.*

We thank the reviewer for the question. Yes the data directly correspond to each other and are represented in two ways: Panel A shows the qRT-PCR raw data for liver load of each parasite strain per humanized mouse using a scientific scale on the y-axis. Panel B shows that magnitude of the DLSA3 defect as a percentage of the total liver load per mouse:

% total parasite liver load = (strain 1 or strain 2 liver load) x100

sum of strain 1 + strain 2 liver loads

The intent of showing both data is to convey the correct magnitude of the difference in two ways to assist the reader in understanding the true defect, both are accurate and both are statistically significant. In revision we detected mislabelling of humanized mouse 2 and 3 in the original graphs that has now been corrected and we sincerely thank the reviewer for helping us identify this error.

> *(9) Line 482: Please add references for this debate.*

These have been added.

> ***Reviewer #2 (Recommendations for the authors):***

> *Major Comments:*
>
> *(1) In general, the authors have taken care not to overstate conclusions from their study. Nonetheless, while not technically inaccurate, the title might misleadingly suggest LSA3 is exported in the liver stage (this was my initial impression on reading it until I looked at the data). I suggest the authors revise the title to avoid confusion by clarifying that export was only observed in the blood stage.*

We sincerely appreciate the reviewer's point. As this article was posted as a preprint that has now been cited several times, we have carefully weighed the comment and in the end decided to retain the current title for the above reason.

> *(2) While the ability to generate the ΔLSA3 parasites clearly shows that the protein is not essential in the blood stage, the impact on parasite fitness is never tested but simply assumed (for instance, in lines 163-164: "...this demonstrates that LSA3 is important...for blood-stage growth..."). Do the ΔLSA3 parasites have a fitness defect in the blood stage consistent with the previous GIA data that would support this claim? Since the rabbit anti-LSA3-C antibodies produced by Morita et al did not have GIA activity against the blood stage, it is possible that the GIA observed with the human and mouse antibodies might have been due to reactivity with a different protein. If ΔLSA3 does cause a fitness defect, it would be interesting to know if the endogenous GFP-tagged line, which alters protein trafficking/membrane association, also produces this effect.*

We agree with the reviewer and would like to clarify that this omission was not intended to create confusion but was by design, due to a separate collaborative study that was underway to address such questions. While this eLife preprint was in revision, our accompanying manuscript on characterising NF54 D*LSA3* at the blood stage was published (PMID:41135800). The findings are now summarized and the citation included in the revised version of this eLife preprint. In sum, LSA3 is not critical for erythrocyte invasion but its deletion perturbs the rate and efficiency of merozoite invasion, at the step(s) of resealing of the PVM/host cell, resulting in aberrant accole forms that protrude from the infected erythrocyte.

> *(2) Figure 1D: While the images are compelling and I don't doubt the claim that LSA3 is exported in the blood stage (also supported by the fractionation/Pk experiments), the authors should provide quantification of the difference in exported signal between the WT and ΔLSA3 parasites in these IFAs to rigorously support this conclusion. Also, please include details about how many independent experiments are represented by the microscopy data throughout the manuscript (Figures 1, 2, 3, and 5).*

We understand the reviewer's request and wish to indicate that the export signal was absent in all cells infected with D*LSA3* that was imaged. The microscopy performed was from n=2-3 experiments except for Figure 5 which was from n=1 humanized mouse per time point in which multiple EEFs from the liver were imaged. This has been indicated in the figure legends.

> *(3) Careful inspection of the z-series images in Figure 5A shows that most of the LSA3-C signal seen outside the PVM (beyond the boundary delineated by EXP1) is closely associated with DAPI puncta, suggesting these are merozoites. Together with the prominent gap in the EXP1 signal, this suggests the schizont has already ruptured. Thus, anti-LSA3-C signal beyond the PV seems best explained as coming from merozoites or other material released by PV rupture, not from export across the PVM, and this should be added to the text in place of comments about localization to PV extensions or potential export (lines 358-359, 422-423).*

We do appreciate the reviewer's careful eye and caution and are in complete agreement. We have added the comment as requested.

> *Minor Comments:*
>
> *(1) The authors may want to denote the disordered repeat region in the LSA3 schematic in Figure 1A that is mentioned in the text.*

We have added the residue boundaries of the predicted domain from AlphaFold into both the schematic and the text and included a link to the LSA3 pages in PlasmoDB and

AlphaFold in the Methods section.

> *(2) The authors use rabbit anti-LSA3-C antibodies previously generated by Morita et al. These polyclonal antibodies were raised against a recombinant C-terminal region of LSA3 (residues 750-1433), but the schematic in Figure 1A indicates the antibodies recognize a smaller region between residues 1154-1433. Please adjust the figure accordingly, or if this is not the same antiLSA3-C antibody reported by Morita, please provide details about its production.*

The figure is corrected.

> *(3) The authors use Alphafold to identify a region of LSA3 with similarity to the substrate binding domain of DnaK, but the data is not shown. Please include the Alphafold prediction in supplementary figures and provide information about how the predicted structural homology was determined.*

We have added a link to the AlphaFold page for PF3D7_0220000 in the methods.

> *(4) The schematic in Figure 1B indicates that the DHFR cassette was inserted at an internal site within the lsa3 gene. If this is the case, it seems possible that an N-terminal portion of the protein is still expressed, but I was unable to find details about the boundaries of the homology flanks to determine the precise insertion site. Please clarify the knockout strategy and indicate the specific insertion site.*

The *LSA3* gene was disrupted using the flanks as shown. The DHFR selection cassette comprises its own promoter and terminator such that insertion into the coding sequence completely disrupts expression of the protein thereafter, including the C-terminus within which the LSA3-C antibody binds. The new LSA3-T antibody described in our recently published accompanying manuscript that binds more N-terminally than LSA3-C also does not label the truncated protein. The original 5' and 3' flanks used for integration of the disrupted *LSA3* allele by double cross-over recombination were then looped out into the original knockout plasmid and this was negatively selected against using exogenous 5-fluorocytidine (5-FC) via the suicide gene cassette *CDUP* (cytosine deaminase and uracil phosphoribosyl transferase that also contains a 5' promoter and 3'UTR terminating element) in the construct. These features should provide clarification and have now been indicated in the figure and legend.

> *(5) Line 162: I think this should read "antibodies that react with LSA3 were...".*

Corrected.

> *(6) Figure 1D: The merge with the transmitted light channel is missing for the third panel in the ΔLSA3 IFAs. Also, please define the scale bar length in the legend.*

Corrected.

> *(7) Lines 744-746: The IFA fixation panel order description (top, bottom) in the Figure 2A legend is reversed from what is shown in the actual figure. Also, please define the scale bar length.*

Corrected.

> *(8) Lines 184-186: Since the fractionation/PK protection assays suggest most of LSA3 is in the PV, it would be interesting to know if the strong peripheral/PV signal observed in the PFA-fixed IFAs in Figure 2A is also present in the ΔLSA3 parasites, or is this non-specific?*

Thank you for the suggestion. We agree this would be an interesting result to know but do not have the capacity at the present time.

> *(9) Lines 219-225: It is unclear to me why these results are interpreted to suggest that the majority of LSA3 is peripherally associated with the luminal leaflet of the PVM. Wouldn't an integral membrane configuration in the PVM (with the C-terminus facing the host cytosol) or PPM (with the C-terminus facing the parasite cytosol) also account for the data? Adding a carbonate extraction would help clarify this point.*

Several pieces of evidence combined led us to this conclusion in Figure 2B. i) if LSA3 was on the outer PVM leaflet, it would be substantially degraded in the EQT Pellet + PK fraction but a substantial population remained insensitive to PK, indicating much of the total protein pool was protected by the PVM (and possibly the parasite membrane; PM), ii) yet saponin, which leaves the PM intact, allowed PK to access and almost completely degrade LSA3 (see Saponin Pellet + PK), indicating that a substantial population of LSA3-C is located inside the boundary of the PVM, and this is membrane associated as saponin did not liberate it, rather, it remained in the Saponin Pellet before PK was added, iii) the TX-100 Super fraction confirmed LSA3 is membrane associated, as more is present in the TX-100 Super than the Saponin Super fractions, iv) if LSA3 was inside the PM, the Saponin Pellet fraction should be resistant to PK (as was the case for the cross-reactive band indicated with a red asterisk) but LSA3 (green asterisk) in the Saponin Pellet was PK sensitive. Altogether, our best conclusion from these data is that LSA3 is likely to be PVM-associated with the LSA-C-binding domain facing internal to the PV, and a fraction is also exported beyond the PVM into the erythrocyte. If the question is whether LSA3 is an integral PVM protein, we agree that use of carbonate in the future would answer that question.

> *(10) Figures 3D and E: There are some problems with some of the text wrapping in these panels.*

We apologise, this was a formatting issue as the manuscript was converted to PDF.

We have corrected this error.

> *(11) Line 422-423: In fact, the Z-sections shown in Figure 5 appear to indicate that the LSA3-C signal is predominantly located within the parasite, not at the PVM.*

We do appreciate the reviewer's careful eye and caution and are in complete agreement. We have corrected the final conclusion to be more accommodating of this.

> *(12) Lines 468-470: Since cross reactivity of anti-LSA3-C is substantial in the blood stage but was not defined in the liver stage by analysis of unmixed infections, how do the authors know that they were not observing ΔLSA3 parasites in their IFAs? I think what they mean here is that parasites lacking anti-LSA3-C reactivity were not observed, which is an important distinction.*

The reviewer is correct and this has been corrected.

> *(13) Lines 478-479: The authors should also mention that the P. berghei PEXEL proteins evaluated in Fougere et al are exported in the blood stage, similar to LSA3. Moreover, other studies have shown something similar for additional endogenous PEXEL proteins or reporters in P. berghei (PMIDs 22329949, 26347246, 34956312).*

We have added the additional text regarding export into the infected erythrocyte and the reference to IBIS1.

> *(14) Line 491: The data here don't support that LSA3 is "required" for liver stage development, only that it is important to it. Since the authors have not defined the cross-reactivity of anti-LSA3C in unmixed infections, it is not clear that ΔLSA3 parasites are arrested early in the liver stage, only that they show a reduced number of genome copies relative to the parental control.*

We have amended the sentence to “required for normal liver stage development”.

> *(15) Line 530: I think NGF54 should be NF54.*

Corrected.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Antibody specificity in liver stage IFA experiments:*

The specificity of the anti-LSA3 antiserum (LSA3-C) used in liver stage IFA is not fully convincing. While the KO parasites were used effectively to validate specificity in blood stages, the same is not true for liver stages.

> *(a) It is essential to repeat IFA with ΔLSA3 parasites in liver stage infections to determine whether the observed PVM staining is truly specific.*

We appreciate the reviewer’s point, however at a cost of over $5000 per humanized mouse, we do not have the capacity to conduct this experiment at the present time. We highlight that, as the blood stage IFAs confirmed the specificity of LSA3-C for LSA3, the possibility remains open that LSA3 is specifically recognized at the PVM.

> *(b) If the antibody is the same polyclonal serum used in Morita et al. (2017), why did the authors not employ a monoclonal antibody, which they presumably have access to and which would provide greater specificity?*

We have included new data confirming that LSA3 is exported using LSA3-T, in addition to LSA3-C.

> *(c) Given that rabbit antisera often show non-specific staining at the PVM in liver stage parasites, co-localization with PVM markers is not sufficient. Inclusion of the ΔLSA3 parasites in liver stage IFA is critical. It will also show whether there is any cross-reaction of the antiserum in liver stage parasites, as seen by IFA for blood stage parasites.*

We thank the reviewer for their feedback.

> *(d) To validate the serum further, the authors should infect HC-04 cells in vitro with GFP-LSA3 parasites and stain with LSA3-C to confirm overlap between the tagged protein and the antibody signal.*

We thank the reviewer for their feedback.

> *(e) For higher-resolution co-localization, expansion microscopy - now commonly used even in malaria research - would substantially improve the analysis.*

We thank the reviewer for their feedback.

> *(2) The localization of LSA3 in this study differs notably from Morita et al. 2017, who reported localization to dense granules in merozoites and staining in ring-stage parasites at the PVM.*
>
> *(a) The authors confirm DG localization, but they do not examine ring-stage parasites. They should include the IFA of ring stages to clarify whether they can replicate the previous findings.*

We thank the reviewer for their feedback.

> *(b) Additionally, the differences in Western blot banding patterns between the two studies should be addressed. Do the authors have an explanation for these discrepancies?*

We thank the reviewer for their feedback.

> *(3) The authors report a ~40% reduction in liver parasite load using qPCR, which is statistically significant. However, this phenotype is modest and should not be interpreted as showing that LSA3 is essential.*
>
> *(a) Please avoid terms like "required" or "essential" and instead describe the protein as "contributing to normal development" or "influencing fitness."*

We have used the term “required for normal liver stage development”.

> *(b) Since the authors generated liver sections, they should take advantage of these to quantify the number and size of liver stage parasites, which would help determine whether the phenotype reflects fewer infected cells or reduced parasite growth.*

We did check this via microscopy of liver sections, but all mice were co-infected with LSA3 mutant and control liver-stage parasites, as we shared the reviewers line of enquiry. Unfortunately we could not detect parasites without LSA3 signal at the 5 day post infection time point. This type of analysis does sound straightforward on paper but in reality is more challenging owing to several factors i) identifying sufficient individual parasites in an entire liver by microscopy can be challenging and variable from lobe to lobe and mouse to mouse, ii) the number of parasites required for a meaningful statistical analysis is increased due to coinfection of the liver (see Figure 4B as an illustration of this), iii) day 5 is a rather late liver-stage time point and so if there was a growth defect the defective parasites may be very small or sparse, iv) we cannot exclude that the LSA3 antibody may cross-react at the liver-stage, v) definitive conclusions are thus challenging and we feel require individual co-infections to be clear in the future. Nonetheless, the detailed qRT-PCR analyses identify a significant reduction in D*LSA3* parasite liver load on day 5, indicating this protein is important for the human malaria parasite’s growth within human hepatocytes.

> *(c) It would also be valuable to include IFA from singly infected ΔLSA3 livers (rather than co-infected), and possibly at earlier timepoints, to identify the developmental window affected.*

We agree it would be valuable.

> *(4) The manuscript suggests that LSA3 may be exported beyond the PVM into the hepatocyte, based on a small number of peripheral puncta.*
>
> *(a) This claim is not convincingly supported by the data. The punctate signals shown in Figure 5 are weak and may rather reflect PVM extensions or TVN. In fact, one punctum even overlaps with the DAPI signal (figure 5, middle panel), which raises further doubt about the localization.*

We appreciate the reviewer's careful eye and caution and have added the comment regarding DAPI.

> *(b) Given the lack of KO controls in these liver stage IFAs, the authors should not describe LSA3 as "exported beyond the PVM". The language should be revised to reflect that the protein localizes predominantly to the PVM, and any extra-PVM signal remains unconfirmed and could be non-specific.*
>
> *(c) This is especially important given the well-known tendency of rabbit antisera to produce background PVM staining in liver stage parasites.*

Corrected.

> *(e) In an earlier report (McConville et al, 2024, PNAS), they clearly state that LSA3 is NOT exported beyond the PVM. Actually, the staining in the previous report looks quite different from the images provided for Figure 5. The authors might wish to comment on this.*

We thank the reviewer for their feedback.

> *Minor comments:*
>
> *In some sections, the manuscript uses "exported" to refer to trafficking to the PVM. This terminology should be used more carefully and consistently, since "export" often implies translocation into the host cytosol*

We understand that export involves a protein localizing within the host cell and so protrusion through the PVM may also be considered exported, however, we have not confirmed this for LSA3 in liver stages.

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