

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 conclusions about antibody specificity, liver stage localization, and phenotype remain **incomplete**. A key gain is the use of mosquito and humanized mouse models to access life cycle stages rarely studied in most laboratories.

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Abstract

Plasmodium falciparum, which causes the most severe malaria, remodels infected erythrocytes by exporting several hundred 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 unknown; these proteins may serve important biological 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 rarely detected beyond this membrane. Finally, genetic disruption of *LSA3* in *P. falciparum* NF54 attenuated fitness at the liver stage, manifest as a 40% reduction in parasite liver load by day 5 postinfection of humanized mice. The identification of LSA3 as a previously unrecognized member of the *P. falciparum* exportome, essential for normal liver-stage development and capable of eliciting

protective pre-erythrocytic immunity, confirms the hypothesized potential of exported proteins as promising malaria vaccine candidates, underscoring the need for continued investigation into their discovery and biological characterization.

Introduction

Plasmodium species are protozoan protists responsible for more than 247 million cases of malaria and >619,000 deaths each year (WHO, 2024 [↗](#)). While major progress has been made in reducing the incidence of malaria over the last decade, these advances are threatened by the development of resistance to front-line antimalarials, such as the artemisinin combination therapies. The development of new therapeutics and improved vaccines requires a better understanding of strategies used by these parasites to survive and proliferate in the human/mammalian host, as well as the mosquito vector. These include mechanisms employed to inhabit specific host cell types and evade the immune system, especially in the liver, as well as the ability of parasites to acquire sufficient nutrients to sustain their extraordinary growth rates in the liver for onward transmission. The liver-stage is currently the target of the only approved vaccines to treat malaria, though they offer 36-70% efficacy, highlighting the importance of continuing research into the immunogenicity of this stage.

Human infections are initiated by *Plasmodium* sporozoites injected into the skin by an infected mosquito that migrate to the liver and develop asymptomatically within hepatocytes for approximately one week. Liver merozoites subsequently exit the liver and initiate cycles of infection in red blood cells (RBC) that are responsible for the clinical manifestations of malaria. Both the liver and RBC stages of *Plasmodium* replicate within a unique parasitophorous vacuole membrane (PVM) and have evolved mechanisms to remodel their host cells. During the *P. falciparum* blood stage, this is achieved by the export of parasite proteins to or across the PVM into the infected erythrocyte. For many exported proteins, their export is dependent on the *Plasmodium* export element (PEXEL) / host targeting (HT) motif consisting of the pentameric sequence (RxLxE/Q/D) (Hiller et al, 2004 [↗](#); Marti et al, 2004 [↗](#)). The PEXEL is recognized by the endoplasmic reticulum (ER)-resident aspartyl protease plasmepsin V (Boddey et al, 2010 [↗](#); Russo et al, 2010 [↗](#)) that processes the PEXEL between the third and fourth residues (Boddey et al, 2009 [↗](#); Chang et al, 2008 [↗](#)), licensing the matured proteins for export. A subset of PEXEL-negative proteins (PNEPs) is also exported into the erythrocyte (Heiber et al, 2013 [↗](#); Spielmann et al, 2006 [↗](#)). Export of proteins 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 to the erythrocyte, including a complex secretory network and cell autonomous immune responses including interferons, lysosomes and autophagy as well as MHC presentation pathways for recruiting adaptive responses, that 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 effector protein into or across the PVM to diminish host defences remains a largely unanswered question. However, several lines of evidence suggest that a protein export pathway, similar to that characterized in blood-stages, is expressed and functionally important in liver-stage parasites, but with some important differences.

Firstly, components of the PTEX translocon, PTEX150 and EXP2, are expressed at the parasite periphery and are required for normal *P. falciparum* growth in hepatocytes, suggesting export may be important (McConville et al, 2024 [↗](#)). Similarly, the cargo unfoldase HSP101 is reportedly absent from the liver-stage translocon in the rodent malaria parasite *P. berghei* (Kreutzfeld et al, 2021 [↗](#);

Matz et al, 2015 [\[1\]](#)) but has been observed by microscopy at the parasite periphery in *P. falciparum*-infected hepatocytes (Zanghi et al, 2025 [\[2\]](#)). Secondly, multiple PEXEL proteins are expressed by liver-stage parasites (Zanghi et al., 2025 [\[2\]](#)), and LISP2 in *P. berghei* is efficiently exported beyond the PVM into the infected hepatocyte cytoplasm and nucleus (Orito et al, 2013 [\[3\]](#)), although LISP2 was limited to the PVM of *P. falciparum*-infected hepatocytes (McConville et al., 2024 [\[4\]](#)). 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 [\[5\]](#); McConville et al., 2024 [\[4\]](#); Zanghi et al., 2025 [\[2\]](#)). Genetic disruption of PTEX translocon 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 [\[4\]](#)).

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 [\[5\]](#)) and RBCs (Morita et al, 2017 [\[6\]](#)). LSA3 contains an N-terminal putative PEXEL motif (McConville et al., 2024 [\[4\]](#); Morita et al., 2017 [\[6\]](#)), suggesting it may be an exported protein, yet whether the PEXEL is functional has not been definitively proven. LSA3 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. It 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 [\[7\]](#); Perlaza et al, 2008 [\[8\]](#)), although, notably, functional characterization of *lsa3* deletion mutants have not yet been reported to confirm an important function. 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 [\[5\]](#)).

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 sera from infected individuals living in endemic areas, with erythrocyte invasion efficiency reduced by ~24% by antibodies that bound to LSA3 (Morita et al., 2017 [\[6\]](#)). In schizonts, LSA3 localizes to dense granules (DGs) yet it is not known where it localizes earlier during infection. 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 [\[5\]](#); Perlaza et al., 2008 [\[8\]](#)). Immunofluorescence analysis of *P. falciparum* sporozoites and liver-stage parasites developing in monkey livers five days following infection detected LSA3 at the sporozoite surface and at the periphery of the parasite, suggesting it localizes at the PVM (Daubersies et al., 2000 [\[5\]](#)) but no markers have yet been used to validate this location conclusively.

Here, we have undertaken a biochemical and functional study of LSA3 in *P. falciparum* NF54. We demonstrate that LSA3 has a *bona fide* PEXEL sequence that is proteolytically processed by plasmepsin V and is targeted both to the PVM and into the erythrocyte cytoplasm where it forms punctate structures and is therefore a previously unappreciated member of the malarial exportome. Antibodies to LSA3 co-localized with EXP1 and EXP2 at the PVM of infected hepatocytes, though rarely beyond this membrane. Genetic disruption of *lsa3* in *P. falciparum* NF54 caused a reduction of parasite fitness by day 5 of the liver stage in humanized mice; together with LISP2 published recently (Zanghi et al., 2025 [\[2\]](#)), 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.

Results

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

LSA3, encoded by [PF3D70220000](#), is conserved in all primate-infective *Plasmodium* species and contains an N-terminal hydrophobic signal anchor likely for ER entry, a PEXEL motif, disordered or flexible repeat regions and a predicted C-terminal hydrophobic region; AlphaFold also predicts a domain with similarity to the substrate binding domain of the chaperone, DnaK ([Daubersies et al., 2000](#) ; [Morita et al., 2017](#) ; [Prieur & Druilhe, 2009](#)) (**Figure 1A**). The presence of a hydrophobic sequence and PEXEL in the N-terminus suggested LSA3 may be a substrate for the ER-resident aspartyl protease, plasmepsin V, raising the possibility that it is exported ([McConville et al., 2024](#)). Despite initially being discovered as a liver-stage protein ([Daubersies et al., 2000](#)), LSA3 is also expressed in asexual blood stages where it localised to dense granules in schizonts; LSA3-binding antibodies inhibited merozoite invasion by ~24%, confirming the protein is expressed in merozoites but expression in ring and trophozoite stages has not yet been characterized ([Morita et al., 2017](#)).

Given the possibility of being exported and the strong immunogenicity of LSA3, we functionally characterised it by generating transgenic Δ LSA3 clones, in which the *LSA3* gene was disrupted in *P. falciparum* NF54 blood stages by homologous recombination (**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 investigated by immunoblot with antibodies recognizing the LSA3 C-terminus (LSA3-C) ([Morita et al., 2017](#)). Loss of LSA3 protein expression was observed in both knockout clones (**Figure 1C**) and a non-specific band at ~75 kDa was present, which was of parasite origin as it was absent in uninfected RBCs alone. While LSA3 has a predicted molecular weight of 175 kDa, it migrates at a larger size in SDS-PAGE ([Morita et al., 2017](#)).

To further validate the loss of LSA3 expression, we performed immunofluorescence microscopy (IFA) of erythrocytes infected with either NF54 parental or Δ LSA3 E2 parasites 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** , *left*). By contrast, in 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 (**Figure 1D**), likely representing the cross-reactive species observed by immunoblot at ~75 kDa (**Figure 1C**). Interestingly, while antibodies that react with LSA3 was shown previously to reduce merozoite invasion of erythrocytes by ~24% in growth inhibitory assays ([Morita et al., 2017](#)), we were able to generate deletion mutant clones. Altogether, this demonstrates that LSA3 is important but not critical for blood-stage growth of *P. falciparum*, and in addition to DG localization, that LSA3 is exported into the infected erythrocyte, where it 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, and 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 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.

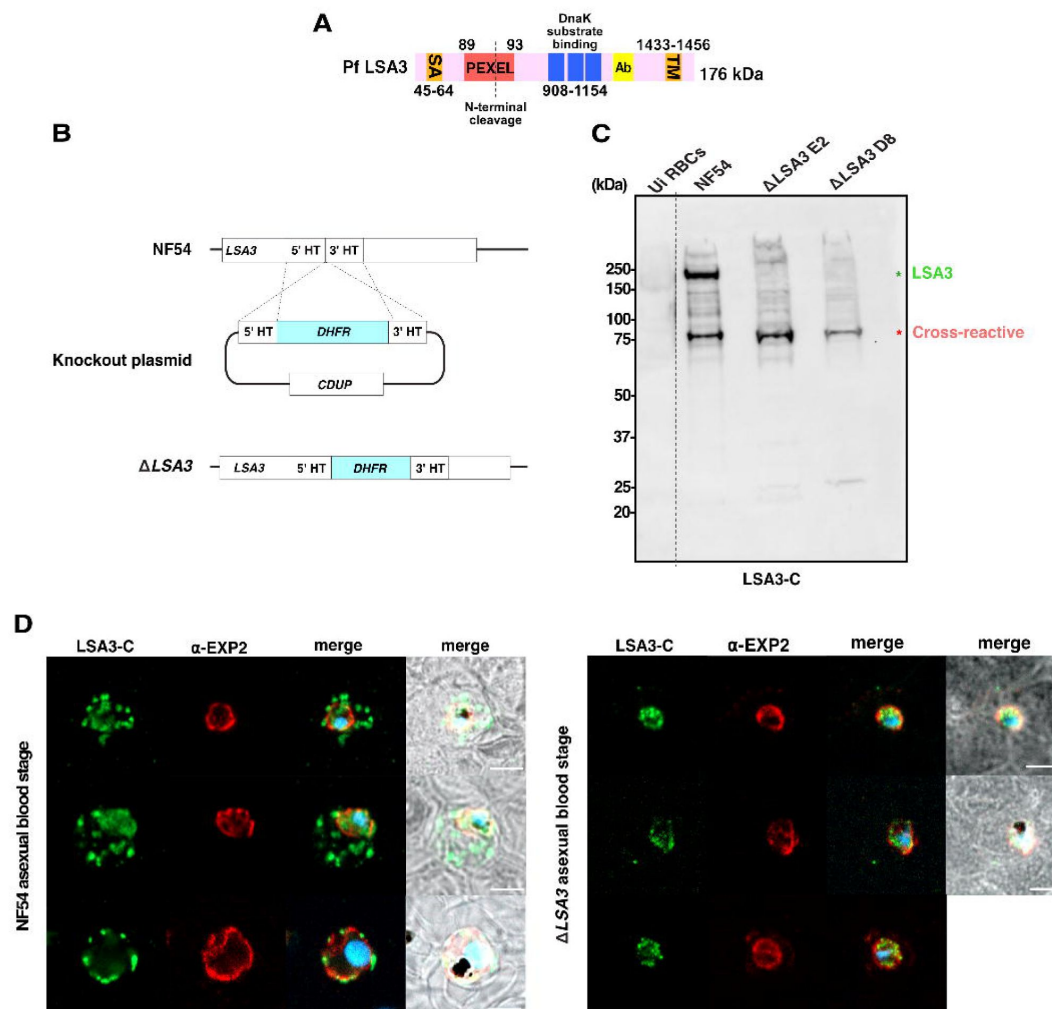


Figure 1.

Genetic disruption of *lsa3* and validation in *P. falciparum* blood-stages.

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 predicted 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 replacement with the DHFR selectable marker cassette flanked by homology targets (HT). **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.** Immunofluorescence microscopy (IFA) of NF54 parental parasites following methanol/acetone fixation and staining with LSA3-C 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.

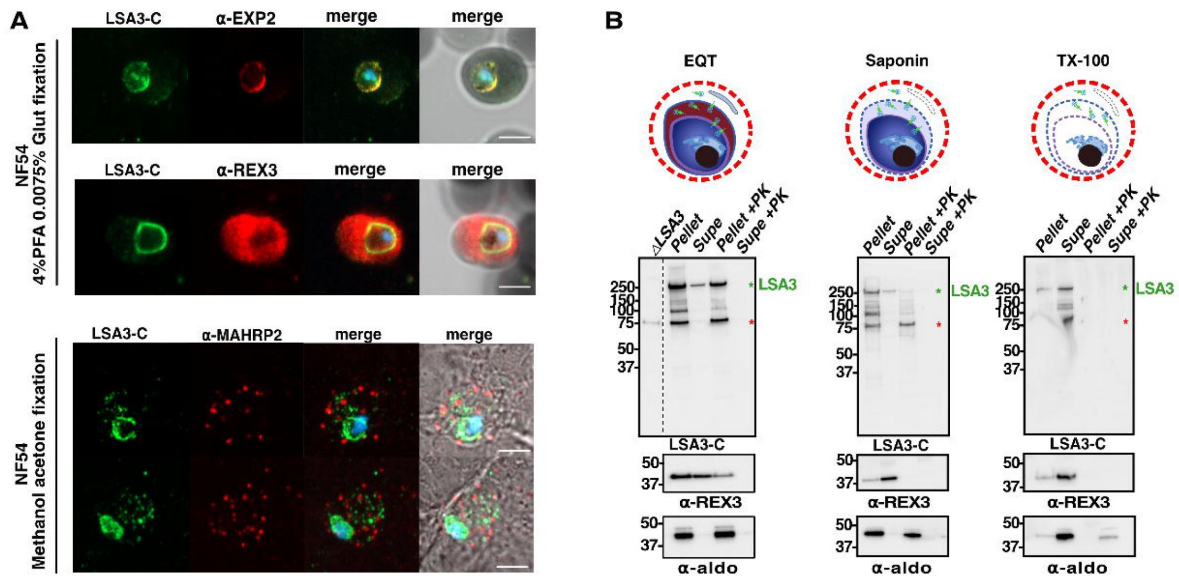


Figure 2.

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

A. Immunofluorescence microscopy of NF54 parental parasites following fixation with methanol/acetone (top) and paraformaldehyde/glutaraldehyde (bottom) staining with anti-LSA3 (LSA3-C) and anti MAHRP2 (Maurer's cleft tethers) antibodies (top). IFA of NF54 parasites following PFA fixation and staining with either anti-LSA3 (LSA3-C), anti-EXP2 or anti-REX3 antibodies (below). **B.** Proteinase K (PK) protection assays to localize LSA3. Erythrocytes infected with NF54 parental parasites were enriched by Percoll centrifugation and permeabilized with either equinotoxin 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.

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

To further understand the export 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, instead the protein had considerable co-localization with EXP2 at the PVM (**Figure 2A**). Therefore, the epitope recognized by LSA3-C was 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*. 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 (**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 supernatant 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 remaining protein in the pellet representing a subpopulation more tightly bound to detergent-resistant structures or incomplete solubilization due to limited detergent accessibility, which was

also observed for aldolase that may be bound as a complex (**Figure 2B**). Altogether these data suggest that LSA3 localizing 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* (Morita et al., 2017) that is conserved in LSA3 from *P. malariae*, *P. ovalis curtisi* and *P. reichenowi* (McConville et al., 2024). 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. These mini genes did not encode the C-terminal sequences recognized by LSA3-C 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 IFAs 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 largely absent confirming the protein was not secreted correctly (**Figure 3B**) consistent with inhibited processing (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 *bona fide* PEXEL, in agreement with its export to the PVM and 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. Live IFAs 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., 2013). Interestingly, while mLSA3-GFP containing the native PEXEL motif was 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 live 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 of GFP

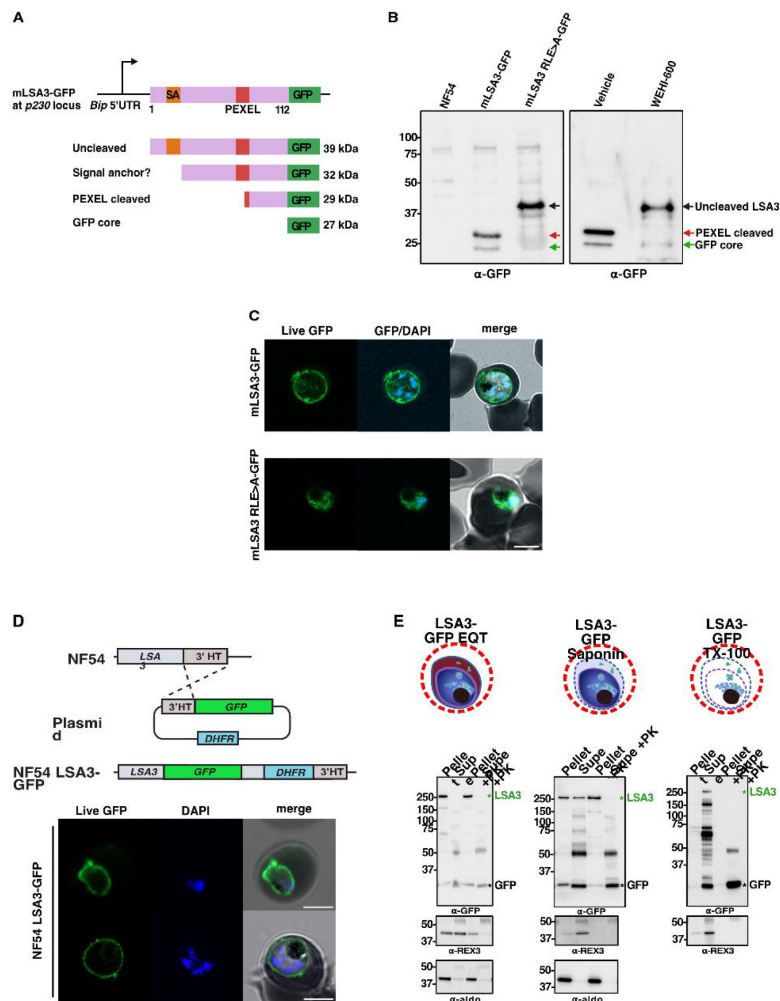


Figure 3.

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 blot 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 μ m. **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 μ m. **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 equinotoxin 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 $^{\circ}$ 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.

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 (Orito et al., 2013). 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 IFAs. Endogenous LSA3-GFP was expressed by blood-stage trophozoites and localized at the parasite periphery, suggestive of the PV / PVM. Interestingly, like the mLSA3-GFP reporter above, no GFP signal was observed beyond the PVM (Figure 3D). This demonstrated by genetic epitope tagging of endogenous *lsa3* 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 the trafficking, export 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 transmission to or development within *Anopheles* mosquitoes

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 for transmission and no significant differences were detected in the formation of stage V gametocytes across parasite lines (Figure 4A). Gametocytes were transmitted to *An. stephensi* mosquitoes by standard membrane feeding assays 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 transmission to or development of *P. falciparum* within mosquitoes or reduce the rate of entry into the salivary glands.

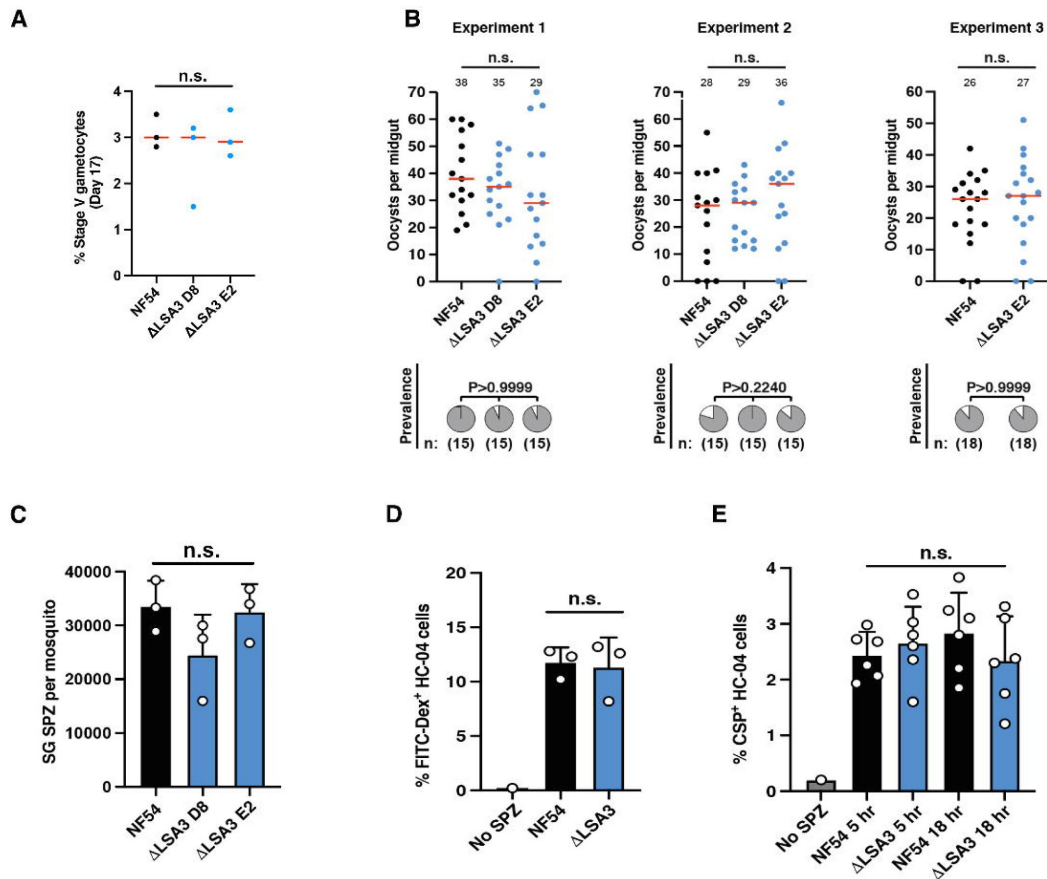


Figure 4.

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 freshly 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 $\pm$ SEM from n=3 (A to D) or n=6 (E) independent experiments; n.s., not significant (P>0.05).

LSA3-deficiency does not perturb cell traversal or invasion of HC-04 hepatocytes

Expression of LSA3 on the surface of *P. falciparum* sporozoites has been reported (Daubersies et al., 2000). To investigate the function of LSA3 in sporozoites, the kinetics of cell traversal and invasion of HC-04 human hepatocytes were analysed. Firstly, cell traversal was investigated by incubating Δ LSA3 clones and 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 and these can be quantified by flow cytometry (Mota et al, 2001; Yang et al, 2017b). Both Δ LSA3 clones and NF54 sporozoites were able to traverse HC-04 hepatocytes at similar rates (Figure 4D) indicating that LSA3 was not critical for sporozoite motility or their capacity to wound human cells, which is a natural process of infection as sporozoites journey from the skin to blood vessels and eventually traverse hepatocytes before productively invading one.

We next investigated whether LSA3 was important for sporozoite invasion of human HC-04 hepatocytes using an invasion assay that quantifies the number of intracellular parasites at different times of incubation for CSP-positive parasites by flow cytometry. 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). 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 sporozoite motility or infection of cultured hepatocytes using this assay (Figure 4E).

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

While LSA3-C specifically recognizes its cognate protein, LSA3, which we validated during blood-stage infection using Δ LSA3 parasites, it also reacts with an off-target protein during RBC infection located within the parasite (this study). We do not know if the cross-reactive protein is expressed during the liver stage, however, LSA3-C localizes at the periphery of liver stage parasites on day 3 postinfection of humanized mice (McConville et al., 2024) in agreement with LSA3 being expressed at the periphery, suggestive of the PVM, in liver schizonts of *P. falciparum*-infected chimpanzees (Daubersies et al., 2000). To better understand the localization of LSA3-C between these timepoints, 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. On day 5 postinfection, LSA3-C antibodies were clearly 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 may be non-specific labelling or LSA3 within PVM extensions or weakly exported (Figure 5A). These results suggest that LSA3 localizes at the parasite periphery including the PVM throughout liver-stage development. 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.

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

LSA3 has very low sequence polymorphism across diverse geographical isolates and is a protective antigen that elicits pre-erythrocytic immunity in chimpanzees and Aotus monkeys (Daubersies et al., 2000; Perlaza et al, 2003), suggesting it may have an important function during liver-stage

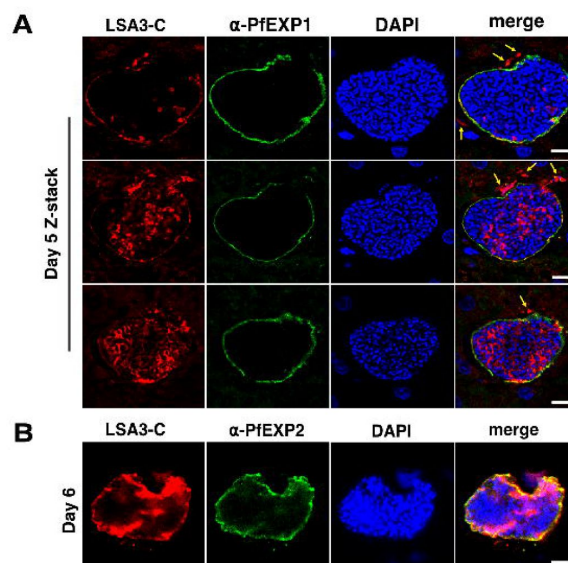


Figure 5.

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 in puncta on PVM extensions or beyond the PVM are indicated (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 μ m.

infection. To investigate this, Δ LSA3 sporozoites were mixed in equal number with parental NF54 sporozoites and inoculated via a single 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 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 reduction in the liver load for Δ LSA3 that averaged a 2.3-fold decline in fitness 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 humanised 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* causes a significant loss of fitness in each of four humanized mice, indicating that this PEXEL-containing protein is required for normal intrahepatic development of the human malaria parasite.

Discussion

Historically, LSA3 was considered a protein expressed only in pre-erythrocytic stages of the *P. falciparum* lifecycle (Daubersies et al., 2000 [↗](#)). More recently, LSA3 was shown to be expressed in DGs of merozoites during invasion of erythrocytes (Morita et al., 2017 [↗](#)). Here, we have further confirmed the expression of LSA3 in blood-stage parasites by endogenous GFP tagging of the *LSA3* gene and performed a biochemical and functional investigation of LSA3 essentiality in *P. falciparum* across the lifecycle, prompted by the presence of a previously uncharacterized PEXEL motif (McConville et al., 2024 [↗](#); Morita et al., 2017 [↗](#)). This revealed that LSA3 is processed by plasmepsin V, which can be inhibited by a small molecule PEXEL-mimetic inhibitor (Nguyen et al., 2018 [↗](#)), and is targeted to the PV, where it is membrane associated. LSA3 is also targeted into the infected erythrocyte where it forms soluble aggregates that were distinct from Maurer's clefts and had a biochemical profile reminiscent of the exported chaperone HSP70-x, suggesting LSA3 could be associated with mobile structures known as J-dots that include chaperone complexes or facilitate interaction of parasite exported effectors (Hanssen et al, 2008 [↗](#); Kulzer et al, 2012 [↗](#); Kulzer et al., 2010 [↗](#); Petersen et al, 2016 [↗](#); Taraschi et al, 2003 [↗](#)). Consistent with this notion, AlphaFold predicts a domain within LSA3 (Jumper et al, 2021 [↗](#)) that has similarity to the substrate binding domain of DnaK, a chaperone with roles in stabilizing unfolded proteins, folding of proteins, and translocation of proteins across membranes. Limitations of our fixation protocol and cross-reactive antibody species so far prevented the definitive localization of LSA3 in the erythrocyte but elucidating this and interacting partners will help define a role for this protein during blood-stage infection and may lend insights into the function of LSA3 during liver-stage development. LSA3 is therefore a previously unappreciated member of the exportome of *P. falciparum* that may function as a molecular chaperone at the parasite periphery and in the host cell.

LSA3 was shown previously to localize in DGs in blood-stage merozoites (Morita et al., 2017 [↗](#)), and this is in good agreement with several exported proteins, including RESA (Boddey & Cowman, 2013 [↗](#)). Following schizont egress, merozoites invade a new erythrocyte and rapidly discharge DGs to establish the PTEX complex at the PVM and to begin exporting proteins previously stored

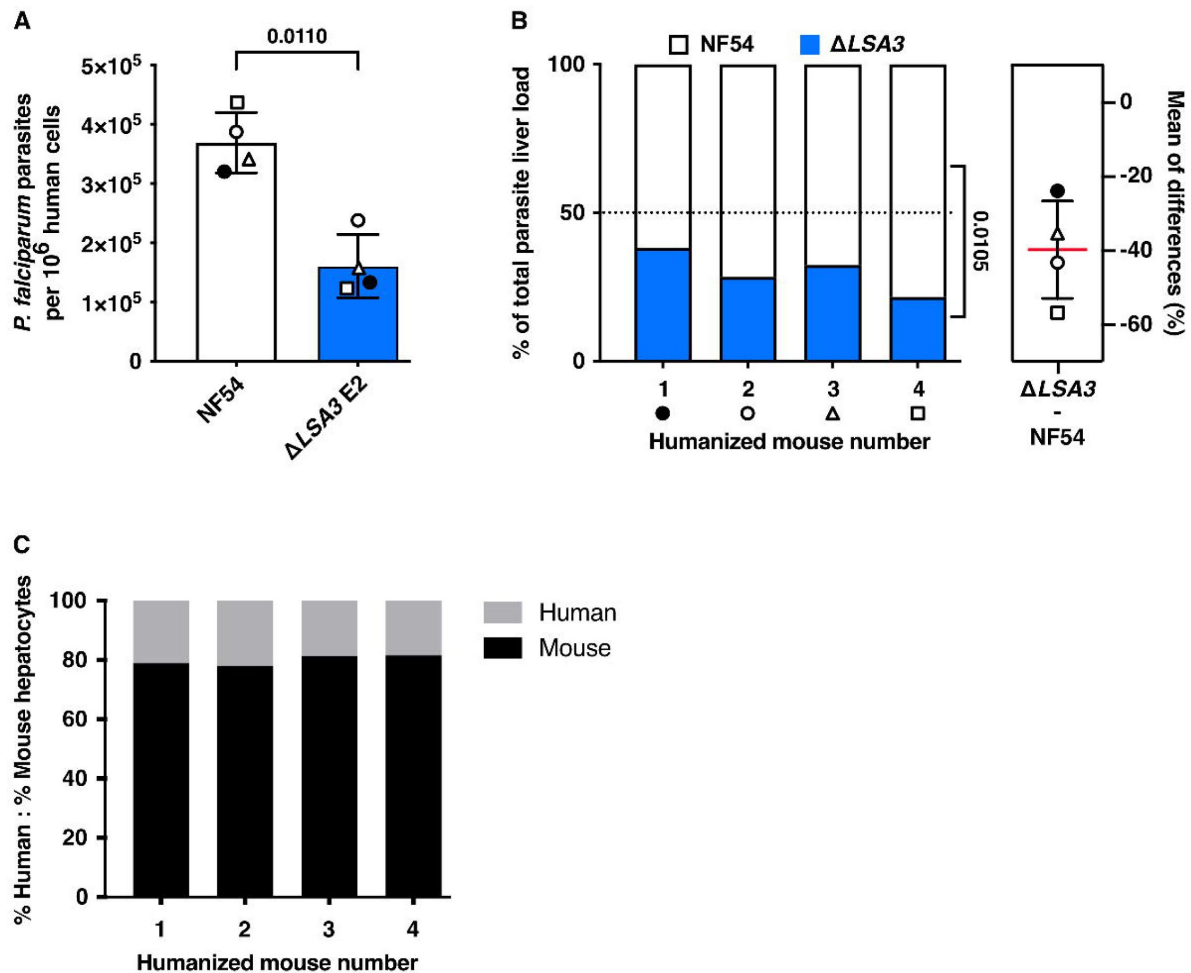


Figure 6.

LSA3 is required 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 $\pm$ SD, statistically analysed by paired *t*-test. Each symbol corresponds to the same coinfecting 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 is shown on the right $\pm$ SD. Each symbol corresponds to the same coinfecting 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.

with them, including RESA (Riglar et al., 2013 [DOI](#)). Therefore, by virtue of its location in DGs and identification recently as a potential blood-stage vaccine target, LSA3 may play a role during or after erythrocyte invasion (Morita et al., 2017 [DOI](#)).

Further work will be required to determine whether LSA3 has similar or distinct functions at the PVM and host cytoplasm or whether the PVM association is a transient trafficking step. LSA3 antibodies localized predominantly at the PVM in infected hepatocytes as well, with very little export beyond this membrane observed using LSA3-C. Interestingly, recent studies have revealed PEXEL processing by plasmepsin V can act as a more general secretory maturase for particular proteins, resulting in their trafficking to invasion organelles like rhoptries or into the PV of the developing blood-stage parasite (Fierro et al., 2024 [DOI](#); Freville et al., 2024 [DOI](#)). The PV is also a terminal destination for proteins of the closely related apicomplexan parasite, *Toxoplasma*, that encodes a similar export element (called TEXEL; RRLxx), that is cleaved by the plasmepsin V ortholog, ASP5, located in the Golgi apparatus (Bougourd et al., 2014 [DOI](#); Coffey et al., 2015 [DOI](#); Hammoudi et al., 2015 [DOI](#)). In *T. gondii*, multiple substrates of ASP5 localise to the PV and are not translocated beyond the PVM (Hsiao et al., 2013 [DOI](#)), whilst others are targeted all the way into the host cell, including the nucleus. It remains unclear with current tools whether a proportion of LSA3 is exported into the hepatocyte, as it is in the erythrocyte.

Trafficking of LSA3 mini-reporter proteins was dependant on processing of the PEXEL motif by plasmepsin V; however, correct targeting was blocked by C-terminal GFP fusions. This was confirmed by GFP-tagging endogenous LSA3, where the protein appeared predominantly retained at the parasite plasma membrane and as a soluble pool in the PV. The negative impact of GFP tagging in malaria pre-erythrocytic biology has been reported previously and can be protein specific (Singer & Frischknecht, 2021 [DOI](#)). This suggests that the conformation of LSA3 is important for trafficking and localization at the PVM and in the erythrocyte.

Relative to infection of erythrocytes, our understanding of *P. falciparum* liver-stage development remains limited. The liver-stage constitutes a critical bottleneck in the parasite's life cycle, and antigens expressed during hepatocyte infection can elicit powerful protective immune responses. This stage therefore presents a prime opportunity to target and eliminate the parasite before it causes clinical disease. However, malaria antigen discovery remains a significant challenge. We postulate that identifying proteins exported during the liver-stage may accelerate antigen discovery and characterization of important biology to refine promising antigens for immune priming in the future. It is thus important to determine which parasite proteins are available for presentation by infected hepatocytes, with exported proteins – those within either the PVM or host cell - being obvious candidates.

LSA3 is well recognised for its immunogenicity and ability to illicit protective immune responses to liver-stage parasites (Daubersies et al., 2000 [DOI](#)), and more recently blood-stage parasites (Morita et al., 2017 [DOI](#)), highlighting its potential as a vaccine candidate. Despite being immunogenic at the liver-stage, the sequence of LSA3 remains largely conserved across diverse geographical isolates, suggesting it has a functionally important role in parasite development. Structural analysis of LSA3 by AlphaFold (Jumper et al., 2021 [DOI](#)) revealed not only a DnaK-like substrate binding domain, but also large unstructured repeat region; previous studies have also identified a large repeat region, with different geographical isolates showing variation in the number of repeats (Priour & Druilhe, 2009 [DOI](#)). Unstructured repeats are common features of other PEXEL proteins exported by *Plasmodium* (Orito et al., 2013 [DOI](#)) and disordered regions are a key feature of exported proteins in Apicomplexa generally (Bougourd et al., 2013 [DOI](#); Dumaine et al., 2021 [DOI](#); Huang et al., 2022 [DOI](#); Rodrigues et al., 2025 [DOI](#); Rosenberg & Sibley, 2021 [DOI](#)).

Despite being an immunogenic protein with a likely important function given the lack of sequence diversity across geographical isolates, the function of LSA3 has not been investigated previously. This study reveals for the first time through genetic disruption, that the expression of LSA3 was

necessary for normal liver-stage development in humanized mice *in vivo*, with attenuation occurring at some point(s) between days 1 and 5 postinfection. IFA of the co-infected humanized mouse livers did not detect Δ LSA3 liver-stages at day 5, suggesting most of these parasites may have been too small to detect or were not present in sufficient numbers to detect in the samples examined, or that LSA3-C was cross-reactive. Future studies will focus on investigating this phenotype using singularly infected humanized mice and looking at earlier time points to determine when LSA3 is necessary for development in the liver.

Previous work localising PEXEL proteins in *P. falciparum* liver-stages has not yet identified translocation of an effector protein beyond the PVM (McConville et al., 2024 [Zanghi et al., 2025](#)), as was observed for the *P. berghei* protein LISP2, which is exported efficiently into the hepatocyte cytosol and nucleus (Orito et al., 2013). LISP2 in *P. falciparum* infected hepatocytes has so far only been detected at the periphery of the liver-stage parasite (McConville et al., 2024 [Zanghi et al., 2025](#)). Several PEXEL-containing proteins also localize to the PV/PVM during hepatocyte infection by the rodent-infective parasite *P. berghei* (Fougere et al., 2016). The lack of clear export into the hepatocyte has created debate as to whether liver-stages export proteins beyond the PVM or, by contrast, down regulate export to reduce MHC presentation of parasite effectors, instead remodelling the PVM to alter and subvert functions of the host cell. If translocation of proteins beyond the PVM is limited during liver-stage development, then the expression of PTEX and a large number of PEXEL-containing proteins during the liver stage (McConville et al., 2024 [Zanghi et al., 2025](#)) may facilitate alterations of the PVM for the parasite's benefit, including to defend against cell-autonomous innate immune attack (Liehl et al., 2014 [Marques-da-Silva et al., 2022](#) [Marques-da-Silva et al., 2025](#) [Prado et al., 2015](#)).

Despite being exported at the blood stage, the LSA3 loss-of-function mutation engineered in this study caused a circa 40% reduction in parasite fitness at the liver-stage, highlighting an important function for LSA3 during hepatocyte infection. LSA3 represents just the second PEXEL protein identified that is required for liver-stage development by *P. falciparum*, with LISP2 being the other protein identified so far, whose genetic disruption caused arrest at a later time point (Zanghi et al., 2025) than for LSA3-deficiency.

Processing of the PEXEL motif of proteins during the blood stage most often results in protein export to the host cytoplasm; in liver-stages, targeting to the PVM is the far more common outcome (Fougere et al., 2016 [McConville et al., 2024](#)). This opens the door for further investigations into the nature and evolution of the PEXEL motif and protein export between these two stages of development and strongly suggests a role for the protein export pathway in influencing the PVM, and possibly beyond this membrane, during the liver-stage. The discovery of LSA3 as a novel component of the *P. falciparum* exportome, crucial for normal liver-stage development and capable of inducing protective pre-erythrocytic immunity, reinforces the notion that exported proteins are promising targets for malaria vaccine development. Defining which parasite proteins are trafficked to the PVM and/or into the hepatocyte may facilitate the identification of novel drug targets and importantly, potential vaccine candidates to help fight malaria.

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% NaHCO₃, 7% heat-inactivated human serum (Melbourne Red Cross), and 3% AlbuMAX (ThermoFisher Scientific). Parasites were maintained at 37 °C in 94 % N₂, 5 % CO₂ and 1 % O₂. Gametocytes were cultured in RPMI-HEPES supplemented with 0.2 %

NaHCO₃, 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*

Highly synchronized schizonts were enriched using 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). Transfections were performed using Amaxa Basic Parasite Nucleofector Kit 2 (Lonza). Purified plasmid DNA (100 µg, Life Technologies) from each plasmid was transfected into *P. falciparum* NF54 and stable transfectants selected and cloned out by serial dilution (Wilde et al, 2019). Integration of plasmid DNA constructs was confirmed by diagnostic PCR. The pCas9-PfLSA3 plasmid was generated using a modified pUF-Cas9 vector that encodes both Cas9 and single guide RNA (sgRNA) (Ghorbal et al, 2014; Volz et al, 2016). The BtgZI adaptor sequence was replaced with the LSA3 guide DNA sequences 5'-CCACCGTTATTAAGATCCAATT -3' and 5'-CCTACTTGTAAGTATGCCTGGAAA -3' (Ghorbal et al., 2014). The LSA3 KO construct was generated by ligating the hDHFR sequence to 5' and 3' homology regions of the NGF54 endogenous LSA3 sequence using primers; respectively. The final construct was sequenced and linearized and then transfected into NF54 *P. falciparum* parasites following standard procedures (Voss et al, 2006). Parasites that had correctly integrated the repair template were selected for after 10 days of incubation with 4 nM WR99210 (WR).

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. 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

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 (1:1000), mouse anti-MAHRP (1:1000), mouse anti-EXP2 (1:500), rabbit anti-LSA3-C (1:1000), in 3% normal donkey serum (NDS/ PBS). Secondary antibodies were goat anti-rabbit Alexa 594 and anti-mouse or anti-rat Alexa 488 (1:1000; Invitrogen). For liver-stage immunofluorescence microscopy: 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), rat anti-EXP1 (1:1000), mouse anti-EXP2 (1:500), diluted in 3% BSA PBS. Secondary antibodies were goat anti-rabbit Alexa 594 and anti-mouse or rat Alexa 488 (1:1000; Invitrogen). 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 mins, washed once with PBS and 4 µL 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 resuspended in 40 μ L PBS + 5 μ L EQTII (0.5mg/ml) per 5 μ L of pellet and incubated at RT for 6 min. The supernatant was collected, the pellet washed in PBS and resuspended 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 μ L 0.03% saponin, incubated at RT for 3 min. Supernatant was collected, the pellet washed in PBS and resuspended 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 resuspended in 45 μ L 0.1% TX-100, incubated at RT for 3 min. Supernatant was collected, the pellet washed in PBS and resuspended 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 [\[link\]](#)). Proteinase K solution (20 μ g/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 (1:1000), mouse anti-REX3 (1:1000), rabbit anti-aldolase (Baum et al, 2006 [\[link\]](#)) (1:4000) followed by horse radish peroxidase-conjugated secondary antibodies (1:1000 (mouse) and 1:4000 (rabbit); Cell Signalling Technology) and viewed by enhanced chemiluminescence (Amersham) (Jennison et al, 2019 [\[link\]](#)).

Generation of the LSA3 minigene

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 [\[link\]](#)) using restriction sites NheI and PstI, for integration into the P230 locus. The resulting plasmid was transfected into 3D7 PEMs schizonts in conjunction with Cas9-U6.2-hDHFR_P230p (50 μ g of each) following standard procedures (Voss et al., 2006 [\[link\]](#)) and correct integration was selected for following addition of 4 nM WR99210 for 10 days.

Plasmeprin 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 μ M of the plasmeprin V inhibitor WEHI-600 (Nguyen et al., 2018 [\[link\]](#)) 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 (1:1000), followed by horse radish peroxidase-conjugated secondary antibodies (1:1000 (mouse) and 1:4000 (rabbit); Cell Signalling Technology) and viewed by enhanced chemiluminescence (Amersham) (Sleebbs et al, 2014 [\[link\]](#)).

Hepatocyte culturing

HC-04 hepatocytes (Sattabongkot et al, 2006 [\[link\]](#)) 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 [\[link\]](#)). 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 [DOI](#)). Briefly, HC-04 hepatocytes (1×10⁵) 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.

Humanised 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 10⁶ 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 [DOI](#); Yang et al, 2017c [DOI](#)). For humanized mice co-infections: 5 x 10⁵ ΔLSA3 clone E2 and 5 x 10⁵ parental NF54 sporozoites (1×10⁶ total) were injected into each of 4 mice by the intravenous route. Livers were isolated 5 days postinfection from CO₂-ethanized mice and individual lobes were cut as described (Lopaticki et al., 2017 [DOI](#)), 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 [DOI](#); Lopaticki et al., 2017 [DOI](#); Yang et al., 2017b [DOI](#)). To specifically differentiate NF54 from

ΔLSA3 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 [DOI](#)). 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: TCCGCTAATTCTACTGTTTCCTCT
- 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: TCAACTACGAACGTTTAACTGCAAC
- 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.

Supplementary figures

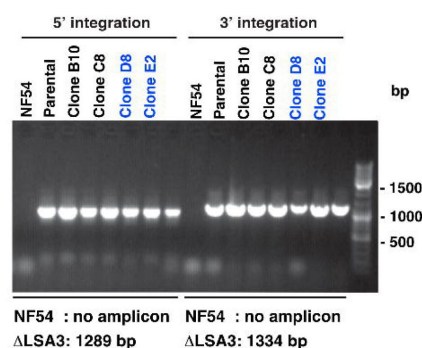


Figure S1.

Genotyping *P. falciparum* NF54 and Δ LSA3 clones.

Diagnostic PCRs (MO545/aw560) confirm correct double cross-over integration of the Δ LSA3 construct in *P. falciparum* NF54 clones. Mutants used in this study are shown in blue and molecular weight sizes in base-pairs (bp) are shown.

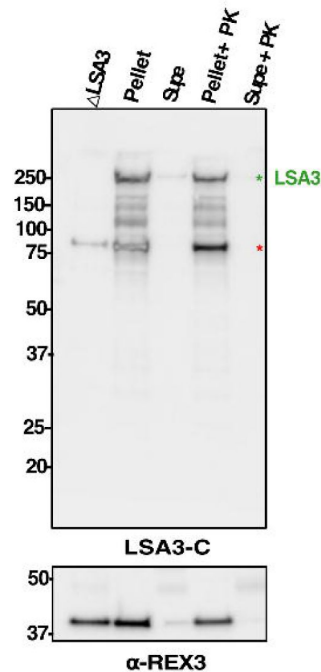


Figure S2.

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.

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Additional information

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.

Data availability

The biological data generated in this study are provided in the Source Data file available in Supplementary Information. Biological tools produced in this study are available from the corresponding author upon request.

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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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.

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.

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.

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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 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.

(2) 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.

Weaknesses:

(1) 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.

(2) 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 Δ LSA3 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.

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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.

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Author response:

We thank all three reviewers for their positive comments and valuable suggestions for improving the manuscript. A detailed blood stage analysis of LSA3-deficient parasites was conducted with, and led by, collaborators at Ehime University in a separate study that is currently in revision at another journal and will be published separately. We intend to cite the complementary publication once it is accepted for publication and to revise the wording in the current manuscript in accordance with suggested feedback. These changes will be reflected in the revised manuscript to be submitted as the eLife Version of Record.

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