Export of malaria proteins requires co-translational processing of the PEXEL motif independent of phosphatidylinositol-3-phosphate binding

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Plasmodium falciparum exports proteins into erythrocytes using the Plasmodium export element (PEXEL) motif, which is cleaved in the endoplasmic reticulum (ER) by plasmepsin V (PMV). A recent study reported that phosphatidylinositol-3-phosphate (PI(3)P) concentrated in the ER binds to PEXEL motifs and is required for export independent of PMV, and that PEXEL motifs are functionally interchangeable with RxLR motifs of oomycete effectors. Here we show that the PEXEL does not bind PI(3)P, and that this lipid is not concentrated in the ER. We find that RxLR motifs cannot mediate export in P. falciparum. Parasites expressing a mutated version of KAHRP, with the PEXEL motif repositioned near the signal sequence, prevented PMV cleavage. This mutant possessed the putative PI(3)P-binding residues but is not exported. Reinstatement of PEXEL to its original location restores processing by PMV and export. These results challenge the PI(3)P hypothesis and provide evidence that PEXEL position is conserved for co-translational processing and export.
Properties, making it more rigid and gaining adhesive properties for parasite survival. This includes alterations of the cell’s physical structural and morphological changes of the host cell central to replication (reviewed in ref. 1). Remodelling results in profound changes of the erythrocyte play an important role in pathogenesis of malaria4.

The properties of P. falciparum-infected erythrocytes are conferred by export of hundreds of parasite proteins. To reach their destination, they traverse membranes of the parasite and parasitophorous vacuole (PV) and, in some cases, the erythrocyte membrane to reach the cell surface. Consequently, the intracellular parasite exports many proteins establishing a trafficking network in the host cell. Most exported proteins contain a signal sequence for entry into the endoplasmic reticulum (ER) followed by 15–30 residues and a motif for Plasmodium export element (PEXEL)/vacular transport signal6–7. The PEXEL is cleaved by the aspartyl protease plasmaspin V (PMV) in the ER, to uncover the export signal for trafficking to erythrocytes1–8–11. The PMV active site appears to be on the ER luminal side12. There are hundreds of proteins with a PEXEL predicted to be exported to the host erythrocyte6,7,13 via the Plasmodium translocon of exported proteins (PTEX) parasite translocon14,15.

Another group of exported proteins lack a PEXEL, the PEXEL-negative exported proteins16,17. This includes skeleton-binding protein 18, membrane-associated histidine-rich proteins 1 and 2 (refs 19,20), ring-exported proteins 1 and 2 (REX1 (ref. 21) and REX2 (ref. 22)), and the MSP-7-related proteins17, as well as P. falciparum erythrocyte membrane protein 1 (PfEMP1)1,17. PfEMP1 is trafficked to the surface of the erythrocyte where it adheres to host receptors on the microvasculature (reviewed in ref. 23). PEXEL-negative exported proteins are not cleaved by PMV and are selected for export through an unknown pathway, but translocated via PTEx1,15.

Recently, a study reported phosphatidylinositol-3-phosphate (PI(3)P) binds PEXEL with high affinity and this phospholipid is concentrated on the ER luminal membrane in P. falciparum24. In addition, PI(3)P binding in the ER, via the PEXEL Arg, is required for export independent of cleavage by PMV24. It was also reported that PEXEL is functionally interchangeable with RxLR of effector proteins from oomycetes that are translocated into host cells24,25. Owing to the implications of these findings, our laboratories have examined key findings of Bhattacharjee et al.24,25. In contrast, we find PEXEL does not bind PI(3)P, this phospholipid is not concentrated in the ER of P. falciparum but on the food vacuole and apicoplast membranes, that the PEXEL is not functionally interchangeable with the RxLR motif of oomycetes and the PEXEL is located at a conserved location to ensure co-translational processing by PMV for export. Therefore, PI(3)P binding of PEXEL in the ER is not the selective mechanism for export of malarial effector proteins to the P. falciparum-infected erythrocyte.

**Results**

**P. falciparum exported proteins do not bind PI(3)P.** It has been suggested that PI(3)P specifically binds PEXEL of exported proteins24. This hypothesis was tested using proteins containing a PEXEL, or with this motif mutated. The amino terminus of exported proteins, histidine-rich protein II (HRPII)26, knob-associated histidine-rich protein (KAHRP)6 and glycoprophin binding protein 130 (GBP130)26,26, which all contain a wild-type or mutant PEXEL motif, were expressed as green fluorescent protein (GFP)/6His fusions (Fig. 1a,b). The N terminus of PfEMP1, which does not have a PEXEL, has also been shown to bind PI(3)P24 and it was expressed fused to GFP/6His (Fig. 1a,b). The p40PX protein, which contains a PX domain that specifically binds PI(3)P, was used as a positive control24.

To determine whether p40PX bound PI(3)P, surface plasmon resonance (SPR) was performed following methods of Battacharjee et al.24. Proteins were flowed over a chip coated with phosphatidylincholine (PC):phosphatidylethanolamine (PE):C16PI(3)P (75:20:5) liposomes (Fig. 1c). As expected, p40PX bound quantitatively to liposomes (Kd 195 nM). HRPII showed very little binding and the control protein HRPIIplexRLE4, which in the PEXEL had been mutated, showed similarly low binding, demonstrating PEXEL of HRPII does not mediate specific binding. Both proteins bound with a similar response to background binding of BSA (Fig. 1c). HRPII-GFP proteins were fluorescent, indicating they were folded, and gel filtration analysis confirmed they were monomers (Supplementary Fig. 1a). Similarly, KAHRPWT, GBP130WT, KAHRPplexRLE4 and GBP130plexRLE4 binding was too weak to quantitate (Supplementary Fig. 1b). Although the purchased p40PX bound quantitatively to PI(3)P (Kd 196 μM), the Req was less than p40PX expressed and purified by ourselves (Fig. 1c). This demonstrated that proteins expressed and purified by our laboratories could bind PI(3)P strongly; however, neither the PEXEL motif nor the surrounding sequences of exported P. falciparum proteins produced specific binding to PI(3)P above background.

As we could not detect specific PEXEL binding to PI(3)P by SPR, we tested other formats. Liposomes containing either PI(3)P or control (PC/PE) were tested for binding in solution24 and proteins sampled by ultracentrifugation. p40PX protein bound strongly to PI(3)P liposomes in HEPES buffer and could be blocked using PBS (phosphate competes with phosphorylated lipids for lipid binding). Some p40PX could be detected in the pellet of control liposomes, suggesting PI(3)P-independent binding (Fig. 1d). Nevertheless, KAHRPW7, KAHRPplexRLE4, GBP13066–88, GBP13084–196 and PIEMPT showed no PEXEL-specific binding. HRPIIT showed binding to PI(3)P and PC/PE liposomes but the HRPII protein, in which the PEXEL was mutated (HRPIIplexRLE4), bound at a similar level and PBS had no effect, providing strong evidence this motif was not responsible for binding. Our results demonstrated no correlation between the presence of PEXEL and specific binding to PI(3)P liposomes, suggesting they do not specifically interact.

To further test whether HRPIIT, HRPIIplexRLE4, KAHRPW7, KAHRPplexRLE4, GBP130WT, GBP130plexRLE4 and PIEMPT bound PI(3)P, they were incubated with PI(3)P-coated beads or controls. The p40PX protein bound strongly to PI(3)P (68% bound) but the P. falciparum proteins showed no binding (Fig. 1e). Similar results were obtained with GBP13066–88, GBP13084–196 and GBP13098–196; confirming these proteins did not bind PI(3)P (Supplementary Fig. 2a).

To analyse whether PEXEL could bind PI(3)P in solution, isothermal titration calorimetry (ITC) was performed with HRPIIT, HRPIIplexRLE4, KAHRPW7, KAHRPplexRLE4 and PIEMPT bound PI(3)P, they were incubated with PI(3)P-coated beads or controls. The p40PX protein bound strongly to PI(3)P (68% bound) but the P. falciparum proteins showed no binding (Fig. 1e). Similar results were obtained with GBP13066–88 and GBP13098–196 confirming these proteins did not bind PI(3)P (Supplementary Fig. 2a).

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Finally, lipid binding was assessed using spot membranes as described previously\(^{28}\). GBP130\textsubscript{66–196} and GBP130\textsubscript{66–196,RLE} > A containing a mutated PEXEL showed binding to PI(3)P, PI(4)P and PI(5)P on membranes; however, the PEXEL mutant also bound, suggesting it was not responsible for the interaction (Supplementary Fig. 2d). Binding to lipid spot membranes can occur by a small proportion of misfolded proteins and the interaction blocked by sub-stoichiometric amounts of DnaK, a chaperone that specifically binds misfolded proteins\(^{28}\). To test whether GBP130 proteins were binding lipids in this way, DnaK was incubated with GBP130\textsubscript{66–196} and this blocked binding (Supplementary Fig. 2e), confirming PEXEL of GBP130 does not specifically interact with PI(3)P, and that misfolded PEXEL proteins interact with lipids.

In summary, we did not detect specific binding of HRPII, KAHRP, GBP130 or PIEMP1 to PI(3)P using methods published previously\(^{24}\), as well as alternative approaches.

**PEXEL and RxLR proteins do not bind PI(3)P.** It was reported that PEXEL protein HRPII binds PI(3)P in the *P. falciparum* ER before cleavage by PMV, as determined by immunoprecipitation and measuring bound lipid by enzyme-linked immunosorbent

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**Figure 1 | Exported *P. falciparum* proteins do not bind PI(3)P.** (a) Recombinant proteins expressed in this study as fusions to GST or GFP/6His. (b) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (c) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 2** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 3** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 5** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 6** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 7** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.

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**Figure 8** | Lipid binding of HRPII, KAHRP, GBP130 or PIEMP1.

(a) Recombinant proteins stained with Coomassie blue stain. Molecular weights (kDa) are shown. (b) Binding of p40PX and HRPII with native PEXEL (WT) or mutant PEXEL (RLE ⇒ A) to PI(3)P liposomes measured by SPR. Binding to control PC/PE liposomes was subtracted to give the sensorgrams\(^{24}\). BSA was used to measure nonspecific binding to the liposome-coated chip (red). Inset: a similar level of low binding of HRPIIWT and HRPIIRLE.
assay (ELISA)\textsuperscript{24}. We followed the same protocol using proteins, to measure bound PI(3)P. We immunoprecipitated (IP) proteins that either had a PEXEL and were cleaved and exported or contained mutations that blocked cleavage and export. We expected PEXEL-cleaved proteins would not co-IP PI(3)P, owing to rapid removal of lipid-binding residues as reported (that is, conserved PEXEL Arg)\textsuperscript{24}; however, if the PI(3)P-binding hypothesis was correct, that proteins containing such N-terminal lipid-binding residues such as KAHRP\textsubscript{38–48} (containing KTLAQ), HRPIIss-AVR3a (containing RLLR) and KAHRP R>K (containing KTLAQ; the PEXEL-like lysine residue in PIEMP\textsubscript{1} previously reported to bind PI(3)P\textsuperscript{24}), would co-IP PI(3)P. However, although PI(3)P ELISA controls were detected, allowing the generation of a standard curve, the quantity of PI(3)P on purified chimeras was minimal and no pattern was evident between PEXEL/RxLR-containing and PEXEL mutant proteins (Supplementary Fig. 3a). One possibility for this discrepancy is Bhattacharjee et al.\textsuperscript{24} used HIV protease inhibitor lopinavir to inhibit PMV, to retain more uncleaved HRPII precursor before IP. Lopinavir is a poor PMV inhibitor and lethal to \textit{P. falciparum}, probably acting on other aspartyl proteases\textsuperscript{10}. To test whether lopinavir affects viability under the conditions described\textsuperscript{24}, we treated parasites and assessed translation. This showed lopinavir had a more potent effect on inhibiting translation than antimalarials, strongly suggesting the parasites were dying (Supplementary Fig. 3b). Under these conditions, it is possible that Bhattacharjee et al.\textsuperscript{24} detected anomalous lipid binding to their reporters.

PI(3)P is present on the food vacuole membrane. Bhattacharjee \textit{et al.}\textsuperscript{24} localized PI(3)P to the inner leaflet of ER membranes in \textit{P. falciparum} by expressing fluorescent reporters fused to PI(3)P-binding domains of two endosomal proteins, p40-phox-PX and early endosomal antigen 1 (EEA1)\textsuperscript{24}. To localize PI(3)P, we made transgenic parasites expressing p40PX fused to GFP (p40PX-GFP) in different parasite subcellular compartments (Fig. 2a,b). The p40PX-GFP protein, lacking a signal sequence, was expressed in the cytoplasm and the fluorescence was concentrated around the food vacuole, as determined by co-localization with \textit{P. falciparum} chloroquine resistance transporter\textsuperscript{28}, and the apicoplast, as determined by co-localization with acyl carrier protein (ACP) (Fig. 2c). This was consistent with previous localization of PI(3)P at the food vacuole and apicoplast\textsuperscript{10}, and the lysosomal membrane and endocytic pathway in other organisms\textsuperscript{35}. To determine whether PI(3)P was present on the luminal side of ER membranes, we fused p40PX-GFP to a signal sequence from STEVOR (STEVOR\textsubscript{ss}-p40PX-GFP) (Fig. 2c)\textsuperscript{36}. This targeted the protein into the ER, where it would be retained bound to PI(3)P as reported\textsuperscript{24}. However, a circumscribed localization around the parasite was observed, indicating it was secreted to the parasite periphery and PV. This was confirmed by the presence of a ‘GFP core’ species by immunoblotting (Fig. 2b), which results from endocytosis from the PV to the food vacuole during haemoglobin uptake; this confirms the chimera was secreted and not ER retained. Experiments using antibodies to the ER resident protease PMV\textsuperscript{33} failed to show co-localization with GFP, indicating the reporter was not retained in the ER (Fig. 2c,e)\textsuperscript{34}. The GFP localization was similar to EXP2 (ref. 34), a marker of the PV confirming secretion. To exclude the possibility that the STEVOR signal sequence influenced this localization, we fused p40PX-GFP to the ACP signal sequence and it trafficked to the PV and was not retained in the ER (Supplementary Fig. 4). This PV localization was also observed when the PI(3)P-binding residue of p40PX was mutated (R58Q, Supplementary Fig. 4). This suggests p40PX did not bind to the inner leaflet of the ER, consistent with a lack of PI(3)P. Only when an SDEL retention signal\textsuperscript{35} was fused to the carboxy terminus of STEVORss-p40PX-GFP did it accumulate in the ER, as shown by co-localization with the ER calcium-binding protein (ERC) (Fig. 2c,e). Therefore, PI(3)P is present on the food vacuole and apicoplast membranes of \textit{P. falciparum} but not within the ER\textsuperscript{24}.

To further assess the location of PI(3)P in \textit{P. falciparum}, we expressed Hrs with two FYVE fingers\textsuperscript{36}, in different subcellular compartments of the parasite, using a STEVOR signal peptide and GFP (Fig. 2a,b). When Hrs2xFYVE was expressed in the cytoplasm, Hrs-GFP was concentrated around the food vacuole and apicoplast, consistent with PI(3)P at these membranes (Fig. 2d). However, when it was directed through the ER (STEVORss-Hrs-GFP), the protein co-localized with the ER marker, ERC, similar to when an ER retention signal was added (Fig. 2d,e), and was not secreted to the PV (note the absence of ‘GFP core’ Fig. 2b). When, however, residues needed for PI(3)P binding were mutated, the protein remained in the ER. Therefore, ER retention observed for STEVORss-Hrs-GFP and STEVORss-HRS\textsubscript{255SS}GFP was not due to PI(3)P-specific binding, suggesting another interaction(s) was retaining reporters in the ER, possibly involving unfolded proteins and ER chaperones. These results are consistent with the lack of PI(3)P in the parasite ER.

The RxLR motif cannot mediate export in \textit{P. falciparum}. The RxLR amino acid motif in effectors of oomycetes has been implicated in export to host cells\textsuperscript{27}. The RxLR motif from \textit{Phytophthora infestans} also reportedly allows export from \textit{P. falciparum} and \textit{Plasmodium berghei} into erythrocytes\textsuperscript{24,25}.
Expression of PEXEL in P. infestans proteins may also aid in translocation of chimeric effector proteins. This suggested RxLR was functionally transferable with PEXEL in Plasmodium spp. We therefore tested whether RxLR could mediate export in P. falciparum and if it was a substrate for PMV, as is the PEXEL. To examine this, we used the same constructs reported by Bhattacharjee et al., where the signal sequence of P. falciparum HRP II was fused to 47 or 49 amino acids of the P. infestans proteins, Avirulence protein 3a (AVR3a) or PH001D5, respectively, which contained RxLR in an equivalent position as PEXEL in P. falciparum. These were fused to GFP (HRPIiss-AVR3a and HRPIiss-PH001D5) and expressed in P. falciparum. Both proteins were retained in the parasite and PV, and not exported, in contrast to KAHRP1–96, which contains a PEXEL and was exported.

**Graph and Table Details:**

- **Graph a:** Shows a comparison of GFP localization in different constructs.
- **Graph b:** Displays a bar graph comparing the subcellular localization of different proteins.
- **Table:** Illustrates the subcellular localization of various proteins in P. falciparum.

**Image Descriptions:**

- **Figure 1:** Illustrates the use of different constructs to study protein localization.
- **Figure 2:** Demonstrates the expression of constructs in P. falciparum.

**Key Points:**

- RxLR is functionally transferable with PEXEL in Plasmodium spp.
- RxLR can mediate export in P. falciparum and is a substrate for PMV.
- Constructs with RxLR are retained in the parasite and PV.
- KAHRP1–96, containing a PEXEL, is exported.
**Figure 3** | Oomycete RxLR does not mediate export in *P. falciparum*. (a) KAHRP1–96 is exported to the *P. falciparum*-infected erythrocyte; however, HRPIiss-AVR3a, HRPIiss-PHO01D5, REX3ss-AVH5 and REX3ss-AVR1b are not exported but accumulate in the parasite and PV. Scale bar, 5 μm. (b) Quantification of cells with exported GFP determined using 20 *P. falciparum*-infected erythrocytes per construct (10 cells per experiment, performed twice). (c) Immunoblot with anti-GFP antibodies of each chimera from the tetanolysin pellet (P) and supernatant (S) shows that KAHRP1–96 is exported but RxLR chimeras are not. Tetanolysin inserts pores in the erythrocyte membrane, allowing sampling of the host cell cytosol. Aldolase was included as a control, as described previously. Full-length gels shown in Supplementary Fig. 8. (d) RP-HPLC shows the KAHRP peptide is cleaved when incubated with PMV (red). AVR3a and PH001D5 peptides are not cleaved by PMV. PEXEL/RxLR sequences are labelled green. (e) MS/MS-extracted ion chromatograms of KAHRP and PH001D5 peptides after incubation with PMV shows that KAHRP peptides were cleaved after the PEXEL Leu (GNGSGDSFDFRNKRTL/C0) but PH001D5 was not cleaved.

To further examine RxLR, we made two constructs where the signal sequence of RE3, a *P. falciparum* exported protein, was fused to the RxLR region of *Phytophthora sojae* AVH5 (REX3ss-AVH5) or AVR1b (REX3ss-AVR1b) followed by GFP. When expressed in *P. falciparum*, REX3ss-AVH5 and REX3ss-AVR1b entered the ER and were trafficked to the PV, but not exported (Fig. 3a,b). These data show RxLR from different *Phytophthora* spp. cannot mediate export to the host cell by *P. falciparum.*

Export of PEXEL proteins requires cleavage by PMV. This was confirmed using KAHRP1–96, which was exported into the erythrocyte (Fig. 3a) and the cleaved protein detected in the tetanolysin supernatant (Fig. 3c). Tetanolysin inserts pores in the erythrocyte membrane, allowing sampling of proteins in the host cell cytosol. Cleavage of KAHRP by PMV in *P. falciparum* occurs after the PEXEL Leu. In contrast, HRPIiss-AVR3a, HRPIiss-PHO01D5, REX3ss-AVH5 and REX3ss-AVR1b showed no signal.
in the tetanolysin supernatant, despite evidence of some N-terminal processing, demonstrating these proteins are not exported nor cleaved in a manner competent for export (Fig. 3c). To understand the mechanism, we examined whether RxLR is a substrate for PMV (Fig. 3d,e). Peptides containing RxLR from AVR3a or PH001D5 were incubated with PMV and analysed by mass spectrometry. In contrast to KAHRP peptides that were cleaved after the PEXEL Leu by PMV, neither AVR3a nor PH001D5 peptides were affected. To confirm this, we affinity purified HRPII-PH001D5 from P. falciparum-infected erythrocytes and examined the proteins by mass spectrometry (MS). We detected peptides N-terminal to RxLR and the intact peptide as well (Supplementary Fig. 5). Thus, RxLR is not a substrate for PMV, consistent with it not mediating export in P. falciparum.

Export of PEXEL proteins is not independent of PMV. It was reported that PEXEL proteins HRPII and PfEMP3 can be exported via an alternative mechanism if PEXEL is mutated when four alanines are inserted immediately after the signal sequence. To test this, we generated similar constructs of HRPII with a signal sequence and PEXEL (RLLHE) fused to GFP.

Figure 4 | PEXEL-independent export of PEXEL proteins does not occur in P. falciparum. (a) HRPII 4A is exported to the P. falciparum-infected erythrocyte. HRPII 4A RLE> A and HRPII 4A RLE> A Down are not exported. Scale bar, 5 μm. (b) PfEMP3 4A RLQ> A is not. Scale bar, 5 μm. (c) Quantification of cells with exported GFP determined using 20 P. falciparum-infected erythrocytes per construct (10 cells per experiment, performed twice). (d) Immunoblot with anti-GFP antibodies of chimeras from the tetanolysin pellet (P) and supernatant (S) shows that HRPII 4A and PfEMP3 4A RLQ> A are cleaved at the PEXEL (blue arrow) and exported. Signal peptide-cleaved HRPII 4A was also visible (red spot). HRPII 4A RLE> A, HRPII 4A RLE> A Down and PfEMP3 4A RLQ> A were not exported and bands corresponding to signal peptide-cleaved (various positions possibly due to insertion of four alanines; red spots/arrow) and uncleaved protein (black spots/arrow) are present. Full-length gels shown in Supplementary Fig. 9.
having four alanines inserted immediately after the signal sequence (Supplementary Table 1). HRPII 4A had a PEXEL and was exported (Fig. 4a). However, when the PEXEL was mutated (HRPII 4A RLE > A), no export was observed and it accumulated in the parasite and PV. Mutation of residues C-terminal to the mutant PEXEL (HRPII 4A RLE > A Down)14,45 did not allow export (Fig. 4a,b). The HRPII proteins were analysed by tetanolysin fractionation and immunoblotting. The HRPII 4A protein with a PEXEL was present in the supernatant fraction and processed by PMV, confirming it was exported (Fig. 4d). However, both HRPII 4A RLE > A and HRPII 4A RLE > A proteins were absent from supernatant fractions and not exported, despite evidence of some processing N-terminal to the mutant PEXEL sequence (Fig. 4d). The lack of export of HRPII 4A RLE > A was in contrast to previous work with this construct24.

Similarly, PfEMP31–82 with a PEXEL was exported (Fig. 4b,c) and processed by PMV (Fig. 4d). Cleavage of PfEMP3 by PMV in P. falciparum occurs after the PEXEL Leu10. However, mutation of residues having four alanines inserted immediately after the signal sequence (Supplementary Table 1). HRPII 4A had a PEXEL and was exported (32 kDa; blue arrows), KAHRP16Ala was of a size corresponding to cleavage at the PEXEL and it was exported (32 kDa; blue arrow); however, cleavage was inefficient as bands consistent with signal sequence cleaved (red arrow) and uncleaved (black) species were present in the saponin supernatant, confirming it was exported.

**Spatial positioning of the PEXEL is important for export.** It has been reported that PEXEL proteins can be exported independent of PMV activity by binding PI(3)P. We sought to generate a protein containing a PEXEL not cleaved by PMV, to assess whether it could be exported by an alternative mechanism such as PI(3)P. The PEXEL is located at a conserved position in exported proteins (15–30 amino acids C-terminal to the signal sequence)13 and we investigated the effect of shifting this position towards the N-terminal signal sequence. KAHRP1–96 containing a PEXEL in the native position was efficiently exported (Fig. 5a,b). Deletion of 11 amino acids N-terminal to the PEXEL (KAHRPA38–48) had no negative effect on export, consistent with these amino acids having no essential role (Fig. 5a,b). However, deletion of 16 amino acids abutting the PEXEL (KAHRPA38–53), which placed the motif 3 residues beyond the signal sequence cleavage site after the signal sequence (Fig. 4b–d). In contrast to earlier work24,45, these results with HRPII and PfEMP3 demonstrate export occurs only when PEXEL was present and processed by PMV.

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**Figure 5 | Correct PEXEL positioning is essential for export.** (a) KAHRP1–96 and KAHRPA38–48 are exported to the P. falciparum-infected erythrocyte but KAHRPA38–53 and KAHRPA38–53 RLE > A accumulate in the PV and are not exported. KAHRP16Ala, KAHRP10Ala and KAHRPSAla are exported to the red blood cell. Scale bar, 5 μm. (b) Quantification of export in a. Data are the mean (± s.e.m.) GFP fluorescence intensity in the host cell from 20 cells per construct (20 replicates), shown as a percentage of KAHRP1–96. Data were analysed by t-test (**P < 0.0001 and *P < 0.003, compared with KAHRP1–96). (c) Immunoblot with anti-GFP antibodies of chimeras from the tetanolysin and saponin pellet (P) and supernatant (S). This shows KAHRP1–96 at a size corresponding to cleavage at the PEXEL and it was exported (32 kDa; blue arrow), KAHRPA38–53 and KAHRPA38–53 RLE > E were cleaved to a size corresponding to cleavage by signal peptidase (32.5 kDa; red spots) and were not exported, but were present in the saponin supernatant, confirming they were secreted to the PV, and that KAHRP16Ala was of a size corresponding to cleavage at the PEXEL and it was exported (32 kDa; blue arrow); however, cleavage was inefficient as bands consistent with signal sequence cleaved (red arrow) and uncleaved (black) species were present in the pellet fraction. HSP70 was used as a control. Full-length gels shown in Supplementary Fig. 10.
(LKC—SNN), determined previously for KAHRP\(^9\), blocked export and the protein was secreted to the PV (Fig. 5a,b). The same PV localization was observed when PEXEL was mutated (KAHRPA\textsubscript{38}–53RLQ>\texttext{A}). Reinstatement of PEXEL to its native position using a 16 alanine spacer (16Ala) restored export, showing that motif positioning, and not primary amino acid sequence N-terminal to the PEXEL, is critical for export. Incorporation of six residues of KAHRP immediately abutting PEXEL (10Ala) did not improve export above 80% of controls; however, inclusion of 11 KAHRP residues abutting PEXEL (5Ala) fully restored export. It is possible that residues in this area assist export by improving the affinity of the substrate for PMV, although not being critical for processing.

To validate export of KAHRP proteins, tetanolysin and saponin fractionation was performed (Fig. 5c). KAHRP\textsubscript{1–96} was present in the tetanolysin supernatant and efficiently processed by PMV. In comparison, KAHRP\textsubscript{A38–53} and KAHRP\textsubscript{A38–53RLQ>\texttext{A}} were absent from the tetanolysin supernatant but present in the saponin supernatant, confirming their localization in the PV but not the erythrocyte. Their identical localization and indistinguishable molecular weight was consistent with cleavage by signal peptidase (Fig. 5a,c). Conversely, KAHRP16Ala was present as a PMV-cleaved protein in the tetanolysin supernatant, confirming it was exported; however, the presence of higher bands in pellet fractions, which correspond to uncleaved and signal peptide-cleaved forms, determined previously\(^9\) indicate the 16Ala mutant form was cleaved inefficiently by PMV, which may explain why export was slightly less than the control (Fig. 5a,b). Collectively, these results show spatial position of PEXEL at the N terminus of KAHRP is important for export and linked to efficiency of cleavage by PMV. Further studies are needed to confirm this with other exported PEXEL proteins.

**Co-translational PEXEL processing occurs before export.**

A paradox of the PI(3)P hypothesis is that the reported lipid-binding residues of PEXEL\(^24\) are removed by PMV in the ER. We investigated the dynamics of PEXEL cleavage to measure the rate of processing by labelling with \(^{35}\)S-methionine/cysteine. HRPII 4A (Fig. 6a) showed three bands of a size corresponding to uncleaved protein and the signal peptidase and PMV cleavage products just 0.25 min after addition of labelled amino acids (Fig. 6b,c). Mutation of the PEXEL RE>\texttext{A} blocked its cleavage, while the uncleaved and the signal peptide-cleaved bands were still observed, as well as an additional band that probably corresponds to a second signal peptide-cleaved species derived from cleavage at the four alanines after the signal peptide. Similarly, for KAHRP\textsubscript{1–70}, there was rapid appearance of the PEXEL-cleaved protein, which was blocked by mutation of the motif RLQ>\texttext{A} (Fig. 6d,e). The KAHRP\textsubscript{1–50}RLQ>\texttext{A} construct was inefficiently cleaved by signal peptidase (cleavage of KAHRP by signal peptidase occurs at \texttext{S}\texttext{LKC}–\texttext{SNN}\textsubscript{37} and this has been confirmed by MS\(^9\) and did not accumulate, raising the possibility that this protein may not efficiently enter the ER, resulting in reduced protein levels either by stalled translation or proteosomal degradation. This was supported by the absence of signal peptidase-cleaved protein in the 3S blot, which was however detected by immunoblot as this methodology detects protein produced over hours. KAHRP RLQ>\texttext{A} has been shown previously to be cleaved by signal peptidase inefficiently; however, a species that had the signal peptide removed was secreted to the PV\(^9\). Pulse-chase experiments with \texttext{PfEMP3}\textsubscript{1–62} also demonstrated that cleavage by signal peptidase and PMV occurred concomitantly with translation, although the product of PMV cleavage was consistently less abundant, raising the possibility this protein may be less co-translationaly cleaved by PMV than HRPII and KAHRP (Fig. 6f,g)\(^43\). The rapid appearance of PEXEL-cleaved and signal sequence-cleaved species of HRPII, KAHRP and PfEMP3 suggest they are co-translationaly processed by PMV and signal peptidase at the ER membrane. Thus, if the PEXEL was available for binding to PI(3)P for export, the Arg residue involved in binding\(^24\) would be very rapidly removed by PMV.

**Discussion**

Bhattacharjee et al.\(^24,25,45\) have suggested the PEXEL directs export by binding to PI(3)P on the inner ER leaflet, and that the RxLR motif of *P. infestans* can functionally replace PEXEL. They also showed RxLR was not cleaved by PMV and concluded export of PEXEL proteins occurs independent of this enzyme. In this study, we show PEXEL proteins and PfEMP1 do not bind PI(3)P, that this lipid is not present inside the ER, and that RxLR cannot functionally complement the PEXEL in *P. falciparum*. Thus, we find no evidence for PI(3)P in recognition of proteins in the ER for export. Rather, it is evident that *P. falciparum* has evolved PEXEL at a conserved spatial position ensuring co-translational cleavage by PMV at the ER for export.

Consistent with our findings, PMV activity previously has been shown to be essential for export and parasite survival. Expression of catalytic PMV mutants in independent laboratories gave a dominant-negative phenotype that reduced export and slowed parasite growth\(^11,43\). A similar but stronger effect on export and parasite lethality was observed using transition-state PEXEL mimetics that specifically inhibit PMV\(^43,46\). The function of PMV has also been determined showing it cleaves PEXEL proteins in the ER at the Leu residue, to expose an export signal necessary for the next step\(^11,11\). PEXEL appears to be cleaved rapidly, as we could not label uncleaved products even using short time points. Cleavage by signal peptidase was also observed at the earliest time points, suggesting both enzymes act co-translationally at the ER membrane.

Although this model is based on the likelihood that the PMV catalytic domain is in the ER lumen, we cannot completely exclude it associates with the cytoplasmic face and PEXEL cleavage occurs on this side\(^47,48\). This would require that proteins are assembled into secretory vesicles on the outside of the ER and trafficked to the PV where the PTEX translocon is located\(^14,15\).

In the context of KAHRP, it is clear that the sequence around PEXEL is not critical but the distance from the signal sequence is important for export, and this was directly linked to the efficiency of PEXEL processing. The steps in export after PMV are not yet clear; however, the Ac-xE/Q/D revealed by cleavage may be a recognition signal for receptors that link cargo inside the ER to coat proteins on forming vesicles outside the ER membrane\(^19\).

A previous study reported *P. falciparum* recombinant exported proteins bind PI(3)P with high affinity and the conserved PEXEL Arg, or Lys in PfEMP1, was responsible\(^24\). When we tested equivalent PEXEL and PfEMP1 proteins using the methods published by Bhattacharjee et al.\(^24\), we did not detect reproducible binding to PI(3)P. Indeed, consistent binding to PI(3)P by p40PX was dependent on the conditions used. Under such conditions, if binding for a recombinant exported protein was detected, it was neither PEXEL dependent nor PI(3)P specific. If the PEXEL did bind PI(3)P as a conserved mechanism for export in *P. falciparum*, it would be expected to occur for multiple proteins and irrespective of the signal peptide chosen or insertion of four unnatural alanine amino acids beyond the signal sequence; however, this was not observed. Interestingly, binding of GBP130 with either a native or mutant PEXEL to PI(3)P, PI(4)P and PI(5)P was seen using spot assays but our data indicate that this was mediated by a minor population of unfolded GBP130, as 1/20 amount of DnaK could block the
interaction. Similar nonspecific binding to PI(3)P was reported for a P. infestans RxLR protein. This raises the possibility that partially unfolded proteins may have contributed to the PI(3)P binding reported previously. We conclude that some experimental conditions may permit some lipid binding but it is not PEXEL dependent, reproducible across all exported proteins or physiologically relevant. PI(3)P is a product of PI3 kinase and is found on endosomal and lysosomal membranes in mammalian cells and yeast. We and others have localized PI(3)P to the food vacuole and apicoplast membranes in P. falciparum. Bhattacharjee et al. reported this lipid is localized on the inner ER leaflet in a steady state for the first time in a eukaryote. However, there is no obvious gene that encodes an ER-resident PI(3) kinase in P. falciparum and PI(3)P recruitment to the ER in eukaryotes is rare, occurring only transiently during autophagy. Autophagosome formation requires a protein complex but orthologues to some others have localized PI(3)P to the food vacuole and the apicoplast, suggesting PI(3)P is located at these membranes, consistent with its widely conserved role in endosomal trafficking. When p40PX-GFP and Hrs, and their expression in P. falciparum showed accumulation at the membrane of lysosome-like food vacuoles and the apicoplast, suggesting PI(3)P is located at these membranes, consistent with its widely conserved role in endosomal trafficking. When p40PX-GFP was directed into the P. falciparum ER, it was not retained but secreted to the PV. In contrast, when Hrs-GFP was directed into the ER, it remained there but mutation of resides required for PI(3)P binding did not alter this localization. This suggests PI(3)P-independent retention of exogenous PI(3)P-binding biosensors can occur in the P. falciparum ER and may explain

Figure 6 | The PEXEL is cleaved co-translationally. (a) Top: schematic of PEXEL proteins and their cleavage positions. (b) 35S-methionine/cysteine-labelling (35S-Met/Cys) of HRPII 4A and HRPII 4A RLE > A in P. falciparum for the indicated times shows bands corresponding to uncleaved (black arrow), signal peptide-cleaved (red arrow) and PEXEL-cleaved (blue arrow) species. Two signal peptide-cleaved species are present for RLE > A (red arrows), possibly due to insertion of four alanines following the signal sequence. (c) Immunoblot with anti-GFP antibodies of the same membrane. Bands corresponding to uncleaved (black arrow), signal peptide-cleaved (red arrow) and PEXEL-cleaved (blue arrow) species are indicated. (d) 35S-Met/Cys-labelling of KAHRP1–70 and KAHRP1–70-RLQ > A in P. falciparum. Bands corresponding to uncleaved (black arrow), signal peptide-cleaved (red arrow) and PEXEL-cleaved (blue arrow) species are indicated. (e) Immunoblot with anti-GFP antibodies of the same membrane. Note the absence of a signal peptidase cleaved product for KAHRP1–70-RLQ > A in the autoradiography that is present in the immunoblot as the latter reveals protein produced over hours. This shows signal peptidase activity was inefficient. (f) 35S-Met/Cys-labelling of PIEMP3₁–₈₂ in P. falciparum. Bands corresponding to cleavage of the indented signal peptide (red arrow) and PEXEL (blue arrow) are indicated. (g) Immunoblot with anti-GFP antibodies of the same membrane. A PEXEL mutant (PIEMP3₁–₈₂ L > A) was included as a size control. Most protein detected with anti-GFP was present before radiolabelling; thus, quantity is not comparable to 35S-Met/Cys. Incorporation of 35S-Met/Cys into HRPII and KAHRP constructs did not increase appreciably over time, in contrast to PIEMP3. The reason for this is unknown but may be due to saturation of anti-GFP agarose in the former experiments, which was used to enrich the GFP proteins. Full-length gels for all panels shown in Supplementary Fig. 11.

Bhattacharjee et al. used fluorescent proteins fused to EEA1 and p40PX to infer PI(3)P is located in the ER. However, the FYVE finger of EEA1 is a poor PI(3)P sensor, because its binding to this lipid is very weak and occurs via strong co-binding to Rab5:GTP. As Rab5:GTP is located on endosomal membranes, EEA1 provides little information about PI(3)P on non-endosomal membranes, and several publications have advised against using this protein for such purposes. We used p40PX and Hrs, and their expression in P. falciparum showed accumulation at the membrane of lysosome-like food vacuoles and the apicoplast, suggesting PI(3)P is located at these membranes, consistent with its widely conserved role in endosomal trafficking. When p40PX-GFP was directed into the P. falciparum ER, it was not retained but secreted to the PV. In contrast, when Hrs-GFP was directed into the ER, it remained there but mutation of residues required for PI(3)P binding did not alter this localization. This suggests PI(3)P-independent retention of exogenous PI(3)P-binding biosensors can occur in the P. falciparum ER and may explain
These results are important, because a primary conclusion of Bhattacharjee et al. was that the PEXEL motif is directed to the ER through a common mechanism. Indeed, it was reported that the PEXEL directs export to the ER in the presence of PMV cleavage, whereas the RXLR motif is directed to the plasma membrane. However, it is possible that the RXLR motif is present at a conserved distance from the signal peptide that permits co-translational export by PMV, whereas the PEXEL motif is present at a conserved distance from the signal peptide that permits co-translational export by PMV.

In conclusion, we find no reproducible evidence that PI(3)P binding in the ER is the mechanism for export of PEXEL proteins. Rather, the PEXEL is present at a conserved distance from the signal peptide that permits co-translational processing by PMV, to reveal a signal for the next step in export to the human erythrocyte.

Methods

Plasmids for recombinant protein expression. P. falciparum exported proteins (PFL1960w) was codon optimized (Genscript) and cloned into the pET11a vector using NdeI/SacII. The pGBP1303CmycGFP6His vector was purchased from Addgene (19008), deposited by Michael Yaffe. See Supplementary Table 1 for more information.

Recombinant protein expression and purification. Escherichia coli BL21 DE3 was grown in Luria broth with 100 μg mL−1 ampicillin, 50 μg mL−1 kanamycin, 37°C, 200 rpm, and optical density (600 nm) 0.5, induced with 1 mM isopropylthiogalactoside (IPTG) at 30°C for 30 min, and supernatant proteins were collected (11,000 g) and supernatants filtered through 5.0- and 0.8-μm filters (Millipore), and proteins were purified using Ni-NTA sepharose (Roche) in 10 mM chromatography columns.

Binding to liposomes by ultracentrifugation. Liposomes and recombinant proteins were subjected to ultracentrifugation at 75,000 rpm for 20 min at 25°C separately. Liposomes and soluble recombinant proteins were reconstituted to a final concentration of 1 mM total lipids and 5 μM recombinant proteins in either PBS pH 7.5 or 150 mM NaCl, 50 mM Hepes pH 7.5 as buffer. Samples were incubated at 37°C for 20 min, and supernatant proteins were reconstituted by injecting 40 μL of 20 mM CHAPS at a flow rate of 30 μl min−1 then re-coating with liposomes as described above. Reactions were performed for 10 min at 25°C before analysis. Supernatant proteins were collected and mixed with reducing sample buffer, pellets were resuspended, and equal proportions were resolved by SDS–PAGE and visualized with Coomassie blue staining. Bands were quantified using a GS-800 Calibrated Densitometer (Bio-Rad).

Binding to PI(3)P-coated beads by pull down. PI(3)P-coated beads and control beads were purchased from Echelon Biosciences (P-B003 and P-B000) and protein binding was performed according to the manufacturer’s instructions.

Binding to liposomes by SPR. SPR experiments were performed as described by Wada et al. and others using a Biacore 3000 at 25°C. Proteins were coated onto an L1 sensor chip by injecting 100–300 μL of a 0.1 mM total lipid solution at 5 μl min−1 using 150 mM NaCl, 50 mM Hepes pH 7.5 as buffer. Control liposomes were injected over lane 1 and PI(3)P liposomes over lane 2. The amount injected was varied so that the total amount bound (typical equilibrium response of 3,000–6,000 response units) was approximately equal between lanes. Before proceeding, 100 μL of 0.1 mg mL−1 BSA was injected over both lanes, to determine the level of residual nonspecific binding to the chip surface.

Recombinant proteins were passed over the liposome-coated L1 chip at a flow rate of 30 μl min−1 in 150 mM NaCl, 50 mM Hepes pH 7.5 buffer for 3 min at concentrations between 62.5 and 2,000 nM, and allowed to dissociate for 3 min. Binding to control liposomes (lane 1) was subtracted to yield the final sensograms, as described. A pulse of 50 mM NaOH was applied at the end of each run to disassociate any bound protein. The chip surface was regenerated by injecting 40 μL of 20 mM CHAPS at a flow rate of 30 μl min−1 then re-coating with liposomes as described above. Reactions were performed for 10 min at 25°C before analysis. Supernatants were collected and mixed with reducing sample buffer, pellets were resuspended in 100 μL of 1 M DTT, and equal proportions were resolved by SDS–PAGE and visualized with Coomassie blue staining. Bands were quantified using a GS-800 Calibrated Densitometer (Bio-Rad).

Preparation of liposomes. Liposomes were prepared as described by Bhattacharjee et al. and others using a Biacore 3000 at 25°C. Proteins were coated onto an L1 sensor chip by injecting 100–300 μL of a 0.1 mM total lipid solution at 5 μl min−1 using 150 mM NaCl, 50 mM Hepes pH 7.5 as buffer. Control liposomes were injected over lane 1 and PI(3)P liposomes over lane 2. The amount injected was varied so that the total amount bound (typical equilibrium response of 3,000–6,000 response units) was approximately equal between lanes. Before proceeding, 100 μL of 0.1 mg mL−1 BSA was injected over both lanes, to determine the level of residual nonspecific binding to the chip surface.

Recombinant proteins were passed over the liposome-coated L1 chip at a flow rate of 30 μl min−1 in 150 mM NaCl, 50 mM Hepes pH 7.5 buffer for 3 min at concentrations between 62.5 and 2,000 nM, and allowed to dissociate for 3 min. Binding to control liposomes (lane 1) was subtracted to yield the final sensograms, as described. A pulse of 50 mM NaOH was applied at the end of each run to disassociate any bound protein. The chip surface was regenerated by injecting 40 μL of 20 mM CHAPS at a flow rate of 30 μl min−1 then re-coating with liposomes as described above. Reactions were performed for 10 min at 25°C before analysis. Supernatants were collected and mixed with reducing sample buffer, pellets were resuspended in 100 μL of 1 M DTT, and equal proportions were resolved by SDS–PAGE and visualized with Coomassie blue staining. Bands were quantified using a GS-800 Calibrated Densitometer (Bio-Rad).

Binding to PI(3)P-coated beads by pull down. PI(3)P-coated beads and control beads were purchased from Echelon Biosciences (P-B003 and P-B000) and protein binding was performed according to the manufacturer’s instructions. Briefly, a protein was incubated in binding buffer (150 mM NaCl, 0.2% NP40, 10 mM HEPES pH 7.4) with 50 μL of beads in 100 μL total volumes for 1 h at 25°C. Beads were collected in Micro Bio-Spin columns (Bio-Rad), washed with 5 × 1 ml of binding buffer and bound proteins eluted in 25 μL reducing sample buffer for 15 min at 25°C. Eluates were resolved by SDS–PAGE and detected by immuno-blotting with anti-GST (p40PX) or anti-GFP (exported proteins) antibodies. Ten per cent of the unbound fraction volumes was loaded to visualize the proteins.
GBP13066–88 and 140 acids (signal sequence) of STEV OR was released from pARL-STEVOR(1–30) and re-digested with KpnI. Hrs-GFP: DNA encoding 2xFYVE from mouse Hrs using KpnI/XmaI (pSTEV-40-GFPSDEL). ACPss-p40PX: human p40PX (residues 38–53, KAHRP 10Ala and KAHRP 5Ala) was synthesized by Epoch Biolabs or Genscript, respectively. These inserts were cloned KpnI/AvrII into pARL2-GFP using HRS_FYVE_AvrII_F and HRS_FYVE_KpnI_R, and cloned into pSTEV-HRS-GFP using AvrII/KpnI (pHRS-GFP). STEVORss-HRS: DNA encoding the first 30 amino acids (signal sequence) of STEVOR was released from pARL2-GFP using AvrII and cloned in-frame with GFP in pGlux.1 using XhoI/XmaI. p40PX-GFP: DNA encoding STEVORss-Hrs was released from pSTEV-HRS-GFP using KpnI-PX40-rev (blunt-KpnI). The amplicon was cloned into pGFPglns29 digested with BglII, blunted using Klenow, and re-digested with KpnI–GFP. DNA encoding 2xFYVE from mouse Hrs separated by QGQGS was amplified from pGFP-2xFYVE50 using primers HRS_FYVE_AvrII_F/HRS_FYVE_KpnI_R and cloned into pARL2-GFP using AvrII/KpnI (pHRS-GFP). STEVORss-HRS: DNA encoding the first 30 amino acids (signal sequence) of STEVOR was released from pARL2-GFP using AvrII and cloned into pHRS-GFP (pSTEV-40-GFP). STEVORss-p40PX-GFPSDEL: DNA encoding GFP SDEL was amplified from pARL2-GFP using GFP_K_F/GFP_SDEL_X_R and cloned into pSTEV-40-GFP using AvrII/KpnI (pSTEV-40-GFP). ACPss-p40PX human p40PX (residues 1–148) was PCR amplified from human complementary DNA using oligo 5′ACPsP-XP40, which also encodes the first 20 amino acids (signal sequence) of ACP, and oligo 3′KpnI-XP40-rev (blunt-KpnI). The amplicon was cloned into pGFPglns29 digested with BglII, blunted using Klenow, and re-digested with KpnI–GFP. DNA encoding 2xFYVE from mouse Hrs separated by QGQGS was amplified from pGFP-2xFYVE50 using primers HRS_FYVE_AvrII_F/HRS_FYVE_KpnI_R and cloned into pARL2-GFP using AvrII/KpnI (pHRS-GFP). STEVORss-HRS: DNA encoding the first 30 amino acids (signal sequence) of STEVOR was released from pARL2-GFP using AvrII and cloned into pHRS-GFP (pSTEV-40-GFP). STEVORss-HRS:p40PX-GFPsDEL: DNA encoding p40PX-HRS SDEL was amplified from pHRS-p40PX using p40PX-HRS-F and p40PX-HRS-R, and cloned into pSTEV-40-GFP using AvrII/KpnI (pSTEV-40-GFP). PMV-agarose was prepared by mixing 5′HRPIIs-PH001D5, HRPII 4A, HRPII 4A RLE with 25 mM Tris HCl, 150 mM NaCl, 0.25% Igepal, 1 mM EDTA to give a 20% solution. Recombinant proteins were incubated with 5′HRPIIs-PH001D5, HRPII 4A, HRPII 4A RLE for 2 h at 4°C before centrifugation (20,800 g) for 10 min at 4°C. Supernatant fractions were resuspended in ethanol/dry ice bath at the indicated times and stored at −80°C. Samples were solubilized in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and Complete inhibitor cocktail (Roche), and GFP species were immunopurified with α-GFP agarose (MBL; catalogue number D153-8) for 4°C for 1 h. Proteins were resolved by SDS–PAGE, visualized by autoradiography and quantified with a GS-800 Calibrated Densitometer (Bio-Rad).

**Protein labelling IPs and densitometry.** Synchronous early trophozoites were magnet purified on CS columns (Milltenyi Biotech) and incubated in Met/Cys-free medium for 30 min at 37°C before addition of 300 μCi ml−1 1S-35S-Met/Cys (Perkin/ Elmer–40-GFPsDEL). Parasites were incubated at 37°C, pelleted and snap frozen in ethanol/dry ice bath at the indicated times and stored at −80°C. Samples were solubilized in 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and Complete inhibitor cocktail (Roche), and GFP species were immunopurified with α-GFP agarose (MBL; catalogue number D153-8) for 4°C for 1 h. Proteins were resolved by SDS–PAGE, visualized by autoradiography and quantified with a GS-800 Calibrated Densitometer (Bio-Rad).

**Lopinavir experiment.** Synchronous early trophozoites were magnet purified on CS columns (Milltenyi Biotech) and incubated with dimethyl sulfoxide, 50 μM lopinavir, 150 ng ml−1 chloroquine or 100 ng ml−1 artemisinin for 7 h before parasite proteins were radiolabelled as described above, but using 800 μCi ml−1 1S-35S-Met/Cys for 15 min. Pellets were saponin treated (0.15%), snap frozen and stored at −80°C. Samples were solubilized in Laemmi buffer, resolved by SDS–PAGE, visualized by autoradiography and quantified as above.

**P3(3) ELISA assays.** ELISA assays were performed using a P3(3) MAP ELISA kit (Echelon Biosciences, K-2500s) according to manufacturer’s instructions and Bhattacharjee et al.24. Synchronous early trophozoites at 10% parasitaemia were magnet purified on CS columns (Milltenyi Biotech) and incubated in lipid-binding buffer (PBS/C176/C176/C176 Tris HCl pH 7.4, 0.3% NaCl, 0.05% Triton X-100, 1 mM EDTA) with Complete inhibitor cocktail (Roche) by rotating at 4°C for 1 h. Insoluble debris was removed by centrifugation (20,800g) for 10 min at 4°C. GFP species were immunopurified as above, beads were washed with lipid-binding buffer and proteins eluted in 100 mM glycine pH 2.6, 500 mM NaCl. Elutions were immediately neutralized in 100 mM glycine pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.25% Igepal, 1 mM ATP, 50 mM DTT performed per sample. As conducted previously24, proteins were quantified by immunoblotting using mouse α-GFP (Roche; 1:1,000, catalogue number 12-17700) and 1× Odyssey imager (LI-COR Biosciences) followed by horseradish peroxidase-conjugated secondary antibodies (1:2,000; Silenus) and visualized by enhanced chemiluminescence (Amersham).
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Author contributions
J.A.B., S.W., P.v.W., D.R., T.S., J.P., J.J.B. and A.F.C. designed the experiments and wrote the paper. M.T.O. and J.J.B. expressed recombinant proteins. J.J.B. performed SPR and gel filtration. J.A.B. performed liposome centrifugation, PI(3)P bead, lipid spot and proteomic experiments. S.W. and J.J.B. performed IT-C. S.W. and J.A.B. performed the lipid spot experiments. J.P., S.F., T.S., Z.E., D.R., S.L. and J.A.B. localized PI(3)P in both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization. J. Biol. Chem. 275, 3699–3705 (2000).

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