PfCERLI1 is a conserved rhoptry associated protein essential for *Plasmodium falciparum* merozoite invasion of erythrocytes

Benjamin Liffner¹,⁸, Sonja Frölich¹,⁸, Gary K. Heinemann², Boyin Liu³, Stuart A. Ralph³, Matthew W.A. Dixon³, Tim-Wolf Gilberger⁴,⁵,⁶ & Danny W. Wilson¹,⁷

The disease-causing blood-stage of the *Plasmodium falciparum* lifecycle begins with invasion of human erythrocytes by merozoites. Many vaccine candidates with key roles in binding to the erythrocyte surface and entry are secreted from the large bulb-like rhoptry organelles at the apical tip of the merozoite. Here we identify an essential role for the conserved protein *P. falciparum* Cytosolically Exposed Rhoptry Leafllet Interacting protein 1 (PfCERLI1) in rhoptry function. We show that PfCERLI1 localises to the cytosolic face of the rhoptry bulb membrane and knockdown of PfCERLI1 inhibits merozoite invasion. While schizogony and merozoite organelle biogenesis appear normal, biochemical techniques and semi-quantitative super-resolution microscopy show that PfCERLI1 knockdown prevents secretion of key rhoptry antigens that coordinate merozoite invasion. PfCERLI1 is a rhoptry associated protein identified to have a direct role in function of this essential merozoite invasion organelle, which has broader implications for understanding apicomplexan invasion biology.
Malaria, caused by infection with *Plasmodium* spp. parasites, results in >400,000 deaths annually with *Plasmodium falciparum* being responsible for the majority of malaria mortality. *Plasmodium* parasites have a complex lifecycle with human infection beginning with transmission of the liver-cell invading sporozoite from a female Anopheline mosquito to the human host. Thousands of daughter merozoites develop in the liver-stages and are released into the blood stream where they invade erythrocytes. In the case of *P. falciparum*, the blood-stage parasite multiplies over the next 48 h, forming 16–32 merozoites that rupture out of the schizont stage parasite and infect new erythrocytes. This asexual blood-stage of the parasite lifecycle causes all disease symptoms.

Erythrocyte invasion is an essential and highly co-ordinated process that begins with reversible attachment of the merozoite to the erythrocyte surface. The merozoite then reorients such that the apical tip, containing specialised invasion organelles known as the rhoptries and micronemes, contacts the erythrocyte membrane. Invasion ligands are released from the micronemes and rhoptries prior to formation of an irreversible tight-junction that binds the merozoite to the erythrocyte surface. The merozoite then engages an acto-myosin motor complex, anchored in an inner membrane complex (IMC) that lies under the merozoite plasma membrane, to pull the erythrocyte membrane around itself, thus completing invasion.

The merozoite invasion organelles harbour essential antigens that are the targets of therapeutic interventions aimed at preventing blood-stage parasite replication. The contents of micronemes are released first and are responsible for coordinating initial attachment events. The rhoptries, which are the largest of the invasion organelles, adopt a dual club-shape with bulbs further towards the basal side of the merozoite and necks at the apical tip. During invasion, rhoptry neck contents are released first and initiate early interactions with erythrocyte receptors. Rhoptry bulb contents are secreted later in the invasion process and are typically involved in parasitophorous vacuole establishment.

It is unclear what differentiates the rhoptry bulb from the neck, as no membrane separates them. Because the neck and bulb remain differentiated after plasma membrane fusion, it has been hypothesised that the structure of the rhoptry may be supported by a scaffold, but the components of such a scaffold are yet to be elucidated. Secretion of rhoptry contents may be partially coordinated through changes in the organelle’s structure during invasion. The membranes at the tip of the rhoptry neck fuse with the parasite membrane early in invasion and the two rhoptries themselves also fuse beginning at the neck. At the point of erythrocyte entry, the rhoptries fully fuse into a single rhoptry (neck and bulb). The mechanisms leading to rhoptry fusion and secretion are not understood.

Approximately 30 proteins have been linked to the rhoptry. Despite the rhoptries being membrane-bound organelles with dynamic functions during secretion of invasion ligands, there remains only a single protein reported to localise to the luminal face (Rhoptry associated membrane antigen) and two to the cytosolic face (Armadillo repeats only) of the *P. falciparum* rhoptry membrane. Therefore, the drivers of rhoptry secretion and compartmentalisation are unknown. Here we describe a highly conserved *P. falciparum* protein, Pf3D7_0210600 (henceforth named *Plasmodium falciparum* Cytosolic Exposed Rhoptry Leaflet Interacting protein (PICERLI1)), which lies on the cytosolic face of the rhoptry bulb and is essential for rhoptry secretion and merozoite invasion.

### Results

**PICERLI1 is conserved and required for blood-stage growth.** PICERLI1 is a protein of 446 amino acids that is predicted to contain a signal peptide (SP) but no transmembrane domains or glycosphatidylinositol (GPI) anchor (Fig. 1a). The RNA expression profile and predicted SP of PICERLI1 has led to speculation that it has a role in merozoite invasion that could be targeted by vaccines. PICERLI1 is highly conserved among *Plasmodium* spp., with >90% identity between *P. falciparum*, *P. reichenowi* and *P. gaboni* orthologues, >75% amino acid identity across human infecting species (including the zoonotic *P. knowlesi*) and >70% when compared to distantly related species (Fig. 1b). To determine whether this conservation was due to a function essential for blood-stage survival, we attempted to disrupt the *Pcerli1* gene using the selection linked integration-targeted gene disruption system (SLI-TGD) (Supplementary Fig. 1a). Our attempts to disrupt *Pcerli1* were unsuccessful, suggesting PICERLI1 is essential for *P. falciparum* blood-stage growth.

**Loss of PICERLI1 is inhibitory to blood-stage growth.** In order to analyse protein expression levels and function of PICERLI1 during blood-stage development, we made transgenic *P. falciparum* parasites with a C-terminal haemagglutinin tagged (HA) PICERLI1 with control of protein expression achieved through a glucosamine (GLCN) inducible *GlmS* ribozyme knockdown system (PICERLI1*HA*GlmS). The resulting PICERLI1*HA*GlmS parasites were cloned and analysed by PCR to confirm plasmid integration (Fig. 1d); cloned parasites were used in all subsequent experiments. Using lysates prepared from synchronised blood-stage parasite cultures (harvested at rings, early trophozoites, late trophozoites and schizonts) probed with anti-HA antibodies, we determined that HA-tagged PICERLI1 was most highly expressed in late schizont stages, concordant with previously published transcriptomic data.

To validate that the integrated *GlmS* ribozyme could control PICERLI1 protein expression, we treated PICERLI1*HA*GlmS parasites with 2.5 mM GLCN and quantified changes in HA-tagged protein levels using Western blot. Treatment of synchronous parasites with 2.5 mM GLCN for ~44 h from early ring stage led to a >80% reduction in PICERLI1 expression, whereas expression of the loading control EXP2 was unaffected (Fig. 1f; Supplementary Fig. 8c). To determine whether loss of PICERLI1 protein expression affected parasite growth, early ring stage PICERLI1*HA*GlmS parasites were treated with increasing concentrations (0.125 to 5 mM) of GLCN for 48 h and parasite growth assessed the following cycle. GLCN treatment led to dose-dependent growth inhibition, with 5 mM GLCN leading to an approximate 55% reduction in parasite growth (Fig. 1g). At 2.5 mM GLCN, which we found to have minimal non-specific growth inhibitory activity against 3D7 WT parasites even after 96 h of treatment (Supplementary Fig. 2a), PICERLI1*HA*GlmS blood-stage parasite growth was reduced by ~40% after 48 h treatment. PICERLI1’s high level of conservation and being refractory to disruption suggested an essential role in parasite growth with the stage of protein expression indicating it could be involved in erythrocyte invasion.

**PICERLI1 has an important role in merozoite invasion.** In order to assess whether PICERLI1 has a role in merozoite invasion, we co-transfected the PICERLI1*HA*GlmS parasites with a cytosolic green fluorescent protein (GFP) reporter plasmid (PICERLI1*HA*GlmS/GFP) that allowed accurate quantitation of new merozoite invasion events by flow cytometry. Since
PfCERLI1 was expressed only at schizont stages, we limited the amount of time PfCERLI1HAGlmS/GFP parasites were exposed to increasing concentrations of GLCN (0.125 to 5 mM) by only treating from early trophozoite stages (24 hpi) for 24 h before assessing parasite rupture and invasion. There was a dose-dependent inhibition of merozoite invasion (Fig. 2a), which was of similar magnitude to the growth inhibition recorded with longer term treatment, strongly suggesting that knockdown of PfCERLI1 restricts parasite growth by preventing invasion.

To determine whether PfCERLI1 knockdown was inhibiting invasion by interfering with merozoite production, the number of merozoites that fully developed per E64 treated PfCERLI1HAGlmS/GFP schizont was counted using light-microscopy of Giemsa-stained thin smears. There was no difference in the number of merozoites or stage of merozoite development evident between GLCN treated and untreated PfCERLI1HAGlmS/GFP parasites (Untreated 18.9 merozoites per schizont; treated 18.8 merozoites per schizont, p = 0.5; Fig. 2b). Furthermore, flow cytometry
PfCERLI1 knockdown does not inhibit merozoite development but does prevent merozoite invasion. a Flow cytometric detection of GFP-expressing PfCERLI1HA-GM/GFP ring stage parasites after merozoite invasion indicated a direct inhibition of merozoite invasion with protein knockdown (results presented as a % of media control, n = 4 biological replicates). PfCERLI1-GM Growth is replicated from Fig. 1g for direct merozoite invasion (4NATURE COMMUNICATIONS| https://doi.org/10.1038/s41467-020-15127-w | www.nature.com/naturecommunications of total erythrocytes (n = 4 biological replicates). b Mean number of fully segmented merozoites per schizont was determined by microscopy analysis of Giemsa-stained thin blood smears. Smears were blind and counted with each data point representing the mean number of merozoites per schizont pooled from 20 schizonts, n = 3 biological replicates. c The percentage of schizont rupture that occurred over a 6-h window either with, or without, GLCN treatment (n = 3 biological replicates). d The number of free merozoites after GLCN treatment was assessed using flow cytometry, with results presented as % of total erythrocytes (n = 4 biological replicates). e Giemsa-stained PfCERLI1HA-GM schizonts, either GLCN treated or untreated, matured normally in the presence of E64. Scale bar = 2 μm. All error bars = SEM. *p < 0.05, ns = p > 0.05 by unpaired t-test.

Fig. 2 PfCERLI1 knockdown does not inhibit merozoite development but does prevent merozoite invasion. a Flow cytometric detection of GFP-expressing PfCERLI1HA-GM/GFP ring stage parasites after merozoite invasion indicated a direct inhibition of merozoite invasion with protein knockdown (results presented as a % of media control, n = 4 biological replicates). PfCERLI1-HA-GM Growth is replicated from Fig. 1g for direct merozoite invasion (4NATURE COMMUNICATIONS| https://doi.org/10.1038/s41467-020-15127-w | www.nature.com/naturecommunications of total erythrocytes (n = 4 biological replicates). b Mean number of fully segmented merozoites per schizont was determined by microscopy analysis of Giemsa-stained thin blood smears. Smears were blind and counted with each data point representing the mean number of merozoites per schizont pooled from 20 schizonts, n = 3 biological replicates. c The percentage of schizont rupture that occurred over a 6-h window either with, or without, GLCN treatment (n = 3 biological replicates). d The number of free merozoites after GLCN treatment was assessed using flow cytometry, with results presented as % of total erythrocytes (n = 4 biological replicates). e Giemsa-stained PfCERLI1HA-GM schizonts, either GLCN treated or untreated, matured normally in the presence of E64. Scale bar = 2 μm. All error bars = SEM. *p < 0.05, ns = p > 0.05 by unpaired t-test.

When the number of free merozoites between GLCN and non-treated cultures was compared, there were significantly more free merozoites in the GLCN treated cultures (p < 0.05) (Fig. 2d). When the number of lost invasion events (lost ring stage parasitaemia) was subtracted from the number of free merozoites, there was no difference between GLCN treated and untreated parasites (p > 0.99, Supplementary Fig 2c), indicating that the increase of the free merozoite population was proportional to the loss in successful merozoite invasion events. In addition, GLCN treated schizonts appeared morphologically normal when Giemsa-stained (Fig. 2e), suggesting PfCERLI1 knockdown does not result in schizont developmental defects. These data indicate that knockdown of PfCERLI1 is associated with a build-up in the number of free merozoites in culture, a pattern consistent with a possible early invasion defect prior to formation of the tight-junction and engagement of the acto-myosin motor.

We also assessed whether knockdown of PfCERLI1 protein expression could lead to reduced or inhibited growth in merozoites that managed to invade a new erythrocyte. We found that there was no difference between the number of early ring stages progressing through to late trophozoite stages (36 h post-invasion) between GLCN treated and untreated cultures, indicating that PfCERLI1 knockdown merozoites that do invade do not have reduced viability (Supplementary Fig. 2b). The similarity between growth and invasion inhibition, the normal development of merozoites and the build-up of free merozoites in the culture media strongly supports a direct role for PfCERLI1 in erythrocyte invasion.

PfCERLI1 localises to the rhoptry bulb. Confocal microscopy of immuno-labelled schizonts was used to assess the spatial positioning of HA-tagged PfCERLI1 relative to the micronemal marker Cysteine-rich protective antigen (CyRPA), inner membrane complex marker glideosome-associated protein 45 (GAP45), rhoptry neck marker rhoptry neck protein 4 (RON4) and rhoptry bulb marker rhoptry-associated protein 1 (RAP1) (Fig. 3a). Using thresholded Pearson’s correlation coefficient to calculate relative spatial proximity between compartments, semi-quantitative colocalisation analysis indicated that the signal of PfCERLI1 was associated most frequently with the rhoptry bulb marker RAP1 (Fig. 3b). Given the resolution limits of conventional confocal microscopy (~200 nm XY and ~500 nm Z)24 relative to the size of the double-bulbous rhoptries,11,25 this approach was deemed unsuitable for the analysis of PfCERLI1 subcellular compartmentalisation within the rhoptries. To overcome this, we utilised Airyscan super-resolution microscopy, which revealed both PfCERLI1 and RAP1 displaying a double doughnut-shaped bulbous structure (Fig. 3c). This fluorescence pattern is analogous to the canonical structure of the rhoptry bulb and, combined with the close colocalisation between PfCERLI1 and RAP1 suggested that PfCERLI1 localises on or within the rhoptry bulb.

In order to assess whether PfCERLI1 is on the inside or the outside of the rhoptry bulb, we biochemically characterised the location of this protein in relation to the membrane of the rhoptries using a proteinase K protection assay. This assay determines the subcellular location of a protein as determined by its sensitivity to proteinase K after selective permeabilisation of non-organellar membranes with detergents. Since the rhoptry membrane is resistant to the detergent digitonin, proteins that lie inside the rhoptry are protected from proteinase K cleavage. Digitonin lysis of parasite membranes resulted in proteinase K cleavage of PfCERLI1HA as detected by Western blot (Fig. 4a), indicating that PfCERLI1 lies outside the rhoptry and is exposed to the parasite cytosol. By contrast, RAP1, was not degraded significantly by proteinase K treatment, confirming that rhoptry luminal proteins are refractory to digestion. Despite the level of colocalisation between PfCERLI1 and RAP1, this difference in proteinase K sensitivity suggested that PfCERLI1 localised to the cytosolic face of the rhoptry bulb.

Having identified that PfCERLI1 lies on the cytosolic face of the rhoptry bulb membrane, we assessed how HA-tagged PfCERLI1 was bound to the rhoptry membrane through sequential, differential solubilisation of lysed parasite membranes.

The confocal microscopy results indicated that PfCERLI1 was bound to the rhoptry bulb, with the signal for PfCERLI1 associated more frequently with the rhoptry bulb than with the rhoptry neck. This was confirmed by a double-dye super-resolution microscopy approach, where both HA-tagged PfCERLI1 and RAP1 were detected in the rhoptry bulb using the fluorescent dyes Cy5 and Cy3, respectively (Fig. 3c). Additionally, the signal for PfCERLI1 was stronger in the rhoptry bulb than in the rhoptry neck, with the signal for PfCERLI1HA associated more frequently with the rhoptry bulb than with the rhoptry neck (Fig. 3c).

To further characterise the location of PfCERLI1 within the rhoptry bulb, we used a proteinase K protection assay. This assay determines the subcellular location of a protein as determined by its sensitivity to proteinase K after selective permeabilisation of non-organellar membranes with detergents. Since the rhoptry membrane is resistant to the detergent digitonin, proteins that lie inside the rhoptry are protected from proteinase K cleavage. Digitonin lysis of parasite membranes resulted in proteinase K cleavage of PfCERLI1HA as detected by Western blot (Fig. 4a), indicating that PfCERLI1 lies outside the rhoptry and is exposed to the parasite cytosol. By contrast, RAP1, was not degraded significantly by proteinase K treatment, confirming that rhoptry luminal proteins are refractory to digestion. Despite the level of colocalisation between PfCERLI1 and RAP1, this difference in proteinase K sensitivity suggested that PfCERLI1 localised to the cytosolic face of the rhoptry bulb.

Having identified that PfCERLI1 lies on the cytosolic face of the rhoptry bulb membrane, we assessed how HA-tagged PfCERLI1 was bound to the rhoptry membrane through sequential, differential solubilisation of lysed parasite membranes.
Saponin pellets from PfCERLI1HAGlmS schizont-stage parasites were hypotonically lysed (to release non-membrane associated proteins), treated with sodium carbonate (Na$_2$CO$_3$; to release peripheral membrane proteins) and Triton X-100 (Tx100; to release integral membrane proteins). The supernatants containing solubilised proteins with the different treatments, as well as the final Tx100 insoluble fraction (containing covalently lipid-linked proteins$^{26,27}$) were used in Western blots (Fig. 4b). PfCERLI1 was detected primarily in the carbonate treatment, indicating that PfCERLI1 is likely to be peripherally associated with the cytosolic face of the rhoptry bulb membrane.

PfCERLI1 is closely juxtaposed to the rhoptry bulb marker RAP1. Since biochemical analysis showed that PfCERLI1 associated with the cytosolic face of the rhoptry membrane whereas RAP1 appears confined to the rhoptry lumen, we used super-resolution microscopy to quantify the degree of physical proximity between PfCERLI1 and RAP1. To do this, we measured the radial positioning of all voxels that define the compartments of interest and plotted the signal intensities for both PfCERLI1 (anti-HA) staining is defined as the region of interest, with the following merozoite organelle markers: GAP45, CyRPA, RON4, and RAP1. ($^{***}p < 0.0001$ by Analysis of variance (ANOVA), $n = 3$ biological replicates, with 6 merozoite containing schizont images taken from each, error bars = SEM).

Fig. 3 PfCERLI1 localises to the rhoptry bulb of merozoites. a Immunofluorescence microscopy showing 3D reconstructed projections of confocal images with anti-HA (PfCERLI1) in green, colocalising more strongly with the rhoptry bulb marker RAP1 than with the micronemal marker CyRPA, rhoptry neck marker RON4 or the inner-membrane complex marker GAP45. b Quantification of the colocalisation, by Pearson’s correlation coefficient when PfCERLI1 (anti-HA) staining is defined as the region of interest, with the following merozoite organelle markers: GAP45, CyRPA, RON4, and RAP1. ($^{***}p < 0.0001$ by Analysis of variance (ANOVA), $n = 3$ biological replicates, with 6 merozoite containing schizont images taken from each, error bars = SEM). c Maximum intensity projections of super-resolution immunofluorescence microscopy (Airyscan) of PfCERLI1HAGlmS schizonts stained with anti-HA and anti-RAP1 antibodies. Yellow box (top left panel) indicates the zoom area for the free merozoite depicted in the other three panels.
Fig. 4 PfCERLI1 is peripherally-associated with the cytosolic face of the rhoptry membrane. a PfCERLI1HA schizont cultures were used for a proteinase K protection assay. Parasites were treated with either saponin alone, saponin and digitonin or saponin, digitonin and proteinase K and probed with anti-HA antibodies (PfCERLI1). GAP45 (inner-membrane complex, exposed to the cytosol) and RAP1 (rhoptry lumen) serving as positive and negative controls, respectively, for proteinase K digestion. Selected images representative of three independent experiments. b To determine membrane association, PfCERLI1HA schizont cultures were subjected to a solubility assay. Saponin lysed parasite cultures were hypotonically lysed before being treated with sodium carbonate and then Triton-X-100 (Tx100), with supernatants and the Tx100 insoluble fraction being reserved after each treatment. Resulting samples were probed with anti-HA antibodies (PfCERLI1). The presence of a strong band in the carbonate treatment indicates release of the majority of PfCERLI1 protein into the supernatant with this treatment. Membranes were also stained with anti-EXP2 (transmembrane domain containing), anti-GAPDH (cytosolic) and anti-RH5 (peripheral) solubility controls. Selected images representative of three independent experiments. c A single z-slice of the double-bulbous rhoptries displaying the scheme that was used to measure the fluorescence intensity peaks (d) and diameter (e) of anti-HA and anti-RAP1 staining across the rhoptry (****p < 0.0001 by unpaired two-tailed t-test, n = 5 biological replicates, 1139 rhoptries measured for PfCERLI1HA and 1040 for RAP1, Error bars = SEM). f 3D rendered image of a free merozoite showing the nucleus (DAPI) localising at the basal surface, PfCERLI1 (HA) wrapping around RAP1 at the rhoptry bulb and RON4 localising in the rhoptry neck at the far apical tip. g Representative image of Compound 1 treated PfCERLI1HA GlmS schizonts that were fixed, labelled with anti-HA antibodies and probed with 18 nm colloidal gold secondary antibodies, before being imaged using transmission electron microscopy. White arrows mark rhoptry bulb (RB) and neck (RN), while black arrows mark PfCERLI1 foci. Scale bar = 500 nm.
PfCERLI1 contains lipid binding domains. To investigate how PfCERLI1 may be binding to the surface of the rhoptry bulb and provide insights on protein function, we used protein structural prediction software Phyre229, I-TASSER30–32, and COACH33,34. These analyses suggested that PfCERLI1 contains a C2 domain, which are known lipid binding regions (amino acids ~50–170), a pleckstrin homology (PH) domain that typically binds calcium (amino acids ~250–360; Supplementary Fig. 3a) and an N-terminal alpha helix that is currently annotated as a SP. Outside of these domains PfCERLI1 was predicted to be partially disordered. Like most canonical C2 domains35, the PfCERLI1 C2 domain was predicted to bind ionic calcium and phospholipids (Supplementary Fig. 3b). Of the models tested against, the PfCERLI1 C2 domain was most structurally similar to the C2 domain of human itchy homolog E3 ubiquitin protein ligase (ITCH) but the ligand(s) of this protein have not been elucidated36. The protein modelled to the C2 domain of PfCERLI1 with highest confidence was that of C2-domain ABA-related protein 1 (CARI) from Arabidopsis thaliana, which has been shown to bind phospholipids in a calcium ion dependent manner37,38. The PH domain of PfCERLI1 was most structurally similar to the PH domain of Protein kinase B/Akt, and like Akt, was predicted to bind to inositol 1,3,4,5-tetrakisphosphate (IP4) (Supplementary Fig. 3c). IP4 forms the head-group of the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which was predicted to bind to inositol 1,3,4,5-tetrakisphosphate (IP4). These domains PfCERLI1 was predicted to be partially disordered. Like most canonical C2 domains35, the PfCERLI1 C2 domain was predicted to bind to inositol 1,3,4,5-tetrakisphosphate (IP4) (Supplementary Fig. 3b). Of the models tested against, the PfCERLI1 C2 domain was most structurally similar to the C2 domain of human itchy homolog E3 ubiquitin protein ligase (ITCH) but the ligand(s) of this protein have not been elucidated36. The protein modelled to the C2 domain of PfCERLI1 with highest confidence was that of C2-domain ABA-related protein 1 (CARI) from Arabidopsis thaliana, which has been shown to bind phospholipids in a calcium ion dependent manner37,38. The PH domain of PfCERLI1 was most structurally similar to the PH domain of Protein kinase B/Akt, and like Akt, was predicted to bind to inositol 1,3,4,5-tetrakisphosphate (IP4) (Supplementary Fig. 3c). IP4 forms the head-group of the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which commonly coordinates protein membrane localisation39,40. In addition, PfCERLI1 has previously been predicted to be palmitoylated41. Collectively, this suggests that PfCERLI1 contains potentially three different lipid-interacting moieties that may work cooperatively or independently to coordinate the binding of PfCERLI1 to the rhoptry bulb membrane.

PfCERLI1 residing on the cytosolic face of the rhoptry bulb membrane appeared to contrast with its predicted SP, as golgi-mediated trafficking of SP-containing proteins should place them on the luminal side of the golgi-derived rhoptries25,42. The putative SP displayed on PlasmoDB43 is predicted using SignalP-3.044, however, the latest version of this program (SignalP-5.045) no longer predicts that PfCERLI1 contains an SP (Supplementary Fig. 4a). To determine whether the putative SP is essential for rhoptry targeting by directing the protein into the secretory system, we generated a PfCERLI1 SP deletion construct (PfCERLI1NoSP) that lacked the N-terminal 26 amino acids. PfCERLI1NoSP was then cloned into the pAr11a vector, conjugating PfCERLI1NoSP to GFP and placing PfCERLI1NoSP under the merozoite-specific ama1 promoter (Supplementary Fig. 4b). The overexpressed PfCERLI1NoSP::GFP fusion protein, like its full-length counterpart16, localised to the apical tip of merozoites (Supplementary Fig. 4c); suggesting that the putative SP plays no role in PfCERLI1 trafficking.

PfCERLI1 knockdown alters rhoptry architecture. Having identified PfCERLI1’s confinement to the cytosolic face of the rhoptry bulb, we investigated whether PfCERLI1 may have a role in invasion ligand processing and distribution, or development of rhoptry architecture. Since a number of rhoptry antigens have been shown to be proteolytically cleaved inside the rhoptry lumen, we reasoned that if loss of PfCERLI1 function inhibited transport of rhoptry antigens to the lumen during biogenesis of the organelle, then we would be able to detect altered protein cleavage patterns in rhoptry antigens that undergo processing within this organelle. To assess this, GLCN treated and untreated PfCERLI1HAGlmS schizont lysates were prepared for Western blot. PfCERLI1 knockdown did not result in any consistent changes in processing of the rhoptry bulb marker RAP1, the rhoptry neck antigen RON4, or a control protein that is a component of the inner membrane complex (GAP45) that all undergo proteolytic cleavage inside the parasite prior to invasion commencing (Supplementary Fig. 1b,c). In addition, total protein levels of both RAP1 and RON4 remained consistent following PfCERLI1 knockdown suggesting there is no relationship between PfCERLI1 and total expression levels of these rhoptry lumen antigens in the parasite.

In order to investigate whether PfCERLI1 caused changes in rhoptry architecture, we visualised rosettes using TEM (Supplementary Fig. 5). Analysis of C1 matured schizonts failed to reveal any obvious ultrastructural differences between the rhoptries of either GLCN treated or untreated PfCERLI1HAGlmS parasites. While the qualitative EM assessment indicated that PfCERLI1 knockdown was not causing clear changes in rhoptry architecture, we investigated whether there was altered rhoptry antigen distribution with PfCERLI1 knockdown using semi-quantitative super-resolution microscopy. GLCN treated and untreated PfCERLI1HAGlmS (Supplementary Fig. 6a) schizonts were imaged in 3-dimensions to estimate shape (sphericity, oblate (flattened sphere), prolate (elongated sphere)), staining intensity, volume and area for the rhoptry bulb marker RAP1 (Supplementary Fig. 6b). A highly significant decrease in the intensity (50% reduction) and increase in the size of RAP1 staining with PfCERLI1 knockdown was observed. These changes were associated with a significant elongation of the RAP1 signal, but no corresponding change in the RAP1 diameter, indicating that the change in RAP1 staining intensity and size is largely due to elongation of the protein’s distribution in the rhoptry (Fig. 5e). Collectively, these changes in intensity and size suggest that knockdown of PfCERLI1 alters the distribution of RAP1 within the rhoptries, and/or rhoptry shape more grossly.

To further investigate the spatial positioning of rhoptry antigens, we quantified spatial proximity between RAP1 and RON4 markers in super-resolved GLCN treated and untreated PfCERLI1HAGlmS schizonts (Fig. 5a–e). We first assessed whether distribution of RON4 changes with PfCERLI1 knockdown as described for RAP1. There was a trend towards greater RON4 intensity (12% increase), probably as a result of a less elongated signal in the rhoptry neck (Supplementary Fig. 6c,d). We next investigated whether changes in RON4 and RAP1 staining altered their localisation relative to each other. Using Mander’s correlation coefficient to calculate association frequency between markers, semi-quantitative colocalisation analysis indicated that the proportion of RAP1 signal that overlapped with RON4 after knockdown of PfCERLI1 was significantly reduced (Fig. 5a–d). These data indicate that PfCERLI1 knockdown causes changes in the distribution of antigens within the rhoptry, most notably for...
PARASITEs. Each data point represents a single schizont image (GLCN schizonts). Data point represents a single rhoptry (RON4 was observed to be more concentrated in the GLCN (Fig. 6b). In contrast, quantitation of Western blots revealed a

tents had been triggered for both GLCN and untreated parasites relative to untreated controls (Fig. 6b). In addition, knockdown of PfCERLI1 did not signifi
can change in the distribution of the rhoptry bulb protein RAP1, we investigated whether knockdown of PfCERLI1 changed apical organelle secretion. Ring-stage cultures were either treated with 2.5 mM GLCN or left untreated before enzymatic removal of erythrocyte surface receptors at trophozoite stages to prevent reinvasion. Schizonts were allowed to rupture, the supernatant material before analysis by Western blot (Fig. 6a). Blots were probed with anti-RON4 and anti-RH4 antibodies to assess rhoptry secretion, anti-EBA175 to assess microneme secretion, and anti-HA (PfCERLI1), anti-RAP1 (rhoptry bulb) and anti-RON4 (rhoptry neck) antibodies. According to the image analysis pipeline detailed in Supplementary Fig. 7, the % of RON4 that colocalised with RAP1, Pearson’s correlation coefficient (PCC) of the amount of RON4 colocalising with the RAP1 signal, and thresholded Mander’s correlation coefficient (MCC) of RON4 overlap with RAP1 signal was compared between PfCERLI1HAGlmS knockdown and control parasites. Each data point represents a single schizont image (n = 5 biological replicates, 38 schizonts imaged for untreated and 36 for +2.5 mM GLCN schizonts). Rhoptry bulb (RAP1) diameter was measured between untreated and PfCERLI1HAGlmS knockdown (+2.5 mM GLCN) parasites. Each data point represents a single rhoptry (n = 5 biological replicates, 1220 rhoptries counted for untreated and 1217 for +2.5 mM GLCN). (ns = p > 0.05, **p < 0.01, ***p < 0.001 by one-way unpaired t-test). All error bars = SEM.

**Loss of PfCERLI1 disrupts merozoite rhoptry antigen function.** After identifying that PfCERLI1 knockdown caused a significant change in the distribution of the rhoptry bulb protein RAP1, we investigated whether knockdown of PfCERLI1 changed apical organelle secretion. Ring-stage cultures were either treated with 2.5 mM GLCN or left untreated before enzymatic removal of erythrocyte surface receptors at trophozoite stages to prevent reinvasion. Schizonts were allowed to rupture, the supernatant collected, and lysates were made from saponin lysed parasite material before analysis by Western blot (Fig. 6a). Blots were probed with anti-RON4 and anti-RH4 antibodies to assess rhoptry secretion, anti-EBA175 to assess microneme secretion, anti-HA to assess PfCERLI1 knockdown and anti-ERC as a loading control. Knockdown of PfCERLI1 did not significantly alter the level of EBA175 (microneme) secretion relative to untreated controls, confirming that release of microneme contents had been triggered for both GLCN and untreated parasites (Fig. 6b). In contrast, quantitation of Western blots revealed a decrease in secreted RON4 and RH4 for PfCERLI1 knockdown parasites relative to untreated controls (Fig. 6b). In addition, RON4 was observed to be more concentrated in the GLCN treated PfCERLI1HAGlmS parasite pellet relative to untreated control parasite material. These data suggest that PfCERLI1 knockdown prevents secretion of essential invasion antigens from the apical tip of the rhoptry after initiation of the invasion process, providing a probable mechanism by which PfCERLI1 knockdown inhibits invasion.

It has previously been demonstrated that RAP1 is processed by Plasmsperin IX and Subtilisin-like protease 1 (SUB1), beginning as an 84 kDa precursor and being processed to a 67 kDa product that is contained in free merozoites46–48, with this processing likely required for function. The anti-RAP1 antibody used in this study typically detects three bands, the full-length unprocessed RAP1, a 67 kDa processed RAP1 contained in merozoites, with the middle band suspected to be an intermediate cleavage product or a product of non-physiological processing47,48. While we were unable to detect RAP1 in the supernatant in rhoptry secretion assays (Fig. 6a), we noticed that there was an increase in unprocessed RAP1 for PfCERLI1 knockdown treatments. We quantified the relative abundance of each of the three RAP1 bands present, relative to total RAP1 signal, for both untreated and PfCERLI1 knockdown free merozoites with PfCERLI1 knockdown causing a significant increase in the proportion of unprocessed RAP1 and a corresponding decrease in the proportion of processed RAP1 (Fig. 6c). No change in this trend was observed for 3D7 WT free merozoites in parallel assays treated with 2.5 mM GLCN, suggesting the loss in RAP1 processing is a direct result of PfCERLI1 knockdown (Supplementary Fig. 9a). In addition, RAP1 processing in C1 arrested schizonts was quantified for both PfCERLI1HAGlmS and 3D7 parasites (Supplementary Fig. 9b,c). There was no change in RAP1 processing seen for C1 arrested schizonts with 2.5 mM

**Fig. 5 PfCERLI1HAGlmS knockdown alters rhoptry antigen distribution.** a 3D reconstructions of super-resolution (Airyscan) micrographs for PfCERLI1HAGlmS schizont cultures that were either left untreated or treated with glucosamine (+2.5 mM GLCN) before being stained with DAPI (nucleus), anti-HA (PfCERLI1), anti-RAP1 (rhoptry bulb) and anti-RON4 (rhoptry neck) antibodies. According to the image analysis pipeline detailed in Supplementary Fig. 7, the % of RON4 that colocalised with RAP1, Pearson’s correlation coefficient (PCC) of the amount of RON4 colocalising with the RAP1 signal, and thresholded Mander’s correlation coefficient (MCC) of RON4 overlap with RAP1 signal was compared between PfCERLI1HAGlmS knockdown and control parasites. Each data point represents a single schizont image (n = 5 biological replicates, 38 schizonts imaged for untreated and 36 for +2.5 mM GLCN schizonts). Rhoptry bulb (RAP1) diameter was measured between untreated and PfCERLI1HAGlmS knockdown (+2.5 mM GLCN) parasites. Each data point represents a single rhoptry (n = 5 biological replicates, 1220 rhoptries counted for untreated and 1217 for +2.5 mM GLCN). (ns = p > 0.05, **p < 0.01, ***p < 0.001 by one-way unpaired t-test). All error bars = SEM.
GLCN treatment for either PfCERLI1HAGlmS or 3D7 WT parasites, suggesting the inhibition of processing occurs after release of merozoite antigens. This observation is consistent with prior observations that this cleavage event occurs very late in schizont development or after merozoite egress.

Discussion

Previous studies have identified PfCERLI1 (P3D7_0210600) as being a potential vaccine target on the basis of its predicted SP and late schizont stage of expression, a transcription profile typical of proteins involved in merozoite invasion. In addition, PfCERLI1 was identified as a member of the ‘invadome’, an interaction network of putative invasion-associated proteins, and was localised to the merozoite apical tip in this study. Here we show that PfCERLI1 localises to the rhoptries and is confined to the cytosolic face on the rhoptry bulb membrane, a localisation that would prevent exposure to antibodies during the natural course of invasion. Semi-quantitative super-resolution fluorescence microscopy showed a difference between the mean diameter of PfCERLI1 and the rhoptry bulb marker RAP1 around the bulb structure, indicating they occupy distinct compartments on/within the rhoptry. PfCERLI1 was sensitive to proteinase K after selective membrane solubilisation and RAP1, which appears to lie along the luminal face of the rhoptry bulb and is protected from cleavage, confirming that PfCERLI1 compartmentalises to the cytosolic face of the rhoptry bulb membrane. PfCERLI1 has been shown to be palmitoylated, but was predominantly present in the carbonate fraction of the membrane solubility assay (Fig. 4b), suggesting that PfCERLI1 predominantly associates with the cytosolic face of the rhoptry membrane through protein-protein or protein-lipid electrostatic interactions in schizonts. PfCERLI1 was also detected in the Tx100 insoluble fraction. While the contents of this fraction are poorly understood in Plasmodium, they have been previously...

Fig. 6 PfCERLI1HAGlmS knockdown alters rhoptry antigen processing and secretion. a PfCERLI1HAGlmS parasites were either treated with 2.5 mM GLCN (+) or left untreated (−), with the erythrocyte surface receptors of the culture cleaved by enzyme treatment to prevent invasion but allow release of merozoite antigens. The parasite lysate (P) and culture supernatant (SN) were stained with anti-RON4 (rhoptry neck), anti-EBA175 (micronemes), anti-RH4 (rhoptry neck), anti-RAP1 (rhoptry bulb), anti-HA (PfCERLI1) and anti-ERC (loading control) antibodies and analysed by western blot. Selected blots representative of five independent experiments (M = size markers). b Western blots of rhoptry antigens, and secreted EBA175, were normalised to the loading control (ERC) and quantified, with data represented as ±2.5 mM GLCN signal expressed as a % of the signal for the untreated control (n = 5 biological replicates, source data are provided as a source data file). c Using the parasite lysates from the rhoptry secretion experiment, each of the three individual RAP1 bands present on the western blots were quantified and presented as a percentage of the total RAP1 signal (n = 5 biological replicates, ns = p > 0.05, *p < 0.05, **p < 0.01 by two-way ANOVA). All error bars = SEM.
identified as containing covalently lipid-linked (palmitoylated and myristoylated) members of the IMC31–33, C2 domains, as present in PfCERLI1, are predicted to bind calcium and to form ionic bridges with lipid phosphates in membrane bilayers that are not easily disruptible19, providing a second potential membrane binding site for PfCERLI1. The presence of two distinct moieties that are predicted to interact with membranes may explain why PfCERLI1 partially solubilises in carbonate buffer while the remainder stays in the TX100 insoluble fraction.

Our attempts to knock-out Pfcerli1 proved unsuccessful, confirming a previous random mutagenesis study that identified Pfcerli1 as essential20. However, we successfully integrated a Glns-ribozyme sequence at the 3′ end of Pfcerli1 and were able to knockdown protein expression by >80% leading to >45% inhibition of parasite growth. While we were unable to achieve complete growth inhibition, it is possible that loss of PfCERLI1 function is compensated for by other proteins with similar function or that enough PfCERLI1 remains to enable invasion of some parasites. Indeed, bioinformatic searches identified a second protein, PSTD7_0405200 (homologous to PfCERLI2), with similar structure and shared predicted epitopes that had previously been implicated in merozoite invasion36, providing a possible functional homologue that could compensate for loss of PfCERLI1.

Knockout and knockdown studies implicate PfCERLI1 as having an important role in asexual stage parasite growth and we further confirmed that loss of this protein led to a defect in invasion. Initial investigation of the PfCERLI1 knockout parasites showed that the number and morphology of merozoites was unaffected by loss of PfCERLI1 and parasites that did invade with PfCERLI1 knockdown developed normally. We determined that PfCERLI1 knockdown led directly to a significant reduction in newly invaded ring-stage parasites and a proportional build up in free merozoites in the culture, possibly through stopping invasion at a point prior to the formation of the tight junction and engagement of the acto-myosin motor. Quantitative assessment of immunofluorescence signal revealed a significant decrease in RAP1 staining intensity with PfCERLI1 knockdown, corresponding to increases in RAP1 volume and area, indicating knockdown of PfCERLI1 changes RAP1 distribution. While changes in area and volume for RAP1 were observed, the diameter of RAP1 rhoptry bulb staining and TEM analysis indicated that there were no large morphological changes in the rhoptry bulb itself. Thus, changes in the ultrastructure of the rhoptry with PfCERLI1 knockdown appear to be minimal and the strongest indication of a modification rhoptry shape achieved to date is increase prolate staining for RAP1, which may indicate a direct association with release of rhoptry antigens RH4 and RON4 but no change in secretion of GlmS.

PfCERLI1 partially solubilises in carbonate buffer while the remainder stays in the TX100 insoluble fraction. The ionic bridges with lipid phosphates in membrane bilayers that are present in PfCERLI1, are predicted to bind calcium and known to form a clear rhoptry bulb localisation using both fluorescence and immuno-EM localisation in our study and a rhoptry tip localisation reported by Suarez et al. It is possible that PfCERLI1 and TgRASP2 have different localisations/functions, as while these organisms are related, they display markedly different host and cellular tropisms. However, further investigation will be required to tease out the specifics of these differences.

Taking all the data obtained together, we propose that PfCERLI1 has an important role in the function of the rhoptries during invasion, with PfCERLI1 knockdown leading to abnormal distribution, secretion and processing of rhoptry antigens that are likely to be essential for merozoite invasion. While further studies need to be undertaken to determine the fine detail of how PfCERLI1 knockdown causes these changes in rhoptry function, identification of PfCERLI1’s direct association with release of rhoptry antigens is a key step in understanding the complex molecular events that control rhoptry secretion during invasion. By understanding how rhoptry secretion is controlled and the key proteins involved, we can identify targets for drug development that will stop merozoites invading and prevent replication of disease-causing parasites. In addition, this study makes extensive use of semi-automated quantitative immunofluorescence microscopy and highlights how this powerful tool can be used to study the process of invasion.

Methods

Bioinformatic analyses. PfCERLI1 (PSID7_0210600) and orthologous sequences in P. reichenowi (PRCDC_0209500), P. gaboni (PGAB01_0208200), P. malariae (PmUG01_04021700), P. ovale (PocGH01_04019500), P. knowlesi (PKNH_0410600), P. vivax (PVX_0035900), P. berghei (PRANKA_0307000), P. yoelli (PY17X_0308180) and P. chabaudi (PCHAS_0309700) were obtained by searching within the PlasmoDB.org database43. Sequence similarities were determined using Geneious 9.1.3 (Biomatters) and performing multiple pairwise alignments using the global alignment with free end gap alignment algorithm with the Blosum62 cost matrix.

Continuous culture of asexual stage P. falciparum. P. falciparum (3D7, 3D7 ΔPfcerli1(ΔHAGlmS) and 3D7 ΔPfcerli1(ΔAdiglN)(ΔGFP)) parasites were cultured in human O+ erythrocytes (Australian Red Cross blood service)38. Parasites were grown in RPMI-HEPS culture medium at pH 7.4 (Gibco) supplemented with 50 μM hypoxanthine, 25 mM NaHCO3, 20 μM gentamicin and either 0.5% Alumax II (Gibco) or 0.25% w/v Alumax II, 5% v/v human serum (Australian Red Cross blood service). Parasite cultures were maintained in an atmosphere of 1% O2, 4% CO2 and 95% N2 at 37 °C.

Plasmid construction and transfection. The PfCERLI1-HAglmS riboswitch transfection vector was prepared from the pTEX150-HAglmS vector20,29. The final 767 base pairs of the 3′ end of the Pfcerli1 genomic sequence (excluding the stop codon) was PCR amplified using the primers Pfcerli1 5′ F R1Bw (GGTAGATTCCTAT CAAATTTGTTGCTGAAG) and Pfcerli1 3′ R R1Bw (GGTCCTGACAGCATCT ATAGTGTAATTTTGGC). The resulting PCR fragment was cloned into the pTEX150-HAglmS vector using the restriction enzyme cloning sites Bgl II and Pst I (restriction enzyme sites in bold). To generate the cytosolic GFP expressing 3D7
**Proteinase K protection assay.** For proteinase protection assays, three 5 mL aliquots of high-parasitaemia E64 treated schizonts were centrifuged at 14,000 rpm for 10 min. Saponin lysis and western blot. For protein samples, 10 mL of high-parasitaemia lysate was lyophilized over 0.1% w/v saponin on 10 min ice on ice, protein material was pelleted by centrifugation before being resuspended once in 0.1% SDS and 0.1% w/v sodium dodecyl sulfate (SDS) in PBS. In the presence of protease inhibitors (Complete, Roche), parasite lysates were resuspended in reducing sample buffer (0.125 M Tris- HCl pH 7, 4% v/v SDS, 20% v/v glycerol, 10% v/v β-mercaptoethanol (Sigma-Aldrich), 0.002% v/v bromophenol blue (Sigma-Aldrich)) and separated by size using SDS-PAGE (4-12% Bio-Tris Cond, Bolt, Invitrogen) at 130 V for 60 min. Proteins were then transferred to a nitrocellulose membrane (iblot, Invitrogen) at 20 V for 7 min, before blocking the membrane for 1 h at room temperature in Odyssey Blocking Buffer (TBS) (LI-COR Biosciences). Primary (mouse 12CA5 anti-HA: 1:4000, Roche), rabbit anti-EXP2 (1:5000), rabbit anti-GAP45 (1:10000), mouse anti-RH4 2E8 (1:2500), mouse anti-RCA1 (1:10000) and secondary (IRDye 800CW goat anti-mouse (1:4000, LI-COR Biosciences), IRDye 680RD goat anti-rabbit (1:4000, LI-COR Biosciences)) antibodies were incubated with membranes for 1 h at room temperature (prepared in Odyssey Blocking Buffer (TBS)), before washing in 0.1% v/v PBS Tween followed by a PBS wash after the secondary antibody incubation. Western blots were visualised using an Odyssey Infrared imaging system (LI-COR Biosciences). Western blot quantification was performed using Image Studio Lite 5.2.5 (LI-COR Biosciences). Uncropped images of all western blots can be found in Supplementary Figs 11-13.

**Protein expression.** For parasite gDNA extraction and plasmid integration assessment. To extract parasite gDNA and confirm whether transfected constructs were stably integrated, schizont stage parasite cultures were sonicated. Parasite gDNA was extracted from saponin lysed parasites using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s protocol. To confirm integration of PfCeri1-HAGlms into the parasite genome, the Fcerli1 locus was amplified with the forward primer Fcerli1 5’ FBW (GTCAATCGCATATAGTGTTACATTGGAGAA) and the reverse primer Fcerli1 3’ RBW (GCCGCCTCACTGAGTCCAAGCTTATTTGC) reverse primer (amplifies wildtype gDNA sequence for the glms R gene) and the reverse primer Glsms (GigAATCCTTACGCCTGTAGTCTGC) reverse primer (amplifies DNA only upon PfCeri1-HAGlms integration).
Airyscan super-resolution microscopy. Sub-diffraction microscopy was performed on a Zeiss LSM880 AxioObserver Z1 microscopy (Carl Zeiss, Oberkochen, Germany), fitted with an Airyscan detector and a Plan-Apochromat ×63 (NA 1.4) M27 oil objective. TetraSpeckTM Fluorescent Microspheres (Life Technologies) were mounted on #1.5H coverslips (Carl Zeiss, Oberkochen, Germany) with a density of ~2.3 × 10^10 particles/ml and used as the 200 nm bead calibration sample to correct for chromatic and spherical aberrations. The super-resolution reconstruction of multi-labelled PRCERLI1HAGlmS labelling structures were acquired, sequentially in four channels, as follows: channel 1 = 633 nm laser, channel 2 = 561 nm laser, channel 3 = 488 nm laser, channel 4 = 405 nm laser, with exposure times optimised from positive and negative control samples (HA blocking peptide, or omitted primary antibody) to avoid saturation. Three-dimensional (3D) Z-stacks were acquired at a pixel resolution of 0.04 μm in XY and 0.16 μm intervals in Z using piezo drive prior to being Airyscan processed in 3D using batch mode in ZEN Black (Zeiss).

Diameter measurements of super-resolved rhoptries. For further spatial exploration of the colocalised signals, 3D volumes captured with super-resolution microscopy after Airyscan processing were imported into ZEN Black (Zeiss) for object-based colocalisation analysis. In this approach, volumetric colocalisation relies on manual identification of structures of interest and a subsequent measurement of their fluorescence intensity curves. Each rhoptry bulb immunolabelled with anti-RAPI and anti-HA antibodies was manually quantified by a researcher blinded to experimental conditions, and the organelle diameter measured by drawing a vector through the centre of these structures and plotting the fluorescence intensity curves for the green and red channel against the length of the vector. Fluorescence intensity profiles of overlapping subcellular structures were then analysed in successive single sections from an image stack representing the two RAPI and PRCERLI1HAGlmS labelled structures. Fluorescence curves between the two antipodal points on the surface of the ring-like organelle were exported as .csv Excel files and plotted against the distance in nanometres. Only rhoptries with homogeneously stained ring-like structures containing no gaps in RAPI or PRCERLI1HAGlmS labelling and >100 nm in size were used for diameter measurements. Schizonts densely packed with merozoites with overlapping rhoptries were excluded from scoring. More than 800 individual organelles were scored and data from biological replicates plotted in Prism for comparison of diameters between untreated and glucosamine treated parasites.

Immunogold labelling and transmission electron microscopy. Schizont stage parasites were Percoll purified and fixed in 1% glutaraldehyde in RPMI-HEPES buffer. Samples were dehydrated in increasing concentrations of ethanol and embedded in LRGold resin. Ultrathin sections were cut and then labelled with anti-membrane antigen 1 and the rhoptry neck protein complex de
tex005 (2019). Briefly, synchronous ring-stage cultures were

Rhoptry and microneme secretion assays. Apical organelle secretion assays were modified from a previous protocol228. Briefly, synchronous ring-stage cultures were obtained using sorbitol and either treated with 2.5 mM GLCN or left untreated and incubated in standard culture conditions for 24 h until early trophozoite stage. Cultures were then enzyme treated with neuraminidase (0.067 U/ml), chymotrypsin (1 mg/ml) and trypsin (1 mg/ml). Parasites were then allowed to rupture over the following 24 h. Following rupture, cultures were centrifuged at 13,000 rcf for 10 min in a benchtop centrifuge with some culture supernatant reserved, stored on ice, and the rest removed. The remaining pellet was then also placed on ice and saponin lysed to form a lysate containing the parasites that did not rupture and free merozoites, with this pellet and the culture supernatant analysed by Western blot.

Statistical analysis. Graphs and statistical analyses were performed using GraphPad PRISM 7 (GraphPad Software Inc.). In all figures where p-values were calculated, the corresponding statistical test is listed in the figure legend along with the number of experiments results are pooled from.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request. The source data underlying Figs. 1g, 2a, 6b and Supplementary Fig. 2a are provided as a source data file. Unprocessed Gel and Blot images are provided in Supplementary Fig. 11 for blots in Figs. 1d–f, 4a, b, Supplementary Fig. 12 for blots in Fig. 6a, and Supplementary Fig. 13 for blots in Supplementary Fig. 1b, 9a,c.

Received: 23 May 2019; Accepted: 15 February 2020; Published online: 16 March 2020

References
1. World Health Organization. World Malaria Report 2017. who.int/malaria/publications/world-malaria-report-2017/en/ (2017).
2. Cowman, A. F., Healer, J., Marapana, D. & Marsh, K. Malaria: biology and disease. Cell 167, 610–624 (2016).
3. Cowman, A. F., Tonkin, C. J., Tham, W. H. & Duraisingh, M. T. The molecular basis of erythrocyte invasion by malaria parasites. Cell Host Microbe 22, 232–245 (2017).
4. Beeson, J. G. et al. Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. FEMS Microbiol. Rev. 40, 343–372 (2016).
5. Boyle, M. J., Wilson, D. W. & Beeson, J. G. New approaches to studying Plasmodium falciparum merozoite invasion and insights into invasion biology. Int. J. Parasitol. 43, 1–10 (2013).
6. Burns, A. L. et al. Targeting malaria parasite invasion of red blood cells as an antimalarial strategy. FEMS Microbiol. Rev. https://doi.org/10.1093/femsec/fizu005 (2019).
7. Riglar, D. T. et al. Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. Cell Host Microbe 9, 9–20, https://doi.org/10.1016/j.chom.2010.12.003 (2011).
8. Counihan, N. A., Kalanon, M., Coppell, R. L. & de Koning-Ward, T. F. Plasmodium rhoptry proteins: why order is important. Trends Parasitol. 29, 228–236 (2013).
9. Richard, D. et al. Interaction between Plasmodium falciparum apical membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. J. Biol. Chem. 285(19), 14815–22 (2010).
10. Counihan, N. A. et al. Plasmodium falciparum parasites deploy RhopH2 into the host erythrocyte to obtain nutrients, grow and replicate. eLife 6, e23217 (2017).
11. Hanssen, E. et al. Electron tomography of Plasmodium falciparum merozoites reveals core cellular events that underpin erythrocyte invasion. Cell. Microbiol. 15, 1457–1472 (2013).
12. Topolska, A. E., Lidgett, A., Truman, D., Fujioka, H. & Coppell, R. L. Characterization of a Membrane-associated Rhoptry Protein of Plasmodium falciparum. J. Biol. Chem. 279, 4648–4656 (2004).
13. Cabrera, A. et al. Dissection of minimal sequence requirements for rhoptry membrane targeting in the malaria parasite. Traffic 13, 1335–1350 (2012).
14. Geiger, M. et al. Structural Insights Into PIARO and characterization of its interaction with PIAIP, J. Mol. Biol. https://doi.org/10.1016/j.jmb.2019.12.024 (2019).
15. Mueller, C. L. et al. Structural and functional dissection of Toxoplasma gondii armadillo repeats only protein. J. Cell Sci. 129, 1031–1045 (2016).
16. Hsu, G. et al. Transcriptional profiling of growth perturbations of the human malaria parasite Plasmodium falciparum. Nat. Biotechnol. 28, 91 (2009).
17. Zenonos, Z. A., Rayner, J. C. & Wright, G. J. Towards a comprehensive Plasmodium falciparum merozoite cell surface and secreted recombinant protein library. Malar. J. 13, 93, https://doi.org/10.1186/1475-2875-13-93 (2014).
18. Suarez, C. et al. A lipid-binding protein mediates rhoptry discharge and invasion in Plasmodium falciparum and Toxoplasma gondii parasites. Nat. Commun. 10, 4041 (2019).
19. Birnbaum, J. et al. A genetic system to study Plasmodium falciparum protein function. Nat. Methods 14, 450 (2017).
20. Pommpana, P. et al. Inducible Knockdown of Plasmodium Gene expression using the glmS ribozyme. PLoS ONE 8, e73783 (2013).
21. Lopez-Barragan, M. J. et al. Directional gene expression and antisense transcripts in sexual and asexual stages of Plasmodium falciparum. BMC Genomics 12, 587 (2011).
22. Wilson, D. W., Crabb, B. S. & Beeson, J. G. Development of fluorescent Plasmodium falciparum for in vitro growth inhibition assays. Malar. J. 9, 152 (2010).
23. Wilson, D. W., Langer, C., Goodman, C. D., McFadden, G. I. & Beeson, J. G. Defining the timing of action of antimalarial drugs against Plasmodium falciparum. Antimicrob. Agents Chemother. 57, 1455–1467 (2013).
24. Ficz, G. & Heintzmann, R. Breaking the resolution limit in light microscopy. Brief. Funct. Genomics 5, 289–301 (2006).
25. Bannister, L. H., Hopkins, J. M., Fowler, R. E., Krishna, S. & Mitchell, G. H. Ultrastructure of rhoptry development in Plasmodium falciparum erythrocytic schizonts. Parasitology 121, 273–287 (2000).
26. Peskan, T., Westermann, M., Fau–Oelmüller, R. & Oelmüller, R. Identification of low-density Triton X-100-insoluble plasma membrane proteins in higher plants. *Eur. J. Biochem.* 267(24), 6989–95 (2000).

27. Kübler, E., Dohlan, H. G. & Lisanti, M. P. Identification of Triton X-100 insoluble membrane domains in the Yeast Saccharomyces cerevisiae: lipid requirements for targeting of heterotrimERIC G-protein subunits. *J. Biol. Chem.* 271, 32975–32980 (1996).

28. Taylor, H. M. et al. The malaria parasite cyclic GMP-dependent protein kinaseplays a central role in blood-stage schizogony. *Eukaryot. Cell* 9, 37 (2010).

29. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. E. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* 10, 845 (2015).

30. Roy, A., Kauczurak, A. & Zhang, Y. I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725 (2010).

31. Yang, J. et al. The I-TASSER Suite: protein structure and function prediction. *Nat. Methods* 12, 7 (2014).

32. Zhang, Y. I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9, 40 (2008).

33. Roy, A., Yang, J. & Zhang, Y. Protein–ligand binding site recognition using complementary binding-specific substructure comparison and sequence profile alignment. *Bioinformatics* 29, 2588–2595 (2013).

34. Yang, J., Roy, A. & Zhang, Y. BioLiP: a semi-manually curated database for biologically relevant ligand–protein interactions. *Nucleic Acids Res.* 41, 2596–2606 (2013).

35. Corbalan-Garcia, S. & Gomez-Fernandez, J. C. Signaling through C2 domains: more than one lipid target. *Biochimica et Biophysica Acta* 1838, 1536–1547 (2014).

36. Liu, Y.-C. The E3 ubiquitin ligase Itch in T cell activation, differentiation, and tolerance. *Semin. Immunol.* 19, 197–205 (2007).

37. Rodríguez, L. et al. C2 domain asblic acid-related proteins mediate the interaction of PYR/PYL/RCAR asblic acid receptors with the plasma membrane and regulate asblic acid sensitivity in Arabidopsis. *Plant Cell* 26, 4802 (2014).

38. Diaz, M. et al. Calcium-dependent oligomerization of CAR proteins at cell membrane modulates ABA signaling. *Proc. Natl Acad. Sci. USA* 113, E396 (2016).

39. Czech, M. P. PIP2 and PIP3: complex roles at the cell surface. *J. Cell Biol.* 128, 2581–2586 (2010).

40. Roy, A., Yang, J. & Zhang, Y. BioLiP: a semi-manually curated database for biologically relevant ligand–protein interactions. *Nucleic Acids Res.* 41, 2596–2606 (2013).

41. Jones, M. L., Collins, M. O., Goulding, D., Choudhary, J. S. & Rayner, J. C. Analysis of protein palmitoylation reveals a pervasive role in Plasmodium development and pathogenesis. *Cell Host Microbe* 12, 246–258 (2012).

42. Deponte, M. et al. Wherever I may roam: protein and membrane trafficking in *P. falciparum*-infected red blood cells. *Mol. Biochemical Parasitol.* 186, 95–116 (2012).

43. Aurrecoechea, C. et al. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Res.* 37, D539–D543 (2008).

44. Dyrlev Bendtsen, J., Nielsen, H., von Heijne, G. & Brunak, S. Improved prediction of signal peptides: signalP 3.0. *J. Mol. Biol.* 340, 783–795 (2004).

45. Almagro Armenteros, J. J. et al. SignalP 5.0 improves signal peptide predictions using neural networks. *Nat. Biotechnol.* 37, 420–423 (2019).

46. Silmon de Monerri, N. C. et al. Global identification of multiple substrates for the Plasmodium falciparum translocation complex in the rhoptries of *Plasmodium falciparum*. *Mol. Biochemical Parasitol.* 151, 1097–112 (1988).

47. Schofield, L. et al. The processing of Plasmodium falciparum rhoptry-associated protein 1 and localization of PfRhop to schizont rhoptries and p67 to free merozoites. *Mol. Biochemical Parasitol.* 122 (1998).

48. Gilson, R. et al. PTEX150HAGlmS transfection vector. Confocal microscopy was performed with the PTEX150HAGlmS transfection vector. *Eukaryot. Cell* 9, 1117–1117 (2010).

49. Absalon, S., Robbins, J. A. & Dvorin, J. D. An essential malaria protein depletes the spontaneous uptake and expression of DNA from human erythrocytes. *Nat. Protoc.* 71 (2016).

50. Absalon, S., Robbins, J. A. & Dvorin, J. D. An essential malaria protein defines the architecture of blood-stage and transmission-stage parasites. *Nat. Commun.* 7, 11449 (2016).

Acknowledgements

We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies. We thank the Australian Red Cross Blood Bank for the provision of human blood. We thank Prof. Alan Cowman for provision of CyRPA, RON4, EBA175 and GAP45 antibodies, Dr. Paul Gilson for EXP2 antibodies, A/Prof. Wai-Hong Tham for RH4 antibodies, and Dr. Paul Gilson for EXP2 antibodies.

Author contributions

Study design and planning: D.W.W., B.L., S.F., M.W.A.D., S.A.R. and T.G. Performed experiments and generated reagents: B.L., S.F., D.W.W., G.K.H., S.A.R. and B. Liu. Data analysis: S.F. and D.W.W. Manuscript writing: B.L., S.F., T.G. and D.W.W. Manuscript was drafted with input from all authors.

Competing interests

The authors declare no competing interests.
