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A novel protein export machine in malaria parasites

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Abstract

Several hundred malaria parasite proteins are exported beyond an encasing vacuole and into the cytosol of the host erythrocyte, a process that is key to the virulence and viability of the causative *Plasmodium* species. The trafficking machinery responsible for this export is unknown. Here, we identify a *Plasmodium* Translocon of EXported proteins (PTEX), which is located in the vacuole membrane. The PTEX complex is ATP-powered and comprises HSP101, which is a ClpA/B-like AAA+ ATPase of a type commonly associated with protein translocons, a novel protein termed PTEX150 and a known parasite protein EXP2. EXP2 is the potential channel as it is the membrane-associated component of the core PTEX complex. Two other proteins, a novel protein PTEX88 and a thioredoxin known as TRX2, were also identified as PTEX components. As a common portal for numerous crucial processes, this novel translocon offers an exciting new avenue for therapeutic intervention.

Malaria remains one of the world's most important infectious diseases and is responsible for enormous mortality and morbidity1. The most lethal form of human malaria is caused by the protozoan parasite *Plasmodium falciparum*. Central to the capacity of this organism to grow inside red blood cells and to thrive inside the blood-stream is its ability to export ~5% of its encoded genome (200-300 proteins) into the cytosol of its host cell2-5. While the function of most of these exported proteins is unknown, those that have been investigated appear to have virulence-associated roles such as promoting infected cell adhesion and/or rigidity6-9. Apart from these functions, a recent gene knockout screen of 85 exported proteins implicated an essential blood-stage survival role for ~25% of exported proteins9. Moreover,

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parasite proteins are also exported beyond a vacuolar membrane during the liver stage of infection10.

To gain access to the host cell cytosol, exported parasite proteins must cross two membranes, the parasite plasma membrane and the parasitophorous vacuole membrane (PVM) that envelops the parasite. Despite its importance, the mechanism of protein export is not known although export initially requires proteins to enter into the secretory pathway11-13. This is mediated by a recessed N-terminal hydrophobic endoplasmic reticulum (ER) signal sequence, which is sufficient to transport proteins beyond the plasma membrane to but not across the PVM12. For most exported proteins, trafficking across the PVM requires an additional sequence element, known as a Plasmodium EXport ELement (PEXEL) or a Vacoular Transport Signal (VTS), which is located approximately 25-30 amino acids downstream of the ER signal sequence2,3. The discovery of this element was a major advance as it allowed predictions of the exported proteome of *P. falciparum* and other *Plasmodium* species2-5 and it also suggested the presence of a central portal through which most or all exported proteins must pass2,3.

Identification of two candidate translocon proteins

Export of PEXEL-containing proteins most likely requires a proteinaceous translocon within the PVM2,3,14, however, homology searches for relatives of known members of translocon systems has failed to predict its identity. As an alternative, we combined two approaches to identify candidates: proteomic analysis of relevant parasite membranes and the establishment of predictive criteria. With respect to the first approach, we found that lipid-raft like detergent resistant membranes from ring-stage parasites were strongly enriched in proteins that are known or suspected to localise to the PVM (Table S1). Hence, it seemed likely that translocon components would be represented amongst these proteins.

Based on existing knowledge we established five specific criteria to systematically predict putative PEXEL-protein translocon components (Table S2). Briefly, we postulated that such proteins should: (1) be restricted to the *Plasmodium* genus only because PEXEL motifs appear to be absent from even closely-related Apicomplexan parasites, (2) incorporate a power source, which previous evidence suggests is likely to be an ATPase15, (3) have dual apical merozoite and ring-stage PVM localisation and also be expressed in the liver stage, (4) be essential for blood-stage growth and (5) specifically bind to their exported protein cargo.

The *P. falciparum* ring-stage detergent resistant membrane proteome was analysed for potential translocon components. Of interest, was HSP101 (PF11_0175; Fig. S1), which belongs to the dual AAA+ ATPase domain containing HSP100/ClpA/B chaperone family. HSP100 proteins are core components of numerous protein translocon systems, such as type VI secretion in gram-negative bacteria16 and the translocon at the inner membrane of chloroplasts17. Importantly, *Plasmodium* HSP101 encodes an N-terminal ER signal sequence for export into the PV and orthologs were only found within the *Plasmodium* genus as expected for a role in PEXEL-protein trafficking (Fig. S1). Members of the HSP100 family form, hexameric ring-shaped complexes that generally translocate proteins

through a central pore of the ring in an ATP-dependent manner. Structural modelling of *P. falciparum* HSP101 predicts that it forms a typical ring-shape hexamer (Fig. S1).

P. falciparum HSP101 has an identical late schizont/early ring transcriptional profile to another protein that was also present in both the ring- (Table S1) and schizont-stage proteomes of *P. falciparum* (Fig. S2). This hypothetical protein, which we have termed PTEX150, also has a putative ER signal sequence and is found throughout the *Plasmodium* genus but not in other genera (Fig. S2). Moreover, data from our previous proteomic study identifying large molecular weight complexes in *Plasmodium* membranes demonstrated that HSP101 and PTEX150 were present in two distinct high molecular weight (> 650 MDa) bands18 (Fig. S2), suggesting they form a large complex.

A series of reagents were generated including PTEX150 and HSP101-specific antibodies and two transgenic *P. falciparum* (3D7) parasite lines termed 3D7-150HA and 3D7-101HA where the respective endogenous genes were modified at their C-termini to include a triple hemagglutinin (HA) epitope tag (Fig. 1A, Fig. S3). Antibodies raised against *P. falciparum* PTEX150 recognised a single protein species of 150 kDa in parasite extracts (predicted molecular weight is 112 kDa), while a slightly larger species of 155 kDa was observed in 3D7-150HA due to the addition of the tag (Fig. 1A). The PTEX150 antibody was further verified by its specific reactivity with the 155 kDa HA-tagged species (Fig. 1A). Similarly, antibodies raised against HSP101 recognised a single protein species of the expected molecular weights in both 3D7 and 3D7-101HA parasites (Fig. 1B). The temporal expression patterns of HSP101 and PEX150 showed that both proteins were strongly expressed in late schizogony and remained at similar levels through the 48 hr blood-stage cycle (Fig. 1C). Since transcription of these proteins peaks in late schizogony/early ring stages (www.PlasmoDB.org), this indicates that these proteins are stable, with little protein turnover.

Immunofluorescence analysis (IFA) showed that PTEX150 and HSP101 co-localise and are found in discrete foci in the membranes surrounding the ring-stage parasite (Fig. 1D). This membrane is the PVM as fluorescence surrounds the parasite membrane marker MSP1(19). Also, it is predicted that the PEXEL protein trafficking machinery would already be made in the merozoite and injected into the newly forming vacuole during erythrocyte invasion so that it can immediately traffic similarly injected proteins such as the ring-infected surface antigen (RESA). Consistent with this, both PTEX150 and HSP101 are strongly expressed late in schizogony (Fig. 1C) and reside at the apical end of free merozoites presumably in secretory organelles (Fig. 1D). Hence, PTEX150 and HSP101 fulfil the first three criteria expected of a PEXEL-protein translocon. With respect to the fourth criterion, we have been unsuccessful in repeated attempts to generate gene knockouts of *P. falciparum* PTEX150 and its rodent malaria orthologue *P. berghei* PTEX150 (data not shown) suggesting that this protein is essential to blood-stage development.

Identification of a novel 5 member putative translocon complex; PTEX

To investigate if PTEX150 and HSP101 associate and to identify other potential binding partners, pull-down experiments were performed using HA antibodies on 3D7-150HA and

3D7-101HA parasites. To control for non-specific interactions, precipitations were also performed on the 3D7 parent (Figs. 2A and B). SDS-PAGE bands visually unique to pull-downs using transgenic parasites by Coomassie staining were excised, as were the corresponding gel regions of the negative control, and analysed by LC-MS/MS based sequencing (see bands 1-5 on Figs. 2A and B and Table S3). These data indicated HSP101 and PTEX150 co-precipitated in a specific and reciprocal manner with 150-HA (Fig. 2A) and 101-HA (Fig. 2B) respectively. Combined with western-blotted extracts of HA-immune-precipitations (Fig. 2C), this data confirmed they form a stable complex. Three additional proteins, identified as the top parasite-specific peptide hit in bands 3-5, were also specifically affinity purified with both 150-HA and 101-HA species (Figs. 2A and 2B and Table S3). These proteins include one known PVM protein EXP2 (PF14_0678)19, a novel protein termed PTEX88 (PF11_0067) and a thioredoxin-like, TRX2 protein (MAL13P1.225)20. The interactions of these proteins were specific since either none or very few peptides were recovered from pull-downs performed in parallel on parental parasites (Table S3). To determine whether other less abundant proteins that co-precipitate with PTEX150 and HSP101 were present, the remaining bands were excised from the gel and analysed by mass spectrometry (Table S4). No other proteins specific to both 150-HA and 101-HA pull-downs were found.

EXP2 has been previously characterised in *P. falciparum* as a 35-kDa ring-stage protein that has been demonstrated to be associated with the PVM19,21. By immune-precipitation we confirmed that EXP2 interacts with the PTEX150 complex (Fig. 2C). Furthermore, dual labelling IFA experiments demonstrated that EXP2 co-localises with PTEX150 and HSP101 in large foci in the ring-stage PVM (Fig. 2D). Similar experiments were not performed for PTEX88 or TRX2 as specific reagents are not available. While we are cautious about assigning these members to the PTEX complex, we emphasise that both proteins have ER signal sequences, are co-regulated at an mRNA level with other PTEX members and are unique to *Plasmodium* (Fig. 2E and Fig. S4). In addition, PTEX88 and TRX2 were specifically detected in both PTEX150 and HSP101 affinity purification experiments. Hence both are likely components of the PTEX complex. This is somewhat surprising in the case of TRX2 as a GFP-tagged version of this protein has been previously shown to localise to the mitochondrion20. However, the presence of an ER signal sequence and the absence of a mitochondrial-targeting motif in TRX2 are consistent with a location outside of the mitochondrion. Indeed, while the TRX2-GFP fusion protein described by Boucher and colleagues localises to the mitochondrion in the majority of parasites, in others it shows a pattern surrounding the parasite consistent with a parasitophorous vacuole localization (S. Muller, personal communication). It is possible that the heterologous heat shock promoter used for this transgene experiment contributed to this dual localization. Hence, although specific antibodies are required to firmly resolve the localization of TRX2, existing data is consistent with it comprising a component of the PTEX machinery.

In summary, we have identified a core macromolecular complex comprising HSP101, PTEX150 and EXP2 with two additional, potentially accessory, proteins PTEX88 and TRX2 (Fig. 2E). Each of these is found throughout the *Plasmodium* genus and obvious orthologues are not present in any other organism. We note, however, that reciprocal BLAST analysis

suggests that EXP2 is related to the dense granule family of secreted proteins known as DG32 in other Apicomplexan parasites22, although the relatively low level of identity and absence of conserved cysteine residues means it is unclear if DG32 proteins represent orthologs of EXP2. Hence, the PTEX complex is a novel PVM-associated, ATPase machine that satisfies the first four essential criteria for a PEXEL-protein translocon.

PTEX specifically interacts with exported proteins consistent with translocon function

We investigated the capacity of PTEX proteins to interact with their anticipated PEXEL-protein cargo. Proteomic analysis of the affinity purifications performed on 3D7-150HA parasite lysates revealed that whilst 53% of the peptides identified were PTEX components, another 8% were from exported proteins, presumably the translocon's cargo (Table S4). No peptides of exported proteins were recovered from control 3D7 parasite lysates analysed in parallel suggesting a specific interaction between PTEX components and exported proteins. The PEXEL protein peptides recovered were from just two, presumably abundant, exported proteins, RESA (PFA0110w) and a previously uncharacterised protein PF08_0137 (see Fig. S5). To confirm the specificity of this interaction we performed the reverse experiment using RESA and PF08_0137 antibodies to pull-down cross-linked parasite protein extracts. Under these conditions both antibodies specifically pulled-down PTEX components HSP101 and PTEX150 to a level 4-6 fold above background levels of binding seen in the bead-only negative control (Fig. S5). To further address whether exported proteins specifically interact with the PTEX complex, we tested whether HSP101 antibodies could precipitate different GFP-tagged reporter proteins. In two separate experiments, we show that the exported GFP reporter protein K+GFP12 showed superior binding to HSP101 (between 4-6 fold in different experiments) than the non-exported GFP-reporters (Fig. 3A). These negative controls include a reporter associated with the parasite membrane; GFP-M1923, and two soluble reporters that localise to the parasitophorous vacuole; SS-GFP24 and K-GFP12 (see Fig. S5). Despite possessing a PEXEL motif that is recognised and cleaved in the ER25, the K-GFP fusion protein is not exported due to the lack of a spacer region between the PEXEL element and the GFP reporter26. To further test the specificity of this and other PEXEL binding protein interactions with the PTEX complex, we performed the immune-precipitations in the K+GFP expressing parasite line with antibodies against GFP and an additional PEXEL protein PF11_00379. Both the K+GFP and PF11_0037 exported proteins, as well as PF08_0137 pulled down the PTEX components HSP101, PTEX150 and EXP2 far more strongly (5-200 fold) than did the negative control pre-immune IgG (Fig. 3B). In summary, we have shown that 4 different PEXEL-motif containing exported proteins demonstrate specific binding to the PTEX machinery.

A model for PTEX function

So how might this apparatus function? Protease sensitivity under differential membrane permeabilisation conditions suggest that HSP101 and PTEX150 reside entirely on the vacuolar face of the PVM (Fig. S6). Indeed, it is already known that EXP2 predominately resides in this location21. Hence, we propose that the PTEX complex is injected directly from apical merozoite organelles into the vacuole where it associates with the PVM to form

large macromolecular foci. It has been shown that PEXEL-containing proteins are recognised and cleaved after the leucine residue in the RxLxE/Q/D PEXEL motif in the parasite's ER25,27. This processing step is likely to free the cargo from the ER membrane27. The xE/Q/D remaining after cleavage is essential for mature cargo to reach the host cell. This may involve either recognition of this sequence in the PV by the translocon and/or the interaction of xE/Q/D with proteins in the ER or Golgi that sort cargo into vesicles that traffic proteins to the specific regions of the PV that contain the PTEX translocon27. Once in the vacuole, proteins destined for export are known to be unfolded presumably prior to being fed through a membrane pore28. HSP101 is most likely to be responsible for unfolding and feeding denatured proteins through its central channel, however, the identity of the transmembrane pore itself is not obvious. Although it does not possess a predicted transmembrane domain, evidence that EXP2 is membrane-associated has been published19,21. Here we show that EXP2 is the most resistant of the three core PTEX components to extraction with carbonate, consistent with it being the direct membrane-associated component of the PTEX complex (Fig. 4A). Notably, using the FUGUE structural homology software29 EXP2 is predicted to fold similarly to the pore forming toxin *Escherichia coli* hemolysin E (HLyE; Fig. S7). Although the `top hit' in this analysis, the predictive score is in the `moderate' range of confidence and other structural homology searches did not yield significant hits. However, adding weight to the prospect that EXP2 folds similarly to HLyE includes the prediction in the structural homology model of EXP2 built from the HLyE structure of an intramolecular disulphide bond with the predicted C24-C113 bond in EXP2 to be in a very similar location to the conserved cysteine pair at the base of HLyE30 (Fig. S7). Indeed, EXP2 must fold in a manner that allows intramolecular disulphide bond(s) as EXP2 migration by SDS-PAGE is sensitive to reduction21. Taken together, EXP2 is the most likely of the identified components to constitute the membrane pore although we caution that direct evidence for this is lacking at present and indeed it remains possible that other, perhaps yet to be identified PTEX components, perform this role. (See Fig. 4B for a model of PEXEL-protein translocation).

The function of the other PTEX components is unclear although scaffolding and cargo-binding functions are likely for some members. TRX2 has a number of potential roles including aiding the unfolding of PEXEL-proteins prior to passage through the translocon or regulating PTEX function. As for the red blood cell cytosolic side of the membrane, it is notable that we detected a 6-fold enrichment of human HSP70 family protein, HSC70, in pull-downs from the HA-tagged parasites compared to parental parasites (Table S4). Host cell HSP70 may be recruited to the translocon, perhaps in conjunction with exported parasite proteins (such as PF08_0137, which we also suggest binds to the PTEX complex; Fig. 3 and Fig. S5), to assist in the passage and/or refolding of exported proteins. Consistent with such a role, erythrocyte HSP70 transforms from a soluble to a membrane-bound state in parasitised cells31.

While many mechanistic questions surrounding our model for *Plasmodium* protein export remain to be addressed, this study reveals the identity of novel protein trafficking apparatus that serves as a common nexus for proteins involved in virulence and survival and hence provides a powerful `Achilles heel' for anti-malaria drug developers. The evolutionary origin

of this machinery remains a mystery since it is absent from even closely related parasites and is distinct from other characterised protein translocon machineries such as the secretion systems in bacterial cell membranes or translocons in eukaryotic organelles. However, the conserved use of hexameric ring-forming AAA+ ATPases in many of these systems, including the PTEX apparatus we describe here, hints at an ancient origin of this key biological process.

Methods summary

Transgenic lines 3D7-150HA and D37-101HA were generated by transfecting *P. falciparum* strain 3D7 with plasmids pPTEX150-HA/Str3' or pHSP101-HA/Str3' respectively. Parasites containing integrated forms of the plasmid were obtained by repeated drug cycling in the presence and absence of WR99210. Antibodies were also raised against recombinant hexa-his fusion proteins derived from either PTEX150 (amino acids 181-236) or HSP101 (amino acids 68-170). These transgenic lines and antibodies, along with other antibodies described in this study, were used in immunoprecipitation experiments as well as western blot and immunofluoresence analysis. Immunoprecipitations were performed on mixed ring and trophozoite-stage infected erythrocytes in which the erythrocyte had been permeabilised with tetanolysin or first cross-linked with 2mM DSP followed by saponin lysis. Proteins present in parasite lysates (generated by incubation of the parasites on ice in 1% (v/v) Triton-X 100 or RIPA buffer) that were pulled down with anti-HA antibodies or other antibodies described in this study were identified either by LC-MS/MS based sequencing or by Western blot analysis. For IFA, parasites were fixed with ice-cold 90% acetone/10 % methanol prior to incubation with various primary and secondary antibody combinations.

Methods

Ring-stage proteome

MudPIT analysis of proteins present in detergent resistant membranes (DRMs) prepared from saponin-lysed ring-stage parasites was performed as described previously32.

Plasmid construction and transfection

Plasmids pPTEX150-HA/Str3' and pHSP101-HA/Str3' were constructed by ligating the last 986 bp of the PTEX150 (PF14_0344) or the last 920 bp of HSP101 (PF11_0175) coding sequence (excluding the stop codon) into the unique *Bg*/II and *Pst*I sites (underlined) of p1.233. The 3' targeting sequences were generated by PCR amplification of *P. falciparum* 3D7 genomic DNA using the oligonucleotides PTEX150int F (5'-gaagatctGAAGAAGGTATTTTAGATTATGAAG) and PTEX150R-PstI (5'-aactgcagCATTGTCGTCCTCTTCTTCGTCC) for PTEX150 and HSP101intF (5'-gcagatctCTAAATCCATTATTGGAAATGAAGA) a n d H S P 1 0 1 i n t R (5'-aactgcagCGGTCTTAGATAAGTTTATAACCAAG) for HSP101. This resulted in the fusion of the 3' end of PTEX150 or HSP101 with the 3xHA/Str epitope tags in p1.2 such they formed a continuous open reading frame. pPTEX150-HA3' and pHSP101- HA3' (75 μ-g) were transfected into *P. falciparum* 3D7 parasites as previously described34.

Expression of fusion proteins and generation of antibodies

The DNA sequence corresponding to amino acids 181-236 of PTEX150 was amplified from *P. falciparum* 3D7 gDNA using the oligonucleotides PTEX150Ab2F (5'-cgcggatccGAAATAAAAATGAAGATGAACTCAC) and PTEX150Ab2R (5'-aactgcagATTATCTTTATTCATCATTTTTCC) and ligated into the *Bam*HI and *Pst*I sites (underlined) of a modified version of pMal-c2x (New England Biolabs) that generates a C-terminal 6 x his tag32. The DNA sequence corresponding to amino acids 68-170 of HSP101 was amplified using the oligonucleotides HSP101e-F (5'-cgcggatccCCCGATAATAAGCAAGAGCAAGGA) and HSP101e-R (5 '-cccaagcttTTATAATATTGCTTTAATGGCTTCATCGGTT) and ligated into the *Bam*HI and *Hin*dIII sites (underlined) of pPROEX-HTb (Invitrogen). PTEX150 and HSP101 fusion proteins were expressed in *Escherichia coli* BL21 cells, purified on a Ni-NTA affinity resin (Qiagen) and used to generate monoclonal and polyclonal antibody reagents.

Immunoprecipitations

Mixed ring and trophozoite-stage infected erythrocytes were either treated with 100 U/ml tetanolysin (Sigma) on ice for 30 min or cross-linked with 2 mM DSP (Dithiobis [succinimidyl propionate]) followed by saponin lysis to remove red blood cell material. Pelleted parasites were lysed with 1% (v/v) Triton X-100 in PBS or RIPA buffer (25 mM Tris pH7.6, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) Deoxycholate-Na, 0.1% (w/v) SDS) containing complete protease inhibitors (Roche) and lysates then applied to goat anti-HA antibodies coupled to agarose beads (Sapphire BioScience), or mixed with antibodies (10-20 μ-g) derived against various recombinant malaria proteins for 4 hrs at 4°C. In the cases in which uncoupled anti-malaria antibodies were used, Protein G sepharose was then added and the material incubated for a further 4 hr at 4°C. After triplicate washing with 1% (v/v) Triton X-100 in PBS, bound protein was eluted from the beads with 1x SDS-PAGE sample buffer and electrophoresed under reducing conditions. For Western blotting, the following dilutions of antibody reagents were used - polyclonal rabbit anti-PTEX150, 1:200; polyclonal rabbit anti-HSP101, 1:1000; monoclonal mouse anti-HSP101, 1:500; monoclonal mouse anti-EXP2 (mAb 7.7, a gift from Dr. J. Mc Bride and described previously21,35, 1:2000; monoclonal mouse anti-HA clone 12CA5 (Roche), 1:2000; polyclonal rabbit anti-MSP(19)36, 1:1000; polyclonal rabbit anti-AMA137, 1:200; monoclonal mouse anti-RESA, 1:200038; polyclonal rabbit anti-PF08_0137, 1:1000.

Indirect Immunofluorescence analysis (IFA)

IFAs were performed on parasites fixed with ice-cold 90% acetone/10% methanol using the following antibody concentrations - polyclonal rabbit anti-PTEX150, 1:100; polyclonal rabbit anti-HSP101, 1:100; monoclonal mouse anti-HSP101, 1:100; monoclonal mouse anti-EXP2 (mAb 7.7),1:20; monoclonal mouse anti-HA, 1:50; polyclonal rabbit anti-MSP(19), 1:1000. The appropriate Alexa Fluor 488/595 secondary IgG antibodies (Molecular Probes) were used at 1:1000 dilution. Mounted slides were visualised with a Carl Zeiss Axioskop microscope. Pictures were recorded using a PCO Sensi-Cam and images were produced using ImageJ software (available from the public domain).

Protease sensitivity assays

Transgenic parasite lines (2-5 ml cultures) at ring-trophozoite stage were harvested, washed in PBS and resuspended in either 1HU equinatoxin (5mg/ml in PBS) for 6 min at RT, 0.09% (w/v) saponin for 10 min on ice or in 1% (v/v) Triton X-100 for 10 min on ice. Parasite samples were then resuspended in either 20 μ-g/ml proteinase K (Roche) or in PBS and incubated for 30 min at RT. Proteolysis was stopped by the addition of TCA to a final volume of 15% (v/v) and incubation on ice for 15 min. The pellets generated after centrifugation were resuspended in reducing sample buffer for analysis on 4-12% Bis-Tris gels.

Modelling of HSP101

For *P. falciparum* HSP101, homologs of known structure were identified by sequence-to-hidden Markov model comparison in HHpred39. Residues 1-26 constitute the ER signal sequence and were omitted. The top two matches, covering Pf HSP101 from residue 35 to the C-terminus, were to *Thermus thermophilus* ClpB (1QVR) at 38% pairwise sequence identity and to *Escherichia coli* ClpA (1R6B) at 33%. The structure of the protein was modelled in MODELLER40 with 1QVR, chain B, as a template, except in gapped regions and at the C-terminus, where 1R6B was used as a second template. The guiding alignment is shown in Fig. S1. The two ATPase rings - one comprising domains N and D1, and the other domain D2 - were modelled separately, assembled into hexameric rings using a previously described procedure41, docked against each other with RosettaDock42, and connected in Swiss PDB Viewer 43 to obtain the final model. Coordinates for the model can be downloaded via the “computational models” hyperlink at http://www.eb.tuebingen.mpg.de/departments/1-protein-evolution. Structure figures were rendered in MacPyMOL (DeLano Scientific LLC).

Homology modelling of EXP2

A protein-sequence BLAST search44 identified 5 sequences homologous to that of EXP2, GI:156099352 from *Plasmodium vivax*, GI:193810225 from *Plasmodium knowlesi*, GI: 82596841 from *Plasmodium yoelii*, GI:68069165 from *Plasmodiumberghei*, and GI: 70943327 from *Plasmodium chabaudi*. These sequences were used to identify potential compatible structures for EXP2 using the FUGUE program29. The structure of hemolysin E (PDB code 1QOY) was identified to have the highest level of compatibility with the sequences, albeit marginal i.e. ~90% confidence level. A model of EXP2 using the structure of hemolysin E as a template was constructed using the MODELLER program40 using the sequence alignment presented in Fig. S6A. Twenty-five models of EXP2 were prepared, and the structure with lowest DOPE score 45 was selected for further analysis (Fig. S6B). The sequence of EXP2 contains four cysteine residues; cysteine residues 24 and 113 are predicted to be disulfide bonded, whereas cysteine residues 140 and 210 are predicted to lie at the opposite end of the molecule, near the β-sheet structure, separated from one another by ~20 Å. Analysis of the DOPE score for individual residues indicates the region between residues 220 and 250 are inadequately modelled (the FG-loop and helix-G; Fig. S6C), which is the region that is the most different for the sequences in the various species identified in the above BLAST search.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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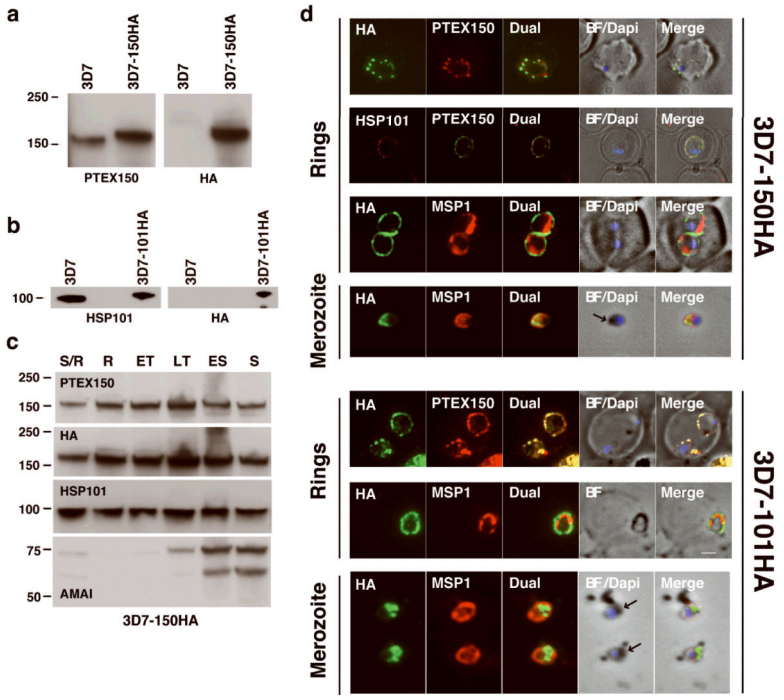

Fig. 1. HSP101 and PTEX150 co-localise and have dual apical merozoite and PVM localisation

Western blot analysis of parasite proteins extracted from: **a,** parental 3D7 and transgenic 3D7-150HA parasites; **b,** 3D7 and transgenic 3D7- 101HA parasites; **c,** 3D7-150HA parasites harvested at mixed schizont/ring (S/R), ring (R), early trophozoite (ET), late trophozoite (LT) and early schizont (ES) stages or from mature magnet purified schizont (S) stages, using the indicated antibodies. AMA1 represents a marker for a protein expressed only at the late-stages of parasite development. Molecular weight standards (kDa) are shown on the left; **d,** Double labelling IFA on fixed 3D7-150HA and 3D7-101HA ring- and merozoite-stage parasites using the antibodies as indicated. The arrowheads indicate the apical end of the merozoite.

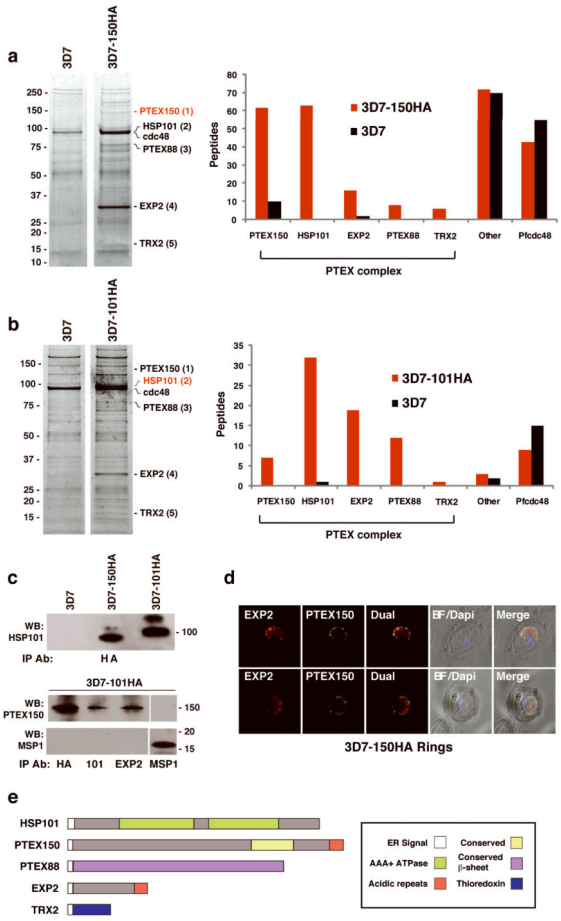

Fig. 2. Isolation of a five-member PTEX complex

Coomassie-stained SDS-PAGE gels of material eluted from immune-precipitations performed using HA antibodies on parasite lysates from: **a,** 3D7 and 3D7-150HA or **b,** 3D7 and 3D7-101HA. The total number of peptides present in all excised bands for those parasite-specific proteins that were the top peptide hit in the visible bands unique to pull-downs using transgenic parasites (labelled 1-5 t are indicated, as is the peptide abundance of Pfcdc48 and all other parasite and host cell proteins identified; **c,** Immunoblot analysis of immune-precipitations performed on 3D7 parental and transgenic HA-tagged parasite lysates using antibody combinations for immuneprecipitation (IP Ab) and Western blot (WB Ab) as indicated; **d,** Double labelling IFA on fixed 3D7-150HA ring-stage parasites using a mixture of antibodies specific for PTEX150 and EXP2; **e,** Schematic of the five members of the PTEX complex showing that each possesses a ER signal sequence and other distinguishing features such as regions that are highly conserved across the genus.

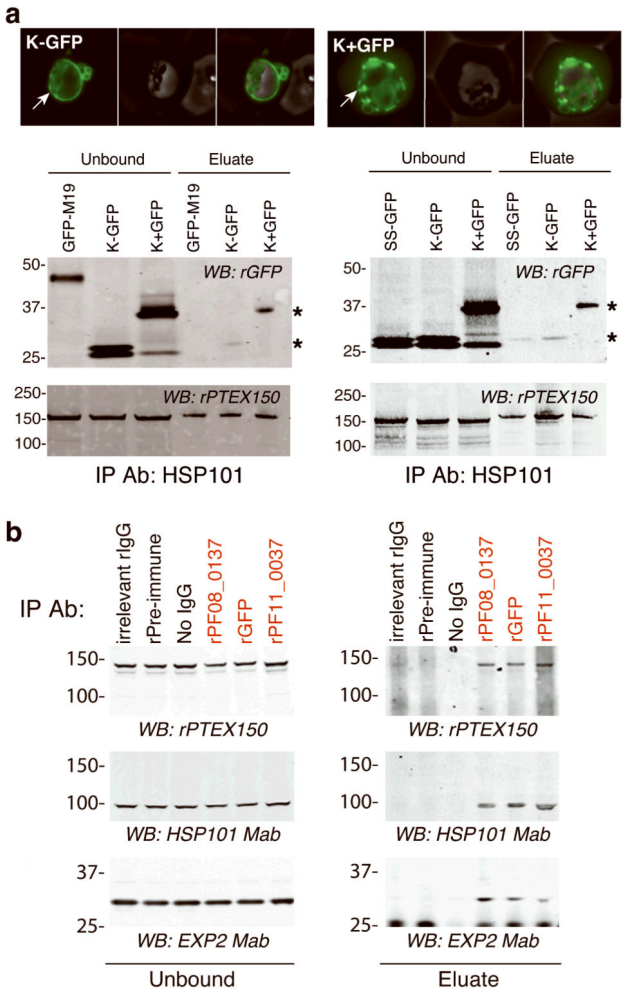

Fig. 3. The PTEX complex interacts with PEXEL proteins

a, Upper panel - GFP fluorescence of non-exported (K-GFP) versus exported (K+GFP) reporters in live transgenic parasites. The arrows highlight the differences in fluorescence at the parasitophorous vacuole, with the exported protein displaying a `necklace of beads' appearance. Lower panel - Western-blot analysis of immune-precipitations with HSP101 antibodies using transgenic parasites, which express various GFP reporter proteins (See Fig. S5). Despite equivalent levels of interaction of PTEX150 with HSP101 in all transgenic parasite lysates (bottom panels), greater amounts of the exported K+GFP protein (asterisks) is pulled down in comparison to any of the non-exported reporters proteins. **b,** Immune-precipitations in the K+GFP transgenic line using antibodies against 3 exported proteins (shown in red) probed in Western blot with antibodies against PTEX components. No antibody, pre-immune and irrelevant (anti-AMA1) IgG were included as negative controls.

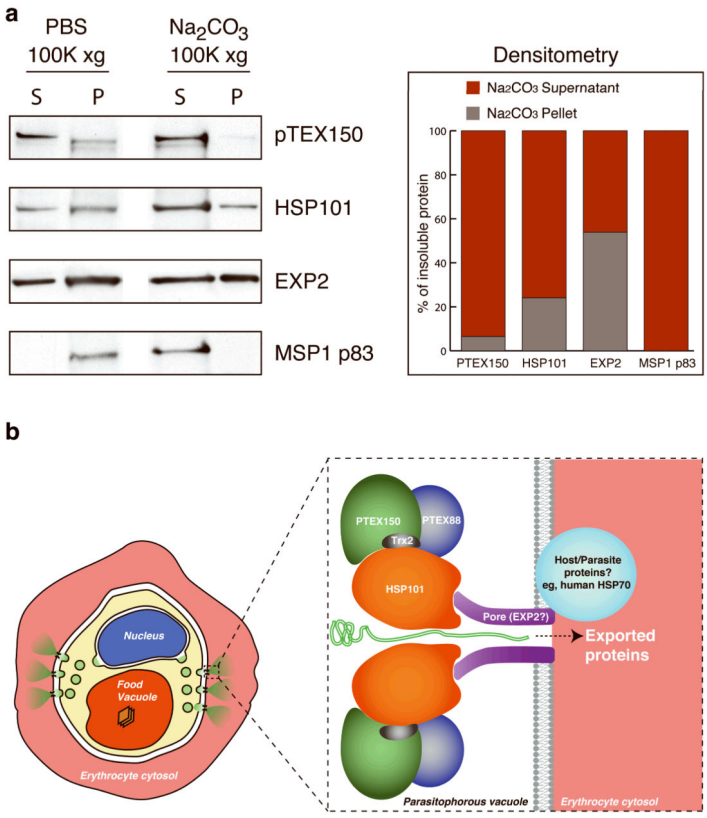

Fig. 4. Model for PTEX function

a, Differential solubilisation of schizont-stage parasites to analyse membrane association. Parasites were first solubilized in PBS into supernatant (S) and pellet (P) fractions and analysed by Western blotting. The PBS insoluble pellet was further solubilized in Na_2CO_3 and similarly analysed. The P83 fragment of MSP1 is included as a control for solubilisation of a peripherally-associated membrane protein; **b**, We propose the following model for PEXEL-protein export. Once deposited into the vaculoar space, proteins destined for export are recognised by some member(s) of the PTEX complex and deposited into the N-terminal domain of HSP101 where they are unfolded. These proteins are fed through the central channel of HSP101 and ultimately through a membrane-associated channel, predicted here to be EXP2. On the cytosolic face, host (eg, human HSP70) and/or exported parasite proteins may assist in energising translocation and/or in re-folding proteins.