

Heterologous expression in *Toxoplasma gondii* reveals a topogenic signal anchor in a *Plasmodium* apicoplast protein

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Glutathione peroxidase-like thioredoxin peroxidase (PfTP_{XGI}) is an antioxidant enzyme trafficked to the apicoplast, a secondary endosymbiotic organelle, in *Plasmodium falciparum*. Apicoplast trafficking signals usually consist of N-terminal signal and transit peptides, but the trafficking signal of PfTP_{XGI} appears to exhibit important differences. As transfection is a protracted process in *P. falciparum*, we expressed the N terminus of PfTP_{XGI} as a GFP fusion protein in a related apicomplexan, *Toxoplasma gondii*, in order to dissect its trafficking signals. We show that PfTP_{XGI} possesses an N-terminal signal anchor that takes the protein to the endoplasmic reticulum in *Toxoplasma*—this is the first step in the apicoplast targeting pathway. We dissected the residues important for endomembrane system uptake, membrane anchorage, orientation, spacing, and cleavage. Protease protection assays and fluorescence complementation revealed that the C terminus of the protein lies in the ER lumen, a topology that is proposed to be retained in the apicoplast. Additionally, we examined one mutant, responsible for altered PfTP_{XGI} targeting in *Toxoplasma*, in *Plasmodium*. This study has demonstrated that PfTP_{XGI} belongs to an emergent class of proteins that possess signal anchors, unlike the canonical bipartite targeting signals employed for the trafficking of luminal apicoplast proteins. This work adds to the mounting evidence that the signals involved in the targeting of apicoplast membrane proteins may not be as straightforward as those of luminal proteins, and also highlights the usefulness of *T. gondii* as a heterologous system in certain aspects of this study, such as reducing screening time and facilitating the verification of membrane topology.

Abbreviations

ACP, acyl carrier protein; BiP, binding immunoglobulin protein; ERAD, ER-associated degradation system; ER, endoplasmic reticulum; HSP70, heat shock protein; PDI, protein disulfide isomerase; PfiTPT, *Plasmodium falciparum* apicoplast innermost membrane phosphate translocator; PfoTPT, *Plasmodium falciparum* apicoplast outermost membrane phosphate translocator; PfTP_{XGI}, *Plasmodium falciparum* glutathione peroxidase-like thioredoxin peroxidase; SELMA, symbiont-derived ERAD-like machinery; TgTPx, *Toxoplasma gondii* thioredoxin peroxidase; TIC, translocon of the inner membrane of the chloroplast; TOC, translocon of the outer membrane of the chloroplast.

The apicoplast is a secondary endosymbiotic organelle, bound by four membranes, found in most members of the phylum *Apicomplexa* [1]. Targeting proteins into and across these multiple membranes is a specialized task that invokes interesting adaptations. For proteins trafficked to the lumen of the apicoplast, a bipartite targeting sequence is employed. A signal peptide that enables co-translational uptake at the ER is cleaved in the ER lumen, thereby exposing the transit peptide. When this preprotein reaches the apicoplast by the endomembrane system, the exposed transit peptide facilitates uptake across its inner membranes by translocons. Transit peptide cleavage then releases the mature protein into the apicoplast lumen [2].

Due to the absence of an apicoplast in the human host of the malaria parasite *Plasmodium falciparum*, this specialized targeting mechanism may be exploited in the development of antimalarials. Once the outermost apicoplast membrane, contiguous with the endomembrane system, is traversed, the protein is transported across the other three membranes by translocons that bear homology to the ERAD system (called the symbiont-derived ERAD-like machinery, or SELMA, in complex plastids) [3–5] and to the TOC and TIC complexes (translocons of the outer and inner membranes of the chloroplast, named similarly in complex plastids) [6] in the periplastid, outer, and inner apicoplast membranes, respectively. Chaperones such as BiP (binding immunoglobulin protein), PDI (protein disulfide isomerase), and HSP70 (heat shock protein) are also involved in these membrane translocations. Given that over 500 nuclear-encoded proteins are targeted to the apicoplast, all of these proteins involved in apicoplast protein trafficking can serve as important drug targets that are capable of disrupting parasite viability when inhibited [7].

In trafficking from the ER to the apicoplast, the scientific community still debates the role of vesicles and the Golgi [8–11]. There also appear to be differences in the trafficking of apicoplast luminal proteins when compared with those trafficked to the membranes. Indeed, a recent paper indicates that the apicoplast outer membrane phosphate translocator of *P. falciparum* (PfoTPT) is devoid of a canonical bipartite targeting sequence and instead uses a signal anchor to localize to the endomembrane system [12].

Another apicoplast protein, glutathione peroxidase-like thioredoxin peroxidase (PFTP_{X_{GI}}, PlasmoDB Gene ID PF3D7_1212000) targets to the apicoplast via the Golgi, unlike the apicoplast luminal acyl carrier protein (ACP), that in the same paper was found to route independently of the Golgi [2]. Additionally, in

another report, PFTP_{X_{GI}} trafficking is inhibited by treatment with tetrafluoroaluminate, which blocks the fusion of heterotrimeric G protein-dependent vesicles to their target membranes, while ACP trafficking is completely unaffected [13]. Incidentally, the bipartite targeting signal of ACP is cleaved during this process, while the size of PFTP_{X_{GI}} remains unaltered [8,10]. Further investigation revealed that PFTP_{X_{GI}} is anchored to the apicoplast membrane [13]. This led us to speculate that membrane anchorage itself might play a role in the altered targeting pathway of this protein when compared with that of luminal ACP.

Kehr *et al.* [14] have shown that the N-terminal 47 residues of PFTP_{X_{GI}} target GFP to the apicoplast and the cytosol in *Plasmodium*. While this work confirmed the adequacy of the N terminus for apicoplast targeting, a deeper understanding of the signals within this region that mediate each step of the pathway requires extensive mutational analysis, a procedure that takes months due to the low efficiencies of transfection in *P. falciparum* [15,16]. In contrast, *Toxoplasma gondii*, an apicomplexan closely related to *P. falciparum*, is easier to transfect, with the efficiency of transfection being orders of magnitude higher than in *Plasmodium*. Consequently, transient transfectants may be studied within hours, while parasites that have integrated foreign DNA under drug pressure revive in a matter of days [17].

Apart from technical benefits, there is another rationale for using *T. gondii* to study the PFTP_{X_{GI}} protein. The ER–Golgi secretory route is broadly conserved across species, more so for closely related apicomplexans such as *P. falciparum* and *T. gondii*. It has been experimentally demonstrated that trafficking to the apicoplast is a branch of the secretory route [2]. In keeping with this theme, the N-terminal apicoplast targeting signal of *Toxoplasma* ACP has successfully targeted GFP to the *Plasmodium* apicoplast [18]. Similarly, the N termini of *Plasmodium* proteins PfiTPT and DOXP reductoisomerase also targeted GFP to the *Toxoplasma* apicoplast [9,19]. Such examples demonstrate that apicoplast signals are conserved, at least for certain proteins, between these organisms.

The factors listed above endorse *Toxoplasma* as a suitable preliminary system for the rapid screening of sequence determinants in *Plasmodium* proteins, such as PFTP_{X_{GI}}, that contribute to biological phenomena that are similar between the two parasites. Therefore, the N-terminal 47 residues of PFTP_{X_{GI}} were codon optimized for expression as a GFP fusion protein in *Toxoplasma* and used to dissect the key sequence features of this region.

Materials and methods

Ethics approval and consent to participate

Human blood for *Plasmodium* culture was collected from a blood bank. The donors gave their written informed consent. All procedures conformed to the standards set by the Declaration of Helsinki.

Plasmid construction

To generate the plasmid expressing PfTP_{XGI}47-EGFP, codon optimized tandem oligonucleotides, encoding residues 1–47 of PfTP_{XGI} and a triple glycine linker, were inserted upstream of EGFP in the vector pCTG-EGFP [20]. As this protein has multiple in-frame methionines, the Kozak context of these methionines was retained so as to match the relative Kozak frequencies between them in *Plasmodium* [21,22]. The plasmid expressing P30-mCherry-HDEL was generated by replacing YFP-HDEL in the ptub-P30-YFP-HDEL vector [23] with mCherry-HDEL amplified from ptub-mCherry. Plasmids encoding PfTP_{XGI}S-EGFP and PfTP_{XGI}ΔS-EGFP were generated by replacing the PfTP_{XGI}47 region in pCTG-PfTP_{XGI}47-EGFP with PfTP_{XGI}S or PfTP_{XGI}ΔS. In the PfTP_{XGI}ΔS-EGFP mutant, a methionine was included at the translation start site. The plasmid encoding ERS1–10 was generated by replacing YFP-HDEL in ptub-P30-YFP-HDEL with GFPS1–10 amplified from the pCMV-mGFP 1-10 Hyg Amp (Sandia Biotech, Albuquerque, NM, USA) including a C-terminal ER retention sequence (HDEL). The plasmid encoding CyS1–10 was generated by replacing EGFP in pCTG-EGFP with GFPS1–10. The plasmid encoding PfTP_{XGI}47-S11 was generated by replacing EGFP in the construct expressing PfTP_{XGI}47-EGFP with GFPS11 amplified from pCMV-mGFP Cterm S11 Neo Kan (Sandia Biotech). Plasmids encoding the PfTP_{XGI} fusion proteins with ACP [ACP_{SP}-PfTP_{XGI}ΔS-EGFP and PfTP_{XGI}S-ACP_{TP}-EGFP] were generated by overlap extension PCR with vectors pCTG-PfTP_{XGI}47-EGFP and pCTG-ACP-HA using Phusion polymerase (NEB) [24]. The plasmid encoding PfTP_{XGI}47-M26A-EGFP was synthesized using a mismatched forward primer to amplify pCTG-PfTP_{XGI}47-EGFP with KAPA HiFi DNA polymerase.

To generate the plasmids expressing GFP fusion proteins in *Plasmodium*, the region encoding the first 47 residues of PfTP_{XGI}, including the start codon context of 12 bp and a triple glycine linker, was amplified from the pET28a-PfTP_{XGI} plasmid containing PfTP_{XGI} cloned from *Plasmodium* mRNA in the case of PfTP_{XGI}47-GFP, and synthesized as tandem oligonucleotides in the case of PfTP_{XGI}47-M26A-GFP. Each of these fragments was inserted upstream of GFP in the pSSPF2 vector [25]. Primers used are listed in Table 1.

Parasite culture and transfection

Methods used for *T. gondii* RH strain maintenance, harvest, transfection, and selection of stable and clonal transfectants were as described previously [17] with minor modifications. For transfections, tachyzoites were electroporated at 1.5 kV/50 Ω/25 μF. Following this, each chamber slide was inoculated with 10⁷ parasites and fixed after 24 h to observe transient transfectants. Stable lines were generated by restriction enzyme-mediated integration using NotI and selecting for growth in 20 μM chloramphenicol.

Plasmodium falciparum strain 3D7 was cultured with 4% hematocrit in RPMI media (Invitrogen, Carlsbad, CA, USA) supplemented with 0.5% albumax using a published protocol [26]. Parasite cultures were synchronized by two consecutive sorbitol treatments, as described by Lambros and Vanderberg [27], 4 h apart. Synchronized *P. falciparum* 3D7 ring stage parasites were transfected with 100 μg of purified plasmid DNA (Plasmid Maxi Kit, Qiagen, Valencia, CA, USA) by electroporation (310 V, 950 μF) [28]. The transfected parasites were selected for growth in 2.5 nM blasticidine.

Microscopy

Toxoplasma tachyzoites were prepared for imaging as described previously [17] with minor modifications. Parasites were incubated in primary antibody (1 : 200 polyclonal rabbit anti-GFP, Invitrogen) for 4 h and in secondary antibody (1 : 400 anti-rabbit Alexa 568) for 1.5 h. Slides were stored at 4 °C and imaged in under 48 h postfixation. Imaging was carried out with the Zeiss LSM 780 NLO confocal microscope (Plan-Apochromat 100X/1.4 NA). Each planar section was averaged eight times. Maximum intensity projections were generated from the Z-stacks. Images were processed using Fiji (IMAGEJ) [29], with only linear adjustments to brightness and contrast applied consistently across all pixels in an image. No nonlinear adjustments, such as changes to the gamma settings or curves, were implemented. Images were processed minimally, only to the extent of reducing background fluorescence external to the parasite, while ensuring that no other features were altered. For each of the transfectants imaged in this study, a minimum of 200 parasites were observed microscopically to ensure consistency in morphology, of which the images of approximately 10 rosettes were captured for the purposes of documentation.

Plasmodium falciparum transgenic parasite lines were prepared for indirect immunofluorescence assays as described previously [30]. Parasites were incubated in rabbit anti-PfClpP (1 : 200) [31] and mouse anti-GFP (1 : 500, Roche, Basel, Switzerland) primary antibodies diluted in 3% BSA (bovine serum albumin), 1 × PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). Subsequently, following incubation in the secondary antibodies goat anti-rabbit Alexa 594 (1 : 250,

Table 1. Primers used in plasmid construction. Restriction sites are underlined and the mismatch regions are italicized. All primer sequences are shown from 5' to 3'.

Construct	Forward primer	Reverse primer
PfTP _{X_G} 47-EGFP (oligonucleotides)	F1: <u>CTAGCAACATGTTCTTCTCAATGTTTCATCAAG</u> TTCATCTTGCCGATCTCTTTCATCTGCTACAACCTTCGG	R1: GAACATGTTGAACTTCTTGC CGAAGTTG TAGCAGATGAAAGAGATCGGCAAGATGAA CTTGATGAACATTGAGAAGAACATGTTG
	F2: CAAGAAGTTCAACATGTTCTTACTTCCAGAAGAT CAAGGTCTCTGAGCAGGAGTTGTTGTCTTCT ATCTACGACTACGGCGGGCGGC	R2: <u>CTAGGGCCGCGCCGCTAGTCGTAGATAG</u> AAGACAACAACCTCTGCTCAGAGACCTTG ATCTTCTGGAAGTAAGA
P30-mCherry-HDEL	GAAC <u>CTAGGG</u> TGAGCAAGGGCGAGGAG	CATT <u>CTTAAG</u> CTACAACCTCGTCGTGCTTGT ACAGCTCGTCCATG
PfTP _{X_G} S1-EGFP	GATCT <u>GCTAGCA</u> ACATGTTCTTCTCAATGTTTCATC	CGC <u>CCTAGGG</u> CCGCGCCGCGCGAAGTTGT AGCA
PfTP _{X_G} ΔS-EGFP	GATCTGCTAGCAACATGAAGAAGTTCAACATGTTCT	CGC <u>CCTAGGG</u> CCGCGCCGCTAGTCGTAG
PfTP _{X_G} 47-S11	GAAC <u>CTAGGG</u> GCGACGGCGGCAGCGGC	CGT <u>TCTGCAG</u> TTTTATGTGATGCCGCGCGGC
ERS1–10	GAAC <u>CTAGGG</u> GCGGTTTCGAAAGGCAGGAG	CGT <u>TCTTAAG</u> CTACAACCTCGTCGTGTTTCT CGTTTGGGTCTTTG
CyS1–10	GAAGCTAGCAAAATGGTTTCGAAAGGCAGG	CGT <u>TCTGCAG</u> GTTATTTCTCGTTTGGGTCT
ACP _{SP} -PfTP _{X_G} ΔS-EGFP	CATTTTTTCTTGAATTCCTTTTAGATCTGCTAGCAA CATGGAGATGCATCCCCG	CTTGATCTTCTGGAAGTAAGAGAACATGTT GAACTTCTTACCGTAGGAAGAAGCAATGG
PfTP _{X_G} S-ACP _{TP} -EGFP	GATCTCTTTCATCTGCTACAACCTTCGGCTTTGTGTCA CCAGGCCTGATC	GCTCACCGCCCTAGGGCCGCGCCATCAGA ACTCGCCTCGTCCG
PfTP _{X_G} 47-M26A-EGFP	AAGAAGTTCAACGCGTTCCTTACTTC	GCCGAAGTTGTAGCAGATGAAAGAG
PfTP _{X_G} 47-GFP (Pf)	GGAAGATCTAATATAAAAAATATGTTCTTCTCAATG TTTATTAATTC	CGC <u>CCTAGG</u> TCTCTCCATAATCATAA ATGGATG
PfTP _{X_G} 47-M26A-GFP (Pf)	F1: <u>GATCTAATATAAAAAATATGTTCTTCTCAATGTTT</u> ATTAAATTCATTTTACCTATATCTTTTATA TGCTACAAT F2: TTTGGAAAGAAATTTAATGCATTAGTATTTTCAAAAA TCAAAGTATCAGAACAGGAATTATTATCATCCATTTA TGATTATGGAGGAGGA <u>C</u>	R1: ATTAATTTCTTTCCAAAATGTAGCAT ATAAAAGATATAGGTAATAATGAATTTAATA AACATTGAGAAGAACATATTTTATATTA R2: <u>CTAGG</u> TCTCTCCATAATCATAAATGG ATGATAATA ATTCTGTCTGATACTTTGATTTTTTTGAA AATAACTAAATGC

Sigma-Aldrich, St. Louis, MO, USA) and goat anti-mouse Alexa 488 (1 : 250), parasite nuclei were stained with DAPI (2 µg·mL⁻¹) for 30 min at 37 °C. The immunostained parasites were viewed using a Nikon A1 confocal laser scanning microscope. Colocalization with the apicoplast marker was verified using methods described previously [32]. Three-dimensional reconstructions of the parasites were generated using the IMARIS 7.01 software by Bitplane (Belfast, Northern Ireland, UK). To assess the extent of colocalization with the apicoplast marker PfClpP, the Pearson's correlation coefficient was calculated for each parasite by using the Nikon NIS-Elements colocalization software. A dot plot of the Pearson's correlation coefficient for colocalization with the apicoplast marker was plotted for parasite lines expressing PfTP_{X_G}47-GFP and PfTP_{X_G}47-M26A-GFP using the GRAPHPAD PRISM 7 software (La Jolla, CA, USA).

Sample preparation for SDS/PAGE and western blotting

Tachyzoites were harvested as described previously [17] and counted using a hemocytometer. Following this, the

parasites were subjected to any necessary extractions as described in Sections 2.5 and 2.6. For whole parasite lysates, the tachyzoites were resuspended in an appropriate volume of PBS. An equal volume of 2X protein loading sample buffer with dithiothreitol [100 mM Tris/Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol] was added to the resuspended tachyzoites. The sample was then boiled for 3 minutes at 100 °C before loading on the gel. For western blotting, a PVDF membrane [Millipore Immobilon-P Transfer Membrane (IPVH00010, 0.45 µm)] was activated in methanol for 5 minutes, following which both the gel and the membrane were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Western blotting by wet transfer was then carried out in a Genetix GX-ZY5 blotting apparatus at 4 °C, either overnight (minimum 12 h) at 40 mA, or for 2 h at 250 mA, with the instrument packed in ice to prevent overheating. Following protein transfer, the PVDF membrane was blocked in 3% BSA-PBS for 1 h, incubated in primary antibodies [1 : 500 mouse anti-GFP (Roche)] for 4 h, and incubated in HRP-conjugated secondary

antibodies for 1.5 h prior to development with diaminobenzidine.

Membrane protein extraction

Extracellular parasites (10^8) were hypotonically lysed in 1 mL of sterile deionized water containing protease inhibitors (Sigma) by six freeze–thaw cycles (5 min each/liquid nitrogen/37 °C). After concentrating over ten times using centrifugal filter units (Amicon ultra-3K, Merck, Kenilworth, NJ, USA), samples were centrifuged (30 000 g/30 min/4 °C) [33]. The supernatant, containing all soluble proteins, constituted the soluble fraction. The pellet was lysed in 1% SDS (1 h/room temperature) to extract all membrane proteins and centrifuged (30 000 g/30 min/4 °C); 30 µL each of the soluble and pellet (1% SDS supernatant) fractions was resolved by SDS/PAGE and subjected to western blotting as described previously. Here, the PVDF membrane was blocked in 3% BSA-PBS for 1 h, incubated in primary antibodies [1 : 500 mouse anti-GFP (Roche) and 1 : 10 000 rabbit anti-BiP (a kind gift from J. Bangs) [34] for 4 h, and incubated in HRP-conjugated secondary antibodies for 1.5 h prior to development with diaminobenzidine.

Differential permeabilization of membranes

All parasites in this assay were freshly harvested to ensure the integrity of all membranes prior to treatment. Extracellular tachyzoites (6×10^7) resuspended in assay buffer (250 mM sucrose, 20 mM Tris/Cl, 0.5 mM CaCl₂, pH 7.4) were aliquoted into six tubes of 95 µL each. Following a 30-minute incubation on ice in digitonin (0.005%), Triton X-100 (1%), and/or EDTA (10 mM) as required, samples were treated with 500 µg·mL⁻¹ thermolysin (5 minutes/37 °C). 10 mM EDTA was then added to the samples to stop the reaction. After lysis in 1% SDS, samples were precipitated by the methanol–chloroform method and subjected to western blotting as described previously.

Results

Immunofluorescence data indicate that glutathione peroxidase-like thioredoxin peroxidase (PfTP_{XGI}) is trafficked not just to the apicoplast, but also to the mitochondrion and the cytosol in *P. falciparum* [10,14]. Small molecule inhibitors have previously been used for the characterization of trafficking pathways used by PfTP_{XGI} to reach the apicoplast and mitochondrion. Inhibition of vesicle fusion by blocking heterotrimeric G proteins showed a complete disruption of PfTP_{XGI} targeting to the apicoplast and only a partial disruption of targeting to the mitochondrion [13].

This suggests that further experiments are needed to understand the mitochondrial trafficking of PfTP_{XGI}. Therefore, this paper is primarily focused on apicoplast targeting alone.

Notably, the homologue of PfTP_{XGI} in *T. gondii* is an alternatively spliced thioredoxin peroxidase (TgTPx). While the shorter splice variant (TgTPx1/1) is localized to the cytosol, the other (TgTPx1/2) is dually targeted to the apicoplast and the mitochondrion [35]. This is very similar to the targeting observed for native PfTP_{XGI} in *Plasmodium* when analyzed by immunofluorescence [10].

Published literature documents the targeting of a C-terminal GFP fusion of the first 47 residues of the antioxidant enzyme PfTP_{XGI} to the apicoplast and the cytosol in *Plasmodium* [14]. The protein is predicted to have an N-terminal signal peptide; however, no clear transit peptide is observed by bioinformatics. Therefore, to study the signals that direct PfTP_{XGI} to the ER, Golgi, and eventually the apicoplast, the first 47 residues of PfTP_{XGI} were expressed as PfTP_{XGI}47-EGFP in *T. gondii* and dissected by mutational analysis.

The N terminus of PfTP_{XGI} possesses a signal sequence

For expression in *T. gondii*, the N-terminal 47 residues of PfTP_{XGI} were codon optimized and expressed as a C-terminal EGFP fusion protein, PfTP_{XGI}47-EGFP, in the RH strain. As this protein has multiple in-frame methionines, the Kozak context of these methionines was retained so as to match the relative Kozak frequencies between these methionines in *P. falciparum* [21,22]. Experiments were performed using clonal lines generated under drug pressure.

In these lines, PfTP_{XGI}47-EGFP was observed to target to the ER in every single parasite, as confirmed by colocalization with the ER marker P30-mCherry-HDEL (Fig. 1A) in a distinctly perinuclear structure characteristic of the ER. Notably, what appeared to be mitochondrial and apicoplast localization was observed in parasites transiently transfected with this construct (data not shown). However, we chose not to pursue these phenotypes as they were not robust and were observed only in very few of several hundred parasites screened.

It is known that entry into the ER is the first step in trafficking to the apicoplast. Additionally, PfTP_{XGI} is known to route via the ER and the Golgi in *Plasmodium* [10]. This motivated us to exploit the ER localization of this protein to understand the signals involved in this first step of organellar targeting by mutational analysis.

Targeting to the ER requires a signal sequence, the length and location of which was predicted using SignalP 3.0 [36] (suitable for apicomplexans [37]) and the Phobius server [38]. The predicted signal peptide was between residues 1–21. A fusion of residues 1–21 with EGFP was expressed in parasites as PfTP_{X_GI}S-EGFP. Interestingly, native EGFP fluorescence was absent in these parasites and anti-GFP antibodies were used to confirm localization, suggesting that EGFP was misfolded. As seen in Fig. 1B, PfTP_{X_GI}S-EGFP colocalized with the ER marker, P30-mCherry-HDEL. This led us to conclude that residues 1–21 were indeed sufficient for ER targeting.

Residues 22–47, when expressed with an N-terminal methionine and C-terminal EGFP tag as PfTP_{X_GI}ΔS-EGFP, did not target to the ER and instead remained in the cytosol (Fig. 1C), as confirmed by colocalization with the cytosolic marker Hexokinase-mCherry (a kind gift from D. Shanmugam) [39]. This implied that the sequence was unable to facilitate uptake at the ER or at either of the endosymbiotic organelles. Notably, the protein PfTP_{X_GI}ΔS-EGFP passively diffused across the nuclear pore complex owing to its small size (<40 kDa) [40].

PfTP_{X_GI} is anchored to the membrane by an N-terminal transmembrane domain

Targeting to the ER lumen typically involves cleavage of the signal peptide. The cleavage site itself is determined by the (–3, –1) rule [41], where small, neutral residues at positions –3 and –1 from a site increase the propensity of cleavage at that site. Western blotting of parasites expressing PfTP_{X_GI}47-EGFP revealed that the band was at the size predicted in the absence of cleavage, ~33 kDa (Fig. 2A). No band was evident in the control lane, which contained total lysates of untransfected parasites of the RH strain. A faint band was visible below the 33 kDa PfTP_{X_GI}47-EGFP band, either a product of GFP degradation that has been reported previously in the literature [18], partial cleavage at residues 21–22, or alternative translation initiation (Met²⁶). Even in light of these other possibilities, a majority of the protein still remains in the unprocessed state, at the full size of ~33 kDa. The lack of processing could be indicative of a signal anchor as proposed elsewhere [12,42]. Additionally, both 1–47 and 1–21 residue EGFP fusion proteins remain in the ER despite the absence of identifiable ER retention signals. It has been suggested that transmembrane domains have the potential to retain proteins in specific compartments [43]. Taken together, these observations led us to test whether the

protein was anchored to the membrane by an N-terminal transmembrane domain. Indeed, PfTP_{X_GI} has been demonstrated to be membrane bound in *P. falciparum* [13].

Multiple freeze–thaw cycles in a hypotonic solution separated the soluble contents from the membrane fraction. Western blots revealed that the ER luminal control protein BiP (binding immunoglobulin protein) partitioned primarily into the soluble fraction (Fig. 2B) [23]. Incomplete partitioning of luminal proteins is a common occurrence documented in other reports [4]. Interestingly, full-length PfTP_{X_GI}47-EGFP partitioned entirely into the membrane fraction, while the shorter protein partitioned into the soluble fraction.

The C-terminal domain of PfTP_{X_GI} lies in the ER lumen

It has been suggested that the orientation acquired by a membrane protein at the ER is retained at its final destination [12,42]. Therefore, understanding the orientation of PfTP_{X_GI} in the ER might reveal its orientation in the apicoplast.

The first method employed to establish PfTP_{X_GI} topology was a protease protection assay. Here, we treated parasites with digitonin, a detergent that selectively permeabilizes the plasma membrane owing to its higher cholesterol content, but not the organelar membranes [44]. These parasites were then incubated with the membrane-impermeant protease thermolysin, which would diffuse across the permeabilized plasma membrane but not the intact ER membrane. Thus, if PfTP_{X_GI}47-EGFP faced the cytosol, it would be susceptible to proteolysis by thermolysin, and if it faced the ER lumen, it would be protected (Fig. 3A).

The concentration of digitonin that permeabilized the plasma membrane, but left the ER membrane intact, was standardized by testing for a lack of thermolysin-induced degradation of the ER luminal protein BiP in parasites expressing cytosolic EGFP (Fig. 3B). At 0.005% digitonin, cytosolic EGFP was degraded, confirming plasma membrane permeabilization, while BiP was intact. This digitonin concentration was used to assay the orientation of PfTP_{X_GI}47-EGFP in the ER membrane.

In parasites treated with digitonin, both PfTP_{X_GI}47-EGFP and the ER luminal protein BiP remained intact in the presence of thermolysin, indicating that they were located in the same compartment and, therefore, that the C-terminal EGFP domain was in the ER lumen (Fig. 3C). In contrast, in the presence of 1% Triton X-100—a detergent that disrupted all

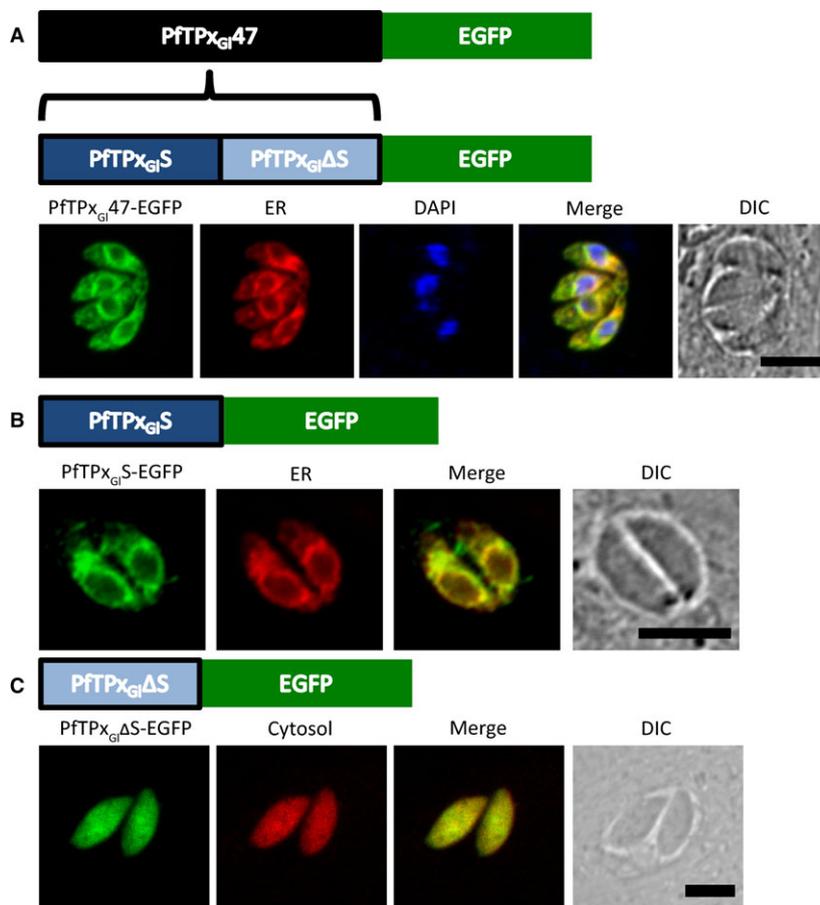


Fig. 1. The PFTP_{G_I}47-EGFP localization in *Toxoplasma gondii*. Microscopic images of parasites expressing (A) PFTP_{G_I}47-EGFP (green), (B) PFTP_{G_I}S-EGFP (green), in which the residues 1–21 of PFTP_{G_I} are fused to EGFP, and (C) PFTP_{G_I}ΔS-EGFP (green), with a methionine residue followed by residues 22–47 fused to EGFP. ER marker protein P30-mCherry-HDEL (red) and cytosolic protein Hexokinase-mCherry (red) have been used to label the respective compartments. Scale bar = 5 μm.

membranes—both PFTP_{G_I}47-EGFP and BiP were degraded, validating the observations in digitonin-treated cells. EDTA was used to inhibit degradation in equivalent control reactions to confirm that the proteolysis was indeed a result of the specific enzymatic activity of thermolysin. The distortion of the PFTP_{G_I}47-EGFP bands in the wells containing thermolysin might be a consequence of the large amounts of thermolysin running at the size of ~34 kDa. Incidentally, the enzymatic degradation of EGFP by thermolysin consistently resulted in a proteolysis-resistant fragment (Fig. 3B), which has been reported elsewhere [45].

The second approach to confirm the PFTP_{G_I}47-EGFP orientation was fluorescence complementation using split-GFP [46]. Split-GFP is GFP that has been engineered as two separate parts—strands 1 to 10 (S1–10) of the barrel and strand 11 (S11)—that can each be expressed as fusions with two separate proteins. While neither S1–10 nor S11 fluoresces independently of the other, self-assembly due to proximity *in vivo* results in fluorescence.

In this report, GFP S1–10 has been expressed either in the cytosol, free of targeting signals, or in the ER, tagged to P30 at the C terminus along with the ER retention/retrieval sequence HDEL. In order to assess the orientation of PFTP_{G_I}47 in the ER membrane, parasite lines expressing PFTP_{G_I}47 with a C-terminal S11 tag were transiently transfected with both GFPS1–10 in the ER—ERS1–10—and GFPS1–10 in the cytosol—CyS1–10. If the C-terminal S11 domain faced the ER lumen, split-GFP complementation would occur in parasites expressing ERS1–10 within the ER (Condition I, Fig. 3D). Complementation in the parasites transfected with CyS1–10 would indicate that the C-terminal S11 domain was located in the cytosol (Condition II, Fig. 3D).

The localizations of both ERS1–10 and CyS1–10 were confirmed by immunostaining with polyclonal anti-GFP antibodies (Fig. 3E,F in red). The distinct perinuclear fluorescence staining pattern observed for ERS1–10 and the diffused fluorescence observed throughout the cell for CyS1–10 are patterns that are consistent with the expected ER and cytosolic

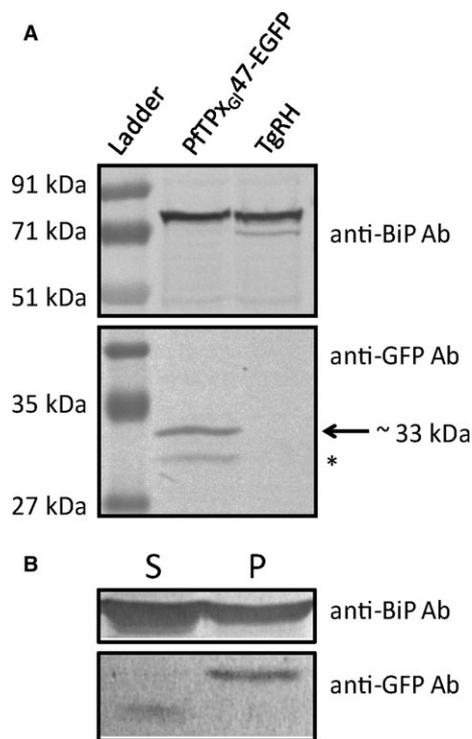


Fig. 2. Western blots of parasites expressing PfTP_{xGI}47-EGFP. Western blot analyses for (A) whole parasite lysates of PfTP_{xGI}47-EGFP transfectants and untransfected *Toxoplasma gondii* RH parasites using anti-GFP and anti-BiP antibodies (B) fractionation of PfTP_{xGI}47-EGFP parasites into soluble [S] and membrane/pellet [P] fractions. The input for the fractionation experiments gave results identical to those of the whole parasite lysate (A).

localizations of these proteins. Green fluorescence was observed in the ER lumen only in parasite lines expressing PfTP_{xGI}47-S11 that had been transfected with ERS1–10 (Fig. 3E), but not in the CyS1–10 transfectants (Fig. 3F). This indicated that complementation had occurred with ER luminal S1-10 (Condition I, Fig. 3D), thereby confirming that the C terminus of the protein was positioned in the ER lumen.

Therefore, using two different approaches, the orientation of PfTP_{xGI}47-EGFP was found to be such that the C-terminal domain of the protein was in the ER lumen, while the N-terminal transmembrane domain was anchored at the ER membrane.

The two sections of the N terminus work in conjunction to ensure membrane anchorage and protein functionality

So far, this study has demonstrated that residues 1–21 of this N terminus are sufficient for targeting the protein to the endoplasmic reticulum, that the C terminus

of this protein resides in the ER lumen, and that the N terminus of PfTP_{xGI}47-EGFP is membrane anchored. If this is indeed the case, then it stands to reason that this transmembrane domain should be able to anchor a different downstream sequence to the ER membrane. Additionally, the role of residues 22–47, immediately preceding the glutathione peroxidase functional domain, remains to be assessed. In order to answer these questions, two chimeric constructs with a known *Toxoplasma* apicoplast-targeted luminal protein, acyl carrier protein (ACP), were generated with EGFP tags. The first was a fusion protein of the signal peptide of ACP with residues 22–47 of PfTP_{xGI} (ACP_{SP}-PfTP_{xGI}ΔS-EGFP). The second was a fusion protein of residues 1–21 of PfTP_{xGI} with the transit peptide of ACP (PfTP_{xGI}S-ACP_{TP}-EGFP).

In parasites expressing ACP_{SP}-PfTP_{xGI}ΔS-EGFP, western blots confirmed cleavage at the predicted site after the ACP signal peptide, resulting in a band that closely correlated with the expected size of 31.3 kDa (Fig. 4A). Upon imaging to assess whether residues 22–47 of PfTP_{xGI} had a role in targeting within the endomembrane system, dispersed structures were observed throughout the parasite, suggesting that the protein had entered the post-ER secretory system (Fig. 4A). This led us to conclude that the protein had escaped the ER lumen with the bulk flow. Indeed, in support of this, no colocalization was observed with the ER marker protein P30-mCherry-HDEL. Additionally, colocalization was not observed even with the apicoplast marker protein FNR-RFP [47], indicating that in this context residues 22–47 possessed no apicoplast targeting information independently of residues 1–21.

In the case of PfTP_{xGI}S-ACP_{TP}-EGFP, the protein was expected to remain anchored to the ER membrane. However, microscopy revealed that the protein had exited the ER and was located in the post-ER secretory system (Fig. 4B), similar to the observation in parasites expressing ACP_{SP}-PfTP_{xGI}ΔS-EGFP. This is supported by the lack of colocalization with the ER marker protein P30-mCherry-HDEL. Western blots revealed that this protein was indeed cleaved (Fig. 4B). While cleavage at the end of the predicted PfTP_{xGI} signal sequence, at residue 21, would have resulted in a 35.4 kDa product, the size of the observed bands was indicative of cleavage further along the polypeptide chain, within the ACP transit peptide sequence, at more than one site. Additionally, the lack of colocalization with the apicoplast marker FNR-RFP suggested that cleavage had taken place within the ACP transit peptide as it was no longer capable of mediating apicoplast localization. As this ACP transit peptide

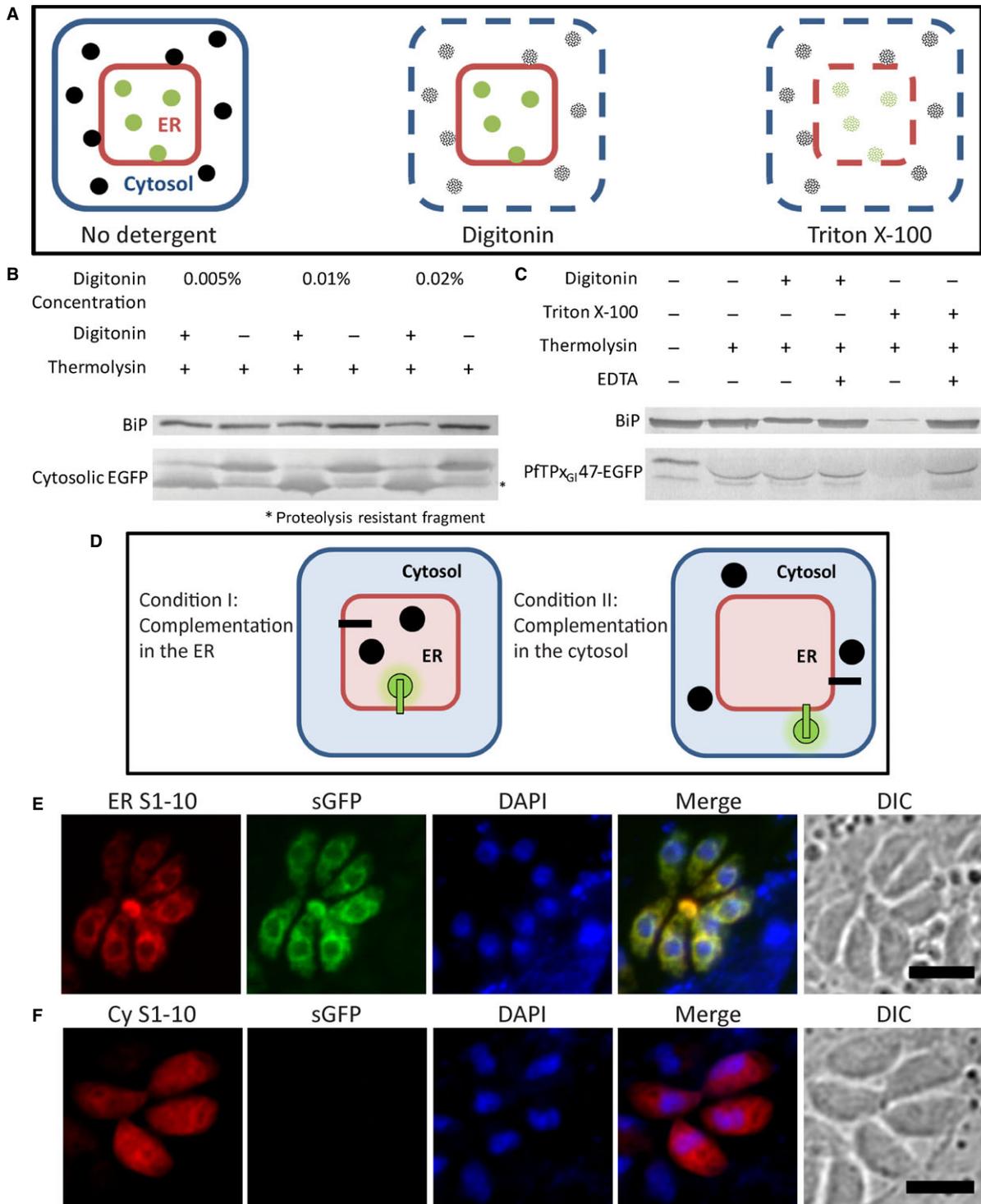


Fig. 3. Assessment of PFTP_{X_G}47-EGFP topology in the membrane. (A) Schematic representation of the protease protection assay. Digitonin permeabilization and protease treatment of parasites expressing (B) cytosolic EGFP for standardization and (C) PFTP_{X_G}47-EGFP. (D) Schematic representation of the possible outcomes of split-GFP complementation in the ER and the cytosol. Split-GFP complementation in parasites expressing PFTP_{X_G}47-S11, and either (E) ER luminal GFPS1-10 or (F) cytosolic GFPS1-10. Scale bar = 5 μ m.

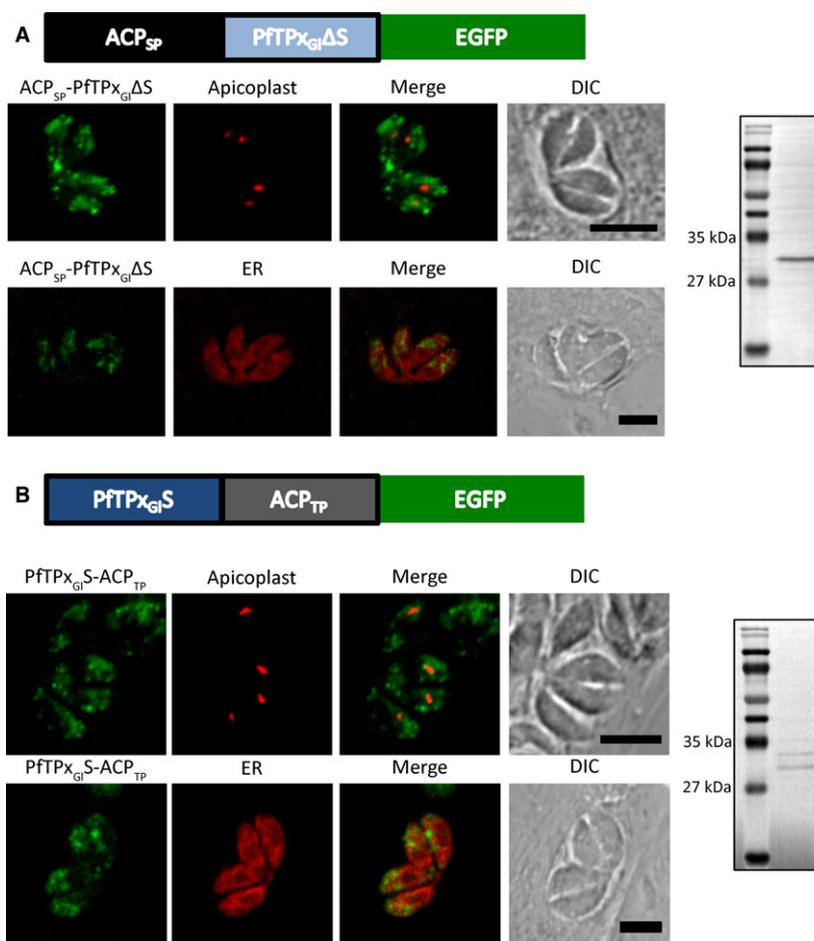


Fig. 4. Mutational analysis of the PfTP_{XGI} N terminus to study the signal sequence. Microscopic images of parasites expressing ACP and PfTP_{XGI} chimeric constructs (A) ACP_{SP}-PfTP_{XGI}ΔS-EGFP (green) and (B) PfTP_{XGI}S-ACP_{TP}-EGFP (green). The marker for the apicoplast is FNR-RFP (red), and for the ER is P30-mCherry-HDEL (red). Scale bar = 5 μm. Western blots of parasites expressing ACP_{SP}-PfTP_{XGI}ΔS-EGFP and PfTP_{XGI}S-ACP_{TP}-EGFP using anti-GFP antibodies have been shown next to the respective constructs.

sequence is not conventionally cleaved, one might gather that in the absence of a strong cleavage consensus in the signal anchor of PfTP_{XGI}, alternative sites are preferred further downstream.

This implies that generating a signal peptide cleavage site downstream of residue 21 of PfTP_{XGI} would result in the cleavage of PfTP_{XGI}47-EGFP. To test this hypothesis, the methionine at position 26 was mutated to an alanine, generating the construct PfTP_{XGI}47-M26A-EGFP. In theory, this should have generated a cleavage site immediately after the serine at position 28. Western blots of lysates of parasites expressing PfTP_{XGI}47-M26A-EGFP indicated that cleavage did actually take place at the predicted site, resulting in a band that closely matched the expected size of 30.4 kDa (Fig. 5A). Imaging of transfectants expressing this protein confirmed that the protein had exited the ER with the bulk flow and was located in the post-ER secretory system. Notably, the protein did not colocalize with the ER marker protein P30-mCherry-HDEL, or with the apicoplast marker protein FNR-RFP (Fig. 5A). From these data, it is evident that in

the presence of a downstream cleavage site, the PfTP_{XGI} signal anchor can serve as a canonical signal peptide.

Transfection of the M26A mutant protein in *Plasmodium falciparum*

Once the signals in the PfTP_{XGI} N terminus that were involved in the first step of the trafficking of this protein were characterized by mutational analysis in *T. gondii*, we decided to test one of the constructs in *P. falciparum*. The revelation that a single point mutation in the PfTP_{XGI} N terminus could drive the protein to a different destination was the motivation for choosing to study the M26A mutant in *P. falciparum*. To ensure that these results were in agreement with the published literature [14], we also expressed the first 47 residues of native PfTP_{XGI} fused to GFP in *P. falciparum*. Notably, in support of the premise of this report, the generation of these two transfectants and the acquisition of data took several months due to the host of complications discussed previously.

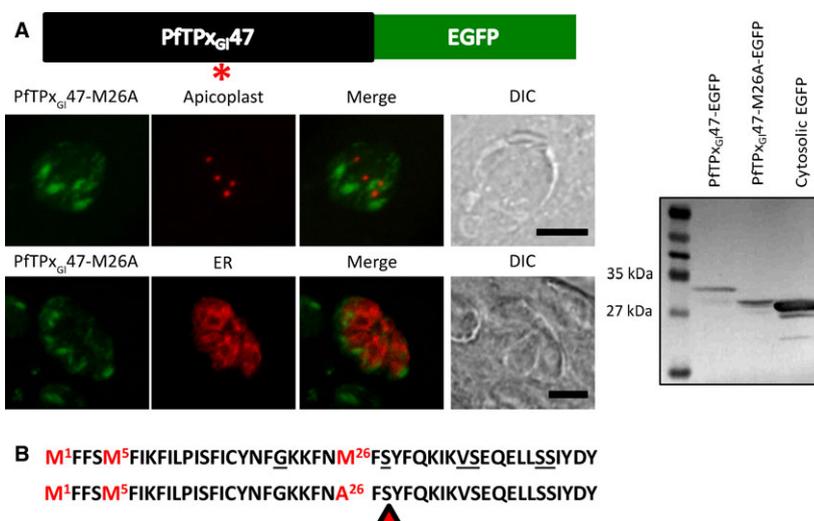


Fig. 5. Mutational analysis of the PfTPx_{GI} N terminus to study cleavage. (A) Microscopic images of PfTPx_{GI}47-M26A-EGFP (green). Apicoplast marker protein FNR-RFP (red) and ER marker protein P30-mCherry-HDEL (red) are used for labeling the respective compartments. Scale bar = 5 μ m. (A) Western blot of parasites expressing PfTPx_{GI}47-M26A-EGFP using anti-GFP antibodies is shown next to the images. (B) The N-terminal sequence of PfTPx_{GI} displays the putative point mutations that can generate cleavage sites (underlined) and the expected site of cleavage in PfTPx_{GI}47-M26A-EGFP (red arrow).

We observe that PfTPx_{GI}47-GFP targeting in the parasite agrees very closely with the published data. In addition to faint, diffused fluorescence throughout the cell, indicative of cytosolic localization (Fig. 6A), the protein was also targeted to apicoplast in most but not all cells observed (Fig. 6C), as confirmed by colocalization with the apicoplast marker PfClpP [31]. Three-dimensional reconstructions using Z-stacked images also showed colocalization of GFP and PfClpP staining (Fig. 6E, PfTPx_{GI}47-GFP). The expression of PfTPx_{GI}47-M26A-GFP, on the other hand, resulted in targeting to punctate structures dispersed throughout the cell, with partial colocalization to the apicoplast (Fig. 6B,D) in a few cells. Here too, the three-dimensional reconstructions were consistent with these observations (Fig. 6E, PfTPx_{GI}47-M26A-GFP).

The Pearson's correlation coefficient for the colocalization of the protein of interest with the apicoplast marker PfClpP was calculated for each parasite that was analyzed in the parasite line expressing the wild-type protein ($n = 28$), as well as in the parasite line expressing the mutant protein ($n = 28$). A dot plot of the correlation coefficients in wild-type and mutant parasite lines is displayed in Fig. 6F. A marked difference was observed between the mean correlation coefficient, representative of the extent of colocalization, of the wild-type protein and that of the mutant protein with the apicoplast marker. There was no overlap in the data points, within one standard deviation of the mean, between the wild-type and mutant protein data

sets. Notably, the mean Pearson's correlation coefficient for the wild-type protein was above 0.3, indicative of a moderate correlation [48], and therefore of the colocalization of the PfTPx_{GI}47-GFP protein with the apicoplast marker [32]. The mean correlation coefficient for the mutant protein, on the other hand, fell below the threshold of 0.1, which is considered the minimum value required for any data to qualify as having a small correlation [48]. This suggested that the extent of colocalization of the mutant PfTPx_{GI}47-M26A-GFP protein with the apicoplast marker was negligible. From these results, it is evident that the M26A mutation alters localization in both *Toxoplasma* and *Plasmodium*, resulting in the respective proteins localizing to structures that are distinct from the apicoplast.

Discussion

Toxoplasma gondii as a heterologous system for studying *Plasmodium* biology

Admittedly, the generation of transfectants in *P. falciparum* makes the screening of multiple mutations and their effects on targeting time-consuming as the low levels of DNA uptake result in longer recovery times under drug pressure [15,16]. In this study, *T. gondii*, a related apicomplexan, has been used to serve as an efficient preliminary screening system to identify a smaller number of relevant mutations whose influence

on trafficking can then be verified in *P. falciparum*. While *Toxoplasma* has been used to express *Plasmodium* proteins for the generation of vaccines and the characterization of enzymes [49–52], to the best of our knowledge, this is the first report outlining the detailed dissection of the targeting signals of a *Plasmodium* protein in *Toxoplasma*. The similarity in the intracellular compartments between the two organisms coupled with the greater clarity in subcellular structure and increased efficiency of transfection makes *T. gondii* an intermediary in understanding certain aspects of protein trafficking in *P. falciparum* [1,53,54]. This study highlights which of these aspects are suitable for study in *Toxoplasma*.

The use of *Toxoplasma gondii* identifies the N-terminal signal anchor in PFTP_{XGI}

Expressing the N terminus of PFTP_{XGI} in *T. gondii* resulted in targeting to the endoplasmic reticulum. This is a fate differing from that in *P. falciparum*, where the N-terminal 47 residues target the protein to the apicoplast and the cytosol. This difference in localization could be due to specific sequence requirements for apicoplast trafficking differing between the two parasites; for example, the lengths of predicted apicoplast targeting sequences are smaller in *P. falciparum* compared to *T. gondii* [55]. The localization of this protein to the ER, however, was favorable as the ER is known to be the first step in trafficking proteins to the apicoplast. We decided therefore to exploit this phenotype to dissect the signals involved in ER uptake.

Mutational analysis of the N terminus of PFTP_{XGI} defined a signal anchor sequence between residues 1–21—a signal sequence that targets the protein to the membrane of the endoplasmic reticulum. Membrane anchorage is consistent with results seen in *P. falciparum* organelles [13], and here, use of the heterologous system was able to define the N terminus of the protein as responsible for membrane anchorage and for targeting.

Localization of the protein in the ER allows identification of membrane topology

Significantly, the C-terminal domain of the protein was located in the ER lumen, indicating that this is most likely the topology retained upon reaching the apicoplast [42]. The fortuitous localization of PFTP_{XGI}47-EGFP to the ER membrane in *Toxoplasma* was extremely useful in determining the orientation of the protein using digitonin permeabilization that

occurs in membranes enriched in cholesterol. The ER membrane contains less cholesterol than the plasma membrane allowing selective digitonin permeabilization while performing the protease protection assays. In contrast, protease protection assays to determine topology would have been challenging with the native protein localized to the apicoplast in *Plasmodium*. Here, as apicoplast membranes are enriched in cholesterol [56], digitonin would have permeabilized the organellar membrane as well as the plasma membrane, allowing the protease to digest PFTP_{XGI} regardless of orientation.

Split-GFP experiments to determine the orientation of the protein in the membrane also gave unambiguous results as markers for only the cytosol and ER lumen were required, and these markers were relatively simple to generate. The larger size and clarity in the ultrastructure of the *Toxoplasma* ER were key to obtaining clean results with split-GFP. In contrast, to have done the same split-GFP experiment in *Plasmodium* would have required a marker for the cytosol and another for the space between the outer membrane and the next membrane of the apicoplast. Only one apicoplast membrane protein has been definitively localized to this outer membrane, PfoTPT in *Plasmodium*. Terminal GFP tagging of this protein would not place the split-GFP in the outermost compartment of the apicoplast because both the N termini and C termini of this protein reside in the cytosol [57]. As little is known in both *Plasmodium* and *Toxoplasma* about the exact orientations of most apicoplast membrane proteins, or the specific membranes in which they reside, it would not have been feasible to conduct this experiment, while the protein was in the apicoplast membrane.

Our results, where the protein of interest was found in the ER, show that this localization provided an unexpected benefit for both the digitonin and split-GFP strategies. We suggest that this approach of forcing a protein into the ER compartment, such as with an HDEL ER retention/retrieval signal, would enable the easier assessment of the membrane topology of Golgi-dependent apicoplast proteins.

Implications of the orientation of the PFTP_{XGI}

It has been implied that the orientation toward the ER lumen suggests a retention of the same orientation in the apicoplast membrane [42]. Thus far, only proteins known to reside in the outermost membrane of the apicoplast have been reported to be devoid of cleavage/processing and a canonical bipartite targeting sequence, such as is observed for PFTP_{XGI}, while

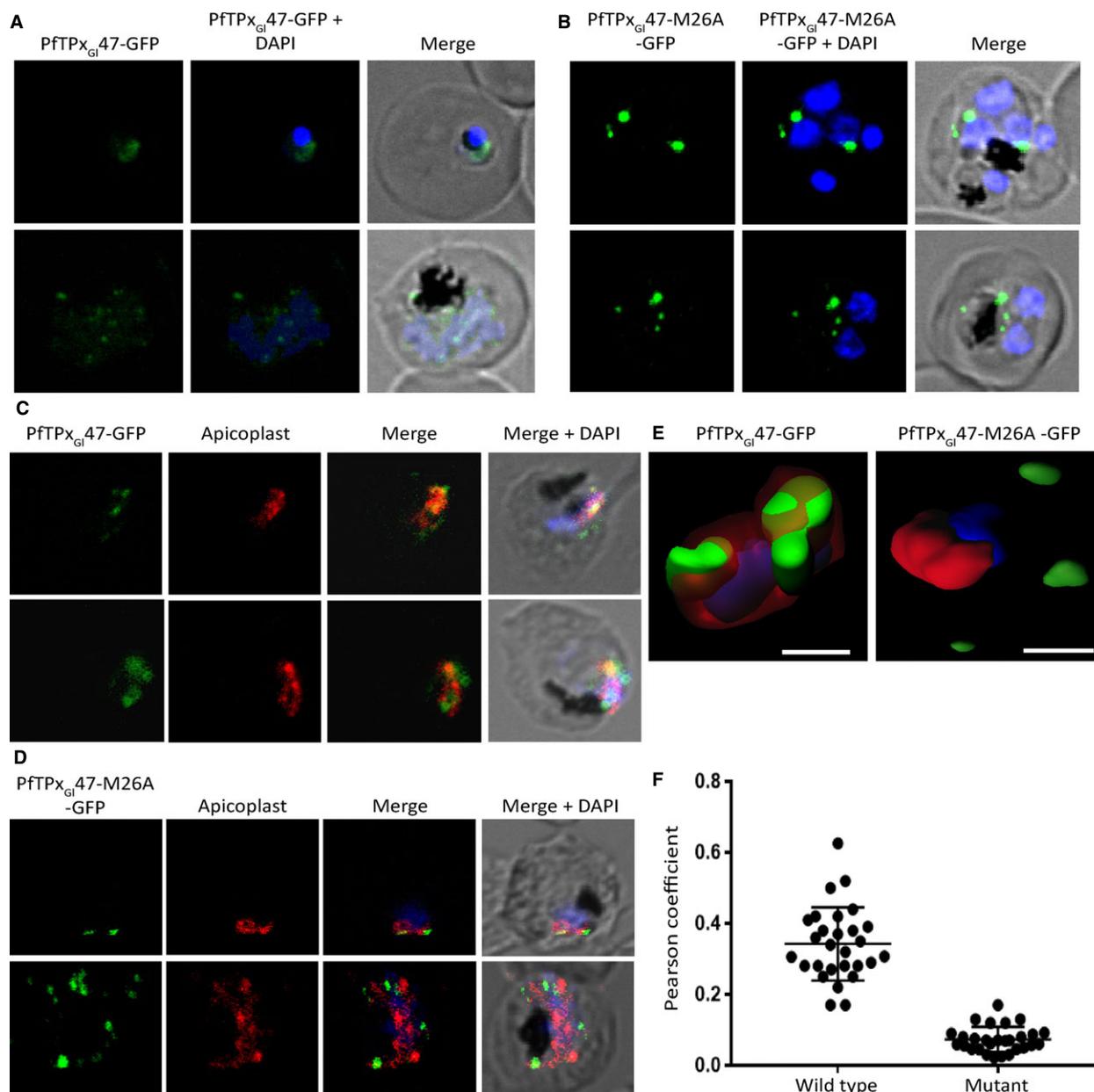


Fig. 6. Expression of GFP fusions of native and mutated PFTP_{GI} N termini in *Plasmodium* transfectants. Immunofluorescence images of parasites expressing (A) PFTP_{GI}47-GFP (green) (B) PFTP_{GI}47-M26A-GFP (green). Localization with the apicoplast marker protein PfClpP (red) is shown in parasites expressing (C) PFTP_{GI}47-GFP (green) and (D) PFTP_{GI}47-M26A-GFP (green). (E) A 3D reconstruction using Z-stack images of parasites expressing PFTP_{GI}47-GFP (green) or PFTP_{GI}47-M26A-GFP (green) with apicoplast marker protein PfClpP (red). Scale bar = 1 μ m. (F) A dot plot of the Pearson's correlation coefficients for colocalization between the protein of interest and the apicoplast marker PfClpP in parasites expressing the N termini of the wild-type ($n = 28$) and mutant ($n = 28$) proteins fused to GFP. The mean correlation coefficient, and therefore colocalization with PfClpP, is significantly higher for the wild-type protein than for the mutant protein.

trafficking to this destination. If the ER orientation is retained, this would mean that in *P. falciparum*, the peroxidase domain of PFTP_{GI} should be located in the outermost compartment of the apicoplast, with the N terminus of the protein anchored to the outermost

membrane. This orientation of the protein is significant as there are a large number of antioxidant proteins in the cytosol, but none reported so far in this compartment of the apicoplast in *Plasmodium* [14,58]. This would make PFTP_{GI} the first antioxidant enzyme

reported that could handle oxidative stress in this niche. Additionally, this protein might serve as a bait in the outermost compartment of the apicoplast that could then be used to identify other proteins in this compartment.

Membrane anchorage of the protein also depends on lack of a signal peptide cleavage site

As the protein is oriented toward the ER lumen, the residues downstream of the signal anchor are available to the signal peptidase. However, the protein is not cleaved, either in *Plasmodium* [10] or in *Toxoplasma* (this report). At a glance, the sequence of the PFTP_{X_{GI}} N terminus reveals enrichment in residues that are part of the signal peptide cleavage consensus [41,59]. Almost all of these residues, as highlighted in the figure, lie in close proximity to the signal anchor (Fig. 5B). This means that although not cleaved, there are many point mutations that can potentially result in any of these residues partaking in the formation of a functional cleavage site. When this hypothesis was tested by mutating the methionine at position 26 to an alanine, the protein was cleaved at the expected site (Fig. 5A). Given the ease of generation of cleavage sites, it might be speculated that the combination of residues in this stretch (residues 22–47) has been selected against cleavage in order to retain the membrane anchorage of the protein. Interestingly, while the M26A mutation resulted in a dramatic change (exit from the ER) in the localization in *Toxoplasma*, the change was more subtle in *Plasmodium*. This difference also suggests that the two apicomplexan parasites do differ in certain aspects of the apicoplast trafficking machinery. Therefore, one must exercise discretion when using *Toxoplasma* as a heterologous system to study the trafficking of *Plasmodium* proteins.

The insights so far might lead one to question why such a long string of residues, with several potential cleavage sites, has been retained at all. The functional glutathione peroxidase domain of PFTP_{X_{GI}} begins at residue 43 (Conserved Domains Database). One might recall that in parasites expressing the PFTP_{X_{GI}} signal anchor (1–21) directly fused to EGFP in the absence of residues 22–47, native EGFP fluorescence was absent. However, anti-GFP antibodies were effective at determining the localization of the protein. This indicated that the protein was targeted correctly but was misfolded, possibly due to steric hindrance from proximity to the membrane. From the data, it appears that residues 22–47 serve to space the functional domain from the membrane to ensure the proper folding of the protein.

Conclusions

While *Toxoplasma* has previously been used to study *Plasmodium* vaccine candidates, enzymes [49], and even protein localization [9,19], this is the first report outlining its utility in the dissection of some of the targeting signals of a *Plasmodium* protein.

This study successfully elucidates that PFTP_{X_{GI}} possesses an N-terminal signal anchor at residues 1–21, with the C-terminal domain in the organelle lumen. Residues 22–47 of the N terminus appear to serve the role of a spacer region lacking a cleavage site, so as to permit proper protein folding while reinforcing membrane anchorage. The use of *T. gondii*, in addition to expediting screening, has been particularly significant in the determination of the membrane topology of PFTP_{X_{GI}}, an exercise that would have been difficult in its native context owing to the factors outlined. PFTP_{X_{GI}} is now in an emerging class of membrane-anchored proteins that target to the *Plasmodium* apicoplast independently of the canonical bipartite sequence involved in the trafficking of apicoplast luminal proteins.

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Author contributions

AN designed and performed experiments, analyzed data, and wrote the paper. PM and VT performed

experiments and analyzed data. PKR analyzed and critically interpreted the data; AM designed experiments and analyzed data. SP designed experiments, analyzed data, and wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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