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Role of the ER and Golgi in protein export by Apicomplexa

Michael J Coffey[1,2], Charlie Jennison[1,2], Christopher J Tonkin[1,2] and Justin A Boddey[1,2]

Apicomplexan parasites cause diseases of medical and agricultural importance linked to dramatic changes they impart upon infected host cells. Following invasion, the malaria parasite *Plasmodium falciparum* renovates the host erythrocyte using mechanisms previously believed to be malaria-specific. This involves proteolytic cleavage of effectors in the endoplasmic reticulum that licences proteins for translocation into the host cell. Recently, it was demonstrated that the related parasite *Toxoplasma gondii*, responsible for disease in immunocompromised individuals and congenital birth defects, has an analogous pathway with some differences, including proteolytic processing in the Golgi. Here we review the similarities and distinctions in export mechanisms between these and other Apicomplexan parasites to reconcile how this group of pathogens modify their host cells to survive and proliferate.

Addresses
[1] The Walter and Eliza Hall Institute of Medical Research, Parkville 3052, Victoria, Australia
[2] Department of Medical Biology, The University of Melbourne, Parkville 3052, Victoria, Australia

Corresponding authors: Tonkin, Christopher J (tonkin@wehi.edu.au) and Boddey, Justin A (boddey@wehi.edu.au)

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Introduction

Apicomplexans are a group of obligate intracellular parasites including species of medical and agricultural significance. *Plasmodium* parasites cause malaria which is responsible for considerable morbidity and ~438 000 deaths in 2015 [1]. *Toxoplasma gondii* is one of the most ubiquitous human pathogens and causes severe disease in immunocompromised individuals and pregnant women and is a leading cause of blindness in some countries [2,3].

One of the key pathogenic mechanisms of Apicomplexan parasites is their ability to modify host cells for their own advantage. *Plasmodium falciparum* imparts dramatic changes onto its infected erythrocyte, renovating this inert cell into one capable of supporting growth of a nutrient-hungry parasite, whilst also mediating cytoadherence to evade immune defences [4]. Conversely, *Toxoplasma* infects almost any nucleated cell and recruits host endoplasmic reticulum (ER) [5] and mitochondria [6] to the vacuolar membrane whilst completely re-wiring the transcriptional output of its host cell [7]. This alters cytokine and chemokine production, preventing apoptosis [8], and causes the up-regulation of genes involved in energy metabolism and down-regulation of MHCII expression in antigen presenting cells [9]. In both parasite species, these processes are geared towards promoting survival and replication in the host for successful transmission.

Over the past 15 years, research into host cell modifications and mechanisms of protein export in *P. falciparum* and *Toxoplasma* has taken different trajectories. *P. falciparum* exports proteins into the infected erythrocyte after invasion [10,11] whereby exported proteins are licensed through a proteolytic maturation step [12–15] followed by transport across the parasitophorous vacuole membrane (PVM) into the erythrocyte [16,17••,18••]. The major virulence factors in *Toxoplasma*, however, were shown to take a different export route. They are transported to the rhoptries, which are club-shaped apical organelles that store proteins involved in invasion, and are secreted into the host cell during parasite entry [7,19–21]. Thus, the dogma was established that *P. falciparum* and *Toxoplasma* used vastly different mechanisms for delivering effectors into host cells.

In this review we will compare and contrast recent advances in knowledge of different protein export pathways in Apicomplexans, as the mechanisms appear more conserved than previously thought. We will explore why there is a need for multiple pathways and discuss the role of cargo maturation in the ER or Golgi and what implications this has on protein trafficking mechanisms across the phylum.

Many paths to the same destination

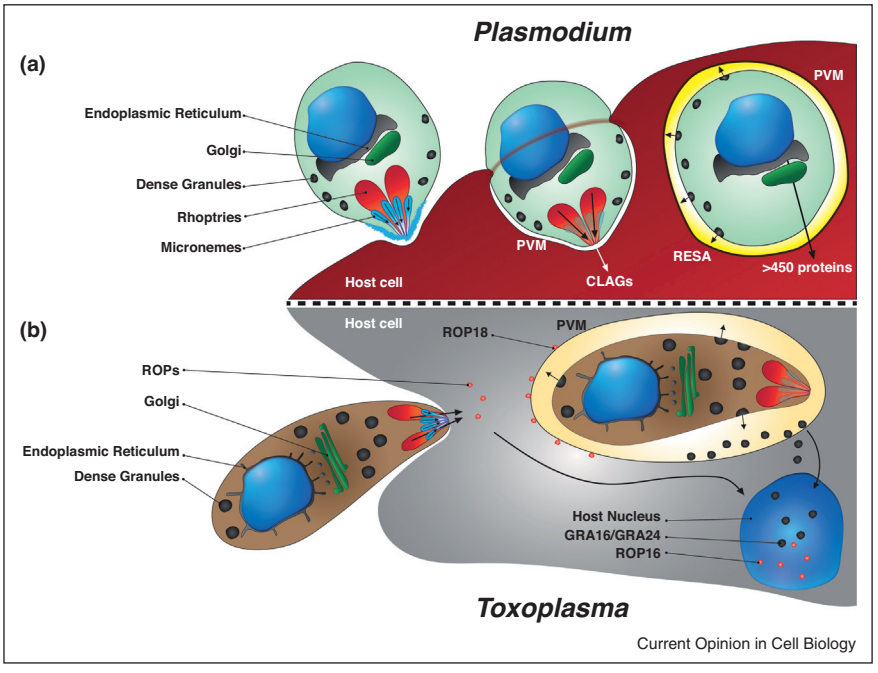
To begin their replicative cycle, Apicomplexan parasites must invade a host cell. This is achieved using a conserved mechanism of invasion that is initiated by sequential secretion of apical organelles [22]. First the

micronemes (organelles whose contents are involved in parasite attachment to, and egress from, the host cell) release adhesins onto the parasite surface, anchoring ligands for intimate host cell attachment (Figure 1). This is followed by rhoptry release, delivering proteins and lipids into the host cell to form the PVM, a barrier that separates the parasite from host cell cytoplasm (Figure 1). Upon completion of invasion, exocytosis of dense granules (DG) delivers proteins into the nascent vacuole and some of these are exported into the host cell (Figure 1).

In *P. falciparum*, the major pathway for export of over 350 proteins [10,11] occurs immediately after invasion [23] and continues for 20–30 hours post-infection in asexual parasites and for the first 2–4 days of gametocytogenesis [24]. Export uses a two-step mechanism where an N-terminal signal peptide (SP) directs entry into the ER, followed by recognition and cleavage of a pentameric amino acid sequence called the *Plasmodium* export element (PEXEL) [10,11], usually located 15–30 amino acids from the SP [25,26,27••]. PEXEL processing is performed by the ER membrane-resident aspartyl protease Plasmepsin V (PMV) [14,15] (Figure 2a). Processing reveals a new N-terminus that is acetylated in the parasite ER, an unusual location for this post-translational modification [12,13], which contains additional unknown information that is important for export [13,28,29]. The steps immediately after cleavage are unknown but PMV contains a unique structural motif that may form part of an ER complex facilitating cargo selection [30••]. An alternate mechanism has been proposed where the PEXEL binds phosphatidylinositol-3-phosphate (PI(3)P) in the ER, which is an unprecedented location for this lipid, independent of PMV activity [31]. However, subsequent studies have failed to confirm PI(3)P in the *Plasmodium* ER or detect PEXEL-lipid binding [27••]. Exported cargo traffic through the Golgi [32] and out of the parasite, where they are unfolded [33] and translocated across the PVM through the *Plasmodium* translocon of exported proteins (PTEX) (Figure 2a) [16,17••,18••].

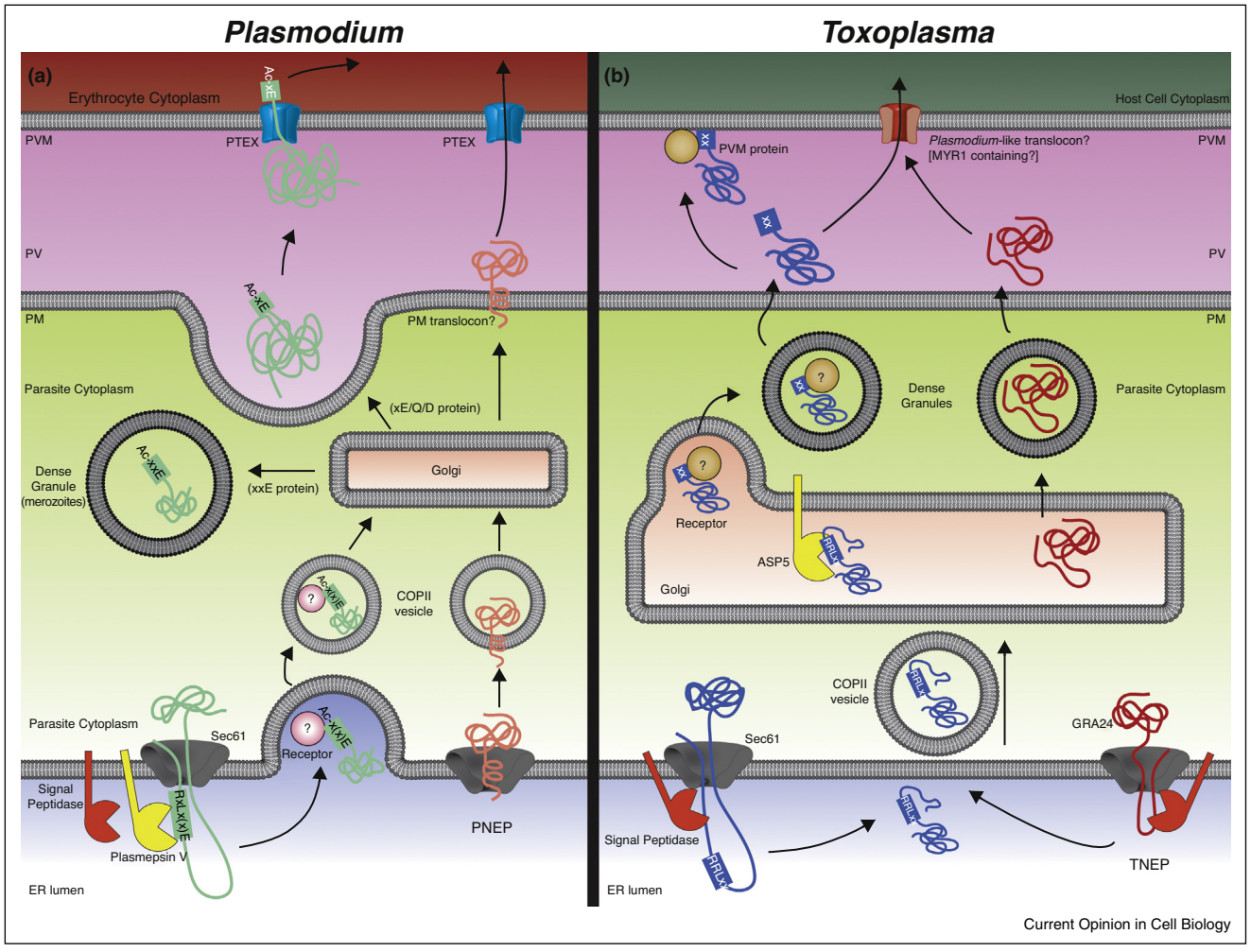
By contrast, the first identified *Toxoplasma* effectors are delivered to the host cell during invasion (Figure 1b). During intracellular replication, the parasite targets

Figure 1



Different paths and timing of protein export to the host cell. **(a)** Left: During schizogony, *Plasmodium* proteins destined for export are trafficked to rhoptries (CLAGs) and dense granules (RESA and others) ready for invasion. Translocon components (PTEX) are also packaged in dense granules. Micronemes and rhoptries containing parasite ligands for host cell recognition, attachment and invasion are secreted onto the parasite's surface and into the host erythrocyte and a tight junction is formed. Center: The merozoite secretes rhoptries into the host cell and enters the erythrocyte creating a parasitophorous vacuole membrane (PVM). Rhoptries release CLAG proteins into the host cell or membrane as well as other proteins and lipids that contribute to the PVM. Right: Following invasion, dense granules are released (small black arrows), PTEX associates at the PVM, and RESA is exported. After DGs are discharged the endoplasmic reticulum-to-PTEX translocon pathway is activated and used to export hundreds of proteins (large black arrow). **(b)** Left: During invasion, *Toxoplasma* injects ROP kinases into the host cell and generates a PVM. ROP16 travels to the host nucleus while ROP18 resides on the surface of the PVM facing the host cell cytosol. Right: Following invasion, tachyzoites synthesize proteins at the ER membrane destined for the PV or host cell and traffic them via the Golgi followed to dense granules. Dense granules then fuse with the parasite membrane releasing their contents (small black spots) into the vacuole (small black arrows). A subset of proteins (including GRA16, 24) is exported across the PVM through an unknown translocon to the host cell nucleus (blue circle in host cell).

Figure 2



Role of the ER and Golgi in protein export. **(a)** Export in *Plasmodium*. Left: Model of the PEXEL pathway. (Apicomplexan parasites cause diseases of medical and agricultural importance. Their success is directly linked to changes imparted upon infected cells.) Proteins containing a signal peptide are co-translationally inserted into the ER through the Sec61 translocon. Proteins containing a canonical or relaxed PEXEL (RxLx(x)E) are co-translationally cleaved by Plasmepsin V (RxL↓) at the ER membrane, revealing a new N-terminus, which is acetylated (Ac-x(x)E-protein), and potentially functions in complex with one or more cargo receptors. These proteins are packaged into COPII vesicles and transit through the Golgi. In developing merozoites, Ac-xxE-proteins are packaged into dense granules for discharge during re-invasion. Ac-xE/Q/D proteins are released by vesicle fusion at the parasite membrane (PM) into the parasitophorous vacuole (PV). At the parasitophorous vacuole membrane (PVM) these proteins are unfolded and funnelled through the *Plasmodium* translocon of exported proteins (PTEX) and delivered into the erythrocyte. Right: Model for PNEP export. A representative PNEP is synthesized and anchored in the ER membrane by a transmembrane domain or signal peptide. The protein diffuses to ER exit sites and buds of with COPII vesicles to the Golgi and is delivered to the PM where it is either removed or translocated through a PM translocon into the PV and then exported through PTEX. **(b)** Export and trafficking via the *Toxoplasma* Golgi. Left: Model of the TEXEL pathway. Proteins containing a signal peptide are co-translationally inserted into the ER through the Sec61 translocon. Following signal peptidase-cleavage of the signal peptide, proteins are packaged into COPII vesicles and trafficked to the Golgi. The TEXEL (RRLxx) is cleaved by aspartyl protease 5 (ASP5) (RxL↓), revealing a new N-terminus (it is unknown whether N-acetylation occurs). Cargo selection occurs in the Golgi and via one or more receptors, before delivery to dense granules. Dense granule contents are released at the PM into the PV. Translocation into the host cell occurs by unknown mechanisms, possibly through a *Toxoplasma*-specific translocon (*Plasmodium*-like translocon), of which MYR1 may be a component. Other cleaved TEXEL proteins (e.g. GRA19) reside in the PV, and TEXEL cleavage is important for efficient PVM localization. Right: Model of TNEP export. A representative TNEP (GRA24) is co-translationally cleaved by SP inside the ER lumen as it passes through Sec61 and is packaged into COPII vesicles for delivery to the Golgi. Following Golgi transit and transport to dense granules, the protein is released into the PV and translocated into the host cell via unknown mechanisms, potentially through the same pathway as exported TEXEL proteins.

ROP16 and ROP18 through the ER and Golgi to the rhoptry organelles, where they accumulate in readiness for reinvasion [7,19,20]. Rhoptries are secreted during invasion, establishing the dogma that *Toxoplasma* only exports proteins at this time, thereby differing substantially from *P. falciparum*.

This recently changed after the discovery of the DG protein GRA16, which is constitutively exported throughout intracellular parasite development, and accumulates in the host nucleus [34] (Figure 1b). This protein possesses an N-terminal PEXEL-like motif that is cleaved by the Golgi membrane-resident Aspartyl Protease 5 (ASP5) in an orthologous manner to PMV cleavage of the PEXEL in the *Plasmodium* ER (Figure 2b) [35••,36••,37••]. This motif was therefore named the *Toxoplasma* export element (TEXEL) [37••]. Trafficking via the dense granules in *Toxoplasma* is the default secretory pathway, and a considerable number of DG proteins have now been identified [38], with many containing a TEXEL motif [35••,36••,37••,39•,40]. Interestingly, unlike in *Plasmodium*, the location of the TEXEL is not always physically constrained to the N-terminus [37••,39•] but whether these particular TEXEL proteins are exported, and which fragment, is yet to be determined.

In *Plasmodium* spp., DGs are synthesized only in merozoites (the invasive form of the malaria parasite that invades erythrocytes) and are not regenerated following invasion [22]. However, developing merozoites do produce and store proteins in these organelles for export following invasion (Figure 2a). These include ring-expressed surface antigen (RESA) [41,42] and approximately 12 other DnaJ-like and PHIST family proteins, which all contain a 'relaxed' PEXEL motif (RxLxxE/Q/D) [25,43]. The relaxed PEXEL is cleaved by PMV in the ER and is necessary for export [43] perhaps by targeting the mature proteins to DGs (Figure 2a). Proteins stored in DGs also include PTEX components [16] and they are released into the vacuole immediately post invasion [16,23] (Figure 1a). RESA is translocated within minutes after invasion [23], demonstrating the DG export pathway is dedicated to the earliest stages of cellular remodelling.

Another class of *Plasmodium* proteins are exported even earlier than those in DGs. Like the ROP proteins in *Toxoplasma*, it has become clear that *P. falciparum* exports proteins from rhoptries. The cytoadherence-linked asexual gene (CLAG) family (also called RopH) [44] are delivered to the erythrocyte surface and modify membrane permeability for the parasite's benefit [45]. The export of CLAG3 was recently shown to be PTEX-independent [18••], demonstrating that this protein likely enters the host cell directly after rhoptry discharge into the host membrane or cytosol (Figure 1a) [46].

A further subset of exported proteins exists in *Plasmodium*, recently also identified in *Toxoplasma*, whereby proteins lacking a PEXEL/TEXEL or a rhoptry-targeting signal [47] possess a hydrophobic stretch (a SP or transmembrane domain) as well as various non-conserved export signals [48–52] that target them through PTEX [17••,18••,28]. These are called PEXEL-negative exported proteins (PNEPs) in *Plasmodium* [48], and we shall refer to them as TEXEL-negative exported proteins (TNEPs) in *Toxoplasma*. They include the dense granule protein GRA24 in *Toxoplasma* [52] and skeleton binding protein 1 [53] and Erythrocyte Membrane Protein 1 (PfEMP1) in *P. falciparum* [54,55]. Although PMV and ASP5 do not cleave PNEPs and TNEPs, respectively [37••,43], export of these proteins can be dependent on substrates of these enzymes. This is the case for the virulence adhesin PfEMP1, which is not cleaved by PMV [43] but requires multiple PEXEL-containing proteins for transport across the PVM and erythrocyte membrane [56,57] and for cytoadherence [58,59••]. In *Toxoplasma*, trafficking of the TNEP, GRA24 [52], is ASP5-dependent [35••,36••,37••]. This also appears to be an indirect effect, where ASP5 matures another protein(s) required for its transport [37••]. One protein is MYR1 (MYc-Regulation 1), recently identified by its role in host c-Myc induction and which is also required for GRA24 export, suggesting it may be a translocon component [60••]. Interestingly, MYR1 localizes to the PVM and may not be exported [60••], but it possesses a TEXEL motif almost two-thirds along its sequence that is cleaved by ASP5 [37••]. The identification of MYR1 highlights a potential mechanism linking export of TNEPs with ASP5. However, MYR1 also illustrates a fundamental difference between *Toxoplasma* and *Plasmodium*, in that TEXEL proteins can be localized at the PVM [37••], which so far has not been the case for *Plasmodium* PEXEL proteins. PVM-targeting was also reported for other TEXEL-containing proteins [40].

Beyond *Toxoplasma* and *Plasmodium* spp., less is understood about protein export in other Apicomplexan species. Recently, it was reported that homologues of PMV are present in other Apicomplexa, plant pathogens and plants [30••]. A PEXEL-like motif (PLM) has also been described in *Babesia bovis* (Rx(x)L) [39•], which is distantly related to *Plasmodium* and infects bovine erythrocytes, causing agricultural loss. Interestingly, *B. bovis* degrades the PVM soon after invasion [61] so export does not require translocation across this membrane. Indeed, a number of *B. bovis* proteins containing a signal sequence but lacking a PLM are exported [62]. However, processing of the PLM appears necessary for optimal targeting to 'spherical bodies' before export, which could ensure regulated release [39•]. A PLM has also been reported for *Cryptosporidium* [39•], though it requires functional characterization. Thus, maturation of the PEXEL is not malaria-specific as previously thought, but is a conserved step in Apicomplexan protein trafficking and export. Inhibitors of PMV [30••,59••,63,64•] and ASP5 [37••] have now been developed and it will be interesting to

see whether blocking these enzymes can be an antiparasitic strategy in future.

Overall, these findings demonstrate that Apicomplexans have many export pathways. The question of why is not yet clear but timing is obviously a factor, since distinct pathways are active at different times. Other reasons could include different protein sizes, solubility, folding, expression levels and final topology. And so, faced with the problem of exporting a complex variety of different effectors at different times, Apicomplexans have evolved diverse pathways as a solution.

Similar decisions at different locations?

The function of proteolytic cleavage in the ER and Golgi for export by Apicomplexans is likely the exposure of a signal located in the new N-terminus that directs the next step in trafficking. The fact that the aspartyl proteases involved are located in different organelles, combined with the differences in the positional constraints of their cleavage sites, raises some interesting possibilities. Currently, the main hypothesis in *P. falciparum* is that PEXEL cleavage by PMV licences export by directing proteins from the secretory pathway into a distinct export pathway [14,15]. Fascinatingly, PMV cleavage of the PEXEL occurs very rapidly, likely during co-translational ER entry [27••,59••] making the decision very early. Shifting the PEXEL in knob-associated histidine rich protein (KAHRP) from its conserved position towards the N-terminal SP blocked cleavage by PMV and export, and the protein was instead cleaved by signal peptidase and secreted to the PV [27••]. This suggests that the PEXEL position is conserved for important reasons that are linked with processing and cargo selection in the early secretory pathway [27••]. Conversely, cleavage by ASP5 is spatially separated from ER import, as it occurs in the Golgi, and the TEXEL is not positionally constrained in *Toxoplasma* [35••,36••,37••,39•]. Is the position of the cleavage site therefore defined by the cellular localization of the protease? Does this influence the selection opportunities and subsequent destinations of cargo, given that the PEXEL mediates export whereas the TEXEL also permits targeting to the PVM? Studies aimed at addressing these questions will help to understand why apparently similar decisions are made at different locations. An in silico analysis of the PLM in *Babesia* and *Cryptosporidium* showed that they are spatially constrained, like in *Plasmodium* [39•]. It will be interesting to see if the PLM-cleaving enzymes are located in the ER or Golgi and whether they control export alone or transport to other destinations.

Conclusions and future perspectives

It has become clear that Apicomplexans harbour multiple pathways that act concurrently and successively to modify the host as needed. Future studies are needed to decipher the steps in trafficking immediately after PEXEL/TEXEL cleavage. Examining the interacting partners of PMV/ASP5 and matured substrates will be important. Indeed, understanding whether different cargo selection occurs for proteins cleaved at different locations seems a worthy cause. Together, these findings have led to some provocative ideas and provided testable hypotheses on the fundamental mechanisms of export in Apicomplexan parasites.

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