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## Review

The roles of intramembrane proteases in protozoan parasites<sup>☆</sup>L. David Sibley<sup>\*</sup>

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## ABSTRACT

Intramembrane proteolysis is widely conserved throughout different forms of life, with three major types of proteases being known for their ability to cleave peptide bonds directly within the transmembrane domains of their substrates. Although intramembrane proteases have been extensively studied in humans and model organisms, they have only more recently been investigated in protozoan parasites, where they turn out to play important and sometimes unexpected roles. Signal peptide peptidases are involved in endoplasmic reticulum (ER) quality control and signal peptide degradation from exported proteins. Recent studies suggest that repurposing inhibitors developed for blocking presenilins may be useful for inhibiting the growth of *Plasmodium*, and possibly other protozoan parasites, by blocking signal peptide peptidases. Rhomboid proteases, originally described in the fly, are also widespread in parasites, and are especially expanded in apicomplexans. Their study in parasites has revealed novel roles that expand our understanding of how these proteases function. Within this diverse group of parasites, rhomboid proteases contribute to processing of adhesins involved in attachment, invasion, intracellular replication, phagocytosis, and immune evasion, placing them at the vertex of host–parasite interactions. This article is part of a Special Issue entitled: Intramembrane Proteases.

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## 1. Introduction to protozoan parasites

Protozoan parasites are extremely diverse and are scattered across many of the major groups of eukaryotic lineages, while by contrast yeast and humans belong to the same major branch (*i.e.* opisthokonts) [1]. The diversity of parasites likely reflects the fact that parasitism has

arisen multiple times evolving independently in groups that were already phylogenetically divergent. Among the major animal and human parasites, the phylum Apicomplexa contains *Plasmodium* spp., the cause of malaria, *Cryptosporidium parvum*, a common cause of diarrheal disease, and *Toxoplasma gondii*, an opportunistic pathogen. This phylum also contains important animal pathogens such as *Theileria* spp., which causes disease in cattle, and *Babesia* spp., parasites of animals that can also cause zoonotic disease in humans. Only distantly related to the apicomplexans, members of the kinetoplastidae also cause important animal and human diseases due to infections by *Trypanosoma brucei* in

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Africa and *T. cruzi* in the New World. Members of the genus *Leishmania*, of which there are multiple species that cause important diseases in humans, are also members of the kinetoplastidae. Other important human pathogens include *Entamoeba histolytica*, a member of the amoeba group, and early branching eukaryotes such as *Trichomonas vaginalis* and *Giardia* spp.

Protozoan parasites contain a wide variety of serine, threonine, cysteine, aspartic, and metalloproteases and many of these have been implicated in important aspects of their biology including development, immune evasion, nutritional acquisition, and maturation of proteins involved in invasion and egress [2–4]. However, most of these proteases do not cleave their substrates within the membrane, and therefore will not be considered further here. Although all three families of intramembrane proteases exist in protozoan parasites, only two have been investigated experimentally: signal-peptide peptidases and rhomboid proteases [5,6]. Site-2 proteases exist in the genomes of protozoan parasites (<http://eupathdb.org/eupathdb/>), yet they have not been studied extensively and so will not be considered here. Signal peptide proteases exist in all protozoan parasites, but have only been examined in *Plasmodium*. Rhomboid proteases have been studied most extensively in the apicomplexans, and on a more limited basis in *Entamoeba*, and as such this review will focus more closely on these later examples.

## 2. Intramembrane proteolysis

Although only recently recognized, intramembrane proteolysis turns out to be a conserved process with an ancient ancestry that stretches across bacterial, archeal, and eukaryotic lineages [7,8]. There are three major types of intramembrane proteases: site-2 metalloproteases, aspartyl proteases consisting of the related signal-peptide peptidase and presenilin families, and rhomboid proteases that have a serine at their active site [9]. Their general functions are briefly reviewed here prior to considering what is known about them in protozoan parasites in the sections below.

Proteins destined for export in eukaryotic cells typically contain a hydrophobic signal peptide at their N-terminus that directs the protein for insertion into the lumen of the ER via the Sec61 complex [10]. Signal-peptide peptidases (SPP) cleave the signal peptide that remains in the ER membrane following protein export [11]. The action of SPP also generates short peptides for recognition of self via MHC class I HLA-E molecules, while cleavage of some substrates by SPP-like proteases can generate signals for activating transcription [11]. SPP also functions in ER quality control of MHC class I molecules in CMV infected cells [12]. SPP share common mechanistic features to presenilins such as  $\gamma$ -secretase, which functions in Notch signaling and in generation of amyloid  $\beta$ -peptide, and the bacterial prepilin IV proteases. These two classes of proteases are defined by an active site containing two conserved aspartate residues that occur within motifs consisting of the residues YD and GXGD, a feature unique to this family of aspartic proteases [11].

Site-2 proteases (S2P) are zinc metalloproteases that cleave within the TMD of their substrate after an initial cleavage, typically by a membrane-tethered site-1 protease that cleaves outside the TMD [6,9]. S2P contain a conserved HExxH motif characteristic of metalloproteases and use a H-H-D motif that coordinates a zinc ion within the active site [13]. S2P are multi-membrane spanning proteases that typically reside in the ER, or other endomembranes. S2P cleave their substrates near the inner leaflet of the membrane, releasing transcription factors that migrate to the nucleus to activate gene expression [6,9]. For example, in eukaryotes, in response to low cholesterol, sterol regulatory element binding protein (SREBP) is processed by the sequential action of site-1 protease and S2P to release a transcription factor that up-regulates sterol biosynthesis [14]. Similarly, the release of ATF transcription factors from the ER in response to the unfolded protein response requires the action of S2P [15]. In prokaryotes, S2P control a variety of responses including stress responses, lipid metabolism, toxin production, and sporulation [16].

Rhomboid proteases were originally identified in *Drosophila* based on a genetic screen for mutants that disrupted development [8]. Rhomboid 1 was shown to cleave Spitz, an EGF-like factor, within its transmembrane domain (TMD) releasing this growth promoting hormone via the secretory pathway to control development in neighboring cells [17]. *Drosophila* rhomboid 1 has 7 TMD and contains a catalytic triad that was originally proposed to contain histidine, aspartate, and an active site serine, based on mutational and inhibitor studies [17]. Rhomboid proteases are unique among intramembrane proteases in not requiring preprocessing of the substrate prior to cleaving within the TMD [8]. Rhomboid proteases are characterized by a conserved domain structure consisting of 6 TMD in most prokaryotes, 6 + 1 TMD in eukaryotes, and 1 + 6 TMD that are found in mitochondrial rhomboid proteases, as well as key catalytic residues including a conserved GxSx active site [7,8]. Although not highly conserved at the amino acid level, rhomboid proteases are phylogenetically very widespread [18]. In addition to catalytically active rhomboid proteases, many organisms contain rhomboid-like genes encoding proteins that lack key catalytic residues (so called inactive rhomboids or iRHOMs); these pseudo-enzymes typically contain a Pro residue upstream of the catalytic Ser and therefore are inactive as proteases [19]. Although originally functional orphans, recent studies suggest that while iRHOMs lack enzymatic activity, they may still be biologically active in influencing the trafficking of single TMD proteins in the secretory pathway, thereby altering signaling [20].

Since their initial discovery, more precise catalytic mechanisms have been worked out based on *in vitro* cleavage assays [21–23] and structural studies on bacterial rhomboid proteases [24–27]. These studies confirmed that the active site serine is found within the membrane where it is located at the top of a short TM helix that places the serine at the base of a cavity that is open to the aqueous environment. They also revealed that the catalytic site involves a dyad of histidine that acts as a base to remove a proton from serine, which then serves as a nucleophile to attack the peptide bond. Several models have been proposed for the insertion of the substrate TMD into this pocket, where upon the helix breaking propensity of typical substrates is important in allowing access to the peptide bond [7]. Various features of rhomboid substrates have been identified, and although there are no universal rules, there are several general features. One prominent feature is the presence of small helix breaking residues in the TM segment adjacent to the site of cleavage [28]. Rhomboid proteases are fairly permissive in cleaving substrates from widely divergent sources, likely reflecting the general features of the cleavage site rather than specific residues. However, other studies have stressed the conservation of small hydrophobic residues at the cleavage site, as well as bulkier hydrophobic flanking residues, as an important sequence determinant for many, but not all rhomboid proteases [29]. These features combine to generate a meta-stable helix that is stabilized in the lipid bilayer, but which can easily be destabilized in other environments, rendering them susceptible to cleavage within the active site of rhomboid proteases [30]. Given these fairly general rules for substrate preference, rhomboid proteases are fairly permissive such that heterologous assays have been very helpful for defining their function, yet identification of native substrates that control important aspects of the biology within their respective systems remains challenging.

## 3. Signal-peptide peptidase in *Plasmodium*

SPP normally resides in the ER, yet surprisingly, in *P. falciparum* this protease was initially reported to be a secretory protein and to interact with Band 3 in the red blood cell, presumably after secretion to the parasite surface [31]. Antibodies raised against PfSPP blocked the invasion of red cells by *P. falciparum*, and purified PfSPP bound directly to a peptide from the 5ABC loop in Band 3 [31]. Collectively, these studies suggested that export of PfSPP to the merozoite cell surface may aid in cell binding via recognition of Band 3 and that this protease may act to process host

receptor parasite ligands involved in invasion. However, the role of PfSPP in cell invasion has been questioned by a more recent study that used C-terminal epitope tagging at the endogenous chromosomal locus [32]. The endogenous protein was found to migrate at 35 kDa, vs. 47 kDa reported previously, a result confirmed by independently generating a new anti-peptide antibody to PfSPP. Moreover, using either the epitope-tagged version or this new peptide antibody, PfSPP was found to be a resident ER protein that did not undergo redistribution during invasion [32]. To reconcile these findings, it was suggested that the initial observation that PfSPP was exported might have arisen due to the generation of a non-specific antibody response that fortuitously recognized a 47 kDa secretory protein of unknown identity [32].

Regardless of the exact cellular location of PfSPP, there is agreement about its essentiality based on its refractoriness to gene disruption and its potential to be inhibited using small molecules. To establish the importance of PfSPP as a target, several groups have tested inhibitors that inhibit SPP (i.e. L-685,458, (Z-LL)<sub>2</sub>-ketone), but not those that exclusively target  $\gamma$ -secretase (i.e. DAPT), blocked growth of *P. falciparum* *in vitro* [33], a result that was shown to be independent of invasion [32]. Using a similar strategy, several presenilin inhibitors were found to block the growth of *P. falciparum* with low nM EC<sub>50</sub>s [34]. Further analysis of one of these inhibitors called NITD731 suggested a role for PfSPP in the ER stress response to unfolded proteins [34], a function that has previously been attributed to SPP in mammalian cells. Isolation of a resistant parasite mutant identified a point mutation in PfSPP, which was in turn capable of imparting limited resistance when expressed as a transgene, supporting the contention that PfSPP is the direct target of this inhibitor in *P. falciparum* [34]. NITD731 blocked the development of liver stages by *P. yoelii* [34], and a similar result was obtained in separate studies using a related inhibitor LY411.575 to treat the rodent malarial parasite *P. berghei* [35].

## 4. Rhomboid proteases in protozoan parasites

### 4.1. Discovery of rhomboid processing in parasite adhesins

The discovery of rhomboid in fly and the realization that the bacterial rhomboid AraA shared sequence requirements for substrate cleavage, and could function in heterologous assays to cleave a wide range of substrates [36], set the stage for identifying rhomboid proteases in other diverse lineages. A cluster of helix breaking residues (typically AG rich) near the external face of the TMD was originally used to identify a number of substrates from the genome of *T. gondii*, which turned out to be good substrates for fly rhomboid 1 [28]. The rhomboid substrates identified in *T. gondii* consisted of secretory micronemal proteins, previously implicated in motility, cell adhesion and invasion [37], and this realization led to a novel role for rhomboid proteases in parasite biology.

Protein secretion is highly specialized in apicomplexans, which contain three classes of regulated secretory organelles that are discharged sequentially during invasion of *T. gondii* [38]. Among these, micronemes are discharged first, releasing adhesins onto the cell surface. Although more challenging to image due to their small size and fragile nature, more recent studies have indicated a similar cascade of events accompanies invasion by *Plasmodium* merozoites [39–41]. Following their discharge onto the cell surface, micronemal proteins are translocated rearward by an actin-myosin motor that lies beneath the parasite membrane [42]. This conveyor-belt process is thought to drive forward motility and power cell invasion [42]. However, in order to maintain the gradient of adhesins and facilitate release from the substratum, it is also necessary for these adhesive proteins to be shed from the membrane once they have completed their journey. Shedding was originally ascribed to an activity called micronemal processing protease 1 (MPP1), although the enzyme responsible was not known at the time [43]. Evidence of the importance of microneme protein shedding was provided by mutations that lie just outside the TMD of MIC2, an important adhesin involved in cell invasion [44]. Mutation of a KK motif to AA

blocked processing of MIC2 and had a dominant negative effect on cell invasion [45]. This result suggested that processing of MIC adhesins might require preprocessing at this dibasic motif. However, it was subsequently shown that processing occurs within the membrane as shown by proteomic studies that identified the cleavage site in MIC6 [46], a finding that was further validated by similar studies on MIC2 [47]. The ability of MIC adhesins to be recognized by heterologous rhomboid proteases, combined with evidence for intramembrane processing at a conserved site, provided strong circumstantial evidence that rhomboid proteases in the parasite are the source of MPP1 activity.

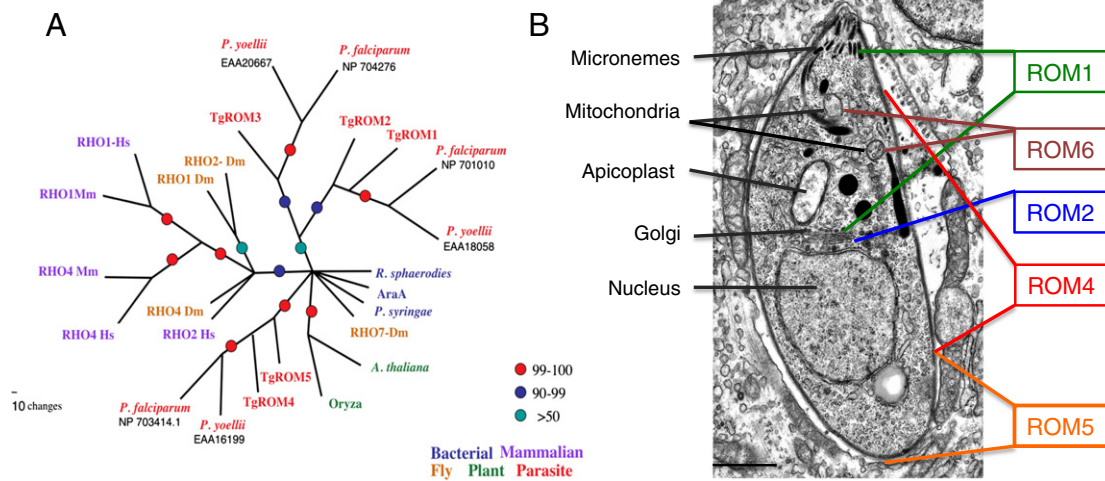
### 4.2. Phylogenetic distribution of rhomboid proteases in parasites

Given their wide phylogenetic distribution, it is not surprising that rhomboid proteases are found in many protozoan parasites. However, the number of rhomboid genes is very different among different groups of parasites. For example, kinetoplastids such as *Leishmania* and *Trypanosoma* spp. typically contain one or two active rhomboid proteases along with at least one that is predicted to be inactive [5]. Aside from observations that some of these are likely to be mitochondrial [5], nothing is known about their biological roles. Rhomboid proteases are also found in *Giardia*, which has two genes that show divergent domains and have only 5 predicted TMD, and *T. vaginalis*, which has nine rhomboid-like genes, several of which are predicted to be inactive [5]. A number of parasitic protozoa also contain rhomboid proteases that are predicted to be inactive, based on lack of conserved catalytic residues [5], although these have not been examined functionally.

By comparison to other groups, rhomboid proteases are abundant in apicomplexans, ranging from eight genes in *P. falciparum*, seven of which are predicted to be active, to four in *C. parvum* and *T. annulata* [5]. Of these, rhomboid proteases have been best studied in *T. gondii*, which contains six rhomboid proteases, and *Plasmodium* spp., which contains eight rhomboid genes, although some of these are unique to each genus (i.e. ROM2, ROM5 in *Toxoplasma* and ROM8, ROM9, and ROM10 in *Plasmodium*). ROM6 in each of these organisms is thought to be a mitochondrial enzyme, both based on domain structure and a predicted mitochondrial import sequence [5]. Phylogenetic comparison reveals that ROM1 and ROM3 are conserved in *T. gondii* and *Plasmodium* spp, while ROM2 is exclusive to *T. gondii* and the closely related *N. caninum* [48], a parasite of cattle and dogs (Fig. 1). By contrast, *Plasmodium* spp. does not have a direct ortholog of ROM5 in *T. gondii*, but rather have a single gene more closely related to ROM4 [48], (Fig. 1). As discussed below, these phylogenetic affinities do not directly mirror enzymatic activities and hence do not always reflect common functions.

### 4.3. Life cycle expression and cellular location of rhomboid proteases in apicomplexans

Studies based on RT-PCR in *T. gondii* indicate that TgROM2 and TgROM3 are almost exclusively expressed in the sporozoite stage [48], which is the product of meiosis following passage through the cat. In contrast, TgROM1 is expressed strongly in tachyzoites, which predominate during acute infection, and more moderately in bradyzoites, which are found in tissue cysts of the chronic infection, and weakly in sporozoites [48]. In *T. gondii*, TgROM2 expression is strongly upregulated in the sporozoites stage, implying that it plays an important role during this life cycle stage. TgROM4 is expressed in tachyzoites and bradyzoites, but is almost absent in sporozoites, while TgROM5 is expressed strongly in tachyzoites and weakly in sporozoites, but not in bradyzoites [48]. Although these findings suggest partitioning of rhomboid function during the life cycle, this hypothesis has not been formally tested. Localization studies, using epitope tagged constructs expressed under their endogenous promoters in *T. gondii*, indicate that TgROM1 is found in the Golgi and micronemes, TgROM4 is found uniformly on the cell surface, while TgROM5 is found primarily at the posterior end of the cell, particularly in extracellular parasites (Fig. 1A) [48]. Although TgROM2 is only



**Fig. 1.** Analysis of conserved rhomboid proteases in Apicomplexan parasites. A) Phylogenetic tree of rhomboid proteases in apicomplexans compared to fly, human bacterial, and plant. Dm, *Drosophila melanogaster*, Hs, *Homo sapiens*, Mm, *Mus musculus*, Tg, *Toxoplasma gondii*, A. thaliana, *Arabidopsis thaliana*, O. sativa, *Oryza sativa*, P. syringae, *Pseudomonas syringae*, P. stuartii, *Providencia stuartii*, R. sphaeroides, *Rhodobacter sphaeroides*, P. falciparum, *Plasmodium falciparum*, P. yoellii, *Plasmodium yoellii* (GenBank accession numbers beneath each entry). Used with permission [1]. B) Cellular localization of rhomboid proteases in *T. gondii* tachyzoite. Image courtesy of Wandy Beatty.

weakly expressed in tachyzoites, when over-expressed with a tubulin promoter, an epitope-tagged form of TgROM2 localized to the Golgi [49] (Fig. 1A). TgROM6 is reported to localize to the mitochondria in unpublished studies [5], while the localization of TgROM3, which is expressed primarily in sporozoites, has not been established.

*T. gondii* has an elaborate system for export of secretory proteins and packaging them into secretory vesicles [50]. Trafficking determinants for several ROM proteins have been studied using chimeric proteins expressed under the tubulin promoter and bearing an epitope tag. These studies established that TgROM4 is oriented in the plasma membrane with its C-terminus outside the membrane and N-terminus in the cytosol [51]. They also established that TgROM2 uses a FF motif in the N terminal segment to mediate export from the ER, yet the mature protein also contains additional targeting information that localizes it to the Golgi [51]. Comparison of TgROM1 orthologs to other parasite ROMs identified a conserved motif of FPHF that was necessary for targeting of TgROM1 to micronemes [51]. Understanding such targeting motifs is of interest for predicting the potential substrates of rhomboid proteases since their activities are likely to be mediated by proximity to substrates within the same membrane, especially given that thus far no other means of regulating rhomboid activity has been described.

Although *Plasmodium* spp. contains a number of rhomboid proteases, only ROM1 and ROM4 have been studied in detail. In *P. berghei* PbROM1 is expressed in micronemes within merozoites, which are the end product of asexual development in the infected red blood cell [52]. PbROM1 expression was not detected in sporozoites found in oocyst in the mosquito, yet it was upregulated in salivary glands where it was found along the entire length of the mature sporozoite [52]. Studies in *P. yoelli* also indicate expression in merozoites and sporozoites and localization to micronemes, yet PyROM1 was not secreted onto the surface of sporozoites during invasion of hepatocytes [53]. In *P. falciparum* PfROM1 has also been reported by one group to occupy a unique secretory organelle called the monome, thought to be important in red cell invasion by merozoites [54]. PfROM4 is also a merozoite surface protein and it is thought to cleave cell surface adhesins during invasion of red blood cells [55]. PfROM4 is also localized to the surface of sporozoites suggesting it is responsible for processing adhesins in the sporozoite, as described below [56].

#### 4.4. Substrate specificity and in vitro activities of parasite rhomboid proteases in apicomplexans

The use of heterologous systems has been very useful for studying substrate preference of parasite rhomboid proteases. Expression of

*T. gondii* rhomboid proteases in COS cells was used to show that several of these parasite proteases were capable of recognizing the TMD of Spitz, the fly Rhomboid-1 substrate, including TgROM1, TgROM3, and TgROM5 [48]. TgROM5 showed a similar requirement for small helix breaking residues (GA) near the outside of the TMD in the heterologous substrate Spitz, and activity was dependent on the predicted catalytic serine residue [48]. In similar studies, expression in HEK293T cells revealed that TgROM1, TgROM2, and TgROM5 were capable of processing Spitz, although TgROM2 was much less active, and only TgROM5 led to secretion of the cleaved product into the medium [49]. Of the *T. gondii* rhomboid proteases, TgROM5 was by far the most active and was the only enzyme capable of cleaving TMD from micronemal proteins MIC2, MIC6, or MIC12 expressed in COS cells [48], while TgROM2 was reported to process the TMD of MIC2 and MIC12, albeit weakly [49]. Interestingly, although TgROM5 was highly active in cleaving the TMD of micronemal proteins in COS cells [48], it did not show a requirement for residues in the extracellular domain of MIC2 where a di-lysine motif (KK) has previously been shown to be required for efficient processing in the parasite ([45] and unpublished data). The reasons for this difference are not known but suggest that when in the membrane of the parasite, the activity of ROM5 may be modulated either by another factor that binds the extracellular domain of MIC2, or alternatively that the suitability of MIC2 as a substrate for ROMs may be influenced by this juxtamembrane segment. Despite evidence for a role *in vivo* as described below, only TgROM4 was inactive against all substrates tested in the heterologous assays [48,49].

*Plasmodium* spp. has a single homolog that is phylogenetically closer to TgROM4, but shows an activity profile more similar to that of TgROM5 [55,57] (Fig. 1A). PfROM4 was shown to process the cell surface adhesin EBA-175, cleaving the protein at a conserved A|GA sequence in the TMD [55]. PfROM4 was refractory to genetic disruption, and it was also not possible to isolate viable parasites with substitutions in the cleavage site within the TMD of the cell surface adhesin EBA-175, arguing that processing is essential to parasite invasion [55]. Further analysis of the substrate specificity of PfROM4 revealed that it was able to process a variety of other parasite adhesins that are members of the erythrocyte and reticulocyte binding ligands (so called EBL and RBL proteins) [57]. The TMD of these malarial adhesins contain bulky hydrophobic, often aromatic, residues that lie just outside of the A|GA cleavage site, and hence they are not good substrates for fly Rhomboid-1 [57]. In contrast, PfROM4 was highly efficient in cleaving EBL and RBL ligands when co-expressed in COS cells, despite

its failure to process Spitz [57]. These findings suggest that PFROM4 has a unique binding pocket that allows for the bulky hydrophobic residues in addition to the small helix breaking residues at the cleavage site. In contrast, PFROM1 was only weakly active on EBL and RBL ligands, but instead was highly active on the heterologous substrate Spitz and the parasite adhesin AMA1 [57], a micronemal protein that plays an important role in invasion [58]. Although these heterologous studies indicate the ability of PFROM1 to cleave AMA1 within its TMD, and intramembrane cleavage of AMA1 has been detected in parasites [59], other studies have stressed the greater importance of extramembrane shedding of AMA1 by the surface protease SUB-2 [60]. Furthermore, it is possible to replace the TMD with mutations that blocked processing by rhomboid proteases, yet parasites are refractory to the introduction of mutations that blocked SUB-2-mediated cleavage outside the membrane [60].

Motility and invasion of *Plasmodium* sporozoites also depend on thrombospondin related anonymous protein (TRAP), and during gliding this adhesin is shed onto the substrate [61]. Heterologous expression in COS cells revealed that PFROM4 is able to process TRAP, and a related molecule CTRP that functions in ookinete motility [57]. Mutations in the TMD of TRAP that alter the rhomboid recognition site disrupt processing and impair gliding motility and cell invasion by sporozoites of *P. berghei*, revealing the importance of this processing step in the parasite [56]. The location of PFROM4 on the sporozoite surface suggests it fulfills this function *in vivo* [56].

Together these findings indicate that PFROM4 has a unique ability to cleave parasite adhesin TMD that contain bulky hydrophobic residues, while PFROM1 is a more conventional rhomboid. Collectively, these rhomboid proteases can process AMA1, the EBL and RBL families, and TRAP and related molecules, thus regulating adhesive proteins during various life cycle stages of the *Plasmodium* life cycle. In contrast, TgROM5 exhibits dual specificity, cleaving both types of *Plasmodium* substrates and a wide variety of *T. gondii* adhesins in heterologous systems. This broad specificity is intriguing since the known micronemal protein adhesins in *T. gondii* lack bulky hydrophobic residues, raising the possibility that additional substrates may exist for this protease.

#### 4.5. Genetic studies on rhomboid function in apicomplexans

Gene disruptions have been described for ROM1 in *T. gondii* and in *Plasmodium* spp, in both cases revealing relatively mild phenotypes. Conditional shut-down of TgROM1 led to a slight reduction in intracellular replication, with almost no effect on cell invasion [62]. Despite this modest phenotype, it was not possible to delete the entire gene by homologous crossover, although newer methods for targeted gene disruption may allow for generation of a complete knockout. TgROM1 is packaged into micronemes along with many cell surface adhesins that are the substrates of rhomboid proteases. When expressed from the endogenous promoter, it was not detected on the parasite surface [62], yet over-expression using a tubulin reporter resulted in surface expression similar to TgROM4 [51]. Hence, it is possible that low levels of ROM1 are released onto the cell surface during invasion, where it may participate in shedding of surface adhesins, similar to TgROM4 described below. Alternatively, TgROM1 may process substrates in the Golgi, where it has also been reported to reside [62]. Whether ROM1 is involved in processing micronemal adhesins in this environment, or as yet undiscovered substrates, is unknown.

In the rodent malaria *P. berghei*, disruption of ROM1 revealed that it is not essential, yet it plays an important role in development as evidenced by the impaired growth of several life cycle stages in mutants lacking this gene [52]. Deletion of PbROM1 in asexual blood cell stages led to normal formation of ookinetes in the mosquito, but these stages were less able to mature to functional oocysts, and those oocysts that did form contained fewer sporozoites [52]. PbROM1 is expressed on the surface of sporozoites and deletion of this gene does not affect sporozoite motility or the ability to invade the salivary glands, yet development in the liver is affected and

this leads to delayed parasitemia and reduced mortality [52]. Although these studies indicate that PbROM1 affects progression through the life cycle, they do not precisely define the role of this protease, nor do they clearly distinguish between a role in invasion and development.

A somewhat different conclusion was reached by disruption of ROM1 in *P. yoelli*, where development in the mosquito was normal, yet infection in the vertebrate host was impaired [53]. Careful analysis of the invasion of hepatocytes by sporozoites failed to reveal a defect in invasion, but rather *Pyrom1*(−) parasites failed to undergo development [53]. Impaired intracellular development was associated with ultrastructural differences in the parasitophorous vacuole and decreased processing of at least one vacuole membrane protein called UIS4, which contains a rhomboid processing site [53]. ROM1 appears to play an accessory role for development in both rodent malaria and *T. gondii*, and its absence does not result in a striking defect in invasion. Hence, ROM1 may prefer additional substrates that extend beyond the known adhesins and/or play a redundant role to other rhomboid proteases that these species express.

Recently, more comprehensive studies of *Plasmodium* rhomboid proteases have been undertaken based on a genetic screen for loss of function [63]. These studies reveal that ROM4, ROM6, ROM7, and ROM8 are refractory to deletion in *P. berghei*, suggesting they play important roles in asexual development [63]. In contrast, ROM1, ROM3, ROM9, and ROM10 were dispensable for growth of asexual stages in red blood cells [63]. Although mutants lacking  $\Delta rom9$  and  $\Delta rom10$  showed no growth defects, mild developmental delay was seen for  $\Delta rom1$  mutants following liver infection and  $\Delta rom3$  mutants developed into oocysts in the mosquito, yet failed to produce sporozoites [63]. These studies reveal both potentially redundant as well as stage-specific roles for rhomboid proteases through out the life cycle. Future studies to define their potential substrates may reveal new pathways controlled by these rhomboid proteases.

The observation that *T. gondii* contains two related rhomboid proteases, TgROM4 and TgROM5 suggests that there might be functional redundancy, or specialized functions that are not conserved among all apicomplexans. Consistent with the latter possibility, these two enzymes have very different activities, at least *in vitro*. Based on the location of TgROM5 at the posterior end of the cell, it was suggested that it might be the crucial protease that releases adhesins from the membrane once they have been translocated rearward by the action of the actin–myosin motor [48]. Alternatively, it has been suggested that the broader phylogenetic distribution of ROM4 supports a critical role in processing adhesins [49]. Genetic assessment of the role of these two genes was initially hampered by difficulties in knocking them out directly, a result that might suggest they are essential. However, we have recently successfully deleted the TgROM5 gene using Cre recombinase to excise the gene after flanking the locus with LoxP sites [64] in a genetic background that is permissive for homologous recombination [65] (B. Shen, L.D. Sibley unpublished). This surprising result indicates that ROM5 is not essential, but leaves open the possibility that it still functions in adhesin processing, yet is functionally redundant.

Initial attempts to delete TgROM4 were also unsuccessful [66], although these studies did not take advantage of the most efficient means of gene disruption that are currently available. Instead, suppression of the TgROM4 gene was achieved using a conditional knockout strategy (cKO) based on a TetOff promoter to suppress expression of a transgene in a background where the endogenous locus was deleted [66]. Although suppression of TgROM4 in the cKO strain did not affect plaque formation, it showed a modest, yet significant reduction in cell invasion. Additionally, gliding motility was impaired when expression was suppressed in the cKO [66]. Despite significant phenotypes in motility and invasion, the maximum level of suppression achieved in the cKO resulted in residual expression at 10–25% of wild type levels [66], leaving open the possibility that a complete gene deletion would show a much more severe phenotype. Nonetheless, a role in processing of cell surface adhesins was suggested by elevated levels of MIC2 on the parasite surface, and decreased shedding of MIC2, AMA1 and MIC3, in

parasites where TgROM4 was suppressed [66]. Consistent with this, TgROM4 cKO parasites were more adherent to host cells, yet show altered motility characterized by extensive twirling, which does not lead to productive cell invasion. Collectively these findings suggest that TgROM4 acts to trim adhesins from the plasma membrane, potentially removing proteins that are not productively engaged with receptors (Fig. 2). This sheddase activity maintains the apical–posterior gradient of cell surface adhesins, which is critical to directional motility and cell invasion (Fig. 2).

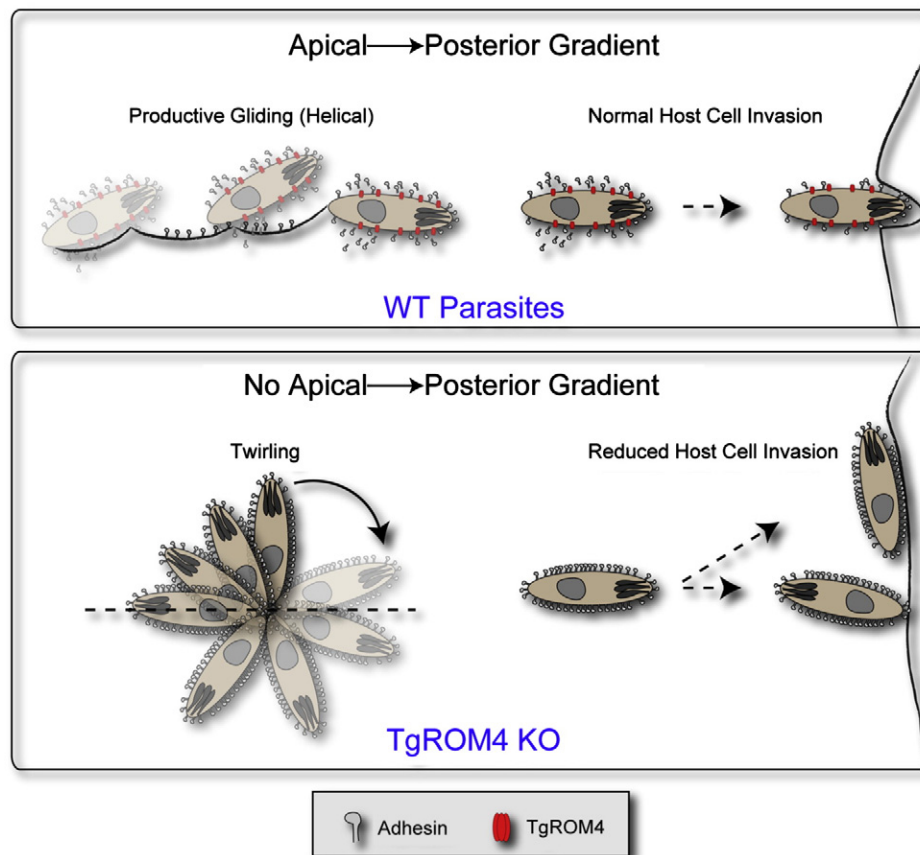
Despite the lack of *in vitro* activity reported for TgROM4 [48], the phenotype of the TgROM4 cKO suggests that several micronemal proteins may be substrates of ROM4 *in vivo*. However, the accumulation of microneme adhesins on the cell surface in the TgROM4 cKO may also be indirect. For example, if the normal substrate(s) of ROM4 accumulates on the cell surface of the cKO following shutdown, it may impair the activity of TgROM5, and indirectly lead to less efficient processing of other cell surface adhesins. Given the lack of apparent growth phenotype observed for the  $\Delta rom5$  mutant and the relatively modest phenotype seen for the cKO of TgROM4, it seems likely there is some level of overlap between these enzymes. Such redundancy may be uncovered by attempts to create double knockouts using improved strategies for targeted gene deletion in *T. gondii* [64,65].

A different role for TgROM4 was suggested by studies using a dominant negative (DN) over-expression mutant that altered the active site Ser to Ala [67]. Dominant negative mutants for rhomboid proteases have not been previously described, so it came as a surprise that the phenotype of this mutant was so striking, especially as the mutated enzyme was not demonstrated to directly bind to and trap substrates or to directly affect the cleavage of any putative substrates. Parasites expressing the DN mutant showed only a modest decrease in invasion, yet had a profound block in intracellular replication

[67]. Differences between this study and the cKO of TgROM4 might result from the incomplete shutdown in the cKO (*i.e.* residual processing of substrates might be sufficient to mask the replication block), and the possibility that the DN differentially affects substrates of ROM4 (*i.e.* certain ligands essential for invasion may be less affected by the DN mutant vs. the cKO). Remarkably the defect in intracellular replication in the TgROM4 DN was rescued by over-expression of the tail of AMA1 [67], suggesting it plays a role in signaling after release from the membrane by ROM4-mediated cleavage. The cleavage site of AMA1 has been mapped previously and it lies near the surface of the TMD in a typical rhomboid-like cleavage site [68]. Moreover, mutants in this cleavage site that reduce processing by > 1000 fold are impaired in invasion, but normal in replication [68], which is at odds with the TgROM4 DN studies. Reconciling these studies is challenging at present, but recent studies reporting inducible deletion of a number of *T. gondii* proteins previously thought to be essential, might resolve whether cleavage of AMA1 is indeed necessary for either invasion and/or replication [69].

#### 4.6. Studies on rhomboid function in *Entamoeba*

Analysis of rhomboid proteases in *Entamoeba histolytica* revealed a single active rhomboid (EhROM1) that shares the unusual substrate specificity with PfROM4 described above [70]. EhROM1 is unable to cleave Spitz, yet it efficiently processes EBL and RBL adhesins that contain bulky hydrophobic residues in their TMD, in addition to the helix-destabilizing residues at the cleavage site [70]. EhROM1 is found on the cell surface, yet it dramatically relocalizes to phagocytic cups during ingestion of red blood cells and to the base of the uroid during capping of cell surface receptors. Searching the amoeba genome for potential substrates identified a family of cell surface adhesins that bind Gal/GalNAc;



**Fig. 2.** Role of TgROM4 in shedding of surface adhesins. A) In wild type parasites, shedding of surface adhesins maintains an anterior to posterior gradient that is critical for directional gliding and cell invasion. B) When TgROM4 is suppressed, adhesins accumulate on the cell surface, resulting in enhanced twirling in place, lack of polarized cell attachment and disruption of invasion. Used with permission [2].

these lectins are implicated in cell attachment, tissue invasion, and immune evasion [71]. Using a heterologous COS cell expression system, EhROM1 was shown to cleave the TMD of the Gal/GalNAc heavy chain, which contains a cluster of helix breaking residues (*i.e.* GA rich) that were shown *via* mutational analysis to be important in substrate recognition [70]. Genetic manipulation of EhROM1 by epigenetic shutdown revealed a role in attachment of live cells and phagocytosis of both live and apoptotic cells [72]. Although these activities might be expected to involve the Gal/GalNAc adhesin, no alteration in the cell surface expression or relocalization of the lectin was noted in the EhROM1 knockdown cells [72]. These findings suggest that additional substrates of EhROM4 are responsible for the observed phenotypes in the knockdown. Additionally, *E. histolytica* contains several ROM-like genes that are predicted to be catalytically inactive, and may participate in regulating the localization and perhaps function of cell surface lectins.

## 5. Conclusions and future directions

The diversification of rhomboid proteases in parasites suggest they play a variety of different functions, perhaps extending beyond the processing of cell surface adhesins involved in cell invasion. Current genetic studies suggest possible redundant function for some rhomboid proteases (*i.e.* TgROM1, TgROM5, PbROM9, and PbROM10), or alternatively, activities that are not required *in vitro* but which may become apparent in various vertebrate hosts. In other cases, specific rhomboid proteases appear to be essential (*i.e.* TgROM4, PFROM4, PbROM4, PbROM6, PbROM7, and PbROM8), although their precise functions remain to be fully defined. Several parasite rhomboid proteases have been shown to cleave cell surface adhesins involved in invasion, yet it remains uncertain how their activities are regulated temporally and spatially. Spatial regulation might be important for regulating some, for example, ROM5 is largely separated from adhesins until they traffic to the posterior end of the cell, while ROM4 presumably has access to potential substrates as soon as they are released onto the parasite plasma membrane. The phenotype of the TgROM4 cKO supports a role as a sheddase to remove unwanted complexes that disrupt the apical–posterior gradient of adhesins needed for polarized attachment and movement [66]. Recent structural studies of TRAP suggest that receptor binding of the ecto-domain may alter the extended structure of the protein [73], which might potentially alter the TMD and hence its preference as a substrate for rhomboid proteases. Consequently, cell surface ligands that are productively engaged may avoid processing while those that are not engaged, or which have completed translocation, might be preferentially susceptible to cleavage. Placing the cleavage site within the membrane might thereby allow the protease to sense the dynamics of the TMD within the membrane, a feature recently shown to be important in rhomboid recognition of substrates [30]. Recent studies have also shown that parasite ROMs are phosphorylated [74], raising the possibility that such modifications may regulate activity. Functions for other ROMs have not been defined (*i.e.* TgROM2, ROM3) and based on their expression in different life cycle stages, they may play similar roles in processing adhesins important for invasion of sporozoites, or function in other developmental pathways, as suggested by the defect in sporozoite development for PbROM3 mutants [63]. Thus far, mitochondrial rhomboid proteases have not been studied in parasites, despite evidence that they are widespread. Parasites also contain several rhomboid-like proteins that are thought to be catalytically inactive, raising the possibility that they function in altering trafficking and perhaps influencing signaling as recently described in humans [20]. Rhomboid proteases have an unusual catalytic mechanism that takes place in a specialized hydrophilic pocket in the membrane; if specific inhibitor could be tailored for this environment they might provide potent inhibition of essential enzymes in parasites, thus offering the promise of new therapies against infections. Additionally, the inhibition of parasite growth by NTDI731 *in vitro* [34], suggests repurposing of inhibitors

developed against mammalian presenilins may be useful for inhibiting SPP and treating infections by a variety of protozoan parasites.

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