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Making the cut: central roles of intramembrane proteolysis in pathogenic microorganisms

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PREFACE

Proteolysis in cellular membranes to liberate effector domains from their transmembrane anchors is a well-studied regulatory mechanism in animal biology and disease. By contrast, the function of intramembrane proteases in unicellular organisms has received little attention. Recent progress has now established that intramembrane proteases execute pivotal roles in a range of pathogens, from regulating *Mycobacterium tuberculosis* envelope composition, cholera toxin production, bacterial adherence and conjugation, to malaria parasite invasion, fungal virulence, immune evasion by parasitic amoebae and hepatitis C virus assembly. These advances raise the exciting possibility that intramembrane proteases may serve as targets for combating a wide range of infectious diseases. I focus on summarizing the advances, evaluating the limitations and highlighting the promise of this newly emerging field.

Keywords

cell signalling; regulated intramembrane proteolysis; rhomboid; site-2 protease; signal peptide peptidase; presenilin; invasion; malaria; hepatitis; cholera; tuberculosis

INTRODUCTION

Pathogens have evolved mechanisms to adapt quickly to the unreceptive environment of the host and establish a flourishing infection. Many of the events to which pathogens must respond occur at cellular membranes as sites of first contact. Work over the past decade has identified a novel class of membrane proteases that function to liberate effector domains from the membrane by cleaving transmembrane segments^{1–3}. These intramembrane proteases are unusual polytopic enzymes; their active sites are assembled from residues on membrane-spanning segments, which embeds their water-dependent catalytic apparatus beneath the surface of the membrane.

Intramembrane proteases are among the most conserved of all membrane proteins known, with members in all kingdoms of life^{4–6}. Despite being widespread in unicellular organisms, without exception every intramembrane protease family was initially discovered through the study of metazoan biology or disease. Early examples of intramembrane proteolysis in unicellular organisms were relatively rare^{7–9}, but this field has gained momentum, especially in the area of pathogens, and is coalescing into a new and distinct field. It is now established that intramembrane proteolysis is a fundamental biochemical mechanism used by a wide variety of pathogens to coordinate their infective cycles.

In this review, I will focus on the roles that intramembrane proteases have been discovered to play in pathogenic microorganisms, starting with bacterial pathogens, moving to pathogenic eukaryotes including protozoa and fungi, and closing with viruses. In comparing these examples, the challenge is to identify emerging common principles, and to expose promising areas for future investigation.

THE PROTEASE CAST

Molecular dissection of a diverse array of cellular processes over the past ~12 years has identified dozens of intramembrane proteases, all of which can be grouped into three families based both on sequence similarity as well as shared properties^{1–3} (Figure 1).

The first intramembrane protease to be discovered was site-2 protease (S2P), the enzyme responsible for catalyzing the second cleavage that releases the transmembrane SREBP transcription factor for cholesterol biosynthesis in mammalian cells¹⁰. S2P also functions in the unfolded protein response¹¹, which is paralleled by the *Escherichia coli* RseP/YaeL homologue in the EXTRACYTOPLASMIC FUNCTION^{12, 13} stress response that also responds to unfolded proteins (see Figure 2). S2P homologues are conserved in all forms of life, from Archaea to humans, although a few organisms lack recognizable members⁶. S2P enzymes are polytopic membrane proteins consisting of a conserved three transmembrane core with a well-defined HEXxH METALLOPROTEASE signature in one transmembrane segment, with the third residue required for zinc coordination (along with the two histidines) supplied by an aspartate on a distal segment⁶ (Figure 1). Individual S2P members elaborate this core structure through the addition of transmembrane segments preceding and/or following the core, as well as insertion of extra-membranous domains.

Intramembrane ASPARTYL PROTEASES were discovered through the analysis of Alzheimer's Disease etiology: presenilin protein within the γ -secretase complex catalyzes intramembrane proteolysis to generate the neurotoxic A β 42 peptide. While γ -secretase is generally absent from unicellular organisms and will not be discussed further, intramembrane aspartyl proteases are widely represented by signal peptide peptidase (SPP), a presenilin-type protein that uses two intramembrane aspartates to catalyze proteolysis^{14, 15} (Figure 1). Detection of signal peptide cleavage following their removal from proteins by signal peptidase led to the discovery of SPP in human cells. Some of the released fragments act as bioactive peptides with a variety of post-targeting functions¹⁶. Unlike γ -secretase, SPPs do not require other protein cofactors for activity^{17, 18}, and are widely conserved as multimember families in multicellular organisms, but they are absent from bacteria^{14, 19}.

Rhomboid proteins are intramembrane SERINE PROTEASES that were identified through the genetic dissection of insect embryogenesis. *Drosophila* rhomboid initiates cell signalling by cleaving the transmembrane EGF precursor, Spitz, releasing it as an active signal from the membrane²⁰ (also see Figure 3). Rhomboid is conserved in all kingdoms of life, with members sharing a conserved core structure of 6 transmembrane segments that contain the catalytic serine-histidine pair, and a membrane-inserted loop^{4, 5, 21–24} (Figure 1). Many homologues contain a variable N-terminal domain that protrudes into the cytosol, and/or a seventh transmembrane segment.

In addition to the catalytic mechanism, intramembrane proteases differ with respect to three main properties that allow them to fulfill distinct roles in the cell. First, both S2P and SPP are dependent on a prior, site-1 cleavage that sheds the substrate ectodomain; this cleavage regulates processing of the substrate since intramembrane cleavage follows automatically^{1, 2}. Conversely, rhomboid proteases cleave full-length proteins and in no circumstance require a prior cleavage^{3, 20}. Secondly, the active site of rhomboid enzymes is situated closer to the

extracellular face of the membrane, and they generally function to release effector domains to the cell exterior²⁵ (Figure 1). Conversely, both S2P and SPP release proteins into the cytosol, and their active sites are accordingly situated closer to the cytosolic face of the membrane. Lastly, rhomboid and γ -secretase cleave transmembrane segments of type I orientation (N-termini outside the cell), while SPP and S2P cleave transmembrane segments of the opposite, type II orientation (C terminus out).

Recently there have been major advances in understanding the biochemistry of these enzymes, including development of pure enzyme reconstitution assays for all three protease classes^{17, 18, 26–28}, and several crystal structures of both prokaryotic rhomboid^{21–24} and S2P homologues²⁹ (Figure 1). This has also resulted in defining new steps in the reaction, including insights into how substrates enter the active site via lateral gating mechanisms^{30–33}. No structure has yet been solved for any aspartyl intramembrane protease, although low-resolution cryo-electron microscopy and particle reconstructions of γ -secretase provide an initial glimpse³⁴. These biochemical advances are beyond the scope of the current discussion, but have been the focus of several recent articles^{33, 35}.

ROLES IN PROKARYOTIC PHYSIOLOGY

In addition to central roles in the development and disease of animals, our understanding of intramembrane proteolysis in microbial cell biology is improving (summarized in Table 1). Investigations over the past few years have revealed direct roles for intramembrane proteolysis in bacterial sporulation⁷, maintenance of cell polarity³⁶, metabolite transport^{37, 38}, stress responses^{12, 13, 39, 40} and cell division⁴¹. It is also noteworthy that all three classes of intramembrane proteases are conserved in Archaea, and some of these homologues have already served as useful models for the study of intramembrane protease biochemistry and structure²⁹. However, while Archaea are widespread ecologically, the biological roles of archaeal intramembrane proteases have not yet been explored in any context, and this topic deserves future investigation. For the sake of clarity, the discussion here will be limited to direct roles of intramembrane proteases in pathogenic events.

ROLES IN BACTERIAL PATHOGENESIS

Bacteria have rhomboid proteases that cleave membrane proteins of type I orientation, and S2Ps that cleave type II membrane proteins, but notably lack intramembrane aspartyl proteases such as SPP. Confusingly, there is an activity that is responsible for cleaving bacterial signal peptides, which was historically also named signal peptide peptidase A (also abbreviated SPP)⁴². However, this bacterial SPP, whose structure has recently been solved, is a serine protease with a single transmembrane segment and an active site that lies in the periplasm⁴³: it bears no sequence or evolutionary similarity to the eukaryotic aspartyl intramembrane SPP.

S2P Circuits and Virulence

The functions of bacterial intramembrane proteases are currently best understood for S2Ps, which, as a rule, regulate membrane-tethered transcription factors: S2Ps cleave transmembrane ANTI-SIGMA FACTORS, or transcription factors that are directly tethered to the membrane. The role of RseP/YaeL in the *E. coli* extracytoplasmic stress response serves as a well-characterized but non-pathogenic paradigm (Figure 2)^{12, 13, 44}, although the recent implication of this pathway in virulence of some pathogens implies that the pathogenic roles of S2Ps are likely to grow in the near future. Currently S2Ps are known to directly regulate four distinct pathways that contribute to virulence of several deadly bacterial pathogens.

Mycobacterium tuberculosis S2P and lipid metabolism

M. tuberculosis is an obligate human pathogen with complex stages of pathogenicity. While it lacks an outer membrane, virulence is known to depend on the unusual waxy cell wall comprised of a hydrophobic envelope⁴⁵. Genome sequencing revealed a multitude of lipid biosynthetic genes, but the mechanism by which they are regulated is not well understood. The precedent of S2P regulating membrane composition in animal cells prompted a similar analysis of the S2P homologue in *M. tuberculosis*. In a striking parallel, the S2P knockout bacteria exhibited altered colony morphology consistent with altered lipid composition of the envelope, a defect that relied on S2P proteolytic activity since a catalytic site mutant failed to rescue the morphology⁴⁶. Although a biochemical analysis failed to reveal major differences in envelope lipids of the S2P knockout strain, switch of S2P knockout cultures to detergent-free media resulted in altered changes in lipid metabolism, suggesting that it is not lipid synthesis, but responses to changing conditions that are defective in S2P mutants. Microarray analysis revealed complex and multi-faceted changes in lipid biosynthetic gene expression, including those for mycolic acid production, an extractable envelope lipid known to contribute to *M. tuberculosis* virulence.

Although the exact basis of the dysregulation awaits further investigation, it is important to note that the S2P knockout bacteria exhibited 100-fold lower acute replication, and 10,000-fold lower persistence in an aerosol infection model of mouse tuberculosis. These defects could be rescued by the S2P transgene, but to date the involvement of a site-1 protease or even the identity of the S2P substrate(s) remain unknown (Figure 2). Nevertheless this work underscores the possibility that S2P may be a viable therapeutic target for tuberculosis, by illustrating that removing S2P activity has dramatic and direct, although not well understood, effects on several phases of the disease.

Pseudomonas aeruginosa S2P and exopolysaccharides

The S2P circuit has been characterized in greater detail in *Pseudomonas aeruginosa*, a Gram negative opportunistic pathogen that colonizes the lungs of cystic fibrosis (CF) patients. Alginate is an EXOPOLYSACCHARIDE that is produced by *P. aeruginosa* and confers a mucoid morphology on bacterial colonies grown in culture, and in the lungs of CF patients renders the bacteria more adherent, less sensitive to antibiotics and host defenses, and contributes to biofilm formation⁴⁷. During colonization, conversion of *P. aeruginosa* strains from non-mucoid to a 'slimy' mucoid appearance correlates with a poor prognosis in CF patients.

The precise molecular pathway regulating alginate biosynthesis was recently revealed to be regulated by an intramembrane proteolysis circuit similar to the *E. coli* extracytoplasmic stress response⁴⁸ (Figure 2). Pathway activation is subject to multiple environmental inputs and intersects with other pathways, but the core of the circuit is sequential site-1 and site-2 protease mediated cleavage of the anti-sigma factor MucA (RseA homologue)⁴⁸. The S1P is AlgW and the S2P is MucP, which is ultimately responsible for cleaving MucA, thereby releasing the σ^E homologue AlgU from the inner membrane to activate the alginate biosynthesis operon. However, unlike RseP/YaeL in *E. coli*, MucP is not an essential gene.

Clinically, MucA is a hot spot for mutations that confer a stable mucoid phenotype to CF patient isolates; as high as 90% of mucoid isolates can carry MucA mutations, the majority of which are nonsense or frameshift mutations that render MucA unable to sequester AlgU⁴⁹. Cell wall-targeting antibiotics also activate the pathway, resulting in alginate production and expression of other factors including cell wall repair enzymes⁵⁰. Although this may be a natural defense mechanism against antibiotic-secreting microbes *P. aeruginosa* encounters in its native soil environment, this response also has implications for treating *P. aeruginosa* infections. It is

presently unclear if biosynthesis of exopolysaccharides is generally regulated by intramembrane proteolysis, as alginates themselves are known to be synthesized by only two bacterial genera, *Pseudomonas* and *Azotobacter*.

Vibrio cholerae S2P and toxin production

It is noteworthy that the opposite situation, although less common, also exists: some transmembrane transcription factors are competent for transcriptional activation while integral to the membrane, but proteolytic release renders them inactive. *V. cholerae* is a devastating diarrhoeal pathogen that has killed millions of people in eight documented pandemics throughout history. Virulence gene expression must be carefully controlled by such a pathogen that also exhibits an aquatic existence outside the human host⁵¹. Intramembrane proteolysis has been implicated in limiting virulence gene expression⁵². The master virulence gene activator ToxT directly activates transcription of the canonical virulence factors cholera toxin and the toxin co-regulated pilus⁵¹. A myriad of inputs including environmental and quorum-sensing signals converge on *toxT* transcription ultimately through two transcription factors, ToxR and TcpP. TcpP appears to be the dominant factor, and is rendered unstable under non-virulence or unfavorable conditions.

A genetic screen for the protease responsible for TcpP instability implicated the S2P protein YaeL⁵² (Figure 2). The stabilized TcpP in a YaeL mutant was smaller, consistent with site-1 cleavage, but despite testing four candidates the identity of the site-1 protease remains elusive. Moreover, the signals that induce degradation of TcpP by activating site-1 cleavage also remain unclear. Like with *M. tuberculosis*, the involvement of an S2P in virulence is recent and the precise molecular pathway has yet to be elucidated, although in this case blocking S2P would be expected to induce virulence genes.

Enterococcus faecalis S2P and pheromones

A rare exception to S2P regulating transcription factor activity is exemplified in mating pheromone production by *E. faecalis*. This Gram positive bacterium is usually a commensal of human intestinal flora, but has also emerged as an important source of NOSOCOMIAL infections. Moreover, *E. faecalis* poses a serious threat beyond its own infective capabilities, because antibiotic resistance is endemic to *E. faecalis*, and vancomycin resistance has now become common in *Enterococci* through horizontal gene transfer⁵³. This has made understanding *Enterococcal* plasmid transfer an important area of research^{54, 55}.

E. faecalis plasmid transfer is regulated by intricate cell-to-cell signalling via pheromones that are produced by plasmid-free, recipient cells^{54, 55}. The pheromones are hepta or octapeptides that are produced by intramembrane proteolysis of latent signal peptides from chromosomally encoded lipoproteins (which are themselves not involved in the process)^{56–58}(Figure 2). These pheromones enter the plasmid-bearing, donor cells and activate transcription of CONJUGATION genes from the plasmid, including an aggregation substance that aids cell clumping, to promote plasmid transfer. Intriguingly, once transferred, the plasmid encodes an inhibiting pheromone precursor of 21 to 23 residues that still relies on intramembrane processing to the active hepta or octapeptide inhibitor to prevent self-induction. A search for the processing enzyme isolated a site-2 protease termed Eep (enhanced expression of pheromone)⁹. Strikingly, *eep* null cells were found to reduce transfer of multiple (but not all) plasmids that use different pheromone systems, indicating that the processing enzyme itself is a common link between multiple pheromone systems.

But recently it has become clear that this form of intramembrane proteolysis also plays a direct role in virulence of this nosocomial pathogen^{59–61}. Induction of conjugal transfer genes was discovered to be triggered upon exposure to human plasma, and the resulting production of the

Asc10 aggregation substance increased resistance to phagocyte killing, as well as bacterial adherence and invasion of epithelial cells^{59–61}. Importantly, Asc10 biosynthesis required pheromone production by the bacterial cells, while serum albumin was found to bind the inhibiting pheromone⁶². Thus, differential sequestration of the inhibitor by albumin alters the pheromone:inhibitor balance, resulting in self-induction and production of the Asc10 aggregation substance. An important implication of these observations is that the autocrine signalling loop that triggers virulence factor production in plasma requires production of the pheromone by the intramembrane protease Eep (Figure 2).

Rhomboid and quorum-sensing

The role of Eep in cell-to-cell communication is exceptional, being the only described case in any organism of an S2P that cleaves proteins to release polypeptides to the cell exterior. This function is normally ascribed to rhomboid proteases, whose active site lies close to the external membrane face. Rhomboid was implicated in cleaving growth factor signals in *Drosophila* development³, and subsequently in QUORUM SENSING by *Providencia stuartii*⁸, a Gram negative pathogen that causes urinary tract infections. Two separate genetic screens isolated the rhomboid homologue AarA (aminoglycoside acetyltransferase regulator A) of *P. stuartii* as a factor involved in density-dependent repression of an aminoglycoside acetyltransferase, and activation of other, uncharacterized genes^{8, 63}. Subsequent study established that proteolytic mechanism and specificity were conserved between *Drosophila* rhomboid and AarA⁶⁴, and remarkably, interchanging their genes could rescue fly and bacterial signalling⁶⁵. Moreover, although its identity remained unknown, the bacterial signal had biochemical properties consistent with being a small peptide⁶⁶. Taken together, these observations dramatically suggested that activation of cell-to-cell signalling through rhomboid-mediated processing of precursor proteins might be the first signalling circuit known to be conserved between animals and bacteria⁶⁵.

Contrary to expectation, this turned out to be both a rare and superficial similarity (Figure 3). In an attempt to clone additional rhomboid homologues, the *aarA* mutant of *P. stuartii* was found to be rescued by the TatA subunit of the twin arginine translocase from several other bacteria, but mysteriously not from *P. stuartii* itself⁶⁷. A sequence comparison revealed that the *Providencia* TatA contains a 7 residue extension on its N terminus. This observation immediately suggested that this extension is not the signal, as occurs in lipoprotein processing by Eep, but rather that this extension acted negatively to prevent TatA activity prior to its proteolytic removal. The role of AarA was clarified in an elegant genetic experiment: production of the signal for quorum-sensing, as well as all known PLEIOTROPHIC phenotypes of *aarA*, were restored in an *aarA* mutant expressing an experimentally truncated TatA lacking the 7 residue extension⁶⁷. Moreover AarA was shown to cleave TatA. Understanding this unusual quorum-sensing system now awaits the identification of the signaling molecule, but it is clear that the only apparent role of AarA in *P. stuartii* is in activating TatA, presumably for signal export, and has no further role in production of the signal itself.

Apart from *P. stuartii*, our knowledge of rhomboid function in bacterial physiology or pathogenesis remains absent. The only known bacterial substrate for rhomboid is TatA, yet only a few bacteria, perhaps less than 1%, encode the rhomboid-dependent form of TatA. This implies that the role of AarA is a rare adaptation rather than a generally applicable paradigm. To date, the genetic deletion of the *E. coli* rhomboid GlpG has no discernible phenotype even when interrogated using a BIOLOG approach⁶⁸, while a filamentous phenotype has been reported in a *B. subtilis* rhomboid knockout, although its molecular basis is unclear³⁷.

It is also at present unclear whether bacterial rhomboid enzymes might constitute potential therapeutic targets. While the Tat system itself has documented roles in pathogenesis in some bacteria and it is possible that AarA could thus turn out to be a target in *P. stuartii*, the rarity

of extended TatA forms requiring rhomboid processing in other pathogens precludes widespread utility⁶⁹. Moreover, the few available examples of rhomboid-deficient bacteria display a modest increase in antibiotic resistance^{63, 68}, suggesting that inhibiting rhomboid might not be a good therapeutic strategy. Conversely, recent identification of rhomboid protease mutants with increased activity might suggest that if this increase can be mimicked pharmacologically, it could lead to a new strategy for lowering antibiotic resistance by stimulating rhomboid activity^{30, 32}.

ROLES IN PROTOZOAN PARASITES

Eukaryotic protozoan parasites are among the most successful and devastating pathogens today. Important non-signalling roles for intramembrane proteases in these pathogens have recently been identified that offer promising targets for therapeutic intervention.

Apicomplexan Parasites

Protozoan parasites of the phylum Apicomplexa include *Plasmodium*, which causes malaria, afflicting 10–40% of the world's population and claiming 2 million lives annually (mainly children). *Toxoplasma gondii* and *Cryptosporidium parvum* are major pathogens of immunocompromised individuals including AIDS patients. The problems that apicomplexans cause are not limited to human health: *Eimeria tenella* infects poultry and causes annual losses in revenue totaling nearly a billion dollars.

Without exception apicomplexans are obligate intracellular pathogens, and this dependence on living inside host cells has focused attention on understanding their invasion mechanism (Box 1). Molecular dissection of *Toxoplasma* invasion revealed that proteolytic shedding of adhesins from the parasite surface is required for invasion^{70, 71} (Figure 4). Although the protease initially eluded identification, many lines of evidence now suggest that a parasite-encoded rhomboid enzyme is responsible. *Toxoplasma* encodes six rhomboid enzymes, four of which are proteolytically active^{72, 73}, but only one, TgROM5, has the capacity to cleave parasite adhesins directly in a defined heterologous assay⁷². Importantly, the cleavage site of numerous adhesins during invasion have been mapped to their transmembrane segments^{74–76}. Lastly, TgROM5 resides on the parasite surface, enriched towards the posterior end, the expected site of adhesin cleavage⁷². Although these observations provide strong, correlative evidence that TgROM5 is the protease that dissolves the moving junction at the end of the invasion program, they should not be viewed as causative. It is important that genetic tests be applied to validate the importance of TgROM5 in invasion, but due to the difficulty of the system this has not yet been possible.

Extension of these observations to *Plasmodium falciparum*, which causes the lethal form of malaria, met unexpected complexity. Although *P. falciparum* encodes up to eight rhomboid proteins, it does not have a direct orthologue of TgROM5^{77, 78}. Moreover, most *Plasmodium* adhesins contain aromatic residues within their transmembrane segments and as a result are refractory to cleavage by typical rhomboid enzymes⁷⁷. While it is clear that other proteases are also involved in invasion, including the membrane-tethered subtilisin PfSUB2⁷⁹, a direct enzymatic analysis of *Plasmodium* rhomboid proteases revealed that one rhomboid, PfROM4, displays an atypical mode of specificity specialized for cleaving *Plasmodium* transmembrane adhesins that cannot be processed by PfSUB2 or canonical rhomboid enzymes^{77, 80}. Indeed, PfROM4 is unable to cleave typical rhomboid substrates, but a second enzyme, PfROM1, provides this activity⁷⁷. Intriguingly, TgROM5 cleaves both standard rhomboid substrates, as well as adhesins with aromatic residues within their transmembrane segments, suggesting that it functions as a 'dual specificity' rhomboid⁷⁷. As such, it is tempting to speculate that when *P. falciparum* lost a TgROM5 homologue, it co-

opted two rhomboid proteases with different substrate specificities that together could cover the repertoire of substrates that were normally cleaved by TgROM5 alone.

Further analysis of over a dozen *P. falciparum* adhesins known to be involved in invasion revealed that they all could be processed by PfROM4, or less commonly by PfROM1, or at times both⁷⁷. These observations potentially broaden the role of rhomboid enzymes to all invasive stages of the parasite life cycle, both in the human host and in the mosquito vector. Analysis of PfROM4 and PfROM1 directly in MEROZOITES, the parasite form that causes the disease, further revealed that they are at the right place at the right time to execute their hypothesized roles during invasion (Figure 4). PfROM4 was found to be on the surface of the merozoite⁸⁰, while PfROM1 was localized internally and defined the existence of a new organelle termed the mononeme⁸¹. However, consistent with a role in adhesin processing, PfROM1 was found to be released onto the merozoite surface prior to or during invasion⁸¹.

Importantly, different adhesins are known to define alternative invasion routes into a host cell, and parasites are able to switch pathways when one becomes blocked⁸². An implication of these early observations is that rhomboid cleavage of most transmembrane adhesins provides a convergence point that might be a weakness of the parasite invasion strategy. In both *P. falciparum* and *T. gondii*, difficulty of working with haploid organisms has thus far impeded genetic tests of this model. Perhaps the most compelling observation currently is the discovery that at least one essential adhesin is cleaved in its transmembrane segment during merozoite invasion⁸⁰. Moreover, a further encouraging observation is failure to generate transgenic parasites harboring a rhomboid-uncleavable adhesin⁸⁰, but this provides only indirect evidence that rhomboid proteolysis is essential. Other apicomplexan parasites also encode rhomboid proteases, including *C. parvum* and *E. tenella*, and although their functions are not yet assigned, they have been identified in several antigen expression cloning screens from specific stages of the parasite lifecycle^{83, 84}. Analogous roles during parasite invasion are anticipated but untested.

In addition to rhomboid proteases, an SPP homologue has recently been implicated in invasion of erythrocytes by *P. falciparum*⁸⁵. Unusual for an intramembrane protease, PfSPP was identified through a yeast two-hybrid screen for parasite proteins that interact with BAND 3 of human erythrocytes. Contrary to metazoan SPPs, PfSPP was not localized in the ER, but internally in MICRONEMES and on the apical surface of the parasite, and an antibody to the surface-exposed region of PfSPP blocked parasite invasion of erythrocytes. Moreover, intramembrane protease activity of PfSPP was shown to be similar to its human counterpart as assayed in a cell-based assay⁸⁶, and two general SPP inhibitors were recently found to block merozoite invasion of erythrocytes, and subsequent parasite multiplication⁸⁷. These observations suggest that PfSPP may have multiple essential proteolytic functions in the erythrocytic stage. PfSPP is also expressed in other phases of the parasite lifecycle, and thus could provide proteolytic activity for other cellular processes. Identifying PfSPP substrates, which based on its topology would be expected to be type II membrane proteins, should help clarify its function(s).

Rhomboid functions beyond invasion

Many parasites including *T. gondii* and *Plasmodium* spp. encode multiple rhomboid enzymes not thought to be involved in adhesin cleavage. Functions beyond invasion are also suggested by the finding that several TgROMs are localized to different organelles^{88, 89}. What are the roles of these rhomboid enzymes?

Recent analyses have started to focus on this question: TgROM1 knockdown revealed no defects in *T. gondii* invasion, but rather impaired multiplication of parasites within host cells⁸⁹. Similarly, ROM1 knockout in the rodent malaria *P. berghei* yielded parasites that were

able to complete the entire lifecycle, both in the mosquito and mouse hosts, although several stages were delayed, or resulted in production of fewer parasites⁹⁰. Removal of PbROM1 activity may thus lead to a general 'unfitness' of parasites, consistent with analysis in *T. gondii*. However, it should be noted that while TgROM1 knockdown parasites displayed mild phenotypes, repeated attempts to generate TgROM1 knockout *T. gondii* failed⁸⁹. This observation raises the possibility that mechanisms partly compensating for ROM1 loss could have emerged in the knockdown parasites. It remains to be seen whether *P. berghei* could also have adapted to PbROM1 loss during the knockout procedure, as has been observed for some genes⁸². Therefore, genetic approaches may not yet have revealed the full spectrum of ROM1 function, and equally the substrate(s) that ROM1 processes in these events also remain unknown.

Despite these limitations, infection of mice with PbROM1 knockout parasites resulted in clearance by the immune system, and protective immunity against subsequent wildtype parasite infection⁹⁰. Collectively, these experiments provide direct evidence that, while some rhomboid enzymes could have broad but not essential roles in the lifecycle of apicomplexan parasites, blocking their activity may still prove to be a successful strategy for anti-parasite therapy.

Role of rhomboid in parasitic amoeba

Intramembrane proteases are also widely conserved in protozoa that do not invade cells. Thus, a direct role in invasion probably reflects a specialization rather than revealing a common function. What is the role of intramembrane proteases in non-invasive pathogens? *Entamoeba histolytica* is an enteric pathogen that causes amoebic dysentery, but can also burrow through the intestine and spread to other organs, causing life-threatening abscesses. Unlike most eukaryotic cells, the amoeba genome encodes only one active rhomboid protease, termed EhROM1, making analyzing non-invasive functions of rhomboid enzymes attractive in this exclusively extracellular pathogen⁹¹.

Biochemical analysis of EhROM1 revealed it to possess atypical substrate specificity analogous to PfROM4⁹¹. But the recent excitement over EhROM1 stems primarily from the protein that it cuts: a substrate search identified a heavy subunit of a family of surface Gal/GalNAc LECTINS known to play decisive roles in parasite virulence⁹². These lectins sit at the crossroads between different pathogenic modes, from establishing a benign, commensal infection, to initiating host attack via cell death and lysis, or differentiation into a dormant cyst. In fact, the lectins themselves are thought to determine the route taken, but how these decisions are made is not clear. At the amoeba surface, lectins contact host cells directly, which is essential both for host cell killing and phagocytosis of erythrocytes⁹² (Figure 4). EhROM1 is also localized to the surface and in undefined internal structures, but relocates to phagosomes along with lectins during phagocytosis⁹¹. During some of these events lectins are cleaved, but the role of the proteolysis and the identity of the responsible protease had remained unclear. It is tempting to speculate that lectin cleavage by EhROM1 may play an instructive role in some of these important events. Interestingly, the function of the amoebic rhomboid enzyme again is to cleave adhesins like in apicomplexans, although this time the outcome isn't host-cell invasion, but rather phagocytosis and virulence.

Analysis of EhROM1 function also revealed a new role for rhomboid enzymes. *E. histolytica* is an extracellular pathogen, and with important proteins such as its lectins prominently displayed on its surface, it must evade the host immune system. One amoebic mechanism for immune evasion is receptor capping; surface proteins that are attacked by the host immune system are translocated to the posterior of the parasite, and jettisoned on a membranous ball⁹³ (Figure 4). Intriguingly, inducing receptor capping experimentally caused EhROM1 to redistribute to the neck of the nascent cap, implying a role in cap release⁹¹ (Figure 4). However, while these intriguing correlations are striking, blocking EhROM1 activity, either

via genetic ablation or pharmacologic interference, is required to test the role EhROM1 might play in these important events. These lines of investigation are challenging, since genetic techniques are rudimentary in amoeba, and no rhomboid inhibitors are yet available.

ROLES IN PATHOGENIC FUNGI

The functions of intramembrane proteolysis in pathogenic fungi have only recently been explored. The only work in this area stems from the study of adaptation to hypoxia by *Cryptococcus neoformans*, an inhaled human pathogen that disseminates to the brain where it causes the most common form of fungal meningitis (Figure 5). *C. neoformans* is an obligate aerobe that is exquisitely sensitive to oxygen levels for growth, and must therefore adapt to very low oxygen during pathogenesis in body tissues. Recent work discovered that the classical SREBP pathway is responsive to oxygen levels, and SREBP itself signals hypoxic adaptation in *Schizosaccharomyces pombe*⁹⁴.

Screens for mutants defective in hypoxic growth⁹⁵, and extension of the SREBP paradigm to *C. neoformans* identified cleavage of its SREBP⁹⁶, Sre1, as a response to low oxygen levels (Figure 5). Microarray analyses identified 50–100 genes regulated by Sre1 in response to oxygen. During infection of a mouse model, fungi harboring deletions of Sre1, or the S2P homologue Stp1 yielded greatly reduced growth in animal tissues, resulting in not only improvement of gross pathology, but a striking increase in survival of infected animals. Thus, S2P-mediated proteolysis plays a key role in virulence of *C. neoformans* by facilitating acclimatization of the fungi to the hypoxic nature of host tissues. Another particularly encouraging observation is that *C. neoformans* mutant for Stp1 is 100-fold more sensitive to common anti-fungal AZOLE DRUGS, suggesting that targeting S2P may at least provide synergistic effects in combination therapy with current drug regimens, if not being an improved regimen on its own. This awaits future investigation, as does identification of other components of this pathway, including a possible site-1 protease.

It should be noted that recognizable S2P homologues appear to be absent in many fungi, some of which may compensate by releasing SREBP using alternative pathways such as RUP⁹⁷. As such, S2Ps may not prove to be broadly applicable as anti-fungal targets. However, most fungi encode other intramembrane proteases, with rhomboid proteases being particularly common, that have not been explored in the context of either biological regulation or fungal pathogenesis. This remains a fertile area of research for the future.

ROLES IN VIRAL MATURATION

Although there are currently no known viral intramembrane protease, several *Flaviviridae* have been found to rely on host-encoded SPP to provide essential functions for viral propagation. Most research has centered on assembly of hepatitis C virus (HCV), a positive strand RNA virus that infects over 150 million people globally and constitutes a major cause of cirrhosis and hepatocellular carcinoma.

The HCV genome consists of a single ORF that codes for one polyprotein of ~3000 residues from which all individual viral factors must be excised⁹⁸. As such, the virus relies heavily on proteases, both host and viral, to process the polyprotein into functional viral components. The N terminal one third contains the structural capsid core protein followed by two envelope proteins. The first signal peptide between core and E1 directs insertion into the ER membrane, and cellular signal peptidase releases the signal peptide from E1. But as a consequence of polyprotein continuity, the released signal peptide of E1 becomes a *de facto* C terminal transmembrane segment of core protein (Figure 6). This signal peptide is processed further by SPP activity, which was recognized well before the gene for SPP was identified⁹⁹. The SPP-processed core remains membrane associated, but now is able to traffic to LIPID DROPLETS

for virus assembly¹⁰⁰. Although the existence of the C terminal, core-processing event was widely documented, whether it was essential for virus production remained unclear until earlier last year.

Initial attempts to address this issue by making core mutants that cannot be processed by SPP yielded conflicting results, which have now been tracked to core polymorphisms among different HCV serotypes. A second hurdle was developing methodologies to assess impact directly on virus production and infectivity. With these advances in hand, several lines of evidence, including siRNA knockdown of SPP¹⁰¹, use of core mutants¹⁰², and SPP inhibitors and dominant negative mutants¹⁰³, all agree that reducing core processing lowers released virus titre, and hinders HCV propagation and infectivity up to 10-fold. Mechanistically, unprocessed core was delayed in trafficking to lipid droplets and subsequent recruitment of replicated viral RNA and non-structural factors to virions¹⁰² (Figure 6). Moreover, capsids self-assembled from unprocessed core expressed in a heterologous system yielded unstable virus-like particles¹⁰⁴.

These studies provide proof-of-principle that inhibiting SPP hinders HCV virus propagation, but it is not clear if the observed reduction is enough to limit or cure a chronic infection, for which a widely effective therapy is eagerly sought. Indeed, early observations suggest that inhibiting SPP results not in a block, but rather a delay, in virus assembly¹⁰². Animal models, which add pharmacokinetic and immune dimensions to the studies, are required to evaluate this possibility further. Recent advances should help to motivate this challenging approach.

Two other *Flaviviridae* of limited medical importance have also been shown to require core processing by SPP^{105, 106} (Table 1), and SPP also plays a role in the infective cycle of an unrelated virus, the herpes virus cytomegalovirus¹⁰⁷. In this context, SPP is required for the dislocation of MHC proteins from the ER as part of a larger viral immune evasion strategy. However, whether SPP requires proteolytic activity for this function, or the impact of blocking SPP on virus infection, remain unexplored.

Notably, in these contexts it is the host SPP enzyme, rather than a viral enzyme, that would serve as a target for antiviral therapy. This has two main implications: first, it could confound therapy with side-effects due to the other roles SPP has in cells, especially in the immune system. Secondly, and on a positive note, targeting host SPP could limit the scope for emergence of drug resistance (since it would be limited to the host cell rather than the virus).

EMERGING PRINCIPLES AND FUTURE DIRECTIONS

Intramembrane proteolysis as a field has its origins in animal biology and disease, but intramembrane proteases are widely conserved in unicellular organisms. The past few years have witnessed major advances in our understanding of the roles these enzymes play in pathogenic organisms of all types. While this can now be viewed as a firmly-established and distinct new field, key challenges remain both in our understanding of the roles these enzymes play, and in evaluating their potential as therapeutic targets.

An important goal is to gain a representative picture of the roles intramembrane proteolysis plays in pathogenic organisms across evolution. Currently the majority of known roles for intramembrane proteases generally fall within pathogen types; S2P figures prominently in bacterial pathogens, rhomboid proteases play central roles in protozoan parasites, and SPP is the only intramembrane protease implicated in viral assembly. Certainly the groupings are not exclusive and at least partly due to the consequence of limited study, as illustrated by well-defined exceptional cases. But it will be important to see to what degree this general trend holds as more examples become characterized. Another major gap is the lack of any information on intramembrane proteolysis in pathogens that infect plants, probably because these pathogens

do not receive comparable levels of effort. Collectively, this information is key to understanding the specialization and evolutionary origins of intramembrane proteolysis in pathogenic microbes.

Secondly, despite the demonstrated importance of intramembrane proteolysis in multiple pathogens, not a single intramembrane protease has been targeted with inhibitors in the context of disease models. Currently this approach is hampered by the difficulty in developing specific inhibitors for both rhomboid and S2P families, but strategies to overcome hurdles should emerge as our biochemical and structural understanding of these enzymes increases. Another potential challenge is the conservation of intramembrane proteases in animal hosts, which could cross-react with inhibitors. While this is a major issue, it is possible that a window of opportunity exists because often these are distantly-related homologues, and because infections tend to require acute rather than chronic treatment. These lines of investigation present tremendous challenges and opportunities for the future, and given the attractive nature of proteases and membrane proteins as drug targets, could even culminate in new strategies for therapeutic intervention into a variety of infectious disease.

BOX 1

Unlike most invasive pathogens, apicomplexa do not enter by triggering endocytic uptake by the host, but rather employ an intricate form of parasite motility to force their way into host cells^{108, 109} (Figure 4). Key molecules in the invasion process are parasite-encoded adhesins that form the interface between the parasite and host. These transmembrane proteins act as bridging molecules that form strong and specific interactions with a variety of receptors on the host surface, and link to the actomyosin contractile system inside the parasite^{108, 109}. Parasites encode a diverse arsenal of adhesins, some of which are specific for invasion of different cell types, others define different entry routes into the same cell, while others may act in concert to facilitate invasion^{82, 110}. The result is formation of a MOVING JUNCTION between the parasite and host surface that sweeps across the parasite from apical to posterior end, propelling the parasite into the host and causing invagination of the host membrane to form the nascent parasitophorous vacuole¹¹¹ (Figure 4). While the moving junction forms the essential tight association that drives invasion, it must be dismantled at the end of the invasion program for parasite internalization and sealing of the host membrane^{70, 71}.

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GLOSSARY

SREBP	<u>sterol regulatory element binding protein</u> , a transcription factor that activates expression of genes required for cholesterol and lipid biosynthesis in animals
Extracytoplasmic function	a variety of conditions that are commonly detected by unfolding of outer membrane proteins, which stimulates proteases that liberate σ^E from the membrane to activate response genes expression
Metalloprotease	an enzyme that cleaves peptide bonds using bound zinc ion to facilitate hydrolysis. The zinc is held in place usually by two conserved histidines and one acidic residue
Aspartyl protease	a hydrolytic enzyme that uses two aspartate residues to activate water for cleaving peptide bonds
Serine protease	an enzyme that uses a serine as a nucleophile for cleavage of peptide bonds. The serine is usually activated by a basic residue, and forms a covalent intermediate with the substrate that is released through attack by water.
Anti-sigma factor	a protein that binds and hinders the transcription-activating function of a bacterial sigma factor
Exopolysaccharide	a linear polymer of modified sugars that is not generally attached to a bacterium; act as an extracellular matrix
Nosocomial	an infection acquired in a hospital
Conjugation	a physical joining of two bacterial cells for the purpose of transferring genetic material

Quorum sensing	a cell-to-cell signalling mechanism by which bacteria monitor their population size and react to it accordingly
Pleiotrophic	having multiple, and seemingly unrelated, phenotypes
Biolog	a commercial, phenotypic testing method conducted using a large number of standardized conditions
Moving junction	a specialized adhesive point of contact between the parasite and host that appears as an electron-dense structure in EM analysis and traverses the surface of the parasite as a tight ring during invasion of the host
Merozoite	the parasite form specialized for invasion of and replication within erythrocytes
Band 3	an integral membrane protein responsible for chloride/bicarbonate exchange on erythrocytes
Micronemes	specialized, apical, tiny, vesicle-like organelles of apicomplexan parasites that house adhesins and other proteins for secretion during invasion
Gal/GalNAc lectin	a protein specialized for binding sugars, in this case containing galactose and N-acetylgalactosamine
Azole drugs	a class of anti-fungal drugs that inhibit the enzyme lanosterol-14α-deme-thylase required for ergosterol biosynthesis
RUP	‘regulated ubiquitin/proteasome-dependent processing’, a form of proteasome degradation that releases intact domains from the membrane into the cytosol
Lipid droplets	lipid storage organelles of cells, in which triacylglycerides in the form of a drop are surrounded by a single leaflet of membrane phospholipid

Figure 2. S2P circuits and bacterial virulence: variations on a theme

The *E. coli* extracytoplasmic function (top left panel) serves as a paradigm for the S2P circuit in bacterial pathogenesis. An input signal (yellow box and arrow), in this case envelope stress, activates the site-1 protease DegS and relief of inhibition by RseB, allowing cleavage of the anti-sigmaE factor RseA at site-1 (top bolt). Intramembrane proteolysis by RseP ensues at site-2 (bottom bolt), resulting in cytoplasmic (bottom) release, degradation of RseA, and liberation of σ^E to activate the output response, in this case transcription of stress response genes (black arrow). The other S2P circuits involved in regulating virulence of various bacterial pathogens (detailed in the text) are depicted in the same way for the sake of comparison.

Components that have been identified are named, while missing components are labeled with a question mark.

Figure 3. Dis-similarity in signalling by rhomboid proteases in *Drosophila* and a bacterium

In *Drosophila* cells (left), the EGF precursor Spitz is transported by Star from the ER to the Golgi apparatus, where intramembrane proteolysis by Rhomboid-1 ensures. The processed Spitz is then secreted from the cell, ready to activate EGF receptor signalling in neighboring cells. In this context, rhomboid directly processes the signalling molecule to activate it from a latent form. Conversely in a bacterium (right), the signal itself is unknown and does not require rhomboid processing. Instead, the rhomboid protease AarA cleaves the TatA component of the twin arginine transporter, presumably to facilitate export of the unknown quorum-sensing signal. Notably, the mechanisms are fundamentally different because AarA does not act on the signal itself. Removal of the N terminal extension of TatA by AarA is shown to be required for TatA oligomerization to form the export pore (along with other subunits), although this is only one model and other possibilities exist.

Figure 4. Function of rhomboid proteases in two different protozoan parasites

An example of parasitism of a human red blood cell by two different protozoan pathogens is shown. In the top panel, a *Plasmodium* merozoite invading the red blood cell using rhomboid enzymes PfROM4 (green) and PfROM1 (red, initially in mononeme) to breakdown the moving junction (formed between parasite adhesins and host receptors) at the end of invasion. The steps and roles of rhomboid enzymes are detailed in the text, but lead to malaria in the host. The invasion mechanism and role of rhomboid is similar in *Toxoplasma*. Bottom, an *Entamoeba histolytica* trophozoite is in the process of internalizing a red blood cell via phagocytosis. The rhomboid EhROM1 (green) relocates from the secretory pathway to the phagosome, colocalizing with its putative substrate the Gal/GalNAc lectin (blue). On the right,

the amoeba translocates antibody-bound surface proteins to the cap, a membraneous vesicle-like structure that it will jettison to evade immune system attack. EhROM1 colocalizes to the neck of the cap during the capping process. Penetration of the intestine by *E. histolytica* and evasion of the immune system can lead to life-threatening abscesses.

Figure 5. S2P mediates adaptation to hypoxia during fungal dissemination

Outside the body, *Cryptococcus* (drawn in blue throughout) experiences a highly aerobic environment, but encounters hypoxia upon inhalation and dissemination into host tissues. Hypoxia is thought to lower sterol production, since biosynthesis requires oxygen. As in human cells, lower sterol levels are thought to result in release of the Scp1:Sre1 complex (SCAP:SREBP in humans) from the ER to the Golgi apparatus (depicted on the right), followed by sequential cleavage by an unidentified *Cryptococcal* site-1 protease (S1P in humans) and Stp1 (S2P in humans). The site-2 cleavage releases the Sre1 transcription factor domain from the membrane, allowing it to enter the nucleus and activate biosynthetic and hypoxia response genes. In humans, SREBP does not participate in a hypoxic response, but if Stp1 activity is blocked by mutation, *Cryptococcus* is unable to adapt to hypoxic conditions and cannot survive in host tissues.

Figure 6. SPP processes core protein for hepatitis C virus assembly

The structural HCV components E1 and E2 envelope proteins, core, and p7 are shown inserted into the ER membrane and already processed by signal peptidase. SPP is subsequently responsible for cleaving the first signal peptide, which following signal peptidase cleavage is attached to the C terminus of core protein. SPP cleavage (depicted by the yellow bolt) is required for core trafficking to lipid droplets (right) and recruitment of viral genomic RNA and factors (left) for capsid assembly. Note the SPP is not encoded by the virus, but rather is an ER-resident protein of the host cell.

TABLE 1

Currently-defined roles of intramembrane proteases in microorganisms.

Organism	Protease (Type)	Substrate	Process	Role	Ref
<i>Bacillus subtilis</i>	SpoIVFB (S2P)	pro- σ^K	sporulation	cleaves pro- σ^K to activate gene expression in the mother cell	7
<i>Bacillus subtilis</i>	YluC/RasP (S2P)	RsiW (anti-sigma)	extracytoplasmic stress response	releases σ^W to activate stress response	39
<i>Bacillus subtilis</i>	YluC/RasP (S2P)	FtsL	cell division	negatively regulates cell division by degrading FtsL	41
<i>Bacillus subtilis</i>	YqgP (Rho)	???	cell division? sugar metabolism?	yqgP mutant has filamentous phenotype	37
<i>Bordetella bronchiseptica</i>	HurP (S2P)	HurR (anti-sigma)	iron uptake	cleaves HurR to activate heme receptor <i>bhuR</i> expression	38
<i>Caulobacter</i>	MmpA (S2P)	PodJ _S	cell polarity	preserves cell asymmetry by cleaving PodJ _S for degradation	36
<i>Escherichia coli</i>	RseP (S2P)	RseA (anti-sigma)	extracytoplasmic stress response	releases σ^E to activate stress response genes	12, 13
<i>Escherichia coli</i>	GlpG (ROM)	???	???	<i>glpG</i> mutant shows a slight antibiotic resistance	68
<i>Streptococcus uberis</i>	Eep (S2P)	MtuA	leader peptidase cleavage	compensates for absence of lipoprotein signal peptidase Lsp	40
<i>Mycobacterium tuberculosis</i>	Rv2869c (S2P)	???	virulence	regulates lipid metabolism genes, envelope composition	46
<i>Pseudomonas aeruginosa</i>	MucP (S2P)	MucA (anti-sigma)	alginate production	regulates alginate biosynthesis genes	48
<i>Vibrio cholerae</i>	YaeL (S2P)	TcpP	toxin expression	Negatively regulates toxin expression, destabilizes TcpP	52
<i>Enterococcus faecalis</i>	Eep (S2P)	Cad, CefA, iAD1, iCF10	pheromone-induced conjugation, cell aggregation/ virulence	produces multiple octapeptide pheromones and inhibitors by processing latent signal peptides	9, 57, 58, 62
<i>Providencia stuartii</i>	AarA (ROM)	TatA	quorum sensing	activates TatA, results in density-dependent gene repression and activation	8, 65, 67
<i>Toxoplasma gondii</i>	TgROM1	???	intracellular growth	knockdown parasites grow less efficiently than wildtype	72, 88, 89
<i>Toxoplasma gondii</i>	TgROM5	AMA1 and MIC adhesins	invasion	breaks down moving junction	72, 74, 75
<i>Plasmodium falciparum</i> or <i>P. berghei</i>	PIROM1/ PbROM1	AMA1, other adhesins?	invasion, growth, oocyst differentiation	cleaves some adhesins, other substrates unknown	77, 81, 90

Organism	Protease (Type)	Substrate	Process	Role	Ref
<i>Plasmodium falciparum</i>	PFROM4	EBLs, RBLs, and TRAP adhesins	invasion	cleaves many families of adhesins known to be essential for invasion	77, 80
<i>Plasmodium falciparum</i>	PFISPP	???	invasion	binds to band 3; protease activity required for invasion and growth of blood stage	85, 87
<i>Cryptosporidium parvum</i>	CpROM4	???	???	protein present in sporozoites	83
<i>Eimeria tenella</i>	EtROM	???	???	protein present in sporozoites	84
<i>Entamoeba histolytica</i>	EHROM1	Gal/GalNAc lectin heavy subunit	phagocytosis, receptor capping	localizes to phagosomes, and cap neck during immune evasion; exact role(s) in these processes are unknown	91
<i>Cryptococcus neoformans</i>	Sip1 (S2P)	Sre1	hypoxia adaptation, lipid metabolism	liberates SREBP homologue Sre1 from the membrane to activate gene expression	95, 96
<i>Saccharomyces cerevisiae</i>	Pcp1/Rbd1 (ROM)	Mgm1, Ccp1	mitochondrial fusion, protein import	removes Ccp1 targeting sequence, generates short form of Mgm1 GTPase	112
Hepatitis C Virus (HCV)	SPP (host)	Core	polyprotein processing	processing facilitates core trafficking to lipid droplets and viral assembly	99-104
GB Virus B (GBV-B)	SPP (host)	Core	polyprotein processing	processing facilitates core trafficking to lipid droplets and viral assembly	106
Classical Swine Fever Virus (CSFV)	SPP (host)	Core	polyprotein processing	processing required for viral production	105
Cytomegalovirus (CMV)	SPP (host)	???	immune evasion	MHC class I heavy chain dislocation from ER; unknown if protease activity required	107

NOTES: Only intramembrane proteases that have been studied are listed; most organisms also encode additional intramembrane proteases that have not been analyzed.

Abbreviations: S2P, site-2 protease; ROM, rhomboid; SPP, signal peptide peptidase; AMA1, apical membrane antigen 1; MIC, microneme; EBL, erythrocyte binding-like; RBL, reticulocyte binding-like, TRAP, thrombospondin-related anonymous protein, ER, endoplasmic reticulum Color code: orange, bacterial physiology; blue, bacterial pathogenesis; green, protozoan parasites; red, fungi; yellow, viruses