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Substrates and physiological functions of secretase rhomboid proteases

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ABSTRACT

Rhomboids are conserved intramembrane serine proteases with widespread functions. They were the earliest discovered members of the wider rhomboid-like superfamily of proteases and pseudoproteases. The secretase class of rhomboid proteases, distributed through the secretory pathway, are the most numerous in eukaryotes, but our knowledge of them is limited. Here we aim to summarise all that has been published on secretase rhomboids in a concise encyclopaedia of the enzymes, their substrates, and their biological roles. We also discuss emerging themes of how these important enzymes are regulated.

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1. Introduction

Classical proteases are some of the most studied and best understood enzymes, but intramembrane proteases are much more recently discovered and quite different from their soluble counterparts. Arising by convergent evolution, and unrelated to the classical proteases, all intramembrane proteases have multiple transmembrane domains (TMDs), active sites buried in the membrane, and they all cut substrate TMDs. In general, proteases can be divided into those whose primary job is to degrade substrates – digestive enzymes, the proteasome, lysosomal enzymes – and those whose job is to separate domains of proteins, thereby modifying their function, and often participating in signalling events. Although an important recurring theme in this review is that we do not yet know enough about rhomboid proteases to draw very sweeping conclusions they, like other intramembrane proteases, appear primarily to act with high specificity and precision to control signalling rather than as general degradation machinery.

Rhomboids are intramembrane serine proteases that were initially discovered in *Drosophila*, where Rhomboids-1 to -3 control EGFR signalling by cleaving and releasing active growth factors from their transmembrane pro-forms [1,2]. It subsequently became clear that rhomboid proteases are members of a much broader superfamily of rhomboid-like proteins, conserved in all kingdoms of life. The wider superfamily consists of both active proteases as well as a significant subset of proteins that appear to have lost their catalytic activity: the iRhoms and a wide range of other inactive rhomboid-like proteins, including derlins [3]. The rhomboid proteases can be further divided into those located along the secretory pathway and plasma membrane, the secretase class, and those in mitochondria, the PARL class [4]. Rhomboid proteases have been extensively studied in *Drosophila* and apicomplexan parasites but, notably, very little is yet known about their functions in mammals. As the primary function of proteases is to cleave other proteins, the key to unlocking the biological role of rhomboids is discovering their substrates.

Here we focus on the most numerous class of eukaryotic rhomboid proteases – the secretase rhomboids. We start with some general principles, but our main goal is to provide a concise encyclopaedia of what is known about them across multiple organisms. We finish with a more speculative discussion about the regulation of these important regulatory enzymes. We do not consider the mitochondrial rhomboids, reviewed in this issue [5], nor the role of prokaryotic rhomboids, about which little is known.

2. Rhomboids – general principles

Like all intramembrane proteases, rhomboids are polytopic membrane proteins with their catalytic site formed of TMD residues within the lipid bilayer; the hydrolysis of the substrate peptide bond occurs within the membrane. They are 'serine' proteases because they use a serine as the reactive nucleophile in the proteolysis reaction [1], although it is important to emphasise that they are evolutionarily unrelated to the classical serine proteases like chymotrypsin: this is an example of convergent evolution. The core conserved rhomboid-like domain has six TMDs, although most eukaryotic rhomboids have an extra TMD – located C-terminally in the secretase rhomboids (Fig. 1), and N-terminally in the mitochondrial ones. Beyond the core six-TMD fold and a few highly conserved residues, it is notable that the similarity amongst members of the family is quite low [4], so it is unsafe to assume that mechanistic and regulatory details will necessarily be conserved. The rhomboids were the first intramembrane proteases to have their atomic structure determined and this has contributed to them being the best understood mechanistically [6–9].

To understand proteases, it is necessary to identify their substrates. Current evidence supports the view that rhomboids predominantly, perhaps exclusively, cleave type I TMDs: those with luminal N-termini. There is also a clear predominance of single pass TMD proteins in the lists of identified substrates, although a few polytopic proteins have been reported. By their nature, intramembrane proteases are expected to cleave their substrates within TMDs and, consistent with the position of the active site, all rhomboids cleavages to date are closer to the luminal rather than the cytoplasmic face of the membrane. There are also a few reports of non-canonical rhomboid cleavage of substrates just outside the membrane or, at least in one case, in more distal luminal parts of a substrate [10–12].

Given the importance of substrate identification, understanding what are the determinants that make a specific TMD a rhomboid substrate is essential. It is clear that there is a high level of specificity – rhomboids do not cleave most TMDs – and we have a partial picture of what is needed. A major property is helical instability: TMDs form alpha helical secondary structure in a membrane but rhomboid substrates tend to have residues that destabilise helices [13]. The importance of this helical instability is highlighted by the observation that insertion of destabilising residues is sufficient to convert otherwise uncleavable TMDs into rhomboid substrates [14]. Beyond helical instability, a motif around the cleavage site has been identified in many substrates [15]. Much greater detail of rhomboid enzyme mechanism and substrate interaction is reviewed elsewhere in this issue [16,17].

3. Eukaryotic secretase rhomboids

In this section we will focus on each secretase rhomboid individually, reviewing what is known so far about their subcellular localisation, substrates and biological roles (Table 1 summarises this information). It is important to note that, as with so many protein families, the nomenclature has grown organically and is not systematic. In particular the numbering of rhomboids in different species does not necessarily imply particular relationships: for example, *Drosophila* Rhomboid-1 is not an orthologue of mammalian RHBDL1.

3.1. *Drosophila rhomboids*

There are five active rhomboids in the secretory pathway in *Drosophila*: Rhomboid-1, -2, -3, -4, and -6 [4]. Rhomboid-1, -2, -3 are very closely related and are adjacent to each other on chromosome 3 [18], suggesting recent gene multiplication. All three have a well established role in EGFR signalling. Rhomboid-4 has proteolytic activity similar to that of Rhomboid-1, -2, -3 in cell culture assays, but has an unknown physiological role, whereas Rhomboid-6 is the most divergent, and nothing is known of its function [4].

3.1.1. *Rhomboid-1*

Rhomboid-1 was the first intramembrane serine protease discovered and, to date, it is still the best-characterised catalytically-active rhomboid. Rhomboid-1 is the primary activator of EGFR signalling in *Drosophila* and thereby regulates many important developmental decisions [18,19]. Its primary substrate is Spitz, a ubiquitously expressed homologue of mammalian TGFα [20]. Spitz is synthesised as a type I membrane protein in the endoplasmic reticulum (ER) and requires the protein Star for export to the Golgi. There it is cleaved by Rhomboid-1, and its ectodomain is subsequently secreted as an activating ligand for the EGFR (Fig. 2) [1,2]. Apart from Spitz, which is broadly expressed, Rhomboid-1 can cleave two other more specifically localised EGFR ligands, Keren and Gurken [21] although, of these, only Keren is likely to be a physiological substrate. Interestingly, Rhomboid-1 was also reported to

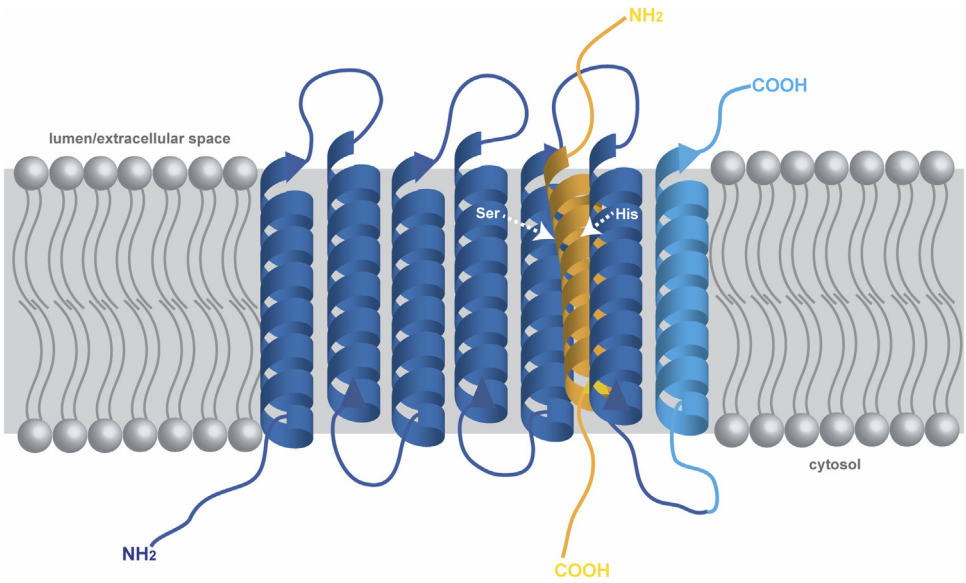

Fig. 1. A secretase rhomboid and its substrate. The core rhomboid domain has six membrane spanning helices (dark blue) – the majority of secretase rhomboids have an extra C-terminal TMD (light blue). The catalytic dyad is formed by the highly conserved serine and histidine in TMD4 and TMD6, respectively. The predominant class of substrate are type I TMD-containing proteins, the majority of which are single-pass (orange).

Table 1
Secretase-type rhomboids and their proposed subcellular localisation, substrates and functions.

	Protease	Proposed cell compartment	Proposed substrate	Proposed function
Drosophila	Rhomboid-1	Golgi [1,2]; endosomes [22]	Spitz [1,2]; Keren [21]; Gurken [21]; Star [22]	EGFR signalling [1,18,19]
	Rhomboid-2	Golgi [21]; ER [85]; endosomes [85]	Spitz [21]; Keren [21]; Gurken [21,25]; Star [85]	EGFR signalling [23–25]
	Rhomboid-3	Golgi [21]; ER [85]; endosomes [85]	Spitz [21]; Keren [21]; Gurken [21]; Star [22,85]	EGFR signalling [18]
	Rhomboid-4	Golgi [21]; plasma membrane [21]	Spitz [21,31]; Keren [21]; Gurken [21]	–
	Rhomboid-6	–	–	–
Mammals	RHBDL1	Golgi [32]	–	–
	RHBDL2	Plasma membrane [12]; ER [82]	Thrombomodulin [32,42]; EphrinB2/B3 [45]; EGFR [46]; EGF [12]; CLEC14A [11]	Wound healing [42]; Angiogenesis [11]
	RHBDL3	Plasma membrane; endosomes; Golgi [32,36],	–	–
	RHBDL4	ER [10]	pTCRα [10]; TCRα [10], opsin-degron [10]; Pkd1ΔN [10]; MPZ-L170R [10]; TSAP6 [52]; proTGFα [53]; BIK1 [57]	ERAD [10]; Exosome secretion [52]; Tumour cell growth [53]; Anti-apoptotic [53,55–58,60]
Yeast	*S. cerevisiae* Rbd2	Golgi; plasma membrane[70]	–	Endocytosis [70]
	S. pombe Rbd2	ER; Golgi [63]	SREBP[63]	Lipid metabolism [63]
Arabidopsis	AtRBL1-12	Golgi (AtRBL1 and AtRBL2) [77]; chloroplast (AtRBL8 and AtRBL9) [78]	–	Flower development, and fertility (AtRBL2) [79]; Jasmonic acid biosynthesis (AtRBL8 and AtRBL9) [78]
Parasites	*Toxoplasma gondii* – multiple rhomboids [86,87]	Microneme; mononemes; plasma membrane; Golgi [86]	Multiple substrates – for review see [86], and [74] in this issue.	Host cell invasion [86]
	Plasmodium spp. – multiple rhomboids [86,87]	Microneme; mononemes; plasma membrane [86]	Multiple substrates – for review see [86], and [74] in this issue.	Parasite development [86]
	Entamoeba histolytica EhROM1 [86,87]	Cell surface [86]; internal vesicles [86]	EhGal/GalNAc lectin [86]	Parasite adhesion [86]

cleave and subsequently inactivate the type II membrane protein Star, thus limiting the amount of substrate that is trafficked and adding another layer of regulation to EGFR activation [22]. If confirmed as a direct substrate, this would be an important and quite surprising result: most analyses of protease active sites imply that they would not accommodate peptides in both orientations and, as described above, all other known rhomboid substrates are type I TMDs.

3.1.2. Rhomboid-2

Rhomboid-2 (Brho/Stet) is expressed exclusively in male and female germ cells and, in a mechanism analogous to Rhomboid-

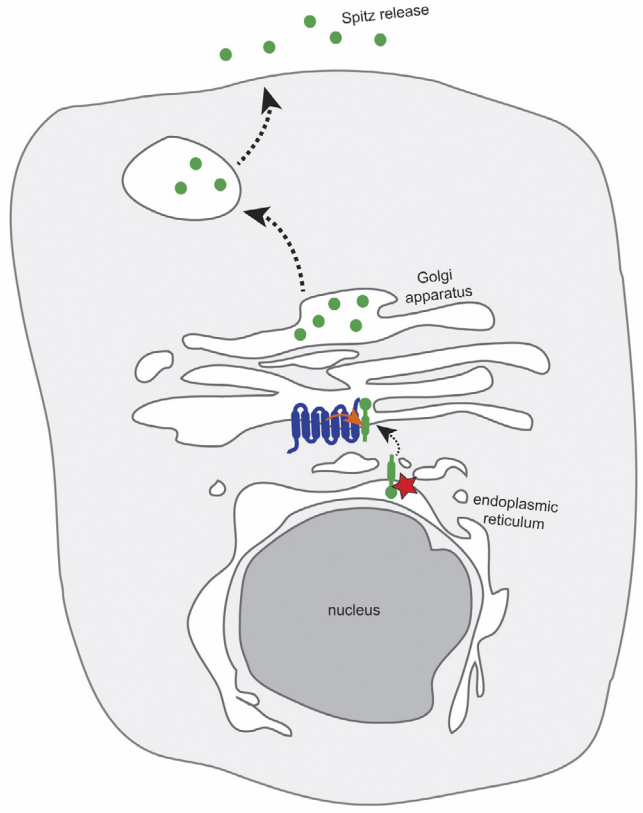

Fig. 2. Rhomboid-1 regulation of Spitz release. Spitz (green) is synthesised in the endoplasmic reticulum and depends on the protein Star (red star) to travel to the Golgi where it encounters Rhomboid-1 (blue). Rhomboid-1 cleaves Spitz (orange arrow), and the resulting ectodomain is secreted as an activating EGFR ligand. This scheme applies to fly Rhomboids-1-3 and the other EGF-like ligands, Keren and Gurken, with some variability in each substrate's requirement for Star.

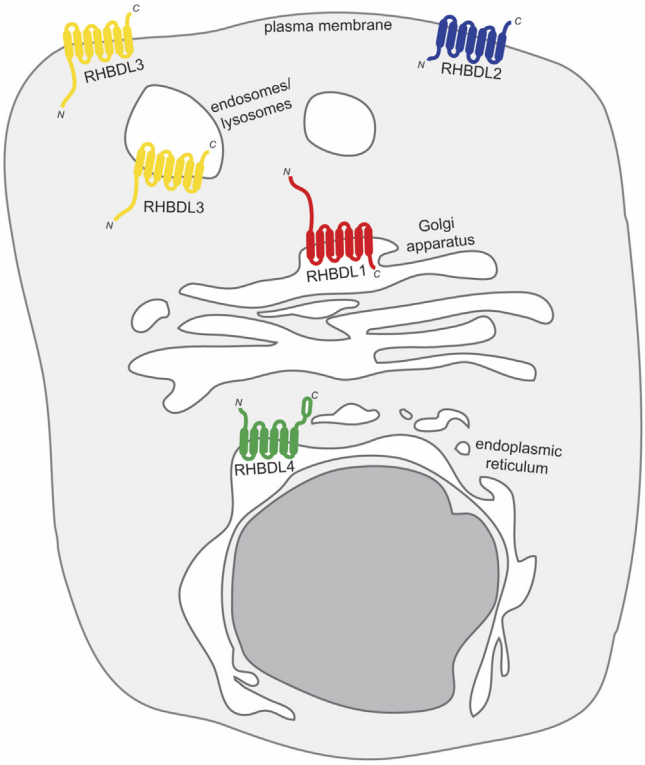

Fig. 3. The predominant subcellular localisation of secretase-type rhomboids in mammals. Rhomboid proteases are located throughout the secretory endomembrane system. RHBDL4 (green) resides within the ER. RHBDL1 (red) mostly localises within the Golgi apparatus. The major observed localisations for RHBDL3 (yellow) are endo-lysosomal membranes and the plasma membrane. RHBDL2 (blue) is principally located at the plasma membrane.

1 activation of Spitz, is required for sending an EGF signal from the germ cells to the surrounding somatic cells that express the EGFR [23]. Spitz and Gurken are the two EGFR ligands expressed in germ cells; Spitz is expressed in both female and male germ cells, whereas Gurken is expressed only in oocytes, where it is required for egg polarization [24–26]. Ectopic expression of Rhomboid-2 together with Spitz or Gurken led to EGFR activation [24], consistent with the observation that Rhomboid-2 can cleave both ligands [21,25]. Altogether, these suggest that Spitz and Gurken are the natural Rhomboid-2 substrates. When misexpressed in the developing wing [24] or follicle cells [25], Rhomboid-2 and Star induce EGFR hyperactivation phenotypes in a synergistic manner implying that, as with Rhomboid-1, Star is needed to supply substrates to Rhomboid-2 *in vivo*. Consistent with this, Star is expressed in oocytes at about the same developmental stage as Rhomboid-2 [24], and it is also expressed at the apical tip of the wild-type testes [23].

3.1.3. Rhomboid-3

Rhomboid-3 (Roughoid) is predominantly expressed in the eye where it contributes to EGFR signalling during development [18], sharing this function with Rhomboid-1. Spitz and Keren are proposed to have overlapping function in the developing eye [27] and they can both be cleaved by Rhomboid-3 in cell culture [21]. Spitz and Keren are functionally similar although processing of Keren is not fully dependent on Star and Rhomboid-1 [21,27,28]. Flies mutant for *rhomboid-3* have a rough eye phenotype [18]. Rhomboid-3 is also expressed in a subset of neurons at the midline of the ventral nerve cord, and mutants have subtle defects in tracheal branching at the midline [29]. More recently, mutations in *rhomboid-3* were shown to result in an enlarged heart and abnormal cardiac function in *Drosophila* adults [30], implying that Rhomboid-3 also has post-developmental roles.

3.1.4. Rhomboid-4

Rhomboid-4, which is sequence divergent from Rhomboid-1, -2, and -3, is expressed ubiquitously at low levels and localised in the Golgi apparatus and plasma membrane [21]. Nothing is known of its biological role, although it can cleave EGFR ligands in cell culture assays [21]. In biochemical and cell biological studies, Rhomboid-4 has been used as an example of a rhomboid regulated by calcium [31] (see Section 4).

3.1.5. Rhomboid-6

We include Rhomboid-6 for completeness, but there is no published information about its function.

3.2. Mammalian rhomboids

Unsurprisingly, there is great interest in the function of rhomboid proteases in mammals. The progress in this key area has been patchy and slow, although the existing data support the importance of rhomboids in developmental and physiological roles. There are four rhomboid proteases, RHBDL1-4, located throughout the secretory pathway in mammals (Fig. 3) [4,32]. An obvious question is whether they, like their *Drosophila* counterparts, control EGFR signalling. The significance of this question is that the EGFR is very widely implicated in cancer, so understanding its regulation is a priority. The simple answer appears to be that mammalian EGFR ligands which, as in flies, are synthesised as transmembrane inac-

tive precursors, are liberated from their TMDs by a different family of proteases, the ADAM metalloproteases [33]. But, as described below, there is a growing body of evidence to suggest that rhomboids may participate, at least in some contexts. Additionally, new activities and roles have been uncovered for the mammalian rhomboids.

3.2.1. RHBDL1

RHBDL1 (also called rhomboid related protein-1) was identified by sequence similarity with *Drosophila* Rhomboid-1 [34]. Within the mammalian rhomboids, RHBDL1 is actually most similar to RHBDL3: the murine proteins share ~55% identity according to Clustal Omega alignment [35]. The main differences are in their cytoplasmic N-termini, which might account for their different subcellular localisation: both are in the Golgi, but RHBDL3 is predominantly in endosomes and at the plasma membrane [32,36]. According to public databases and transcriptome-wide analyses, RHBDL1 is highly expressed in neurons [37,38]. To date, no RHBDL1 substrates have been identified and its biological role is unknown.

3.2.2. RHBDL2

RHBDL2 is the best-characterised mammalian rhomboid protease and a number of substrates have been reported. It is localised at the plasma membrane and shares proteolytic specificity with *Drosophila* Rhomboids-1-3, being able to cleave *Drosophila* Spitz [32] and Gurken [39]. Although this represents a non-physiological activity, it is unique amongst the mammalian rhomboids, and has served as a starting point for substrate identification. We now describe the RHBDL2 substrates that have been identified.

3.2.2.1. EGF. Based upon the *Drosophila* rhomboid role in EGFR signalling, the mammalian proteases were screened for activity against various EGF-like ligands. RHBDL2 cleaves EGF itself, but no other related ligands, at a luminal region close its transmembrane domain. The soluble product which, due to the different cleavage site, is distinct from that produced by its major processing enzyme ADAM10, is sufficient to engage and activate the EGFR [12]. RHBDL2 cleavage of EGF is only observed upon inhibition of metalloprotease activity, suggesting that it may be only a minor contributor to EGF shedding, or occurs only in cells without ADAM10. In the absence of supporting functional evidence, these results formally only prove that RHBDL2 can cleave EGF, not that it does so in a physiologically meaningful way. It is notable, however, that several cancer cell lines have enhanced RHBDL2-driven EGF cleavage, suggesting a potential pathological role in mammalian EGFR signalling [12,40].

3.2.2.2. C-type lectin family members – thrombomodulin and CLEC14a. The first mammalian rhomboid substrate to be identified was the lectin and EGF-like domain containing protein thrombomodulin [32]. Thrombomodulin is an anti-coagulant that converts thrombin from its pro- to anti-coagulant form [41], with roles in angiogenesis and inflammation. Unlike most rhomboid substrates, thrombomodulin does not require helix-destabilising residues in its TMD to be cleaved by RHBDL2: instead, its cytosolic domain acts as a cleavage determinant (Fig. 4A). Indeed, the cytoplasmic region of thrombomodulin is sufficient to transform otherwise non-cleavable proteins into rhomboid substrates [32]. More recently, the biological significance of this cleavage was suggested by observations that RHBDL2 is required for thrombomodulin shedding during *in vitro* scratch-wound assays. Moreover, in a mouse model, RHBDL2 is upregulated after transdermal wound formation, and thrombomodulin shedding is instrumental to closure of the wound and subsequent healing [42], suggesting that RHBDL2 may have a role in endothelial and immune cells.

A potential role for RHBDL2 in endothelia is further supported by the cleavage of C-type Lectin 14A (CLEC14a) [11], another member

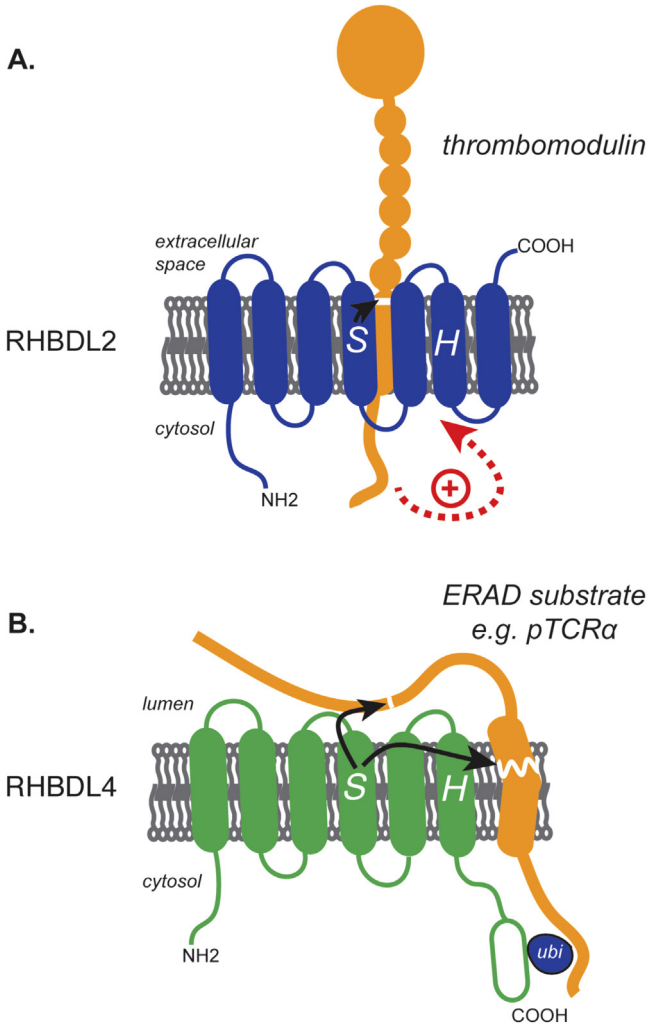

Fig. 4. Regulation of rhomboid proteases by cytosolic regions of substrates. Thrombomodulin (orange) is cleaved within its TMD by RHBDL2 (blue). The black arrow indicates cleavage. An essential determinant for proteolysis is the cytosolic domain of thrombomodulin. (B) RHBDL4 (green) binds ubiquitinated ERAD substrates (orange) via a C-terminal ubiquitin-interaction motif. RHBDL4 cleaves at least some substrates at multiple sites, promoting their degradation (black arrows).

of the thrombomodulin family. CLEC14a is specifically and highly expressed in endothelial cells and mouse vasculature, where it plays roles in cell-cell adhesion and angiogenesis [43]. CLEC14a is cleaved by RHBDL2 in a luminal region close to its transmembrane domain, mirroring RHBDL2 cleavage of EGF. Similar to thrombomodulin, the cytoplasmic domain of CLEC14a is essential for its cleavage by RHBDL2. A role in angiogenesis for RHBDL2 in relation to CLEC14a cleavage has been postulated, and in cell culture, RHBDL2 depletion strongly affects endothelial cell migration [11].

3.2.2.3. B-type ephrins. B-type ephrins are ligands for Eph receptors, which are the largest family of receptor tyrosine kinases [44]. Based upon amino acid identity between ephrin B2 and the luminal region of Spitz, it was found that membrane anchored B-type ephrins are RHBDL2 substrates, and are cleaved within their TMDs [45]. In this case, the cleavage follows the conventional pattern: the TMDs of B-type ephrins contain all the required information for RHBDL2 cleavage; their cytoplasmic domains are not required. Despite the efficiency of this cleavage by RHBDL2, no physiological significance for it has yet been reported.

3.2.2.4. EGFR. The EGFR itself has also been shown to be cleaved by RHBDL2 [46], further elaborating the complex relationship between rhomboids and EGFR signalling. EGF stimulation triggers cleavage of the EGFR. This process is increased by over-expression of RHBDL2 and is sensitive to the serine protease inhibitor dichloroisocoumarin [46], which has previously been used for inhibition of rhomboid protease activity [1,39,47]. Importantly, as in most other cases, the biological significance of EGFR cleavage by RHBDL2 still needs to be clarified.

3.2.3. RHBDL3

RHBDL3 (also called ventrhoid) localises to the Golgi apparatus, as well as endosomes and plasma membrane [32,36]. RHBDL3 is highly expressed in the nervous system [48] and, consistent with this, was identified as a candidate gene involved in the onset of mental retardation in neurofibromatosis type 1 (NF1) microdeleted patients [49]. Furthermore, in a study searching for genes that change expression levels in association with chronological age in human brain, *RHBDL3* showed the most consistent age-associated change in expression [50]. No substrates have been reported for RHBDL3. RHBDL3 has predicted EF-hands in its cytoplasmic N-terminal domain [31], suggesting the possibility that it is regulated by calcium binding, similarly to *Drosophila* Rhomboid-4.

3.2.4. RHBDL4

RHBDL4 (also called RHBDD1) is the only mammalian rhomboid protease resident in the ER and is upregulated upon ER stress [10]. It is phylogenetically divergent from RHBDL1-3 and, unlike them and *Drosophila* rhomboids, RHBDL4 has six TMDs instead of seven [4]. Several functions have been reported for RHBDL4.

3.2.4.1. RHBDL4 and ER-associated degradation. Fleig et al. [10] showed that RHBDL4 binds via a ubiquitin interaction motif to ubiquitinated substrates of ER-associated degradation (ERAD), including the α chain of the pre-T cell receptor (pTCRα). This binding is coupled to substrate proteolysis and degradation. RHBDL4 cleaved pTCRα within its TMD but also at other sites within the ectodomain, far from the membrane. RHBDL4 also cleaved other unstable membrane proteins – mutants containing helix-destabilizing amino acids or degron motifs in their TMDs – but not their wild-type counterparts (Fig. 4B). Uncommonly for rhomboid proteases, among the destabilized substrates some were polytopic proteins, which were cleaved within loops or juxtamembrane regions of their TMDs. These data suggest a role for RHBDL4 in ERAD although, since it is not part of the core ERAD machinery, it is not yet clear what the normal substrates are, nor in what context RHBDL4 acts.

3.2.4.2. RHBDL4 and exosomal secretion. Another potential RHBDL4 substrate is TSAP6, a six-TMD protein that plays a role in exosomal secretion [51]. It has been suggested that RHBDL4 regulates exosomal secretion by cleaving and inactivating TSAP6 [52]. RHBDL4 cleaved TSAP6 at three sites and, given the more recently proposed role for RHBDL4 in ERAD, this raises the question of whether TSAP6 is an ERAD substrate. Not only is TSAP6 a potential polytopic substrate, but also the primary cleavage site is in a TMD with type II topology. As in the case of Star [22] described above, this orientation of the substrate potentially implies that some rhomboids can cleave type II TMDs.

3.2.4.3. RHBDL4 and TGFα. Recently, Song et al. [53] reported that RHBDL4 is implicated in colorectal cancer (CRC). Elevated RHBDL4 expression correlated with poor prognosis in human CRC, and RHBDL4 depletion led to a decrease of tumour growth in a mouse model. Elevated RHBDL4 correlated with EGFR activation. It was proposed that this was caused by RHBDL4 promoting the cleavage and subsequent secretion of the EGFR ligand TGFα, a mechanism that would be analogous to *Drosophila* Rhomboid-1 cleaving Spitz. This mechanism, but not RHBDL4's implication in CRC, has subsequently been challenged in a paper reporting that RHBDL4 cannot cleave TGFα, but instead promotes the cellular export of full-length pro-TGFα in exosomes [54]. These very recent papers on TGFα clearly need further resolution but the message that RHBDL4 participates in some way in CRC fits into a growing pattern of links between rhomboid-like superfamily and cancer.

3.2.4.4. RHBDL4 and apoptosis. High RHBDL4 expression levels have been reported in a range of cancer cell lines or tissues. Conversely, loss of RHBDL4 is associated with a decrease in cell proliferation, colony formation, and tumour growth, as well as increased apoptosis in human glioblastoma, hepatoma HepG2 cells, and human colorectal cancer [53,55–58]. Anti-apoptotic activity is a trait of cancer [59], however, RHBDL4 has not been proved to regulate this postulated role directly, although Wang et al. [57] reported a role for RHBDL4 in degrading the pro-apoptotic protein BIK, and Ren et al. [60] proposed that RHBDL4 can regulate apoptosis by influencing the levels and activity of c-Jun and subsequently of Bcl-3.

3.3. Yeast rhomboids

Two predicted rhomboid proteases are present in *Saccharomyces cerevisiae*, Rbd2p and Pcp1p/Rbd1p (the mitochondrial rhomboid) [61]. In the distantly related fission yeast, *Schizosaccharomyces pombe*, there are four predicted rhomboid proteases according to the public source database PomBase [62], two of which are predicted to be mitochondrial [63].

3.3.1. S. cerevisiae *Rbd2p*

RBD2 is not an essential gene, and its loss in *S. cerevisiae* leads to no obvious growth defects [61]. According to two transmembrane domain prediction servers, Phobius [64] and TMHMM [65], Rbd2p is predicted to have an unusual five TMD structure, with a conserved catalytic serine in its fourth transmembrane helix. No substrate has been discovered for Rbd2p but, if these predictions are accurate (note that such prediction methods are not very reliable), this unusual topology would suggest distinct enzymology. Multiple large-scale screens have revealed that Rbd2p interacts genetically and physically with regulators of vesicular traffic and the actin cytoskeleton [66–69]. These observations fit well with recent data demonstrating that Rbd2p negatively regulated the timing of actin assembly during clathrin-mediated endocytosis [70] by altering the distribution of phosphatidylinositol 4,5-bisphosphate (PIP2), a key determinant of this process. This is the first demonstration that a rhomboid can regulate the distribution of a specific species of lipid. The cytoplasmic tail of Rbd2p was sufficient to bind PIP2 and regulate actin assembly *in vivo*, implying that this role is independent of potential proteolytic activity. Notably, general endocytic uptake was not drastically affected in RBD2 knockout strains.

3.3.2. *S. pombe Rbd2*

S. pombe Rbd2 is not an orthologue of the rhomboid in *S. cerevisiae* with the same name, and is predicted to have a classical six-TMD structure with a conserved catalytic dyad (according to TMHMM [65] and Phobius [64]). It has recently been reported that *S. pombe* Rbd2 has a key role in lipid homeostasis by processing the sterol regulatory element-binding protein (SREBP) [63,71]. SREBP is a membrane tethered transcription factor that in metazoans is released from the Golgi membrane by site-2-protease (S2P) (an intramembrane metalloprotease, unrelated to rhomboids) to become an active transcription factor [72]. There are no homo-

logues of S2P in *S. pombe*; instead Rbd2 performs this function [63].

3.4. Rhomboids in apicomplexan parasites

Sequence similarity to the *Drosophila* rhomboid substrate Spitz, identified *Toxoplasma gondii* surface antigens involved in parasite adhesion and invasion as rhomboid substrates [13]. Much has subsequently been published about the role of rhomboids in apicomplexan parasites, including the malaria parasite Plasmodium [73]. Parasite species tend to have a large number of rhomboids, and multiple substrates have been identified. The functional roles of these rhomboids are complex and varied and are described in detail in another review in this issue [74].

3.5. Plant rhomboids

The number of rhomboid genes in plants ranges from four in the green alga *Ostreococcus lucimarinus* to twenty-five in soybean, *Glycine max* [75]. Recent sequence analysis indicates that, as in animal rhomboids, some contain recognisable additional motifs, including cysteine-rich, ubiquitin-binding or RanBP-type Zinc finger motifs [75]. *Arabidopsis thaliana* has 17 rhomboid genes, of which twelve, *AtRBL1-12*, are predicted to encode for active, non-mitochondrial rhomboids [4,76]. AtRBL1 and AtRBL2 are in the Golgi apparatus, and AtRBL2 has similar enzymatic activity to *Drosophila* rhomboids in that it can cleave Spitz and Keren [77]. AtRBL8 and AtRBL9 are localised in the chloroplast inner envelope [78]; the localisation of the rest has not been reported. AtRBL8 has a role in flower development, and fertility [79]. The double knockout of AtRBL8 and AtRBL9 decreases allene oxide synthase (AOS), an enzyme involved in biosynthesis of jasmonic acid [78]. Jasmonate regulates auxin synthesis and transport [80], providing a possible explanation of the flower and fertility phenotypes in *AtRBL8* mutants [79].

4. Regulation

Proteolysis is an irreversible process and its consequences range from protein degradation to the production of important functional molecules such as untethered transcription factors and signalling components. This implies that proteases are potentially dangerous and must be tightly regulated. We currently have only a patchy knowledge of rhomboid regulation, but some themes do emerge. A key difference between intramembrane proteases and classical soluble proteases is that in the case of the former, both the enzyme and the substrate are membrane proteins. This implies that they can only interact if they are present in the same membrane, and this allows for regulation by compartmentalisation. The best example of this is the case of *Drosophila* Spitz being trapped in the ER, and therefore inaccessible to the Golgi-localised rhomboids, until Star supports its onward trafficking [2]. *Drosophila* Rhomboids-1-3 are also tightly transcriptionally regulated [18], and although this cannot provide the kind of acute control needed in cells, when combined with compartmentalisation, these two mechanisms appear to provide the core regulation of the most studied *Drosophila* rhomboid proteases. Interestingly, in the case of the *S. cerevisiae* mitochondrial rhomboid, Rbd1p, its substrate, the GTPase Mgm1p, has developed a different way of segregating its substrate TMD from the rhomboid. Mgm1p has a primary TMD that is not a substrate for Rbd1p and a secondary hydrophobic domain, containing the cleavage site, which resides out of the bilayer until it is pulled into the plane of the membrane by an ATP-dependent process. Only then can Mgm1p be cleaved [81]. Although a very different mechanism to that regulated by Star in *Drosophila*, both are examples of regulating substrate accessibility to the protease.

A second theme in rhomboid regulation is the role of their cytoplasmic domains in modulating enzyme activity. The cytoplasmic N-terminal domain of *Drosophila* Rhomboid-1 is not required for enzyme activity [1] and much further evidence has accumulated that the conserved six-TMD rhomboid-like domain is sufficient for proteolysis. The cytoplasmic domains are less well conserved and frequently have recognisable other motifs, making them attractive candidates for regulatory modules. In one case, this has been shown explicitly to be the case. *Drosophila* Rhomboid-4, whose biological function is not yet known, is an example of a rhomboid protease with EF-hands in its cytoplasmic N-terminus. Baker et al. [31] showed that calcium binding by the cytosolic loops controlled substrate cleavage by modulating access or gating to the active site.

Another way of regulating enzymes is by post-translational modifications. It has been reported that proteolytic processing of RHBDL2 is required for its activity against Spitz [82]. A cleaved form of RHBDL2 is detected in cells, and an uncleavable mutant has no catalytic activity and is retained in the ER. The cleavage depends on a highly conserved tryptophan-arginine motif in the first luminal loop. However, current evidence does not support the interpretation that this cleavage is equivalent to activation of an inactive zymogen form of the enzyme: the L1 loop, which is separated from the catalytic core of the enzyme upon this cleavage, is necessary for stabilising the core rhomboid-like fold. It therefore appears more likely that the mutations in L1 loop destabilise RHBDL2, leading to its inactivity and retention in the ER.

The intimate relationship between polytopic membrane proteins and the lipid bilayer indicates that lipids might also regulate rhomboid activity. This is an understudied area but there is good evidence to support the idea that membrane lipids can regulate rhomboids. Rhomboid activity was modified by varying lipid constitution *in vitro* [47]. The interaction with the membrane also affects the conformation of both the rhomboid active site and the substrate TMD, thereby influencing cleavage [14]. In addition, compounds that perturb the lipid composition of the membrane bilayer reduce the specificity of rhomboids, allowing non-substrates to be cleaved [14]. These experimental observations are supported by molecular dynamics analysis [83]; they are also consistent with the structural observation that the hydrophobic core of at least the *E. coli* rhomboid GlpG is unusually thin, suggesting unusual interactions with surrounding lipids [84].

5. Conclusion

The secretase rhomboids are the most numerous eukaryotic class of this universally conserved family of enzymes, yet we still know quite little about the range and significance of their biological functions. This knowledge gap is perhaps most striking in the case of mammalian rhomboids, where there is mounting evidence to implicate them in human disease. In this review we have attempted to present a concise encyclopaedia of secretase rhomboids. Beyond the trivial point that they need more study, perhaps the main conclusion that emerges is the importance of identifying their natural substrates: that will be the key to understanding their biological significance. Knowing which proteins rhomboids can cleave is a good start but is not the same as knowing which proteins are substrates in real biological contexts. Unfortunately, there is no simple pipeline for protease substrate identification. Instead, a combination of genetic, proteomic, biochemical, and bioinformatic approaches are needed. The good news is that, as methods in all these areas become more sophisticated, there is a good prospect of making substantial progress in the near future. Of course, the possibility that rhomboids will participate in pathological events underlies the search for specific inhibitors – reviewed in this issue [17]. Whole new families of enzymes – inherently more drug-

gable than protein-protein interactions – are not discovered very frequently, and the rhomboids remain one of the most recently identified. It may not be long before we can begin to review their medical utility.

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