Rhomboid proteases in human disease: mechanisms and future prospects

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Abstract

Rhomboids are intramembrane serine proteases that cleave the transmembrane helices of substrate proteins, typically releasing luminal/extracellular domains from the membrane. They are conserved in all branches of life and there is a growing recognition of their association with a wide range of human diseases. Human rhomboids, for example, have been implicated in cancer, metabolic disease and neurodegeneration, while rhomboids in apicomplexan parasites appear to contribute to their invasion of host cells. Recent advances in our knowledge of the structure and the enzyme function of rhomboids, and increasing efforts to identify specific inhibitors, are beginning to provide important insight into the prospect of rhomboids becoming future therapeutic targets.

1. Introduction

Rhomboid proteases are intramembrane serine proteases present in all kingdoms of life. Intramembrane proteases (IMPs) are multi-pass membrane proteins, which, by contrast to other proteases, have their catalytic residues embedded in the lipid bilayer. Therefore, IMPs have the unique ability to cleave membrane proteins within their transmembrane helices (TMH). Rhomboid proteases constitute one of the best characterised classes of IMPs, which also include aspartyl IMPs (presenilins and signal peptide peptidase(-like)), metallo IMPs (site-2-protease) and, most recently discovered, glutamyl IMPs (Rce1) [1].

The first rhomboid gene was identified in a mutant screen in *Drosophila melanogaster*, where loss of *rhomboid-1* caused a pointed head skeleton in embryos [2]. Multiple genetic analyses demonstrated that *Drosophila* Rhomboid-1 acts as a regulator of epidermal growth factor receptor (EGFR) signalling in *Drosophila*: Rhomboid-1 cleaves the membrane-bound proform of the *Drosophila* EGFR ligand Spitz leading to its release and the subsequent activation of EGFR signalling [3, 4]. Biochemical studies of Rhomboid-1 revealed its function as an intramembrane serine protease [3], the first to be identified. Bioinformatics analyses showed that rhomboid proteases are present in most prokaryotes and all eukaryotes and, intriguingly, that several eukaryotic rhomboid homologues lack catalytic residues [5, 6]. It is now

known that there is a broader superfamily of rhomboid-like proteins consisting of rhomboid proteases and catalytically inactive pseudoproteases [7, 8].

The characteristic feature of all rhomboid-like proteins is the conserved core of 6 TMHs, the rhomboid-fold, which is frequently extended by a seventh TMH added to the N- or C-terminus (Fig.1A). The catalytic serine and histidine residues that form the active centre of the rhomboid proteases are located in TMH 4 and TMH 6 of the rhomboid fold, respectively. In mammals the rhomboid-like superfamily comprises five rhomboid proteases, RHBDL1-4 and PARL, as well as nine pseudoproteases (Fig.1B).

In this review, we aim to describe what is known about rhomboids in human disease, and to discuss how recent structural and functional results underpin their potential as future drug targets. An increasing number of reports point towards a role of rhomboids in cancer, neurodegenerative, metabolic and infectious diseases, suggesting that rhomboids may be valuable drug targets. The development of potent and specific rhomboid protease inhibitors requires knowledge of the protein structure, and these issues will also be discussed.

2. Mammalian rhomboid proteases

Mammals express five rhomboid proteases - RHBDL1-4 in the secretory pathway, and PARL in mitochondria. The primary question that must be answered when investigating the function of a protease is *what is the substrate?* An increasing number of substrates have been identified for PARL, RHBDL2 and RHBDL4 (reviewed in [9, 10]), and these have provided strong clues about the functions of these rhomboids in health and disease (Table 1). Two of the mammalian rhomboid proteases, RHBDL1 and RHBDL3, however, have only been poorly characterised, mainly because of a lack of identified substrates.

RHBDL2 controls cell migration and proliferation

RHBDL2 is primarily localised at the plasma membrane [11, 12], and appears to regulate cell migration and cell proliferation at multiple levels. RHBDL2 can cleave the *Drosophila* EGFR ligand Spitz [3]. However, unlike in *Drosophila*, where

rhomboid proteases are the main regulators of EGFR signalling pathway with Rhomboids-1-4 cleaving the membrane-tethered forms of the EGFR ligands Spitz, Keren and Gurken [13], in mammals ADAM10 and ADAM17, members of the ADAM metalloproteases, are the main sheddases of EGF and EGF-like ligands [14]. Nevertheless, upon ADAM metalloprotease inhibition, RHBDL2 (but not RHBDL1, 3, 4) cleaves proEGF in its luminal region just outside of the TMH [11]. The soluble EGF thus released by RHBDL2 is able to activate EGFR signalling with similar efficiency to EGF generated by ADAM proteases. This suggests that EGF cleavage by RHBDL2 may either be a canonical pathway in some tissues with low ADAM10 expression, or constitutes an alternative pathway important under certain physiological situations. Since hyperactivation of the EGFR has been reported in many types of cancer [15], the fact that RHBDL2 can release active EGF suggests a potential role for it in cancer biology. Indeed, one study showed a correlation between RHBDL2 overexpression and increased cell proliferation and resistance to anoikis, a form of apoptosis in cells detached from the extracellular matrix, suggesting that RHBDL2 may serve as an interesting drug target for cancer metastasis [16]. Other studies have further implicated RHBDL2 in cell migration and proliferation: loss of RHBDL2 impaired wound healing *in vitro* and *in vivo* due to reduced cell proliferation and cell migration [17]; the authors of this work suggested that this might be caused by a reduction of soluble thrombomodulin, another RHBDL2 substrate [12]. Another study implied that RHBDL2 might reduce angiogenesis *in vitro* and *in vivo* [18]: RHBDL2 cleaves the lectin CLEC14A in its extracellular region and exogenous addition of the CLEC14A extracellular domain reduced cell migration and sprouting of endothelial cells [18]. Although these data suggest a role of RHBDL2 in the inhibition of endothelial cell migration and angiogenesis, this has not yet been directly demonstrated. Finally, RHBDL2 was reported to cleave B-type ephrins [19], which can also play a role in cell migration, although, again, the physiological significance of cleaved B-type ephrins has yet to be shown.

A role of RHBDL4 in cancer

RHBDL4 localises to the endoplasmic reticulum (ER) [20, 21] where it can assist in ER-associated degradation (ERAD). RHBDL4 is upregulated upon ER stress and

recognises and cleaves membrane proteins with unstable TMHs, which are then degraded by the ERAD machinery [20]. Moreover, RHBDL4 expression has been linked to tumour growth in various studies [22-25]. Knockdown of RHBDL4 reduced proliferation and colony formation in liver carcinoma cell lines [22], glioblastoma cell lines [23], and colorectal cancer cell lines [24]. In colorectal cancer patients, lower RHBDL4 expression is correlated with better disease-free survival. Conversely, increased expression of RHBDL4 was found in colorectal cancer tissues [25]. RHBDL4 has been reported to reduce apoptosis [26, 27], which could also contribute to the described role of RHBDL4 in cancer. These indirect experiments need to be treated with caution, and the molecular mechanism that underlies the contribution of increased RHBDL4 levels to tumour growth is currently speculative. Song et al [25] suggested that the membrane-tethered proform of the transforming growth factor alpha (TGFα) is cleaved by RHBDL4 in a colorectal cancer cell line, thereby releasing active TGFα and activating EGFR signalling. However, these findings have been challenged by a recent study that concluded that RHBDL4 does not cleave proTGFα [21]. Instead, RHBDL4 was shown to promote the transport of proTGFα through the secretory pathway, followed by proTGFα secretion in exosomes. This process was dependent on RHBDL4 protease activity. The authors speculated that RHBDL4 may regulate proteins at the ER exit sites, thereby increasing trafficking. Significantly, this potential RHBDL4 function was not exclusive to TGFα. In summary, although a role of RHBDL4 in cancer has been reported in various studies, the pathological significance of these observations and the molecular mechanism by which RHBDL4 might promote tumour growth remains unclear.

A link between RHBDL4 and neurodegenerative diseases?

Recently, RHBDL4 was shown to cleave amyloid precursor protein (APP) [28], a key molecule in Alzheimer's disease (AD). The pathologic processing of APP by the proteases BACE and γ-secretase results in the formation of 38 to 43 acid long β-amyloid (Aβ) peptides, ultimately causing amyloid plaque formation, a hallmark of AD [29]. RHBDL4 cleaves APP within its ectodomain and reduced the production of Aβ38, Aβ40 and Aβ42 peptides. This processing of APP by RHBDL4, which is presumed to happen in the ER, might constitute an alternative mechanism to the cleavage by γ-secretase. The physiological relevance of RHBDL4-mediated APP

processing needs to be further investigated, but the possibility that RHBDL4 activity can contribute to APP processing is an important new direction of study for rhomboid proteases.

Beyond AD, RHBDL4 might also be linked to inherited neuropathies caused by mutations in the TMD of myelin protein zero (MPZ), an adhesion protein in the peripheral nervous system. RHDBL4 promotes ERAD of the Charcot-Marie-Tooth disease-associated MPZ^{L170R} mutant, but not of wild-type MPZ [20]. However, whether RHBDL4-mediated ERAD of MPZ occurs in vivo and contributes to inherited neuropathies has not been investigated yet.

Biological function of RHBDL1 and RHDBL3 have yet to be discovered

Substrates or biological functions for RHBDL1 and RHBDL3 have not been described yet. Given the prenatal (RHBDL1) or pre-weaning lethality (RHBDL3) described by the International Mouse Phenotyping Consortium [30] and the high expression of both proteases in the brain [31] and central nervous system [32], it is likely that these proteases play and important role in the nervous system that has still to be discovered. As with other contexts, the mechanistic entry point into the physiological roles of these uncharacterised rhomboids is likely to be the identification of their substrates.

PARL is linked to Parkinson's disease and type 2 diabetes

PARL, the mammalian nomenclature for the mitochondrial rhomboid protease [33], is located in the inner mitochondrial membrane and conserved in all eukaryotes. The mitochondrial rhomboids were first studied in yeast, where the PARL homologue Rbd1p/Pcp1p was shown to cleave the membrane tethered GTPase Mgm1p, thereby regulating membrane fission and mitochondrial morphology [34]. Subsequently, PARL in mammals has been investigated intensively, and is involved in the cleavage of a variety of mitochondrial membrane proteins, linking it closely to mitochondrial homeostasis and diseases with mitochondrial dysfunction (reviewed in [10]).

Firstly, PARL has been linked genetically to Parkinson's disease (PD), the second most common neurodegenerative disease. A polymorphism in the PARL gene leading to a Ser77Asn missense mutation in the N-terminus of PARL was discovered in two PD patients [35]. PARL mutations might, however, be very rare in PD, as two other studies have failed to find the PARL variant Ser77Asn in PD patients [36, 37], or an association of other PARL gene variants with PD [37]. The molecular basis for a role of PARL in PD is suggested by several reports that PARL cleaves the Ser/Thr kinase PINK1 [35, 38-41], itself frequently mutated in familial cases of PD, and an important player in the regulation of mitophagy, a degradation mechanism to clear defective mitochondria from cells. Defects in mitophagy have been suggested to play an important role in the development of PD [42]. PINK1 is targeted to the inner mitochondrial membrane where it is processed by PARL leading to the release of PINK1 into the intermembrane space or the cytosol [35, 40]. In damaged mitochondria, PINK1 accumulates on the outer mitochondrial membrane, where it recruits, phosphorylates, and activates the cytosolic E3 ligase parkin, which initiates mitophagy [43-45]. Loss of PARL and the disease mutant $PARL^{Ser77Asn}$ impaired Parkin recruitment to mitochondria [35], suggesting a role of PARL in inducing mitophagy, and protecting from PD. However, two other studies made an opposing observation by showing that PARL knockdown induces the accumulation of PINK1 on the outer mitochondrial membrane [40, 46], consequently leading to the activation of parkin and mitophagy [46]. A second substrate of PARL, the Ser/Thr phosphatase PGAM5 [47], might also be linked to PINK1/parkin-mediated autophagy. Loss of PGAM5 led to reduced mitophagy due to decreased PINK1 stability [48]. Interestingly, PINK1 and PGAM5 are cleaved by PARL under distinct physiological conditions: PINK1 is processed in healthy mitochondria, whereas PGAM5 cleavage by PARL is significantly increased by loss of membrane potential [47]. This suggests that the role of PARL could be distinct in healthy and defective mitochondria. A recent study further elucidated the changes of PINK1 processing by PARL upon mitochondrial stress [49]. It was reported that mitochondrial stress increases autocatalytic cleavage (β cleavage) of the PARL N-terminus. The N-terminally truncated PARL thus generated cleaves PINK1 less efficiently than full-length PARL and can lead to more parkin recruitment to mitochondria; in this way, β cleavage has a positive effect on mitophagy. PDK2, a kinase involved in energy metabolism in the mitochondria, regulates PARL β cleavage by phosphorylating the PARL N-terminus.

PDK2 phosphorylation inhibits β cleavage and therefore mitophagy, and was shown to be decreased upon mitochondrial damage [49]. This study further underlines the role of PARL as an important linker between mitochondrial damage and mitophagy.

Beyond PD, mitochondrial dysfunction has been suggested to play an important role in the development of insulin resistance, which precedes the development of type 2 diabetes mellitus (T2DM), a very common metabolic disease. The first link between PARL and T2DM was discovered in expression studies in *Psammomys obesus*, sand rats that are prone to develop diet-induced T2DM. In obese, diabetic *P. obesus* PARL expression in muscles was reduced by 50% compared to lean, glucose tolerant animals. Exercise of obese sand rats restored PARL expression levels and improved insulin sensitivity [50]. In mice, muscle-specific knockdown of PARL resulted in reduced mitochondrial DNA content, impaired mitochondrial cristae, reduced OPA1 protein levels (the mammalian counterpart of yeast Mgm1p), and impaired insulin signalling [51], suggesting a link of PARL expression to mitochondrial dysfunction in insulin resistance. In humans, too, PARL mRNA levels and mitochondrial DNA content were reduced in skeletal muscle of T2DM patients [51]. Furthermore, a polymorphism in human PARL leading to a Leu262Val point mutation (located at the interface of TMH 4 and the mitochondrial matrix) has been associated with increased plasma insulin concentrations, a marker of insulin resistance, in white Americans [50]. However, other three other studies in European and African American cohorts failed to associate the $PARL^{Leu262Val}$ variant with insulin resistance [52-54].

In line with the physiological roles of PARL in mitochondrial biology, PARL-deficient mice die before the age of 3 month due to muscular atrophy and excess apoptosis in spleen and thymus [55]. It was suggested that this was caused by increased release of cytochrome c in PARL deficient cells, suggesting that PARL also plays a role in apoptosis [55]. Furthermore, a recent study identified the pro-apoptotic mitochondrial protein Smac (DIABLO) as a novel PARL substrate in a proteomics approach [56]. PARL cleaves Smac within its TMH leading to the generation of an inhibitor of apoptosis (IAP)-binding motif at the N-terminus of Smac. In apoptotic cells, processed Smac is released from mitochondria into the cytosol where it binds and inhibits IAP protein XIAP, thereby initiating the caspase cascade. PARL-deficient cells were resistant to apoptosis, as they did not release Smac into the cytosol upon

treatment of cells with apoptosis-inducing drugs suggesting that PARL has a pro-apoptotic function.

Overall, it is absolutely clear that PARL and, more widely, mitochondrial rhomboids, have important functions in mitochondrial function and homeostasis. There is increasing data to support this both at the level of mechanistic cell biology and also disease association. Despite this rapid progress, however, we still lack a full and detailed explanation of either the mechanism or its pathophysiological significance, although the intense recent interest provides hope that clearer insight will soon be forthcoming.

3. Parasite rhomboid proteases

Rhomboid proteases are found in many protozoa parasites and are best studied in the intracellular apicomplexan parasites *Toxoplasma gondii* and *Plasmodium spp,* where rhomboid proteases have been implicated in parasite cell growth, motility and host cell invasion (reviewed in [57]). Apicomplexan parasite motility and host cell invasion requires adhesion proteins, like micronemal proteins (MICs) and the apical membrane antigen 1 (AMA1), on the surface of parasites. These proteins engage with host cell receptors and lead to the attachment of the parasite, but only the shedding of the adhesion molecules facilitates the efficient invasion of the host cell [58, 59]. The link between rhomboid proteases and parasite invasion was first established when several *T. gondii* MICs were identified as substrates for bacterial, mammalian and *Drosophila* rhomboid proteases [60]. Infections with *T. gondii*, a widely spread protozoan parasite in mammals, are usually asymptomatic, but can cause serious health problems in immunosuppressed individuals and in the foetus upon infection of pregnant women. *T. gondii* expresses six rhomboid proteases (TgROM1-6) with TgROM1-5 located in the secretory pathway [61] and TgROM6 presumably targeted to mitochondria [62]. TgROM1, 4 and 5 are highly expressed in tachyzoites, the acute infection stage of *T. gondii* [61], and have therefore obtained particular attention in the parasite field. TgROM4 and TgROM5 localise to the cell surface [61, 63] and were shown to cleave the adhesion protein AMA1, as well as several MICs [61, 64, 65]. Loss of TgROM4 impairs host cell invasion, and changes the gliding motion of the parasite [64-66]. TgROM5 knock-out (KO) parasites showed

no defects in gliding or host invasion [65, 66], suggesting that TgROM4 is the main rhomboid protease in processing adhesion proteins *in vivo*. Inducible knockdown of TgROM1, the third rhomboid protease expressed in *T. gondii* tachyzoites, led to slight growth and replication defects [67], however, the underlying molecular mechanism is unclear due to a lack of known substrates. Notably, none of the three rhomboids expressed in tachyzoites, TgROM1, 4, 5, are essential for parasite survival. Triple KO parasites showed significant growth defects, but were viable and virulent. Mice injected with triple KO parasites died as quickly as mice infected with wild-type (WT) parasites [66].

Plasmodium, another apicomplexan parasite, causes malaria, a severe infectious disease that affects over 200 million people each year and caused 429 000 deaths in 2015 [68]. In humans, five different *Plasmodium spp,* transmitted by mosquitos*,* cause malaria, with *P. falciparum* having the most severe disease progression. Among the eight rhomboid proteases (ROM1, 3, 4, 6, 7, 8, 9, 10) expressed by *P. falciparum* and other *Plasmodium spp*, ROM1 and ROM4 are the best characterised ones. PfROM1 and PfROM4 appear to have a crucial role in host invasion as they cleave several adhesion molecules such as AMA1, and members of the erythrocyte binding-like family (EBL), reticulocyte binding-like family (RBL) and thrombospondin-related anonymous protein (TRAP) family in heterologous cell-based assays [69, 70]. Mutations in the TMH of TRAP that block rhomboid cleavage *in vitro*, also prevented its cleavage *in vivo* and impaired parasite gliding and infectivity [71]. Attempts to delete ROM4 in two different *Plasmodium spp* were not successful, indicating an indispensable role of PfROM4 [70, 72]. Loss of the PbROM1, the PfROM1 homologue in *P. berghei*, a parasite that causes malaria in rodents, led to viable parasites with an impaired growth in the blood stage. Notably, mice infected with PbROM1-deficient parasites had a significantly increased survival rate and were immune against an infection with a lethal dose of WT *P. berghei* [73].

In addition to their crucial role in the invasion of the mammalian host, it has been speculated that PfROM1 and PfROM4 might have a role in cell invasion in the mosquito as both rhomboids have the ability to cleave CTRP and MAEBL, adhesins essential for the invasion of the mosquito mid-gut and salivary gland [69]. PbROM1 and PbROM4 were indeed reported to be expressed in the mosquito stage of the parasite [72, 73], where PbROM1 might be important in the efficient formation of

oocystes in the mosquito mid-gut [73]. The systematic deletion of the rhomboid proteases in *P. berghei* and the phenotypic analysis of the mutants [72] also revealed potential roles of other *Plasmodium* rhomboids in parasite life cycle. Attempts to delete PbROM4, 6, 7, 8 were unsuccessful hinting on an essential role of these rhomboids in parasite survival. Loss of PbROM9 and PbROM10 did not show any defects in the parasites, whereas PbROM3 loss impairs the formation of sporozoites in the mosquito salivary glands.

Beyond apicomplexa, rhomboids in other protozoan parasites have also been shown to be involved in cleavage of adhesion proteins. The extracellular parasite *Entamoeba histolytica* causes diarrhoea, colitis and liver abscess. EhROM1, the only catalytically active rhomboid encoded in *E. histolytica*, cleaves lectins, which play a role in cell adhesion [74]. Consequently, EhROM1-depleted parasites showed a reduced host cell adhesion, and a decreased phagocytosis of host cells [75, 76], a process required for proliferation and virulence of the parasite. In line with this, EhROM1 loss or the overexpression of a catalytically inactive EhROM1 mutant showed reduced cytotoxicity and haemolysis. Furthermore, EhROM1 loss significantly decreased motility [76], resembling the role of rhomboids in apicomplexa. In *Trichomonas vaginalis,* an extracellular parasite that causes sexually transmitted infections, the rhomboid protease TvROM1 was suggested to play a role in the attachment to and lysis of host cells, by cleaving parasitic cell surface proteins [77]. In conclusion, rhomboid proteases in parasites are involved in the attachment, host cell invasion, or host cell lysis, and therefore constitute potential drug targets for infectious diseases. In particular, several *Plasmodium* rhomboid proteases have been well characterised and might be valuable drug targets for the treatment of malaria due to their important roles in parasite survival and host cell invasion.

4. Bacterial rhomboid proteases

Many bacteria express one or more rhomboid proteases, but very little is known about the biological function of bacterial rhomboids, largely due to the lack of known endogenous substrates. *In vitro* assays showed that various bacterial rhomboids are able to process eukaryotic rhomboid substrates [78], implying common enzyme

mechanisms. The only endogenous bacterial rhomboid substrate reported to date was identified in *Providencia stuartii*, a gram-negative bacterium causing urinary tract infections in humans. The *P. stuartii* rhomboid protease AarA cleaves TatA, a type 1 single pass transmembrane protein, that is a component of the twin-arginine translocation (Tat) system [79]. *P. stuartii* TatA contains an N-terminal extension that is removed by AarA [79] to enable TatA to assemble with TatC, another component of the Tat system [80]. The assembled Tat system allows protein export from bacteria. These findings provide a mechanistic explanation for the role of *P. stuartii* AarA in activating quorum sensing [81], an intercellular signalling mechanism in bacteria. A screen for a rhomboid cleavage recognition motif identified five additional *P. stuartii* proteins as *in vitro* substrates of AarA[82]. In *Bacillus subtilis,* loss of the rhomboid GluP impairs bacterial cell division and slightly reduces glucose uptake [83], and one of the two rhomboids expressed in *Mycobacterium smegmatis* is implicated in biofilm formation and susceptibility of antibiotics [84], although in both cases the underlying molecular mechanisms are not yet understood since the endogenous substrates have not been identified.

5. Rhomboid structure and function

Members of the rhomboid-like superfamily share basic structural characteristics and motifs despite very low sequence identity [6].

Topology

Membrane topology is a defining key feature of all members of the rhomboid-like superfamily. All known members have at least six TMHs. Within the rhomboid proteases, this represents the catalytic core. Throughout evolution, many rhomboid proteases have acquired an additional seventh TMH, either N- or C-terminal to the 6 TMH core (Fig. 1). Based on the number and position of TMHs, rhomboids are categorised in different topology classes [6]. Rhomboids with only 6 TMHs are found in prokaryotes, while most eukaryotic rhomboids have a seventh TMH, and these are divided into the 1 + 6 TMH topology class (with an N-terminal additional TMH), which seem to be limited to eukaryotic organelles such as plastids and mitochondria; and

the 6 + 1 TMH topology class (with a C-terminal additional TMH), which are the main group of rhomboids in the eukaryotic secretory pathway.

Conserved motifs within the structure of the rhomboid core

A huge leap was made in the rhomboid field when the structures of *E. coli* rhomboid GlpG (ecGlpG) and its equivalent in *Haemophilus influenza* (hiGlpG) were solved [85-96] – the first atomic resolution structures of any intramembrane protease. The crystal structures of the native enzyme, as well as in complex with various inhibitors, revealed the composition of the catalytic site, and provided initial insight into the mechanism of intramembrane proteolysis (Fig. 2). The 6 TMH rhomboid core forms a compact helical bundle mostly embedded in the membrane. Unlike their soluble counterparts rhomboids do not possess the classical serine protease catalytic triad of amino acids (D-S-H) within the active site, but instead have a catalytic dyad (S-H) [6, 85, 97]. The use of a dyad instead of a triad is consistent with the phylogenetic conclusion that rhomboids have evolved independently from soluble serine proteases. The fourth transmembrane helix of the rhomboid core harbours the catalytic serine of the S-H dyad within a GxSx motif, while the histidine is located in an A/GH motif in TMH6 [85, 97]. As in soluble serine proteases, the nucleophile, which attacks the peptide bond during the catalysis, is formed by the catalytic serine activated by abstraction of a proton from the OH group by the catalytic histidine.

Further clarifying the enzymatic mechanism, crystal structures revealed the position of the oxyanion hole [85, 89], which stabilises the negative charge of the substrate transition state during catalysis. Essential for the oxyanion hole formation are the backbone amides of the catalytic serine and its adjacent residue, as well as the side chains of a histidine and an asparagine within a HxxxN motif located in TMH2. A more detailed description of the catalytic mechanism can be found in these reviews [98-100]. Overall, it is not surprising that the HxxxN motif of the oxyanion hole as well as both motifs, which form the catalytic dyad, are highly conserved in rhomboid proteases, while these motifs are mutated in their inactive counterparts (rhomboid-like pseudoproteases).

There are other highly conserved features in rhomboids worth mentioning. The GxxxG sequence, a well-known helix dimerisation motif [101], is crucial for stabilising

the rhomboid structure [102]; it is found in TMH6 of the rhomboid core and is conserved both in active rhomboids as well as in the pseudoproteases [6, 102]. The second motif is a short W/xR sequence, which is conserved in active rhomboids, and sits within the long L1 loop between TMH1 and TMH2. The L1 loop is partially immersed in the membrane and seems to play a crucial role for overall structural integrity [85, 88]. Although there is no direct physical link between L1 or the WR motif and the active site, mutations of the WR motif abrogate protease activity, probably by loss of structural integrity [88, 103]. The L1 loop was also highlighted in recent work that suggested it was involved in observed rhomboid dimerisation [95, 104, 105]. Recently, a study demonstrated that residues of the L1 loop also participate in forming the S4 subsite for the P4 position in the substrate recognition motif [95].

Substrate recognition

As discussed above, physiological substrates for many rhomboids are not yet identified. However, there are substrates that can be cleaved by many evolutionarily divergent rhomboids [84, 106, 107]. Since this cross-reactivity points to a shared mechanism of substrate recognition and proteolysis, it provides the opportunity to study rhomboid-mediated proteolysis even if true substrates are unknown. For example, *E. coli* GlpG, whose structure is solved but whose physiological substrate(s) are not known, binds and cleaves *Providencia stuartii* TatA, which is a substrate of the *Providencia* rhomboid AarA [79, 80].

The identity of known rhomboid substrates indicates that most are single pass type I transmembrane proteins, although this conclusion needs to be treated with caution: the first confirmed substrates had this topology, and this has probably biased subsequent searches. The cleavage site usually lies within in the N-terminal region of the substrate TMH. However, there are also reports of cleavage sites in the luminal stalk region, either close to the membrane [11, 20, 108], or, in one interesting exception, further from the TMH [20, 28]. The usual cleavage in or close to the membrane is consistent with the location of the catalytic rhomboid core in a cavity facing the extracellular/luminal part of the membrane [85]. This cavity is easily accessible for unstructured parts of substrate stalk regions with cognate cleavage

sites. There is, however, an additional structural challenge caused by the substrates being transmembrane helices: alpha helices are not favoured to enter active sites [109] and the hydrogen bonds that form the helix shield the peptide backbone from the catalytic site of the enzyme. Therefore, the helix around the cleavage site has to unfold. Consistent with this, rhomboid substrates typically harbour helix destabilising residues around their cleavage sites [60]. Indeed, in some cases the introduction of helix-destabilising residues in otherwise uncleavable TMHs is sufficient to convert them into rhomboid substrates [110].

Despite the significance of helical destabilisation, there are clearly other determinants of physiological rhomboid substrates. An important feature of many substrates is a specific motif around the cleavage site, originally discovered in an extensive site-directed mutagenesis screen [82]. This motif consists of an amino acid with a small side chain at the P1 position (in line with an earlier study [111]), a large hydrophobic residue at P4, and a small or large hydrophobic residue at P2' (in a nomenclature where P1 is the residue immediately N-terminal to the cleavage site, and P1' the residue immediately C-terminal). Although this motif is found in many prokaryotic as well as eukaryotic rhomboid substrates, it is not universal. For example, it is absent from mitochondrial rhomboid substrates [112, 113], and only one of two rhomboids *in Mycobacterium tuberculosis* seem to be able to complement loss of the rhomboid AarA [84], suggesting that one may use different substrate determinants. Recent studies have elucidated the mode of binding of the recognition motif region into the rhomboid active site by analysing X-ray structures of GlpG bound to peptidyl chloromethylketone [95] and peptidyl aldehyde [96] inhibitors, and enzymological studies have revealed key role for these interactions in determining the k_{cat} of rhomboid catalysis and the selectivity of substrates [94, 114].

Rhomboid-substrate interaction is facilitated by exosites within the rhomboid core

Interestingly, enzymological studies indicated the existence of other substrate-rhomboid interaction sites (exosites) beyond the active site [82, 94]. Although we now have much information about the enzyme-substrate complex, the mechanism of substrate binding is still poorly understood. Several reports imply that the interaction between substrate and rhomboid consists of two steps [82, 94, 96]. First, the

substrate TMH binds to site distinct from the active site. This exosite binding step [82] is called “interrogation” [94] or “docking” [115]. In the second stage, scission, the unwound substrate helix around the recognition motif enters the active site and cleavage occurs (Fig. 3) [82].

There are currently two apparently distinct models of the substrate-rhomboid interaction, although it is not clear whether they are really incompatible. The first model suggests that there is lateral access of a substrate TMH between a ‘gate’ comprising TMH2 and TMH5 of the rhomboid core (Fig. 2) [86, 103]. In this model TMH5, as well as the L5 loop, which caps the active site, have to be displaced to make space for the substrate TMH. Subsequently, the substrate TMH can enter the rhomboid core, leading to the unfolding of the recognition motif and its alignment with the active site. In contrast, the second proposed model downplays the significance of a gate requiring major TMH movement, instead suggesting that only minor displacement of the L5 loop is required to grant access to the active site [88, 95, 111, 116]. In this case, the ‘lower’ part of the substrate TMH binds to the exosite [82, 95]. Structural considerations hint that this exosite most likely consists of TMH5 and/or TMH2. It has also been suggested that the interaction between the enzyme and substrate is promoted by thinning of the surrounding lipid bilayer, caused by the TMHs of rhomboids being short, thereby facilitating the exit of the recognition site out of the lipid environment into the enzyme active site [117].

Extra-membranous domains and regions

Beyond the membrane-immersed rhomboid core, most rhomboids contain N- or C-terminal accessory regions in the cytoplasm and extracellular/luminal compartment. These regions are not essential for catalytic activity [3, 5]. They are also highly diverse, and contain additional protein interaction motifs, suggesting that they might provide clues about regulation and substrate selectivity, although few have been well characterised.

For example, in RHBDL4, these accessory domains can function as additional exosites for substrate interaction. RHBDL4 is involved ERAD and contains a ubiquitin interacting motif (UIM) in its cytosolic region [20]. This motif is required for the recognition and cleavage of its ubiquitinated substrates. Another example is

RHBDL2 and its recognition of thrombomodulin as a substrate. In this case, the interaction of both proteins via their cytosolic regions is crucial for the cleavage to take place [12]. *Drosophila* Rhomboid-4 has calcium binding EF hands [5] in its cytoplasmic domain, and it has been shown that calcium binding to these EF-hands promotes the recognition and cleavage of substrates by controlling the access to the active site [118]. These additional rhomboid domains are not restricted to eukaryotes. *E. coli* GlpG contains a N-terminal cytosolic domain (DUF3582), which was recently shown to not be directly involved in the activity of GlpG [85, 119], but instead to drive its dimerisation [119, 120], although the functional significance of this dimerisation remains to be determined.

5. Inhibitors

As described above, the likely pathological role of rhomboid protease in a wide range of disease has gradually emerged in the last years. Rhomboid inhibitors look increasingly attractive, not only as valuable tools to investigate structure and physiological functions of rhomboids, but also as foundations for drug development. The unusual properties of rhomboids do present challenges when trying to develop inhibitors that are both potent and selective. The general principle of serine protease inhibition is the use of electrophilic compounds, which covalently and hence irreversibly bind to the active site [121, 122]. The first identified rhomboid inhibitors, some of which also helped to get structural insight into the proteolytic mechanism, were tosyl phenylalanyl chloromethyl ketone, diisopropylfluorophosphate, phosphonofluoridate as well as isocoumarins [60, 90, 93]. However, the high reactivity of their electrophilic groups is also the major disadvantage of these inhibitor classes, since they are prone to inhibit other serine proteases quite widely. Second generation rhomboid inhibitors were the monocyclic β-lactams [123] and β-lactones [124, 125]. Although these inhibitor classes show higher selectivity, their inhibitory potency is rather weak. In the case of β-lactams, the inhibitor is slowly turned over by the protease, probably explaining why β-lactams are unable to inhibit rhomboid proteases fully in vivo [123].

Beyond small molecule inhibitors, another successful strategy of protease inhibition has been the coupling of substrate related oligopeptides with electrophilic groups

[126]. This approach has recently yielded two classes of inhibitors, peptidyl chloromethylketones [95] and peptidyl aldehydes [96]. Although these inhibitors are not very potent, they interact with the rhomboid active site in a substrate-like manner, which made them valuable tools in the structural determination of rhomboid-substrate complexes [95, 96]. Interestingly, these structures revealed that, although the P1 site prefers small residues [82, 95], the corresponding S1 subsite in the enzyme is located in a large cavity, which would in principle allow larger residues at the P1 position [95]. However, it is postulated that this cavity is also the water retention site enhancing catalysis [95, 127]. Therefore, large residues in the substrate P1 position might block the water transfer and hence impair catalytic activity; this hypothetical mechanism could perhaps be exploited for the design of future inhibitors [100].

The exosite(s) that mediate the initial interaction between enzyme and substrate are other untried potential inhibitory targets. Indeed, γ-secretases, which are intramembrane aspartyl proteases, can be inhibited by membrane immersed, helical peptides [128] that may interfere with exosite binding. However, the interaction between rhomboid exosites and substrates seems to be relatively weak and to have low selectivity [94], suggesting that this approach might not be successful. Finally, given that selectivity is a desirable characteristic of inhibitors, there may be value in targeting functionally important regions of the extra-membrane domains of rhomboids. Although not part of the core protease activity, they represent the least well conserved part of rhomboids and, at least in the case of RHBDL4 and *Drosophila* Rhomboid-4, are functionally important, making them potentially promising targets.

6. Outlook/summary

Since their discovery and their identification as intramembrane serine proteases, rhomboids have increasingly emerged as important factors in disease-related processes. Although the biology of rhomboids is still not fully understood, it seems likely that they will present therapeutic opportunities. For example, the link between EGFR hyperactivation in cancer and RHBDL2 may represent a new therapeutic target; overexpression of RHBDL4, too, is associated with cancer. Of course, until

the underlying biology of their physiological and pathological roles is properly understood, for example by more detailed studies of knock-out mice, the possibility remains speculative. Other future therapeutic targets might include mitochondrial rhomboids (PARLs), with their increasingly extensive links to Parkinson's disease and diabetes, and the parasite rhomboids. The quite intense focus on rhomboids in protozoan parasites has been driven by indications that they are involved in the invasive mechanisms by which the parasite enters the host cell and also perhaps the parasite's own replicative cycle. These efforts have included parasites associated with major public health concerns (*Toxoplasma, Plasmodium Trichomonas* and *Entamoeba*), and have led to insights into mechanisms of host cell invasion. This work has provided encouragement that rhomboid inhibitors might provide a basis for therapeutic intervention, including in major diseases like malaria. It is also interesting to speculate that the mitochondrial rhomboids in parasites might represent useful targets, although they have not yet been well characterised.

If rhomboids are indeed validated as significant therapeutic targets, there will be great incentives to identify molecules that can modify their function, most obviously inhibitors. As we have described, although there has been some success in identifying rhomboid protease inhibitors [95, 96, 123, 124, 129], we are still far from having realistic templates for drug development. Inhibitor design has been and will be aided by the structural and mechanistic understanding derived from crystal structures of rhomboids. In this regard, the field is currently limited to structures of prokaryotic 'ancestral' rhomboids with six TMHs, while no structures of a rhomboid of the common topologies 1 + 6 or 6 + 1 TMHs, particularly a eukaryotic one, have been reported. Beyond the 6 TMH core, the accessory cytoplasmic and luminal regions of many rhomboids, which may often have roles in regulating enzyme activity and substrate interaction [12, 20, 118], are also structurally and functionally rather poorly explored and understood. Since they also represent targets for further investigation, and for future attempts to alter rhomboid activity for therapeutic purposes, their structures will also be of significant interest.

Finally, we have not in this review described the function or mechanism of the inactive relatives of the rhomboid proteases, the rhomboid-like pseudoproteases [130, 131]. Some of these proteolytically inactive members of the rhomboid-like superfamily have already been shown to be associated with pathophysiological

processes. Most prominently, the mammalian iRhoms and their client ADAM17, are now well established as important regulators of inflammatory signalling [130, 132]. Since rhomboid-like pseudoproteases evolved from their protease ancestors [6, 131], have the same topology as rhomboids, and are likely to be structurally similar (although they also represent an important gap in our structural insight), it is tempting to speculate that the underlying mechanism of pseudoprotease:client interaction might be similar to the mechanism of substrate recognition in rhomboid proteases. We therefore suspect that advances in our understanding of rhomboid proteases and their substrates, and the development of inhibitors, will in parallel accelerate our knowledge of rhomboid-like pseudoproteases. This should allow the development of molecular tools to modulate the interaction between pseudoproteases and their clients which, in turn, may also have future therapeutic value.

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Figure Legends

Figure 1) Overview of rhomboid topology in mammals. A) Shown is a schematic depiction of the three rhomboid topology classes as well as of the relative positions of the following evolutionary conserved motifs. The GxSx and A/GH motif form the catalytic S-H dyad within the active site, while the HxxxN motif is part of the oxyanion hole. The other two highly conserved motifs are the GxxxG sequence, a helix dimerisation motif, and a W/xR motif, which is crucial for the protease activity of rhomboids with the 6 or 6 + 1 topology. In contrast, the W/xR motif cannot be found in PARLs (1 + 6 topology). B) Schematic representation of the different branches of the rhomboid superfamily in mammals with the corresponding rhomboid topology classes. Active rhomboid proteases are written in bold.

Figure 2) Structure of *E.coli* GlpG. Shown is the structure of GlpG (pdb: 3zmh) in ribbon cartoon illustration as side and top view. The residues forming the catalytic dyad (histidine, serine) are depicted in red.

Figure 3) Schematic model of substrate recognition. Shown is a simplified illustration of the substrate recognition mechanism with the cleavage site being either outside or inside the substrate TMH.

Table 1

Most significant disease associations of eukaryotic rhomboid proteases.

Rhomboid	Disease or pathology	Observed linked to disease	Proposed underlying molecular mechanism
Mammals			
RHBDL2	Wound healing	RHBDL2 inhibition or knockdown impairs wound healing [17]	Reduced cleavage and release of RHBDL2 substrate Thrombomodulin upon RHBDL2 knockdown or inhibition [17]
	Tumour metastasis	RHBDL2 overexpression in epithelial cells increases cell proliferation and resistance to anoikis [16]	unclear
	Tumour angiogenesis	Addition of CLEC14A extracellular domain (ECD) reduces cell migration and sprouting angiogenesis *in vitro* and *in vivo* [18]	CLEC14A is cleaved by RHBDL2 leading to the release of CLEC14A ECD [18]
RHBDL4	Cancer	RHBDL4 mRNA unregulated in colorectal cancer patients [25]; RHBDL4 knockdown reduces colony formation in and proliferation in different cancer cell lines [22-24]	Cleavage of TGFα by RHBDL4, followed by EGFR activation is controversial [21, 25]
	Alzheimer's disease (AD)	Aβ38, Aβ40, and Aβ42 levels are reduced upon RHBDL4 wt overexpression compared to RHBDL4 S/A overexpression [28]	RHBDL4 cleaves APP within its ectodomain [28]
PARL	Parkinson's disease (PD)	PARL^{Ser77Asn} mutation was found in two PD patients [35]	PARL^{Ser77Asn} impairs autocatalytic β cleavage of PARL and parkin recruitment to mitochondria [35]
		PINK1 mutations are a risk factor for familial PD [133]	PARL cleaves PINK1 [35, 38-41], however, PARL processing of the PD-associated PINK1^{I111S} and PINK1^{R98W} mutants is reduced [40]
	Type 2 diabetes mellitus (T2DM)	Reduced PARL expression correlates with insulin tolerance and T2DM [50, 51]	unclear
Protozoa			
TgROM4	Toxoplasmosis	Loss of ROM4 in *T. gondii* impairs ability of the parasite to invade host cells [64-66]	TgROM4 cleaves the *T. gondii* adhesion proteins AMA1 and several MICs [61, 63-65]
***Plasmodium spp* ROM1, ROM4, and ROM6-8**	Malaria	Mice infected with PbROM1-defienct *P. berghei* have a highly increased survival rate [73]; attempts to delete PbROM4, 6, 7, 8 were not successful hinting on an essential role of these rhomboids [72]	PfROM1 and PfROM4 cleave various *Plasmodium* cell adhesion molecules [69, 70] required for mosquito and host cell invasion; substrates of ROM6-8 have not been identified yet
EhROM1	Amoebiasis	EhROM1-deficient *E. histolytica* show reduced phagocytosis of host cells [75], reduced motility and cytotoxicity [76]	EhROM1 cleaves lectins on the surface of *E. histolytica* [74]

Figure 1

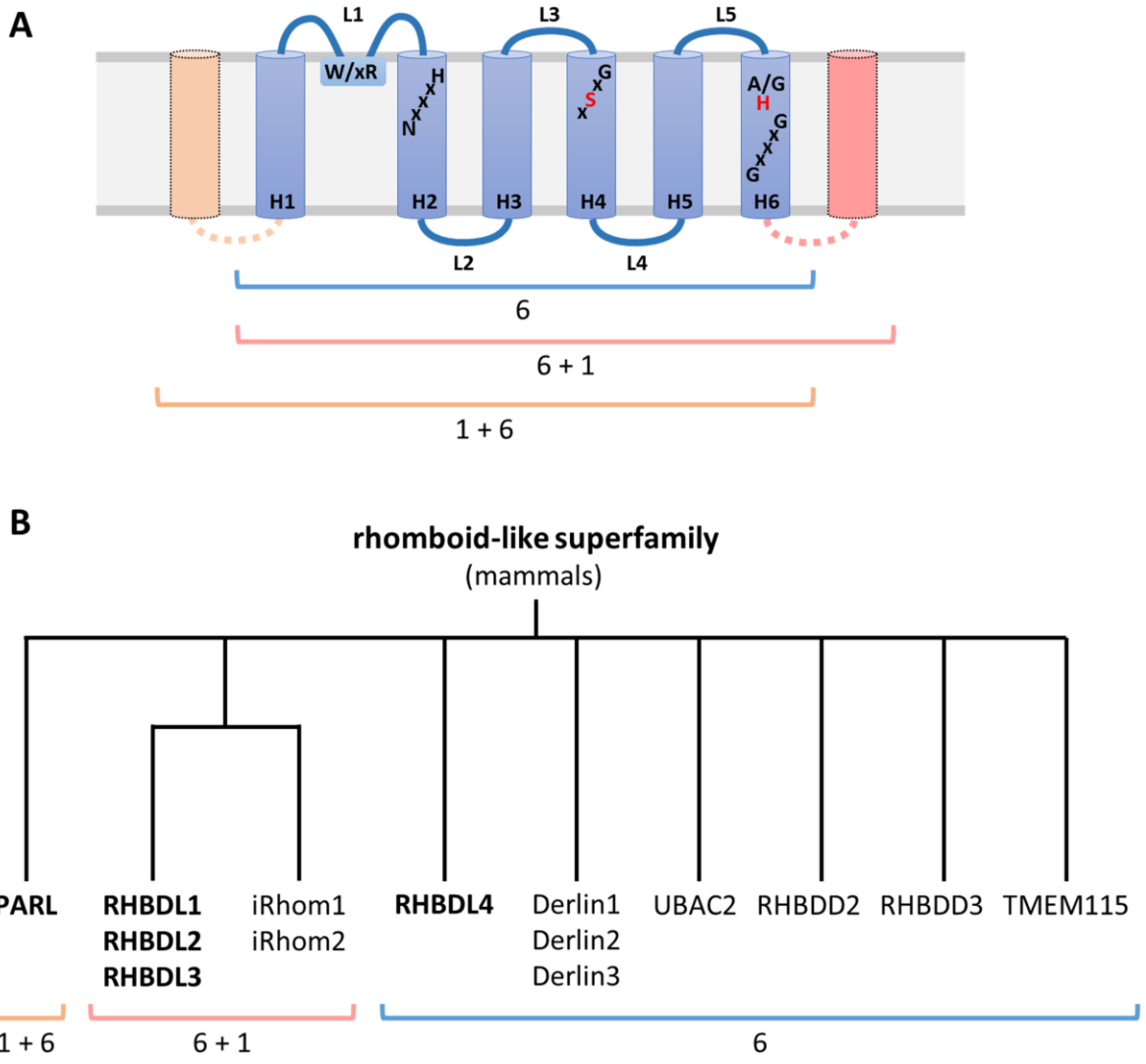

A
L1
L3
L5
W/xR
H
N
G
S
A/G
H
G
G
H1
H2
H3
H4
H5
H6
L2
L4
6
6 + 1
1 + 6
B
rhomboid-like superfamily
(mammals)
PARL
RHBDL1
RHBDL2
RHBDL3
iRhom1
iRhom2
RHBDL4
Derlin1
Derlin2
Derlin3
UBAC2
RHBDD2
RHBDD3
TMEM115
1 + 6
6 + 1
6

Figure 2

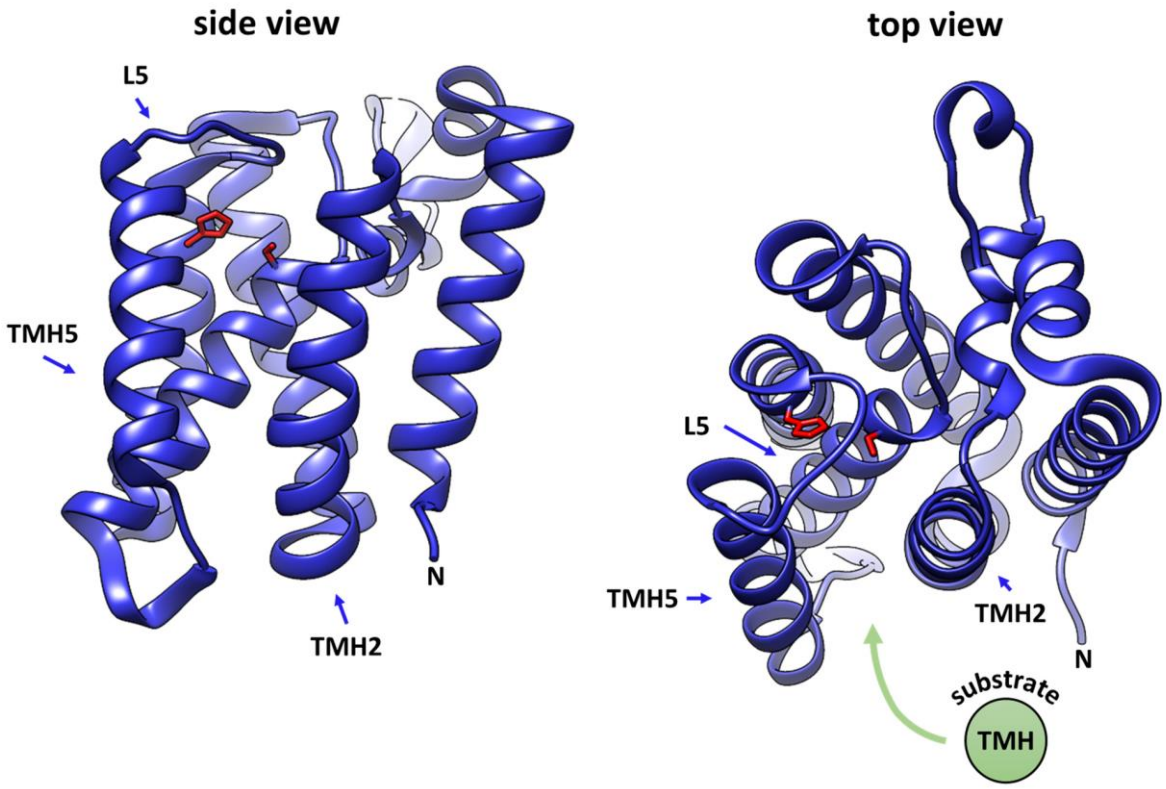
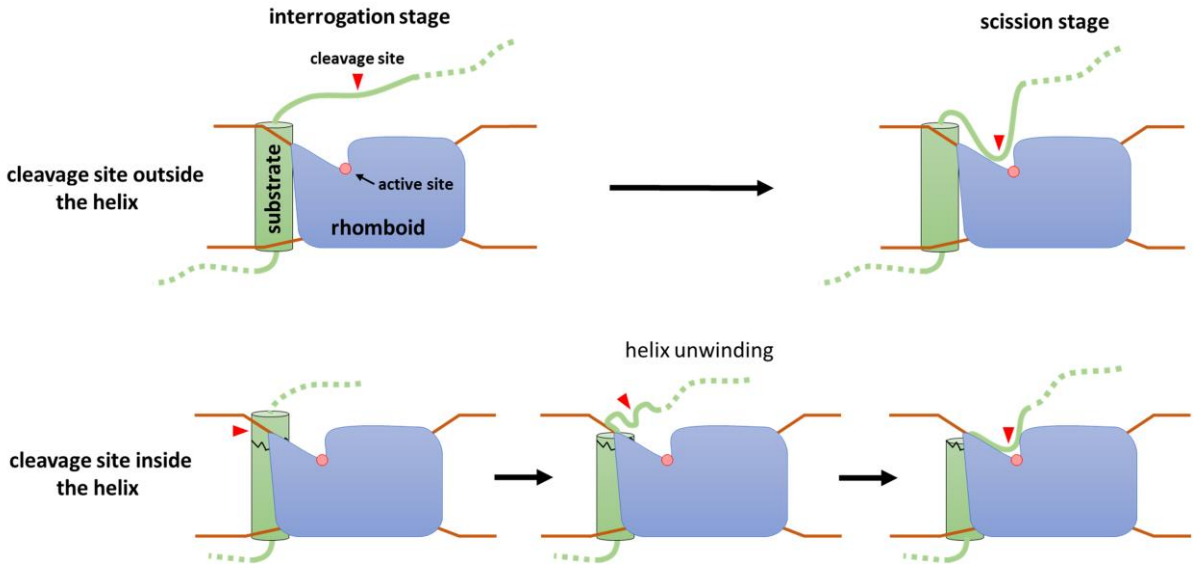


Figure 3


interrogation stage
cleavage site
scission stage
cleavage site outside the helix
substrate
active site
rhomboid
helix unwinding
cleavage site inside the helix