The rhomboid-like superfamily: molecular mechanisms and biological roles

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Abstract

The rhomboid proteases were first discovered as regulators of Drosophila EGF receptor signalling; soon after, it was recognised that they represented the founder members of a widespread family of intramembrane serine proteases, conserved in all kingdoms; more recently still, the family was promoted to a superfamily, encompassing a wide variety of distantly related proteins. One of the surprises has been that many members of the rhomboid-like superfamily are not active proteases. Given the size of this clan, and its relatively recent discovery, there is still much to learn. Nevertheless, we already understand much about how rhomboid proteases perform their surprising function of cleaving transmembrane domains. We also already know that members of the rhomboid-like superfamily participate in biological functions as diverse as growth factor signalling, mitochondrial dynamics, inflammation, parasite invasion, and the machinery of protein quality control. Their potential medical significance is now becoming apparent in several areas.

Introduction

Rhomboid-like proteins are now known to comprise a diverse superfamily of polytopic membrane proteins with a remarkable range of biological functions across evolution. This wide significance belies their relatively recent discovery and the even more recent appreciation that the rhomboid intramembrane proteases, after which the clan is named, are just one branch of a much larger group of evolutionarily related proteins of largely unknown function. Although a rapidly growing field, it is inevitable that we are still in a position more of ignorance than knowledge. Nevertheless, what we already know about the superfamily has highlighted not only its range of functions but also its biological significance and in some cases medical potential, and this provides a strong incentive to learn more about the rhomboid-like proteins.

Classification of rhomboids

Unfortunately, as in most protein families, rhomboid-like proteins suffer from confusing and inconsistent naming, multiple synonyms, and little or no systematic nomenclature across species (Lemberg and Freeman, 2007). The very wide sequence divergence across the whole superfamily also leads to uncertainties about their phylogenetic relationships (Koonin et al., 2003; Lemberg and Freeman, 2007; Kinch and Grishin, 2013). Nevertheless, there is an emerging consensus that allows at least a broad organisation of the superfamily into a plausible classification (Fig. 1). For the purpose of this review it is important that the common ancestor of all extant members of the clan was probably an active intramembrane protease; that despite this, the majority of existing members, at least in metazoans, are not active proteases; that the iRhoms are ‘dead’ rhomboids that evolved from the active rhomboids but which have maintained quite high similarity to the rhomboid proteases; that the rhomboid proteases themselves fall into distinct sub-groups; that the mitochondrial rhomboids are more closely related to some of the bacterial rhomboids than to the ancestral eukaryotic rhomboid; and that the more distant branches of the superfamily (TMEM115, UBAC2, RHBDD2, RHBDD3) have diverged very extensively from the rhomboid/iRhom branch (Adrain and Freeman, 2012; Lemberg, 2013). Indeed, although it is now clear that these more distant relatives are genuinely members of the superfamily, and therefore evolutionarily related, they are not easily recognisable as such without quite sophisticated analysis (Greenblatt et al., 2011; Finn et al., 2014). Despite these complexities and uncertainties, the

promotion of the previously recognised rhomboid family to the much larger rhomboid-like superfamily has been important in revealing the variety of biological roles that are mediated by rhomboid-like proteins.

The rhomboid proteases

Although intramembrane proteolysis is now known to control a great many biologically and medically important processes across evolution, its discovery came relatively recently. This was at least partly due to its oddness. Two things were known about proteases: that they all fitted into well defined and phylogenetically clear families, and that since proteolysis is a hydrolytic reaction, their active sites need easy access to water molecules. When diverse research groups, none of whom had backgrounds in protease biochemistry, started to propose that their favourite polytopic membrane proteins were completely novel proteases and that, moreover, their active sites were buried in the lipid bilayer of cell membranes, where they cleaved transmembrane domains, this was therefore quite heretical. Nevertheless, it soon became clear that intramembrane proteases were real; that they mainly fall into mechanistic classes that mimic the classical soluble proteases (metallo, aspartyl and serine); but that they are phylogenetically unrelated to the classical proteases – i.e. they have evolved convergently. They are also unrelated to each other except that they all share the common characteristics of having multiple TMDs, membrane embedded active sites, and cleaving TMD substrates.

The three major classes of intramembrane proteases (excluding the very recently discovered glutamate protease, Rce1 (Manolaridis et al., 2013)) were discovered in quick succession between 1997 and 2001. The first was the intramembrane metalloprotease site 2 protease (Rawson et al., 1997), followed by the aspartyl protease presenilin (Esler et al., 2000), and finally the serine protease rhomboid (Urban et al., 2001). The focus of this review, rhomboid, was initially identified as a Drosophila mutation disrupting development (Mayer and Nusslein-Volhard, 1988; Bier et al., 1990), subsequently shown to be involved in generating the active ligand for EGF receptor signalling in flies (Guichard et al., 1999; Wasserman et al., 2000), and finally identified as the founder of a novel family of intramembrane serine protease by a combination of genetic and biochemical approaches (Lee et al., 2001; Urban et al., 2001) (Fig. 2). Rhomboids are now the best understood of known intramembrane proteases, and appear to be the most biologically diverse.

Rhomboid protease mechanisms

An area of great interest surrounding all intramembrane proteases is how these unconventional enzymes actually work. This has been intensively studied in rhomboids and several broad principles have emerged. As an already well-reviewed area, I will restrict my description here to a broad overview. For more specialised recent reviews of rhomboid enzymology, see (Brooks and Lemieux, 2013; Strisovsky, 2013; Vinothkumar and Freeman, 2013). After the initial genetic phase of rhomboid research, understanding of the enzyme rapidly accelerated once in vitro reconstitution of protease activity was achieved (Lemberg et al., 2005; Urban and Wolfe, 2005), followed by high resolution crystal structures of the bacterial rhomboid GlpG (Wang et al., 2006; Wu et al., 2006; Ben-Shem et al., 2007; Lemieux et al., 2007) – the first of any intramembrane proteases. These structures resolved important mysteries. First, they discovered that the serine protease active site of rhomboids shares important characteristics with soluble serine proteases, while at the same time being architecturally distinct, most notably by relying on a catalytic dyad instead of the classical Ser/His/Asp triad. Second, structures explained how water molecules get access to the active site: the catalytic serine sits at the base of a water-accessible indentation in the plane of the membrane, protected from the bilayer lipids by surrounding TMDs. But these initial structures did not answer everything. They left open, for example, how substrate TMDs are recognised, how the TMD alpha helices are unwound to allow access to their peptide bonds, and the mechanism by which substrates approach and enter the active site.

Genetics and cell biology made it clear that most TMDs were not cleaved by rhomboid proteases, so what defines a rhomboid substrate? Several early papers identified the involvement of amino acids that tend to destabilise TM helices (Urban and Freeman, 2003; Maegawa et al., 2005; Akiyama and Maegawa, 2007). This principle has received further support from detailed biophysical analysis of substrate TMDs (Moin and Urban, 2012). An alternative perspective on substrate specificity derives from the discovery of a substrate determining motif (involving residues P4, P1 and P2' surrounding the cleavage site) in a natural substrate of a bacterial rhomboid, and confirmed in a variety of model rhomboid substrates (Strisovsky et al., 2009). This result is consistent with subsequent structural analysis of the GlpG active site bound to mechanism-based inhibitors, in which cognate binding pockets for the P1 and P2' residues have been proposed (Vinothkumar et al., 2010; Vinothkumar et

al., 2013). Given the vast diversity of rhomboid proteases, this particular motif is unlikely to be universal, but the important point is that the site of cleavage and the efficiency of cleavage can be determined by sequence preferences around the scissile bond. Of course these two principles of substrate determination, helical instability and a primary sequence motif, are not mutually exclusive: the best current guess is that both are necessary, perhaps to a greater or lesser extent in different substrates. For example, mammalian EGF appears to be cleaved by RHBDL2 just outside its TMD (the cleavage site apparently looping back into the rhomboid active site) (Adrain et al., 2011) and in this case helical instability appears unnecessary (Strisovsky et al., 2009). In other cases, the P4, P1, P2' motif appears not to be essential, perhaps when helical instability is particularly prominent (Strisovsky et al., 2009). Elegantly proving that classical and potentially quite old fashioned techniques still have currency, a recent enzyme kinetic analysis has shown that E coli GlpG has unusual characteristics: the cleavage reaction is driven by the catalytic rate, while the affinity between enzyme and substrate is remarkably low. This implies that the substrate motif and proposed cognate enzyme binding pockets cannot be sites of tight interaction but presumably instead as transient drivers of catalytic rate (Dickey et al., 2013). It is speculated that these unexpected kinetics gives the enzyme the ability to 'sample' many potential TMDs while only cleaving the correct ones (Dickey et al., 2013).

A related topic of much debate has been the question of how substrates gain access to the active site of rhomboids. Early structural work suggested two apparently contradictory models: either the substrate entered between TM helices 2 and 5, or from the opposite side of rhomboid, between TM helices 1 and 3 (Wang et al., 2006; Wu et al., 2006; Ben-Shem et al., 2007; Lemieux et al., 2007). Subsequently, most evidence has tended to support the TM2/5 approach but the details remain far from clear. There have been two primary pieces of evidence in favour of TM helices 2 and 5 acting as a 'lateral gate' to regulate substrate entry. The first is structural: TM5 has been seen in some structures to substantially displaced or unstable (Wu et al., 2006; Ben-Shem et al., 2007; Brooks et al., 2011). The importance of TM2 and 5 in substrate gating was also supported by a series of site directed mutants in which alterations predicted to enhance gate opening led to increased enzyme activity (Baker et al., 2007). More indirectly, a detailed biophysical analysis of the thermodynamic stability of GlpG concludes that TM5 does not contribute the overall structural integrity of the protein, which would consistent with its freedom to move substantially (Baker and Urban, 2012). In opposition to the TM5

lateral gate model, many other GlpG structures have not included an open form, including several in which the active site is bound to inhibitors that are thought to mimic substrate binding (Vinothkumar et al., 2010; Xue and Ha, 2011; Xue et al., 2012; Vinothkumar et al., 2013), and one in the presence of lipids (Vinothkumar, 2011). It remains possible, therefore, that the open structure is some kind of artefact. A more direct challenge comes from a recent paper that concludes that crosslinking to 'lock' the putative TM5 lateral gate and thereby prevent potential substrate TMD access, does not inhibit GlpG activity (Xue and Ha, 2013). Over the last years, this debate has swung back and forward, and it is probably fair to conclude that, as with the question of substrate determinants, we won't get a fully satisfying answer until there are high resolution structures of rhomboid/substrate peptide complexes.

Biological roles of rhomboid proteases

First identified as enzymes that release the active signalling ligands for EGF receptor signalling in Drosophila (Wasserman et al., 2000; Lee et al., 2001; Urban et al., 2001), once it became clear that rhomboids existed in all branches of evolution, there was intense speculation about their other biological roles. The size and complexity of the rhomboid protease family means that our knowledge it still very patchy, but a fuller picture is now beginning to emerge.

Mammalian rhomboid proteases

Beyond the mitochondrial rhomboids described below, there are four rhomboid 'secretase' proteases in mammals (Lemberg and Freeman, 2007). Although there is great interest in their function, especially since their Drosophila homologues have such a central role in regulating growth factor signalling, little is really known about their function in mammalian cells. RHBDL1-4 are all located in the secretory pathway (Lohi et al., 2004; Fleig et al., 2012) hinting at functions in regulating release of secreted proteins, but no natural substrates for any of them have yet been unambiguously identified. RHBDL2 can cleave thrombomodulin, ephrinB3, EGF and EGFR (Lohi et al., 2004; Pascall and Brown, 2004; Adrain et al., 2011; Liao and Carpenter, 2012) but in none of these cases has cleavage been proved to be physiologically meaningful. On the other hand, the fact that the vast majority of TMD containing proteins cannot be cleaved by the mammalian rhomboids hints that those few that can be, may be significant. In the case of EGF (Adrain et al., 2011), this hint is further supported by the conservation in Drosophila.

A recent development in this rather preliminary picture is RHBDL4 (aka RHBDD1; for discussion of nomenclature see Lemberg and Freeman, 2007), a rhomboid that is distinct in a number of ways from the other secretory pathway rhomboids. It is phylogenetically divergent from RHBDL1-3; it has only six TMDs, as opposed to seven in the other mammalian rhomboids; and it is the only one to be ER resident. In a number of papers published over the last 6 years, the group of Linfang Wang has described RHBDL4 to be highly expressed in spermatogonia, to cleave the Bcl-2 family member BIK (Wang et al., 2008; Wang et al., 2009), as well as the polytopic membrane protein TSAP6, and to have a potential role in regulating apoptosis, possibly via c-Jun, and exosome secretion (Wan et al., 2012; Ren et al., 2013). Although these data have not yet been assimilated into a unified picture, they represent important clues about RHBDL4 function. Intriguingly, though, a different model has recently been proposed. Showing that RHBDL4 is upregulated by the unfolded protein response (UPR), the Lemberg group proposed that the ER-localised RHBDL4 might participate in ER-associated degradation (ERAD) (Fleig et al., 2012). Indeed, they found that orphan T cell receptor subunits can be cleaved by RHBDL4 and then subsequently degraded by the proteasome. They also show that RHBDL4 can bind ubiquitin and the ERAD factor p97. There is currently no easy way of reconciling these two ideas of RHBDL4 function, and it is probably fair to conclude that neither yet draw a fully compelling and comprehensive picture of how this unusual ER-localised rhomboid functions. This work does, however, represent the most detailed analysis yet of the function of a mammalian secretase rhomboid.

Mitochondrial rhomboids

The mammalian mitochondrial rhomboid, called PARL, has been the subject of intense recent research because of proposed links with type 2 diabetes (Walder et al., 2005; Hatunic et al., 2009; Civitarese et al., 2010) and Parkinson's Disease (PD) (McQuibban et al., 2006; Deas et al., 2011; Shi et al., 2011). The first identification of a mitochondrial rhomboid – located in the inner mitochondrial membrane – was in yeast, where Rbd1 (aka Pcp1) was shown to cleave two single-pass TMD proteins, Ccp1 and Mgm1 (Esser et al., 2002; McQuibban et al., 2003; Herlan et al., 2004; Tatsuta et al., 2007). Mgm1, the substrate most studied, is a large GTPase, whose cleavage is necessary for mitochondrial membrane fusion. In Drosophila, the mitochondrial rhomboid, Rhomboid-7, similarly cleaves the Mgm1 orthologue OPA1, causing mitochondrial defects associated with disrupted membrane dynamics

(McQuibban et al., 2006). In addition, Drosophila *rhomboid-7* mutations interact genetically with the PD associated genes *pink1, htrA2* and *parkin*; it is also required for cleavage of Pink1 and HtrA2 proteins (McQuibban et al., 2006). As in yeast and flies, knockout of the mouse mitochondrial rhomboid, PARL, causes disruptions in mitochondrial function and morphology, specifically defects in cristae remodelling and cytochrome c release (Cipolat et al., 2006). Despite initial evidence that, like in flies and yeast, this was mediated by OPA1 (Cipolat et al., 2006), the current consensus is that PARL is not a major OPA1 protease (Duvezin-Caubet et al., 2007; Guillery et al., 2008), and its precise role in mammalian mitochondrial dynamics, and how this relates to its function in yeast and flies, remains to be determined. The biological significance of a reported autocatalytic cleavage of an N-terminal PARL fragment is also not yet understood (Sik et al., 2004; Jeyaraju et al., 2006).

Much more attention has been paid to the potentially exciting medical significance of PARL's role in the cleavage of PINK1, and its consequent implication in Parkinson's Disease. The relationship between PARL, PINK1 and HtrA2/Omi is clearly conserved between flies and mammals (Chan and McQuibban, 2013). Indeed, both PINK1 and HtrA2/Omi are substrates of mammalian PARL (Jin et al., 2010; Deas et al., 2011; Meissner et al., 2011; Shi et al., 2011). The current consensus view is that PARL activity turns over PINK1 in healthy cells, thereby preventing its accumulation. Mitochondrial damage, leading to disruption of membrane potential, inhibits this turnover, allowing PINK1 to recruit the E3 ubiquitin ligase Parkin, which in turn ubiquitinates a number of mitochondrial proteins, ultimately triggering mitophagy, a form of autophagy that protects cells by destroying damaged mitochondria (Gomes and Scorrano, 2013). Significantly, reduced mitophagy has been proposed to contribute to the pathology of PD (Imai and Lu, 2011).

Another link between PARL, apoptosis and sensing mitochondrial damage occurs via the recently discovered PARL substrate PGAM5 (phosphoglycerate mutase 5) a serine/threonine phosphatase located in the inner mitochondrial membrane (Sekine et al., 2012). Upon loss of mitochondrial membrane potential, PGAM5 cleavage is triggered, almost certainly by PARL, releasing a soluble fragment that can promote apoptosis. These data imply a pro-apoptotic role for PARL, which is at odds with the protective role implied by PARL's cleavage of PINK1, and this illustrates that despite the recent intensity of research into PARL's role in mitochondrial function, regulation of cell death and mitophagy, and ultimately

human disease, the real physiological and pathological details remain uncertain. Nevertheless, the discovery of the significance PARL by different routes, combined with human genetic data that support a role in PD (Heinitz et al., 2011; Shi et al., 2011), and an association between reduced PARL, ageing and type 2 diabetes (Civitarese et al., 2010), adds up to a strong case for a role in these processes.

Other biological functions of rhomboid proteases

Three other important areas round up this survey of recent advances in our understanding of the biological role of rhomboid proteases: plants, bacteria and apicomplexan parasites. It has long been recognised that, compared to animals, plants have an expanded number of rhomboid proteases (Knopf and Adam, 2012), but their biological functions have not yet been extensively studied. Recently, however, two chloroplast localised rhomboids have been investigated and shown to affect flower development, possibly by regulating the levels of allene oxide synthase (Knopf et al., 2012; Thompson et al., 2012). Another area about which very little is known is the bacterial rhomboids. Most but not all bacteria have one or more rhomboids (Rather, 2013), but in very few cases is anything known about them. The best studied case is the rhomboid AarA in Providencia stuartii, which is involved in quorum sensing and antibiotic resistance (Clemmer et al., 2006). At a molecular level, AarA cleaves the TMD of TatA, a component of the twin arginine translocation system that transports fully folded proteins across the cytoplasmic membrane (Stevenson et al., 2007). This cleavage of TatA is required for its activity. Loss of rhomboids in other bacteria have been associated with a variety of specific phenotypes (Rather, 2013), but in no other case is a substrate known or the biological role appreciated.

Compared to the paucity of plant and bacterial rhomboid data, their study in parasites has been quite extensive. It is more than 10 years since the first indication that rhomboids can cleave some of the surface antigen proteins that play important roles in apicomplexan parasite adhesion and invasion (Urban and Freeman, 2003). Since then, there has been substantial effort put into investigating their function in parasites (Brossier et al., 2005; Carruthers and Blackman, 2005; Dowse et al., 2005; Baker et al., 2006; O'Donnell et al., 2006; Baxt et al., 2008; Baxt et al., 2010; Santos et al., 2011; Vera et al., 2011; Parussini et al., 2012; Lin et al., 2013). As in plants, many parasite species have an amplified number of rhomboid proteases, suggesting complex biological roles (Sibley, 2013). That they can cleave a variety of substrates

has been shown clearly, as have some of the determinants of cleavage (Brossier et al., 2005; Baker et al., 2006). However, the genetics needed to define their role has been less conclusive, at least partly due to the practical difficulty of genetic analysis in these organisms. This is illustrated by the case of the best studied example, TgROM4 from Toxoplasma gondii which cleaves the apical membrane antigen 1 (AMA1) (Howell et al., 2005). When TgROM4 was knocked down by a dominant negative strategy it was concluded that it acted in parasite replication but not host cell invasion (Santos et al., 2011). In another approach, TgROM4 cleavage of AMA1 was prevented by mutating the rhomboid cleavage site; transgenic parasites in which the wild type AMA1 was replaced by this non-cleavable form were found to have impaired invasion but normal replication (Parussini et al., 2012). These two results cannot be easily reconciled: both point to the importance of TgROM4 cleavage of AMA1, but the biological significance remains to be discovered. Nevertheless, these current uncertainties about the precise roles of rhomboids in parasites ought not to obscure the primary reason that they have been extensively studied: the unusual enzymology of rhomboid proteases makes them promising targets that might represent therapeutic leads in the search for drugs against serious and widespread parasitic diseases.

Non-protease members of the rhomboid superfamily

As described above, the key to unlocking the biochemical, physiological and pathological significance of rhomboids was the discovery that they are a previously unknown class of intramembrane proteases. It is ironic, therefore, that the most exciting recent progress has occurred in the study of members of the rhomboid-like superfamily that are not proteases. Soon after the recognition of the rhomboid family of proteases, it became clear that there were members that lacked essential catalytic residues and could not, therefore, be active proteases (Koonin et al., 2003; Lemberg and Freeman, 2007). Their prevalence and widespread conservation strongly suggested that these ‘iRhoms’ (for inactive rhomboids) must have some function. More recently, in depth bioinformatic analysis of rhomboid-like proteins, coupled to the identification of highly conserved residues in polytopic proteins that only weakly resemble rhomboids, has led to the identification of the rhomboid-like superfamily, a quite diverse group of proteins that appear to have derived from a common rhomboid-like ancestor (Greenblatt et al., 2011; Finn et al., 2014). Not only do these include the rhomboid proteases and iRhoms, but also derlins and several other uncharacterised proteins (see Fig 1). The rest of this review will focus on what has

recently been discovered about the non-protease members of the rhomboid-like superfamily.

iRhoms

Of the non-protease rhomboid-like proteins, the iRhoms are both phylogenetically the closest to the rhomboid proteases and the most studied (Lemberg and Freeman, 2007). As with the rhomboids themselves (and a huge number of other biologically significant proteins), the first discovery of their function came from Drosophila genetics (Zettl et al., 2011). Genetic interactions implied that they counteracted the function of the active rhomboids by inhibiting EGF receptor signalling. This was shown to be caused by their ability to bind to rhomboid substrates in the endoplasmic reticulum (ER), in this case membrane tethered EGFR activating ligands, and promote their degradation before they were released from the signalling cell (Zettl et al., 2011). This degradation of growth factor proteins occurs by ER associated degradation (ERAD), a process in which ER proteins are 'retrotranslocated' from the ER back into the cytoplasm to be destroyed by the proteasome (Fig. 3). ERAD is primarily a quality control process that that removes misfolded proteins in the ER (Vembar and Brodsky, 2008), but Drosophila iRhom has evolved to exploit this fundamental cell biological process to regulate intercellular signalling (Zettl et al., 2011). Of course, the justification for using simple model organisms like Drosophila is that fundamental biological processes are conserved; so what do iRhoms do in mammals? Perhaps unsurprisingly, it was shown that, as in flies, mammalian iRhoms (of which there are two) can promote ERAD of EGF (Zettl et al., 2011), but the physiological significance of this remains uncertain because mouse genetics has highlighted a quite different but equally important function.

Mouse knockouts of iRhom2, which is expressed predominantly in macrophages, appear healthy but closer examination proved that they have profound immune defects. Most substantially, macrophages from these mice are unable to produce the primary inflammatory cytokine TNF, in response to challenge with lipopolysaccharide (LPS), the bacterial cell wall component that triggers many immune and inflammatory responses (Adrain et al., 2012; McIlwain et al., 2012). Further analysis revealed the underlying molecular and cellular explanation for this inflammatory defect (Fig. 4). TNF is synthesised as a transmembrane protein and the active extracellular cytokine is released from the TMD at the plasma membrane by the protease TACE (TNF alpha converting enzyme, also called ADAM17), itself a

membrane protein. In iRhom2 mutant macrophages, TACE cannot exit the ER, and therefore is not activated by removal of its inhibitory prodomain, nor can it get to the plasma membrane where it would normally process TNF. iRhom2 thus acts as an essential and specific TACE cargo receptor, promoting its exit from the ER (Adrain et al., 2012). Consistent with the failure of iRhom2 mutant mice to mount a normal inflammatory response (Adrain et al., 2012; McIlwain et al., 2012; Siggs et al., 2012), they are do not mount a toxic shock reaction to LPS and are hypersensitive to bacterial infection (McIlwain et al., 2012).

iRhom1 also specifically binds to and traffics TACE from the ER, but its expression is much wider than iRhom2 (Christova et al., 2013), and iRhom1 knockout mice have multiple defects and die soon after birth (Christova et al., 2013). TACE maturation was completely abolished in cells in which both iRhoms were mutated (Christova et al., 2013) or knocked down with siRNA (Issuree et al., 2013), leading to the conclusion that all TACE activity requires iRhom function (iRhom1 or iRhom2 or both, depending on cell type). iRhom1/2 double knockout mice die at around E10 in embryogenesis (Christova et al., 2013). This is significant because loss of TACE is less severe (Peschon et al., 1998), providing strong genetic evidence that iRhoms may have other clients beyond TACE, potentially extending their biological role.

The discovery that iRhoms are essential components of TACE activation, and therefore TNF release, has major implications and raises important questions. TNF inhibition is a successful therapeutic strategy in the treatment of multiple inflammatory diseases – indeed, TNF inhibitors are currently the world's largest grossing drugs. These inhibitors are mostly monoclonal antibodies and are therefore expensive and inconvenient. In principle, at least, a small molecule inhibitor that could block the interaction between iRhom2 and TACE would inhibit TNF release specifically from macrophages and might represent a cheaper drug with fewer side effects (as non-macrophage TNF would not be inhibited). In practice, there would be many major hurdles to surmount for this to be a realistic strategy, but it does illustrate the broad medical significance of identifying iRhoms as potential new targets. At a more fundamental level, high priority questions include the identity of the other iRhom clients implied by genetics; how iRhoms interact with the cellular machinery of trafficking; how their role in trafficking relates to their role in ERAD (which has only been shown to be important in Drosophila, but can be triggered by mammalian iRhoms); how they are regulated; and their physiological and pathological

significance in contexts other than inflammation. The importance of this last issue is highlighted by the multiple functions of TACE (Saftig and Reiss, 2010). Having discovered that iRhoms are needed for TNF release (Adrain et al., 2012; McIlwain et al., 2012; Siggs et al., 2012), it is no surprise that they are implicated not only in bacterial infection but also rheumatoid arthritis (Issuree et al., 2013) (which is TNF dependent). But TACE is also the enzyme responsible for the shedding of other cytokines, growth factors and adhesion molecules (Saftig and Reiss, 2010), so the extent and biological significance of iRhom involvement in a much broader variety of processes remains to be determined.

There are fewer insights into other questions about iRhom function. For example, how they engage with other ER machinery remains unknown: they act in a way that resembles specific ER cargo receptors but we know nothing about what this means at a molecular level; nor about how they interact with the components of ERAD. Even more fundamentally, there is no clear picture of how to reconcile their apparently distinct functions of promoting ERAD or promoting forward trafficking. It is possible that ERAD is only physiologically significant in Drosophila, but mammalian iRhoms can also do it (Zettl et al., 2011). A particularly interesting question is how iRhoms are regulated and, indeed, whether they represent a significant point of regulation of, for example, inflammatory or growth factor signalling. In support of this possibility, iRhoms are required for the indirect activation of EGFR signalling by GPCR stimulation, a process known as transactivation (Zou et al., 2009; Christova et al., 2013; Maretzky et al., 2013).

The relatively simplicity picture of the relationship between iRhoms, TACE and TACE substrate cleavage is challenged by a recent study in which the release of multiple TACE substrates was assayed in iRhom2 mutant embryonic fibroblasts (Maretzky et al., 2013). In these cells, mature TACE levels were unaffected (probably due to the presence of iRhom1) but nevertheless, the rapid, stimulus induced release of some TACE substrates was impaired. Thus, EGFR transactivation and the shedding of HB-EGF, amphiregulin and epiregulin was inhibited, while the shedding of TGFa was normal. This distinction between the induced shedding of some TACE substrates being affected and others not, also extended beyond the EGFR ligands. The cytoplasmic N-terminus of iRhom2 was shown to be involved in what was interpreted to be an iRhom2 function in determining substrate selectivity of TACE-dependent shedding (Maretzky et al., 2013). Although there is not yet a full molecular picture to explain these results, it is an intriguing idea that iRhom2 may have a role in

TACE regulation, beyond acting as a cargo receptor. It is possible, for example, that in binding to TACE, iRhom can modulate the interaction between the enzyme and substrate, although this speculative idea would imply that iRhom remains in complex with TACE after its activation.

More broadly, the regulation of iRhom activity and how they integrate into signalling pathways is not yet understood in much detail. It is tempting to speculate that the iRhom cytoplasmic domains may receive signals (e.g. from GPCR activity) and then translate those signals into modulating iRhom function. This general view of a possible role in regulated signalling is supported by the observation that iRhom2 can be phosphorylated in response to GPCR signalling in hepatocytes, and that this correlates with increased TACE-dependent shedding of the TNF receptor (Chanthaphavong et al., 2012). It is important to note, however, that none of this work yet provides definitive mechanistic insight into the process; for example, we have no idea what effect phosphorylation might have on influencing iRhom function. This is an exciting area and it seems likely that progress will be rapid.

The work described above casts a spotlight onto the cytoplasmic N-terminal domain of iRhoms, a strong candidate to be involved in receiving and/or transducing signals, as well as perhaps in modulating TACE substrate selectivity. This domain has also been highlighted by a very different set of experiments. Tylosis is a rare human autosomal dominant syndrome characterised by palmoplantar keratoderma, coupled with a late onset oesophageal cancer (Kelsell et al., 1996). The genetic cause of tylosis has now been shown to be mutations in the iRhom2 gene, and in all four families identified these are amino acid substitutions in residues 186-189 in the cytoplasmic N-terminus (Blaydon et al., 2012; Saarinen et al., 2012). The molecular cause of the syndrome is not yet clear, so the mechanistic significance of this tight clustering of mutations remains obscure, but it seems likely that it identifies a significant site of modification or interaction with another protein. Intriguingly, the same genetic interval has been associated with sporadic oesophageal cancer and also with breast and ovarian cancer (Iwaya et al., 1998; Fukino et al., 1999; Presneau et al., 2005; Wojnarowicz et al., 2012), although is not known whether the iRhom2 gene is responsible for this association with more common cancers.

Other non-protease members of the rhomboid-like superfamily

The iRhoms are the best characterised of the non-protease rhomboid-like proteins; they are also phylogenetically the closest to the rhomboid proteases. Of the more distant branches, none of which are predicted to be proteases, only the derlins have been studied in any depth. Their identification as possible members of the same superfamily was suggested by the recognition of a conserved pair of rhomboid-characteristic amino acids (WR) in their luminal domain (Lemberg et al., 2005), but was only confirmed by a thorough structure and function approach that proved that derlin topology was more similar to rhomboids than had been appreciated and that the rhomboid-like aspects of derlins were essential for their function (Greenblatt et al., 2011). Derlins were first discovered in yeast to be a essential for ER quality control (Knop et al., 1996; Needham and Brodsky, 2013). Their mammalian homologues were subsequently also found to be involved in ERAD (Lilley and Ploegh, 2004; Ye et al., 2004; Oda et al., 2006), although there are apparent differences in their function: yeast Der1 is primarily required for ERAD of luminal proteins, for example, while mammalian derlins 1-3 participate in ERAD of luminal and membrane proteins (Hampton and Sommer, 2012). Despite their importance, the actual role of derlins remains unclear. Their multiple TMD structure led to the proposal that they might be at least part of the elusive channel, through which proteins must be retrotranslocated prior to ubiquitination and degradation in the cytoplasm (Lilley and Ploegh, 2004; Ye et al., 2004). But there are also several other strong candidates for to be the channel (Hampton and Sommer, 2012), and the discovery that derlins are related to rhomboids, whose known structures are inconsistent with channel-like properties (Ha et al., 2013), tended to suggest that derlins instead had accessory roles in retrotranslocation. Very recently, the pendulum has swung back in favour of the possibility of derlins contributing the channel (Mehnert et al., 2014). It was shown that the yeast Der1 oligomerises (one solution to the question of how a derlin could form a channel), that mutations in conserved polar TMD residues blocks client dislocation, that clients crosslink to both the luminal and TMD regions of Der1, and that Der1 is located in close proximity to both Hrd3, the client receptor, and Hrd1, the cytoplasmic ligase. Beyond the obvious question of whether this is, indeed, the correct model of Der1 function, the question remains of whether this relates to the two other known functional intersections between rhomboid-like proteins and ERAD: iRhom promoting ERAD of EGFR ligands (Zettl et al., 2011), and the proposal that RHBDL4 might cleave ERAD substrates (Fleig et al., 2012).

The other identified members of the superfamily include UBAC2 which, significantly, is another protein associated with the ERAD machinery (Christianson et al., 2011), although nether its precise function is yet understood, nor how its ERAD association might fit with its other known role in regulating lipid droplet synthesis (Olzmann et al., 2013). The least known members are TMEM115, RHBDD2 and RHBDD3. TMEM115 is a Golgi protein whose loss has been associated with renal clear cell carcinomas (Ivanova et al., 2008). RHBDD2 is upregulated in breast and colorectal cancer and is an indicator of a poor prognosis in breast cancer (Abba et al., 2009; Lacunza et al., 2012; Canzoneri et al., 2013). A homozygous RHBDD2 mutation also cosegregates with disease in a family with autosomal recessive retinitis pigmentosa (Ahmedli et al., 2013). The cellular function of RHBDD2 is not known, although a recent proteomics study associated it with the unfolded protein response and cell stress (Lacunza et al., 2013). Very little has been reported about RHBDD3, beyond its possible association with pituitary tumours (Bahar et al., 2004). Recently, however, a role for RHBDD3 in inhibiting TLR3-inducued acute liver cell injury has been reported (Liu et al., 2013). Although the cellular details of this function are not yet clear, RHBDD3 was shown to promote the proteasomal degradation of DAP12, an adaptor protein that associates with several activating receptors on NK cells, thereby acting in a negative feedback loop to inhibit signalling.

Concluding remarks

When rhomboids were first discovered to be intramembrane serine proteases that regulate EGF receptor signalling in Drosophila it would have been impossible to predict the breadth of the functions associated with related proteins. At that stage, they were remarkable because they were the first members of a new class of unexpected proteases. Despite being the third family of intramembrane protease discovered (after site 2 protease and presenilin) they fairly quickly became the best understood mechanistically, mainly because they were the first to be structurally solved. In some ways, the progress in revealing the full range of the biological functions of the rhomboid proteases has been slower, perhaps because once the eureka moment of identifying them as intramembrane proteases had occurred, the questions of their physiological significance inevitably became a case by case process: we know it's a protease so we have to find its substrate and biological role. Nevertheless, there have been major insights of a more conceptual kind, for example the demonstration that intercellular signalling can be regulated by intramembrane proteolytic release of growth factors, the discovery of the existence and function of

mitochondrial rhomboids, the implication of rhomboids in the life cycle of apicomplexan parasites and, more broadly, a growing understanding of the likely medical significance of rhomboid proteases.

Probably the most important recent milestone in our understanding of the overall biological significance of rhomboids came from the twin discoveries of the proteolytically inactive members of the family, and that rhomboids actually belonged to a much wider superfamily that included many other more distantly related proteins. These two insights have hugely expanded the horizons of the field and has led to a series of issues that extend far beyond the initial questions that arise from the rhomboid proteases. This expansion is highlighted by the fact that, despite the phylogenetic evidence implying that the common ancestor of the superfamily was likely to be a protease, only one branch of the extant superfamily includes active proteases: they are in the minority in the clan (see Fig. 1).

So can we draw any conclusions about overall functional themes that link members of the entire superfamily? The simple answer is that our knowledge is too incomplete to make many firm statements. But there are some reasonable speculations. If it is correct that the whole superfamily evolved from an ancient intramembrane protease, then the link between rhomboid-like proteins and interactions with TMDs is ancient and likely to still be widespread. Indeed, our working model is that the interaction between an iRhom and its client protein mimics that of between a rhomboid protease and its substrate. That is, we suspect that client TMDs will bind to iRhoms at, or close to, the region equivalent to the active site of the proteases.

Several broad biological themes emerge from the study of the rhomboid-like superfamily. The first is the extent to which the cell biology of protein synthesis, maturation and trafficking turns out to be important for regulating signalling between cells. Intercellular signalling, for example by growth factors and cytokines, has largely been studied as a biochemical, physiological, or developmental problem and has therefore often been distilled into a series of schematic arrows linking biochemical steps. This perspective is valuable and informative but ignores the ‘cell biological’ regulation of signalling components, both in signal emitting and signal receiving cells. Perhaps of most relevance to the rhomboid-like superfamily, many signalling components are transmembrane proteins and, as discussed in this review, they can be subject to TMD cleavage and to regulated trafficking, compartmentalisation or

degradation by rhomboid-like proteins. For example, the Drosophila EGF ligands are held in the ER until active trafficking allows their exit to the Golgi, where they encounter the rhomboid proteases that will activate them (Lee et al., 2001); or iRhoms regulate the access of TACE to furin, the trans-Golgi network enzyme that removes its inhibitory prodomain (Adrain et al., 2012); or apicomplexan rhomboids appear to be delivered to the membrane in which their substrates reside as part of a tightly regulated developmental programme (Brossier et al., 2005); or, in a non-rhomboid-related example, the ER protein Unc93B1 regulates the trafficking of some of the Toll-like receptors (Kim et al., 2008; Lee et al., 2013).

Another broad principle that is highlighted by recent advances in the rhomboid-like family is the likely widespread significance of pseudoenzymes. These are proteins that are evolutionarily related to enzymes – usually derived from them – but which do not themselves have the enzyme activity associated with their cognates (Todd et al., 2002; Pils and Schultz, 2004; Adrain and Freeman, 2012). There have been few systematic studies of these kinds of 'dead enzyme', probably because they are often assumed to be evolutionary debris. But they are widespread – most enzyme families include them – as well as often being highly conserved. The case of iRhoms illustrates clearly how they have lost their original function but gained a new role, but one that it functionally related to the original enzyme function. The general significance of this idea that pseudoenzymes may well be a rich source of pathway regulators and thereby represent an interesting new set of targets for investigation, is strongly supported by analyses of the known cases (Todd et al., 2002; Pils and Schultz, 2004; Adrain and Freeman, 2012).

The appropriate conclusion about the emerging medical significance of the rhomboid-like superfamily is probably best described as 'watch this space'. There are no fully validated therapeutic opportunities ripe for immediate exploitation, but there are a very wide range of possibilities. For the active rhomboid proteases these already include the mitochondrial rhomboids and their likely role in Parkinson's Disease and type 2 diabetes (Chan and McQuibban, 2013); the apicomplexan rhomboids as possible targets for anti-parasitic drugs (Sibley, 2013); the potential involvement of mammalian rhomboids in regulating growth factor signalling and therefore possible relevance to cancer (Adrain et al., 2011); and the possible involvement of bacterial rhomboids in antibiotic resistance (Rather, 2013). This list will presumably grow substantially as we learn more about the biological functions of a wider range of rhomboids. It is worth noting that there are a growing number of new

classes of rhomboid inhibitors (Pierrat et al., 2011; Wolf et al., 2013), although none yet have the potency, selectivity and structure to be compelling leads for drug development. Nevertheless, the important principle of the drugability of rhomboids has clearly been established. The non-protease members of the rhomboid-like family may also have exciting therapeutic futures. Most prominently, the discovery that iRhoms control TNF release catapults them into the limelight in the anti-inflammatory sector. As discussed above, though, the iRhoms have much wider functions through their control of the multifunctional shedding enzyme TACE, not to mention the likelihood that they have other clients. It is too early to guess the medical significance of the more distant and less well understood rhomboid-like proteins but the precedents within the clan are strong, and there are strong incentives to learn more about these still rather mysterious proteins.

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Figure legends

Figure 1

Classification of the rhomboid-like superfamily. This scheme provides a general classification of extant members of the rhomboid-like clan, based on best current fucntional and sequence evidence, and is a useful framework for understanding their relationships. Importantly, this does not represent an evolutionary model. For example, derlins, UBAC2 and TMEM115 are present in yeast, whereas RHBDD2 and RHBDD3 are vertebrate specific.

Figure 2

Drosophila Rhomboids 1-3 activate EGF receptor signalling. Rhomboids 1-3 in Drosophila are located in the Golgi apparatus where they cleave the membrane tethered, immature form of Spitz, the Drosophila homologue of TGFalpha. Once released from the membrane, mature Spitz is secreted to activate the EGFR in surrounding cells.

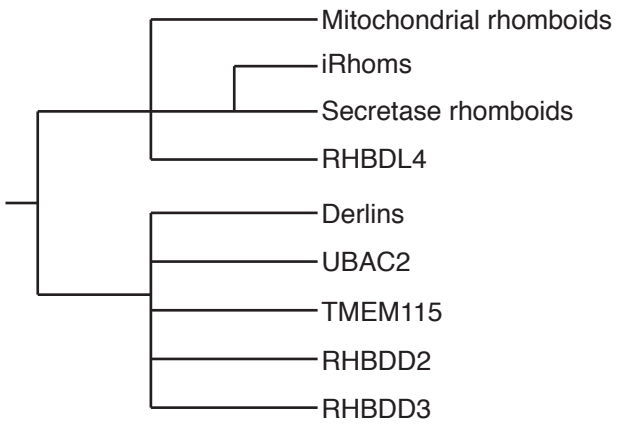
Figure 3

Drosophila iRhom promotes ERAD of EGF family ligands. iRhom binds to client proteins in the ER, thereby triggering their degradation by the ERAD machinery (blue). It is not clear whether how iRhoms interact with the essential components of ERAD, which are here depicted schematically and not to scale as a single retrotranslocation channel, but which in fact comprise multiple subunits that mediate recognition, retrotranslocation, ubiquitination, and proteasomal degradation.

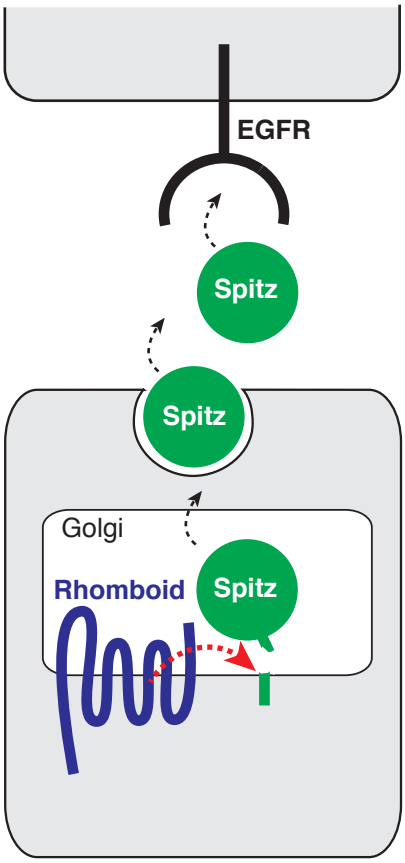
Figure 4

Mammalian iRhom is essential for release of the inflammatory cytokine TNF. iRhom is mainly located in the ER where it binds TACE and supports its onward trafficking to the Golgi apparatus (1). It is not known whether iRhom remains bound to TACE in the Golgi or is released (2); furin removes the inhibitory prodomain (red triangle) of TACE in the late Golgi (3). Active mature TACE is then released to the plasma membrane, where it can cleave and activate TNF (4).

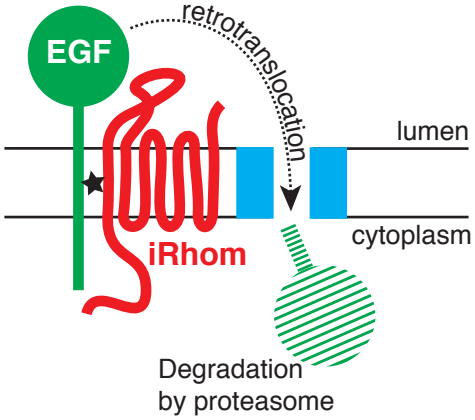
Freeman Figure 1



Freeman Figure 2



Freeman Figure 3



Freeman Figure 4