

## Inactive Rhomboid Proteins: New Mechanisms with Implications in Health and Disease

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## **Abstract**

Rhomboids, proteases containing an unusual membrane-integral serine protease active site, were first identified in *Drosophila*, where they fulfill an essential role in epidermal growth factor receptor signaling, by cleaving membrane-tethered growth factor precursors. It has recently become apparent that eukaryotic genomes harbor conserved catalytically inactive rhomboid protease homologs, including derlins and iRhoms. Here we highlight how loss of proteolytic activity was followed in evolution by impressive functional diversification, enabling these pseudoproteases to fulfill crucial roles within the secretory pathway, including protein degradation, trafficking regulation, and inflammatory signaling. We distil the current understanding of the roles of rhomboid pseudoproteases in development and disease. Finally, we address mechanistically how versatile features of proteolytically active rhomboids have been elaborated to serve the sophisticated functions of their pseudoprotease cousins. By comparing functional and structural clues, we divine common principles shared by the rhomboid superfamily, and make mechanistic predictions.

## **Key words**

Rhomboid pseudoproteases, endoplasmic reticulum-associated protein degradation, vesicular trafficking control, innate immunity, cancer, catalytically inactive enzyme homologs.

## **Abbreviations**

ADAM17, a disintegrin and metalloproteinase 17; COG, conserved oligomeric Golgi; Der1, degradation in the ER 1; Dfm1, Der1-like family member 1, DKO, double knockout; EGFR epidermal growth factor receptor; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERAD-R, regulatory ERAD; IRHD, iRhom homology domain; iRhom, inactive rhomboid; KO, knockout; L1, loop 1; RHBDD, rhomboid domain-containing; RHBDL, rhomboid-like protein; SELMA, symbiont specific ERAD-like machinery; SPP, signal peptide peptidase; SREBP, sterol regulatory element-binding protein; TNF, tumor necrosis factor; TACE, TNF $\alpha$ -converting enzyme; TM, transmembrane; TMEM, transmembrane protein; UBAC2, ubiquitin-associated domain-containing protein 2.

## 1. Introduction

Rhomboid proteases are conserved group of polytopic membrane proteins that cleave substrates within the plane of cellular membranes. First described in *Drosophila melanogaster*, Rhomboid-1 is a key activator of the epidermal growth factor receptor (EGFR) by cleaving its activating ligands within their transmembrane (TM) anchor, triggering their secretion, enabling signaling to neighboring cells [1]. Rhomboids actually fulfill diverse functions in all kingdoms of life, including: pro-peptide removal of the *Providencia stuartii* TatA protein translocase, host cell invasion by apicomplexan parasites, and protein degradation along the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway [2, 3]. The rhomboid active site is located within a conserved six-pass TM domain core (the 'rhomboid domain', Fig. 1). Some rhomboid homologs include additional TM segments and extended tails harboring diverse functional domains, fused either to the amino- or carboxy-terminus (Fig. 1) [4]. The crystal structures of the *Escherichia coli* rhomboid protease GlpG, solved to atomic resolution, revealed a serine-histidine dyad active site located, several ångstroms beneath the membrane surface, in the center of a six TM helix-bundle [5]. Other conserved features include the tryptophan-arginine (WR) motif, of important but unknown function, that contributes to the prominent L1 loop extending sideways into the upper leaflet of the lipid bilayer (Fig. 1).

In eukaryotes, several tightly clustered lineages of rhomboid homologues that lack key catalytic residues were identified, and are predicted to be proteolytically inactive [4, 6]. As we highlight below, the functions of these rhomboid pseudoproteases are as diverse and fundamental as their

active relatives. Our synthesis reveals principles concerning how rhomboid pseudoproteases functionally interact with protein clients in the membrane environment, and mechanistically how they relate to their active counterparts. For clarity, we define the ‘rhomboid superfamily’ as the universe of rhomboid homologues containing the six-TM rhomboid fold [5], and operationally subdivide these into ‘rhomboid proteases’ versus ‘rhomboid pseudoproteases’.

## **2. Mysterious inactive protease homologues**

Extensive sequence comparisons indicated that rhomboid proteases share certain features with the ERAD factor derlin [7]. Moreover, automated gene annotation by hidden Markov models, and BLAST searches, identified several more distantly related catalytically inactive rhomboid relatives amongst the entire eukaryotic kingdom including, fungi, plants and red algae [4, 6, 8-10]. Crucially, most genes encoding these mysterious ‘dead’ rhomboids are well conserved, indicating that they are not random mutants or pseudogenes [3, 11]. Indeed, all rhomboid pseudoproteases are expressed widely, supporting the notion that they have genuine and universal functions. We may reason, that these diverse set of pseudoenzymes evolved via several independent gene duplication events of different rhomboid proteases, followed by diversification, and loss of proteolytic activity [12]. The easiest way to envisage loss of proteolytic activity during evolution involves loss of the catalytic serine or histidine. However, certain iRhoms contain an intact catalytic dyad, but have acquired a conserved proline in the x-position of the ‘GxSG’ rhomboid protease active site consensus motif. This destroys the

active site geometry, making them inactive (Fig. 1) [4, 13]. Common amongst membrane protein families, a low sequence consensus between different lineages poses the question of whether all rhomboid homologues form a superfamily, or whether distinct groups better represent them. Since currently structural similarity cannot be demonstrated experimentally, the best evidence for a shared fold is the apparent conservation of the six-TM rhomboid domain, containing conserved key 'anchor' residues such as an Engelmann di-glycine helix-helix interaction motif (GxxxG) in TM segment 6 (Fig. 1). Homology modeling based on the *E. coli* GlpG rhomboid protease structure indicates that human Derlin1 and the *Schizosaccharomyces pombe* Dsc2 rhomboid pseudoprotease (see below) indeed form a rhomboid fold, with the characteristic L1 loop clamping a six-helix bundle [6, 14].

Overall, there is growing evidence that the diverse set of rhomboid homologues can be seen as a superfamily, implying potentially strong structural and functional parallels and potentially a shared mechanism in recognition of substrates and pseudosubstrates. However, this remains speculative until high-resolution structures of rhomboid pseudoproteases become available.

### **3. Trafficking control**

The rhomboid pseudoproteases comprise iRhoms, derlins and UBAC2, which localize to the ER; and RHBDD2, RHBDD3 and TMEM115, which localize to the Golgi apparatus and endosomes, respectively [3, 6, 11, 15-18]. As discussed below, an emerging theme is trafficking control, whereby the

interaction between a rhomboid pseudoprotease and its client enables a triage decision, trafficking, degradation, or compartmental signaling.

### *3.1 Pseudoprotease function in growth factor signaling and inflammation*

iRhoms are metazoan-specific rhomboid pseudoproteases that differ from their catalytically active counterparts in several respects [3, 4, 11]. First, the ‘vestigial’ catalytic site of iRhoms follows the ‘GPxx’ consensus, described above. Second, iRhoms contain an extended cytoplasmic amino-terminus that fulfills a regulatory role (see below). Third, iRhoms have a unique structure called the ‘iRhom homology domain’, a cysteine-rich globular domain, inserted within the L1 loop (Fig. 1) [4].

We will now contemplate how iRhoms relate to rhomboid proteases, using *Drosophila* iRhom as a reference point. Fly iRhom localizes to the ER and is expressed in the nervous system and brain [13]. iRhom deficient flies develop normally, but exhibit a pronounced defect in daytime activity. As mentioned above, rhomboid proteases control EGFR signaling in *Drosophila*. Specifically in the central nervous system, it modulates wakefulness. Interestingly, iRhom knockout (KO) flies exhibit a phenotype similar to that caused by overexpression of active rhomboids in the central nervous system [13]. Although the molecular mechanism remains to be defined, *Drosophila* iRhom fulfills a triage role by binding to EGFR ligands and passing them over to the ERAD machinery thereby limiting their availability to active rhomboids [13]. Hence, in the case of *Drosophila* iRhom, pseudoprotease function counteracts the role of active rhomboids, a common principle in pseudoprotease biology [11].

The functional relationship between active rhomboids and iRhoms is harder to decipher in mammals. The role of rhomboid proteases in the control of EGFR signaling is limited in mammals; instead, the metalloprotease ADAM17 (for 'a disintegrin and metalloproteinase 17', also known as 'TNF $\alpha$ -converting enzyme', TACE) cleaves multiple mammalian EGFR ligands, fulfilling an analogous role to *Drosophila* rhomboid [19]. However, in an evolutionary twist, mammalian iRhoms fulfill an indirect but essential role in EGFR ligand release [12, 20-22]. Mammalian iRhoms act as trafficking factors that escort ADAM17 from the ER to the later secretory pathway (Fig. 2A). iRhom mutant cells are defective in trafficking of ADAM17, resulting in the retention and inactivation of ADAM17 in the ER [12, 20-22].

Mammalian genomes contain two iRhoms that fulfill redundant, but also, specialized, roles in ADAM17 activation. As well as being the sheddase for EGFR ligands, ADAM17 has numerous substrates, including the inflammatory cytokine, TNF (tumor necrosis factor) [23]. Similar to ADAM17 KO mice, mice deficient for iRhom2 are defective in TNF release from myeloid cells. iRhom2 KO mice respond less severely to sepsis, are protected from experimental arthritis, but exhibit increased sensitivity to *Listeria* infection, hallmarks of TNF biology [12, 22-24]. iRhom redundancy is illustrated by the fact that iRhom single KO mouse embryonic fibroblasts retain substantial ADAM17 activity, whereas ADAM17 is inactive in double knockout (DKO) cells [20, 21]. The phenotype of iRhom1 KO mice and iRhom1/2 DKO mice themselves is controversial: a recent study reported that iRhom1 mice are aphenotypic [20], whereas a previous report found that iRhom1 KO mice exhibited a cachectic phenotype, defects in several professional secretory



tissues, and brain hemorrhages [21]. Concerning iRhom DKO mice, one study found that DKO embryos were lethal, whereas the later study reported perinatal lethality, similar to ADAM17 KO mice [20, 21, 25].

In addition to its importance in leukocytes, iRhom2 is required for ADAM17 activity in microglia and potentially in the liver [20]. Likewise, iRhom1 is expressed highly in many regions of the brain [20] potentially explaining the brain hemorrhages observed in ADAM17 KO mice and in one iRhom1 KO study [21, 26].

The implication of the rhomboid superfamily in innate immunity is not restricted to iRhom2. Mice deficient for the rhomboid pseudoprotease RHBDD3, exhibit spontaneous autoimmune disease, driven by defects in T cell homeostasis caused by hyperactivation of the inflammatory transcription factor NF $\kappa$ B, in dendritic cells [18]. Rather than a trafficking role, RHBDD3 localizes to endosomes, acting as a scaffold for ubiquitin-dependent recruitment of the NF $\kappa$ B pathway kinase IKK $\gamma$  to the deubiquitinating enzyme A20, enabling the latter to blunt IKK $\gamma$ -driven NF $\kappa$ B responses [18] (Fig. 2B).

### *3.2 Association of iRhoms and RHBDD2 with disease*

In spite of the implication of rhomboid pseudoproteases in processes connected to inflammation, autoimmunity and cancer, with the exception of iRhoms, evidence for an association with disease is currently lacking. As deregulated autocrine EGFR signaling drives tumors [19], it is interesting to note that iRhom2 mutations are causal for an autosomal dominant disease called Tylosis with esophageal cancer [27]. In several families, iRhom2 mutations [27] cluster within a conserved hotspot in the iRhom N-terminus.

Tylosis is characterized by keratinocyte hyperproliferation and affected patients develop oesophageal cancer in later life [27]. A potential explanation for this phenotype is that the iRhom2 mutants are gain of function mutants, thereby enhancing their ability to activate ADAM17, which subsequently increases release of EGFR ligands, a key proliferation and differentiation signal for keratinocytes. Consistent with this, tylotic keratinocytes display increased EGFR ligand secretion and have increased migratory and proliferative potential [28].

Finally, a point mutation in a region close to the cytoplasmic loop between TM helices 2 and 3 of another rhomboid pseudoprotease, RHBDD2 [16], has been identified in a familial form of retinitis pigmentosa, a disease causing progressive vision loss. Whether the disease is caused by a loss of functional RHBDD2 (which is expressed in mouse photoreceptors [16]), or is a secondary consequence of ER stress caused by ER retention of misfolded RHBDD2, remains to be ascertained.

### *3.3. Mechanistic roles of iRhoms*

We will now discuss the mechanism whereby mammalian iRhoms control ADAM17 trafficking. Notably ADAM17 binds to iRhom2, implying that its effect may be direct [12]. iRhom could modulate ADAM17 biogenesis in the ER, cargo recognition, or its trafficking through the secretory pathway. However, as ER exit is contingent on protein folding, it is not trivial to separate these roles. Sidestepping this, notably, purified ADAM17 from iRhom KO extracts can be activated by incubation with the pro-protein convertase responsible for ADAM17 maturation in the *trans*-Golgi (Fig. 2A) [12]. This argues that

ADAM17 folding largely proceeds normally, invoking the other mechanisms discussed above.

Another way to address function is to reflect upon the subcellular localization of iRhom, but this too is controversial: endogenous iRhom2 in primary macrophages has EndoH-insensitive glycans, suggesting that can traffic at least to the *medial*-Golgi [12]. On the other hand, overexpressed iRhom localizes variously to the ER, Golgi, and a fraction is found at the cell surface [13, 28, 29]. These observations provoke the question of whether iRhom associates with ADAM17 throughout its journey to the cell surface. Speculatively, the corollary of this is that ADAM17-iRhom complexes could exist for purposes beyond trafficking, such as an allosteric regulator, or substrate recruitment platform.

The identification of gain-of-function mutations in the iRhom N-terminus implies an important regulatory role for the cytoplasmic tail. This is interesting because membrane protein tails, including iRhom, contain sorting motifs that determine ER exit or retrieval. Hence, cofactor binding to the iRhom tail could govern its ER exit, and signals that impinge on ADAM17 trafficking [30] may control ER exit of iRhom. Notably, N-terminally truncated versions of iRhom promote ADAM17 activity more efficiently, as do the tylosic mutants discussed above [28], suggesting that the iRhom tail contains an autoinhibitory moiety. The structural basis for this, and how the putative de-repression is achieved, are unclear.

Given that some iRhom1 KO phenotypes are not explicable purely by ADAM17 biology [21] this could imply that additional iRhom clients exist. Notably, a genome wide screen identified iRhom1 as a stimulator of

proteasome activity [31] and iRhom1 has been implicated in regulation of the hypoxic transcription factor Hif1 [32]. Notably, mice null for Hif1 $\alpha$  exhibit vascular defects, as do ADAM17 KO mice [26].

#### *3.4. Emerging role of TMEM115 in retrograde trafficking*

Continuing the theme of trafficking control, we turn to TMEM115, a Golgi-localized rhomboid pseudoprotease [17]. TMEM115 knockdown cells exhibit a delay in experimentally-induced Golgi fragmentation, implicating TMEM115 in retrograde transport of COPI vesicles from the *cis*-Golgi to the ER [17]. This is significant because the retrograde machinery is crucial to maintain homeostasis of the secretory pathway, returning escaped ER-resident proteins from the *cis*-Golgi [33]. COPI vesicles also ensure spatial fidelity of Golgi-resident proteins, against a flux of anterograde movement [34].

The COG (conserved oligomeric Golgi) complex facilitates COPI vesicle tethering [35]. Notably, the cytoplasmic tail of TMEM115 interacts both with COPI vesicles, and the COG complex [17]. Similar to COG mutant cells, TMEM115 knockdown cells exhibit defects in O-glycosylation, consistent with mislocalization of glycosylation enzymes caused by defective retrieval mechanisms [17]. This implies an important general role for TMEM115 in retrograde trafficking, as a scaffold interconnecting COPI vesicles with other tethering components [35].

## 4. Role in quality control and protein dislocation

### 4.1. *Der1* defines a versatile safeguard of ER protein homeostasis

As the portal to the secretory pathway, the ER harbors a sophisticated quality control machinery that targets misfolded proteins and surplus protein subunits into ERAD (for recent reviews see [36, 37]). In a slightly modified manner, regulatory ERAD (ERAD-R) tunes the flux of natively-folded proteins by targeting them for degradation [37]. Similarly, certain viruses use the ERAD machinery to down regulate host cell factors such as to escape the immune response [38].

The first molecular insights into ERAD were obtained by a genetic screen in *Saccharomyces cerevisiae* that identified Der1 (for 'degradation in the ER 1') as the first component of the ERAD machinery [39]. Yeast Der1 is the founding member of a family called derlins, which as discussed below were recently recognized to be rhomboid pseudoproteases. Subsequently, genetic and functional studies revealed that Der1 interacts with ERAD scaffolding proteins including Usa1 (known as Herp1 in mammals), thereby eventually targeting ERAD substrates, via an unknown mechanism, for ubiquitination by the E3 ubiquitin ligase Hrd1 (Fig. 3A) [40-43]. Downstream of this reaction, a number of other factors mediate transit of ERAD substrates to the proteasome for final destruction [36]. The hexameric AAA+-type ATPase Cdc48 (known as p97 or VCP in mammals), and associated ubiquitin-binding proteins, are thought to provide the major driving force and directionality of these processes [37, 44]. In a related manner, the second yeast derlin protein Dfm1 (for 'Der1-like family member 1') is a key component of several ERAD complexes, functionally interacting with either Hrd1, the second yeast ERAD

E3 ubiquitin ligase Doa10 [45], or with the aspartyl intramembrane protease Ypf1 [37, 46]. Interestingly, whereas Der1 is required for turnover of soluble ERAD substrates but is not essential for degradation of TM proteins [39, 43, 47, 48], the few known Dfm1 clients are all membrane proteins [45, 46]. This indicates that the two yeast derlins define alternative branches of the ERAD pathway specific for distinct protein classes. However, in yeast not all ERAD substrates depend on Der1 or Dfm1 [43, 45, 49], suggesting that redundant degradation routes exist.

#### *4.2. What has identification of derlins as rhomboid pseudoproteases taught us about mechanism?*

Der1-like proteins are in fact conserved and play key roles in ER proteostasis in mammals. The first human Der1 homologue, referred to as Derlin1 (to indicate that it is Der1-like), was identified by probing for factors involved in degradation of MHC class I heavy chains by human cytomegalovirus [50, 51]. Despite clear evidence that derlins interact with aberrant polypeptides, directing them from the ER lumen, or the plane of the ER membrane, towards ubiquitylation and extraction [52-56], the exact molecular mechanism of dislocation is still debated. Whereas initially yeast Der1 was suggested to act as a receptor for ERAD substrates [39], subsequently human Derlin1 was hypothesized to form a protein-conducting dislocation channel [50, 51].

Our perspective on derlin mechanism recently became illuminated when, building upon previous observations [7], the Kopito lab noted that derlins are rhomboid pseudoproteases, providing a homology model for Derlin1 based on the *E. coli* rhomboid GlpG structure [6]. One important

consequence is that the architecture of human Derlin1 is now recognized to conform to the six TM rhomboid domain [6], rather than the 4 TM architecture previously assumed for mammalian and yeast derlins. In fact, confusion of topological architecture has hampered mechanistic studies on many rhomboid pseudoproteases [16, 17, 47, 52]; further biochemical and structural studies are required to resolve this discrepancy. Another important implication of the derlin-rhomboid interrelationship is that the protein-conducting channel hypothesis (mentioned above) becomes implausible. Although it is not entirely clear how rhomboid proteases interact with their substrates [57, 58], the rhomboid fold adopts a compact structure, undermining the likelihood that derlins form an aqueous ERAD membranous pore.

Importantly, the recognition of a common ancestry between rhomboids and derlins helps to rationalize several previous observations. Mutation of what are now recognized to be shared features, namely the 'WR' motif in the L1 loop and the 'GxxxG' helix dimerization motif in TM segment 6 (Fig. 1), block both, protease and pseudoprotease function [6, 7, 59, 60]. Likewise, the serine-59-leucine loss-of-function mutation encoded by the yeast *der1-2* allele found in the original genetic screen [39, 47], affects a conserved residue that derlins share with most rhomboid proteases [7]. These striking similarities in the L1 loop strengthen the hypothesis of structural conservation between secretory pathway rhomboids and derlins. This is important since it potentially indicates a shared mechanism for substrate/client recognition. Further supporting this hypothesis, the ER-resident rhomboid protease RHBDL4 combines features from both sides: ubiquitin-dependent recognition of ERAD substrates, and intramembrane protease activity (Fig. 3B) [61]. Intriguingly,

RHBDL4 is unique amongst rhomboid proteases in that it cleaves most of its substrates at various alternative positions, ranging from the TM region to luminal loops and ectodomains [61]. This is consistent with cleavage by RHBDL4 of ERAD substrates at different stages in the retrotranslocation journey through the membrane, and demonstrates a mechanistic collaboration between RHBDL4 and dislocation. It remains to be investigated whether this proteolytic feature is shared with other rhomboid proteases, or whether it represents a unique adaptation in RHBDL4 – a nexus between rhomboid proteolysis and ERAD.

#### *4.3. Physiological and pathological roles of mammalian derlins*

Whereas in yeast the ERAD pathway is mainly centered around two E3 ligases, mammals have an expanded set of E3s that form a complex protein network in concert with a wide range of ER quality control and ERAD factors [62]. This presumably allows flexible adaptation to the individual properties of misfolded proteins, to cope with proteostatic demands imposed by larger genomes, multicellularity, and longer life spans [36, 37]. This increased complexity is also reflected in three mammalian Der1 orthologs [62, 63]. Derlin1 and Derlin2 are expressed ubiquitously, whereas Derlin3 is restricted to the placenta, pancreas, spleen, and small intestine [63].

Given their importance in safeguarding the ER from misfolded proteins, this predicts that derlin KO mice should phenotypically resemble mutants in key sensors of the unfolded protein response, such as Ire1 $\alpha$ , PERK or ATF6 [64]. Hence, one would expect to observe defects in professional secretory cells and tissues including plasma cells, the pancreas, liver, and the secretory



cells of the intestinal epithelium [65-68]. Unfortunately, consistent with their ubiquitous expression pattern, knockout of Derlin1 [69] and Derlin2 [70] in mice results in early embryonic versus perinatal lethality, respectively, precluding discerning many of these anticipated phenotypes. In contrast, Derlin3 deficient mice appear normal [69]. Nonetheless, tissues from Derlin2 KO mice exhibit constitutive ER stress responses [70], highlighting Derlin2's physiological importance for ERAD. However, with the exception of skeletal abnormalities caused by secretory defects in chondrocytes, most professional secretory tissues are in fact normal in Derlin2 KOs [70]. Future work exploiting conditional Derlin1 mutant and Derlin1/2 double knockout mice will reveal their putative roles in ER homeostasis-associated diseases, viral infection, and other processes requiring retrotranslocation, such as antigen cross-presentation [71].

Concerning the association between derlins with cancer, although there is no causal link, conceptually, upregulation of ERAD may be important to fulfill proteostasis demands in cancer cells, which have elevated translational rates, and suffer hypoxic conditions, conducive to protein misfolding. Some evidence in favor of this comes from a study showing that tumor lines positive for the oncogene HER2 upregulate the ERAD machinery [72]. Crucially, cells unresponsive to HER2-targeting drugs are sensitive to ERAD inhibition, displaying the ERAD 'addiction' alluded to above. However, the opposite of this has been observed for Derlin3, whose expression is silenced in some colorectal cancer lines [73]. In fact, Derlin3 promotes ERAD of the glucose transporter Glut1. Tumor-associated epigenetic silencing of Derlin3 hence elevates Glut1 levels, allowing a shift in tumor cell metabolism,

from oxidative phosphorylation to glycolysis – a characteristic of cancer [73]. There is clearly much more to be learned concerning how derlins impacts on development, homeostasis and disease, but these examples illustrate their potential importance for proteostasis control. For similar reasons, although causal evidence is still lacking, one can envisage the implication of derlins in protein folding diseases of the secretory pathway, included inflammatory bowel disease and type II diabetes [74].

Although there are differences in the mouse KO phenotypes of individual derlins, it is not yet clear whether the three paralogs have different functions at the molecular level. Derlin1, the best characterized, serves a role in multiple arms of the ERAD network, dealing with both soluble and membrane-anchored substrates. First, Derlin1 serves as a key factor of the Hrd1 ERAD complex (Fig. 3A) [62, 75], which is commonly regarded as the major ERAD activity. In this canonical dislocation route, multiple quality control factors such as Sel1L, which mediates interaction with the luminal lectin OS9 and acts as substrate receptor for soluble ERAD substrates [76], or BAP31, a protein sorting factor for TM cargo [77], collectively deliver ERAD substrates to Derlin1. More recently, an alternative dislocation route centered on the E3 ligase TMEM129 has been shown to mediate human cytomegalovirus-induced MHC degradation of MHC class I [78, 79]. Furthermore, Derlin1 also contributes to non-canonical degradation routes, in conjunction with the intramembrane protease SPP (signal peptide peptidase) and the E3 ligase TRC8 [59]. When associated with SPP, Derlin1 interacts with an ER luminal portion of the type II membrane protein substrate XBP1u,

thereby targeting it for SPP-catalyzed cleavage and p97-independent degradation [37, 59].

Notably, derlins are also implicated in a so-called 'pre-emptive' ER quality control pathway. During ER stress, they reroute nascent chains that fail translocation and signal peptidase cleavage, from the signal recognition particle, to the cytoplasmic Bag6 complex and subsequently, the proteasome [80]. This result indicates that derlins provides a general interaction interface for aberrant TM proteins. In summary, derlins are versatile proteostasis regulators that have evolved multiple ways to interact with topologically diverse clients, via a variety of functionally distinct ERAD machineries.

#### 4.4. *UBAC2 and Dsc2*

Another rhomboid pseudoprotease that is predicted to bind ubiquitin via a conserved cytoplasmic, C-terminal UBA domain is UBAC2 (for 'ubiquitin-associated domain-containing protein 2') (Fig. 1) [6, 62]. While its phylogenetic relationship to derlins is unclear, its localization, physical and functional interaction with ERAD factors including the E3 ligase gp78, implicate it within the ERAD network [6, 62]. In a striking contrast, Dsc2, the homologue in the fission yeast *S. pombe*, Dsc2 localizes to the Golgi apparatus and is a component of a stable multi-protein E3 protein ligase complex implicated in activation of the SREBP (sterol regulatory element-binding protein) transcription factors (Sre1 and Sre2), key regulators of lipid biogenesis [14, 81]. On the other hand, human UBAC2 serves as an ER tether for the p97 adaptor protein UBXD8, controlling its trafficking from the ER to lipid droplets. In lipid droplets, UBXD8 represses the activity of adipose

triglyceride lipase, the key enzyme involved in triglyceride turnover. This implicates UBAC2 in control cellular lipid storage [15], suggesting that UBAC2 orthologs share a common role in integrating the cellular metabolic state by recruiting regulatory factors to polytopic E3 ubiquitin ligases. However, the respective metabolic pathways controlled by human versus *S. pombe* UBAC2 orthologs appear distinct, and precisely how UBAC2 and Dsc2 interplay with their clients remains to be investigated. Notably, UBAC2 has been identified, in genome-wide association studies, as a candidate gene for Behçets syndrome, a complex inflammatory condition of unknown etiology [82]. It is difficult to reconcile this with the cell biological observations mentioned above. One tentative possibility is that UBAC2 impacts on lipid droplet homeostasis in inflammatory cells, which utilize triacylglyceride as a precursor for the production of inflammatory lipid species called eicosanoids [83]. Clearly, the precise physiological role of UBAC2 in metabolic control (potentially ERAD-R) awaits knockout mouse studies.

#### *4.5. Variation of the theme: Protein import into complex plastids*

Further insights into the function of rhomboid family proteins in recognition of proteins at the membrane surface has recently been derived from so-called complex plastids of red algae, photosynthetic organisms that are thought have evolved via secondary endosymbiosis of two unicellular eukaryotes [84]. In the second outermost membrane of up to four distinct membrane layers forming these complex organelles, referred to as the periplastidal membrane, a symbiont specific ERAD-like machinery (SELMA) exists that specifically targets certain proteins from the ER lumen, to further traverse into the plastid

interior [9]. Like for higher plants, most plastid proteins are synthesized from nuclear genes and must be imported into the plastid. Depending on the sub-organellar destination, several membranes must be sequentially crossed. SELMA clients first have to be co-translationally translocated in a Sec61-dependent manner across the outermost plastid membrane, which is continuous with the canonical ER, and subsequently they are released from the ER lumen into the periplasmic compartment. This process topologically resembles protein dislocation into the cytoplasm and is mediated by a 480-kDa multiprotein SELMA complex. The complex consists of several ERAD-like components including two symbiont-specific derlins, known as sDer1 and sDer2, an E3 ubiquitin ligase, and the rhomboid protease ptsRhom3 [85, 86]. Consistent with a role of SELMA in protein translocation, two symbiotic copies of the AAA+-ATPase Cdc48 have also been identified [87]. An important distinction to ERAD, however, is that for SELMA translocation is uncoupled from degradation and the imported proteins either stay in the periplasmic compartment, or are further imported into plastid intermembrane space. Although like for ERAD, the molecular mechanism of SELMA is just beginning to unfold, these striking parallels highlight the unique role of rhomboid pseudoproteases and proteases as versatile protein interaction interfaces in the plane of the membrane.

## **5. Conclusions**

Loss of proteolytic activity during evolution has enabled rhomboid pseudoproteases to acquire fundamental additional roles within the secretory pathway, including protein translocation, vesicle tethering, triage, forward

trafficking, and acting as signaling scaffolds (Fig. 2 and 3). Many clues still remain to be unveiled. From the organismal perspective, we lack insights into the physiological role of UBAC2, RHBDD2 and TMEM115, as well as the tissue-specific roles of derlins.

A precedent from studies on pseudoenzymes is that they frequently act as allosteric regulators of their active counterparts [11]. This example is conspicuously absent here; perhaps the discrepancy will be resolved upon learning more about the physiological roles of rhomboid proteases themselves. Meanwhile, this provokes the question of whether the *mechanisms* of rhomboid pseudoproteases diverge radically from rhomboid proteases. iRhoms interact with their TM clients, suggesting that they use a vestigial ‘exosite’ for recognition [19, 58]. This feature, harbored within the rhomboid core, has been reported for active rhomboids [88, 89] and may dependent on dimerization. For fly iRhom, recognition precedes passing a client into ERAD, whereas for mammalian iRhoms, it directs the client protein’s vesicular trafficking. Notably, iRhoms and rhomboid proteases both recognize type I membrane proteins, suggesting broad similarities in the client recruitment interface. This contrasts with the ability of derlins and the ERAD rhomboid protease RHBDL4 to associate with a topologically heterogeneous variety of misfolded proteins, implying an intriguing exosite specialization.

Continuing on the theme of conservation of mechanism, we now address the L1 loop defined by a characteristic WR motif (Fig. 1), a functionally important structure conserved between active rhomboids and derlins. Structural data suggests that by supporting the flexible six-TM helix bundle it stabilizes the active rhomboid core [90], potentially allowing the

coordinated conformational rearrangements necessary for substrate recruitment, partial unfolding of the substrate surrounding the scissile peptide bond, and the proteolytic cycle. Extrapolating this to derlins predicts that the L1 loop stabilizes the rhomboid core to coordinate rearrangements necessary for client unfolding, or threading misfolded ERAD substrates through the bilayer. By contrast, the L1 loop of iRhoms contains a large insertion, the iRhom homology domain (Fig. 1). We await structural and functional studies to elucidate the impact that this disrupted L1 loop has on the iRhom core, and to establish the structure and role of the iRhom homology domain. Very speculatively, this could imply that iRhoms have repurposed the putative L1 loop 'unfoldase' function for something else, perhaps a protein-protein interaction surface. The implication that iRhoms may not be able to engage in client protein unfolding would place them in a class distinct from derlins and rhomboid proteases and frames the role of *Drosophila* iRhom in the triage process described above, rather than directly in ERAD. However, this is speculative, and the model also needs to incorporate the ability of symbiont-specific derlins in the SELMA complex to engage in protein translocation without predisposing clients to degradation. Whether SELMA derlins have lost 'unfoldase' function, or can engage in cycles of partial unfolding followed by refolding, remains to be addressed.

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## Figure Legends

### **Figure 1. Rhomboid family proteins share a membrane-integral core domain.**

Topology models for human rhomboid protease RHBDL2 and pseudoproteases iRhom2, Derlin1, UBAC2, RHBDD3 and TMEM115. The conserved six-pass TM 'rhomboid domain' is shown in blue. Structurally important features such as the 'GxxxG' TM helix-helix dimerization motif and the L1 loop are indicated. Additional domains including TM helices, the iRhom homology domain (IRHD), the p97/VCP-binding motif (VBM) and ubiquitin binding domains (UBA), are highlighted in red. For rhomboid proteases, the active site motifs 'GxSG' and 'H' form a catalytic dyad between TM helices 4 and 6. iRhoms have a 'GPxG' sequence in this position.

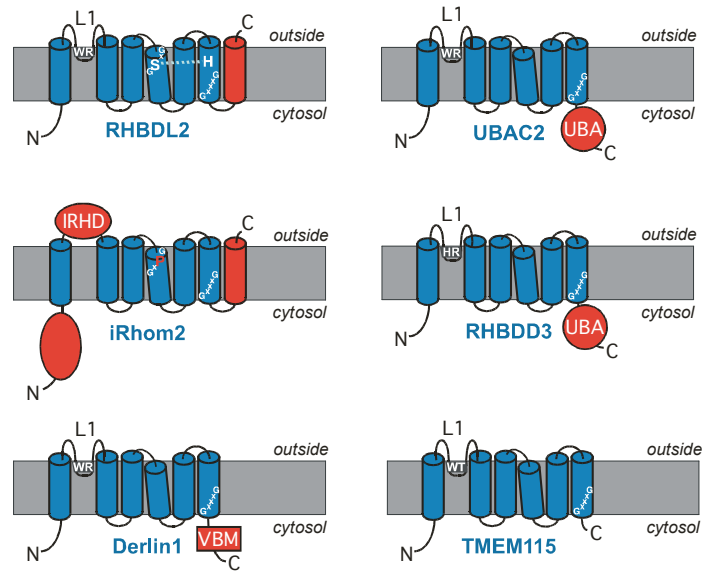
### **Figure 2. Rhomboid pseudoproteases control secretion dynamics.**

Models illustrating the proposed functions of mammalian iRhoms, TMEM115, and RHBDD3 in the secretory pathway. (A) Mammalian iRhom2 binds to ADAM17, mediating its trafficking from the ER to the Golgi apparatus, wherein it is activated by the pro-protein convertase furin (B) RHBDD3 localizes to endosomes. During inflammatory signaling, its UBA domain acts as a scaffold, bringing the NF $\kappa$ B pathway kinase IKK $\gamma$  in contact with the deubiquitinase A20, attenuating NF $\kappa$ B activation. (C) TMEM115 interacts with the COG complex, (indicated in green) and with COPI-coated vesicles, coordinating retrograde trafficking between the *cis*-Golgi and the ER.

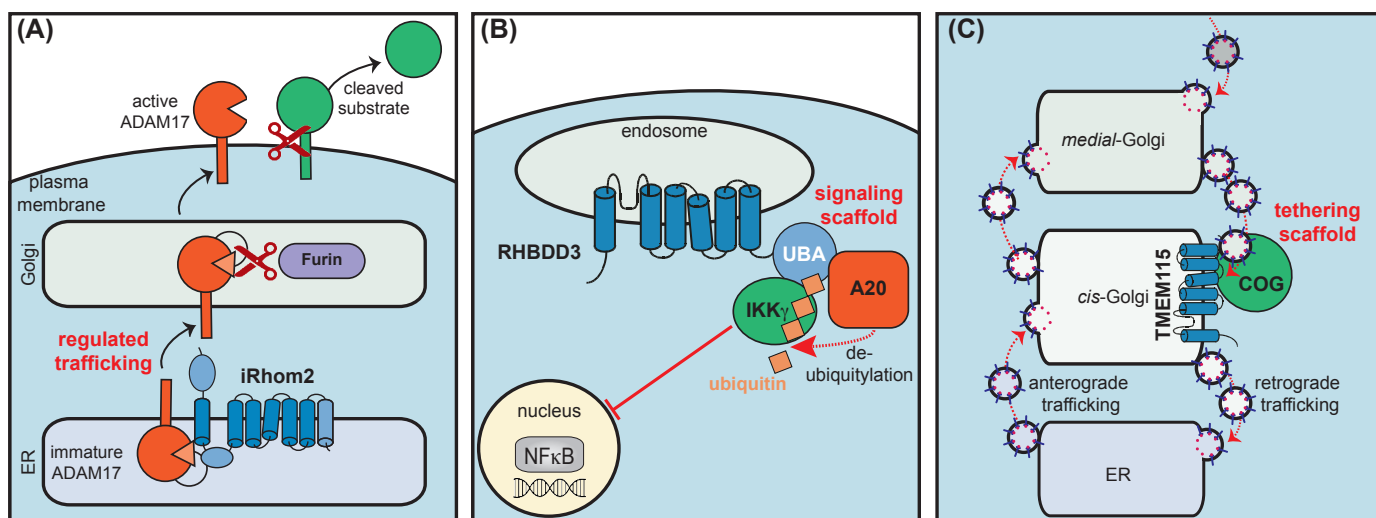
**Figure 3. Rhomboid family proteins are involved in ERAD.**

In the canonical ERAD dislocation pathway (A), misfolded proteins are recognized by the rhomboid pseudoprotease Derlin1 and substrate receptors such as OS9/Sel1L. The ERAD substrate is dislocated across the ER membrane through an unknown mechanism, then ubiquitylated by the membrane-tethered ubiquitin E3 ligase Hrd1. On the cytoplasmic side, the AAA+-ATPase p97 extracts the ubiquitylated substrate from the membrane, targeting it for proteasomal degradation. An alternative ERAD route (B) involves the rhomboid protease RHBDL4, which binds to ubiquitylated unstable membrane proteins by a conserved ubiquitin-interacting motif (UIM) leading to their cleavage within TM helices or luminal domains, and subsequent clearance along the ERAD dislocation pathway in a p97-dependent manner.

Lemberg and Adrain Fig. 1



Lemberg and Adrain Fig. 2



Lemberg and Adrain Fig. 3

