Rhomboid pseudoproteases are catalytically inactive members of the rhomboid superfamily. The founding members, rhomboids, were first identified in *Drosophila* as serine intramembrane proteases that cleave transmembrane proteins, enabling signaling. This led to the discovery of the wider rhomboid superfamily, a clan that in metazoans is dominated by pseudoproteases. These so-called rhomboid pseudoproteases inherited from their catalytically active ancestors a conserved rhomboid-like domain and a propensity to regulate signaling. Lacking catalytic activity, they developed new 'pseudoenzyme' functions that include regulating the trafficking, turnover, and activity of their client proteins. Rhomboid pseudoproteases have preeminent roles in orchestrating immune cell activation, antiviral responses, and cytokine release in response to microbial infection, or in chronic diseases, and have also been implicated in growth factor signaling, cancer, and, more recently, metabolism. Here, we discuss the mechanism(s) of action of rhomboid pseudoproteases, contrasted with rhomboid proteases. We also highlight the roles of rhomboid pseudoproteases in mammalian physiology, which, quite paradoxically among pseudoenzymes, is understood much better than active rhomboids.
deregulated EGFR activity. Overexpressed rhomboid-1 induced the recruitment of additional photoreceptors [2], an effect that was mapped, in genetic interaction experiments, to the epidermal growth factor receptor (EGFR) pathway [3]. Other groups working on different developmental aspects of the fly, including oogenesis [4] and wing development [5], came to the same general conclusion that rhomboid-1 mediated EGFR signaling by some undefined mechanism [6].

Later, in the early 2000s, Freeman’s group provided key mechanistic insights, identifying that rhomboid-1 was the founding member of the fourth class of intramembrane proteases, joining three established families of intramembrane proteases (gamma-secretase and SPP-like proteases, and site 2 metalloproteases). Freeman’s group demonstrated that rhomboid-1 proteolytically activated (cleaved) the EGFR (epidermal growth factor receptor) ligand Spitz (a homologue of TGF-α, a ligand of the mammalian EGFR) in the Golgi apparatus, from where it could be exported, be secreted, and activate signaling on nearby cells [7] (Fig. 1A). Mutational and biochemical studies by Sin Urban in the Freeman Lab first established rhomboid-1 as a serine protease that cleaves Spitz within its transmembrane helix (TMH) [6,8] (Fig. 1A). Unlike classical serine proteases, rhomboids use a serine and histidine catalytic dyad (rather than a catalytic triad) and their catalytic site was found to be located within the lipid bilayer. Hence, rhomboids defined a new family of intramembrane proteases [6–8]. In fact, rhomboid proteases are the most numerous family of intramembrane proteases [9], spanning all kingdoms of life and playing roles in a range of processes including mitochondrial dynamics in yeast, quorum sensing in bacteria, or infectivity of apicomplexan parasites, implying medical relevance in numerous different biomedical areas [10].

Later, in an unexpected twist, rhomboids were found to share some common features with derlins [11,12], membrane proteins lacking any predicted enzymatic activity, best known for their roles in endoplasmic reticulum (ER)-associated degradation (ERAD), a process in which proteins are retrotranslocated from the ER into the cytoplasm, for proteasomal degradation (Fig. 1B). The dawn of genomics ignited the identification of several tightly clustered eukaryotic lineages of catalytically inactive rhomboid-like genes: the rhomboid pseudoproteases in the mid 2000s [13]. Although bacterial genomes contain only genes encoding active rhomboid proteases, in fact, the majority of rhomboid superfamilies members, in eukaryotes, are pseudoenzymes [14]. Currently, 14 members of the mammalian rhomboid-like superfamily are known [15]: five rhomboid proteases (RHBDL1, RHBDL2, RHBDL3, RHBDL4, and PARL) and nine pseudo-proteases iRhoms 1–2, Derlins 1–3, UBAC2, RHBDD2, RHBDD3, and TMEM115 [13,16] (Fig. 2).

Pseudoenzymes are not pseudogenes, (i.e., the non-coding evolutionary remnants of genes or gene duplicates), but are instead the coding products from distinct
(often duplicated) genes [17]. Almost 20 classes of enzymes have been shown to have inactive counterparts [17], present in all kingdoms of life, with an estimated prevalence of 10–15% in a typical genome [17,18]. Similar to other pseudoenzymes, rhomboid pseudoproteases are highly conserved, implying strong selective pressure due to important biological roles [19].

Defining features of the rhomboid superfamily

The defining feature of the rhomboid superfamily is the rhomboid-like domain (Fig. 2), a core of six transmembrane helices (TMHs) with significant sequence conservation, whose structure was first revealed by studies on the bacterial rhomboid protease, GlpG [20]. Rhomboid proteases possess a catalytic dyad composed of the catalytic serine, embedded within a conserved motif (GxSxG) in TMH4, plus a conserved catalytic histidine in TMH6 (Fig. 2). Additional conserved features include a tryptophan–arginine (WR) ‘motif’ found in the L1 loop, and an Engelman helix dimerization motif (GxxxG) in TMH6 [15] (Fig. 2). Structural insights from the bacterial rhomboid protease
GlpG indicate that the rhomboid fold is primarily formed by five TMHs encircling the central TMH4, with the L1 loop, forming a helical hairpin that protrudes sideways into the lipid bilayer. Both the ‘WR’ and ‘GxxxG’ motifs help to stabilize the structure of the rhomboid domain [20,21].

Not all of the hallmarks mentioned above are strictly conserved across the wider rhomboid superfamily, and indeed, many rhomboid-like proteins have elaborations in addition to the rhomboid domain. For instance, many family members have an additional TMH [21] (added to either the N- or C-terminal side of the rhomboid core) and additional globular domains appended at the N and C terminus, or extended loop insertions (Fig. 2). As will be discussed below, these important domains can serve regulatory roles and confer new functions.

**Rhomboid pseudoproteases: evolution and new structural features**

Most (but possibly not all) pseudoenzymes evolved from ancestral enzymes [18,22,23], a conclusion reached through the evolutionarily traceable loss of key catalytic residues but also by the disruption of the active site conformation (i.e., a situation in which the catalytic residues are retained but activity is abrogated by the acquisition of a sterically unfavorable residue such as proline) [13].

The wide sequence divergence amongst rhomboid superfamilies makes it difficult to trace their phylogenetic relationships. However, it is clear that rhomboid proteases occurred first in prokaryotes, while Derlin1, TMEM115, and UBAC2 appeared in early eukaryotes. iRhoms arose later, in metazoans, while Derlins, TMEM115, and UBAC2 appeared in different eukaryotes and are closely related to rhomboid proteases. As noted above, iRhoms are the pseudoproteases most closely related to rhomboid proteases. This complex evolutionary relationship is best illustrated by the seemingly beguiling manner in which iRhoms antagonize EGFR signaling. As noted above, Drosophila rhomboid proteases promote EGFR activation by cleaving the EGFR ligand Spitz, uncoupling it from the membrane, allowing it to be secreted from cells as a soluble growth factor (Fig. 1A). Intriguingly, later studies revealed that in Drosophila, the single iRhom protein antagonizes EGFR signaling by recognizing EGFR ligands and then shunting them into a degradative pathway (ERAD, Fig. 1B).

Although the physiological and mechanistic roles of iRhoms will be discussed extensively in the sections below, in order to highlight some of the principles of rhomboid pseudoenzyme biology, we will briefly introduce the fundamentals of iRhom biology and compare and contrast this with active rhomboid proteases. As noted above, iRhoms are the pseudoproteases most closely related to rhomboid proteases. This complex evolutionary relationship is best illustrated by the seemingly beguiling manner in which iRhoms antagonize EGFR signaling.
pathway (by promoting the degradation of EGFR ligands, preventing EGFR signaling). This type of relationship is common theme within pseudoenzyme biology [19].

Moving next to mammalian iRhom[s], the scenario becomes considerably more baroque. Strikingly, in mammals, rhomboid proteases do not cleave EGFR ligands to promote EGFR signaling. Instead, members of the unrelated ADAM family (A disintegrin and metalloprotease) of membrane-tethered cell surface metalloprotease, most notably ADAM17 (also called TACE, for TNF-α-converting enzyme TACE) and ADAM10 [26,27], act as EGFR ligand sheddases, implying that the mammalian rhomboid proteases play no role in regulation of the EGFR. This apparent evolutionary inconsistency became more confusing when iRhom[s] were identified as key regulators of ADAM17/TACE (but not other ADAM family metalloproteases [28,29]). In brief, as summarized in Fig. 2B iRhom proteins are required for multiple steps in the regulation of ADAM17/TACE biology: They are required for the egress of ADAM17/TACE from the ER, which is coupled to a proteolytic maturation step (prodomain removal) that is catalyzed by proprotein convertases in the Golgi apparatus. As discussed below, iRhom[s] also form the central component, alongside ADAM17/TACE in the ‘sheddase complex’, an assemblage that controls the stimulation of cleavage of ADAM17/TACE substrates on the cell surface (Fig. 2B). Hence, in summary, through an ironic evolutionary twist, whereas rhomboid proteases directly promote EGFR signaling in Drosophila, iRhom[s] play an equally critical role in the promotion of EGFR signaling in mammals as key regulators of ADAM17/TACE.

**Repurposing of the classical enzyme fold: losing proteolytic substrates but gaining ‘client’ proteins**

The principle of a ‘client protein’ is an important one in pseudorhomboid biology. Where their catalytically active counterparts transiently recruit a substrate protein, then cleave, and release the cleaved products as part of the catalytic cycle, pseudoproteases no longer have this capability. Instead, they have acquired (or enhanced) the capacity to recognize and recruit so-called ‘client’ proteins to the pseudoenzyme core. This capability has been co-opted to fulfill a range of roles, described in detail below.

An iRhom (or pseudorhomboid) client protein could have one of two major conceptual origins. First, it is tempting to speculate that for iRhom proteins, EGFR ligands may represent ancestral ‘pseudosubstrates’, since they are proteolytically cleaved by active rhomboids. Extending this nomenclature assignment, to be correct, it would imply that such ‘pseudosubstrates’ are recognized by rhomboid pseudoproteases using the same architectural features within the rhomboid core that rhomboid proteases use to recognize their substrates. A second class of client protein would be an accessory molecule that was never actually proteolytic substrate of an active rhomboid.

 Interestingly, the conserved proline residue predicted to alter the geometry of the rhomboid fold, the lack of the WR domain, and the sheer size of the IRHD (260aa long), suggest that the functions of iRhom[s] and rhomboids have clearly diverged, and by extension, that the rhomboid domain architecture might potentially be different [15], perhaps to accommodate client proteins or indeed accessory proteins with different binding properties (Fig. 3). In fact, while rhomboid–substrate interactions are weak in nature [30], a critical feature of iRhom biology is the ability to form a stable complex with at least some of their client proteins, most notably ADAM17/TACE (Fig. 3B) [29,31–33]. This suggests either that an alternative rhomboid-like domain conformation is exploited by iRhom[s], or that a different binding site recruits client proteins, distinct from that which would have been occupied by proteolytic substrates in ancestral active rhomboids (Fig. 3). As noted above, this has important implications for whether client proteins of rhomboid pseudoproteases are ‘pseudosubstrates’, or are accessory proteins that have an altogether different relationship and mode of binding to rhomboid pseudoproteases.

Thanks to rhomboid protease enzyme–inhibitor structural studies (on bacterial rhomboids), we know rather a lot about the structural basis for rhomboid substrate recruitment to the rhomboid domain. These studies suggest that TMH2 and TMH5 engage with the substrate TMH at an exosite (i.e., a substrate recruitment site distinct from the catalytic site) that facilitates access to the catalytic center [21,34] (Fig. 3A), while the L1 loop can interact with the extramembranous parts of client proteins [35] (Fig. 3A). Unfortunately, the field still awaits equivalent key high-resolution structural information on iRhom[s] (which carries the challenge of expressing recombinant forms of metazoan iRhom[s]), so our understanding of iRhom/client relationships is necessarily more speculative. Nonetheless, by contrast, molecular simulations suggest that TMH1 of iRhom2 binds to ADAM17/TACE [36], a region distinct from the substrate recruitment conduit defined by TMH2/TMH5 within rhomboid proteases. Intuitively, this implies either that ADAM17/TACE did not evolve from
being a proteolytic substrate of an ancestral rhomboid protease, but was recruited as an accessory protein. To take this assumption to a conclusion would require testing the hypothesis that by contrast, EGFR ligands, which can also be recruited to iRhom proteins [25,37], are recruited via the TMH2/TMH5 conduit [21]. This would suggest a scenario whereby iRhoms evolved as regulatory platforms to recruit accessory molecules (e.g., ADAM17/TACE) into proximity with pseudosubstrates (e.g., EGFR ligands, Fig. 3C).

The initial findings that fly rhomboids cleaved EGFR ligands, which are type I transmembrane proteins (Fig. 1A), and the subsequent discovery that the iRhoms bind to type I transmembrane protein clients proteins including EGFR ligands and ADAM17/TACE (Fig. 1A), [16] rooted the belief that the ancestral rhomboid fold is a dedicated sensor of single-pass TMHs and that rhomboid pseudoproteases repurposed their ancestral rhomboid fold into new ‘pseudoenzyme’ functions. However, it is now abundantly clear that both single-pass [38,39] and polytopic [40,41] membrane proteins can serve as clients for the rhomboid-like family (Fig. 4). 

In fact, several lines of evidence suggest that other features beyond the rhomboid core also determine client binding (Fig. 4A). For instance, a mutant form

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**Fig. 3.** Rhomboids and iRhoms promote cleavage of EGF-family ligands. (A) In active rhomboids, an exosite mapping roughly to TMH2, TMH5, and the WR motif in the L1 loop (green asterisk) mediates client loading into the active site (red asterisk). (B) iRhoms inherited the capacity to bind EGF ligands. This suggests an exosite of similar architecture (green asterisk) is used for client loading, with contributions from TMH2, TMH5, and the IRHD. iRhoms forms stable complexes with ADAM17/TACE, possibly through a different exosite, with contributions from TMH1 and IRHD (orange asterisk). After loading, iRhom will decide its client’s fate, for instance, regulating retrotranslocation into the cytoplasm versus ER exit to the Golgi (where the inhibitory prodomain is removed, yellow triangle). Once on the cell surface, ADAM17/TACE is only able to cleave its substrates after stimulation-induced recruitment of 14-3-3 proteins to the phosphorylated iRhom2 cytoplasmic tail (N-term). Stimulation triggers ADAM17/TACE dissociation from iRhom2, allowing proteolytic release of substrates (yellow asterisk). (C) In an alterative model, iRhom2 serves as a platform for ADAM17/TACE engagement with its substrates that upon stimulation undergoes a conformational switch that brings ADAM17/TACE catalytic center (yellow asterisk) and client TMH closer together in the iRhom luminal cavity (see Fig. 5B), allowing cleavage.
of the ADAM17/TACE substrate TGF-α (a ligand for the EGFR) lacking the ectodomain fails to interact with iRhom1 [37]. Moreover, the ADAM17/TACE ectodomain might also interact with the iRhom2 IRHD (Fig. 4H). Mutant iRhom2 lacking the IRHD fails to promote ADAM17/TACE maturation, due to an inability to bind immature ADAM17/TACE in the ER [33]. Concordantly, ADAM17/TACE ectodomain conferred some degree of binding to iRhom2, although much less than TACE TMH, in domain swap experiments with ADAM10, the closest relative of ADAM17 [32].

Bifunctional proteases and adaptive conflict as a driver of pseudoenzyme evolution

The assumption that pseudoenzymes acquire new functions following the loss of catalytic activity in a catalytic ancestor is potentially unsatisfactory. It requires assuming that evolution tolerates a period of time after loss of catalytic activity but before novel functions were elaborated, when the pseudoenzyme concerned effectively had no or minimal functions. One way in which this paradox can be reconciled is in the concept of ancestral enzyme that served two functions: its canonical role as a protease, combined with a nonproteolytic, ‘pseudoenzyme-like’ role. Notably, ‘pseudoenzyme’ functions can be encoded within the rhomboid fold of active rhomboid proteases [41,42]. This was recently shown by the Strisovsky Lab for the Bacillus subtilis rhomboid protease YqgP [41]. A mutant form of YqgP that lacks catalytic activity can promote degradation of the magnesium transporter MgtE, by serving as a substrate adaptor for a downstream (and unrelated) FtsH AAA protease, in a primordial ERAD-like activity [41]. In fact, several other enzyme families are known to harbor ‘pseudoenzyme’ functions [17].
Hence, the most primordial known rhomboid protease function is in ERAD-like activities [42]. Bacterial [39,41] and eukaryotic rhomboids [38,40] assist in the quality control of unstable membrane proteins, for example, orphan T-cell receptors by RHBDL4 [38] (Fig. 1B) and orphan respiratory chain components by RHBDL4 [40]. Pseudoproteases can also serve as adaptors for downstream ERAD-like activities during quality control of misfolded proteins (most notably Derlins, Fig. 4B) and to regulate the abundance of functional clients (Derlins, UBAC2, iRhom2, Fig. 4C–F). Overall, these observations suggest that the core rhomboid-like domain possesses conserved, yet currently unknown structural motifs, beyond those required for catalysis, that act as sensors of misfolded clients or of the abundance of functional clients, serving as adaptors for downstream degradative processes. It is conceivable that within the context of more specialized dislocation machinery in the ERAD pathways of eukaryotic cells, the protease activity of some rhomboid-like proteins (e.g., Derlins) became secondary and indeed eventually irrelevant for its adaptor function, explaining why pseudoproteases are so abundant within the superfamily [41,42].

The model described above, of bifunctional enzymes that unite catalytic and noncatalytic functions, provokes the obvious question of what evolutionary forces drive the process whereby gene duplications result in a bona fide pseudoenzyme. One model that can explain this is ‘adaptive conflict’, whereby an ancestral enzyme that unites both functions results in a trade-off in the limitations of efficiency of either role. Hence, gene duplication enables the enzymatic and nonenzymatic properties to be separated, resolving the adaptive conflict and allowing optimization of both roles [43].

This model does not preclude the possibility of subsequent tuning or enhancement of pseudoenzyme functions. Indeed, as noted above, upon loss of catalytic activity, pseudoenzymes often ‘repurpose’ their substrate-binding capacity to regulate ‘pseudo’-client trafficking and activity, or to compete with active enzyme counterparts within their ‘ancestral’ signaling pathways [17]. Enzymes often function in higher-order complexes, with conformational switches and allosteric regulation, a property that has been maintained by the ‘repurposed’ pseudoenzyme fold, as direct modulators (activators or inhibitors) of related or distinct families of enzymes, or serving as scaffolds that nucleate signaling [44]. Many of these repurposed functions have parallels in the rhomboid superfamily. For instance, iRhoms regulate the activity of both related and unrelated enzymes: They control an unrelated protease ADAM17/TACE [29,32,33,45,46] by multiple regulatory mechanisms (Fig. 4H), and in Drosophila, iRhom regulates active rhomboids, using a mechanism of substrate competition to deplete EGFR ligands in the ER (Fig. 1B) that serve as substrates for Drosophila rhomboids in the Golgi [31] (Fig. 1A). Rhomboid pseudoproteases can also form docking platforms that nucleate, or interfere with signaling events, but this seems to be an acquired function related to their cytoplasmic extensions, rather than a repurposing of the rhomboid-like domain, for example, the cytoplasmic tail of iRhom2 and iRhom1 (Fig. 4H,J) or the RHBDL3 UBA domain (Fig. 4I).

Paradoxically, iRhoms can promote either ER exit or retrotranslocation from the ER back into the cytosol (Fig. 1B). How to reconcile these seemingly opposing roles is currently unclear. While we know little of how they interact with the trafficking machinery, both rhomboids (RHBDL4) and their pseudoprotease counterparts interact with ERAD factors and ERAD client proteins (Fig. 4B–F; see section on ‘Protein turnover’). Some of these interactions could relate back to an ancestral membrane-buried exosite that mediates transient interactions with TMHs, before delivering them to the catalytic site (e.g., active rhomboids) or passing them to the ERAD machinery (rhomboid pseudoproteases) (Fig. 3A,B, green asterisk). Speculatively, by contrast, forward trafficking could be enabled by an altered exosite (Fig. 3B, orange asterisk), which mediates more stable interactions with its client TMH.

Until this point, we have addressed how rhomboid pseudoproteases relate back to active rhomboids both in structure (Figs 2 and 3) and in function (Figs 1 and 3), and how they relate more broadly to the relatively new field of pseudoenzyme biology.

In the following sections, we will explore their mechanism of action, including (but not limited to) how, or if at all, they interact with ERAD and trafficking machinery, and their roles in mammalian physiology.

**Functional and mechanistic roles of rhomboid pseudoproteases**

In the last couple of years, numerous reports have identified novel pseudoprotease interactors [47]. It is tempting to categorize them as ‘clients’ when they have (e.g., ADAM17/TACE binding to iRhoms), and ‘regulators’ when they are cytoplasmic proteins (e.g., FRMD8/iTAP binding to iRhoms). However, this is not always the case, and pseudoproteases can exert an effect on, or conversely, be regulated by, cytoplasmic
proteins (Fig. 4A). In the paragraphs that follow, we will highlight how the mechanisms of action are dependent on their interactions, some seemingly stable, others more transient, in response to signaling events, within multiprotein complexes. These functional roles can be crudely conceptually subdivided into protein turnover, trafficking, and regulation of client activity (Fig. 4), each of which is dealt with below.

**Trafficking regulation**

iRhoms form a stable complex with ADAM17/TACE from the point of their biogenesis in the ER and throughout the secretory pathway *en route* to the cell surface [32,33,45,46]. In reflection of this essential interaction, this complex has been termed the 'shed-dase complex'. This tight interaction serves a dual purpose, it allows ADAM17/TACE to traffic out of the ER to the Golgi apparatus, where the inhibitory pro-domain is removed [29]; on the cell surface, iRhom2 prevents ADAM17/TACE from cleaving its substrates, until activating signals are received (Fig. 3B).

Colocalization and co-immunoprecipitation experiments indicate that iTAP (iRhom tail-associated protein, also known as FRMD8) is a third constitutive component of the sheddase complex that loads unto the complex in the ER and remains there throughout the sheddase complex's journey to the plasma membrane, where iTAP/FRMD8 stabilizes the complex (Fig. 4H). In the absence of iTAP/FRMD8, the sheddase complex is aberrantly sorted into the lysosome and degraded [45,46].

STING (stimulator of interferon genes) is an essential adaptor for the induction of type I interferons in response to DNA viruses. During infection, STING associates with the β-subunit of the translocon-associated protein complex (TRAP β). This enables the trafficking of STING out to the ER, to the Golgi apparatus and perinuclear microsomes. During the process, TBK1 and IRF3 are recruited to STING complexes leading to the activation of type I interferon responses [31]. In close analogy to its effects on ADAM17/TACE, iRhom2 is required for the trafficking of STING out of the ER. Microscopy experiments suggest they colocalize in the ER, and in response to DNA virus infection they are translocated as a stable complex via the Golgi apparatus, to perinuclear microsomes. Mechanistically, iRhom2 binds to and recruits the β subunit of the TRAP–translocation complex to STING, facilitating STING’s ER exit [31] (Fig. 4G). A recent report by the Blobel group, showed that ADAM17/TACE but not STING, regulates the overall stability of iRhom2 (but not iRhom1) [48]. Adding to the fact that pro-ADAM17 can exist in the absence of iRhom2, but requires iRhom2 to be converted into its mature [29,48,49], these results support a model in which pre-existing pro-ADAM17 assembles with newly synthesized iRhom2 in the endoplasmic reticulum (ER), allowing both to exit the ER toward the cell surface.

As discussed above, it is generally accepted that the distinctive function of the rhomboid-like fold is in recognition of specific client TMHs [47]. Domain-mapping experiments have shown that TMH1 of iRhom2 (i.e., the same TMH as required for binding to ADAM17/TACE) is required for the interaction with STING (containing four TMHs), TRAPβ (one TMH), and VISA (one TMH), whereas soluble domains of these clients appear not to play an important role in binding to iRhom2 [31,32,50]. Moreover, an iRhom2 mouse mutant called *sinecure*, harboring a point mutation in the first TMH (I387F), is sufficient to block ADAM17/TACE maturation, possibly due to impaired binding to the ADAM17/TACE TMH [36,51]. In summary, evidence suggests that client TMH interactions with iRhom2 TMH1 are required for trafficking of the iRhom2 sheddase complex and iRhom2-STING-TRAPβ complexes.

As illustrated in Fig. 3, it has been proposed that an ancestral rhomboid exosite (green asterisk in Fig. 3A, B) mediates weak iRhom interaction with EGFR ligands, while TMH1 (orange asterisk, Fig. 3B) mediates a stronger interaction with ADAM17/TACE [21]. This remains to be established by structural studies, but the studies on STING (Fig. 4G) further implicate TMH1 in mediating more stable iRhom–client interactions necessary for trafficking purposes. Whether an equivalent motif exists in active rhomboids for the recruitment of accessory molecules remains to be defined, but notably, the rhomboid protease YqgP can interact with its accessory protease FtsH, providing evidence for such a phenomenon [38].

Of note, soluble appendages also affect trafficking of iRhoms and their client proteins. In addition to the cytoplasmic tail that recruits iTAP (Fig. 4H), mutant iRhom2 lacking the IRHD fails to promote ADAM17/TACE exit from the ER (thereby preventing ADAM17/TACE maturation), due to impaired interaction with immature ADAM17/TACE in the ER [33] (Figs 2B and 4H).

Other rhomboid pseudoproteases have also been implicated in the trafficking regulation of client proteins. For instance, UBAC2 tethers the ERAD factor UBXD8 to the ER, preventing its trafficking to lipid droplets, where it represses lipolysis mediated by adipose triglyceride lipase (ATGL) [52]. Interestingly,
UBXD8 does not contain a TMH but rather possesses an amphipathic alpha-helix that allows it to associate with the outer leaflet of the ER. This highlights that the rhomboid core has an adaptable capacity to recognize and bind to multiple classes of membrane and soluble proteins.

Moreover, TMEM115 is proposed to regulate retrograde trafficking from the Golgi to the ER [53], a key process that ensures spatial distribution of cis-Golgi and ER-resident proteins [53]. In summary, rhomboid pseudoproteases regulate the subcellular localization of proteins directly, by binding to proteins with a single TMH (ADAM17/TACE), multiple TMHs (STING), or no TMH (UBXD8). Furthermore, soluble domains and interactors (iTAP) also regulate the subcellular fate of clients (ADAM17/TACE) (Fig. 4H).

While it is still unclear how they interact with trafficking machinery, their localization to the secretory and endocytic pathways [15,16,45,46], and, as we have recently showed [32], their compartment-specific roles (at least in the regulation of ADAM17/TACE; see Figs 2B and 3B) suggest that further intriguing functions await discovery.

**Protein turnover**

Consistent with a predominant ER localization [16] and induction by ER stress (iRhom1 [54], iRhom2 [55], Derlin 1, Derlin 2, Derlin 3, and Ubac2 [56]), several rhomboid pseudoproteases are involved in ERAD, primarily a quality control mechanism to remove misfolded proteins from the ER, and ERAD has been co-opted by Drosophila iRhom (Fig. 1B), and various other pseudorhomboids (Fig. 4C–F) to control signaling, by shunting EGFR ligands into ERAD.

Derlin 1, the best characterized Derlin paralog [15,57], was hypothesized to form a retrotranslocation channel, potentially connecting misfolded proteins in the ER lumen to the proteasomal degradation machinery in the cytosol, because it is a multipass membrane protein that interacts with ERAD factors on both sides of the membrane [58,59] in the classical HRD1 pathway (Fig. 4B) and noncanonical ERAD routes, for example, to control signaling from the mediator of the unfolded protein response XBP1 [60] (Fig. 4C).

However, the subsequent finding that Derlins adopt a compact rhomboid fold precluded the possibility of channel formation [12], which instead might be conferred by the E3-ligase HRD1 [58,61] (Fig. 4B). Notably, this debate was resolved by a recent cryo-EM structure of the yeast ERAD-L complex, required for the dislocation of substrates with luminal misfolding lesions. It shows that the yeast homologues of Derlins (Der1) and HRD1 (Hrd1) form two hydrophilic ‘half-channels’, composed of cytosolic and luminal cavities, respectively, and lateral gates facing one another [62] (Fig. 5A). While the structure critically lacks a bound ERAD substrate, photocrosslinking experiments with an ERAD-L substrate further support a model for substrate dislocation across the Der1-Hrd1 complex.

The luminal hydrophilic cavity of Der1 (analogous to the active site in rhomboids themselves) (Fig. 5B) receives luminal misfolded substrates from the sensors Hrd3/Yos9 (Fig. 5A). The substrate is then stabilized as a hairpin that is hydrophilic on both sides by the membrane-buried lateral gates of Der1 (TMHs 2 and 5) and Hrd1. The membrane at the cytosolic site between the lateral gates is particularly thin, due to a cytosolic cavity formed by Hrd1, but also by hydrophilic residues within TMH2 of Der1 on the TMH2. These membrane thinning features are believed to decrease the energy barrier for dislocation of a hydrophilic substrate.

Overall, the yeast Der1 structure provides new structural clues that might give mechanistic insights for the wider rhomboid family. It confirms that rhomboid pseudoproteases can adopt a rhomboid fold, very similar to that of *Escherichia coli* rhomboid GlpG, with the lateral gate closed (i.e., TMH2 and TMH5 in close contact). Interestingly, while the GlpG luminal cavity is clamped by the loop between TMHs 5 and 6, in Der1 (Fig. 5B), this cavity seems to be perpetually open, as the loop is substantially shorter. It is plausible that this luminally exposed cavity might be present in other rhomboid pseudoproteases known to interact with client luminal domains (Derlins and iRhoms) and that other rhomboids involved in ERAD (Derlins, Ubac, iRhoms) might harbor ‘half-channel’ and membrane thinning properties for dislocation with HRD1.

UBAC2 is ER-localized, has a conserved ubiquitin-associated (UBA) domain (Fig. 2) binds to the VCP/p97-binding protein UBXD8, and has been shown by proteomics and functional genomics to promote ERAD of model substrates as part of the GP78-ERAD pathway [56]. Although its precise role is unclear, it affects how substrates are delivered to the ER-ligase GP78 for ubiquitination and appears to have a Derlin-like role [16,56]. Recently, it has been shown that UBAC2 and GP78 form an ERAD-like complex with limb region 1–like gene (LMBR1L) [63]. LMBR1L binds and delivers Frizzled-6 (a transducer of Wnt signaling) to the UBAC2-GP78 for ubiquitination and protein degradation, resulting in decreased WNT signaling (Fig. 4E). The significance of this pathway in Ubac2 KO mice remains to be established.
As noted above, the single fly iRhom promotes the ERAD of EGFR ligands [25] (Fig. 1B). A recent development seems to support a role for iRhom2 in the regulation of ERAD, but within the context of viral infection, with iRhom2 acting as an antagonist rather than a promoter of ERAD, shielding the viral response adaptors STING and VISA (virus-induced signaling adaptor, also known as MAVS) from degradation by ERAD-like activities. iRhom2 recruits the deubiquitinase EIF3S5 to STING, to remove K48-linked poly-Ub chains (markers for proteasome degradation [64]), ensuring protection from ERAD [31] (Fig. 4F). As for MAVS/VISA, iRhom2 protects it from ubiquitination by the E3-ligase RNF5 and subsequent engagement with the classical ERAD factors GP78 and VCP/p97. Co-immunoprecipitation experiments suggest that iRhom2 associates with RNF5 and VISA, as well as GP78 and VCP/p97 (but not HRD1). Furthermore, in a function akin to the role of Drosophila iRhom as a promoter of ERAD, iRhom2 drives RNF5 self-association, auto-ubiquitination, and degradation by ERAD. While the molecular sequence of events and what ERAD factors bind iRhom2 directly is yet to be fully understood, the results are consistent with a model whereby iRhom2 promotes RNF5 degradation, which prevents VISA from being delivered to
GP78 and VCP/p97 (Fig. 4D). These data also imply that iRhoms are in proximity to the ERAD machinery and that, depending on an as yet unclear set of governing rules and contexts, they can promote or repress ERAD of specific proteins.

The studies on MAVS/VISA and STING currently represent the most detailed analysis of how mammalian iRhoms interact with the ERAD network. However, it is fair to conclude that there is still not enough mechanistic detail to reconcile the seemingly opposing roles of iRhoms in the ER, where they can both promote and protect a client from being degraded by ERAD, and also promote their forward trafficking (Fig. 1). EGFR ligands and VISA have a single-pass TMH (Fig. 1A), while STING is a multi-pass protein (Fig. 4F), precluding any obvious generalization on the importance of client topology in this decision.

RHBDD3 has also been implicated in the turnover of client proteins, but the precise mechanism is unclear. This endosome-localized pseudoprotease promotes the degradation of the type I membrane protein signaling adaptor DAP12, during activation of T cells by poly I:C [65–68]. In line with the argument that rhomboid pseudoproteases evolved new ‘pseudoenzyme’ functions, conferred by acquired extramembranous domains, iRhoms have also been implicated in the turnover of cytosolic proteins. iRhom1 promotes proteasome biogenesis, increasing the cellular capacity to promote the turnover of soluble proteins (see below). In addition, it prevents ubiquitination of the transcription factor HIF-1 by ubiquitin E3 ligases (Fig. 4J) [54]. In keratinocytes, iRhom2 apparently interacts with and regulates the turnover of cytosolic Survivin, Keratin 16, and Cytoglobin, but the mechanism is unknown [69].

In summary, many of the reported roles for iRhom proteins allow us to envisage a theme around the regulation of trafficking or degradation within the ER. However, beyond the general sense that iRhoms regulate client protein stability and interact with the ubiquitin–proteasome system, a clear mechanistic understanding of many of these mechanisms is unfortunately currently lacking. This precludes any generalization concerning which pseudoprotease features are required to control a client protein turnover and indeed how such features interface with signaling pathways and the trafficking or degradative machinery.

**Regulation of client activity**

In addition to regulating client trafficking and turnover, pseudorhomboids can also more directly impact on client activation and enzymatic activity. NEMO (NF-kappa-B essential modulator, also known as IKKγ) is the regulatory subunit of the IKK kinase complex that regulates NFκB signaling. RHBDD3’s UBA domain binds K27-linked poly-Ub chains within NEMO-Lys302. This mediates the recruitment of the deubiquitinase A20, thereby suppressing NEMO’s activity by removing K63-linked polyubiquitin chains. Consequently, this results in decreased NFκB activity, and hence, IL-6 production in LPS stimulated dendritic cells [67] (Fig. 4I). Of note, unlike K48-linked chains which are associated with proteasomal degradation, K63 and K27 chains are nondegradative [64,70].

Substantiating the emerging theme that pseudoproteases evolved new cytosolic regulatory domains, iRhom1 has been implicated in the regulation of the activity of cytosolic proteins (RACK1 and PAC1/2) by interfering with their dimerization state. The hypoxia-inducible factor-1α (HIF-1α) enables cellular growth under low oxygen tension [71]. iRhom1 has been proposed to stabilize HIF and promote its transcriptional activity, by inhibiting the formation of RACK1 dimers, required to target HIF-1 for ubiquitination by E3 ligases [71,72] (Fig. 4J). Under conditions of ER stress, iRhom1 promotes heterodimerization of the proteasome assembly chaperones PAC1 and PAC2, increasing proteasome assembly to facilitate degradation of soluble substrates (e.g., mutant Huntingtin) [54].

Perhaps the best-established role for iRhom proteins is in the regulation of the cell surface metalloprotease, ADAM17/TACE, as introduced above. In the ER, iRhoms form a stable complex with ADAM17/TACE, promoting its forward trafficking to the Golgi apparatus (where prodomain removal occurs) and then onwards to the cell surface (Figs 2B and 3B). Here, iRhom2 is believed to prevent ADAM17/TACE from cleaving its substrates, until activatory signals are received [32,33] (Fig. 3). Stimuli that activate ADAM17/TACE (e.g., GPCR and TLR agonists) act via MAPK family members (ERK, JNK, and p38) which phosphorylate iRhom2, thereby promoting the binding of 14-3-3 proteins, most predominantly to phospho-Ser60 [32] within iRhom2. Recruitment of 14-3-3 promotes dissociation of ADAM17/TACE from the sheddase complex, which now becomes fully capable of cleaving its substrates on the plasma membrane [32,33] (Fig. 3B).

It is possible to conceive of an alternative model whereby iRhoms act as a platform for the simultaneous engagement of ADAM17/TACE and its ligands (Fig. 3C). In this model, iRhom2 would undergo a conformational switch upon 14-3-3 binding that would
bring ADAM17/TACE and EGF ligands in close proximity. In this model, the EGFR ligand accesses the iRhom2 luminal cavity through the TMH2-5 lateral gate (i.e., potentially through an exosite conserved with active rhomboids) (Fig. 3C; see also Fig. 5B), just like in active rhomboids (Fig. 3A) [16]. As for ADAM17/TACE, it (or at least its catalytic site) would be brought into close proximity with the EGFR ligand in the iRhom2 luminal cavity, enabling substrate cleavage (Fig. 3C). This structural rearrangement might confer an overall weaker affinity of ADAM17/TACE for the iRhom2 site involved in the recruitment of accessory molecules (TMH1) [21], which manifests as a decreased interaction by co-immunoprecipitation. Either model is plausible (Fig. 3B, C) and requires further investigation. Using the iRhom2/ADAM17/TACE sheddase complex as a model, it is possible to envisage that many other subcellular specific roles might exit for the rhomboid superfamily, which may indeed be subject to regulatory themes that are loosely analogous to mechanisms that regulate active rhomboids or their catalytic cycle.

Physiological roles of rhomboid pseudoproteases

Since they lack catalytic activity, it is unsurprising that historically, dead enzymes have been overlooked. Paradoxically, however, we know much more about the physiological roles of mammalian rhomboid pseudoproteases than we currently do of their active counterparts.

Inflammation and tissue damage

The release of TNF by innate immune cells such as macrophages is a key driver in the inflammatory response to infection or injury; however, when unresolved it leads to tissue damage. TNF orchestrates the rapid recruitment of other immune cells and the production of other cytokines and is produced as a membrane-tethered form that can be cleaved by ADAM17/TACE. The first reports of iRhom2 KO mice revealed an inflammatory defect. iRhom2-null macrophages failed to release TNF, due to retention of ADAM17/TACE in the ER and a consequent failure for ADAM17 to undergo prodomain removal within the Golgi apparatus. These mice exhibited hallmark of defective TNF biology: protection from the pathogenic effects of TNF in sepsis (they survive otherwise lethal dose of LPS) and impaired clearance of bacterial infection (*Listeria monocytogenes*) [29,49]. Since then, several studies have substantiated a role for myeloid iRhom2 in pathologies with a strong TNF-driven inflammatory component, most notably chronic inflammation and autoimmunity (obesity [73], colitis [74], rheumatoid arthritis [75], hemophilic arthropathy [76], lupus nephritis [77]) but also tissue damage and repair (fibrosis [77,78], hepatic steatosis [79], ischemic lung injury [80], myocardial infarction [81,82], cardiac damage in obesity [55]), and speculatively also bone homeostasis by osteoclasts, a macrophage-derived skeletal cell type [83].

The ability of iRhom2 to be a friend or a foe (e.g., promote sufficient inflammation to clear a pathogen versus trigger chronic inflammatory disease; or promote inflammation via TNF versus tissue repair via EGFR signaling) critically depends on its regulation of ADAM17/TACE and, very broadly, its capacity to influence inflammatory signaling via the TNF axis or growth factor signaling, via the EGFR. The pathophysiological effects of iRhom2 therefore depend on which substrates ADAM17/TACE cleaves (e.g., TNF, TNFRI, TNFRII, HB-EGF), and indeed which signal-sending versus signal-receiving cell types are involved [47,84]. For example, in a model of surgically induced liver damage, iRhom2 plays a protective role by promoting the ADAM17/TACE-dependent shedding of TNFRI, which reduces TNF signaling and liver fibrosis, an effect that was attributed to iRhom2 expression in hepatic stellate cells [78]. In the autoimmune disease systemic lupus erythematosus (SLE), the EGFR and its ligand HB-EGF are heavily implicated as promoters of kidney damage and fibrosis. Reduced HB-EGF shedding and EGFR signaling in the kidney contributes to the protection of iRhom2 KO mice from a mouse model of SLE [77]. It remains to be clarified whether the source of HB-EGF was myeloid or nonmyeloid cells within the kidney. Conditional KO mice will be needed to dissect nonmyeloid roles for iRhom2 in the complex relationship between tissue damage, inflammation, and repair. Another critical issue, into which several laboratories including the Blobel group are making inroads, concerns whether and specifically how iRhom proteins influence inflammatory signaling via the TNF axis or EGFR signaling) critically depends on its regulation of ADAM17/TACE substrates [85,86].

RHBDD3 fulfills an essential role in immunity, not in the macrophage, but in two major leukocyte populations: natural killer (NK) cells and dendritic cells (DCs). RHBDD3 suppresses production of IL-6 by DCs in response to LPS stimulation, by inhibiting NEMO, a regulatory subunit of the NFkB pathway [67] as mentioned above (Fig. 4I). Loss of this brake on IL-6 production leads RHBDD3 KO mice to develop autoimmune diseases characterized by defects in T-cell homeostasis (increased Th17 T cells,
decreased Tregs). RHBD3 also suppresses the activation of natural killer cells in response to poly I:C (polynosinic–polycytidylic acid, an agonist of TLR3), by promoting the degradation of the signaling adaptor DAP12. Loss of this inhibitor of TLR signaling sensitizes RHBD3 KO mice to poly I:C-induced liver damage, as they fail to control NK cell activation and accumulation in the liver, where they cause acute inflammation [65].

While the physiological role of UBAC2 awaits development of KO mice, it has been associated with Behçets syndrome, a human autoimmune disorder, and it forms a complex with LMBRL1 (Fig. 4E), whose KO mice develop lymphopenia due to aberrant activation of WNT signaling in T cells, causing apoptosis.

**Metabolism**

Mice lacking iRhom2 are protected from the metabolic complications associated with obesity, including weight gain, dyslipidemia, and insulin resistance, when challenged by a high-fat diet (HFD) [73,87]. They burn excess fat more efficiently than WT controls, by increasing thermogenesis in brown adipose tissue (BAT) and by promoting white adipose tissue beiging, a phenomenon whereby so-called brown beige adipocytes which have a higher thermogenic potential by virtue of their expression of the key thermogenic gene, UCP-1 (uncoupling protein-1), are recruited within white adipose tissues. Cultured iRhom2 KO brown adipocytes exhibited increased UCP-1 and mitochondrial proton leak, indicating that the effect on thermogenesis was cell-autonomous. Interestingly, other studies have reported opposing effects on weight gain and insulin resistance [88], highlighting how the roles of iRhom2 in metabolism might be dependent on yet unidentified environmental inputs, that can vary between mouse facilities (e.g., microbiota).

Continuing the theme of metabolic stress, iRhom2 loss conferred protection from obesity-induced cardiac damage [55], an effect that relates to less macrophage activation and inflammation in response to lipid overload. UBAC2 is also implicated with lipid metabolism. HeLa cells lacking UBAC2 have more numerous and larger lipid droplets, as noted above, due to increased trafficking of UBXD8 to lipid droplets where it represses lypolysis [52]. The study of UBAC2 KO mice is required to clarify weather this mechanism is physiologically important.

**Cancer**

A connection to most of the known hallmarks of cancer can be established for rhomboid pseudoproteases, most notably iRhom2 [69,89–96] and iRhom1 [47,71,97–99] but also RHBD2 [100–102] and Derlin 1 [103–106] (Fig. 6). In cancer cell lines, iRhom1 has been reported to activate important and diverse cancer signaling pathways, such as WNT [98], HIF-1 [71], and EGFR signaling [99] as well as tumor xenograft growth [97]. However, only for iRhom2 there is enough supportive evidence from mouse genetics and human disease to implicate it in cancer [89–93,107]. Mutations in iRhom2, which were identified by the Kelsell Lab to affect a conserved region within the cytoplasmic tail of iRhom2, are the cause of a rare autosomal dominant disease called ‘Tylosis with esophageal cancer’ (TOC). Affected individuals have palmoplantar hyperkeratosis (skin thickening) and a higher risk of developing esophageal cancer [89,90,92]. Mutant TOC iRhom2 is proposed to confer a ADAM17/TACE gain-of-function phenotype to keratinocytes, resulting in increased EGFR-ligand shedding, migration, and proliferation [89–93]. Notably, iRhom2 has also been shown to regulate other clients that could contribute to esophageal cancer development (Fig. 6). Survivin, an anti-apoptotic gene, is stabilized by the iRhom2 TOC mutant, conferring resistance to radiation induced cell death in TOC skin [69,108]. Cytoglobin, an antioxidant gene, is destabilized, conferring susceptibility to oxidative stress [69,108]. TOC iRhom2 also interacts with and stabilizes Keratin 16 [69,108], a stress-inducible keratin that facilitates keratinocyte proliferation and migration. Hence, through is various clients, iRhom2 regulates proliferation, migration, and survival, leading to hyperkeratosis (skin thickening) and cancer in TOC patients [69,90,108] (Figs 6 and 7). Whether mutations of iRhoms are drivers in other (common) forms of cancers remains to be fully established.

**Epithelial barrier homeostasis**

Epithelial barriers such as the skin, the gut, and the airways are a critical defense against physical or microbial insults. EGFR signaling, which requires the cleavage and release of its cognate EGFR ligands by ADAM17/TACE, promotes epithelial repair, and maintains mucosal and skin barrier integrity [109]. A deregulated barrier function results in bacterial translocation and inflammation, and is seen as an important contributor to inflammatory disorders such asthma and colitis [110], and to a common side effect associated with EGFR inhibition in cancer patients, an inflammatory skin rash, accompanied by increased susceptibility to *Staphylococcus aureus* infections [74]. In contrast to ADAM17/TACE deficiency, iRhom1 or
iRhom2 KO mice have no barrier defects at homeostasis, presumably due to redundant roles of iRhom1 and iRhom2 in the regulation of pathways that control epithelial regeneration and integrity [110]. Earlier onset and more severe colitis develops in iRhom2 KO mice, on a susceptible genetic background (IL-10 KO mice), correlated with increased intestinal epithelial permeability and early colonization by potentially pathogenic bacteria (E. coli and Bacteroides acidifaciens) [74]. Once again, reflecting as noted above the differential importance and roles of ADAM17/TACE in control of repair versus inflammation, barrier integrity was not compromised due to defective EGFR-ligand shedding and epithelial repair, but rather by a strong pro-inflammatory Th1 immune response, caused most likely by loss of myeloid TNF, which known to suppress Th1 responses during bacterial infection [111].

What we have learned about iRhom2 in skin homeostasis does not come from global KO mice, which have an overtly normal skin barrier function [69], but instead from TOC disease, and other iRhom2 gain-of-function mutants, believed to confer a dominant effect over endogenous iRhom2 (and iRhom1) proteins [75,86]. Human TOC skin, as discussed before, is thicker with keratinocyte hyperproliferation [69,108]. This can be explained by cell-autonomous roles of iRhom2 in keratinocytes, where it activates crosslinking enzyme transglutaminase-1 (TGM1) in the granular layer of the epidermis, downstream of ADAM17/TACE /EGFR signaling [93]. In fact, cultured human TOC keratinocytes are resistant to adhesion and invasion by S. aureus, consistent with iRhom2 activation being barrier protective [93]. The skin response to injury is also affected by iRhom2, as shown by deregulated wound healing in mice harboring iRhom2 alleles (TOC and N-term deletions) thought to act as ADAM17/TACE gain-of-function mutations [47,92].

**Viral infections**

Most nucleated cell express type I interferons, essential cytokines that limit the spread of viral infection [112]. Interferon responses are induced after viral recognition
Fig. 7. Upstream and Downstream of iRhom1s. Interactors, signaling molecules, diseases, and mutations can all serve as ‘Inputs’ that regulate iRhom1s via multiple mechanisms. Through multiple clients, iRhom1s regulate several signaling pathways and cellular processes ‘Outputs’. Positive effects are denoted by ‘+’ sign, arrowheads, and green text, while negative effects are denoted by ‘−’ and red text. IC, immune complexes; CQ, chloroquine; BAF, bafilomycin; GoF, gain of function; CAFs, cancer-associated fibroblasts. See main text and references [29,31,46,47,55,75,79,95,107,108,118–120] for details.
Pseudorhomboids are themselves targeted by viral encoded proteins, implying that they are crucial for the outcome of infection. The protein pp71, expressed from the UL82 gene of the DNA virus human cytomegalovirus, binds iRhom2 (and also STING), preventing assembly of a STING/iRhom2/TRAPβ translocation complex and activation of antiviral responses (Fig. 7) [96]. Derlin 1 is hijacked by the US11 gene product of human cytomegalovirus, to induce ERAD of MHC class I, preventing antigen presentation, hence contributing to viral immune evasion [106].

**Pathophysiological roles of other pseudorhomboids**

Derlins (Derlin 1, Derlin 2, and Derlin 3) were well-described mammalian ERAD factors, before being recognized as rhomboid pseudoproteases [11-13,15]. While Derlins are central to the degradation of a large subset of ERAD clients (Fig. 4B,C), redundant Derlin-independent ERAD routes exist [15,56]. Derlin 2 KO mice die perinatally, with signs of constitutive ER stress, consistent with a role in ERAD [113]. However, with the exception of a chondrocyte secretory defect, most professional secretory tissues, which would be expected to be the most sensitive to defects in ER homeostasis, are normal in Derlin 2 global KO mice [113]. It is currently unclear whether this reflects redundancy for Derlins in ERAD since Derlin 3 KO mice have no overt phenotype while Derlin 1 KO mice are embryonic lethal [16], or a limited physiological role for Derlins in ERAD. A recent study supports a role for Derlin 2 in Schwann cell pathophysiology [114]. These cells are highly secretory, producing key secreted proteins including myelin protein 0 (P0). Mutations in P0 induce its misfolding, induction of ER stress, and are the underlying cause of the human demyelinating Charcot-Marie-Tooth 1B (CMT1B) neuropathy, one of the most common genetic disorders of the peripheral nervous system. Specific ablation of Derlin 2 in Schwann cells worsened the demyelinating phenotype of a mouse model of CMT1B, and caused axon demyelination in WT adult mice, supporting a role for Derlin 2 in maintaining myelin integrity by promoting the ERAD of misfolded myelin proteins.

The specific role of iRhom1 in mammalian physiology is currently debated. We reported that iRhom1 KO mice die shortly after birth, with defects in a range of tissues (including secretory tissues), of as yet unclear etiology. Interestingly, mice lacking both iRhoms die in utero, while ADAM17/TACE KO mice die perinatally. Notwithstanding differences in the genetic background or microbiota between the models employed, this implies that the physiological role of iRhom extends beyond that of ADAM17/TACE [28], which would be consistent with the range of ADAM17/TACE-independent auxiliary roles for iRhoms that many groups have reported (as noted above). By contrast with our studies [28], a study by Li et al. [115] reported iRhom1 KO mice are phenotypically normal, while iRhom1/iRhom2 DKO animals exhibit perinatal lethality, with an ‘eyelids open at birth’ phenotype similar to ADAM17/TACE KOs. As recently highlighted, the differences could be explained by the gene targeting strategy [116]. Finally, the role of RHBD2 is still largely unknown, apart from being overexpressed in breast [100,101] and colon cancer [101,102], and mutated in a familial form of retinitis pigmentosa [117], a disease-causing progressive vision loss.

**Pending questions and concluding remarks**

Members of the rhomboid-like superfamily have a conserved set of 6 transmembrane helices (the rhomboid-like domain) that is generally believed to confer them with a common conformation, the rhomboid fold (Fig. 2). However, we still lack basic knowledge of what structural features defines the rhomboid clients that are recognized by the rhomboid fold, since there are no structures of a ‘physiological’ rhomboid–substrate complex. Structural insights from the bacterial rhomboid GipG bound to an inhibitor suggest an exosite, mapping to TMH2, TMH5, and the WR motif in the L1 loop, mediates loading into the active site defined by TMHs 4 and 6 (Fig. 3). However, several structural features in iRhoms, which share the closest sequence similarity with active rhomboids, such as the IRHD between TMHs 1 and 2, and conserved proline adjacent to the catalytic residues on TMH4 (Fig. 2), suggest they might adopt a deviant fold. In addition, potentially different exosites encoded by TMH1 of iRhoms might mediate a more stable interaction with client proteins (Fig. 3), to regulate their trafficking, activity, or for quality control purposes in ERAD (Figs 3 and 4).
Early work was consistent with a model whereby a similar conformation of the rhomboid fold was central to the biology of this protein family; it allows them to bind to similar client proteins within similar pathways to active rhomboids (e.g., EGFR ligands) albeit to influence distinct outcomes ERAD (Fig. 1). However, more recent evidence suggests that pseudoproteases have evolved new structural features, either within the membrane-embedded region (e.g., TMH1, in Fig. 3), or as soluble appendages (Figs 2–4), that allow them to regulate, and be regulated by various proteins of different membrane topology, and also by soluble factors (Fig. 4A).

Unfortunately, the few available mechanistically defined examples preclude generalization on how rhomboid pseudoproteases regulate client protein activity or function (Fig. 4H–J). Moreover, we still know very little concerning how rhomboid pseudoproteases interact with the cellular machinery to exert trafficking control on their client proteins (Fig. 4G,H). Nonetheless, a clearly emerging theme is how they regulate signaling via interactions with ubiquitin, in the modulation of protein activity (NEMO, Fig. 4I), in the turnover of soluble proteins (Fig. 4J), and in ERAD to tune the levels of important signaling molecules or misfolded proteins (Fig. 4C–F). In sharp contrast to other pseudoenzyme families, we currently know much more about the physiological roles of rhomboid pseudoproteases than we do about their active rhomboid counterparts. However, many unanswered questions remain, not least the physiological roles of TMEM115, RHBD2, and UBAC2, nor the precise physiological roles of Derlins in ERAD in mammals.

While it is now clear that iRhom1s have multiple regulatory inputs and biological outcomes (Fig. 7), still several outstanding questions remain including whether they have completely redundant functions (i.e., do differences in iRhom1 versus iRhom2 mutant phenotypes solely reflect differences in expression patterns [28,75]), or do they in addition indeed have different sets of clients as is currently suspected from cellular and biochemical experiments. Conditional KO models are required to help clarify some of these open questions.

The key to understanding the mechanism of rhomboid pseudoproteases lies in defining how they regulate the fate of their clients. Proteolysis is an irreversible modification; hence, client loading into the rhomboid active site must be a key regulatory step (Fig. 3A). By contrast, the lack of proteolytic activity renders rhomboid pseudoproteases much more intriguing since many more outcomes are apparently possible (Fig. 3B). For instance, what controls client loading and dissociation? What happens to the client when it is in a complex with the pseudoprotease? Is its activity inhibited, is it shielded; is it more or less prone to enter degradation pathways, and why? Does it traffic to other cellular compartments? Overall, what governs the seemingly distinct and sometimes apparently contradictory fate of different client proteins in different contexts? Throughout this review, we have exemplified how all of these are entry points for regulation, and how rhomboid pseudoproteases do not act alone, but rather their effect on client proteins depends on the set of auxiliary proteins that they interact with (their ‘complex life’).

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
MC and CA were involved in the literature review and manuscript generation, illustration generation, and critical revision of manuscript.

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