Inactive rhomboid proteins RHBDF1 and RHBDF2 (iRhoms): a decade of research in murine models

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Abstract
Rhomboid proteases, first discovered in Drosophila, are intramembrane serine proteases. Members of the rhomboid protein family that are catalytically deficient are known as inactive rhomboids (iRhoms). iRhoms have been implicated in wound healing, cancer, and neurological disorders such as Alzheimer’s and Parkinson’s diseases, inflammation, and skin diseases. The past decade of mouse research has shed new light on two key protein domains of iRhoms—the cytosolic N-terminal domain and the transmembrane dormant peptidase domain—suggesting new ways to target multiple intracellular signaling pathways. This review focuses on recent advances in uncovering the unique functions of iRhom protein domains in normal growth and development, growth factor signaling, and inflammation, with a perspective on future therapeutic opportunities.

The rhomboid proteins: rhomboid proteases and rhomboid pseudoproteases
Rhomboid proteins are a highly conserved superfamily of polytopic membrane proteins (Urban and Dickey 2011). Rhomboid proteins can be broadly classified into active (Lastun et al. 2016) and inactive enzymes (Freeman 2014), also called pseudoproteases or iRhoms. Active rhomboid proteins, first discovered in Drosophila as key regulators of epidermal growth factor receptor (EGFR) signaling, are intramembrane serine proteases that hydrolyze peptide bonds within the lipid bilayer (Lemberg et al. 2005). Catalysis is achieved by a histidine-serine dyad, which is submerged 10 Å below the cell membrane surface (Fig. 1A). Conversely, iRhoms lack a catalytic serine residue and hence do not retain any enzymatic protease activity (Fig. 1B). Nevertheless, the iRhoms RHBDF1 and RHBDF2 have been implicated in neurological disorders including Alzheimer’s and Parkinson’s diseases, as well as in cancer, inflammation, and skin diseases (Jager et al. 2014; Raj et al. 2018; Hosur et al. 2014; Hosur et al. 2018; Yan et al. 2008; Zhou et al. 2014; Zou et al. 2009; Blaydon et al. 2012; Young 2019). Nearly a decade of research in mice has emphasized an essential role for these two proteins in normal functioning of the brain, heart, skin, eye, bone, adipose tissue, and the immune system. In line with the role of active rhomboids in EGFR signaling, findings from mouse models demonstrate that iRhoms are also essential regulators of EGFR signaling. While loss-of-function (LOF) mutations in Rhbdf1, Rhbdf2, or both significantly suppress stimulated secretion of EGFR ligands, gain-of-function (GOF) mutations in either Rhbdf1 or Rhbdf2 stimulate enhanced EGFR ligand secretion. iRhoms consist of a long cytosolic N-terminal domain, a conserved cysteine-rich iRhom homology domain (IRHD), a six transmembrane (TM helices 1–6) core (Fig. 1B), and an additional TM segment (TM helix 7). The six TM core harbors the dormant peptidase domain (TM helices 2–6), which has an alanine residue (instead of serine) in the enzyme core. In this review, we describe how insights from mouse models carrying either spontaneous mutations or CRISPR/Cas9-induced gene modifications in Rhbdf1 and Rhbdf2 have been crucial in identifying their physiological targets, in defining the unique roles of iRhom protein domains in development and disease, and in nominating possible novel therapeutic opportunities targeting iRhoms.
Overlapping and discrete functions of RHBD1 and RHBD2 in regulating EGFR signaling

Evidence for shared targets and function

Homozygous *Rhbd1* knockout mice (KO) exhibit multiorgan pathologies, including brain hemorrhage, cardiac fibrosis, and lower body weight compared with heterozygous littermates, and die within two weeks after birth (Christova et al. 2013; Hosur et al. 2020) (Fig. 2A). Conversely, *Rhbd2-null* mice are healthy and fertile and do not present with growth retardation or brain and heart defects (Hosur et al. 2014; Adrain et al. 2012; McIlwain et al. 2012; Siggs et al. 2012) (Fig. 2B). Nevertheless, a combined absence of RHBD1 and RHBD2 results in a more severe phenotype than either *Rhbd1* or *Rhbd2* KO, as evidenced by sub-viability and eyelids open at birth (EOB) observed in *Rhbd1:Rhbd2* double KO mice (Fig. 2C) (Hosur et al. 2020). This phenotype, together with the multiorgan pathology exhibited by *Rhbd1-null* mice, suggests that (1) RHBD1 and RHBD2 have overlapping functions, as the presence of RHBD2 reverses sub-viability and the EOB phenotype in *Rhbd1* KO mice (Hosur et al. 2020), and (2) RHBD1 and RHBD2 share some physiological functions and targets.

Evidence for distinct targets and function

Studies of *Rhbd1* and *Rhbd2* GOF mutant mice (Fig. 2D, E)—*Rhbd1viable* (Hosur et al. 2020), curly bare (*Rhbd2cab*), uncovered (*Rhbd2uncv*), and CRISPR/Cas9-mediated genetically engineered mouse model of tylosis with esophageal cancer (*Rhbd2TOC*)—suggest that RHBD1 and RHBD2 have distinct functions, and thereby distinct physiological targets. *Rhbd1viable* mice, which are generated using CRISPR/Cas9-mediated excision of exons 2 and 3 of the *Rhbd1* gene, are healthy, viable, and fertile. Despite lacking the exons containing the transcription start site (ATG), the mutant viable transcript produces an N-terminal-truncated RHBD1 protein (ΔN1–151) using the next in-frame ATG, which is in exon 4. While most *Rhbd1-null* mice die by postnatal day 14, no abnormalities are observed in *Rhbd1viable* mice (Fig. 2D), suggesting that the viable mutation rescues the severe multiorgan pathologies observed in RHBD1-deficient mice. Additionally, the viable allele rescues the sub-viability of *Rhbd1:Rhbd2* double KO mice (Fig. 2F) and induces enhanced stimulated secretion of EGFR ligands in vitro, suggesting that viable is a gain-of-function mutation. Consistent with the observations that mutations in the N-terminus of *Rhbd1* result in GOF mutants, either loss of the N-terminus (loss of amino acids 1–268 [ΔN1–268], *Rhbd2cab* mutation) (Hosur et al. 2014; Siggs et al. 2014), or missense mutations (p.P159L, *Rhbd2TOC* mutation) (Hosur et al. 2017) in the N-terminus of *Rhbd2*, yields GOF mutant mice. Each of these two mutations induces, through enhanced amphiregulin (AREG) secretion, accelerated wound healing and a loss-of-hair phenotype (Fig. 2E). In addition, uncovered, a recessive mouse mutation (*Rhbd2uncv*) results from a spontaneous loss of 309 bp in the N-terminus of *Rhbd2* (ΔN118–191) (Li et al. 1999). Like *Rhbd2cab* and *Rhbd2TOC*, *Rhbd2uncv* mice exhibit a loss-of-hair phenotype. Loss of RHBD2 does not affect skin architecture or hair development, indicating that *Rhbd2uncv* is a GOF mutation and that mutations in the N-terminus of *Rhbd2* facilitate transmembrane domain (TMD)-mediated enhanced secretion of EGFR ligands. Interestingly, whereas *Rhbd2 GOF* (*Rhbd2cab*, *Rhbd2TOC*, and *Rhbd2uncv*) mutant mice exhibit a loss-of-hair phenotype through enhanced secretion of AREG (Christova et al. 2013; Siggs et al. 2012), *Rhbd1 GOF* (*Rhbd1viable* and *Rhbd1viable2*) mutant mice have a normal hair coat (Hosur et al. 2020), suggesting that RHBD1
Fig. 2 Loss-of-function (LOF) and gain-of-function (GOF) mouse models of iRhoms. A Rhbdf1-null mice exhibit brain hemorrhage, cardiac fibrosis, and lower body weight than control littermates. B Rhbdf2-null mice are healthy and fertile and do not show brain, heart, or growth defects. C A combined loss of Rhbdf1 and Rhbdf2 results in sub-viability and eyelids open at birth (EOB) phenotype. D GOF mutations in the Rhbdf1 gene, such as viable (v) 1, viable 2, and viable 3, produce an N-terminal-truncated protein to rescue the overt phenotype observed in the Rhbdf1-null mice in panel A. E GOF mutations in the Rhbdf2 gene, such as Rhbdf2<sup>v1</sup>, Rhbdf2<sup>uncv</sup>, and Rhbdf2<sup>P159L</sup>, exhibit a loss-of-hair phenotype through enhanced secretion of EGFR ligand AREG via the TMD. F The Rhbdf1<sup>v</sup> allele reverses sub-viability of Rhbdf1:Rhbdf2 double KO mice in panel C. Rhbdf1<sup>v</sup>Rhbdf2<sup>−/−</sup> mice show an EOB phenotype and develop a wavy hair coat; however, no cardiac or brain abnormalities are observed in Rhbdf1<sup>v</sup>Rhbdf2<sup>−/−</sup> double mutant mice. Rectangles indicate deleted regions.
and RHBDF2 have distinct physiological substrates and non-overlapping phenotypes.

**Physiological targets of iRhoms**

**RHBDF1**

In vitro studies in mouse embryonic fibroblasts reveal that RHBDF1 deficiency suppresses the stimulated secretion of EGFR ligands, including AREG, heparin-binding EGF (HB-EGF), and transforming growth factor-alpha (TGFα) (Hosur et al. 2020; Li et al. 2015). Additionally, short hairpin RNA (shRNA) or small interfering RNA (siRNA)-mediated silencing of *RHBDF1* in various human breast cancer cell lines and a human squamous cancer cell line significantly inhibited TGFα-mediated EGFR signaling and, further, showed anti-cancer effects by inhibiting cell proliferation and invasion and, ultimately, tumor growth in vivo (Yan et al. 2008; Zhou et al. 2014; Zou et al. 2009). These studies suggest that RHBDF1 might regulate EGFR signaling through secretion of multiple EGFR ligands, and that RHBDF2 does not compensate for the loss of RHBDF1-mediated signaling underlying the multiorgan pathologies observed in *Rhdfl* KO mice.

Despite the clear biological importance of RHBDF1, the precise molecular mechanisms and the physiological targets of RHBDF1 underlying the multiorgan pathology and anticancer effects resulting from RHBDF1 deficiency remain to be investigated. It is unlikely that the pathology observed in *Rhdfl*-null mice is mediated solely through AREG, HB-EGF, and/or TGFα, as mice lacking either AREG or TGFα are healthy and fertile (Luetteke et al. 1999; Luetteke et al. 1993; Mann et al. 1993). However, RHBDF1 likely regulates the secretion of more than one EGFR ligand. In particular, the cardiac fibrosis observed in *Rhdfl*-null mice resembles the heart enlargement displayed by *Hbegf*-null mice (Iwamoto et al. 2003), and the eyelids open at birth (EOB) observed in *Rhdfl*:Rhdfl* null mice resembles the EOB phenotype displayed by *Hbegf* and *Tgfa* double null mice (Mine et al. 2005). Together, these observations suggest that HB-EGF and TGFα could be the physiological targets of RHBDF1.

If defects in EGFR signaling alone underlie the multiorgan pathology observed in *Rhdfl*-null mice, it is likely that RHBDF2 compensates for the loss of RHBDF1 during early development through secretion of TGFα, particularly during eyelid development. This is because, although *Rhdfl*-null mice exhibit multiorgan pathology (Hosur et al. 2020), they do not exhibit the in utero lethality, EOB phenotype, and epidermal defects observed in *Egfr*-null mice (Miettinen et al. 1999; Sibilia and Wagner 1995). Furthermore, mice lacking both HB-EGF and TGFα show a highly penetrant EOB phenotype that is not observed in *Rhdfl*-null mice, suggesting that RHBDF2 compensates for the loss of RHBDF1 only during early development to facilitate EGFR signaling, including during eyelid development, through secretion of TGFα.

**RHBDF2**

In vitro, loss of RHBDF2 has been shown to result in significantly reduced stimulated secretion of EGFR ligands, including AREG, HB-EGF, and TGFα (Siggs et al. 2014; Maretzky et al. 2013). In vivo studies in mice suggest that AREG is a *bona fide* physiological target of RHBDF2. In humans, dominant mutations in RHBDF2 cause tylosis with esophageal cancer (TOC) syndrome through a hyperactive EGFR signaling pathway. Using spontaneous (*Rhdfl*<sup>cub/cub</sup> and *Rhdfl*<sup>cub/cub Areg<sup>−/−</sup></sup>) (Hosur et al. 2014) and CRISPR/Cas9 (*Rhdfl*<sup>2TOC</sup>) (Hosur et al. 2017) genetically engineered mouse models, we have shown that dominant mutations in RHBDF2 induce a hyperactive EGFR phenotype through enhanced secretion of AREG, and that genetic deletion of *Areg* in *Rhdfl*<sup>cub/cub</sup> or *Rhdfl*<sup>2TOC</sup> mice prevents TOC. Further, shRNA-mediated silencing of *Areg* inhibits the hyperactive EGFR signaling phenotype in *Rhdfl*<sup>cub/cub</sup> embryonic fibroblasts (Hosur et al. 2014). Together, these studies suggest that AREG is a physiological target of RHBDF2.

**Cytosolic N-terminus and TM helix 1 of RHBDF1 are dispensable for normal growth and development**

The *Rhdfl*<sup>viable</sup> mutation generates an N-terminal-truncated RHBDF1 protein (ΔN1–151) (Fig. 3A, B), which rescues the severe multiorgan pathologies observed in RHBDF1-deficient mice (Fig. 2D). Notably, even in the absence of the N-terminal domain, the mutant *Rhdfl*<sup>v/v</sup> transcript generated by the *Rhdfl*<sup>v/v</sup> mutation induces enhanced secretion of EGFR ligands, suggesting that the IRHD and the TMD of RHBDF1 are sufficient to mediate EGFR signaling.

*Rhdfl*<sup>viable</sup> mice, which were first generated by Li X. et al. are *Rhdfl* heterozygous mutant mice lacking exons 4–11 in the *Rhdfl* gene (Li et al. 2015). The resulting *Rhdfl* mutant transcripts yield two variant proteins (~32 and ~29 kDa) that each lack the entire N-terminus, the TM helix 1, and the majority of the IRHD (Fig. 3C). Like *Rhdfl*<sup>v/v</sup> mice, *Rhdfl*<sup>viable</sup> mice are healthy and fertile and do not exhibit the cardiac, brain, or growth defects observed in *Rhdfl*<sup>v</sup> mice. *Rhdfl*<sup>viable</sup> mice are referred to by this name because of the phenotypic similarity to *Rhdfl*<sup>v/v</sup> mice. Since the N-terminus, the TM helix 1, and the majority of the IRHD are lost in *Rhdfl*<sup>viable</sup> mice,
and these mice remain healthy and fertile, we reasoned that the entire N-terminus, the IRHD, and the TM helix 1 might be dispensable. Instead, we found that either of the two variant proteins, each consisting solely of TM helices 2–7, is adequate to rescue the Rhbdf1-null phenotype (Hosur et al. 2020). This result explains the healthy and fertile phenotypes observed in Rhbdf1viable and Rhbdf1viable2 mice, with no brain, heart, or body weight defects.

The Rhbdf1viable2 homozygous mutant mice reported by Li et al. were generated using the KO-first gene disruption strategy. Nevertheless, the Rhbdf1viable2 mutant mice are not homozygous-null because the Rhbdf1viable2 mutant transcript generates truncated proteins through an alternative promoter usage and through exon skipping. Interestingly, DNA sequencing revealed that the Rhbdf1viable allele retains the En2 splice acceptor sequence from the cassette used to generate the KO-first allele, resulting in novel mutant transcripts lacking the N-terminus, TM helix 1, and the majority of the IRHD (72%) (Hosur et al. 2020). Thus, to further validate if the N-terminus, TM helix 1, and the majority of the IRHD are dispensable, we generated Rhbdf1viable3 mice using CRISPR/Cas9-mediated gene editing in C57BL/6 J mice. We excised exons 4 through 13 in the Rhbdf1 gene, resulting in mice lacking the N-terminus, the first TM helix 1, and 72% of the IRHD, but that retain TM helices 2–7 that harbor the dormant peptidase domain (Fig. 3D). We refer to these mice as viable 3 (Rhbdf1v3) (unpublished observations). We observed that homozygous-viable 3 (Rhbdf1v3v3) mice did not display any gross deformities of major organs, which is consistent with both the Rhbdf1viable (Rhbdf1ΔN1-151) mice and Rhbdf1viable2 (lacking exons 4–11) mice, and in contrast to the Rhbdf1null/null mice that show multiorgan pathology and die within two to three weeks. Collectively, these studies suggest that the cytosolic N-terminus, TM helix 1, and possibly the IRHD of RHBD1 are dispensable for normal growth and development.

Cytosolic N-terminus and TM helix 1 of RHBD2 regulate inflammatory signaling through TNFα secretion

While the N-terminus and the TM helix 1 of iRhoms are dispensable for normal growth and development, they nevertheless play an important role in RHBD2-mediated inflammatory signaling. In mice, loss of RHBD2 significantly

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**Fig. 3** Domains of RHBD1 gain-of-function proteins. A A schematic of the full-length mouse RHBD1 protein showing the cytosolic N-terminus domain, IRHD, transmembrane helices, and the peptidase domain. B A schematic of the mouse RHBD1 viable protein showing the loss of 151 amino acids in the N-terminal domain. C A schematic of the mouse RHBD1 viable2 protein showing the complete loss of the cytosolic N-terminal domain and the partial loss of the IRHD (Targeted KO-first allele). D A schematic of the mouse RHBD1 viable3 protein showing the complete loss of the N-terminal domain and the partial loss of the IRHD. Notably, all three mutant proteins, viable, viable2, and viable3 retain the dormant peptidase domain (CRISPR/Cas9 generated allele)
reduces regulated secretion of TNFα following stimulation with bacterial endotoxin lipopolysaccharide (LPS) (Hosur et al. 2014; Adrain et al. 2012; McIlwain et al. 2012; Siggs et al. 2012). Particularly, the N-terminus seems to be essential for TNFα secretion, because Rhbd12<sup>−/−</sup> mice, which lack the N-terminal domain similarly to Rhbd12-null mice, show significantly reduced TNFα secretion upon stimulation with LPS, demonstrating that the N-terminal domain is essential for TNFα secretion (Hosur et al. 2014). Concordantly, Cavadas et al. and Grieve et al. found that phosphorylation of RHBDF2 at the N-terminus is essential for TNFα secretion (Cavadas et al. 2017; Grieve, et al. 2017); upon stimulation with LPS, RHBDF2 serine phosphorylation and binding to 14–3–3 proteins was observed in primary macrophages. Additionally, in Rhbd12 KO macrophages, LPS-induced TNFα secretion was rescued by RHBDF2, but not by N-terminal-truncated RHBDF2 lacking phosphorylation sites (58–361 aa) (Fig. 4A), suggesting that RHBDF2 phosphorylation and binding to 14–3–3 proteins controls TNFα release in macrophages. In addition to binding of 14–3–3 proteins, FRMD8 has been shown to be a binding partner for RHBDF2. Künzel et al. and Oikonomidi et al. suggest that the N-terminus of RHBDF2 forms a tripartite complex with FRMD8 and ADAM17, a metalloprotease essential for ecto-domain shedding of TNFα, to facilitate inflammatory signaling through stimulated secretion of TNFα (Künzel et al. 2018; Oikonomidi et al. 2018). The authors showed that stimulation of Frmd8 KO macrophages with LPS resulted in reduced secretion of TNFα, suggesting that FRMD8-RHBDF2 interaction is necessary for TNFα secretion.

It has been suggested that the TM helix 1 of RHBDF2 is also essential for TNFα secretion in macrophages (Li

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**Fig. 4** RHBDF2 binding partners and iRhom macrophage expression. **A** A schematic of the full-length mouse RHBDF2 protein showing the 14–3–3 and FERM Domain Containing 8 (FRMD8) binding sites in the cytosolic N-terminal domain and the sinecure mutation I387F in transmembrane helix 1. **B** Violin plots showing expression of various mouse and human genes in macrophages. In both mouse (left panel) and human (right panel) macrophages, Rhbd1, Rhbd2, Tnfa, and Adam17 are expressed. Cd19 and Cd3g serve as negative markers, whereas Cd14 and Fcgr1 serve as positive controls, in macrophages.
et al. 2017). Like Rhbd2-null mice, mice homozygous for the sinecure (sin) mutation, a recessive mutation in the Rhbdf2 gene, are viable and fertile (Siggins et al. 2012). The Beutler laboratory identified sinecure during a forward genetic screen of mice for regulators of Toll-like receptor (TLR)-induced TNFα secretion. A single nonsynonymous mutation (A to T) in Rhbd2 results in conversion of isoleucine to phenylalanine at amino acid 387 (I387F) (Fig. 4A). Non-complementation studies validated that sinecure is a mutation at the Rhbd2 locus, as compound mutant Rhbd2sin/null mice and Rhbd2sin/sin Rhbd2-null mice showed similar reductions in TNFα secretion following stimulation with LPS. TNFα secretion was not completely blocked, suggesting that constitutive secretion of TNFα is not affected by RHBD2 deficiency. These data suggest that TM helix 1 of RHBD2 is essential for constitutive TNFα secretion.

Interestingly, the binding partners for the N-terminus of RHBD2—14–3–3 and FRMD8—seem to be dispensable for RHBD1-mediated growth factor signaling. We recently generated Ywhaq−/− mice, homozygous-null mice (Ywhaq−/−), homozygous-null mice (Ywhaq−/−) showed reduced TNFα secretion following stimulation with LPS. However, Ywhaq-null mice exhibit normal body weight, no postnatal lethality, nor any brain or heart defects, as observed in Rhbd1 KO mice. This suggests that YWHAQ could be a binding partner for RHBD2 and that it might be essential for RHBD2-mediated stimulated secretion of TNFα, but not for RHBD1-mediated growth factor signaling (unpublished observations). In line with these findings, FRMD8 also seems to be essential for TNFα secretion, but not for growth factor signaling. Specifically, we generated Frmd8 KO mice and observed that, compared with heterozygous-null mice (Frmd8+/−), homozygous-null mice (Frmd8−/−) showed significantly reduced TNFα secretion following stimulation with LPS, in accordance with the observations of Künzel et al. and Oikonomidi et al. However, Frmd8 KO mice do not phenocopy Rhbd1 KO mice in terms of lower body weight, postnatal lethality, or brain and heart defects (unpublished observations). This suggests that FRMD8 could also be a binding partner for RHBD2 and might be essential for RHBD2-mediated stimulated secretion of TNFα, but not for RHBD1. More importantly, RHBD2-mediated stimulated secretion of TNFα suggests high specificity of RHBD2 for TNFα. Even though RHBD1 is expressed in both mouse and human macrophages (Fig. 4B), it does not compensate for the loss of RHBD2 in regulating stimulated secretion of TNFα, suggesting that TNFα could be a specific target of RHBD2.

### The dormant peptidase domain of iRhoms (TM helices 2–6)

**Peptidase domain of RHBD1 is sufficient for normal growth and development.** In an in vivo screen in mice, we identified the minimal protein domain required for normal growth and development—the transmembrane peptidase domain of RHBD1. The healthy and fertile phenotypes of Rhbd1viableΔN1–151, Rhbd1viable2, Rhbd1viable3, and Rhbd1viable Rhbd2−/− mice (Hosur et al. 2020) (Fig. 2D, F), with no defects in the brain, heart, or in body weight, surprisingly suggest that RHBD2 and the N-terminus, the IRHD, and the first TM helix of RHBD1 are dispensable for normal growth and development (Hosur et al. 2020). However, TM helices 2–6, which harbor the dormant peptidase domain of RHBD1, are adequate and essential for survival and normal growth and development.

**Peptidase domain of RHBD2 facilitates AREG secretion.** The Rhbd22cub spontaneous mouse mutation results from a ~12.5 Kb deletion in the Rhbd2 gene, leading to the loss of exons 2 through 6. Nevertheless, the Rhbd22cub mutant transcript generates an N-terminal-truncated protein using an in-frame ATG in exon 8 resulting in loss-of-hair and rapid wound healing phenotypes. We previously showed that the N-terminal-truncated transcript is sufficient to induce AREG secretion, leading to a hyperactive EGFR phenotype. Furthermore, using site-directed mutagenesis, we showed that mutating residues in TM helices 2, 4, and 6, which harbor the dormant peptidase domain, prevents AREG secretion, and that the N-terminal domain is dispensable for mediating the hyperactive EGFR phenotype observed in Rhbd22cub mice (Hosur et al. 2014).

**Highly conserved amino acid residues in the dormant peptidase domain of iRhoms**

As noted above, the dormant peptidase domain of RHBD2 is sufficient to induce accelerated wound healing in mice through enhanced secretion of AREG (Hosur et al. 2014). Furthermore, the survival of Rhbd1viable2 and Rhbd1viable3 mice indicates that the dormant peptidase domain is sufficient for survival. Nevertheless, the underlying molecular mechanisms are unclear. Here, we perform new sequence analysis of key amino acid residues in the peptidase domain of the rhomboid family using HMM Logos, a widely used tool for visualization of protein families, and uncover highly conserved amino acid residues in TM helices 2, 3, 4, and 6. We thus propose...
that these amino acids could be critical for RHBDF1- and RHBDF2-mediated EGFR signaling (Fig. 5A). We further propose that the dormant peptidase domain could also account for differences in substrate specificity of RHBDF1 and RHBDF2. For instance, even though both Rhbdf1 and Rhbdf2 are expressed in keratinocytes (Fig. 5B), RHBDF2, but not RHBDF1, selectively induces accelerated wound healing in mice through enhanced AREG secretion. Therefore, RHBDF1 and RHBDF2 have high specificity toward target proteins, which might be conferred by the peptidase domain.
Inactive rhomboid proteins RHBD1 and RHBD2 (iRhoms): a decade of research in murine models

How do iRhoms regulate secretion of EGFR ligands and TNFα?

The underlying mechanisms are still emerging, but two hypotheses have been proposed based on available data:

ADAM17 hypothesis

iRhoms have been shown to regulate maturation, trafficking, and activation of ADAM17, a metalloprotease required for ectodomain shedding of EGFR ligands and TNFα (Adrain et al. 2012; McIlwain et al. 2012; Maretzky et al. 2013). Thus, according to this hypothesis, mice lacking Rhbd1f or Rhbd2f or both together fail to promote ADAM17 activity, thereby leading to significantly reduced secretion of EGFR ligands and TNFα following stimulation with phorbol ester or LPS. However, genetic evidence deduced from the mouse models that we and others have generated argue against this hypothesis. First, the necessity of RHBD2 for ADAM17 maturation and trafficking raises an obvious question as to why Rhbd2 KO mice do not phenocopy Adam17 KO mice. Notably, whereas Adam17 KO results in embryonic or perinatal lethality (Veit 2019), Rhbd2 KO mice are viable and fertile. To this end, Issuree et al. (Issuree et al. 2013) suggested that RHBD1 compensates for the loss of RHBD2 in Rhbd2 KO mice and facilitates ADAM17 maturation and trafficking, and hence Rhbd2 KO mice do not phenocopy Adam17 KO mice. Consistent with the Issuree et al. study, Li et al. (2015) showed that, whereas Rhbd1f and Rhbd2f single KO mice are viable and fertile, Rhbd1f/Rhbd2f double KO mice phenocopy Adam17 KO mice, exhibiting perinatal lethality, open eyelids at birth, and heart valve defects. Li et al.’s findings are in direct conflict with the results of a previous study by Christova et al. (2013), who found that Rhbd1f single KO mice have multiorgan pathologies, and Rhbd1f:Rhbd2f double KO mice show early embryonic lethality. To try to resolve this discrepancy, we found that both the Rhbd1f single KO mice and the Rhbd1f/Rhbd2f double KO mice generated by Li et al. are indeed not null for RHBD1F as they retain residual RHBD1 functional activity (Hosur et al. 2020). This brings into question the notion as to whether RHBD1 compensates for the loss of RHBD2 in regulating ADAM17 maturation and trafficking, and the association in general between iRhoms and ADAM17 maturation, trafficking, and activation. Second, transcriptome data suggest that macrophages (human and mouse) express both Rhbd1f and Rhbd2f (Fig. 4B). According to the ADAM17 hypothesis, loss of RHBD2F is compensated by RHBD1F, and vice versa. However, Rhbd2 KO macrophages show significantly reduced stimulated secretion of TNFα (Hosur et al. 2014; Adrain et al. 2012; McIlwain et al. 2012; Siggs et al. 2012), even though Rhbd1f is expressed in Rhbd2-null macrophages, arguing against the iRhoms-ADAM17 hypothesis. Third, Rhbd2-null mice show reduced stimulated secretion of EGFR ligands, including AREG and TGFα, in keratinocytes. It has been suggested that loss of RHBD2F fails to promote ADAM17 maturation, trafficking, and activity, leading to the reduction in stimulated secretion of EGFR ligands. Again, according to the ADAM17 hypothesis, RHBD1F, which is abundantly expressed in keratinocytes (Fig. 5B), compensates for the loss of RHBD2F. However, contrary to this prediction, Rhbd2-null keratinocytes show significantly reduced stimulated secretion of EGFR ligands. Lastly, and more importantly, this hypothesis does not account for target specificity of RHBD1F and RHBD2F. For instance, GOF mutation in Rhbd2f (Rhbd2f<sub>322</sub>), but not in Rhbd1f (Rhbd1f<sub>1addle</sub>), selectively regulates AREG secretion to induce loss-of-hair and wound healing phenotypes. Therefore, it seems unlikely that iRhoms regulate secretion of EGFR ligands and TNFα through direct regulation of ADAM17 maturation, trafficking, and activation.
Target trafficking hypothesis

According to this hypothesis, RHBD1 regulates secretion of EGFR ligand TGFα through delivery of pro-TGFα to the plasma membrane, where pro-TGFα undergoes ectodomain shedding by ADAM17 to release TGFα. In breast cancer cell lines, Li J. et al. showed that RHBD1 is an essential component of the protein trafficking machinery involving clathrin-coated vesicles (Li et al. 2018). Following stimulation with the G-protein-coupled receptor agonist Sphingosine 1 Phosphate (S1P), RHBD1 participates in clathrin uncoating of vesicles to deliver pro-TGFα to the cell surface. Specifically, RHBD1 interacts with a clathrin-coated vesicle protein auxilin-2 to recruit Heat shock cognate protein (HSC70) to the vesicles to initiate clathrin uncoating. Furthermore, siRNA-mediated silencing of RHBD1 inhibits the interaction between HSC70 and auxilin-2, significantly reducing TGFα secretion by preventing uncoating of clathrin and delivery of pro-TGFα to the plasma membrane for ectodomain shedding. Although in vivo validation is required to further support the target trafficking hypothesis, these findings could help address certain unanswered questions, such as the observation that Rhbd2 KO macrophages demonstrate reduced stimulated secretion of TNFα. According to the target trafficking hypothesis, RHBD2 deficiency does not affect ADAM17 activity, but due to target specificity (RHBD2 for TNFα), RHBD2 deficiency in macrophages, regardless of RHBD1 expression, suppresses TNFα secretion.

Concluding remarks

iRhoms are characterized by a cytosolic N-terminal domain, a luminal IRHD, and a transmembrane dormant peptidase domain. Since the initial discovery in Rhbd2<sup>viable3</sup> mice that in the absence of the cytosolic N-terminal domain, the dormant transmembrane peptidase domain of RHBD2 is sufficient to regulate EGFR signaling through secretion of EGFR ligand AREG, a substantial amount of literature has revealed the biological functions of iRhoms domains. Furthermore, the normal development and survival of Rhbd1<sup>viable3</sup> mice demonstrates that whereas the N-terminal domain and the IRHD are dispensable for viability and fecundity, the loss of the transmembrane dormant peptidase domain is associated with developmental defects, indicating that the peptidase domain is essential and is sufficient to regulate the secretion of diverse EGFR ligands. Although in vitro biochemical assays indicate some redundant functions for RHBD1 and RHBD2 in controlling secretion of various EGFR ligands, mouse genetic studies reveal unique signaling pathways and distinct client proteins for iRhoms. Additionally, uncovering the pathological role of iRhoms in epithelial cancers, inflammation, and skin diseases suggests iRhoms as potential therapeutic targets. Particularly, since ADAM17 inhibition to block secretion of EGFR ligands, including AREG, is associated with severe adverse effects (Ieguchi and Maru 2016), identification of selective inhibitors of the dormant peptidase domain—although challenging—could lay the foundation for the development of more selective and effective therapeutics targeting iRhoms to abrogate multiple pathogenic signaling pathways.

Outstanding questions

- **RHBD1 KO mice die of brain hemorrhage and cardiac fibrosis. What are the physiological substrates of RHBD1 and the underlying signaling pathways that maintain brain and heart function? Addressing these questions may have implications for treating neurological disorders, such as Alzheimer’s and Parkinson’s, as well as cardiac abnormalities.**

- **TM helices 2–6, which harbor the dormant peptidase domain, of iRhoms are essential for stimulated secretion of EGFR ligands. Which amino acid residues in the dor-
mant peptidase domain regulate secretion in vivo? These findings will help in rational drug design of potential novel treatments for cancer and skin diseases.

- iRhoms have overlapping functions only during developmental stages. Why does RHBDL2 not rescue mult organ pathology, including brain hemorrhage and cardiac fibrosis, in Rhdf1-null mice? Is RHBDL2 not expressed in the brain and heart during later stages, i.e., postnatal days?

- There is a need to understand tissue-specific regulation of EGFR ligand secretion by iRhoms. Mutations in the N-terminus (Rhbd2^TOC) or loss of the entire N-terminus (Rhbd2^cub) in RHBDL2 enhance AREG secretion to cause a hair loss phenotype. However, mutations in the N-terminus (Rhbd1^visible) or loss of the entire N-terminus (Rhbd1^visible) in RHBDL1 do not result in a similar phenotype.

**Note** Tissue-specific gene expression data were obtained from the ARCHS^4 database, which provides access to gene counts from HiSeq 2000, HiSeq 2500, and NextSeq 500 platforms for human and mouse experiments from GEO and SRA (Lachmann et al. 2018). We downloaded expression files (gene-level) for mouse (mouse_matrix_v10.h5) and human ((human_matrix_v10.h5) and selected the samples with tissue annotation from metadata as macrophages (Fig. 4A) and keratinocytes (Fig. 5B).

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**Declarations**

**Conflict of interest** The authors declare that no competing interests exist.

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