iRhoms; Its Functions and Essential Roles

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Abstract
In Drosophila, rhomboid proteases are active cardinal regulators of epidermal growth factor receptor (EGFR) signaling pathway. iRhom1 and iRhom2, which are inactive homologs of rhomboid intramembrane serine proteases, are lacking essential catalytic residues. These are necessary for maturation and trafficking of tumor necrosis factor-alpha (TNF-α) converting enzyme (TACE) from endoplasmic reticulum (ER) to plasma membrane through Golgi, and associated with the fates of various ligands for EGFR. Recent studies have clarified that the activation or downregulation of EGFR signaling pathways by alteration of iRhoms are connected to several human diseases including tylosis with esophageal cancer (TOC) which is the autosomal dominant syndrom, breast cancer, and Alzheimer’s disease. Thus, this review focuses on our understanding of iRhoms and the involved mechanisms in the cellular processes.

Key Words: iRhom1, iRhom2, TNF-α, TACE, EGFR

INTRODUCTION
Rhomboid protease was initially discovered in Drosophila (Sturtevant et al., 1993; Freeman, 1994). Drosophila rhomboid protease cuts epidermal growth factor receptor (EGFR) ligand Spitz and a homologue for mammalian tumor growth factor (TGF)-α, triggering the secretion of the factors (Rutledge et al., 1992; Schweitzer et al., 1995). Homologs of the fly rhomboid proteases have been identified in most prokaryotic and eukaryotic organisms (Lemberg and Freeman, 2007). Rhomboid proteases comprise a superfamily of proteins consisting of intra-membrane serine proteases and their inactive homologs (Freeman, 2014). The common ancestor of all members of the family is probably an active intra-membrane protease, although the majority of existing members are not active proteases (Freeman, 2014).

The rhomboid protease family members have been shown to have a common structure, six or seven transmembrane domains (Ha et al., 2013) as seen in Table 1. Rhomboid proteases have conserved transmembrane segments of their polytopic rhomboid core domain, in which there are a catalytic motif in forth transmembrane domain, and an Engelman helix dimerization motif in sixth transmembrane domain (Urban et

Table 1. Mammalian rhomboid family proteins

| Rhomboids    | Number of TM domains | Catalase activity | Localization       |
|--------------|----------------------|-------------------|--------------------|
| PARL         | 7                    | Yes               | Mitochondrial inner membrane |
| RHBDL1       | 7                    | Yes (predicted)   | Golgi              |
| RHBDL2       | 7                    | Yes               | Plasma membrane    |
| RHBDL3       | 7                    | Yes (predicted)   | Endosomes          |
| RHBDL4       | 6                    | Yes               | ER                 |
| iRhom1       | 7                    | No                | ER-Golgi           |
| iRhom2       | 7                    | No                | ER-Golgi           |
| Derlin1      | 6                    | No                | ER                 |
| Derlin2      | 6                    | No                | ER                 |
| Derlin3      | 6                    | No                | ER                 |
| UBAC2        | 6                    | No                | ER                 |
| TMEM115      | 6                    | No                | ?                  |
| RHBDD2       | 6                    | No                | Golgi              |
| RHBDD3       | 6                    | No                | ?                  |

Reference: Bergbold and Lemberg et al., 2013.

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As more distant rhomboid family members, many other genes without the key catalytic motif, such as derins, UBAC2, RHBDDs and TMEM115, have also been annotated as rhomboid-like proteins by bioinformatics search based on their sequence similarities (Koonin et al., 2003; Lemberg and Freeman, 2007; Finn et al., 2010). The structural relations for these proteins remain to be investigated because of their limited overall sequence conservation (Bergbold and Lemberg, 2013). Currently, there are 14 rhomboid family members, five rhomboid proteases and nine catalytically inactive homologues (Bergbold and Lemberg, 2013). Among these rhomboids, iRhoms comprise a unique family; not only with the key catalytic motif and the highly conserved sequences between species, but also with the unique iRhom homology domain and cytosolic N-terminal cytosolic domain, suggesting an important biological role for these proteins, despite their lack of protease activity (Koonin et al., 2003; Lemberg and Freeman, 2007; Freeman, 2014). This review focuses on our current understanding of iRhoms and their roles in cellular processes and diseases.

**IRHOMS IN DROSOPHILA AND MAMMALS**

In *Drosophila*, active rhomboids are cardinal regulators of EGFR signaling pathway, and their activity is conserved in mammals (Lui et al., 2003; Zettl et al., 2011). The principal ligand of *Drosophila* EGFR is Spitz, which is homologous to mammalian TGF-α. Spitz must be proteolytically released as a soluble extracellular fragment to be functional, and Rhomboid-1 is directly involved in the proteolytic cleavage of Spitz (Zou et al., 2009). Until now, genetic approaches have been used to investigate iRhom function in both *Drosophila* and mammals. Losses of function in mutated flies and mice have revealed the role of iRhoms in both ER-associated degradation and trafficking of TACE which is known to be responsible for the releases of active TNF and EGF family ligands (Siggs et al., 2014). EGF ligands in *Drosophila* are activated by its cleavage by Rhomboids. This is distinct from the metalloprotease-induced activation of mammalian EGF-like ligands (Siggs et al., 2014).

In mammals, iRhoms are known to regulate trafficking of TACE which is the protease that cleaves membrane-bound substrates including inflammatory cytokine TNF-α as indicated in Fig. 2. Both in *Drosophila* and mammals, losses of function mutations in *Drosophila* and mice have revealed the role of iRhoms in both ER associated degradation, and the control of trafficking of the metalloprotease TACE, the enzyme that releases active TNF-α and ligands of the EGF family (Zettl et al., 2011; Adrain et al., 2012; Mollwain et al., 2012). Especially, it became clear that iRhoms in human are associated with inheritable diseases and cancers (Etheridge et al., 2013). Understanding on the underlying mechanisms would be an important task for using the related pathways as therapeutic targets.

**ESSENTIAL ROLE OF IRHOMS ON TACE REGULATION**

TNF-α converting enzyme (TACE) (also known as ADAM17) is a membrane-anchored metalloproteinase that controls two major pathways, the EGF receptor pathway and proinflamma-

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*Fig. 1. Rhomboid protein iRhoms regulate the EGFR ligands in the ER. iRhoms binds to various EGFR ligands in the ER and facilitate their forward trafficking or ERAD pathway. The mechanism with which the fate determination of the EGFR ligands is not clear yet.*

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Rhomboid protein iRhoms regulate the trafficking and maturation of TNF-α converting enzyme (TACE). iRhoms bind to TACE, which promotes its exit from ER to Golgi. Within the Golgi, TACE is processed by furin into its mature form. At the plasma membrane, TACE cleaves the membrane-bound form of TNF-α to generate soluble TNF-α, which binds to TNFR.

**Fig. 2.** Rhomboid protein iRhoms regulate the trafficking and maturation of TNF-α converting enzyme (TACE). iRhoms bind to TACE, which promotes its exit from ER to Golgi. Within the Golgi, TACE is processed by furin into its mature form. At the plasma membrane, TACE cleaves the membrane-bound form of TNF-α to generate soluble TNF-α, which binds to TNFR.

EGFRs are a family of receptor tyrosine kinases essential for the control of many cellular processes, including proliferation, survival, and differentiation (Lui et al., 2003). The EGFR ligand family includes EGF, TGF-α, amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin, and epigen (Bassik et al., 2013; Sahin and Blobel, 2007). EGFR plays a major role in cancers as an activated oncogene (Lui et al., 2003). Activation of EGFR is frequently detected in a wide variety of carcinomas, including breast, lung, head and neck, and cervical cancers, and has been correlated with their poor prognosis (Zou et al., 2009). Several lines of evidence have implicated iRhoms in the regulation of EGFR signaling pathway. In Drosophila, active rhomboid proteins are cardinal regulators of EGFR signaling pathway which is activated throughout growth and development of wings (Lui et al., 2003). Clear involvement of rhomboid protein in EGFR activity was demonstrated using the sensitive developing wing primordium of Drosophila to reveal ectopic EGFR activity (Sturtevant et al., 1993; Nakagawa et al., 2005). Co-expression of iRhom1 with HB-EGF in Drosophila resulted in the severe wing phenotypes (Nakagawa et al., 2005). Drosophila iRhom deficiency induced sleep-like phenotype (Zettl

IRHOM-MEDIATED REGULATION OF EGFR SIGNALING

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et al., 2011), similar to the phenotype observed in increased activation of the EGFR pathway (Foltenyi et al., 2007). These results indicate *Drosophila* iRhoms are involved with inhibitory regulation of EGFR signaling pathway (Zettl et al., 2011; Freeman, 2014).

TACE can release not only membrane bound TNF-α but also various ligands of the EGFR. Therefore TACE can control a wide range of physiologically and medically important EGFR signaling (Blobel, 2005). An identification of iRhom1 and iRhom2 as key regulators of TACE-dependent EGFR signaling in mice highlighted an important role of iRhoms in the EGFR signaling pathway (Freeman, 2014; Siggs et al., 2014; Li et al., 2015). Coexpression of human iRhom1 or mouse iRhom2 with EGFR family ligands in COS7 cells downregulated all the EGFR ligands (Zettl et al., 2011). On the other hand, in the response of rapid stimulation for release of some TACE substrates in iRhom2 mutant embryonic fibroblasts, shedding of HB-EGF, amphiregulin and epiregulin was downregulated, whereas the shedding of TGF-α2 was not changed. In the condition, there was no change in the mature TACE levels, suggesting that iRhom2 itself may be involved in determining substrate selectivity of TACE-dependent shedding (Maretzky et al., 2013; Freeman, 2014).

siRNA-mediated *RHBDF1* gene silencing in cancer cell lines reduced the levels of cell migration and proliferation and induced apoptosis or autophagy in cancer cells (Zou et al., 2009). Moreover, iRhom1 is necessary for the survival of epithelial cancer cells in humans and may be linked to G protein-coupled receptor (GPCR)-mediated EGFR transactivation (Zettl et al., 2011). Collectively, these results indicate that iRhoms are not only promote forward trafficking of EGFR ligands from ER to Golgi, but also block ER export of the EGFR ligands by ERAD via the proteasome (Zettl et al., 2011; Bergbold and Lemberg, 2013) and that iRhoms may be attractive targets for treatment of TACE/EFGR-dependent pathologies (Li et al., 2015).

Different to an initial hypothesis that the iRhoms directly blocks active rhomboid proteases (Koonin et al., 2003), it reduces the level of growth factor substrates by triggering their degradation (Zettl et al., 2011). iRhom in *Drosophila* genetically interacts with the E3 ubiquitin ligase Hrd1 and the ERAD substrate receptor EDEM (Zettl et al., 2011). In addition, human iRhom1 and mouse iRhom2 have been demonstrated to mediate the down-regulation of EGFR signaling pathway by binding to EGFR ligands in the ER and targeting them for ERAD, which is induced as a result of ER quality control mechanisms (Etheridge et al., 2013). Therefore, both *Drosophila* and mammals share iRhom-mediated ERAD of EGFR family ligands in regulation of EGFR signaling pathway (Urban and Dickey, 2011), although there is no solid evidence for physiological role of iRhoms in regulating ERAD in mammals (Siggs et al., 2014). The exact mechanism whether EGFR ligands are exported or degraded is not clear yet.

**POTENTIAL ROLE OF I RHOMS IN HUMAN DISEASE**

Although iRhoms have no protease activity, they regulate the secretion of several ligands of EGFR and proinflammatory cytokine TNF-α. Therefore, iRhoms can activate the EGFR signaling pathway and inflammation process, which regulate cell survival, proliferation, migration and inflammation, resulting in modifications of disease condition. Two recent studies reported a strong association between Alzheimer’s disease and changes in iRhom2 methylation in the brain (De Jager et al., 2014; Lunnon et al., 2014). They showed that the methylation level in the *RHBDF2* gene was changed in Alzheimer’s disease and the *RHBDF2* expression was increased in the context of Alzheimer’s disease. In their connectivity analysis, *RHBDF2* was connected to *PTK2B* that is a key element gene of signaling cascade involved in modulating the activation of microglia and infiltrating macrophages. Therefore, changes in methylation in iRhom2 gene and its increased expression may be associated with the role of microglia and infiltrating macrophages in Alzheimer’s disease (De Jager et al., 2014).

Missense mutations in *RHBDF2*, iRhom2 encoding gene, were shown to cause tylosis esophageal cancer (TOC), the autosomal dominant condition, in four families from the UK, US, Germany and Finland (Blaydon et al., 2011; Saarinen et al., 2012). TOC is an inherited condition characterized by palmoplantar keratoderm and esophageal cancer (Abbruzzese et al., 2012; Rugg et al., 2002). Palmoplantar keratoderm usually begins around age 10, and esophageal cancer may form after age 20. The mutations occurred in the N-terminal domain of iRhom2, which has a highly conserved region in different species as well as between iRhom1 and iRhom2 (Blaydon et al., 2012). These reports indicate that the N-terminal domain may have some important functions, but little is known yet. On the other hand, unusual distribution of iRhom2 is reported in normal skin. The iRhom2 expression is detected primarily at the plasma membrane in the normal epidermis and is much more diffuse in cells from TOC patients (Blaydon et al., 2012), instead of ER and Golgi localization in macrophages. Alteration of iRhom2 localization was also observed in tylotic and esophageal squamous cell carcinomas (Blaydon et al., 2012). Moreover, there is recent evidence that these TOC-associated mutations in iRhom2 induce TACE activation in epidermal keratinocytes, resulting in increased shedding of TACE substrates including EGFR-family growth factors and pro-inflammatory cytokines (Brooke et al., 2014). These results may explain the high proliferative activity of TOC cells and the predisposed esophageal cancer development in TOC patients. The mutations of iRhom2 in TOC patients may also regulate the EGFR signal pathways by altering pro-EGF targeting of iRhom2 for ERAD (Zettl et al., 2011), instead of activation by EGFR cleavage by RHBDL2 (Adrain et al., 2011). These mutations in iRhom2 are also associated with ovarian cancer (Wojnarowicz et al., 2012). iRhom2 expressions were much lower in a subset of benign and malignant ovarian tumors compared with primary cultured cells from normal ovarian epithelium.

Altered iRhom1 may cause squamous epithelial cancer and breast cancer (Yan et al., 2008; Zou et al., 2009). iRhom1 expression is elevated in breast cancer samples and knockdown of iRhom1 by siRNA has led diminished EGFR transactivation in tissue culture cells (Zou et al., 2009).

**CONCLUSION**

iRhoms are unique members in Rhomboid family with unique domains as well as no catalytic active motif, suggesting an important biological role for these proteins, despite their lack of protease activity (Koonin et al., 2003; Lemberg et al., 2005; Siggs et al., 2014).
and Freeman, 2007; Freeman, 2014). In facts, recent studies started to reveal their diverse roles in TACE maturation and in EGFR signaling pathways. It appears that iRhoms are associated with development of several human diseases including cancers. The fact that iRhoms interact with TACE provides novel therapeutic opportunities for selective and simultaneous inactivation of the major signaling pathways which are closely associated with the disease development (Lisi et al., 2014). Hyperactivity of EGFR is implicated in many tumors with several molecular mechanisms, including the autocrine activation of EGFR by unregulated release of ligands (Buckland, 2013).

Recent research data provide genetic, cellular, and biochemical evidence that the principal function of iRhoms1/2 is regulated by TACE-dependent TNF-α or EGFR signaling pathway, suggesting that iRhoms1/2 could emerge as novel targets for treatment of TACE/TNF-α and TACE/EGFR- dependent pathologies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest to publish the results.

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