BIOMOLECULES & THERAPEUTICS

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 (RHBDD1)	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.
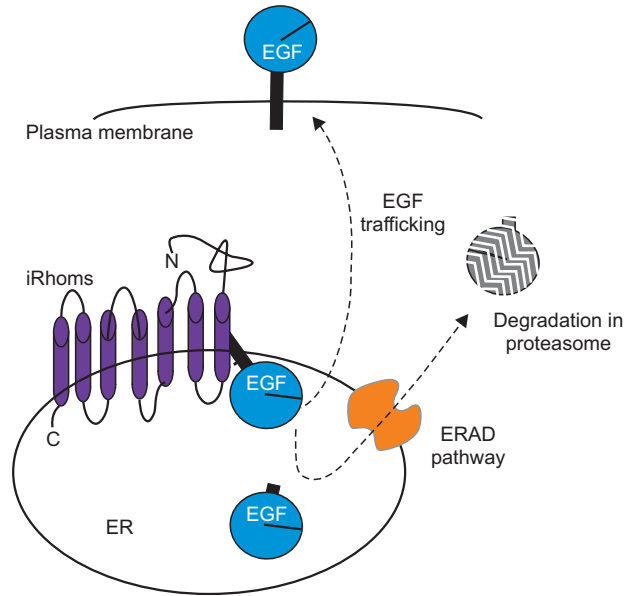
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Fig. 1. *Rhomboid protein iRhoms regulate the RGFR ligands in the ER.* iRhoms binds to various EGFR ligands in the ER and facilitate their forward trafficking or ERAD pathway. The mechanism with which the fate determination of the EGFR ligands is not clear yet.

al., 2001; Urban *et al*., 2002; Lemberg *et al*., 2005; Urban and Wolfe, 2005). A tryptophan-arginine motif in loop 1 present between first and second transmembrane domains is also an invariant structure observed in rhomboid proteases.

Although the first mammalian rhomboid protease was cloned and named *RHBDL1* for rhomboid-like protein1 as early as before *Drosophila* Rhomboid-1 was recognized as intra-membrane protease (Pascall and Brown, 1998), the function is remained elusive yet. RHBDL2, however, was shown to share the catalytic activity of *Drosophila* Rhomboid-1. The localization of the known five mammalian rhomboid proteases is diversely scattered; golgi for RHBDL1, plasma membrane for RHBDL2, endosomes for RHBDL3, endoplasmic reticulum (ER) for RHBDL4 and mitochondrial inner membrane for PARL (Bergbold and Lemberg, 2013), suggesting their distinct and diverse functions (Table 1). Actually only RHBDL2 can cleave and activate the mammalian proEGF (Adrain *et al*., 2011). And EGFR signaling is negatively modulated by RHBDL2-mediated lysosomal degradation of EGFR (Haglund and Dikic, 2012) or EGFR cleavage (Liao and Carpenter, 2012). On the other hand, RHBDL4 localizing to the ER can induce degradation of various substrates (Bergbold and Lemberg, 2013), as a part of ER-associated protein degradation (ERAD) machinery as shown in Fig. 1 (Fleig *et al*., 2012).

There are, however, other subgroups of rhomboid family members, calling iRhoms, with high sequence similarities. These rhomboid family members, iRhom1 and iRhom2, have no the key catalytic motif observed in rhomboid proteases, meaning inactive rhomboid (Lemberg and Freeman, 2007; Ha *et al*., 2013). iRhom1 and iRhom2 have lost their protease activity during their evolution but retained the key non-protease functions, which have been implicated in the regulation of EGF signaling pathway (Adrain *et al*., 2011) and TNF-α signaling pathway (Adrain *et al*., 2012).

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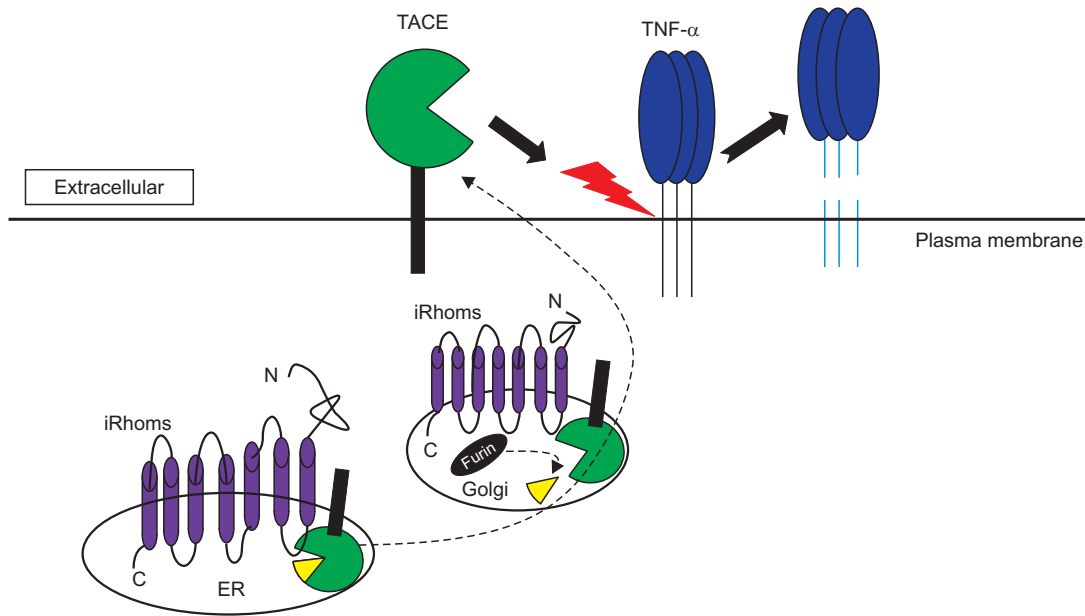
Fig. 2. *Rhomboid protein iRhoms regulate the trafficking and maturation of TNF-a converting enzyme (TACE)*. iRhoms bind to TACE, which promotes its exit from ER to Golgi. Within the Golgi, TACE is processed by furin into its mature form. At the plasma membrane, TACE cleaves the membrane-bound form of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

tory TNF-α pathway, with important roles in development and disease (Black *et al*., 1997; Peschon *et al*., 1998; Sahin *et al*., 2004). TACE is essential for release of EGFR ligands (Sahin *et al*., 2004; Sahin and Blobel, 2007) and TNF-α (Black *et al*., 1997; Issuree *et al*., 2013), which is the primary trigger of inflammation (Adrain *et al*., 2012). Like many extracellular signaling proteins, TNF-α is synthesized as a transmembrane protein, and the active signal of its ectodomain is shed from cells after cleavage by a metalloprotease, TACE (Black *et al*., 1997; Siggs *et al*., 2012). TNF-α is considered as a 'fire alarm' of the body, as it helps body fight against infections. But TNF-α can also cause diseases such as inflammatory arthritis (Haxaire and Blobel, 2014). TACE and its regulator, iRhom2, can be rapidly activated by small amounts of cytokines, growth factors, and pro-inflammatory mediators present in the blood (Hall and Blobel, 2012), substantiating rapid alarm through TNF-α. iRhoms are co-expressed with TACE and are essential for specific regulation of TACE activity as shown in Fig. 2 (Christova *et al*., 2013).

iRhom1 and iRhom2 are both essential for regulation of TACE (Zou *et al*., 2009). iRhom2 is highly expressed in macrophages but not in skin or other tissues (Christova *et al*., 2013) and is a key regulator in myeloid TACE (Lichtenthaler, 2013). Loss of iRhom2 blocks maturation of TACE in macrophages, resulting in defective shedding of TNF-α (Li *et al*., 2015). iRhom2 binds to TACE and promotes its transfer from the ER. Therefore, without iRhom2, TACE is unable to exit the ER and be trafficked to the Golgi apparatus where its inhibitory prodomain is removed by furin (Christova *et al*., 2013). Therefore, inactivation of iRhom2 in mice prevented maturation of TACE in hematopoietic cells but not other cells and tissues (Issuree *et al*., 2013), and targeting of iRhom2 effectively inactivated TACE in immune cells without affecting its function in other tissues (Wasserman *et al*., 2000; Lemberg, 2013). Its protective role in relation to TNF-α has been demonstrated

in mouse models with shock induction by lipopoloysaccharide (Adrain *et al*., 2012) or with *Listeria monocytogenes* infection (McIlwain *et al*., 2012). Inactivation of iRhom2 in human macrophages also prevents maturation of TACE and release of TNF-α from cells, suggesting that the iRhom2/TACE/TNF-α pathway has been conserved in both mice and humans (Puente *et al*., 2003; Issuree *et al*., 2013). Therefore, iRhoms are considered as attractive novel targets for treatment of TACE/TNF-α-dependent pathologies (Issuree *et al*., 2013).

IRHOM-MEDIATED REGULATION OF EGFR SIGNALING

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; Hosur *et al*., 2014). 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

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 EGF 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-α 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 EGF signaling pathway by binding to EGF 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 EGF family ligands in regulation of EGF 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 IRHOMS 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, result-

ing 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*., 2012; Saarinen *et al*., 2012). TOC is an inherited condition characterized by palmoplantar keratoderma and esophageal cancer (Abbruzzese *et al*., 2012; Rugg *et al*., 2002). Palmoplantar keratoderma 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 EGF-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 EFGR signal pathways by altering proEGF targeting of iRhom2 for ERAD (Zettl *et al*., 2011), instead of activation by EFGR 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

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 iRhomes are associated with development of several human diseases including cancers. The fact that iRhomes 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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