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Review

Developmental roles of Rhomboid proteases

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ABSTRACT

Rhomboid proteins have emerged as one of the most tantalizing and diverse families of proteases. Gene duplication events and structural alterations have sculpted the varied roles of this protein family, maintaining a conserved structural core throughout the bacterial, plant and animal kingdoms. Unresolved questions pop up at many junctions. This review will focus on a distinct class of Rhomboid proteins that plays an essential role in development. It will outline the diverse mechanisms by which these proteins are regulated, and the implications on the biological processes they control. While most of the review will deal with Rhomboids in *Drosophila*, a system that has been studied in the greatest detail, it will also explore parallels and differences in the function of Rhomboids in the flour beetle *T. castaneum* and the worm *C. elegans*.

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1. Rhomboid1 intracellular localization and trafficking of EGFR ligands

Rhomboid proteases maintained a conserved structural core throughout the bacterial, plant and animal kingdoms [1,2]. Epidermal growth factor (EGF) receptor signaling plays a pivotal role in determination of cell fates at many junctions during *Drosophila* embryonic and post-embryonic development [3,4]. As a rule, the receptor and its downstream canonical Mitogen activated protein kinase (MAPK) pathway are ubiquitously expressed, “awaiting”

the regulated activation by ligands. The tight spatial and temporal regulation of the pathway is dictated by the ligands. Three components are essential for ligand processing and secretion. The cardinal ligand, Spitz (Spi), is produced as an inactive transmembrane precursor [5], and retained in the endoplasmic reticulum (ER). It is trafficked by bulk flow to the Golgi, but returned to the ER by retrograde trafficking. In order to prevent retrograde trafficking and allow processing and secretion, a second protein, Star, is required. Star is a type II transmembrane protein that associates with Spi in the ER and blocks its retrograde trafficking [6,7]. Following trafficking from the Golgi to a Rab 4 and Rab14 endosomal compartment [8], the Spi/Star complex encounters the Rhomboid1 protein, which cleaves the transmembrane domain of the Spi precursor [9]. This releases the Spi extracellular domain containing the

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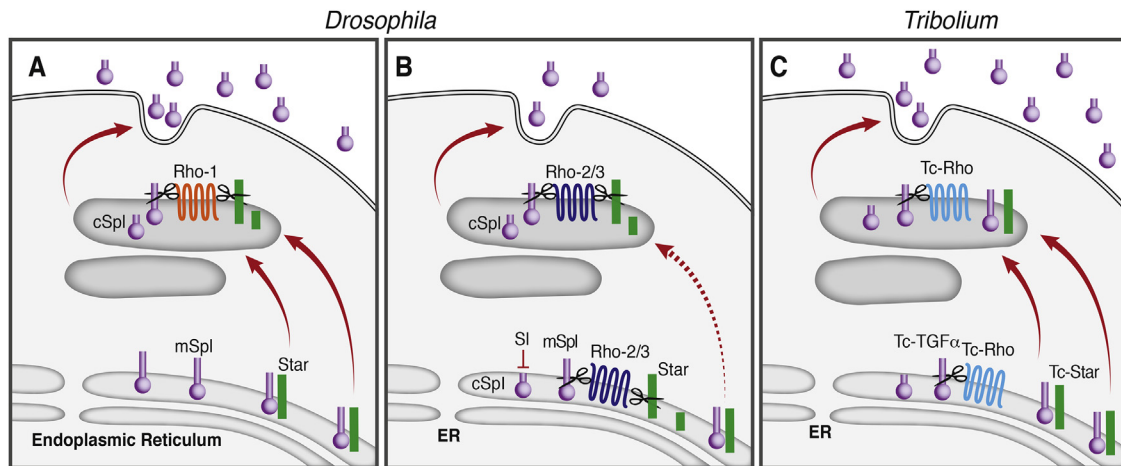


Fig. 1. Diversification of Rhomboid intracellular localization impinges on EGFR ligand processing.

A,B) In *Drosophila*, the existence of multiple Rhomboid proteins enables modulation of the processed ligand levels by differential compartmentalization. Rhomboid1 is located only at the secretory compartment and mediates high signaling levels (A). Rhomboids2/3 display a combined ER and secretory compartment localization and cleavage activity, leading to an attenuated signal, primarily due to cleavage in the ER of the ligand chaperone Star. (B). The cleaved ligand (cSpi) generated in the ER, is retained by Small wing (SI) and is thus inactive. C) In the flour beetle *Tribolium*, the single Rhomboid is active in the combined ER and secretory compartment mode. Signaling levels remain high, however, since Tc-Star is refractive to Rhomboid cleavage and can efficiently traffic the Spi precursor to the secretory compartment.

EGF motif, allowing its subsequent secretion to the extracellular milieu (Fig. 1A).

Three elements, Spi, Star and Rhomboid, comprise a cassette that is sufficient for ligand processing. When expressed in *Drosophila* S2 cells or in mammalian cells, efficient trafficking and cleavage ensues [6,7]. This indicates that the signals that target each of these proteins to distinct cellular compartments are conserved. It also implies that once Rhomboid and Spi are located within the same compartment, efficient cleavage takes place, suggesting that no accessory proteins are required. The primary regulation of the processing cassette is thus executed at the level of intracellular compartmentalization and trafficking. It follows that diversification of function or regulation of Rhomboid proteins may emerge from alterations in their intracellular localization.

2. Rhomboid1 exhibits restricted substrate specificity

Proteins of the Rhomboid family exhibit a diverse array of substrate specificities. For example, members that are involved in quality control in the ER are required to recognize a broad range of substrates [10]. Rhomboid1, on the other hand, displays a very narrow substrate specificity. A powerful approach to gauge the roles and potential substrate specificity of Rhomboid1 is to define the mutant phenotype resulting from its absence. In view of the multiple roles of EGFR throughout development, there are many instances in which the phenotypes can be monitored. In all cases, the phenotype of Rhomboid1 loss-of-function is synonymous with loss of elements in the ligand cassette (Spi, other ligands and Star), or with mutants in EGFR [11,12]. This indicates that Rhomboid1 is dedicated to the EGFR pathway, and does not have substrates outside this cascade. Moreover, Rhomboid1 activity is essential and thus its absence results in a complete shutoff of the pathway.

Within the EGFR pathway, what are the Rhomboid1 substrates? There are four ligands that activate *Drosophila* EGFR [13]. Three of them (Spi, Gurken and Keren) are produced as type I transmembrane precursors that require Rhomboid cleavage for activation [5,14,15]. The fourth ligand, Vein, is produced as a secreted protein that does not require processing [16]. Surprisingly, another member of the ligand-processing cassette, Star, is also a substrate for Rhomboid1 [17]. This highlights the capacity of Rhomboid proteins to cleave both type I and type II transmembrane proteins. It is not clear however, if Star cleavage by Rhomboid1 has a significant

regulatory outcome. The two proteins first “meet” in the late secretory compartment, after Star has already fulfilled its job of bringing Spi to Rhomboid1. Following Star cleavage and inactivation, the prevention of Star recycling may have a regulatory significance. As will be outlined below, the capacity of Rhomboids to cleave Star has substantial regulatory implications for other members of the Rhomboid family.

3. Regulation of rhomboid1 expression

If spatial and temporal regulation of EGFR activation relies on the ligand-processing cassette, which of the three elements in this group is normally limiting? Expression of Spi and Star appears to be ubiquitous. Presumably, in the absence of Rhomboid the Spi/Star complex fails to reach the plasma membrane. Thus, the Spi precursor is protected from fortuitous cleavage by broad-specificity metalloproteases that reside on the plasma membrane. In contrast, expression of *rhomboid1* is extremely restricted and dynamic [18–20]. In fact, there is a strict correlation between the expression pattern of Rhomboid1 and the activation of the EGFR pathway as monitored by staining for phosphorylated MAPK [21,22]. Moreover, ectopic expression of *rhomboid1* in different tissues leads to broad EGFR activation, indicating that Rhomboid is the only limiting element in the ligand-processing cassette [20].

The entire plan for EGF activation during development is essentially “etched” into the enhancer of *rhomboid1*. Dissection of this enhancer has indeed identified discrete regulatory elements that are dedicated to the expression of Rhomboid1 in the different tissues [19,23]. Having the regulatory switch for the EGFR pathway rely on the expression of an enzyme is a “risky” prospect, since low levels of leaky expression of *rhomboid1* could give rise to fortuitous EGFR activation. Nevertheless, activation of EGFR is tight, suggesting that the switch of *rhomboid1* expression is firm. In addition, biochemical studies have implied that intra-membrane proteolysis is a slow inefficient process, and the turnover times for substrates are extremely long [24]. Thus, the number of processed ligand molecules that could be generated by a “stray” Rhomboid1 protein may not be high.

The normal expression of *rhomboid1* defines the cells that are actively processing the ligand. On the other hand, the pattern of phosphorylated MAPK in the tissues where EGFR is activated outlines the distribution profile of the active ligand itself. A comparison

between these two patterns sketches the diffusion pattern of the ligand in each tissue. Indeed, there are instances, such as the early embryonic neuroectoderm, where the two patterns are overlapping, indicating that the active ligand is activating, in an autocrine manner, the same cells where it was produced [25]. In other cases, activation of the pathway is monitored up to several cell rows away from its source. For example, following gastrulation the ligand-processing cassette operates in a single cell row- the midline cells, while activation of EGFR is seen in the neuroectoderm up to six cell rows away [22,26].

4. *rhomboid1* induction by EGFR signaling mediates a ratchet mechanism

rhomboid expression can be viewed as the cardinal trigger for EGFR activation. In many scenarios, expression of *rhomboid* is triggered by a combination of transcription factors that collectively define the correct time and place. For example, activation by the midline transcription factor Single minded triggers *rhomboid1* expression in the midline [27]. This type of expression is spatially “static” and temporally transient, in that it will only last as long as this set of transcription factors is maintained.

It is interesting to consider another mode of induction of *rhomboid1* transcription, which is more dynamic. In this setting, *rhomboid* expression is actually triggered by the EGFR pathway. This mode of regulation provides an expanding, “Ratchet like” mechanism of EGFR activation. An initial source of *rhomboid1* expression and ligand processing is defined by cues that are independent of EGFR signaling. However, once the neighboring cells respond to the EGFR signal they will themselves trigger expression of *rhomboid1*, which will generate active ligand for the next cohort of cells, and so on. The number of cells in which *rhomboid1* expression will be induced at each round depends on the ligand diffusion properties, and the sum of such cycles will depend on the developmental competence window and the available pool of undifferentiated cells.

The tissue which displays this ratchet mechanism most dramatically is the developing compound eye, which harbors ~750 ommatidia. Each ommatidium comprises a unit of 20 cells that include different types of photoreceptors, cone and pigment cells. Development occurs during the larval and pupal stages, where differentiation takes place dynamically in the wake of a progressing “morphogenetic furrow” [28]. Initially, the cells that will form photoreceptor R8 are defined, in an EGFR-independent manner. The precise placement of the R8 cells will determine the spacing between future ommatidia [29,30]. The R8 cell now serves as an anchor to recruit an additional seven photoreceptor cells, as well as cone and pigment cells, by triggering reiteratively the EGFR pathway [31]. Induction of EGFR signaling in the two cells adjacent to R8 will trigger expression of *rhomboid1* [29]. These cells now become the source of ligand for the next two cells, and the process will continue until the full complement of ~20 cells is recruited to each ommatidium.

Different cell types are generated by the repeated activation of the EGFR pathway, due to alteration in the transcription-factor landscape of the responding cells. The duration of each activation cycle is important. Obviously, the time of each EGFR activation cycle has to be matched with the dynamics of this changing landscape. A tight regulation of the diffusion range of the ligand is required at each cycle, in order to define the correct number of cells in each cohort. Induction of inhibitory feedback loops provides one way to limit the number of responding cells [32–34]. Below, we will also discuss a Rhomboid-based mechanism that restricts the amount of ligand secreted by the photoreceptor cells.

5. *rhomboid1* induction by EGFR signaling amplifies the response

Multiple rounds of EGFR activation within the same tissue can be generated by virtue of reiterated induction of *rhomboid1* expression. The capacity to trigger *rhomboid1* expression by EGFR signaling can also promote propagation of EGFR signaling *between* tissues. During oogenesis, migration of the oocyte nucleus defines the future dorsal site along the dorso-ventral axis. The mRNA encoding Gurken, an EGFR ligand precursor, is localized around the oocyte nucleus leading to graded distribution of the secreted Gurken protein [35]. The resulting activation profile of EGFR in the surrounding follicle cells defines distinct cell types that will be generated [36]. Towards the end of oogenesis the follicle cells secrete the vitelline membrane that insulates them from the oocyte. However, by this time, Gurken signals that emanated from the egg have triggered local expression of *rhomboid1* in the follicle cells [37,38]. This leads to processing of the Spi ligand in the follicle cells, and the resulting local EGFR activation in the follicle cells is important for shaping distinct extended structures of follicle cells, termed dorsal appendages.

In the worm *C. elegans*, the induction of vulval cell fates depends on EGFR signaling. Initially, a single cell termed the anchor cell, expresses the ligand Lin-3, which is likely to be processed by a ubiquitous metalloprotease. Once the ligand triggers EGFR activation in the underlying vulval-precursor cells, these cells begin to express a Rhomboid protein [39]. Presumably, the local processing of ligand produced by these cells will amplify EGFR response, and assure a robust induction and activation of more distant cells, thus amplifying the initial signal from the anchor cell.

6. Rhomboid2 and Rhomboid3 have distinct functions

The ancient origin of Rhomboid proteins is reflected by the presence of multiple members in the genomes of different organisms [40]. *Drosophila* is no exception, and in fact seven different members of the Rhomboid family, including the founding member Rhomboid1, were identified, [41]. Within this family one protein is dedicated to mitochondrial functions [42], and one member is a non-functional protease defining the iRhom family of proteins that may facilitate protein trafficking or degradation in the ER and Golgi [43]. Two additional members, Rhomboid2 (also termed Stet or Bro) [14,44] and Rhomboid3 (also termed Roughoid) [41] have an active proteolytic site, and were shown to be able to cleave the EGFR ligands similar to Rhomboid1 [45]. However, they appear to have distinct biological roles that were revealed by analysis of their mutant phenotypes.

rhomboid2 is expressed in the male and female germ line. Ligand processing in germ cells activates Egfr signaling in adjacent somatic cells [44,46]. These somatic support cells encapsulate germ cells, thereby inducing germ cell differentiation. In *rhomboid2* mutants, somatic support cells fail to surround the germ line, resulting in arrested differentiation. Thus, Rhomboid2 activity in the germ line is required for germ cell differentiation, via Egfr activation in neighboring somatic cells.

rhomboid3 is expressed in the developing eye disc, and in its absence rough eyes are formed. Closer examination revealed that both *rhomboid1* and *rhomboid3* are expressed in the eye, and the *rhomboid3* mutant phenotype results from excessive EGFR activation when only Rhomboid1 is operating [47]. *Rhomboid3* is also expressed in the embryo, and plays a role in tracheal cell migration [48]. The presence of distinct tissues where other Rhomboid proteins are required raised the question what is unique about these tissues, and how are Rhomboid2/3 adapted to fulfill these specific requirements.

7. Rhomboid2/3 intracellular localization modulates ligand processing

We mentioned earlier that the activity of the EGFR ligand-processing cassette is intimately linked to intracellular localization and trafficking. While Rhomboid1 protein is restricted to a punctate pattern coinciding with the secretory compartment, Rhomboid-2 and -3 display, in addition to the punctate distribution, a perinuclear pattern consistent with ER localization [47]. How does this distribution impinge on the function of Rhomboid-2 and -3?

Two hints pointed at the possible answer. In both the germline and the eye, the number of cells recruited by EGFR signaling should be tightly controlled. In addition, both tissues are highly sensitive to the level of Star protein. While heterozygosity for Star does not give rise to any phenotypes in tissues where EGFR is activated by Rhomboid1 expression, in the eye it leads to rough eyes (as originally noted by Calvin Bridges in 1919), due to a lower number of recruited photoreceptor cells.

How could the presence of Rhomboid proteins in the ER affect the ligand-processing outcome? When Rhomboid proteins meet their substrate in a common compartment, cleavage ensues. This indicates that no accessory proteins are required for the cleavage, and the convergence or segregation of Rhomboid from its substrates is the key. The two other elements in the ligand-processing cassette are also transmembrane proteins that are trafficked through the ER, and are potential substrates for Rhomboid in this compartment. Upon encounter with Rhomboid in the ER, Star and Spi may undergo premature cleavage. In both cases, an inactive component will be generated. In the case of Spi, it was shown that cleaved Spi is trapped in the ER and does not undergo secretion [49]. In the case of Star, the cleaved protein cannot facilitate Spi trafficking [17]. The haploinsufficiency phenotype for Star suggested that this element is compromised in the tissues where Rhomboid is also located in the ER. Conversely, overexpression of Star in these tissues leads to excessive EGFR activation, indicating that the level of Star modulates the amount of secreted ligand.

The emerging model is that in tissues where Rhomboid is localized at both ER and secretory compartments, the level of secreted ligand is attenuated. In the ER, the cleavage of Star reduces the amount of ligand precursor that can be effectively trafficked to the secretory compartment. Hence, Rhomboid in the ER has a mitigating role. Once the ligand precursor reaches the secretory compartment and encounters Rhomboid, it will be effectively processed and secreted. The net effect is thus a reduction in the level of secreted ligand, impinging on the range of EGFR signaling and the number of cells that are recruited [47] (Fig. 1B).

The diversity of Rhomboid localization in *Drosophila*, and the capacity of Star to undergo Rhomboid-dependent cleavage generates two modalities of signaling. In the case of Rhomboid1 that is restricted to the secretory compartment, high levels of cleaved ligand are produced. Conversely, Rhomboids 2/3 that are also present in the ER, reduce the level of active Star in the ER and hence compromise the amount of ligand that is secreted.

8. Rhomboid3 axonal trafficking is facilitated by ER localization

EGFR signaling in the developing eye disc is perhaps the most elaborate and convoluted scenario for this pathway. Following the reiterated activation of the pathway within the disc epithelium, to recruit the different elements of the ommatidia, the photoreceptor cells send long axonal projections towards the brain. The processed Spi ligand that these cells produce now serves to induce neuronal cell fates in the brain [50,51]. In view of the central role of Rhomboid3 in recruitment of photoreceptor cells, it was interesting to

examine what functions it may have in the subsequent phase, when EGFR is activated within the brain.

It was shown that Rhomboid3 is absolutely essential for the capacity of the axonal projections to trigger EGFR in the brain. Moreover, it is the ER localization of Rhomboid3 that is crucial. While in the epithelium the ER localization attenuated the ligand-processing capacity, in the axonal projections it has an essential facilitating role. The ER extends throughout the axon. Thus, the localization of the three elements of the ligand-processing cassette within the ER enables their effective trafficking through the axon until they reach the axon terminus where they are delivered to the cell membrane, in a similar manner to their earlier trafficking in the cell body [8]. Local trafficking and processing of ligand at the axon terminus will then generate a Spi signal that is required to activate EGFR in the adjacent brain cells.

9. Evolution of Rhomboid proteases

It was interesting to investigate how the repertoire of Rhomboid localization sites within the cell was generated during evolution, and which form of Rhomboid may represent the more ancestral type. The flour beetle *Tribolium castaneum* diverged from *Drosophila* ~250 million years ago. In *Tribolium* each of the three elements of the ligand-processing cassette is represented by only a single member. Examination of these elements indicated that the *Tribolium* Rhomboid displays the dual intracellular localization. In addition, the *Tribolium* Star chaperone cannot be cleaved by Rhomboid proteins [52] (Fig. 1C). It is interesting to note that the properties of the Rhomboid catalytic domain appear to be maintained throughout evolution. For example, *Drosophila* Spi and Star can be cleaved efficiently by the *Tribolium* Rhomboid [52]. Thus, the critical changes during evolution take place at the level of alteration in the propensity of substrates to be cleaved, and by the intracellular localization of Rhomboid proteins.

We can tentatively reconstruct the following evolutionary sequence: the ancestral Rhomboid protein displayed a dual intracellular localization. However, it was still able to produce large amounts of the processed ligand since the Star protein was refractive to cleavage, and could effectively transport the ligand to the late secretory compartment. A duplication of the Rhomboid protein gave rise to two different patterns of intracellular protein localization. In parallel, the Star protein has acquired the capacity to be cleaved by Rhomboid [52]. Thus, two modes of ligand processing were generated, giving rise to different levels of processed ligand that is eventually secreted (Fig. 1).

10. Conclusions

In conclusion, the Rhomboid intramembrane proteases provide the key to regulation of the EGFR pathway in *Drosophila*. Rhomboids 1–3 are dedicated to the ligands of the EGFR pathway. Their dynamic expression pattern defines the resulting profile of EGFR activation, and their intracellular localization demarcates the amount of ligand that will be released from the cells. It is interesting to note that while the EGFR pathway is highly conserved, the strategy of ligand processing in vertebrates and to some extent also in *C. elegans* is different, relying mostly on restricted expression of the ligand precursor, followed by processing mediated by ubiquitous ADAM metalloproteases. However, the capacity of two mammalian Rhomboid homologues to process the EGFR ligand precursor TGF α , indicates that this mode of processing may also be playing a role in mammalian cells and contributes to tumor growth in pathological situations [53,54].

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