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Versatility of EGF receptor ligand processing in insects

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ABSTRACT

Processing of EGF-family ligands is an essential step in triggering the EGF receptor pathway, which fulfills a diverse set of roles during development and tissue maintenance. We describe a mechanism of ligand processing which is unique to insects, and possibly to other invertebrates. This mechanism relies on ligand precursor trafficking from the ER by a chaperone, Star (S), and precursor cleavage by Rhomboids, a family of intra-membrane protease. Remarkably, the ability of Rhomboids to cleave S as well, endows the pathway with additional diversity. Rhomboid isoforms which also reside in the ER inactivate the chaperone before any ligand was trafficked, thus significantly reducing the level of ligand that will eventually be processed and secreted. ER localization also serves as a critical feature in trafficking the entire ligand-processing machinery to axonal termini, as the ER extends throughout the axon. Finally, examination of diverse species of insects demonstrates the evolution of chaperone cleavability, indicating that the primordial processing machinery could support long-range signaling by the ligand. Altering the intracellular localization of critical components of a conserved signaling cassette therefore provides an evolutionary mechanism for modulation of signaling levels, and diversification of the biological settings where the pathway functions.

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Conservation of the EGF receptor signaling pathway

The EGF receptor signaling pathway has emerged as one of the central modes by which cells communicate with their neighbors in all multicellular organisms. The pathway is initiated by ligands of the TGF alpha family, which are produced as inactive transmembrane precursors. Cleavage generates the secreted form of the ligand which is active, and upon binding gives rise to receptor dimerization. Dimerized receptors contain an active cytoplasmic tyrosine kinase domain, triggering trans-phosphorylation of key tyrosine residues, to generate docking sites for SH2-domain proteins. Most importantly, the recruitment of Ras GEF to the membrane triggers activation of Ras and a cascade of three cytoplasmic kinases, culminating in the activation of MAP kinase (ERK) that is translocated to the nucleus to activate transcription factors (Citri and Yarden, 2006; Deribe et al., 2010; Schlessinger, 2002).

The pathway was shown to be remarkably conserved, from *C. elegans* and *Drosophila* to vertebrates (Shilo, 2003). The presence of a single receptor in invertebrate species, in contrast to the four members in vertebrates, simplifies the analysis of the pathway and its functions, since a single homodimeric receptor pair represents the only form of activation. At the level of the ligands, while *C. elegans* displays a single ligand, there are four ligands in *Drosophila* (Shilo, 2005).

Remarkably, in spite of the conservation in ligand structure, the mechanisms generating the active processed ligand are distinct between vertebrates and *Drosophila*. In vertebrates, the sites of ligand expression are restricted, while the processing itself is carried out by ubiquitously expressed metalloproteases (Lee et al., 2003). Genetic and biochemical dissection has identified a novel strategy for ligand processing in *Drosophila*.

EGF ligand processing in *Drosophila*

The cardinal *Drosophila* ligand is termed Spitz (Spi), but two additional ligands, Gurken and Keren are similarly regulated in the tissues where they are utilized (Neuman-Silberberg and Schupbach, 1993; Reich and Shilo, 2002; Schweitzer et al., 1995). Grouping of *S* and *rhomboid* together with *spi* as loci giving rise to similar mutant phenotypes (Mayer and Nusslein-Volhard, 1988) has upon further analysis defined two central components in ligand processing. Spi is broadly expressed, and is retained in the ER, in order to prevent its accessibility to non-specific metalloproteases at the cell surface. Star is a type II transmembrane protein that is also located in the ER. Association between the Spi precursor and S allows trafficking of the ligand to a late secretory compartment, where the complex meets the Rhomboid protein (Fig. 1A) (Lee et al., 2001; Tsruya et al., 2002). Rhomboid encodes a seven-pass transmembrane protein, and represents a highly conserved family of serine proteases that carry the key active site residues within transmembrane domains. Rhomboid activity leads to intra-membrane proteolysis of Spi (Urban et al., 2001).

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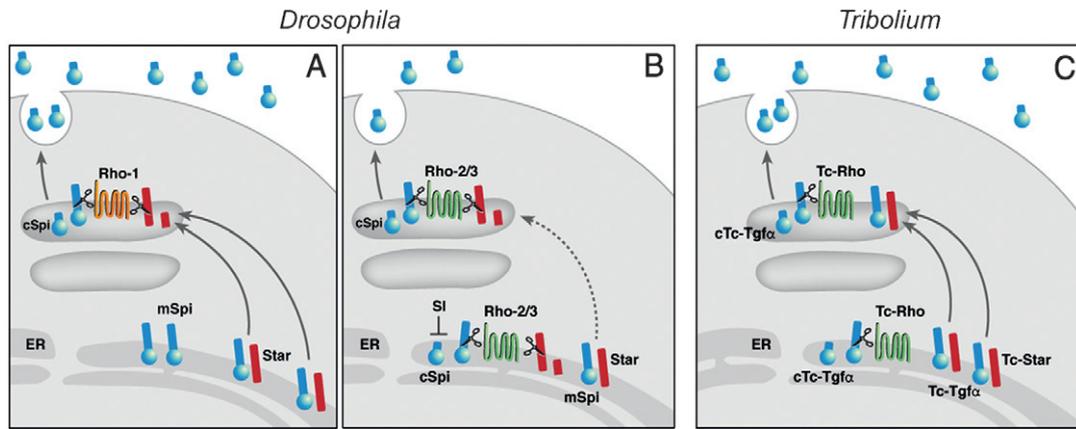*Drosophila*   *Tribolium*

Fig. 1. Variations in the ligand-processing machinery generate distinct modes of EGFR signaling. A,B) In *Drosophila*, multiple Rhomboids enable modulation of the signaling levels by differential compartmentalization. Rho-1 is distributed only to the secretory compartment and mediates high signaling levels (A). Rho2/3 employ the combined ER and secretory compartment cleavage, leading to an attenuated signal, primarily due to cleavage of the ligand chaperone, Star, in the ER (B). The cleaved ligand (cSpi) generated in the ER, is retained by a Small wing (Sl) dependent mechanism. C) In *Tribolium*, the single Rhomboid is active in the combined ER and secretory compartment mode. Signaling levels are high, however, since Tc-Star is refractive to Rhomboid cleavage in the ER.

While Spi and S are ubiquitous, it is the dynamic expression pattern of Rhomboid that determines the sites and timing of EGF receptor activation. Essentially, the information embedded within the promoter of *rhomboid* is the blueprint for the pattern of EGF receptor activation throughout development. Rhomboids have a limited range of substrates (Strisovsky et al., 2009). One surprising finding was that the ligand chaperone protein, S, is also cleaved by Rhomboid (Tsruya et al., 2007). Since the transmembrane topologies of Spi and S are inverted, it is not clear mechanistically how Rhomboid can cope with the two types of substrates. The biological significance of S cleavage by Rhomboid became apparent when specific tissues, in which EGF signaling plays a critical role, were analyzed.

ER localization of Rhomboids reduces the signal level

Three Rhomboids mediate EGFR signaling in *Drosophila*. Despite high sequence similarity between them and similar catalytic activities, they differ in their subcellular distribution. Whereas Rho-1, the founding and best studied member of the family is exclusively localized to a secretory, punctate compartment, Rho-2 and -3 are distributed between the ER and the Rho-1 compartment (Yogev et al., 2008).

What is the biological significance of the differential compartmentalization? A revealing observation is that Rho-2 and -3 are dedicated to tissues where Spi is secreted over a very short range, the developing gonad and the eye, respectively. This suggests that ER localization provides an attenuating function. Indeed, it was discovered that cleavage in the secretory compartment mediates effective Spi secretion, whereas the ER activity of Rho-2 and -3 attenuates signaling (Yogev et al., 2008). Thus, all three proteases act as activators of EGFR signaling via late compartment cleavage. However, Rho-2 and -3 are milder activators than Rho-1 due to their additional attenuating ER activity. This was evident by observation that tissues in which both Rho-1 and -3 are expressed secrete higher levels of Spi upon removal of Rho-3.

Since Rhomboids can cleave both Spi and S in the ER, the question arises as to which is the relevant substrate to their attenuating activity. ER cleavage could prime Spi for retention, or prematurely inactivate the chaperone, before it accomplishes Spi trafficking. While the effects of the first possibility may still contribute to ER based attenuation, it was found that premature S cleavage is the main mechanism by which Rho-2 and -3 dampen signaling (Fig. 1B) (Yogev et al., 2008). This finding also explains the long standing observation of defective eye development in S heterozygous flies (Bridges and Morgan, 1919): the developing eye, in which Rho-3 is active, is very sensitive to S levels, while most other tissues, where Rho-1 mediates signaling and EGFR activation, are not affected by reduction of S levels.

Correspondingly, it was more recently observed that the developing male and female gonads, where Rho-2 is expressed, are also sensitive to S levels (Kitadate and Kobayashi, 2010; Yogev et al., 2008).

The identification of compartmentalization as a tier of regulation illustrates how a signaling pathway's output can be adjusted by the cellular machinery, without resorting to novel components.

Spi processing by Rho-1 occurs in Rab4 and Rab14 positive endosomes

The role of compartmentalization and trafficking in regulating EGFR activation raise the issue of the identity of the late secretory compartment in which the active EGFR ligand is produced. In mammalian cell culture, *Drosophila* Rho-1 localizes to the Golgi (Lee et al., 2001). However, this localization is not observed in *Drosophila* cells or *in-vivo*. Instead, in both these settings Rho-1 displays significant co-localization with Rab4 and Rab14 positive endosomes (Yogev et al., 2010). Interestingly, this colocalization can be with either or both Rabs, indicating that the Rho-1 steady-state distribution is dynamic.

Rab14 was shown to mediate trafficking between the Golgi and endosomes (Junutula et al., 2004; Kitt et al., 2008), and accordingly, Rho-1 passes through the Golgi on its way to the late compartment. Rab4 regulates a fast recycling route for endocytosed cargo (van der Sluijs et al., 1992; Zerial and McBride, 2001). This raises the possibility that endocytosis regulates the steady-state distribution of Rho-1, and may play a role in ligand maturation, as is the case for the Notch signaling pathway. Indeed, blocking endocytosis alters the Rho-1 intracellular distribution (our unpublished observations). The Rho-1 endosomal distribution is critical to EGFR ligand emission, as cells in which this distribution is perturbed fail to correctly secrete Spi.

Endosomal trafficking may also assist in preventing ectopic EGFR activation. In fact, Spi molecules which arrive at the cell surface can be cleaved by non-specific metalloproteases, leading to Rho independent signaling (Reich and Shilo, 2002). The dynamic localization of Spi and S to an endosomal compartment may ensure that uncleaved ligand is delivered to degradation in lysosomes, without reaching the cell surface

ER localization of Rho-3 controls secretion from photoreceptor axons

Repetitive activation of the EGFR via Spi triggers recruitment of photoreceptor neurons into ommatidia. Upon differentiation, these neurons grow basal axons, which traverse the eye disc field, funnel into the optic stalk, and arrest their growth at the first optic neuropil

in the brain—the lamina. Photoreceptor neurons secrete the Hh signal, followed by Spi, from their axonal termini, triggering neurogenesis in lamina precursor cells (Chu et al., 2006; Huang and Kunes, 1996; Huang et al., 1998). Thus, the Spi processing machinery is employed at two distinct locations within the same cell: initially within the cell body to pattern the ommatidia, and later at the distant axonal growth cones.

Rho-1 and Rho-3 function together in the developing eye to process and influence Spi secretion from the neuronal cell bodies. As discussed above, ER localization of Rho-3 in this context serves an attenuating role. While this aspect of Rho-3 function influences proper patterning of the eye field, it is not essential, since Rho-1 is present to ensure Spi secretion from cell bodies and eye development. Lamina neurogenesis, on the other hand, relies exclusively on Rho-3, as secretion of Spi from axons completely fails in *rho-3* mutants (Yogev et al., 2010). In this instance, ER localization of Rho-3 is a critical feature underlying generation of an active Spi signal at the right time and place.

What is the role of the ER in mediating the axonal signal? In *Drosophila* photoreceptors, as in many neurons, the ER extends throughout the axon. A key observation is that Rho-3, along with Spi and S, are enriched in axons, whereas Rho-1 is not. Artificial relocalization of Rho-1 to the ER also induces its axonal localization. The ER thus serves as a selective conduit of the Spi processing machinery, promoting its axonal translocation.

Spi, which is eventually secreted from axons, is processed at the growth cones, after the complex has exited the ER (Fig. 2) (Yogev et al., 2010). Thus, while the ER component of Rho-3 facilitates axonal trafficking, the secretory compartment pool, which is also present at axonal tips, ensures productive cleavage. Furthermore, the joint travel of the processing machinery through the ER has a "cost": S is continuously exposed to ER cleavage, rendering lamina development even more sensitive to its levels than the developing eye.

It is remarkable how compartmentalization of Rho-3 is utilized twice in the same cells: Initially to inhibit excessive ligand release in the eye disc, and again to direct polarized secretion at the axon termini. Whereas in the first case S cleavage is the means by which ER localization attenuates signaling, in the second it is a byproduct of the trafficking mode, and potentially renders signaling sensitive to perturbations.

A switch in Star cleavability by Rhomboids alters EGFR signaling range during evolution

Studying the EGFR ligand-processing cassette in the flour beetle *Tribolium castaneum* has provided an opportunity to assess regulation of ligand processing in a simplified system, which contains only one member of each pathway component. The central features of ligand retention, trafficking by the chaperone and cleavage by Rhomboid have been conserved in *Tribolium*. However, while in flies multiple Rho proteins enable modulation of signaling levels, a single EGFR-related Rho protein in *Tribolium* and other early-diverged insect species generates a single activation mode.

Consistent with the highest sequence similarity to *Drosophila* Rho-2, the single Tc-Rho displays combined ER and secretory compartment localization, and accordingly mediates attenuated levels of Spi signaling when expressed in *Drosophila* tissues (Rousso et al., 2010). In spite of the attenuated EGFR activation that is mediated by Tc-Rho in the *Drosophila* system, active-MAPK detection in the ventral ectoderm of *Tribolium* embryos displays a long-range pattern, indicative of high levels of released ligand. This discrepancy is reconciled by the striking finding that the *Tribolium* Star molecule is refractive to cleavage by Rho proteases, a feature that is shared by Star proteins of additional early-diverged insect species such as the wasp *Nasonia vitripennis* (Fig. 1C) (Rousso et al., 2010). This comparative analysis therefore highlights the capacity to diversify the features of EGFR signaling by modulating the sub-cellular localization and biochemical features of key proteins in the ligand-processing cassette.

Concluding remarks

Although diverse regulatory circuits are operating in the cells receiving the EGF receptor signal, the spatial and temporal pattern of receptor activation is primarily determined by the production and secretion of the receptor ligands. In invertebrate species, the protease Rhomboid was shown to play the most critical role among the pathway components involved in generation of an active ligand. The Rhomboid expression pattern dictates the corresponding pattern of receptor activation, and modulation of its intracellular localization directly impinges on the level of signal that is released. The ability to
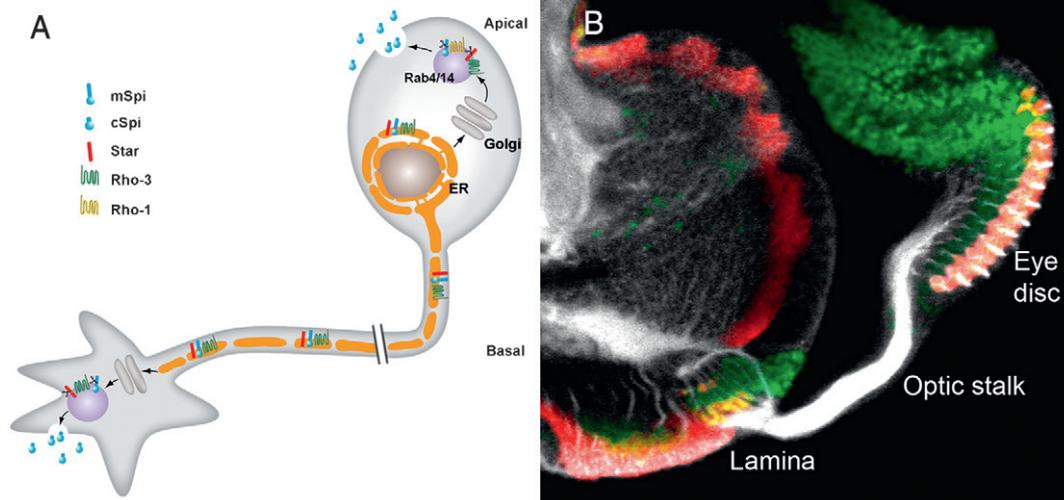

Fig. 2. ER facilitated trafficking of the ligand-processing machinery to axon termini, promotes ligand secretion from the axons to the lamina. A) In *Drosophila* photoreceptor cells, both Rhomboid-1 and -3 are expressed. The ER localization of Rhomboid-3 attenuates the level of ligand that is secreted apically, to induce photoreceptor cell fate in adjacent cells within the eye disc epithelium. However, ER localization of Rhomboid-3, the ligand, Spitz, and the chaperone, Star, facilitate trafficking of the processing machinery within photoreceptor axons. At the axon termini, the processing machinery is transferred to a secretory compartment, where productive ligand processing and secretion takes place. The released ligand triggers neuronal cell fates within the lamina. B) Image of photoreceptors in the eye disc (upper right), projecting their axons through the optic stalk to the lamina (lower left). Neural cell fates (ElaV positive) are marked in red, and axon fascicles in gray.

modulate signaling levels by altering the intracellular localization of critical pathway components, allows to diversify a conserved signaling cassette according to the distinct biological settings where it functions.

Acknowledgments

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