

## 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).

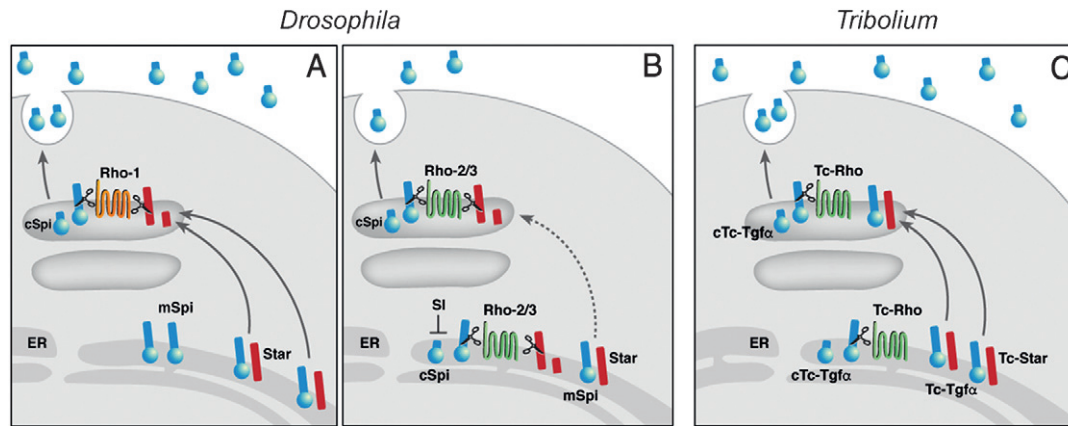
Remarkably, in spite of the conservation in ligand structure, the mechanisms generating the active processed ligand are distinct between vertebrates and *Drosophila*. In vertebrates, the sites of ligand expression are restricted, while the processing itself is carried out by ubiquitously expressed metalloproteases (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 Spi (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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**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). Rho-2/3 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 (S) 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

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