Regulating the dynamics of EGF receptor signaling in space and time

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Summary

The epidermal growth factor receptor (EGFR) signaling cascade represents one of the cardinal pathways that transmits information between cells during development in a broad range of multicellular organisms. Most of the elements that constitute the core EGFR signaling module, as well as a variety of negative and positive modulators, have been identified. Although this molecular pathway is utilized multiple times during development, the spatial and temporal features of its signaling can be modified to fit a particular developmental setting. Recent work has unraveled the various mechanisms by which the EGFR pathway can be modulated.

Introduction

A common paradigm of the cardinal signaling pathways that direct the development of all multicellular organisms is the repeated use of the same signaling cascades at numerous developmental decisions. This strategy raises several problems. First, how can the same pathway dictate a multitude of different cell fates? The prevalent solution seems to lie in the combinatorial context of the promoters that are activated in each setting. For example, different combinations of signaling pathways and tissue-specific enhancers allow epidermal growth factor (EGF) receptor signaling to activate distinct promoters in different tissues (Flores et al., 2000; Halfon et al., 2000; Xu et al., 2000).

A second problem posed by the use of the same signaling pathway in different tissue settings is how to modulate signaling parameters according to the unique requirements of each tissue. For example, for each signaling scenario, the duration of signaling could be extended or restricted, the pathway may be activated only once or multiple times, and the range of activation could be limited or widespread. Because each of the signaling pathways relies on a ‘hard-wired’ cascade of signaling modules, how can these different signaling features be achieved?

This review focuses on the EGF receptor signaling pathway in *Drosophila*, and highlights the common aspects and differences between the *Drosophila* pathway and the pathway in *C. elegans* and in vertebrates. [For a recent review of the diverse functions of the *Drosophila* EGFR pathway during development see Shilo (Shilo, 2003).] Although the receptor is activated by secreted ligands, the pathway predominantly mediates short-range signaling, i.e. activation is restricted either to the cells producing the signal or to cells positioned 1-2 cell diameters adjacent to the signal source. A variety of regulatory modes have evolved to maintain this restricted signaling range. Here, I discuss the mechanistic features that underlie how the EGFR pathway can be activated in different modes: as a single burst, as reiterative activation cycles within the same tissue, or by relay to another tissue. Central to these modes is the distinction between the cell(s) that provides the signal and the cells that are activated. Different strategies for achieving this distinction are discussed. The ability to compartmentalize the responses to EGFR activation within the receiving cells and its implications are also addressed, as is the inter-relationship between the EGFR and Notch pathways in *Drosophila* and *C. elegans*.

The EGFR pathway in *Drosophila*: the basic hardware

In *Drosophila*, EGFR is the sole receptor of the pathway. The cascade downstream of the receptor is the canonical RAS/RAF/MEK/MAPK pathway. In most instances, the cascade downstream of the receptor appears to be unbranched. As such, induction of gene expression by EGFR, mostly through the Pointed ETS transcriptional activator, represents the universal output of the pathway (Gabay et al., 1996; O’Neill et al., 1994). Only two outputs that are not transcriptional have been described so far. In the developing eye and in embryonic midline glial cells, EGFR antagonizes apoptosis by MAPK-induced phosphorylation and inactivation of the pro-apoptotic protein HID (Bergmann et al., 1998; Bergmann et al., 2002). In the migrating follicle border cells of the egg chamber, EGFR mediates guided cell migration in response to attraction by the ligand Gurken produced in the egg (Duchek and Rorth, 2001).

A key way in which the EGFR pathway is regulated is through the generation of activate ligands of the pathway. There are four EGFR ligands in *Drosophila*: Spitz, Keren, Gurken and Vein (Table 1). Vein is produced as a secreted protein that does not require processing for its activity (Schnepp et al., 1996). The other three ligands are produced as inactive membrane-bound precursors. Only upon cleavage and the release of the extracellular EGF-containing domain is the active ligand generated (Schweitzer et al., 1995). Spitz represents the cardinal ligand that is used in the numerous developmental contexts in which EGFR operates.

Although the expression of the ligands Gurken and Vein is

Table 1. Core elements in EGFR activation

	Species and function		
Element	*Drosophila*	*C. elegans*	Human/Mouse
EGF receptor	EGFR	LET-23	EGFR ERBB2 (does not bind ligand) ERBB3 (has an inactive kinase domain) ERBB4
EGF ligands	Spitz (acts in most EGF signaling situations) Keren (function unknown) Gurken (functions in oogenesis) Vein (functions in a restricted manner)	LIN-3 (activates LET-23 in vulval development)	EGF (activates EGFR) TGFα (activates EGFR) Heparin-binding EGF (activates EGFR) Amphiregulin (activates EGFR) Betacellulin (activates EGFR) Epiregulin (activates EGFR) Epigen (activates EGFR) Neuregulin 1 (activates ERBB3 and ERBB4) Neuregulin 2 (activates ERBB3 and ERBB4) Neuregulin 3 (activates ERBB3 and ERBB4) Neuregulin 4 (activates ERBB3 and ERBB4)
Ligand processors	*Rhomboid 1 (commonly cleaves Spitz) *Rhomboid 2/BRHO/STET (cleaves Spitz in male and female germline) *Rhomboid 3/Roughoid (functions predominantly in eye development) †Star (traffics ligand precursors from the ER)	*ROM-1 (enhances EGFR activation in primary vulval cells)	ADAM17/TACE (cleaves TGFα)

*A seven-pass transmembrane, intramembrane protease.
†A Type II transmembrane protein.
References for the above data can be found in the main text.

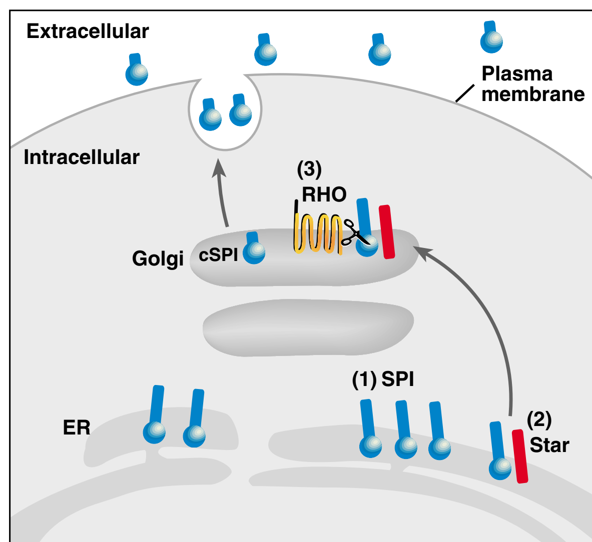
restricted (Golembo et al., 1999; Neuman-Silberberg and Schupbach, 1993; Simcox et al., 1996; Wasserman and Freeman, 1998), the primary ligand Spitz is broadly expressed (Rutledge et al., 1992). Regulated processing, rather than restricted expression, provides the primary cue for the spatial and temporal activation of the pathway by Spitz. This mode of regulation constrains where and when the active ligand is processed, prevents the inappropriate production of a potent ligand in tissues not requiring activation, and involves controlled intracellular trafficking (see Box 1). The processing of Spitz requires two proteins that are an integral part of EGFR signaling in all *Drosophila* tissues: Star and Rhomboid (Bier et al., 1990; Kolodkin et al., 1994; Schweitzer et al., 1995). In accordance with their central role in processing, the phenotypes of *Drosophila* embryos homozygous for mutant alleles of *Star* or *rhomboid* are highly similar to those of *spitz* (Mayer and Nusslein-Volhard, 1988).

Rhomboids and EGFR activation

The characterization of the key molecules that process Spitz has provided a deeper understanding of how EGFR activation is spatially and temporally controlled. Like *spitz*, *Star* is also broadly expressed in most developmental settings, although in some cases its expression domain is confined (Heberlein and Rubin, 1991). Conversely, the expression of *rhomboid* is extremely dynamic (Bier et al., 1990), and precedes the appearance of EGFR-induced MAPK activation (dpERK) (Gabay et al., 1997). Ectopic *rhomboid* expression leads to EGFR activation in a wide range of tissues (Golembo et al., 1996a; Sturtevant et al., 1993), indicating that Rhomboid is the limiting factor and all other components are ubiquitous. Thus, the complex array of enhancers that regulate the *rhomboid* gene contains the ‘blueprint’ for the dynamic pattern of EGFR activation throughout *Drosophila* development. An example of this can be seen in denticle-belt specification, where the expression of *rhomboid* in defined cell rows determines the position of the future denticle belts in each abdominal segment. Expression of *rhomboid* is induced in two rows by Hedgehog, and in another row by Serrate, triggering Notch signaling. Conversely, Wingless restricts the domain of *rhomboid* expression (either directly or indirectly) (Alexandre et al., 1999).

Rhomboid is the founding member of a conserved gene family (Wasserman and Freeman, 1998), the function of some of its members being an intramembrane protease (Urban et al., 2001). The highest degree of conservation among this family lies within the transmembrane domains, which contain the catalytic site. Active intramembrane proteases belonging to this family have been identified in species from bacteria to humans (Koonin et al., 2003). In *Drosophila*, seven members of the family have been identified (Wasserman et al., 2000). Only three have so far been shown to be involved in EGFR signaling. Rhomboid 1 is the cardinal player in this context. Rhomboid 2/BRHO/STET is expressed in the germline and was suggested to be required for Gurken processing during oogenesis (Guichard et al., 2000). An analysis of *stet* mutants has shown that STET is required in germline cells at the early stages of both oogenesis and spermatogenesis, and that, in its absence, somatic cells fail to enwrap the germline cells and to provide them with a microenvironment for their differentiation (Schulz et al., 2002). Rhomboid 3/Roughoid is expressed in the eye, where it is partially redundant to Rhomboid 1 (Wasserman et al., 2000). In addition, it is required in the embryo to facilitate the repulsion from the midline of the tracheal

Box 1. The regulation of Spitz cleavage by intracellular trafficking



The core cleavage machinery was originally deciphered by recapitulating the processing of Spitz in cell culture (Lee et al., 2001; Tsruya et al., 2002). This process was found to require the following elements.

(1) The Spitz precursor (SPI), which contains a signal peptide and a transmembrane (TM) domain. SPI is retained in the endoplasmic reticulum (ER) by coat protomer I (COPI)-mediated retrograde trafficking from the Golgi, as demonstrated by RNAi-based knockdown (Schlesinger et al., 2004). This retention prevents the non-specific release of an active ligand upon cleavage at the plasma membrane by metalloproteases, which have a broad substrate specificity.

(2) Star, a type II TM protein, which associates with SPI (Tsruya et al., 2002) and facilitates its translocation from the ER to a cellular compartment where it can be cleaved by Rhomboid (RHO).

(3) RHO, a seven-pass TM protein. RHO is an intra-membrane protease that cleaves SPI (Urban et al., 2001) to yield the active form, cleaved Spitz (cSPI), which is destined for secretion. Although the identity of the compartment that contains RHO can depend on the cell type, one established route entails RHO cleavage in the Golgi (Lee et al., 2001).

ganglionic branch (Gallio et al., 2004), and it might also cooperate with Rhomboid 1 to promote the viability of smooth cuticle-producing cells in the ventral epidermis (Urban et al., 2004).

In cell culture, all three Rhomboid proteins, as well as Rhomboid 4, can cleave the three EGF ligands (Ghiglione et al., 2002; Urban et al., 2002). In accordance with their different expression patterns, only *rhomboid 1* mutants are lethal. It will be interesting to explore whether there are functional differences between them, beyond their patterns of expression. As will be discussed below, the intracellular sites of action of Rhomboid proteins may be distinct, giving rise to interesting twists in the regulation of EGFR signaling.

EGFR ligand processing in vertebrates

In vertebrates, four EGF receptors participate in signaling. The first, EGFR, is activated by a set of seven ligands and undergoes homodimerization. The remaining receptors (ERBB2, ERBB3 and ERBB4) are usually activated as heterodimers by four ligands termed neuregulins. All ligands are produced as precursors with a single transmembrane domain (Table 1) (reviewed by Falls, 2003; Harris et al., 2003). The more-prevalent heterodimeric pair, ERBB2/ERBB3, is complementary, as the former does not bind ligands, while the latter has an inactive kinase domain. Phosphorylation of tyrosines on ERBB3 by ERBB2 following dimerization leads to signaling (reviewed by Citri et al., 2003).

TGFα is an EGFR ligand that has been studied in detail. It contains a signal peptide, EGF domain, transmembrane and cytoplasmic domains. In contrast to previous reports, recent work demonstrates that although the precursor form of TGFα can mediate interaction between cells by virtue of its binding to EGFR, it does not lead to activation of the receptor (Borrell-Pages et al., 2003). Thus, cleavage is essential to produce a potent ligand. The cleavage machinery, however, appears to be different in vertebrates. The accumulating evidence suggests that membrane metalloproteases of the ADAM family, which are active on the cell surface, cleave the ligand immediately above the transmembrane domain. Especially revealing has been the observation that mice in which the gene encoding TACE/ADAM17 metalloprotease has been inactivated exhibit a TGFα mutant phenotype (Blobel, 2005; Peschon et al., 1998). Fibroblasts from these knockout mice are defective in the shedding of several EGFR ligands (Merlos-Suarez et al., 2001; Peschon et al., 1998; Sunnarborg et al., 2002). Other ADAM proteins have also been implicated in EGFR ligand processing (Fischer et al., 2004). Because the ectodomain shedding machinery is located at the cell surface, trafficking of the ligand precursors is essential. Two PDZ-domain proteins that interact with the extreme C terminus of TGFα are required for its trafficking to the membrane (Fernandez-Larrea et al., 1999).

ADAM metalloproteases have a fairly broad substrate specificity that depends upon the domain(s) that mediates their association with the substrate, rather than on a defined consensus sequence for cleavage. For example, TACE/ADAM17 cleaves not only TGFα, but also tumor necrosis factor (TNF), the TNF receptor and L-selectin. It remains to be determined whether particular metalloproteases have a preference for distinct EGFR ligands (Harris et al., 2003). In addition, the ability to modulate the activity of metalloproteases has a decisive regulatory effect on EGFR ligand cleavage. Activated G-protein coupled receptors (GPCRs) stimulate the activity of ADAM proteins, leading to the processing of the EGFR ligand heparin-binding EGF (HB-EGF), and thus to EGFR activation (Prenzel et al., 1999; Wetzker and Bohmer, 2003). Osmotic and oxidative stress may also induce EGFR activation by stimulating metalloprotease-mediated cleavage of EGFR ligands (Fischer et al., 2004).

Taken together, the emerging strategies for controlling the activation of EGFR/ERBB signaling in vertebrates are based on the restricted expression of ligands and on the particular combination of ligands that are expressed by a given cell type. ADAM metalloprotease-induced cleavage of the plasma membrane generates the potent ligands. The repertoire of ADAM proteins, as well as the stimulation of ADAM activity by external signals, may modulate the efficiency of ligand

cleavage. However, as neither the ligand trafficking machinery (Urena et al., 1999), nor the ADAM proteins are dedicated just to EGFR/ERBB activation, the ligand processing system in vertebrates is less stringently regulated. In cases where spatially restricted activation of EGFR is required, such as in the definition of the feather inter-bud territory in chick, the restricted expression of EGF has been observed (Atit et al., 2003).

Positive- and negative-feedback responses

The transcriptional induction of modulators of the pathway by EGFR signaling plays a major role in shaping responses to this pathway. This section discusses such modulators and their modes of action, whereas the subsequent section addresses their roles in shaping the spatial and temporal features of activation. In the *Drosophila* embryonic ventral ectoderm and follicle cells, the original activation of the EGFR pathway is amplified by inducing the expression of the ligand Vein (Golembo et al., 1999; Wasserman and Freeman, 1998). It is interesting to note that because Vein represents a ligand that is weaker than Spitz [as measured by its capacity to activate EGFR in cell culture (Schnepp et al., 1998) or to induce the appearance of phosphorylated MAPK (dpERK) and target genes in embryos (Golembo et al., 1999)] this feedback response may allow the spread of lower levels of activation to more distant cells. A second positive-feedback response in *Drosophila* and *C. elegans*, as discussed below, is the induction of *rhomboid* expression, which occurs only in restricted instances, as it relies not only on EGFR activation, but also on the convergence of additional signaling pathways (Dobens et al., 2000; Dutt et al., 2004; Peri and Roth, 2000; Sapir et al., 1998; Wasserman and Freeman, 1998).

Implicit in the short range of action of the EGFR pathway in *Drosophila*, is the presence of multiple negative regulators of the pathway. One class includes constitutively expressed elements such as CBL, an E3 ligase that recognizes the activated, endocytosed EGFR by virtue of its P-Y motifs, and induces its ubiquitination and degradation. CBL may also enhance the endocytosis of EGFR, following ligand binding. Although CBL is broadly expressed, it only modulates EGFR signaling in the follicle cells, which receive the Gurken signal from the oocyte. In *cbl*-mutant cells, EGFR is hyperactivated, leading to the repression of genes such as *pipe* (Pai et al., 2000). Another constitutive repressor is YAN (AOP – FlyBase), an ETS-domain transcriptional repressor that blocks the DNA-binding site of Pointed. Following the activation of MAPK, the phosphorylation of YAN leads to its nuclear export and degradation (Rebay and Rubin, 1995; Tootle et al., 2003). Finally, a recent report has suggested that a class II Phosphoinositide 3-kinase may be constitutively recruited to activated EGFR to attenuate signaling (MacDougall et al., 2004).

The other known negative regulators of EGFR signaling in *Drosophila*, including Argos, Kekkon and Sprouty, are transcriptionally induced by the pathway. Their role is to inhibit signaling in cells that are more distant from the source (Fig. 1). The inducibility of negative regulators endows the EGFR signaling pathway with a short-range signaling mode, irrespective of the nature of the tissue in which signaling takes place.

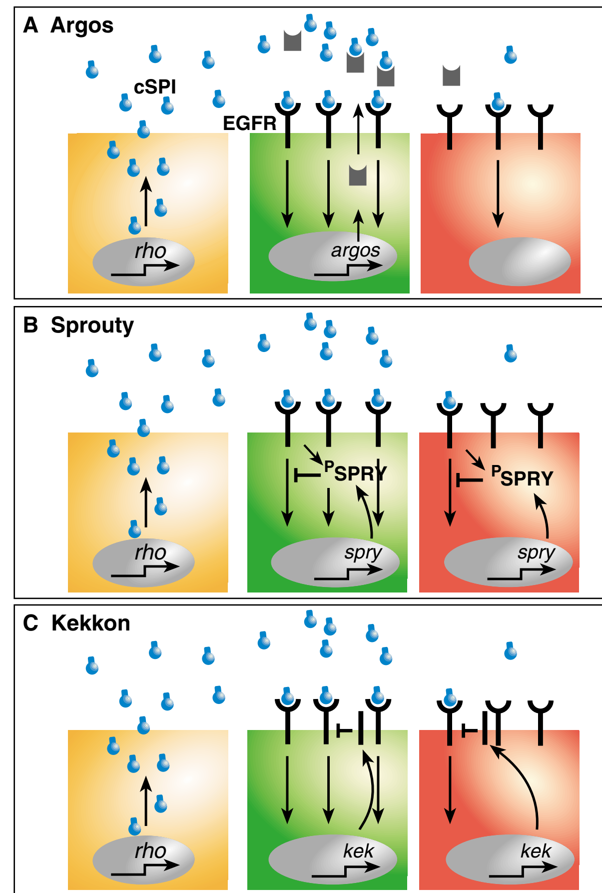


Fig. 1. Inducible negative regulators of EGFR signaling. Activation of the EGF receptor elicits the transcription of negative regulators, such as Argos, Kekkon and Sprouty, that restrict the range of signaling. EGFR activation usually leads to Argos and Kekkon induction, and in some settings also to the induction of Sprouty. Sprouty is also induced by, and inhibits, other signaling pathways, such as the FGF pathway. The cell expressing Rhomboid (RHO) and releasing cleaved Spitz (cSPI) is colored orange, the cell where prominent EGFR activation takes place is green, and the cell where EGFR activation is repressed is red. (A) Argos is induced only in the cells receiving the highest levels of the EGFR signal cSPI, i.e. those closest to the source of ligand processing. Argos is secreted from the cells where it is produced and associates with cSPI, thus restricting the levels of cSPI molecules that diffuse beyond the expression domain of Argos. Because Argos associates with cSPI, the actual range of Argos diffusion is not crucial for its long-range inhibitory effect. (B) Sprouty is induced in the cells receiving high and intermediate levels of EGFR activation. Following its production, Sprouty undergoes tyrosine phosphorylation, which is induced by the activated receptor, to produce a potent inhibitor. (C) Kekkon is induced in the cells receiving high and intermediate levels of EGFR activation. The protein localizes to the plasma membrane, where it forms heterodimers with EGFR.

Argos is a secreted protein that has an EGF domain (Freeman et al., 1992). It is induced only in the cells where pronounced EGFR activation takes place (Golembo et al., 1996b). Based on the atypical EGF domain of Argos and on its capacity to bind EGFR, it was initially assumed to function as a competitive inhibitor of ligand/receptor binding (Jin et al., 2000). Recent detailed biochemical studies have demonstrated that Argos associates predominantly with Spitz, to form non-

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functional heterodimers (Klein et al., 2004). The mechanism is similar to the known inhibitors of the bone morphogenetic protein (BMP) pathway, such as Chordin/SOG, Noggin and Follistatin, which sequester the BMP ligands (De Robertis and Kuroda, 2004). This mode of action has two appealing features. First, it provides an effective way of restricting the range of Spitz action. One can actually consider Argos as a ligand 'sieve', which allows only a small number of Spitz molecules to reach and activate more distant cells. Recent computational analysis has demonstrated that the short-range activity of Argos is sufficient to restrict the range of Spitz diffusion and buffer fluctuations in the levels of Spitz or EGFR (Reeves et al., 2005). Second, because Argos binds the ligand and not the receptor, it is tempting to consider the possibility that Argos may specifically sequester some ligands but not others. For instance, in the ventral ectoderm, the source of Spitz is restricted to a single row of cells, the midline glial cells. In the adjacent ectodermal cells, which receive the highest level of signal, both *argos* and *vein* are induced (Gabay et al., 1996; Golembo et al., 1999). Argos may restrict the amount of Spitz that reaches more distant cells, while allowing Vein to diffuse readily and elicit a lower level of EGFR activation. Argos-like genes have not been identified in vertebrates to date.

The inhibitors Kekkon and Sprouty are conserved in vertebrates. Kekkon is a transmembrane protein that forms heterodimers with *Drosophila* EGFR and with each of the known members of the mammalian ERBB family (Ghiglione et al., 2003; Ghiglione et al., 1999). The binding of Kekkon to EGFR is mediated by its leucine-rich repeats and juxta-membrane domain; its cytoplasmic tail is required for its apical subcellular localization (Alvarado et al., 2004; Ghiglione et al., 2003). Interestingly, the transcription of a mammalian homolog of Kekkon, termed LRIG1, is also upregulated upon EGFR stimulation. The physical association of LRIG1 with the four ERBB members, which does not depend on ligand binding, leads to the enhanced ubiquitination and degradation of these receptors by the recruitment of CBL (Gur et al., 2004; Laederich et al., 2004).

Sprouty proteins have a conserved carboxy-terminal cysteine-rich domain that is necessary for their specific localization and function. The amino terminus of Sprouty is divergent among organisms, except for a conserved tyrosine residue. Sprouty exerts its inhibitory effect on receptor tyrosine kinase (RTK) signaling by intercepting essential elements of the RAS/MAPK cascade through diverse mechanisms (Kim and Bar-Sagi, 2004). It is interesting to note that, in addition to the transcriptional induction of *sprouty* by EGFR activation, the protein must also undergo tyrosine phosphorylation at a distinct site, in order to carry out its repressive activity (Hanafusa et al., 2002; Rubin et al., 2003). The double switch for Sprouty activation may ensure that it functions only in regions where pronounced activation by RTKs takes place. In parallel, the tyrosine phosphatase SHP2, which is a positive element in signaling by RTKs, has been shown to attenuate the repression of Sprouty by dephosphorylating the crucial residue (Hanafusa et al., 2004).

Recently, Sprouty family proteins have been shown to not only attenuate the response to RTK signaling, but also to mediate a developmental switch (Sivak et al., 2005). In *Xenopus tropicalis*, two members each of the *sprouty* and the related *spred* genes were identified. This study showed that their spatial patterns of expression during FGF-induced mesoderm differentiation are similar. However, *sprouty* RNA levels are high during the gastrula stage, whereas *spred* RNA levels are high in the subsequent neurula stage. Sprouty selectively inhibits mesoderm spreading, whereas SPRED inhibits mesoderm specification. The different activities of the two inhibitors stem from their capacity to selectively attenuate different branches of FGF-induced signaling. Sprouty inhibits FGF-receptor induced PLCγ activation, whereas SPRED selectively attenuates MAPK signaling.

Another interesting tier of negative regulation to consider may operate at the level of the cells sending the signal, to restrict the amount of ligand that is released. When the cleaved form of Spitz was expressed in a variety of tissues, it was retained in the ER and was not secreted to activate neighboring cells. This retention requires the activity of phospholipase Cγ (termed Small wing, Sl). In *sl* mutants, hyperactivation of EGFR is observed due to the excessive release of the ligand, specifically in the eye (Schlesinger et al., 2004; Thackeray et al., 1998). It is possible that, in this tissue, some of the cleaved ligand is normally generated in the ER, rather than in a more advanced secretory compartment. Both Rhomboid 1 and Rhomboid 3 are required for Spitz processing in the eye (Wasserman et al., 2000). In contrast to Rhomboid 1, Rhomboid 3 may generate the cleaved ligand already in the ER. For example, when Rhomboid 3 was expressed with the Spitz precursor in cultured cells, the cleaved ligand was observed to accumulate within the cells, indicating that Spitz could be cleaved in the absence of Spitz trafficking by Star (Urban et al., 2002). The biological impact of cleaving and retaining Spitz in the ER for eye development is not known. The retention of cleaved Spitz may simply prevent the secretion of ligand generated in the ER. However, if the ER cleavage mode in the eye is predominant, the retention of cleaved Spitz may affect the overall level of ligand that is eventually secreted by these cells.

Different modes of EGFR signaling

This section discusses how the common EGFR signaling cassette can be adjusted to generate different modes of signaling that are suited to each tissue. In many cases, the signaling event is executed once. In other instances, the capacity to process the ligand is relayed to adjacent cells of the same tissue, or to a neighboring tissue, leading to reiterated EGFR signaling. Finally, there are cases where low level, trophic EGFR signaling is necessary to maintain cell viability, for example in the eye disc (Baonza et al., 2001).

Single burst

Most cases of EGFR signaling fall into this category. In the typical case, the restricted expression of Rhomboid in a distinct cell(s) provides a source of active ligand that will activate EGFR in the same cells or in adjacent cells. Cells undergoing the highest level of activation induce expression of Argos to modulate the response. Kekkon, and in some instances Sprouty, are also induced, usually at a broader range (Fig. 1, Fig. 2A). Patterning of the embryonic ventral ectoderm, as described above, is an example of a single burst situation. The cSPI signal emanates from a single row of midline glial cells, and the induction of Argos expression in the cells receiving the highest

levels of EGFR activation ensures the spatially restricted induction of EGFR target genes.

Multiple activation cycles

Multiple EGFR activation cycles within the same tissue

This scenario involves the most complex setting in terms of signaling, as it is based on discrete and successive bursts of EGFR activation within the same tissue. How the range of each burst is controlled, how the final cumulative outcome of all bursts is restricted, and how discrete bursts, rather than a continuous signal, can be obtained are central issues. Below, I outline several mechanistic solutions to these challenges.

In the *Drosophila* embryonic ectoderm, oenocytes (secretory cells of epidermal origin) are formed in the dorsal-most cluster of sensory organ precursors. A single cell expressing Rhomboid provides a signal to induce and recruit up to six oenocytes. Time-lapse movies show that this induction takes place in two bursts, and that three cells are recruited in each round (Brodu et al., 2004). A recruitment burst has a typical duration of ~1 hour. The generation of these signaling bursts does not stem from the discontinuous production of EGFR ligand, as the enhanced, continuous production of ligand from the original source cell maintained the cyclic EGFR activation pattern, while giving rise to extra bursts. Remarkably, it is the capacity of the activated cells that are immediately adjacent to the source to express the secreted inhibitor Argos, and the topology of these cells, that provides the basis for generating discrete activation bursts. Once these cells delaminate, the ligand can effectively activate the next group of cells (Fig. 2B).

In the above example, the signal source was restricted to the same cells throughout the induction process. In other cases of cyclic EGFR activation, the signal source itself expands within the tissue. In the leg imaginal disc, the induction of chordotonal stretch receptors takes place through multiple cycles of EGFR induction, which generate up to 70 chordotonal cells (zur Lage and Jarman, 1999). The most elaborate system of multiple cycles of EGFR activation occurs during the development of the *Drosophila* compound eye. Once the initial photoreceptor in each ommatidium (the R8 cell) is defined, the recruitment of the additional seven photoreceptor cells, the cone and pigment cells, the G2/M transition after the morphogenetic furrow and the rotation of ommatidia, are all controlled by reiterative cycles of EGFR activation (Baker and Yu, 2001; Freeman, 1996; Freeman, 1997; Strutt and Strutt, 2003).

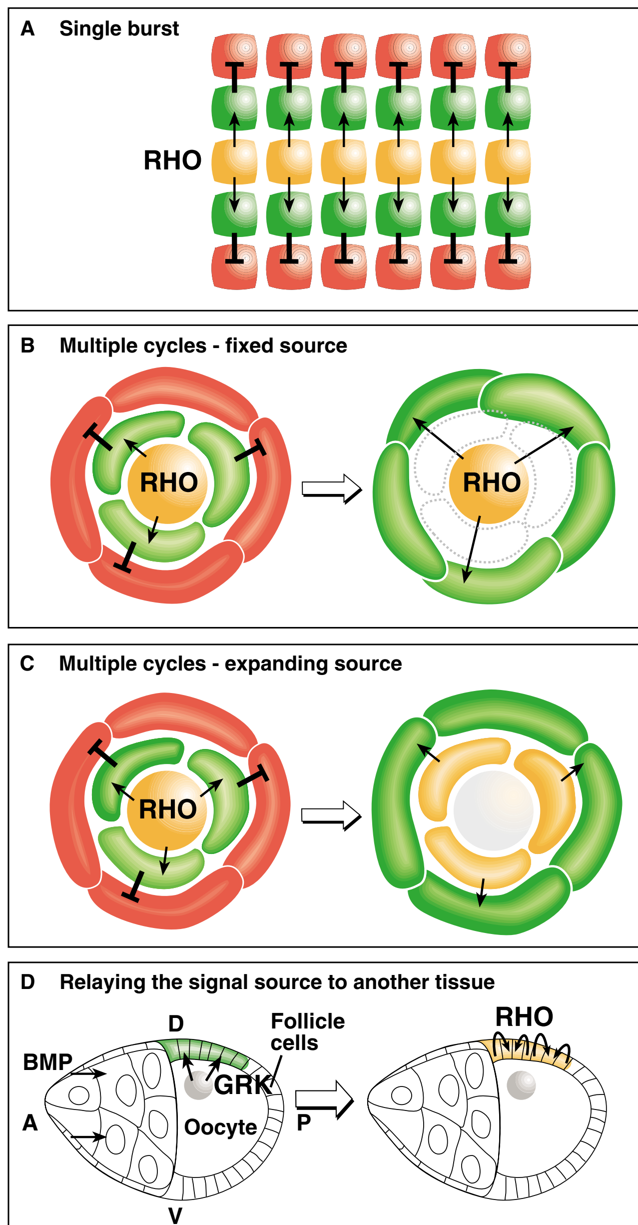

Fig. 2. Different modes of EGFR signaling. Although the same canonical EGFR signaling pathway is used in numerous developmental settings, subtle alterations in its regulatory circuitry lead to distinct modes of signaling. (A) A single signaling burst. In this mode, ligand processing is confined to the cells expressing *rhomboid* (RHO, orange). Secreted ligand is presented to neighboring cells, and the induction of negative-feedback loops (red) confines the signaling zone (green). This is the simplest, and most commonly used, EGFR signaling mode. The figure depicts EGFR signaling in the *Drosophila* embryonic ventral ectoderm, where *rho* is expressed in the midline glial cells. (B) Multiple activation cycles from a fixed source. Induction of Argos expression in the cells immediately adjacent to the cleaved ligand source limits the range of activation. Once these cells delaminate, the signal from the original source can now reach the next ring of cells, giving rise to a cyclic pattern of EGFR activation. This mode was first described in the induction of embryonic oenocyte cell fates. (C) Multiple activation cycles from an expanding source. This mode operates during the development of the *Drosophila* eye, and relies on the expansion of *rho* expression. A different cell type is induced following each round of EGFR activation, by combining EGFR signaling with distinct transcriptional cues that are unique to each cell type. Each burst of EGFR activation has to be discrete in space and time. (D) The relay of signal source. When signaling takes place between two cell types, *rho* induction in the cells where EGFR is activated converts them to a signaling source. This response leads to amplification of the original signal, and extends signaling over time, even after the original signal source can no longer be detected. This type of signaling occurs during *Drosophila* oogenesis, and in *C. elegans* during vulval cell fate induction. The figure shows a *Drosophila* egg chamber, where Gurken signal emanating from the oocyte leads to EGFR activation in the follicle cells. The convergence of EGFR and BMP (DPP) signaling from the stretch follicle cells induces *rho* expression in the dorsal anterior follicle cells, which generates cleaved Spitz in these cells to amplify the signal. Anteroposterior (AP) and dorsoventral (DV) axes are indicated.

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Because the number of undifferentiated precursor cells is restricted, only the required number of cells should be induced in each round of activation.

Distinct cell types are induced in the eye, depending upon the combination of transcription factors within the cells being activated, and on the signaling pathways that converge on EGFR signaling in these cells (Flores et al., 2000; Xu et al., 2000). Thus, every round of activation must be discrete in space and time. In other words, the new signal should emanate only from the newly differentiated cells, rather than be provided continuously from the primary cells. The prevailing notion is that the reiterative EGFR activation cycles in the eye stem from expanding the expression of Rhomboid 1 and Rhomboid 3 following EGFR signaling (Baonza et al., 2001) (Fig. 2C).

What mechanisms keep the range of Spitz signaling tightly restricted in the eye? One mechanism might involve the induction of negative regulators such as Argos and Sprouty, which are indeed necessary for correct ommatidial differentiation (Casci et al., 1999; Freeman et al., 1992; Kramer et al., 1999). It is also possible that the original cells that generated the signal stop producing the cleaved ligand, after the new cohort of cells begin to express *rhomboid*.

Relaying the signal source to another tissue

In some developmental contexts, the response to the signal in the receiving cells needs strengthening. One way to achieve this is to endow the receiving cells with the capacity to process their own ligand, by inducing the expression of *rhomboid* in response to EGFR signaling.

An example of this strategy can be found in the differentiation of follicle cells in the ovary, where the Gurken signal, emanating from the oocyte, activates EGFR in the follicle cells. Gurken first activates EGFR in follicle cells at the posterior part of the egg-chamber. Subsequently, following the migration of the oocyte nucleus, Gurken activates the dorsoanterior cells where *rhomboid* becomes a target gene of the pathway (Sapir et al., 1998; Wasserman and Freeman, 1998). This induction of Rhomboid by EGFR signaling also requires activation of the BMP pathway, which synergizes with EGFR activation only when the follicle cells have completed their migration over the oocyte nucleus (Dobens et al., 2000; Peri and Roth, 2000). Once Rhomboid is expressed, it facilitates the processing of Spitz expressed by the follicle cells (Fig. 2D). This type of signaling relay can fulfill several functions. First, it might amplify the signal, by expanding the ligand source. Second, it can perpetuate the signal after the original source has faded.

Another example of signal relay has been recently identified during EGFR-controlled vulval development in *C. elegans* (Dutt et al., 2004). Interestingly, the original expression of the EGF-like ligand LIN-3 in the inducing anchor cell of the developing gonad does not require the Rhomboid homolog ROM-1 for its cleavage nor for the induction of the proximal vulval precursor cells. ROM-1 is required, however, in the vulval precursor cells, which receive the primary LIN-3 signal, to increase the range of EGFR signaling to more distal cells. An intriguing model to explain this invokes the metalloprotease-based cleavage of a short splice variant of LIN-3 in the anchor cell, which induces primary vulval cell fates in the adjacent cells, and is followed by the relaying of the signal to secondary cells in a ROM-1-dependent fashion.

Compartmentalization within signal-receiving cells

The previous sections have highlighted the importance of intracellular localization and trafficking for the correct processing of EGFR ligands in *Drosophila*. In this section, examples of the asymmetric segregation of EGFR within the cells that receive the signal will be discussed.

In the ventricular and sub-ventricular zones of the mouse embryonic forebrain, EGFR is distributed asymmetrically between daughter cells during mitosis, by an actin-dependent mechanism (Sun et al., 2005). The resulting progenitor cells respond differentially to EGFR activation in terms of migration, proliferation and marker expression: the cells with high EGFR levels give rise to astrocytes, whereas cells with low EGFR levels generate oligodendrocytes. This asymmetric segregation of a signaling receptor, which was previously described for Notch, provides a mechanism for generating further diversity within developing neurons, by altering their sensitivity to the same external cues according to the levels of a receptor that they display.

The intracellular segregation of the EGFR ligand Neuregulin and the EGF receptors ERBB2 and ERBB4 is crucial in human airway epithelia. Neuregulin is present exclusively on the apical membranes of this epithelium, whereas the receptors are restricted to the basolateral surface. When the epithelium is intact, the receptors are not activated and the proliferation rate of the tissue is low. Upon disruption of epithelial integrity, the receptors encounter the ligand at the wound edges and cell proliferation ensues, leading to the restoration of epithelial integrity (Vermeer et al., 2003).

Conversely, the same basolateral localization of an EGF receptor is used in other biological settings to enhance the signal, when the ligand and receptor are present on adjacent surfaces of interacting cells. In *C. elegans*, the LET-23 EGFR localizes to the basolateral membranes of polarized vulval epithelial cells (Kaech et al., 1998). The anchor cell secretes LIN-3 into the basal extracellular space that abuts the vulval precursor cells. The juxtaposition of the receptor thus sensitizes the receiving cells to the signal. Mutations in *lin-2*, *lin-7* and *lin-10* compromise LET-23 localization and lead to reduced signaling. The encoded proteins contain PDZ domains and form a protein complex that binds the LET-23 cytoplasmic tail (Kaech et al., 1998). The PDZ proteins and the interactions between them are conserved in vertebrates and are necessary for the localization of ERBB receptors at the basolateral epithelial surface of polarized MDCK cells (Shelly et al., 2003). Targeting the receptors to the basolateral domain is achieved by the N-terminal part of human LIN7, which binds the kinase domain of the receptors. Once targeted to the basolateral surface, the human LIN7 PDZ domain stabilizes ERBB2 at this position.

Inter-relationship between EGFR and Notch signaling

In most developmental settings, signaling from EGFR is integrated with signaling from other pathways. The most detailed studies have addressed the interaction between EGFR and Notch signaling. In some cases, these interactions reinforce signaling. For example, induction of the *Pax2* gene in the

Drosophila cone cell requires the simultaneous binding of Pointed (triggered by EGFR) and SU(H) (triggered by Notch) (Flores et al., 2000) (Fig. 3A). In parallel to the activation of EGFR in the future cone cell, Spitz also induces the expression of Delta in the photoreceptor cell, providing the Notch signal to the cone cell (Tsuda et al., 2002) (Fig. 3B). This mode of activation represents a 'feed-forward' loop, which is used in multiple transcriptional settings (Milo et al., 2002). The activation of EGFR signaling at two junctions to produce the final output buffers the system against transient fluctuations in signaling to ensure that only sustained EGFR activation will lead to a response.

In *C. elegans* vulval development, mutual repression between the EGFR and Notch pathways contributes to the generation of distinct cell types. EGFR activation in the primary vulval-precursor cells induces the expression of Delta-like ligands (DSL) and repressors of Notch signaling. In parallel, activation of Notch in the secondary cells restricts the expression of DSLs, and induces repressors of EGFR signaling (Yoo et al., 2004) (Fig. 3C). In addition to Notch, another mechanism has recently been shown to be involved in restricting EGFR activation. The DEP-1 receptor tyrosine phosphatase binds to and dephosphorylates activated EGFR. Expression of DEP-1 is repressed by EGFR activation in the primary cells, and is induced by a Notch-independent mechanism in the secondary cells (Berset et al., 2005). The use of parallel mechanisms that restrict EGFR activation in the secondary cells converts the graded activation by the ligand LIN-3 into a binary EGFR-activation response.

The repression of EGFR responses by Notch has been demonstrated in the differentiating *Drosophila* photoreceptor cells upon ectopic expression of activated Notch (Fortini et al., 1993). Strategies that parallel the induction of vulval cell fates in *C. elegans* are also found during the induction of vein cell fates in the *Drosophila* wing (Fig. 3D). Following the localized expression of *rhomboid*, activation of EGFR in the veins reinforces the expression of *rhomboid* and induces the expression of Delta (de Celis et al., 1997; Sotillos and De Celis, 2005). These cells may also be refractive to Notch signaling.

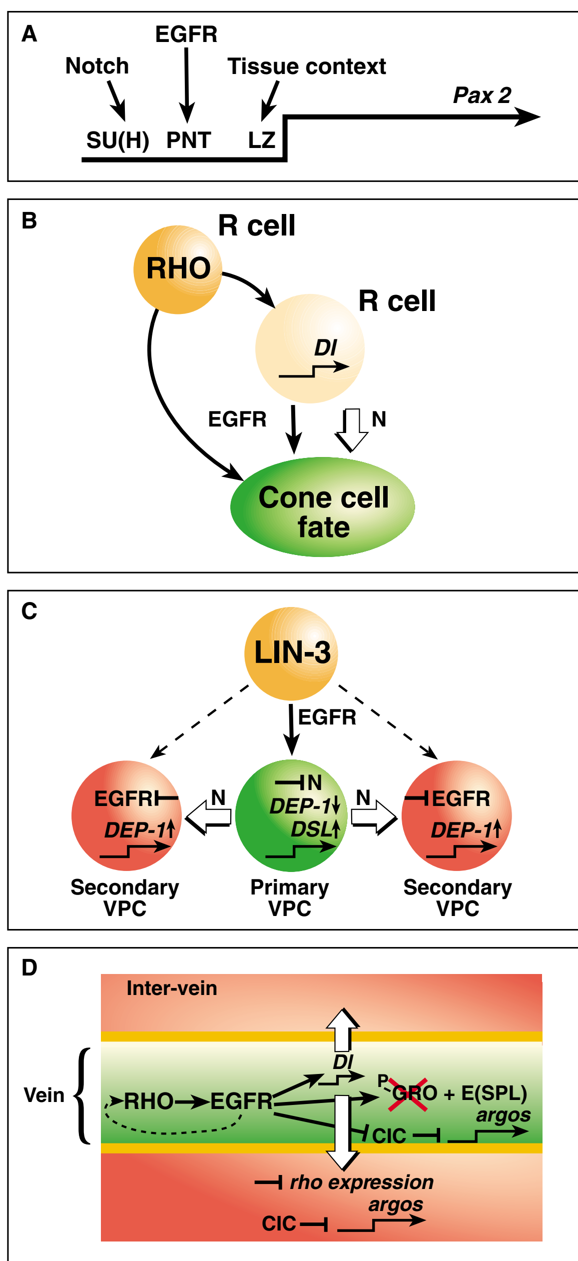

Fig. 3. Diverse interactions between EGFR and Notch signaling. (A) During the development of distinct cell types in the *Drosophila* eye, combined inputs from the EGFR pathway (through Pointed) and the Notch (N) pathway [through SU(H)], in conjunction with distinct transcription factors, induce the relevant target genes. For example, the two pathways in conjunction with Lozenge (LZ), induce *Drosophila Pax2* (*shaven* – FlyBase) expression in the future cone cells. (B) The combined activities of EGFR and Notch signaling integrated in a 'feed-forward' loop. EGFR activation in the *Drosophila* photoreceptor (R) cells induces Delta (DL) expression. The combination of the Spitz and DL ligands presented by the R cells induces the cone cell fate, by triggering target gene expression, such as that of *Drosophila Pax2*. N pathway activation is marked by an open arrow. (C) Mutual repression between the EGFR and Notch pathways refines cell fates during *C. elegans* vulval development. The anchor cell provides the EGFR ligand (LIN-3) to the primary vulval precursor cell (VPC, green). EGFR signaling in this cell leads to DSL expression (a Notch ligand) and reduces the capacity of the cell to respond to N activation. This cell displays DSL to the secondary VPCs. N signaling in these cells triggers repressors of EGFR signaling (red), thus eliminating their responses to lower levels of the EGFR ligand presented by the anchor cell. In parallel, EGFR activation represses the expression of the receptor tyrosine phosphatase DEP-1 in the primary VPC, whereas a Notch-independent mechanism induces *DEP-1* expression in the secondary VPCs. (D) A similar circuit of mutual repression during the determination of vein versus inter-vein fates in the *Drosophila* wing. Restricted expression of *rhomboid* only in the future vein cells leads to localized, autocrine EGFR activation (green) and induction of DL expression. EGFR signaling also reinforces the expression of *rhomboid*. In parallel, MAPK activation by EGFR in these cells can phosphorylate and attenuate the activity of Groucho, which is involved in executing the transcriptional repression responses elicited by N signaling. EGFR activation also eliminates the HMG-box transcriptional repressor Capicua (CIC). In the adjacent inter-vein cells, *rhomboid* expression is repressed by N signaling, and CIC represses other vein-specific genes. Thus, EGFR signaling is confined to the veins, whereas N signaling is restricted to the inter-vein cells.

One of the primary outputs of the Notch pathway is the transcriptional induction of the bHLH proteins E(SPL), which recruit the transcriptional repressor Groucho to their target sites on the DNA. It was recently shown that the activity of Groucho is attenuated in response to MAPK activation (Hasson et al., 2005). Thus, in cells where EGFR is activated, Groucho and hence E(SPL)-dependent repression, should be less potent. In the same cells, EGFR activation removes the HMG-box protein Capicua, which is a potent repressor of vein-specific genes such as *argos*, *dpp* and *vvl* (Roch et al., 2002). Because Capicua represses target genes by recruiting Groucho (Jimenez et al., 2000), activated EGFR attenuates both of these components in the veins. Conversely, activation of Notch in the inter-vein cells may restrict the domain of EGFR activation by repressing the expression of *rhomboid* (de Celis et al., 1997; Sotillos and De Celis, 2005). Again, two distinct cell types (vein versus inter-vein) are generated by the mutual repression of the Notch and EGFR pathways. In this setting, because Delta is a target gene of EGFR, it is indeed imperative to maintain autocrine activation and to restrict EGFR activation to the cells that express Rhomboid, in order to preserve the restricted zone of Delta expression.

In contrast to the autocrine activation of EGFR in the wing veins, activation of EGFR is paracrine in many biological settings. In most, if not all of these cases, the cells that produce the signal are refractive to it. From a developmental viewpoint, this refractivity may allow the sending and responding cell populations to maintain distinct identities. Several mechanisms underlying the refractivity of the sending cells to EGFR activation have been identified, the most prevalent of which is the suppression of the transcriptional response. For example, the R8 photoreceptor cells in *Drosophila* are the first cells to differentiate in the eye. Their differentiation does not require EGFR activation, and they provide the initial source of ligand for the recruitment of additional cells. It was shown that in the R8 cells, expression of the transcription factor Senseless prevents the nuclear transduction of EGFR activation, by blocking the transcriptional responses to Pointed (Frankfort and Mardon, 2004). The transmembrane protein Echinoid, which contains L1 repeats, associates with EGFR and is phosphorylated by it. Echinoid attenuates EGFR signaling either by promoting receptor endocytosis, or by recruiting phosphatases to the receptor complex. In the absence of Echinoid, extra R8 cells are induced (Rawlins et al., 2003; Spencer and Cagan, 2003).

In other cases, the mechanistic basis for refractivity is not known. For example, in the C1 sensory organ precursor cell in *Drosophila*, which provides the ligand for oenocyte differentiation, no activation of MAPK is observed, as monitored by dpERK antibodies, whereas prominent activation is detected in the adjacent cells (Brodu et al., 2004). In this case, the importance of preventing EGFR activation in the ligand-producing cell may stem from the necessity to maintain it as a stable signaling source that will not produce Argos.

Concluding remarks

This review has explored the ability to apply subtle twists to the highly conserved EGFR pathway, to generate a wide and varied array of signaling modes that are adapted to the particular constraints of each tissue. Given the central role of EGFR signaling in development, it is conceivable that evolutionary selection for variation in these 'subtle twists' could change the morphology of tissues. For example, in any tissue where the *rhomboid* promoter is induced by EGFR activation, the outcome is dramatic because of the reiterative activation of the EGFR pathway. Restricting the intracellular distribution of the receptor or the ligands in polarized cells is also a strategy to modulate the signaling level.

Although the confined range of activation is a hallmark of EGFR signaling, little is known about the conversion of graded EGFR activation into sharp transcriptional response borders. Do these thresholds rely on multiple binding sites for the MAPK-activated transcriptional activators on the promoters of target genes, or are other mechanisms involved? As outlined above, cooperation with pathways such as Notch can facilitate sharp EGFR-response borders.

It is interesting to consider the evolution of distinct ligand-cleavage modes. Clearly, the EGFR ligands share a common ancestral molecule. In some organisms, one mode of ligand cleavage was replaced by another. Whether this change was driven by selection pressures that make one strategy advantageous in a particular setting, or whether it was a random event is a question that is likely to remain open. Interestingly, both strategies of cleavage appear to co-exist in *C. elegans*. Future studies should reveal whether the Rhomboid family of proteins are involved in the processing of EGFR ligands in vertebrates, or whether the two modes of cleavage are completely distinct.

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