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There Must Be 50 Ways to Rule the Signal: The Case of the Drosophila EGF Receptor

Minireview

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One common route by which extracellular signals regulate gene expression is via activation of receptor tyrosine kinase (RTK) signaling pathways. The processes regulated by these receptors and their ligands are diverse and include the regulation of cell growth, differentiation, migration, viability, and homeostasis. Interestingly, while some RTKs are cell type specific and devoted to a single specific function, others are implicated in multiple processes (reviewed by Perrimon, 1994). In Drosophila, single function RTKs include Sevenless (Sev), which specifies the differentiation of one photoreceptor in the adult eye, and Torso (Tor), utilized exclusively for determination of cell fates at both embryonic termini. In contrast, the Drosophila epidermal growth factor (EGF) RTK (Egfr) is involved in a myriad of developmental decisions; just in the embryo, Egfr is involved in the establishment of ventral cell fates, maintenance of amnioserosa and ventral neuroectodermal cells, germ band retraction, cell fate specification in the central nervous system, and production of cuticle.

A few years ago, the examination of RTK signaling pathways culminated with the realization that most RTKs regulate $p21^{ras}$ activity, which subsequently activates a kinase cascade that sequentially involves Raf, MEK, and MAPK (see Perrimon, 1994). Central to the establishment of this "universal cassette" dogma were studies of the single function Drosophila RTKs (Sev and Tor) that provided excellent genetic paradigms to identify molecules involved in transduction of the signals, as well as to characterize their relative positions within the signaling cascade. Recently, a number of studies have focused on understanding how a single RTK, such as Egfr, can regulate multiple developmental responses. In this review, we discuss how a multiplicity of ligands, positive and negative tissue specific feedback loops, and cooperativity between different RTKs offer a diverse array of strategies to regulate the actions of the same receptor. Many of these mechanisms will likely be conserved during evolution; therefore, it is anticipated that critical lessons learned by examination of Drosophila Egfr signaling will contribute significantly to our understanding of vertebrate RTK signaling.

Multiple Ligands Regulate Egfr Function During Development

In addition to its multiple functions during embryogenesis (listed above), Egfr activity, which correlates with its broad expression pattern, is critical for proliferation of imaginal tissues, and in the determination of both the antero-posterior and dorso-ventral polarities of the oocyte. The seemingly global requirement of Egfr during development raises the question of how one RTK regulates each of these developmental processes.

One answer is that there are multiple Egfr ligands that activate the RTK in a tissue-specific manner. Characterization of mutants that exhibit only a subset of the *Egfr* mutant phenotypes led to the identification of three candidate Egfr ligands, Spitz (Spi; Rutledge et al., 1992), Gurken (Grk; reviewed by Ray and Schupbach, 1996) and Vein (Vn; Schnepp et al., 1996). All three proteins contain an EGF repeat similar to that of transforming growth factor (TGF)-α, a known ligand of the vertebrate EGF RTK.

Two scenarios have emerged by which these ligands activate the Egfr. In the case of Grk, at one point during oogenesis, *grk* transcripts become localized to the antero-dorsal corner of the oocyte resulting in the production of a spatially restricted ligand, which presumably is secreted into the perivitelline space and activates Egfr in the adjacent dorsal follicle cells (reviewed by Ray and Schupbach, 1996).

In contrast, Spi is uniformly expressed (Rutledge et al., 1992), suggesting that an additional level of regulation must be involved to explain how Spi activates Egfr in a tissue-specific manner. Characterization of Spi in tissue culture has revealed that it is produced as a transmembrane precursor which is processed into a secreted form (Schweitzer et al., 1995a). The ubiquitous nature of Spi expression suggests a model whereby one or more factors required for Spi processing must be spatially restricted to specify where Spi is processed, secreted, and subsequently activates Egfr.

Golembo et al. (1996b) obtained evidence for such a model by examining Egfr signaling in the embryonic ventral ectoderm. During embryogenesis, it has been proposed that graded Egfr signaling patterns the ventral ectoderm with highest activity defining the ventral-most epidermal cell fates. In both *Egfr* and *spi* mutant embryos, defects in dorso-ventral (D/V) patterning are observed. The signal that triggers graded activation of Egfr appears to originate from the ventral midline since in *single minded* (*sim*) mutant embryos, where the ventral midline does not develop, patterning of ectodermal cells along the D/V axis is abnormal and exhibits a nearly identical ventral ectodermal mutant phenotype as *spi*. Golembo et al. (1996b) found that the production of secreted Spi only in the ventral midline was able to rescue ventral ectoderm cell fates of *spi* mutant embryos suggesting that localized processing of Spi at the midline organizes the graded activation of Egfr. To demonstrate that secreted Spi is the only factor essential for ventral ectoderm determination that is produced by the midline, Golembo et al. (1996b) ectopically expressed the secreted form of Spi in the ectoderm of *sim* mutant embryos. In this case, secreted Spi not only induces ventral cell fates but also gives rise to ventralized embryos. Together, these results provide compelling evidence that Spi acts as a processed, secreted ligand; however, it remains to be shown that a processed form of Spi exists in vivo.

The third putative Egfr ligand, Vn, is expressed in highly dynamic patterns that are consistent with the

embryonic mutant phenotypes of *Egfr* (Schnepp et al., 1996). For example, beginning at blastoderm stages, *vn* transcripts are expressed as two broad ventro-lateral stripes in cells patterned by the Efgr. Although *vn* mutant embryos only exhibit weak D/V patterning defects, genetic evidence that Vn operates in Egfr signaling stems from embryos doubly mutant for null alleles of both *spi* and *vn* that exhibit more severe mutant phenotypes than *spi* mutant embryos alone. This finding suggests that Spi cooperates with Vn in establishing the gradient of Egfr activity during embryogenesis, thus illustrating the possibility that multiple ligands operate in concert for proper spatial and temporal activation of Egfr.

Interestingly, the embryonic phenotype of *spi; vn* double mutants is not as severe as the *Egfr* null mutant phenotype, suggesting that a basal, ligand-independent Egfr activity exists, or that a third, unknown ligand regulates Egfr during embryogenesis, or that maternally provided Spi activity masks the interaction.

The processing of Spi from an inactive to an active form is in apparent contrast to Vn and possibly Grk. Vn contains, in addition to the EGF repeat, an Ig-like domain with homology to the neuregulins and lacks a transmembrane domain (Schnepp et al., 1996). The mechanism by which Vn is processed, if at all, is not understood. Grk encodes a potential transmembrane domain but it has not yet been determined whether Grk functions as an unprocessed, membrane-localized signal or is processed into a secreted active ligand.

Collectively, the studies on the candidate Egfr ligands have revealed that one of the mechanisms by which Egfr activity can be modulated is by the existence of multiple ligand activities. Diversity in the expression patterns of these ligands as well as in the molecular mechanisms deployed to activate them have increased the repertoire of functions of this RTK.

Regulation of Egfr Activity by Positive and Negative Feedback Loops

Recent studies on two proteins, Argos (Aos) and Rhomboid (Rho), have provided evidence that Egfr can also be regulated via two feedback loops, suggesting that Egfr itself can regulate its own set of regulators (Figure 1).

Rho Positively Regulates Egfr Signaling. The *rho* gene encodes an integral membrane protein, is dynamically expressed throughout embryogenesis in a tissue-specific fashion, and exhibits a nearly identical embryonic mutant phenotype to *spi*. Furthermore, *rho* expression is required during development of the wing disc for formation of distal wing veins (Sturtevant et al., 1993) and during oogenesis for cell fate specification of anterodorsal follicle cells. Gene dosage and genetic interaction experiments have revealed stong interactions between Rho and Egfr during wing vein formation. For example, in animals doubly mutant for gain-of-function alleles of both *rho* and *Egfr*, formation of extra wing veins is enhanced. Conversely, vein loss is greatly enhanced in animals doubly mutant for loss-of-function alleles of both *rho* and *Egfr*. Together these results suggest that Rho may function to facilitate Egfr signaling (Sturtevant et al., 1993).

The expression of *rho* in the ventral midline of wild-type embryos and its absence in *sim* mutant embryos are consistent with its hypothesized role in production

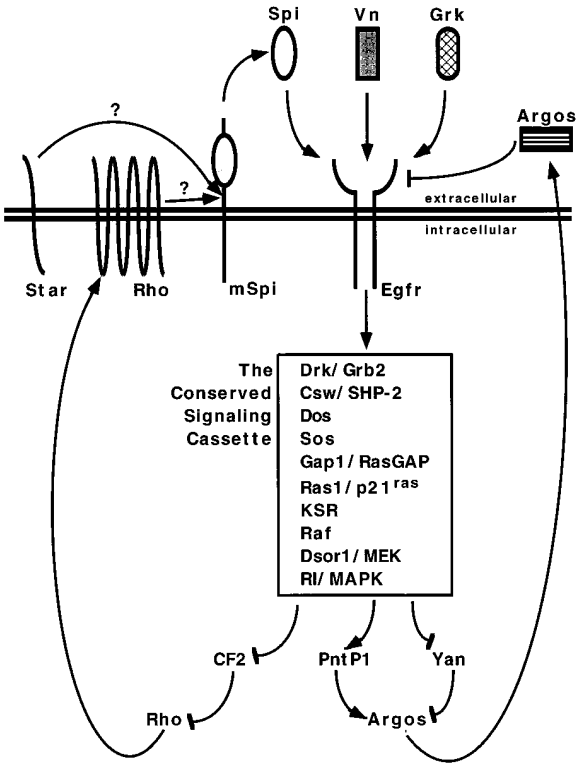

Figure 1. Egfr Signaling Pathway

or processing of the transmembrane form of Spi. Ectopic expression of *rho* only in the ventral midline of *rho* mutant embryos is sufficient to rescue the ectodermal defects observed in these animals, suggesting that Rho function is required in the midline where it acts nonautonomously to pattern the ventral ectoderm (Golembo et al., 1996b). In addition, another transmembrane protein, Star (S), behaves similarly to Rho in the embryonic ventral midline and may also be involved in the production or processing of Spi (Golembo et al., 1996b), a model that remains to be tested biochemically.

rho expression can also be regulated by Egfr. During oogenesis, *rho* expression in follicle cells is expanded in ovaries in which *grk* gene dosage is increased (reviewed by Ray and Schupbach, 1996). *rho* expression is repressed by CF2, a zinc finger transcription factor that is itself repressed by Egfr signaling. Therefore, in follicle cells, a feedback loop likely exists whereby Grk activates Egfr signaling which suppresses *CF2* expression, thus allowing *rho* expression and subsequent Egfr hyperactivation (Hsu et al., 1996). However, such a feedback loop does not appear to exist in all tissues. For example, in the embryo the initial zygotic expression of *rho*, including expression in the lateral neuroectoderm and subsequently in the ventral midline is induced by Dorsal and Twist and repressed by Snail.

Aos Negatively Regulates Egfr Signaling. Aos encodes a secreted protein with a signal sequence, no apparent transmembrane domain and a cysteine-rich region that resembles an EGF motif. Unlike Spi, Vn, and Grk, which regulate Egfr positively, Aos, as assessed by in vivo genetic interaction studies, inhibits Egfr signaling. For

example, *aos* loss-of-function mutations suppress *Egfr* loss-of-function phenotypes, while the *aos* gain-of-function phenotype is enhanced by *Egfr* loss-of-function mutations (Schweitzer et al., 1995b). In vitro, Aos can interfere with Egfr activation in a manner that is both saturable and competitive. Given these latter results and the fact that Aos contains an EGF domain, it is proposed that Aos acts as an inhibitor of Spi by directly binding to Egfr (Schweitzer et al., 1995b), a model that remains to be demonstrated biochemically.

Interestingly, the expression of *aos* is dependent upon Egfr activation (Schweitzer et al., 1995b; Golembo et al., 1996a). During embryogenesis, *aos* expression in the ventral ectoderm is not observed in *Egfr* loss-of-function mutant embryos. Conversely, in embryos where *Egfr* is activated by ectopic expression of secreted Spi, *aos* expression is greatly expanded. Furthermore, two transcription factors of the ETS family, PointedP1 (PntP1) and Yan, affect *aos* expression (Gabay et al., 1996). *aos* is not expressed in *pntP1* mutant embryos, and conversely, *aos* is expressed in cells where PntP1 is ectopically expressed. Together, these results suggest not only that PntP1 is sufficient to positively regulate *aos* expression, but also that it is likely a direct transcriptional activator. In embryos mutant for Yan, a negative regulator of ETS transcriptional activators, *aos* expression in the ventral ectoderm is expanded, and in a complementary experiment, expression of an activated form of Yan greatly reduces expression of *aos*.

The Aos Negative Feedback Loop. The fundamental observation, that transcription of both *rho* and *aos* can be regulated by Egfr itself, demonstrates that this RTK can activate both negative and positive feedback loops (Figure 1). What is the meaning of these intricate relationships between activating ligands and feedback loops? While the Rho feedback loop during oogenesis requires further clarification, for Aos, one potential answer is provided by studies of patterning of the embryonic ventral ectoderm as well as the ommatidia of the adult eye.

During patterning of the embryonic ventral ectoderm, high levels of Egfr activity induce the most ventral cell fates while lowering activities induce more ventro-lateral cell fates. Golembo et al. (1996b) propose a model whereby graded Egfr activation is established and maintained by competition between the activating activity of Spi and the repressing activity of Aos. In the ventral midline, production of Spi, Rho, and S depends on Sim. Rho and S participate in the production or processing of Spi from an inactive, membrane form to an active, secreted form, which then diffuses from the midline, presumably forming a gradient. In cells where Egfr is maximally activated (cells immediately adjacent to the midline), Aos is expressed and secreted, where it, like Spi, diffuses to form a gradient. Along the Aos diffusion gradient, competition with secreted Spi may result in either termination or reduction of Egfr signaling, thus preserving the initial graded effects of Egfr activation. Alternatively, the capacity of Aos to block Egfr signaling may be incomplete. Thus only in the more lateral cells, where lower levels of Egfr activation are encountered, is Egfr signaling terminated.

A second explanation that could account for the observation that different cells fates are determined in the ventral ectoderm by distinct signaling thresholds is that Egfr signaling could serve only as an on–off switch that triggers distinct cellular responses in cells with different histories or prepatterns. In support of this model, several genes, including *orthodenticle* (*otd*), are expressed in distinct D/V domains of the ventral ectoderm prior to Egfr signaling. When the production of secreted Spi along the ventral midline is increased, the expression pattern of *otd* is not displaced dorsally (Golembo et al., 1996b) as would be expected if a graded signal triggered defined threshold responses. This finding suggests that ventral ectodermal cells are not equivalent at the time they are presented with the midline signal. Thus the issue of whether the Egfr trigger acts as a morphogen gradient needs to be further substantiated.

Freeman (1996) has described a similar competition between the Aos and Spi signals during eye development. He proposes that the establishment of various cell types in the ommatidium invokes successive waves of recruitment by secreted Spi and the secreted inhibitor Aos. According to this model, active Spi is first produced by the central cells of the ommatidia (R8, R2, and R5) and this leads to the recruitment of neighboring cells (R1, R3, R4, R6, and R7) as photoreceptors. As cells differentiate they express Aos, which diffuses outward to prevent other cells from activating the Egfr signaling pathway.

In summary, studies of Egfr signaling have identified proteins that act either positively or negatively to regulate receptor activation by specific ligands. These proteins can be transcriptionally regulated by Egfr signaling revealing intricate relationships between the factors that activate the receptor and these feedback loops.

Cooperation between Egfr and Sev

A recent report by Freeman (1996), examining the differentiation of the R7 photoreceptor cell, describes a situation whereby activation of two different RTKs, Egfr and Sev, is required for proper establishment of cell fate. This unique case, whereby Egfr cooperates with another RTK, represents yet an additional mechanism by which the Egfr can participate in a multitude of developmental processes.

Sev is highly specific to the differentiation of only one of the eight photoreceptor cells in each ommatidium, the R7 cell. In a *sev* mutant, R7 fails to differentiate and instead becomes a lens-secreting cone cell. *sev* is expressed transiently in 8 of the 20 cells of the ommatidium and Sev specificity is regulated by a more localized signal, the transmembrane protein Boss, which is expressed only in R8, a cell that physically touches R7.

In contrast to Sev, Egfr is expressed uniformly in the eye, and clones of *Egfr* mutant cells are not recovered in the eye indicating that Egfr is required for cell survival. To circumvent the role of Egfr in cell survival, Freeman (1996) expressed a dominant negative form of Egfr (DN-Egfr) in the developing eye after completion of cell proliferation. In this event loss of Egfr affects determination of all neuronal and nonneuronal cells of the ommatidia, even in R7 where Sev is required. Realizing that R7 development requires both Sev and Egfr, the specificity of these RTKs was tested. Interestingly, Freeman found that overexpression of activated Egfr in R7 can bypass the requirement for Sev, indicating that these RTKs have no inherent specificities.

Why are two RTKs required in R7 for its differentiation?

One model proposes that, following activation of Egfr in R7, the sole function of Sev is to further increase the level of MAPK activation in the cell. Such a scenario would be reminiscent of the observation in PC12 cells that different levels and durations of MAPK activation lead to different cell fate outcomes (Traverse et al., 1994). Mechanistically, both Egfr and Sev activation would be required to reach a sufficiently high level of MAPK activation to trigger R7-specific genes. Since both Egfr and Sev appear to regulate similar downstream events, this combinatorial model would explain why upregulation of Egfr activity can bypass the requirement for Sev. Freeman argues that this is unlikely because the strength of RTK signaling does not appear to regulate the choice of cell fates in the eye. Instead, he favors a second model whereby both RTKs are required temporally in R7. According to this model, R7 differentiation requires two separate bursts of Ras activation, an early one triggered by Egfr and a later one triggered by Sev.

Role of SHP-2/Csw in RTK Signaling

So far, this review has focused on the modulation of Egfr activity at extracellular and membrane levels. In addition, it is possible that specific cytoplasmic signal transducers of RTKs exist. In the case of Egfr, one such example may be the nonreceptor protein tyrosine phosphatase SHP-2/Corkscrew (Csw). Csw has been implicated in multiple RTK signaling pathways, including the Tor, Egfr, and Sev pathways (Allard et al., 1996; Perkins et al., 1996). Collectively, these studies have led to the proposal that Csw is an essential component of the evolutionarily conserved cassette of molecules that transduce signals received by RTKs (Figure 1). This model, however, may have to be reexamined in light of the findings of Freeman (1996).

The current model, which is supported by findings on the mammalian PDGF RTK, is that SHP-2, through at least one of its SH2 domains, binds to a specific phosphotyrosine residue on the activated RTK and becomes tyrosine phosphorylated, thereby creating a docking site for Grb2 (see Discussion in Perkins et al., 1996). Consistent with this model, studies on Tor have identified a specific tyrosine residue (Y630) as a Csw binding site (Cleghon et al., 1996). However, Csw does not bind to the activated Sev RTK (Allard et al., 1996), as the activated Sev molecule contains a single phosphotyrosine residue (Y2546) outside the kinase domain that corresponds to a Grb2 binding site. Mutation of this site only partially abolishes Sev signaling (Raabe et al., 1995).

The genetic evidence that Csw is involved in Sev signaling stems from the observation that a dominant negative form of Csw suppresses activated Sev (Allard et al., 1996). Considering the role of Egfr in R7 development and the lack of physical association between Sev and Csw, it is possible that the ability of a dominant negative form of Csw to interfere with Sev signaling is indirect. A dominant negative form of Csw could deplete the pool of Grb2 necessary for activated Sev to exert its dominant effect. These considerations suggest that Csw may not be involved in Sev signaling and that SHP-2/Csw may be specific to a subclass of RTKs. If true, this model will have further implications in understanding the functions of other signal transducers such as DOS, which has been implicated in Sev signaling as a substrate for Csw (Herbst et al., 1996). Finally, if this model is correct we are left with a paradox because results of experiments in the eye have argued that Sev and Egfr have similar specificities. Thus, proteins such as SHP-2 may not contribute to RTK specificity per se but may alternatively serve to facilitate or amplify signals.

Perspectives

We have discussed some of the mechanisms that can regulate the activity of the Egfr in Drosophila. However, from studies of this receptor in other species, additional mechanisms that modulate the activity of this protein have been identified and may also play a role in Egfr regulation. Among these are the regulation of RTK activity by endocytosis, control of receptor turnover, subcellular localization of the RTK within the membrane, and cross-talk with other signaling pathways.

A detailed understanding of RTK regulatory mechanisms may have important therapeutic applications. Many cancers are caused by misregulation of RTK pathways, and some of the strategies to design drugs that cure malignancies have focused on targeting drugs against components of the RTK conserved signaling cassette, such as $p21^{ras}$. However, because these molecules are shared by multiple RTKs, it may be difficult to achieve specific therapeutic effects. An alternative strategy is the design of drugs that interfere with the activities of molecules, such as Aos, Rho, and S, that function in modulating specific RTK signaling pathways.

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