rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development

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SUMMARY

Genes of the ventrolateral group in Drosophila are dedicated to developmental regulation of Egfr signaling in multiple processes including wing vein development. Among these genes, Egfr encodes the Drosophila EGF-Receptor, spitz (spi) and vein (vn) encode EGF-related ligands, and rhomboid (rho) and Star (S) encode membrane proteins. In this study, we show that rho-mediated hyperactivation of the EGFR/MAPK pathway is required for vein formation throughout late larval and early pupal development. Consistent with this observation, rho activity is necessary and sufficient to activate MAPK in vein primordium during late larval and early pupal stages. Epistasis studies using a dominant negative version of Egfr and a ligand-independent activated form of Egfr suggest that rho acts upstream of the receptor. We show that rho and S function in a common aspect of vein development since loss-of-function clones of rho or S result in nearly identical non-autonomous loss-of-vein phenotypes. Furthermore, miss-expression of rho and S in wild-type and mutant backgrounds reveals that these genes function in a synergistic and co-dependent manner. In contrast, spi does not play an essential role in the wing. These data indicate that rho and S act in concert, but independently of spi, to promote vein development through the EGFR/MAPK signaling pathway.

Key words: rhomboid, Star, Egfr, spitz, MAP-K, Drosophila, wing vein development

INTRODUCTION

Receptor tyrosine kinases (RTKs) mediate diverse processes including cell fate determination during development and control of cell proliferation in response to growth factors. A wealth of data indicates that RTKs activate the shared and well-conserved RAS/MAPK pathway culminating in altered gene expression (Perrimon, 1994; Bier, 1998a). In contrast to other Drosophila RTKs, which seem to fulfill single and defined roles during development, the Drosophila EGF receptor (Egfr) plays crucial roles in a wide spectrum of cell fate decisions throughout development (Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997, Bier, 1998b). For example, in embryos, Egfr signaling is involved in patterning the ventral epidermis (Kim and Crews, 1993; Raz and Shilo, 1993), differentiation of midline glial cells, specification of sensory organ precursor cells (Lage et al., 1997), formation of somatic muscle (Buff et al., 1998) and morphogenesis of trachea (Wappner et al., 1997). During larval and pupal development, Egfr activity is involved in photoreceptor specification (Freeman, 1996), wing vein development (Díaz-Benjumea and García-Bellido, 1990, Sturtevant et al., 1993) and oogenesis (Price, 1989). In addition to these various roles in cell fate determination, low basal levels of Egfr activity provide a general function necessary for cell survival and cell size control in most epidermal cells (Schejter and Shilo, 1989; Díaz-Benjumea and García-Bellido, 1990; Díaz-Benjumea and Hafen, 1994).

Because of its multiple roles, EGFR must be subject to precise spatial and temporal regulation. A set of genes, referred to as the ‘ventrolateral’ or ‘spitz’ group genes, seems to be exclusively dedicated to the developmental regulation of EGFR signaling. These genes were initially identified by virtue of a shared embryonic mutant phenotype (Mayer and Nüsslein-Volhard, 1988) similar to that observed in embryos mutant for strong, but not null, alleles of Egfr (Schejter and Shilo, 1989; Price, 1989). Among the ventrolateral group genes, spitz (spi) and vein (vn), encode EGF-related ligands, in the TGFα and neuregulin families, respectively (Rutledge et al., 1992; Schejter et al., 1991; Schnepf et al., 1996), whereas rhomboid (rho) and Star (S) encode membrane proteins of yet unknown function (Bier et al., 1990; Schenodkin et al., 1994). The similar phenotypes of ventrolateral group mutants and the strong synergistic genetic interactions observed among members of this group suggest that these genes collaborate to promote Egfr signaling (Rutledge et al., 1992; Sturtevant et al., 1993; Kolodkin et al., 1994). Consistent with Egfr activity being generally required for cell survival, Egfr, spi and S are expressed ubiquitously
relationship between development. In contrast to synergistically and in a co-dependent fashion to promote vein downstream of the receptor and requires the integrity of its or dominant-negative forms of during which ectopic fates, as indicated by temperature-shift experiments and throughout the first 30 hours of pupariation to establish vein extra-vein phenotypes. Co-expression of during which ectopic phenotypes. Additionally, we show that function synergistically and in a co-dependent fashion to promote vein development. In contrast to rho, S and Egrf, spi does not play an essential role in the wing. These results reveal an intimate relationship between rho and S during vein development, which is independent of spi. We discuss these results in terms of models for how ventrolateral group genes activate Egrf signaling.

**MATERIALS AND METHODS**

**Fly stocks and crosses**
To produce marked rho- clones, we crossed mwh rho selv FRT72D males to Flp1; FRT72D females. The progeny of this cross and of similar crosses to generate other clones were heat shocked as described below. To generate large rho- clones, we used the Minute technique. We crossed mwh rho selv FRT80B/TM6 females to Flp2 Bc; M(3)55 FRT80B males, and searched for clones among [Bc, M, non Tb] F1 progeny. For S- clones, we crossed S1535 ck FRT80B males to Flp1; FRT80B females. To generate S- clones with the Minute technique, we crossed S1535 ck FRT80B/ CyrO females to Flp1/Y; M(2)36F FRT80B/+ males. We used the same strategy to generate clones homozygous for another strong allele S218. To produce rho- clones in flies ectopically expressing S, we crossed GAL4 MS1096 Flp1/Y; M(3)55 FRT80B/+ males to UAS-S; mwh rho selv FRT80B/TM6 females, and searched for mwh rho selv clones among [M non Tb] females. The M(3)55 FRT80B stock was kindly provided by Pascal Heitzler (University of Strasbourg). To produce S- ck clones in flies ectopically expressing rho, we crossed GAL4 MS1096 Flp1/Y; M(2)36F FRT80B/+ males to S1535 ck FRT80B/CyrO; UAS-rho females, and identified ck marked clones in [M, non Cy] females. For ectopic expression of rho, we used an inducible hs-rho stock (described in Sturtevant et al., 1993). For overexpression of S, we crossed hs-S/Cyo flies (kindly provided by Utpal Banerjee, UCLA) to wild-type controls, or to the weak non-inducible rho S1535 line (Sturtevant, 1993), which provides low levels of constitutive rho expression. Similarly, for overexpression of spi, we crossed hs-spi flies either to wild-type or to heat-inducible hs-rho flies, or to a weak hs-rho; hsS/Cyo stock. To generate spi- clones, spi ck FRT80B/CyrO females were crossed to Flp1; FRT80B males, and clones were examined among [non Cy] female progeny (the spi allele was kindly provided by Kevin Moses, Emory University). To produce spi- clones in a vn1 mutant background (Lindsley and Grell, 1968; Lindsley and Zimm, 1992), we crossed a spi ck FRT40A/Cyo; vn1 stock to a Flp1; FRT80B, vn1 stock. For temperature-shift experiments to analyze Egrf function in the wing, we used the Egrf126 allele in trans to the Egrf80 allele. We refer to the Egrf126 allele as Egrf- (Lindsley and Zimm, 1992). To generate a hs-1-top line, we crossed A2-35B/TM6 males to hs-1-top (X-linked insert) females. The [Sb w+] male progeny were then crossed to w- females, and we recovered new insertions with extra-vein phenotypes among the [non Sb, w+] male progeny. The UAS-DN-Egrf and UAS-Egrf stocks were kindly provided by Allam Michelson (Harvard University), the UAS-S-top and hs-1-top (activated Egrf) stocks were kindly provided by Trudi Schüpbach (Princeton University). All other stocks were obtained from the Bloomington stock center.

**Heat shock conditions**
For overexpression experiments, pupae were collected every 3 hours at 25°C, placed on a humid filter paper in plastic Petri dishes and aged to the appropriate time. Heat shocking was performed by floating the dishes in a covered water bath at 38°C for 1 hour, followed by a 1 hour rest at room temperature, and then by one more hour at 38°C. For heat shocks during larval stages, mixtures of larvae of various ages were collected and submitted to the same regimen in a glass vial containing a small amount of food. The pupae that formed after the heat shock were then collected every 24 hours.

**Generation of mitotic clones**
Appropriate crosses were transferred to fresh vials every 24 hours. Each batch of progeny was then aged for 24 hours and the first and second instar larvae were submitted to a sequential heat-shock regimen at 38°C.

**Temperature-shift experiments**
White pre-pupae were collected onto moistened filter paper, placed in...
vented plastic Petri dishes, and incubated at 18°C or 29°C for appropriate periods at permissive or non-permissive temperatures respectively.

In situ hybridization and antibody staining

In situ hybridization experiments were performed with digoxigenin-labeled antisense RNA probes as described previously (O’Neill and Bier, 1994). For in situ detection of MAPK activation, pupal and larval carcasses were dissected and fixed in 8% formaldehyde/PBS, washed and stored in 100% methanol at −20°C. They were then exchanged into PBT, washed in PBT at room temperature, and incubated in anti-activated MAPK (MAPK*) antibody (Sigma) overnight at 4°C (1/200 dilution). To visualize the MAPK* staining pattern, the tissues were incubated in biotinylated secondary antibodies, followed by treatment with avidin/HRP (Vecstain ABC elite kit). MAPK activation was detected as a brown peroxidase reaction product. For the double rho/MAPK* labeling experiment, carcasses were fixed in 8% formaldehyde/PBS. The anti-MAPK* antibody was applied followed by in situ hybridization. MAPK* staining was detected as a brown HRP reaction product and rho RNA was visualized as a blue alkaline phosphatase precipitate as previously described (Sturtevant et al., 1993, O’Neill and Bier, 1994).

Transgenic stocks

UAS-Star and hs-m-spi constructs were generated by cloning cDNAs containing the full open reading frame of Star and spi into the pUAST vector (Brand and Perrimon, 1993), and in the hs-CaSpeR vector (Bang and Posakony, 1992). Details of these constructs are available on request. Transgenic lines carrying these insertions were obtained by standard methods of P element-mediated transformation.

RESULTS

Expression of ventrolateral genes in larval discs and pupal wings

As a starting point for analyzing the requirement for Egfr, rho, S and spi function during vein formation, we determined the RNA expression pattern of each of these genes throughout the course of vein development during the third instar larval and early pupal development to fill gaps in the existing data (Fig. 1, and Sturtevant et al., 1993, 1994; Herberlein et al., 1993). rho is the only ventrolateral group gene expressed specifically in vein primordia throughout larval and pupal wing development (Fig. 1A,B, and Sturtevant et al., 1993, 1994). S is expressed ubiquitously in the disc (Fig. 1C) and pupal wing (Fig. 1D, and Heberlein et al., 1993), but is expressed at higher levels in stripes overlapping the L3 and L4 vein primordia of larval wing discs and in all vein-competent domains during pupal stages (Fig. 1D). Similarly, spi RNA is present at low ubiquitous levels in both imaginal discs and pupal wings, and is enriched in vein competent domains, particularly in pupal wings (Fig. 1E,F). S and spi also are expressed in a subset of sensory cells along the wing margin (Fig. 1D,F). Egfr is expressed ubiquitously at high levels during all stages of development, but is downregulated between the L3 and L4 veins in late larval discs (Fig. 1G) and becomes strongly downregulated in veins during pupal stages (Fig. 1H and Sturtevant et al., 1994). Finally, vn is expressed in a stripe between the L3 and L4 primordia in late third instar wing discs

Fig. 1. Wild-type expression patterns of rho, Star, spi, and Egfr. (A) rho is expressed in vein primordia in third instar imaginal wing discs and in vein primordia throughout early pupal development. Longitudinal veins are labeled L2-L5. (B) rho expression in a pupal wing at 25 hours APF = stage P2. The margin (m) is an extension of L1. (C) Star expression in third instar imaginal disc is ubiquitous, but is elevated in stripes, corresponding to the L3 and L4 (double-label experiment not shown). (D) In a P2 pupal wing, S expression is stronger in provein domains. spi expression in (E) discs and (F) pupal wings is low and ubiquitous, and elevated in provein domains during pupal stages. (G) Egfr is expressed ubiquitously at high levels in discs, but is downregulated along the margin and between the L3 and L4 vein primordia. Double-labeling experiments indicate that the edges of strong Egfr expression coincide with the L3 and L4 primordia (data not shown). In pupal wings (H), Egfr expression is strong in intervein regions and downregulated in vein primordia (Sturtevant et al., 1994).
and expands to occupy all intervein regions during pupal stages (Simcox et al., 1996).

**rho activates EGFR signaling throughout early pupariation**

In order to assess the temporal requirement for **Egfr** activity during vein development, we used the temperature-sensitive combination of **Egfr** alleles, **Egfr**\(^{top}\)/**Egfr**\(^{ts}\), which results in a large L4 truncation when flies are raised at the non-permissive temperature (29°C) during the first 30 hours of pupariation (Fig. 2B). This vein-loss phenotype is very similar to that observed in **Df**/**Egfr**\(^{top}\) animals (Sturtevant et al., 1993). Temperature-shift experiments reveal that **Egfr** function is required primarily between 6 and 18 hours APF (after puparium formation) for L4 development (summarized in Fig. 2J).

**Fig. 2.** **Egfr** and **rho** are required during early pupariation. (A) A wild-type adult wing. Veins L1 (= m) to L5 are indicated. (B) A wing from an **Egfr**\(^{top}\)/**Egfr**\(^{ts}\) fly, raised at the non-permissive temperature (29°C) from 0 to 30 hours APF. (C) A wing from an **Egfr**\(^{top}\)/**Egfr**\(^{ts}\); **rho**\(^{ve}\)/**rho**\(^{ve}\) individual raised at 18°C (the phenotype is similar to that of a **rho**\(^{ve}\)/**rho**\(^{ve}\) fly (Sturtevant et al., 1993). (D) A wing from an **Egfr**\(^{top}\)/**Egfr**\(^{ts}\); **rho**\(^{ve}\)/**rho**\(^{ve}\) individual raised at 29°C from 0 to 30 hours APF. The vein truncation phenotype is similar to that of a **Egfr**\(^{top}\)/**Df**/**Egfr**; **rho**\(^{ve}\)/**rho**\(^{ve}\) fly (Sturtevant et al., 1993). (E) A hs-**rho** wing with a blister separating the dorsal and ventral surfaces. (F) A hs-**rho** wing with ectopic cross-veins (arrows). (G) A hs-**rho** wing with an blister separating the dorsal and ventral surfaces. (H) A hs-**rho** wing with a thickened L3 vein. (I) Temporal sensitivity of the wing to **rho** mis-expression during larval and pupal development. The frequency of **rho**-induced vein phenotypes is plotted for heat shocks applied at various times throughout larval and pupal development. (J) The temporal requirement for **Egfr** and **rho** function in wing vein development is revealed by temperature-shift experiments, using the **Egfr**\(^{top}\)/**Egfr**\(^{ts}\) and the **Egfr**\(^{top}\)/**Egfr**\(^{ts}\); **rho**\(^{ve}\)/**rho**\(^{ve}\) allelic combinations.
We also were interested in establishing the temporal requirement for \textit{rho} in vein development. Several previously reported observations indicated that \textit{rho} is required during the third larval instar (see Discussion). To determine whether \textit{rho} also functions during pupariation, we combined \textit{Egfr}^{ts}/\textit{Egfr}^{top} with \textit{rho}^w/\textit{rho}^w. When raised at 18°C, these individuals display a phenotype similar to that of \textit{rho}^w flies in which L4 and L5 have prominent distal truncations, but L2 and L3 remain largely intact (Fig. 2C). However, when \textit{Egfr}^{ts}/\textit{Egfr}^{top}; \textit{rho}^w individuals are raised at 29°C during early pupariation (0-30 hours APF), sections of all veins are deleted (Fig. 2D), resembling the severe phenotype of \textit{Df}^{Egfr}/\textit{Egfr}^{top}; \textit{rho}^w wings (Sturtevant et al., 1993). The critical period for the enhanced vein-loss phenotype is broader than that for \textit{Egfr}^{ts}/\textit{Egfr}^{top} alone (from 0 to 24 hours APF, Fig. 2J), suggesting that \textit{rho} functions in concert with \textit{Egfr} during much of the first 24 hours of pupal development.

\textbf{\textit{rho} mis-expression during early pupariation induces extra-vein phenotypes}

Mis-expression of \textit{rho} generates several ectopic vein phenotypes (Sturtevant et al., 1993; Noll et al., 1994). To define the temporal sensitivity of the wing to \textit{rho} mis-expression, we performed a series of staged heat-shock experiments. These experiments reveal a broad phenocritical period for \textit{rho} mis-expression (Fig. 2I). Four categories of ectopic vein

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{Activated MAPK is detected in veins throughout wing development. (A) A wild-type third instar imaginal disc single stained with the anti-MAPK* antibody (left insert), or double labeled with a \textit{rho} antisense RNA probe (center and right). Arrows in the left insert indicate that MAPK* is concentrated in nuclei at this stage. Arrows in the right insert indicate that there are sporadic cells staining for MAPK* which do not express \textit{rho} (blue staining). (B) A \textit{rho}^w/\textit{rho}^w disc, stained with the anti MAPK* antibody. (C) A third instar larval disc from the \textit{rho}^{HS-sld} enhancer piracy line double stained with the anti MAPK* antibody and a \textit{rho} antisense probe. Brackets indicate domains of ectopic \textit{rho} expression. The arrow points to cells stained for MAPK* not expressing \textit{rho}. MAPK* staining in (D) an early wild-type pupal wing (stage P1 ~18 hours APF), (E) an early P2 (~22 hours APF), and (F) a late P2 pupal wing (~25 hours APF). MAPK* is detected in vein primordia until about 24 hours APF and then fades. At the same time, diffuse low level intervein staining appears. (G) Ectopic \textit{rho} expression (from an inducible hs-\textit{rho} transgene) in an early P2 pupal wing causes strong ectopic activation of MAPK, which is particularly elevated at the borders of vein-competent domains.}
\end{figure}
phenotypes were observed: (1) blisters separating the dorsal and ventral surfaces of the wing (Fig. 2E), (2) ectopic cross-veins (Fig. 2F), (3) an ectopic vein spur running between L3 and L4 near the margin (Fig. 2G), and (4) a thickened L3 vein (Fig. 2H). Although there are peak periods for inducing these various ectopic vein phenotypes, they were all formed throughout the period of vein development, between 0 and 30 hours APF (see Fig. 2I). Thus, the sensitive periods for rho mis-expression and for rho loss-of-function during pupariation are largely overlapping (Fig. 2J).

**rho is necessary and sufficient for MAPK activation in developing wings**

As an independent measure of EGFR pathway activity during early pupariation, we used an antibody that specifically recognizes the phosphorylated (activated) form of MAP kinase (Gabay et al., 1997a). MAP kinase is a key downstream component of RTK signaling. As no other RTK is known to be involved in wing development, the pattern of MAP kinase activation (MAPK*) is likely to reflect the activation pattern of EGFR. In wild-type third instar wing discs, MAPK* is present in the margin, and in the L3, L4 and L5 vein primordia (Fig. 3A, and Gabay et al., 1997a). A weaker signal also is detected in the L2 primordium in late discs (Fig. 3A). Much of the MAPK* staining in third instar wing discs is concentrated in nuclei, consistent with the role of MAPK* in regulating transcription (see left insert of Fig. 3A). Double-label experiments reveal that the strongest MAPK* staining is in cells expressing rho. However, staining is also observed sporadically at reduced levels in adjacent cells (see right insert of Fig. 3A). rho expression is necessary and sufficient for MAPK activation in wing discs since, in rho<sup>ve</sup> mutants, which selectively lack rho expression in longitudinal vein primordia (Sturtevant et al., 1993), MAPK* is not detected in longitudinal vein primordia (Fig. 3B), although staining is still observed along the wing margin. Consistent with rho-expressing cells having peak levels of MAPK activation, localized rho mis-expression in the rho<sup>Sld</sup> enhancer piracy line (between L2 and L3 and between L4 and L5, Noll et al., 1994), induces a similar pattern of ectopic MAPK* staining (Fig. 3C). In addition, MAPK* staining is detected in cells adjacent to cells mis-expressing rho (Fig. 3C, arrow). The short-range activation of MAPK in neighboring cells is consistent with the local cell non-autonomy of rho function observed in rho<sup>-</sup> clones (see below), and with similar results observed during embryogenesis (Gabay et al., 1997a). During early pupal stages, MAPK is activated in all vein primordia, but this staining disappears 24 hours APF (Fig. 3 D,E). Later, diffuse...
MAPK* is detected in intervein regions (Fig. 3F). As in larval wing discs, ectopic expression of rho (from a heat-shock transgene) in early pupae induces MAPK* staining throughout the wing blade, with a more intense staining along the borders of vein-competent domains (Fig. 3G). Egfr, vn and Star are also more strongly expressed in these vein border cells. The

Fig. 5. rho− and S− clones have similar non-autonomous phenotypes. Dorsal rho− (A) and S− (B) clones (dotted red outlines) in which the vein-loss phenotype extends outside the clone area (solid arrows). − indicates homozygous mutant cells, + indicates +/- or −/+ wild-type tissue. Dorsal rho− (C) and S− (D) clones in which the vein-loss phenotype within the clone is rescued near the border of the clone (dotted arrows, see also in B). (E) Large overlapping rho− clones generated with the Minute technique covering the entire anterior or posterior (G) compartment. (F) Overlapping clones homozygous for SX155 covering the entire wing blade, and (H) composite vein-loss map for clones for overlapping dorsal and ventral rho− and S− clones. Green and black lines correspond to veins that are not affected by rho− and S− clones, respectively. Dotted lines represent vein segments that are sometimes missing in rho− or S− clones.
prolonged localized activation of MAPK in veins, and the ability of ectopic rho to activate MAPK, is consistent with genetic experiments indicating that Egfr and rho are required for vein development throughout larval and early pupal stages.

**rho does not interact with a ligand-independent activated form of Egfr**

It has been proposed that rho acts upstream of the EGFR (Ruohola-Baker et al., 1993; Golembo et al., 1996). Alternatively, rho could activate a separate pathway that intersects and enhances EGFR signaling. According to the first hypothesis, rho would not be expected to interact synergistically with an activated ligand-independent version of Egfr (λ-top) (Queenan et al., 1997). In order to distinguish between additive and synergistic effects of combined ectopic rho and λ-top, we chose two pirate insertions, λ-top^HS-mod and rho^ HS-mod, that induce weak extra-vein phenotypes when heterozygous (data not shown, and Sturtevant et al., 1993), and moderate ectopic vein phenotypes when homozygous (Fig. 4A,B). The rho^ HS-mod pirate line interacts strongly with several known extra-vein mutants (Sturtevant et al., 1993; Sturtevant and Bier, 1995). In contrast to these extra-vein mutants, transheterozygotes containing the rho^ HS-mod pirate line and the λ-top^HS-mod insertion have an extra-vein phenotype similar to that of the rho^ HS-mod or λ-top^HS-mod homozygous insertions, revealing no more than an additive effect of these two elements (Fig. 4C). Also, we observed that a rho^ HS-wk pirate insertion does not enhance the moderate extra-vein phenotype caused by expression of a UAS-λ-top under the control of the GAL4 CY2 line (data not shown). The lack of synergism between rho and ligand-independent activated Egfr is consistent with Rho requiring the integrity of EGFR extracellular domain in order to function and supports models in which rho acts upstream of Egfr.

**rho-induced ectopic vein formation is suppressed by DN-Egfr**

To investigate further the interaction between rho and Egfr, we examined the effect of rho mis-expression in situations where EGFR signaling has been compromised. One way to reduce EGFR signaling is to overexpress a dominant negative version of EGFR (DN-EGFR) consisting of only the extracellular and transmembrane domains of the receptor. It has been proposed that DN-EGFR acts by forming heterodimers with the wild-type receptor that are unable to signal (Kashles et al., 1991; Freeman, 1996; Buff et al., 1998). To mis-express various genes in the wing, we employed the GAL4-UAS system (Brand and Perrimon, 1993). In this system, GAL4 produced in a particular pattern acts in trans to activate expression of UAS-transgenes in a corresponding pattern. We expressed UAS-DN-Egfr using the GAL4 driver MSl1096, which is strongly expressed on the dorsal surface of the wing primordium (Capdevila and Guerrero, 1994; Lunde et al., 1998). Such mis-expression of DN-Egfr results in narrow wings, which consistently lack veins having their major component on the dorsal surface (e.g. L3 and the distal parts of L4 and L5, see Fig. 4E). Mis-expression of rho using the same GAL4 driver converts almost the entire wing blade into solid vein material and dramatically reduces the size of the wing, presumably reflecting the smaller size of vein-versus-intervein cells (Fig. 4D). When we co-expressed rho and DN-Egfr constructs in the wing, the resulting phenotype was nearly identical to that observed with Egfr alone, namely a vein-loss phenotype (Fig. 4F). This strong loss-of-vein phenotype could not be substantially suppressed by co-expression of vn (Fig. 4G) or S transgenes (Fig. 4H), two vein-promoting genes that interact intensely with rho (see below). In contrast, the strong rho-induced extra-vein phenotype was fully rescued by co-expression of a wild-type Egfr transgene (i.e. rho + Egfr + DN-Egfr in Fig. 4I). These data lend further support to the view that Rho and S act upstream of EGFR.

**rho- and S- clones cause nearly identical vein-loss phenotypes**

Because rho^ve mutants lack only distal parts of veins, it is not clear whether rho activity is required in other regions of the wing. Similarly, the involvement of S in vein development has not been characterized in detail (Heberlein et al., 1993). In order to explore further the requirement for rho and S during vein development, we generated marked mitotic clones lacking either rho or S function. We observed that homozygous rho^− and S^− clones cause vein-loss phenotypes in nearly identical regions of the wing. Vein-loss phenotypes associated with either rho^− or S^− clones sometimes extend a few cells outside of the clone boundary (Fig. 5A,B, plain arrows). In other cases, vein truncation is rescued within the border of the clone (Fig. 5B,D, dotted arrows). These reciprocal forms of local cell non-autonomy appear to depend on the shape of the clone. For example, vein formation is often rescued in a clone surrounded by wild-type cells. On the contrary, vein-loss most often extends into genetically wild-type regions when a mutant clone surrounds wild-type cells. A third form of cell non-autonomy involves signaling between the dorsal and ventral surfaces of the wing (García-Bellido, 1977). Typically, dorsal rho^− or S^− clones erase both dorsal and ventral components of the vein, even when the main component of the vein is ventral (Fig. 5B). In contrast, ventral clones generally affect only the ventral component of the vein (data not shown). In order to determine which portions of veins require rho and S to develop normally, we used the Minute technique to produce large overlapping mutant clones. The Minute strategy is useful for generating clones completely covering veins since smaller clones tend to have boundaries that run along veins and avoid crossing over them (González-Gaitán et al., 1994). We generated overlapping dorsal and ventral clones filling either the anterior (Fig. 5E) or posterior compartments (Fig. 5G), and occasionally encompassing the entire wing blade (Fig. 5F). A composite tabulation of this clonal analysis is presented schematically in Fig. 5H. The anterior marginal vein L1 is never affected by either rho^− or S^− clones. The L2 and L5 veins, however, can be almost completely eliminated by large rho^− or S^− clones. In the case of L3 and L4, only distal halves of the veins are typically affected. Clones lacking S seem to have slightly stronger vein-loss phenotypes extending more proximally than clones lacking rho (Fig. 5H). Nevertheless, the composite overall pattern of vein-loss is strikingly similar for rho^− and S^− clones. In addition, rho^− and S^− clones display a cell-non-autonomous behavior that is distinct from the strictly cell-autonomous behavior of Egfr^− or Ras^− clones (see Discussion).

**rho and S function co-dependently**

To determine whether overexpression of S is able to induce
extra-vein phenotypes similar to those generated by mis-expression of rho, we used a heat-inducible hs-S line to supply staged pulses of strong S expression. In contrast to rho, overexpression of S does not induce wing phenotypes during any stage of development tested (Fig. 6B). Because previous data revealed strong genetic interactions between rho and S mutations (Sturtevant et al., 1993), we asked whether there might be a synergistic effect of overexpressing S in the presence of ectopic rho. To avoid generating a significant extra-vein phenotype with rho alone, we used a rho_HS-Wk pirate line that causes a weak non-inducible ectopic vein phenotype (Fig. 6C). When flies carrying both the hs-S and rho_HS-Wk constructs were heat shocked between 6 and 9 hours APF, we observed strong synergism between rho and S manifested by entire

![Image of wing phenotypes](image-url)

**Fig. 6.** S and rho function in a co-dependent manner. (A) A wild-type wing. (B) A wing from a hs-S/+ individual, subjected to a heat-shock treatment started between 6 and 9 hours APF. Ectopic expression of S throughout larval life and pupariation has no effect. (C) A wing from a rho_HS-Wk individual heat shocked between 6 and 9 hours APF. This non-inducible rho_HS-Wk line exhibits only a very weak extra-vein phenotype with or without heat shock. Similar very weak ectopic vein phenotypes are observed when rho_HS-Wk are heat shocked between 9 and 24 hours APF (data not shown). (D) A wing from a hs-S/+; rho_HS-Wk individual, heat shocked between 6 and 9 hours APF, (E) 9 and 12 hours APF, (F) 21 and 24 hours APF. Co-expression of S with a low dose of rho results in a massive ectopic vein phenotype in the 6-9 hours APF time window, indicating a strong synergism between rho and Star. (G) A GAL4 MS1096; UAS-S wing. Strong ectopic expression of Star induces faint extra-vein material located near longitudinal veins (arrow). (H) Anterior overlapping rho− clones, and (I) two overlapping rho− clones covering the entire wing blade in a GAL4 MS1096; UAS-S background. The rho− phenotype is not rescued in any respect by overexpression of Star (compare to rho− clones in wt background in Fig. 5E and 5G). (J) A GAL4 MS 1096; S^{Y155}X155 ck FRT40A; UAS-rho control wing without S− clones. Ectopic rho converts the entire wing into a solid vein territory when expressed under the control of GAL4 MS1096, although the phenotype is slightly suppressed in a S+/+ background (compare with Fig. 4D). (K) A GAL4 MS1096 FLP1; S^{Y155}X155 ck FRT40A/M(2)36F FRT40A; UAS-rho wing containing two overlapping S− clones covering the anterior compartment of the wing. Ectopic veins do not form within the S− clone. (L) A GAL4 MS1096/ FLP1; S^{Y155}X155 ck FRT40A/M(2)36F FRT40A; UAS-rho, with two overlapping clones covering the entire wing blade. No ectopic veins form and the loss-of-vein phenotype within the S− clone is indistinguishable from that seen in Fig. 5F, where two overlapping S− clones similarly fill the entire wing.
intervein sectors being converted into veins (Fig. 6D). From 9 to 12 hours APF, ectopic expression of \( S \) and \( rho \) induced moderate extra-vein phenotypes, consisting of ectopic cross-veins and some additional longitudinal veins (Fig. 6E). Between 12 and 24 hours APF, combined ectopic expression of \( S \) and \( rho \) induced diffuse extra-vein material between L1 and L2, and between L4 and L5, as well as a thickened L3 vein (Fig. 6F). No significant phenotype was generated by co-expressing \( Srho \) and \( S \) at later stages. We conclude that \( S \) and \( rho \) collaborate intimately to promote the vein-versus-intervein cell fate choice throughout pupal vein development.

The above experiments show that overexpression of \( S \) can only generate ectopic vein phenotypes in the presence of \( rho \). This observation raises the question of whether \( S \) also requires endogenous \( rho \) to function in veins. To address this possibility, we asked whether strong expression of \( S \) could rescue the \( rho \) vein-loss phenotype in the wing. In this experiment, we generated \( rho \) clones in wings overexpressing \( S \) driven by GAL4 MS1096. Control \( S \) mis-expressing wings have very faint traces of ectopic vein material, which are always located along longitudinal veins (Fig. 6G, arrow). We observed that \( S \) mis-expressing wings containing \( rho \) clones have vein-loss phenotypes that are not rescued in any respect by overexpression of \( S \) (compare \( rho \) clones of Fig. 6H,I with \( rho \) clones generated in a wild-type background in Fig. 5E,G). We conclude that \( S \) strictly depends on \( rho \) to function in the wing.

Given that \( S \) requires \( rho \) to function, we asked the converse question of whether \( rho \) can function in the absence of \( S \). This question cannot be addressed by a simple overexpression experiment because endogenous \( S \) is ubiquitously expressed in the wing. Therefore, we generated large homozygous marked clones of the strong loss-of-function allele \( S^{X155} \) in wings expressing high levels of ubiquitous \( rho \) on the dorsal surface of the wing. Mis-expression of \( rho \) in a wild-type background converts almost the entire wing blade into a solid vein territory (Fig. 4D). This solid vein phenotype is partially suppressed in a \( S \) heterozygous background (Fig. 6J), consistent with previous observations that \( S^{X155} \) is a potent suppressor of \( rho \)-induced ectopic vein phenotypes (Sturtevant et al., 1993). We recovered wings mis-expressing \( rho \) and carrying homozygous \( S \) clones covering anterior or posterior compartments (Fig. 6K) or the entire wing (Fig. 6L). In all cases examined, ectopic \( rho \) had no effect within \( S \) clones, nor could it rescue the \( S \) loss-of-vein phenotype (e.g. compare a wing lacking \( S \) and overexpressing \( rho \) in Fig. 6L to a \( S \) wing in a wild-type background in Fig. 5F). These results demonstrate that \( rho \) strictly requires \( S \) to function. We conclude that \( rho \) and \( S \) act in an obligate co-dependent fashion.

**Spitz does not play any detectable role in wing development**

Although it is well established that \( Egfr \) plays a central role in

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**Fig. 7.** \( spi \) has no detectable function in the wing. (A) A wild-type wing. (B) A \( vn^1/vn^1 \) wing. Vein truncation affects the L4 vein, with partial penetrance at the proximal part of L3. (C) An FLP1; \( spi^I \) FRT40A/FRT40A wing with two overlapping \( rho \) clones covering the distal part of L5. Red dotted lines indicate the border of dorsal clones, blue dotted lines indicate the border of ventral clones. (D) An FLP1; \( spi^I \) FRT40A/ FRT40A; \( vn^1/vn^1 \) wing, with two overlapping clones covering the proximal part of L3. The \( vn^1 \) phenotype is not exacerbated within the \( spi^I \) clone. (E) A hs-\( rho^+ \) wing from an inducible line subjected to heat-shock treatment between 6 and 9 hours APF. Ectopic \( rho \) induces a modest ectopic vein phenotype. (F) A wing from a hs-\( rho^+ \); P(hs-\( m-spi^+ \); individual, submitted to heat-shock treatment between 6 and 9 hours APF; ectopic \( m-spi \) does not enhance \( rho \)-induced ectopic vein phenotype. The ability of the hs-\( m-spi \) transgene to induce high levels of \( m-spi \) expression in the wing was confirmed by in situ hybridization (data not shown). (G) A wing from a hs-\( rho \); hs-\( Star \) individual, submitted to a heat shock between 6 and 9 hours APF, with moderate diffuse ectopic veins. (H) A wing from a hs-\( rho \); hs-\( Star \); hs-\( m-spi \) individual, submitted to a heat shock between 6 and 9 hours APF. The expression of \( m-spi \) enhances and sharpens the pattern of ectopic veins caused by ectopic \( rho + S \).
vein development (Diaz-Benjumea and García-Bellido, 1990; Sturtevant et al., 1993), the relative contribution of different EGF-related ligands remains unclear. One putative EGFR ligand, which plays a prominent role in vein development, is the neuregulin family member encoded by vein (Schnepp et al., 1996; Simcox et al., 1996a). It is not known, however, whether \( \text{vn} \) is the only ligand responsible for the localized activation of EGFR during vein development. \( \text{spi} \) is another obvious candidate EGFR ligand since it is expressed in the developing covering the wing, which were homozygous for the null allele \( \text{EGFR} \) during vein development.

In order to assess the possible contribution of \( \text{spi} \) to vein development, we generated a collection of marked clones covering the wing, which were homozygous for the null allele \( \text{spi}^2 \). We observed that \( \text{spi}^- \) clones, in contrast to clones of \( \text{rho} \), \( \text{S} \) or \( \text{Egfr} \), have no effect on vein patterning (Fig. 7C). We considered the possibility that a function of \( \text{spi} \) may have been masked by a compensatory effect provided by the other known EGFR ligand \( \text{Vn} \). Thus, we generated \( \text{spi}^- \) clones in \( \text{vn}^1 \) homozygous mutants that lack detectable expression of \( \text{vn} \) in the third instar larval discs (Simcox et al., 1996). We found that \( \text{spi}^- \) clones similarly have no effect on the vein loss phenotype of \( \text{vn}^1 \) mutants, even when overlapping dorsal and ventral clones fall within particularly sensitive areas of the wing where the \( \text{vn}^1 \) phenotype is not fully penetrant (compare Fig. 7B and D). We conclude that there is no detectable requirement for \( \text{spi} \) during wing development.

The Spitz protein is thought to be produced initially as an inactive membrane-bound precursor (\( \text{m-Spi} \)), which, in principle, could be cleaved to generate a more active secreted peptide (\( \text{s-Spi} \)). Consistent with this possibility, expression of a constitutively secreted version of \( \text{Spi} \) is able to activate EGFR in cell culture experiments (Schweitzer et al., 1995), and induces formation of extra-veins when expressed in the wing (Schnepp et al., 1998). It has been proposed that \( \text{Rho} \) and \( \text{S} \) might participate in the activation of the m-Spi precursor, possibly by facilitating its proteolytic cleavage (Golembi et al., 1996). In order to examine the functional relationship between \( \text{spi} \) and \( \text{rho} \), we overexpressed \( \text{m-Spi} \) in the presence or absence of ectopic \( \text{rho} \). We found that heat induction of a hs-\( \text{m-Spi} \) transgene alone throughout larval life and pupariation resulted in no detectable phenotype (data not shown, see also Pickup and Banerjee, 1999). Expression of this same transgene in \( \text{spi}^- \) embryos, however, could partially rescue lethality of the \( \text{m-Spi} \) embryos and reduced the severity of the \( \text{spi}^- \) phenotype in remaining dead embryos, indicating that the hs-\( \text{m-Spi} \) construct encodes a functional protein. We then provided moderate levels of ubiquitous \( \text{rho} \) (from a hs-\( \text{rho} \) transgene) in addition to hs-\( \text{m-Spi} \), in order to express \( \text{rho} \) and \( \text{m-Spi} \) in the same cells, and performed the same staged mis-expression experiments. In these experiments, we observed no effect of elevated \( \text{spi} \) expression in hs-\( \text{m-Spi} \) + hs-\( \text{rho} \) wings beyond that attributable to \( \text{rho} \) mis-expression (Fig. 7F compare with E). In order to test the possibility that the effect of \( \text{m-Spi} \) expression might have been hidden by some negative feedback loop, we determined the activation state of MAPK and the induction of argos briefly after heat induction of \( \text{rho} \) or \( \text{rho} + \text{m-Spi} \) in imaginal discs. Again, no difference in the induction of \( \text{MAPK}^* \) and argos was detected when hs-\( \text{m-Spi} \) was co-expressed with \( \text{rho} \) (data not shown). We also co-expressed hs-\( \text{m-Spi} \) + hs-\( \text{Star} \) during larval or early pupariation and used the strong 1096 GAL4 driver to mis-express UAS-\( \text{Star} \) in the presence of hs-\( \text{m-Spi} \). In each of these cases, we observed no effect (data not shown).

To provide the most sensitized background possible to detect an activity of \( \text{m-Spi} \) in the wing, we mis-expressed \( \text{star} + \text{rho} + \text{m-Spi} \) together. For this experiment, we used a combination of hs-\( \text{rho} \) and hs-\( \text{Star} \) insertions that generates a weak to moderate ectopic vein phenotype upon pupal heat shock (Fig. 7G). We crossed a hs-\( \text{rho} \); hs-\( \text{Star} \) line to the hs-\( \text{m-Spi} \) line and heat shocked the progeny at various stages of development. In this highly sensitized background, we did uncover an activity of overexpressed \( \text{m-Spi} \) in the wing during early pupariation (mostly between 6 and 12 hours APF) since the resulting ectopic vein phenotype was enhanced relative to that induced by \( \text{rho} \) and \( \text{Star} \) (Fig. 7H). Interestingly, the most notable feature of ectopic veins induced by \( \text{rho} + \text{Star} + \text{m-Spi} \) is that they differ in quality from those produced by \( \text{rho} + \text{Star} \) alone. In contrast to the diffuse ectopic veins induced by \( \text{rho} + \text{Star} \), a sharp pattern of well-defined forked longitudinal veins is observed in \( \text{rho} + \text{Star} + \text{m-Spi} \) wings. As mis-expression of \( \text{lambda-top} \) (Queenan et al., 1997), or \( \text{s-Spi} \) (Schnepp et al., 1998), induces formation of diffuse rather than sharp ectopic veins, the phenotype arising from co-expressing \( \text{rho} + \text{Star} + \text{m-Spi} \) is unlikely to result from the production of \( \text{s-Spi} \). Rather, these data suggest that \( \text{m-Spi} \) can collaborate with ectopic \( \text{Rho} \) and \( \text{Star} \) to transform a graded vein-promoting activity into a sharp on-off response. A phenotype resulting from ectopic expression of \( \text{rho} + \text{Star} + \text{m-Spi} \) also could be observed in the eye, in which cell death was localized at the anterior part of the eye, when heat shock was provided between 0 and 6 hours APF (not shown).

The absence of any phenotype detected in \( \text{spi}^- \) clones, and the failure of \( \text{m-Spi} \) to potentiate the effect of ectopic \( \text{rho} \) alone (even in presence of ubiquitous endogenous \( \text{Star} \) expression) argues against the idea that \( \text{Rho} \) acts through the activation of the m-Spi ligand. We conclude that both \( \text{rho} \) and \( \text{Star} \) function independently of \( \text{spi} \) during wing vein development.

DISCUSSION

**Egfr and rho function throughout vein development**

Egfr signaling plays multiple sequential roles during embryonic and adult development (Perrimon and Perkins, 1997; Schweitzer, 1997, Bier, 1998b). For example, in the wing, Egfr provides an early function in cell proliferation and cell survival during early larval stages (Diaz-Benjumea and García-Bellido, 1990), and then acts to promote the vein-versus-intervein cell fate choice. It has not been established, however, at what step in vein development Egfr activity is required. A variety of previously published data indicates that Egfr and \( \text{rho} \) are required during the third instar larval disc to initiate vein development (Sturtevant and Bier, 1995; Roch et al., 1998; Biels et al., 1998). For example, \( \text{rho}^{\text{vco}} \) homozgyous mutants, which lack \( \text{rho} \) expression in third instar discs, have significant truncations of longitudinal veins (Sturtevant et al., 1993). Reciprocally, in the \( \text{rho}^{\text{std}} \) mutant, localized ectopic \( \text{rho} \) expression in the third larval instar disc and early prepupae prefigures the final adult pattern of ectopic veins (Noll et al., 1994). Furthermore, vein-specific expression of several genes is partially dependent on \( \text{rho} \) function in third instar discs (Sturtevant and Bier, 1995; Biels...
et al., 1998). In this study, we show that Egfr and rho also promote vein fates during early pupal stages. For example, temperature-shift experiments indicate that Egfr and rho function are required from 0 to 24 hours APF, and staged mis-expression of rho induces ectopic vein phenotypes any time between 0 and 30 hours APF. The pattern of MAPK activation supports the view that Egfr and rho function throughout the period of vein development, as MAPK* staining follows the pattern of rho expression from the mid-third larval instar until approximately 25 hours APF. Furthermore, we show that rho expression is necessary and sufficient for activating MAPK throughout the extended course of vein development. Together, these data reveal a continuous requirement for rho and Egfr function during vein development to promote vein fates mediated by localized activation of MAPK. An unexpected feature of later pupal wings (25-30 hours APF) is that MAPK* staining disappears from vein primordia and is expressed diffusely in all intervein cells. Although there is no evidence for EGFR/MAPK playing any role in the late differentiation of intervein cells, the observed MAPK* staining and the strong expression of Egfr (Fig. 1H, and Sturtevant et al., 1994) and vein (Simcox et al., 1996) in these cells raises this possibility. Alternatively, Egfr-expressing cells might produce a secondary signal required for vein fate maintenance in neighboring vein cells.

**Rho acts upstream of EGFR**

As MAPK is a key downstream component transducing signals from tyrosine kinase receptors to the nucleus, the observation that ectopic rho expression can rapidly induce activation of MAPK conforms with previous genetic data indicating that rho is dedicated to promoting EGFR/MAPK signaling. This observation, however, does not address the question of whether Rho acts upstream of or in parallel with EGFR. Our present data suggest that rho acts upstream of EGFR in the wing. First, we observe that the strong ectopic vein phenotype caused by rho mis-expression is completely suppressed by co-expression of DN-Egfr, even when vn or S are co-expressed along with rho to potentiate its effect. Second, we observe no synergism between a ligand-independent activated form of EGFR and ectopic rho expression, suggesting that Rho requires the integrity of the EGFR extracellular domain to activate it. These observations strengthen the idea that Rho acts upstream of and through EGFR. In contrast to the results obtained with DN-Egfr, the vein-loss phenotype associated with Egfr\textsuperscript{top}/DfEgfr mutant combination is unable to suppress the extra-vein phenotype caused by the rho\textsuperscript{HS-SG} pirate line (Sturtevant, 1993).

Molecular analysis of the Egfr\textsuperscript{top} allele indicates that it results from a point mutation in the extracellular domain of the receptor (Clifford and Schüpbach, 1994), which may reduce its affinity for ligands. The ability of rho to overcome the effect of this mutation can be understood if ectopic rho creates a surplus of ligand or ligand co-factor(s) that can compensate for the reduced affinity of the receptor for its ligand(s).

**rho and S function co-dependently**

rho and S were initially identified based on similar embryonic loss-of-function phenotypes, suggesting that they are involved in a common molecular process (Mayer and Nüsslein-Volhard, 1988). Moreover, S is the most potent known dominant suppressor of rho-induced extra-vein phenotypes (Sturtevant et al., 1993). In this study, we have provided further support for a close partnership between these two genes. Critically, we show that, in the wing, loss-of-vein phenotypes caused by rho\textsuperscript{–} or S\textsuperscript{–} clones are very similar, but different from phenotypes associated with clones of mutants in the EGFR signalling cassette. For example, as mentioned above, rho\textsuperscript{–} and S\textsuperscript{–} clones exhibit local cell non-autonomy, in contrast to Egfr\textsuperscript{–} clones. Furthermore, cells in rho\textsuperscript{–} or S\textsuperscript{–} clones have normal viability and size, whereas mutant clones lacking EGFR or downstream components have reduced cell size and viability (Díaz-Benjumea and Garcia-Bellido, 1990; Díaz-Benjumea and Hafen, 1994). Thus, loss-of-function analysis reveals that rho and S define a subgroup of genetic functions required for strong EGFR signaling, distinct from activities in the EGFR/MAPK pathway proper.

We also addressed the relationship between rho and S in series of epistasis experiments. We found that overexpression of S, which alone is unable to cause any phenotype in the wing, generates a strong ectopic vein phenotype only when ectopic rho is provided, showing that S needs rho to function in intervein regions. Clonal analysis indicates that S also depends on endogenous rho expression in veins, since overexpression of S in the absence of rho cannot rescue vein formation. Reciprocally, strong ectopic rho expression cannot generate any phenotype in wing clones lacking S. Collectively, these data indicate that rho and S function co-dependently, and collaborate to activate EGFR signaling by a common molecular mechanism.

Although rho and S mutant phenotypes are similar during many stages of development, the co-dependence of rho and S does not seem to apply to the eye. In the eye imaginal disc, S is required for cell viability and generates dominant morphological defects in the heterozygous condition (Heberlein et al., 1993), while clones lacking rho have no obvious phenotype (Freeman et al., 1992). This difference between rho and S function in the embryo and wing versus in the eye could be explained by the possible existence of other Rho-like proteins interacting with S during eye development, or might reflect the ability of S to function in the absence of Rho in certain cellular contexts.
**Which EGF-ligands function during vein development?**

Several ligands are known to activate EGFR in various developmental situations. In the embryo, *spi* and *vn* cooperate to promote localized *Egfr* activity (Rutledge et al., 1992; Schnepp et al., 1996). In the developing wing, *vn* is involved in cell proliferation during early larval stages, and later in promoting vein-versus-intervein cell fates. In contrast to *vn*, *spi* does not play any detectable role in the wing for cell survival or cell proliferation, nor is it involved in initiation of vein development (Simcox, 1997). Here, we show that, although *spi* is expressed in the wing disc and in the pupal wing at elevated levels in vein competent domains, it similarly has little if any function in vein development. A key unanswered question is whether *vein* is the only EGF ligand involved in vein differentiation. Since *vn* mutant clones lack detectable *vn* expression in the third instar larval disc, but exhibit only a truncation of L4 in adults, it is possible that additional EGF ligand(s) or cofactors function in concert with *vn*. Such ligand(s) and/or cofactors may require *rho* and *S* to be active, since the double mutant *rho*<sup>-</sup> *vn*<sup>1</sup> entirely lacks veins.

**Possible models for *rho* function**

Two classes of models have been proposed to explain the activity of *Rho* at the molecular level. In the first type of model, *Rho* acts as a separate signaling pathway, that ultimately converges on the RAS/MAPK pathway. This class of models accounts for the fact that in most situations, *rho* is required in the cells in which it is expressed (Bier et al., 1990; Sturtevant et al., 1993; Ruohola-Baker et al., 1993). An exception to this rule is in embryonic chordotonal organs, where *rho* is expressed in the sensory organ precursor cell (Lage et al., 1997), but activates MAPK only in surrounding epidermal cells (Gabay et al., 1997a). In a second class of models, *Rho* produces an extracellular signal that activates EGFR (Golembo et al., 1997; Pickup and Banerjee, 1999). According to this latter model, *spi*<sup>-</sup> loss-of-function clones would be expected to induce phenotypes equivalent to or stronger than those observed in *rho*<sup>-</sup> or *S*<sup>-</sup> clones, particularly in a *vn* mutant background. Since *spi*<sup>-</sup> clones have no detectable effect, our data argue against the ‘*Spi* processing’ model in the context of wing vein development. Even if *Spi* produced in wild-type cells was able to diffuse and rescue the vein-loss phenotype of *spi*<sup>-</sup> clones, the fact that small *rho*<sup>-</sup> or *S*<sup>-</sup> clones induce vein-loss phenotypes argues strongly against the possibility that *Rho* acts through the processing or the activation of m-***S***. On the other hand, the ability of m-***S*** to enhance and sharpen the vein phenotype caused by ectopic *Rho* and *Star* proteins suggests that *Rho* and *Star* can collaborate with m-***S*** to generate a stronger and more localized signal. The inability of m-***S*** to induce any phenotype in absence of ectopic *Rho* and Star proteins suggests that m-***S*** may require a prior action of *Rho* and *Star* to activate the EGFR in this artificial situation, whereas s-***S*** or *Vein* do not. It is also very unlikely that *Rho* promotes the processing of *Vein*, since the *Vein* ligand is not transmembrane bound and does not require a cleavage to function (Schnepp et al., 1996). Nevertheless, it is possible that *Rho* and *S* support the processing or facilitate the presentation of an unknown EGF ligand in the wing, or promote transcytosis of EGF ligands or ligand-receptor complexes. The drawback to these last two hypotheses is that they involve a yet unidentified molecule(s). Alternatively, *S* may constitute the missing link between *Rho* and EGFR. *S* could be a precursor for a diffusible factor, which ultimately activates EGFR, either as a ligand, or as a co-ligand. Such a co-ligand would reinforce or coordinate the effect of EGF ligands, possibly by promoting the formation of receptor oligomers. According to this scenario, *Rho* might act by promoting the processing of a membrane tethered S precursor into an active diffusible form. Finally, *Rho* and *S* could be acting directly on the EGFR (i.e. on the extracellular domain of the receptor itself) of the cells expressing *Rho*, and sometimes also of the adjacent cells (e.g. *Rho* could promote receptor dimerization or aggregation and thereby enhance the EGFR signal). Although this last hypothesis would not account for *rho* action over more than one cell diameter, it could explain why *rho* generally has a greater effect and ability to promote MAPK activation in cells expressing *rho*. Additional biochemistry experiments will be required to understand the basis for the non-autonomous action of *Rho*, and its predominant action in cells in which it is expressed.

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