RESEARCH COMMUNICATION

EGF domain swap converts a *Drosophila* EGF receptor activator into an inhibitor

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In *Drosophila* the function of the epidermal growth factor (EGF) receptor is modulated zygotically by three EGF-like proteins: Spitz (Spi), which is a potent activator; Vein (Vn), which is a moderate activator; and Argos (Aos), which is an inhibitor. Chimeric molecules were constructed in which the EGF domain of Vn was swapped with the EGF domain from each factor. The modified Vn proteins behaved both in vitro and in vivo with properties characteristic of the factor from which the EGF domain was derived. These results demonstrate that the EGF domain is the key determinant that gives DER inhibitors and activators their distinct properties.

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The *Drosophila* epidermal growth factor (EGF) receptor (DER) is a member of the ErbB family of receptor tyrosine kinases (RTKs) and, like its vertebrate counterparts, controls cell differentiation, survival, and proliferation in many tissues throughout development (Perrimon and Perkins 1997; Schweitzer and Shilo 1997). DER function is modulated by four candidate ligands each of which possesses a predicted EGF-like domain. Gurken (Grk) and Spitz (Spi) are TGF α -like proteins (Rutledge et al. 1992; Neuman-Silberberg and Schüpbach 1993). *grk* is a maternally active gene involved in establishing egg polarity (Neuman-Silberberg and Schüpbach 1993; González-Reyes et al. 1995), whereas *spi* is zygotically active and functions in the embryo, adult eye, and wing (Rutledge et al. 1992; Freeman 1994b). Vn resembles the vertebrate neuregulins in that both possess an Ig-C2 domain in addition to the EGF-like domain (Schnepf et al. 1996). Vein (Vn) functions zygotically in the embryo and the adult wing (Schnepf et al. 1996; Simcox et al. 1996; Simcox 1997; Yarnitzky et al. 1997). Here we show that Vn is a moderate activator of DER signaling in comparison

with Spi, which is a potent DER activator. Argos (Aos) has been recognized recently as an inhibitor of the DER pathway and was the first extracellular factor shown to inhibit an RTK in vivo (Schweitzer et al. 1995a). Aos functions in the embryo, adult eye, and wing (Freeman et al. 1992; Sawamoto et al. 1994; Golembo et al. 1996).

Considerable effort has been made to understand the structure-function relationships of vertebrate EGF-like mitogens to aid in the development of ErbB receptor superagonists or antagonists; however, to date these studies have not led to the design of effective factors (Groenen et al. 1994). The fly system offers a unique opportunity to define the molecular basis for the distinct properties of three natural ligands with different effects on a receptor and could facilitate the development of vertebrate factors with similar relative properties.

Spi, Vn, and Aos are structurally unrelated except within the EGF domain (Fig. 1A). The EGF domain contains a series of six cysteines, which form three disulfide bonds to generate a looped structure, and a number of other highly conserved residues that are known to be required for binding and activating members of the vertebrate ErbB receptor family (Groenen et al. 1994). The EGF domains of Vn and Spi are not highly related (38% conserved) but have more sequence conservation with each other than with Aos (Fig. 1A). Additionally, the length of the predicted B loop that forms from the region between cysteines 3 and 4 is significantly longer in Aos than in the activating ligands (Fig. 1A). The low level of sequence homology and the structural differences in the EGF domain could account for the different effects that the proteins have on DER signaling. To determine whether the EGF domain is sufficient to confer these distinct properties we generated chimeric molecules by exchanging the EGF domain of Vn for those of Spi or Aos (Fig. 1A). The activity of these chimeras was compared with the native factors in vitro and in vivo.

Results and Discussion

Activation of DER pathway by Vn in vitro

Upon ligand binding, ErbB receptors dimerize, cross-phosphorylate on carboxy-terminal tyrosine residues, and transmit a signal to the nucleus through the Ras/Raf/ERK pathway (Egan and Weinberg 1993). Thus, DER tyrosine phosphorylation is the initial indication of pathway activation, and ERK phosphorylation is a result of the signal transduction. Secreted Spi (sSpi, the active form of Spi) and Aos have been shown to increase or decrease, respectively, the level of DER tyrosine phosphorylation in *Drosophila* S2-DER tissue-culture cells (Schweitzer et al. 1995a,b). We applied Vn produced by transfected S2 tissue-culture cells to S2-DER cells and showed that Vn is a DER activator and induced DER tyrosine phosphorylation in a dose-dependent fashion with a concomitant rise in ERK activation (Fig. 1B). These in vitro results provide biochemical evidence that the newly discovered Vn protein, which had been linked

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Figure 1. (A) Schematic of native and chimeric EGF-like proteins. The domain structure of native Vn, Spi, Aoi, and chimeric proteins constructed between Vn and the Vn, Spi, or Aoi EGF domains is shown at the top. The manipulation to produce the chimeras results in the addition of 4 residues flanking the EGF domain in each chimera (shown only in the Vn:Vn EGF cartoon). (SP) Signal peptide; (Ig) immunoglobulin-like domain; (EGF) EGF-like domain; (TM) transmembrane region. The alignment of the EGF domains of Vn, Spi, and Aoi is shown below. The six conserved cysteines are boxed. The spacing between cysteines 3 and 4 is significantly longer in Aoi than any of the other proteins. (B) Activation of DER signaling by Vn in vitro. S2 cells expressing DER were exposed to increasing concentrations of Vn in conditioned medium. DER activation as measured by tyrosine phosphorylation (anti-p-tyr) increased relative to DER protein level (anti-DER) as Vn concentration increased. The level of ERK phosphorylation (anti-dp-ERK) also increased relative to ERK protein level (anti-ERK) as Vn concentration increased. The maximum level tested was a 60x concentration of Vn-conditioned medium, and the preceding lanes are dilutions that differ by 0.125 increments. (C) Activation of DER signaling by sSpi, Vn, and the chimeras. S2-DER-expressing cells were exposed to control medium and medium conditioned with sSpi, Vn, Vn:Vn EGF, and Vn: Spi EGF. Control cells (-) show that the basal level of DER tyrosine phosphorylation is low relative to DER protein level (anti-DER). sSpi-conditioned medium results in an elevation of DER phosphorylation (anti-p-tyr). Native Vn and the Vn:Vn EGF chimera result in a modest elevation of DER tyrosine phosphorylation, whereas, the Vn: Spi:EGF chimera results in a high level of DER tyrosine phosphorylation. Corresponding relative increases in ERK activation (anti-dp-ERK) were seen with the factors. Similar levels of Vn and the Vn chimeras were added to the S2-DER cells as determined by Western analysis with anti-Vn. This concentration was equivalent to the highest used in the dose-response-experiment (B). (D) Vn: Aoi EGF inhibits ligand-independent ERK activation. S2-DER cells exhibiting a high level of ligand-independent DER activation also had high levels of ERK activation (anti-dp-ERK) relative to ERK protein level (anti-ERK). Addition of Vn: Aoi EGF results in a lowering of the level of ERK activation.

to the pathway genetically (Schnepf et al. 1996; Yarnitzky et al. 1997), is a DER ligand. A direct comparison of the potency of native Vn and sSpi in vitro cannot be made because the proteins have not been purified and the absolute levels of each protein in the media are thus unknown; however, we infer that sSpi is the more potent factor because the Vn: Spi EGF chimera has stronger ac-

tivity than Vn and because sSpi is more potent than Vn in vivo (see below).

In vitro activity of Vn chimeras resembles the factor from which the EGF domain derives

The level of activation of the chimeric proteins was monitored. The Vn:Vn EGF chimera, which serves as a control for the effect of the additional residues introduced during construction of the chimeras, behaved like native Vn (Fig. 1C). In contrast, possession of the Spi-EGF domain converted Vn into a stronger DER activator (Vn: Spi EGF) (Fig. 1C). The Vn: Aoi EGF chimera behaved as an inhibitor, rather than an activator and caused a reduction in ERK activation resulting from the ligand-independent activation of DER (Fig. 1D). These results show that the properties of Vn are changed when its EGF domain is swapped with that of Spi or Aoi so that the chimeras behave like the factors from which the EGF domain is derived.

Vn: Spi EGF chimeras behave as strong activators in vivo

In the embryo, ectopic activation of the DER pathway by sSpi using the Gal4-UAS system [Krüppel (*Kr-gal4*; UAS-sSpi)] causes an expansion of ventral cell fates that can be monitored by expression of the ventral cell marker *orthodenticle* (*otd*) (Wieschaus et al. 1992; Brand and Perrimon 1993; Schweitzer et al. 1995b; Gabay et al. 1996) (Fig. 2D). Ectopic expression of native Vn (*Kr-gal4*; UAS-vn) caused no change in the expression of *otd* (Fig. 2B). The Vn:Vn EGF chimera (*Kr-gal4*; UAS-vn:vn EGF) caused a very mild expansion of *otd* expression (Fig. 2C). This slight effect could be the result of higher expression of the transgene (cf. insets in Fig. 2, B and C). In contrast, ectopic expression of the Vn: Spi EGF chimera

(*Kr-gal4*; UAS-vn:spi EGF) caused a dramatic expansion of *otd* expression that was similar to that seen with ectopic expression of sSpi (Fig. 2E,D).

In the wing, ectopic activation of the DER pathway is characterized by the appearance of extra veins (Sturtevant et al. 1993). Ectopic expression of native Vn in pupal interveins (*1348-gal4*; UAS-vn) produced a mild or mod-

Figure 2. Ectopic expression of native and chimeric factors in the embryo. Each panel shows the expression of *otd*, which is a marker for ventral fates. (Insets in B, C, and E) Endogenous *vn* expression in two ventrolateral domains and ectopic expression of *vn* and the *vn* chimeras in a circumferential band spanning the *Kr* expression domain [second thoracic segment (T2)–fourth abdominal segment (A4)]. These controls show the level of ectopic expression of the factors. (A) Wild type. One to two rows of cells on either side of the ventral midline express *otd*. (B) *Kr-gal4; UAS-vn*. There is no change in *otd* expression following ectopic expression of native *Vn* in the *Kr* domain. (C) *Kr-gal4; UAS-vn:vn EGF*. There is a very mild expansion of *otd* expression following ectopic expression of the *Vn:Vn EGF* chimera. This may be the result of higher ectopic expression of this factor than native *Vn* (cf. insets in B and C). (D) *Kr-gal4; UAS-sSpi*. Ectopic expression of *sSpi* causes ventral cell fates to spread laterally in T2–A4 as seen by the expanded expression of *otd*. (E) *Kr-gal4; UAS-vn:spi EGF*. Ectopic expression of the *Vn:spi EGF* chimera causes an expansion of *otd* expression similar to that seen after ectopic expression of native *sSpi*. Arrowheads in A–E indicate segment T2.

erate extra-vein phenotype, whereas ectopic expression of *sSpi* (*1348-gal4; UAS-sSpi*) caused a strong extra-vein phenotype (Fig. 3G). A direct role for *Vn* in normal vein development has been shown; such a role has not been demonstrated for *sSpi* but is likely to be involved (Simcox et al. 1996; Schweitzer and Shilo 1997). Ectopic expression of the *Vn:Vn EGF* chimera (*1348-gal4; UAS-vn:vn EGF*) gave extra-vein phenotypes similar to those seen after ectopic expression of native *Vn* (Fig. 3F). In contrast, ectopic expression of the *Vn:spi EGF* chimera (*1348-gal4; UAS-vn:spi EGF*) produced a strong extra-vein phenotype like that seen following ectopic expression of *sSpi* (Fig. 3H).

In the eye, ectopic activation of the DER pathway is characterized by loss of ommatidia, over-recruitment of cell types, and blistering (Baker and Rubin 1989; Freeman et al. 1992; Freeman 1996). Ectopic expression of native *Vn* posterior to the morphogenetic furrow in the eye disc (*GMR-gal4; UAS-vn*) had no effect on the adult

eye phenotype (Fig. 4E), whereas, ectopic expression of *sSpi* (*GMR-gal4; UAS-sSpi*) and the *Vn:spi EGF* chimera (*GMR-gal4; UAS-vn:spi EGF*) produced small disorganized eyes with blisters (Fig. 4G,H). Surprisingly ectopic expression of the *Vn:Vn EGF* chimera (*GMR-gal4; UAS-vn:vn EGF*) also showed a strong eye phenotype (Fig. 4F).

These *in vivo* data corroborate the biochemical data that *Vn* is a less potent activator of DER than *sSpi*. Additionally they show the EGF domain is a key feature that differentiates *Vn* and *sSpi* because *Vn* can be converted into a more potent DER activator if its EGF domain is swapped with that of *Spi*. The ability to differentially regulate signaling depending on whether *Vn* or *sSpi* is utilized may be one mechanism by which DER elicits specific cell responses during development.

Figure 3. Ectopic expression of native and chimeric factors in the wing. (A) Wild-type wing showing the normal pattern of five longitudinal veins and two crossveins. (B) *69B-gal4; UAS-vn*. Extra vein phenotype produced by overexpressing native *Vn* in the wing disc. The wing is smaller than wild type because vein cells that replace intervein cells are more compact. (C) *69B-gal4; UAS-aos*. (D) *69B-gal4; UAS-vn:aos EGF*. Ectopic expression of native *Aos* (C) and the *Vn:Aos EGF* chimera (D) causes loss of wing veins and the wings are smaller than wild type due to a reduction in the number of vein and intervein cells. Ectopic expression of *Vn:Aos EGF* gives a milder vein loss phenotype than native *Aos*. The fusion between L1 (the margin) and L2 is unlikely to be due to expression of *UAS-vn:aos EGF* as it is also seen in flies expressing the *69B-gal4* gene alone (arrowhead in D). (E) *1348-gal4; UAS-vn*. (F) *1348-gal4; UAS-vn:vn EGF*. In E and F there is a moderate extra-vein phenotype produced by ectopic expression of factors in pupal intervein regions. (G) *1348-gal4; UAS-sSpi*. (H) *1348-gal4; UAS-vn:spi EGF*. In G and H there is a strong extra-vein phenotype produced by ectopic expression of *sSpi* and the *Vn:spi EGF* chimera in pupal intervein regions. All wings are shown at the same magnification.

Figure 4. Ectopic expression of native and chimeric factors in the eye. All panels show scanning electron micrographs of adult eyes. (A) Wild-type eye showing the highly structured array of ommatidia, some of which are shown at higher magnification in the *inset*. (B) *69B-gal4; UAS-vn*. Ectopic expression of native Vn in the eye disc produces eyes that are indistinguishable from wild type. (C) *69B-gal4; UAS-aos*. (D) *69B-gal4; UAS-vn:aos EGF*. (C,D) A rough eye phenotype caused by ectopic expression of native Aos and the Vn:Aos EGF chimera in the eye disc. The eyes are smaller than wild type and some ommatidia are fused (see *insets*). (E) *GMR-gal4; UAS-vn*. Overexpression of native Vn in cells behind the morphogenetic furrow apparently has no effect. The occasional bristle duplications and depressions in the lenses are also seen in eyes of flies expressing the *GMR-gal4* gene alone (not shown). (F) *GMR-gal4; UAS-vn*. (G) *GMR-gal4; UAS-sspi*. (H) *GMR-gal4; UAS-vn:spi EGF*. (F,G,H) Ectopic expression of factors produces disorganized eyes with loss of ommatidia and blisters. (A–H) *Insets* are fivefold magnification of a part of the whole eye image.

The Vn:Vn EGF chimera appears to be a more potent factor than native Vn in the eye because unlike ectopic Vn, which had no effect, ectopic Vn:Vn EGF produced a strong phenotype. This suggests that regions outside the EGF domain can affect the activity of a factor because the manipulation used to create the chimeras changed 4 residues flanking the EGF domain (Fig. 1A). This observation also underscores the importance of assaying for ligand function in a number of cell types because such modulating effects may only lead to appreciable differences in phenotype in some cell types.

Vn:Aos EGF chimeras behave as inhibitors in vivo

To test whether Vn could be converted into an inhibitor by swapping its EGF domain with that of Aos, we compared the effects of ectopic expression of Vn, Aos, and the Vn:Aos EGF chimera in larval wing and eye discs. Native Vn produced an extra-vein phenotype when expressed ectopically in larval wing discs as expected for an activator of DER signaling (*69B-gal4; UAS-vn*; Fig. 3B). In the wing, ectopic suppression of the DER pathway is characterized by vein loss (Sawamoto et al. 1994; Schweitzer et al. 1995a) and ectopic expression of native Aos (*69B-gal4; UAS-aos*) or the Vn:Aos EGF chimera (*69B-gal4; UAS-vn:aos EGF*) resulted in vein loss (Fig. 3C,D). The vein loss phenotype associated with ectopic expression of Vn:Aos EGF was not as severe as that caused by native Aos.

In the eye, reduction in activity of the DER pathway is characterized by loss of cell types and fusion of ommatidia (Clifford and Schüpbach 1989; Freeman 1994a). There was no observable effect on adult eye phenotype following ectopic expression of native Vn in eye discs (*69B-gal4; UAS-vn*), but ectopic expression of the Vn:Aos EGF chimera (*69B-gal4; UAS-vn:aos EGF*) produced a rough eye phenotype with fused lenses similar to, but not as severe as, that produced by ectopic expression of native Aos (*69B-gal4; UAS-aos*) (Fig. 4C,D). These results show that the EGF domain is a key determinant responsible for the difference between Vn and Aos and that the EGF swap is sufficient to convert a DER activator into an inhibitor. The Vn:Aos EGF chimera is apparently not as potent an inhibitor as native Aos in the eye or the wing, suggesting that other regions of the proteins (Vn and/or Aos) may play modulating roles.

Concluding remarks

We have shown that Vn is a moderate activator of DER signaling and can be converted into a strong DER activator by exchanging the Vn EGF domain with that of Spi or into an inhibitor by exchanging the Vn EGF domain with that of Aos. This demonstrates that the EGF domain is the key feature that gives each ligand its distinct property. The result is important for understanding the function of the novel extracellular inhibitor Aos, as it suggests that the EGF domain is sufficient for DER inhibition while other regions of the protein are not essential (but may play modulating roles). There is currently no definitive evidence that Aos elicits its effect on DER signaling by binding DER directly; however, our results suggest that the EGF domain is a critical region for mediating the effect.

The fly system, which can efficiently test the function of chimeras in vivo, may be a powerful tool for precisely defining the region within the EGF domain that is characteristic of an inhibitor. Human EGF chimeras with substitutions or insertions of sequence from the B loop of the Aos EGF domain functioned as agonists rather than antagonists (van de Poll et al. 1997). Nevertheless, these results demonstrate that fly/vertebrate chimeras are able to bind and elicit a response from a vertebrate EGF re-

ceptor and suggest that inhibitory sequences identified in the fly system could be used to modify vertebrate factors to function as antagonists. The development of ErbB antagonists has significant clinical implications, as these receptors and their ligands function in growth of tumor cells (Groenen et al. 1994).

Materials and methods

Generation of chimeric genes

The chimeras were made by combining a *vn* cDNA lacking the EGF motif with fragments encoding the EGF motif of *vn*, *spi*, or *aos*. To generate the cDNA lacking the EGF motif, a *vn* type 1 cDNA corresponding to nucleotides 1679–3980 was used as a template to amplify 5' and 3' regions extending to 10 amino acids before the first cysteine and from 10 amino acids after the sixth cysteine, respectively. For the 5' fragment, the primers 5'-ATTAACCTCACTAAAG-3' (this corresponds to the T3 region in pBS, the vector in which the *vn* cDNA is inserted) and 5'-ACCCGGGAAAGTGAAGTGGTGAGGCCTTG-3', which incorporates an *Xma*I site at the 3' end, were used. For the 3' fragment the primers 5'-GACTAGTGTTCGAATCTACGGCCAAATAC-3', which incorporates the *Spe*I site at the 3' end, and 5'-AATACGACTCACTATAG-3' (T7 region in pBS) were used. Clones encoding the EGF motif from *vn*, *spi*, and *aos* were also generated by PCR amplification and incorporated 5' *Xma*I and 3' *Spe*I sites for insertion into the *vn* cDNA lacking the EGF motif. The primers to amplify the *vn* EGF motif from a *vn* cDNA were 5'-ACCCGGGGCCACGACCGGTGAGCCTCG-3' and 5'-GACTAGTAAATATCTACTGTCTGGGCC-3'. The primers used to amplify the *spi* EGF domain from genomic DNA were 5'-ACCCGGGAGGCCCCAATATTACATTCCTCC-3' and 5'-GACTAGTCAGGTAAGTATTGTCTGATCTC-3'. The primers used to amplify the *aos* EGF domain from an *aos* cDNA (kindly provided by K. Sawamoto, University of Tokyo, Japan) were 5'-ACCCGGGGACAGTCCGGGCTACAGATATC-3' and 5'-GACTAGTTATCACGCCGATTGCGTGTG-3'. All reactions contained 100 ng of template, 10 pmoles of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 2 mM dNTPs, and 5 units of *Taq* DNA polymerase (GIBCO BRL). Cycling conditions were 1 cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. For in vitro studies, cDNAs encoding native Vn and the Vn chimeras were cloned into the *Xho*I and *Bam*HI sites of pMK33 (Schnepf et al. 1996). For in vivo studies, the genes were cloned into the *Xho*I and *Xba*I sites of the P-element vector pUAST (Brand and Perrimon 1993).

In vitro assays of DER signaling

Stable *Drosophila* S2 cell lines were established that express and secrete the Vn and the Vn chimeric proteins (Vn, Vn:Vn EGF, Vn:Spi EGF, and Vn:Aos EGF) (Schnepf et al. 1996). Receptor phosphorylation assays were performed according to Schweitzer et al. (1995b): Medium collected from stably transfected S2 cells expressing native Vn, native sSpi, or the Vn chimeric proteins was added to a monolayer of S2 cells expressing DER. After a 5-min incubation, DER was immunoprecipitated from the cells and samples were run on duplicate SDS-polyacrylamide gels and blotted. One blot was probed with anti-phosphotyrosine antibodies (kindly provided by M. Coggeshall, The Ohio State University) to show the level of DER tyrosine phosphorylation, and one blot was probed with anti-DER antibodies to show that the amount of protein loaded in each lane was similar. The relative amounts of Vn and the Vn chimeras were determined using an anti-Vn antibody (Schnepf et al. 1996). For the dose-response experiment, Vn-conditioned serum-free medium was concentrated 60× by centrifugation through a Millipore Biomax 10K column. The concentrate was subsequently diluted over an eightfold range with 0.125 increments. ERK assays were performed according to Gabay et al. (1997): Conditioned medium was added to S2-DER cells for 14 min. The cells were lysed, and samples were run on duplicate gels and blotted. One blot was probed with anti-diphosphorylated ERK (Sigma M8159) to show the level of ERK phosphorylation, and one blot was probed with anti-ERK (Sigma M-5670) to show that the amount of protein loaded in each lane was similar. To show the ligand-independent inhibition by Vn:Aos EGF, S2-DER cells were incubated with Vn:Aos EGF-conditioned medium for 14 min. Controls were incubated with medium from untransfected S2 cells.

Transgenic cell lines

Multiple transgenic lines were established for each construct: 8 lines of

UAS-vn; 6 lines of *UAS-vn:vn EGF*; 17 lines of *UAS-vn:vn spi EGF*; and 12 lines of *UAS-vn:aos EGF*. Single transgenic lines of *UAS-aos* (kindly provided by R. Howes and M. Freeman, MRC, Cambridge, UK) and *UAS-spi* were examined.

Ectopic expression in the embryo

To examine the effect of ectopic expression of the factors in the embryo the *UAS* transgenes were expressed with the *Kr-gal4* driver (ectopic expression in segments T2–A4) and *otd* expression was monitored. *otd* expression serves as a marker for ventral cell fate. Embryos were collected for 2 hr from a cross between *Kr-gal4/TM3* flies and flies with the native or chimeric EGF-like genes (*UAS-vn*, *UAS-vn:vn EGF*, *UAS-spi*, or *UAS-vn:spi EGF*). The embryos were aged for 5.5 hr at 25°C (about stage 11) and hybridized with a digoxigenin-labeled *otd* antisense RNA probe. In situ hybridization to young embryos (1–3 hr) with a *vn* probe was used to screen for lines in which *vn* and the *vn* chimeras were ectopically expressed in the *Kr* pattern at similar levels. Lines expressing comparable levels of *vn* and *vn:spi EGF* were found. The line with the lowest expression of *vn:vn EGF* was used, but it had a slightly higher expression level than *vn* and *vn:spi EGF*.

Ectopic expression in the wing

To analyze the effect of ectopic expression of the factors in the wing the *UAS* transgenes were expressed with the *1348-gal4* or *69B-gal4* drivers, and the venation pattern was examined (25°C). When ectopically expressed in pupal intervein regions (*1348-gal4*) *UAS-vn* produced a mild (4 lines) or moderate (4 lines, Fig. 3E) extra-vein phenotype; *UAS-vn:vn EGF* produced a mild (2 lines), moderate (2 lines, Fig. 3F), or strong (2 lines) extra-vein phenotype; *UAS-spi* produced a strong extra-vein phenotype (1 line, Fig. 3G); and *UAS-vn:spi EGF* produced a moderate (6 lines) or strong (11 lines, Fig. 3H) extra-vein phenotype. We used the *69B-gal4* driver, which causes ectopic expression in the larval discs, to test the effect of ectopic expression of *UAS-aos* and the *UAS-vn:aos EGF* chimera in the wing because these transgenes did not produce a phenotype when ectopically expressed with the *1348-gal4* driver. When ectopically expressed in the wing *UAS-aos* (1 line that shows strong expression, Fig. 3C) and *UAS-vn:aos EGF* (12 lines that showed similar phenotypes, shown in Fig. 3D) caused loss of veins. Most *69B-gal4*; *UAS-vn* animals die, but a few adults survived and these had a strong extra-vein phenotype (Fig. 3B).

Ectopic expression in the eye

To analyze the effect of ectopic expression of the factors in the eye the transgenes were expressed with the *GMR-gal4* (17°C) or *69B-gal4* (25°C) drivers, and the adult eyes were examined. Eye defects were visible with the light microscope for flies expressing *UAS-vn:vn EGF* (6 lines tested, 1 line showed a weak phenotype), *UAS-vn:spi EGF* (8 lines tested), and *UAS-spi* (1 line tested). No defects were seen following expression of *UAS-vn* with *GMR-gal4* (2 lines tested) or *69B-gal4* (8 lines tested). We used the *69B-gal4* driver to determine the effect of ectopic expression of *UAS-aos* (1 line) and *UAS-vn:aos EGF* (12 lines) because the latter did not produce a phenotype when ectopically expressed with *GMR-gal4*. A rough phenotype was seen in all lines tested. For SEM analysis of eyes by scanning electron microscopy, one representative line was examined for each construct. Fly heads were dehydrated through an ethanol series (25%, 50%, 75%, 2× 100%), followed by 50% hexamethyldisilazane (HMDS) in ethanol and 2× 100% HMDS for 2 hr each. The heads were air-dried overnight, sputter coated with gold/palladium, and analyzed with a Phillips XL30 scanning electron microscope at 20.0 kV.

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EGF domain swap converts a *Drosophila* EGF receptor activator into an inhibitor

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