DOI: 10.1534/genetics.103.019588

Regulation of the Drosophila Epidermal Growth Factor-Ligand Vein Is Mediated by Multiple Domains

Timothy Donaldson,[1] Shu-Huei Wang, Thomas L. Jacobsen, Bruce Schnepp,[2] Jessica Price and Amanda Simcox[3]

Department of Molecular Genetics, Ohio State University, Columbus, Ohio 43210

Manuscript received July 2, 2003
Accepted for publication December 31, 2003

ABSTRACT

Vein (Vn), a ligand for the Drosophila epidermal growth factor receptor (Egfr), has a complex structure including a PEST, Ig, and EGF domain. We analyzed the structure-function relationships of Vn by assaying deletion mutants. The results show that each conserved domain influences Vn activity. A PEST deletion increases Vn potency and genetic evidence suggests that Vn is regulated by proteasomal degradation. The Ig deletion causes toxic effects not seen following expression of native Vn, but the Ig domain is not required for Vn localization or for the activation of Egfr signaling in wing vein patterning. Remarkably, when the EGF domain is deleted, Vn functions as a dominant negative ligand, implying that Vn normally physically interacts with another factor to promote its activity. We identified additional highly conserved sequences and found several regions that affect Vn potency and one that may mediate the effect of dominant negative Vn molecules. Together the results show that the activity of Vn is controlled both positively and negatively, demonstrating the existence of additional levels at which Egfr signaling can be regulated.

INTERCELLULAR communication is fundamental to the development of multicellular organisms and facilitated by a number of signaling systems. The ErbB receptor family has four vertebrate members, the epidermal growth factor receptor (Egfr)/ErbB1, ErbB2/neu, ErbB3, and ErbB4, which play key roles in cell communication by acting as receptors for epidermal growth factor (EGF)-like signals including EGF, transforming growth factor (TGF)-α, the neuregulins, and others (Olayioye *et al.* 2000; Falls 2003; Harris *et al.* 2003). Drosophila has a single member of the ErbB family, Drosophila Egfr, and its activity is modulated by five ligands. The TGF-α-like molecules, Gurken (Grk), Spitz (Spi), and Keren, and the neuregulin-like molecule Vein (Vn) function as receptor activators (Rutledge *et al.* 1992; Neuman-Silberberg and Schüpbach 1993; Schnepp *et al.* 1996; Reich and Shilo 2002; Urban *et al.* 2002). The fifth Egfr ligand, Argos (Aos), is a receptor antagonist (Freeman *et al.* 1992; Schweitzer *et al.* 1995a). ErbB receptors regulate many different cellular processes such as proliferation, cell survival, cell migration, and differentiation. Not surprisingly, aberrant activity of the receptors or their signaling components leads to a number of pathological outcomes (Olayioye *et al.* 2000; Holbro *et al.* 2003).

Signaling through ErbB receptors is initiated when ligands bind to the extracellular domain, which relieves autoinhibition and exposes a dimerization loop within the receptor (Garrett *et al.* 2002; Ogiso *et al.* 2002; Ferguson *et al.* 2003). Thus the activity of a ligand and the regulation of its production and presentation are key to signaling control as they precede all other events in the pathway.

The activity of a ligand is determined in part by the sequence of the EGF motif, which is required for receptor binding and *in vitro* is sufficient for activation. For example, the vertebrate neuregulin-1 gene (*NRG*-1) encodes isoforms that differ in the EGF-like domain such that the β-form is 10–100 times more potent than the α-form (Lu *et al.* 1995; Falls 2003). In Drosophila, the Spi EGF motif is a stronger activator of Egfr than the Vn EGF motif (Schnepp *et al.* 1998). EGF motifs are composed of six conserved cysteine residues, which form three disulfide bonds to generate a three-looped structure (the A, B, and C loops), as well as a few other highly conserved residues. Whereas the overall sequences of the EGF motifs of Spi and Vn are ~40% conserved, the Aos EGF motif is significantly different. Notably, the Aos B loop (the region between cysteines 3 and 4) is 20 amino acids long, compared to 10–12 amino acids in the activating ligands. Biochemical studies showed that Aos competes with agonists and prevents receptor dimerization (Jin *et al.* 2000). Although there is currently only one known homolog of Drosophila Aos, also in an insect, *Musca*

[1]*Present address:* Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599.

[2]*Present address:* Center for Gene Therapy, Columbus Children's Hospital, Columbus, OH 43205.

[3]*Corresponding author:* Department of Molecular Genetics, Ohio State University, 484 W. 12th Ave., Columbus, OH 43210. E-mail: simcox.1@osu.edu

domestica (Howes *et al.* 1998), understanding how Aos functions is of considerable interest and may lead to the development of vertebrate Egfr inhibitors that could have therapeutic use in human disease.

Howes *et al.* (1998) investigated structure-function relationships of the ligands Spi and Aos by creating a set of chimeras between the two proteins. They found that swapping the EGF domains of these proteins eliminated their function; neither the Spi EGF domain in Aos nor the Aos EGF domain in Spi had activating or inhibiting properties. In contrast, a chimeric molecule in which the Aos EGF domain was swapped with that of Vn (Vn::Aos-EGF) resulted in the conversion of the activator Vn into an inhibitor (Schnepp *et al.* 1998). The simplest interpretation of this result is that the Aos EGF motif is sufficient for receptor inhibition. However, here we report surprisingly that a Vn molecule completely lacking an EGF domain is also an inhibitor. Thus both molecules function as dominant negative ligands, suggesting that the Aos EGF domain may not play a significant role in the inhibitory properties of Vn::Aos-EGF. The creation of such dominant negative EGF-like ligands has implications for both normal regulation of ligand activity and development of therapeutic inhibitors.

In addition to the intrinsic properties of a given EGF motif, ligand activity is regulated by transcriptional and post-translational mechanisms. Feedback loops involving transcriptional regulation of the ligand genes *vn* and *aos* have been discovered in Drosophila (Golembo *et al.* 1996b; 1999; Wasserman and Freeman 1998; Wessells *et al.* 1999). These function to spatially refine signaling and ensure robustness (Casci and Freeman 1999; Shilo 2003). Post-translational processing of ligands is also important in Drosophila Egfr signaling, where proteolytic cleavage activates Spi and the other TGF-α-like ligands. The trafficking and cleavage of the ligands is mediated by the membrane proteins Star and Rhomboid (Rho; Bang and Kintner 2000; Lee *et al.* 2001; Urban *et al.* 2001, 2002; Ghiglione *et al.* 2002; Reich and Shilo 2002; Tsruya *et al.* 2002). Star is required for transporting the membrane-tethered ligands from the endoplasmic reticulum to the Golgi where they are cleaved by the intramembrane serine protease Rho and then secreted by the normal route.

Unlike the TGF-α agonists, Vn is made as a secreted molecule and is not dependent on proteolytic activation. *vn* is expressed in a spatially restricted pattern (Schnepp *et al.* 1996; Simcox *et al.* 1996); however, it is clear that transcription of *vn per se* does not always lead to an active ligand. Ectopic expression of *vn* in the early wing disc, where it acts as the key activator of Egfr signaling, does not mimic the transformations induced by ectopic expression of a consitutively active receptor (Wang *et al.* 2000; Zecca and Struhl 2002). This implies other factors are required to promote or inhibit Vn activity. Understanding the structure-function relationships of Vn will give insight into possible domains through which these factors may act.

Structurally, Vn resembles the vertebrate neuregulins because it possesses an Ig domain in addition to the EGF motif. The neuregulins exist in multiple different isoforms and those containing an Ig domain are essential for viability (Meyer and Birchmeier 1995; Kramer *et al.* 1996). Here we sought to understand the roles of the EGF, Ig, and other key domains in Vn. The results suggest Vn interacts with multiple factors that control its activity both positively and negatively, thus providing additional levels at which Egfr signaling can be regulated.

MATERIALS AND METHODS

Sequencing of *vein* EMS alleles: The *vein* alleles (allele name/synonym) sequenced were: *L6, WA178, ddd-2/RD310, ddd3/RG436, ddd-7/UH5, ddd-10/VK97, ddd-11/VU288, ddd-12/VW100,* and *ddd-13/WB240.* Mutant larvae were either homozygous for a given allele or *trans*-heterozygous with *Df(3L)vn-γ3.* Genomic sequences were obtained by PCR amplification using Platinum Taq polymerase (Invitrogen, Carlsbad, CA). Exon 1 of *vn* was amplified as a 1.68-kb PCR product. Exons 2, 3, 4, and 5, comprising the remaining 523 bp of the *vn* coding sequence, were amplified as a contiguous 1.76-kb PCR product that included 1.24 kb of intronic sequence. Three clones for each allele, as well as *vn* sequences from w^{1118} and *mwh red e* controls, were sequenced. Primers used are listed in supplemental material at http://www.genetics.org/supplemental/.

Cloning *D. virilis vn:* Reverse transcription was performed by standard procedures using a RETROscript (Ambion, Austin, TX) kit and 2 μg total *D. virilis* RNA as a template and oligo(dT) as the primer. Degenerate primers were used to amplify *vn* sequences and a FirstChoice RLM-rapid amplification of cDNA ends (RACE) kit (Amicon, Beverly, MA) was used to complete the 3′ end of the gene. Primers used are listed in supplemental material at http://www.genetics.org/supplemental/.

Generation of Vn transgenes: *Vn::Aos/Spi-EGF chimeras:* Recombinant PCR was employed using Vn::Aos-EGF or Vn::Spi-EGF in pBS (Schnepp *et al.* 1998) as templates to take advantage of the *Xma*I and *Spe*I sites flanking the EGF motif. The inside primers (1–8; see supplemental material at http://www.genetics.org/supplemental/) corresponded to sequences within the EGF motif flanking the junctions between the A, B, or C loops and contained both Aos and Spi sequence; the outside primers corresponded to sequences in the Vn backbone or pBS T7.

*Vn:*Δ*EGF:* pBS-Vn1 was used as a template for two PCR reactions, one using primers pBS T3 and DEGF-R and the other using pBS T7 and DEGF-F. The products, corresponding to residues 1–564 and 599–622, respectively, were cut with *Xma*I, ligated, and cloned into the *Eco*RI site of pBS.

*Vn:*Δ*Ig:* pBS-Vn1 was used as a template for PCR with primers pBS T7 and DIg-F. The PCR product, corresponding to residues 522–622, was cut with *Bgl*II/*Not*I and used to replace the corresponding fragment in pBS-Vn1.

*Vn:*Δ$MR^{93\text{-}213}$*:* The orientation of the Vn1 cDNA in pBS was reversed and the resulting construct (named pBS-$Vn1^R$) was used as a template for PCR with primers T7 and MR3-R. The PCR product, corresponding to residues 1–92, was cut with *Eag*I and used to replace the corresponding fragment in pBS-$Vn1^R$.

*Vn:*Δ$MR^{177\text{-}395}$*:* pBS-Vn1 was cut with *Sph*I, purified to remove

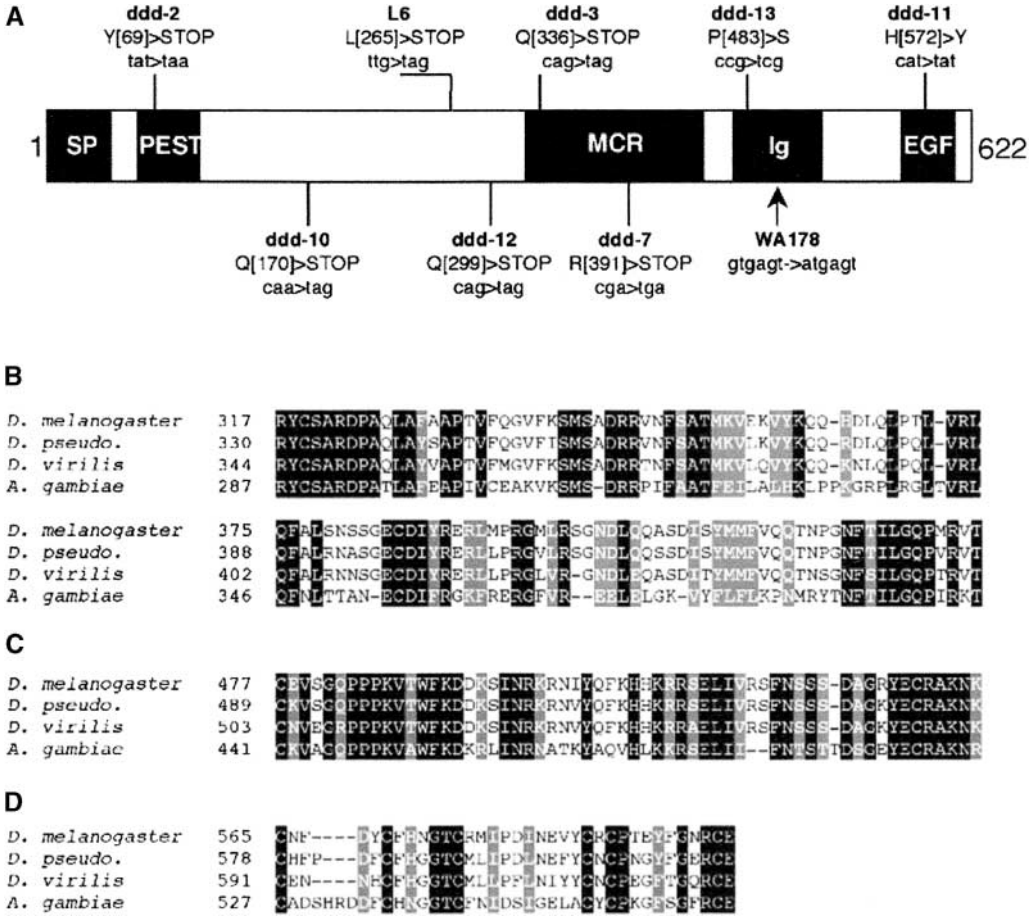

FIGURE 1.—The Vn protein. (A) Structure of the Vn protein showing conserved domains and mutations associated with various alleles. (B–D) Alignment of Vn protein showing conserved regions from three Drosophilids and the mosquito (*A. gambiae*); (B) novel conserved region (MCR); (C) Ig domain; (D) EGF domain. Similar (light shading) and identical (dark shading) amino acids are indicated.

an internal *Sph*I fragment corresponding to residues 177–395, and religated.

*Vn:*Δ*MR*$^{395\text{-}476}$*:* pBS-Vn1 was used as a template for PCR with the primers pBS T7 and MR2-F. The PCR product, corresponding to residues 477–621, was cut with *Sac*II and used to replace the corresponding fragment in pBS-Vn1.

*Vn:*Δ*PEST:* pBS-Vn1 was used as a template for PCR, using primers pBS T3 and DP-R. The product, corresponding to residues 1–41, was cut with *Afl*II/*Xho*I and used to replace the corresponding fragment in pBS-Vn1.

Constructs were excised from pBS and inserted into the transformation vector pUAST. Transgenic stocks were generated by standard techniques and multiple transgenic lines for each construct were examined (see below). Primer sequences are listed in supplemental material at http://www.genetics.org/supplemental/.

Drosophila stocks and cultures: All crosses were performed at 25°, unless otherwise noted. All Gal4 lines (*71B, 69B, bs-1348, en, Kr*, and *ptc*) and the *DTS5* and *DTS7* proteasome subunit alleles were obtained from the Bloomington Stock Center. To account for differences in expression due to position effects, we analyzed at least five independent transgenic lines for each construct, except for the Aos/Spi chimeras, for which a minimum of two lines were examined (Table 1). In only two cases (Vn:ΔIg and Vn:ΔMR$^{93\text{-}213}$) did an individual line exhibit a phenotype that was somewhat weaker than the others in that group. The stronger lines were used unless otherwise indicated.

Expression analysis: Embryos were prepared and processed for *in situ* hybridization using standard procedures (TAUTZ and PFEIFFLE 1989) and mounted in Aquapolymount (Polysciences, Warrington, PA) for analysis by brightfield microscopy. Immunostaining of embryos was performed using standard procedures (PATEL 1994). A rat polyclonal to Vn (kindly provided by T. Volk; YARNITZKY *et al.* 1997) was diluted 1:200 and Cy3-conjugated goat-anti-rat secondary (Jackson) was diluted 1:500. Embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and analyzed with a Bio-Rad (Richmond, CA) MRC 1024 confocal laser microscope.

Bromodeoxyuridine labeling: Larvae were dissected in Schneider cell medium and inverted anterior ends were incubated in Schneider cell medium containing 50 μg/ml bromodeoxyuridine (BrdU; Roche Molecular Biochemicals, Indianapolis) for 30 min to label proliferating cells. BrdU detection was as previously described (HARTENSTEIN and POSAKONY 1989) with mouse anti-BrdU (Becton-Dickinson) used 1:20 and a goat-anti-mouse HRP-conjugated secondary (Jackson) used 1:300. Tissues were mounted in Aquapolymount for analysis by brightfield microscopy.

RESULTS

Temperature-sensitive mutations map to the Ig and EGF domain: Nine EMS-induced *vn* alleles were sequenced (Figure 1A). The six nonsense mutations map to exon 1 and thus either produce truncated proteins or would be subject to nonsense-mediated mRNA decay. The *WA178* allele contains a change at the first position of the intron at the exon 2/intron 2 splice junction, a position that is normally invariant (MOUNT *et al.* 1992). If splicing at this site does not occur, the readthrough

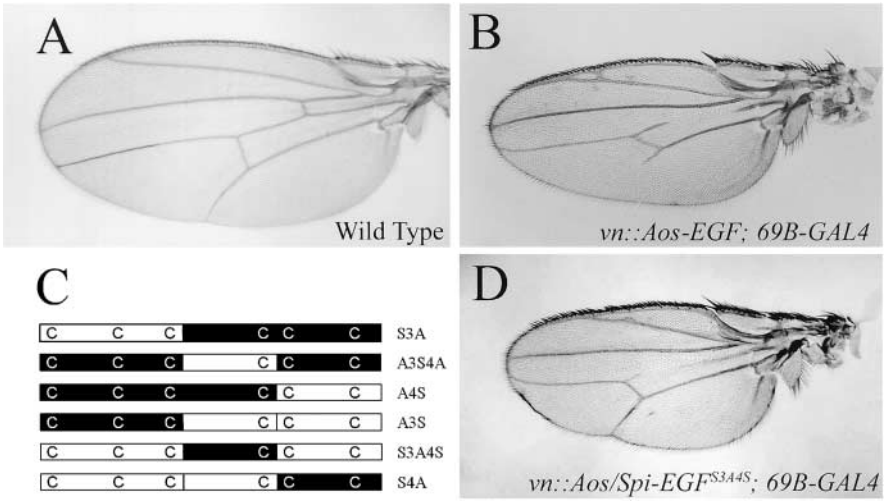

Figure 2.—Vn molecules with chimeric EGF domains function as inhibitors. (A) A wild-type wing showing the normal pattern of wing veins. (B) Expression of *UAS-vn::Aos-EGF* with *69B-GAL4* inhibits Egfr activity and causes vein loss. (C) Cartoon showing chimeric EGF domains composed of all combinations of the A, B, and C loops of Aos (solid) and Spi (open). Each chimeric EGF domain was inserted in place of the Vn EGF domain and the resulting constructs were tested for effects on Egfr activity (Table 1). (D) Expression of *UAS-vn::Aos/Spi-EGF^{S3A4S}* with *69B-GAL4* causes vein loss (29°, two copies of the transgene).

product would terminate at a premature STOP codon in intron 2. The two missense mutants, *ddd-13* and *ddd-11*, each contain a single amino acid change, in the Ig domain and the EGF domain, respectively. Both *ddd-13* and *ddd-11* are temperature-sensitive mutations, suggesting that these two regions are key determinants of Vn structure and function. This has been confirmed by examining deletion mutants as described below.

Comparison with *Anopheles gambiae* and other Drosophila species reveals additional highly conserved regions: To determine if there are conserved regions in addition to the Ig and EGF domains, we compared the Vn sequences from other Drosophilids and a mosquito. The genomes of *A. gambiae* and *Drosophila pseudoobscura* were recently sequenced (Holt *et al.* 2002; Human Genome Sequencing Center, Baylor College of Medicine, http://hgsc.bcm.tmc.edu/projects/drosophila/). The *A. gambiae vn* cDNA sequence was further confirmed by reverse transcriptase (RT)-PCR (data not shown). We also cloned and sequenced the *D. virilis vn* gene using degenerate RT-PCR and RACE (see materials and methods).

The structure of the *vn* gene is conserved between the Drosophilids and *A. gambiae*, with an exon coding for most of the protein followed by one large intron and several small exons that encode the Ig and EGF domains (data not shown). Interestingly, the EGF domain in each *vn* gene is divided by an intron located between the fourth and fifth cysteines. Stein and Staros (2000) report that genes for vertebrate ErbB ligands contain a splice site in the same position. The placement of this intron appears to be unique to ErbB ligands and is not generally seen in other EGF-domain-containing proteins (Stein and Staros 2000), suggesting that the insect and vertebrate genes share a common ancestry.

The overall identity of the *D. melanogaster* Vn protein is 70% with *D. pseudoobscura*, 58% with *D. virilis*, and 26% with *A. gambiae*. The sequences of the Ig (Figure 1C) and EGF (Figure 1D) domains from these species are strongly conserved. The proline triplet at the beginning of the Ig domain that is affected in the conditional *ddd-13* mutant is conserved between all four proteins. There is also a high degree of similarity in the region just N terminal to the Ig domain, which we term the mosquito-conserved region (MCR; Figure 1B). The N-terminal portion of the gene has lower conservation (data not shown). As expected for a region with strong evolutionary conservation, deletions removing parts of the MCR impair the function of Vn (see below).

Vn::Aos/Spi-EGF chimeras function as inhibitors: A chimeric molecule composed of the Aos EGF domain inserted into the Vn backbone (Vn::Aos-EGF) inhibited Egfr signaling (Schnepp *et al.* 1998; Figure 2B). In an attempt to define the region within the Aos EGF motif conferring this property, we created a set of chimeric Vn molecules with EGF motifs corresponding to all possible combinations of the A, B, and C loops from the Spi and Aos EGF motifs (Vn::Aos/Spi-EGF chimeras, Figure 2C). We chose to test chimeras between the Spi and Aos EGF motifs rather than between the Vn and Aos EGF motifs because it has been shown that Spi is a stronger activator than Vn (Schnepp *et al.* 1998; Golembo *et al.* 1999), and thus the difference in activity of the chimeric EGF motifs would be more apparent.

We tested the activity of each chimera using the Gal4-UAS system (Brand and Perrimon 1993). *UAS-transgenes* encoding the chimeras were misexpressed in the wing and their ability to produce vein loss, characteristic of Egfr inhibitors, or ectopic veins, characteristic of Egfr activators, was assessed. Surprisingly, every one of the chimeras functioned as an inhibitor (Table 1). Each EGF motif chimera had approximately the same activity (one shown in Figure 2D), which was also similar to that of Vn::Aos-EGF (Figure 2B), except A3S4A (Aos A and C loops with the Spi B loop, Table 1) that had weaker activity. We suspect that this chimera acts as a weak inhibitor due to a nonspecific defect rather than to an effect of the Spi B loop because the two other chimeras that include this region (A3S and S4A) are potent inhibitors.

TABLE 1

Vn::Aos/Spi-EGF chimeras function as inhibitors of Egfr signaling

UAS-transgene[a]	No. lines tested (60–130 wings scored/line)	% missing anterior crossvein (range)[b]
lac-Z (control)	1	0
Vn::S3A	2	90 (85–94)
Vn::A3S4A	3	5 (0–9)
Vn::A4S	4	85 (79–98)
Vn::A3S	2	72 (62–82)
Vn::S3A4S	5	81 (62–95)
Vn::S4A	4	83 (74–92)
Vn::Aos-EGF	3	92 (87–99)
Vn::Spi-EGF	3	Lethal[c]

[a] *ptc-GAL4* flies were crossed to each of the transgenic lines. *ptc-GAL4/UAS-x* female flies were examined for the presence of the anterior crossvein. The chimeric EGF domains are shown in Figure 2.

[b] The anterior crossvein is sensitive to Egfr signaling and lost when signaling is inhibited.

[c] This construct is a receptor activator and causes extra veins when expressed with *1348-GAL4*.

Vn:ΔEGF functions as an Egfr inhibitor: Finding that all Vn::Aos/Spi-EGF chimeras tested functioned as inhibitors suggested that the sequence of the Aos EGF motif was not critically important for mediating the inhibitory effect of Vn::Aos-EGF and that the mechanism of inhibition was distinct from that of native Aos. One explanation could be that all chimeras possess nonfunctional EGF domains that cannot bind Egfr but interfere with signaling in a dominant negative fashion by affecting a pathway component other than the receptor. Therefore we tested whether an EGF domain was required for inhibition by creating a form of Vn lacking the EGF domain (Vn:ΔEGF). If the inhibition occurred through a dominant negative mechanism not involving receptor binding, then Vn:ΔEGF would also be expected to function as an inhibitor. Indeed this was found to be the case.

Misexpression of Vn:ΔEGF with *69B-GAL4* produced a vein-loss phenotype in the wing similar, although somewhat milder, to that produced by misexpression of Vn::Aos-EGF and the Vn::Aos/Spi-EGF inhibitors (Figure 3A). These phenotypes also closely resemble that of a hypomorphic mutation of *vn* (Puro 1982; Figure 3B), further suggesting that the transgenes are functioning as inhibitors through a dominant negative mechanism, possibly by interfering with the activity of endogenous Vn. In this model, Vn activity would be compromised because the inhibitors compete with Vn for a factor required to promote ligand-receptor interaction.

One prediction of this model is that in addition to being able to prevent endogenous Vn from activating Egfr, Vn:ΔEGF and Vn::Aos-EGF (hereafter referred to collectively as DN-Vn ligands) would also be able to inhibit misexpressed, native Vn. To test this, wild-type Vn and DN-Vn transgenes were coexpressed using *69B-GAL4* and the resulting wing phenotypes were analyzed. We found that expression of Vn alone caused a moderate extra-vein phenotype (Figure 3C) and this phenotype was suppressed by coexpression of DN-Vn (Figure 3D). (Coexpression with an unrelated UAS-transgene had no effect, confirming that the suppression was not due to a dilution of GAL4.)

Vn:ΔIg has normal activity in wing vein patterning but is toxic in early development: Misexpression of Vn:ΔIg in pupal interveins (with *1348-GAL4*) produced an extra-vein phenotype similar to that caused by misexpression of native Vn (Figure 4, A and B). This indicates that the Ig domain is not required for Vn-mediated receptor activation and that without an Ig domain, Vn has a similar ability to activate the receptor. (Vn:ΔIg also had similar activity as native Vn in a tissue-culture assay; see supplemental Figure 1 at http://www.genetics.org/supplemental/.) In the neuregulins, the Ig domain is required for anchoring to the extracellular matrix (ECM; Loeb and Fischbach 1995; Loeb *et al.* 1999; Li and Loeb 2001), but this does not appear to be the case for Vn as Vn:ΔIg is concentrated at the surface of cells in which it is expressed (Figure 4C).

However, the Ig domain clearly has a function because the effect of Vn:ΔIg differed from wild-type Vn when misexpressed earlier in development. Misexpression of Vn:ΔIg resulted in complete lethality with *71B-GAL4*, whereas misexpression of wild-type Vn only slightly affected viability. Expression of wild-type Vn with *69B-GAL4* Vn induced partial lethality but this occurred at the pupal stage, whereas misexpression of Vn:ΔIg was embryonic lethal (data not shown). We found one transgenic line of Vn:ΔIg that produced adults with *69B-GAL4*, presumably because the insertion site of the transgene supports only relatively low expression levels. These exhibited a moderate extra-vein phenotype (Figure 4F) similar to that caused by ectopic expression of native Vn, but this Vn:ΔIg line also consistently produced serrated wing margins, which were only rarely seen following Vn misexpression, demonstrating an abnormal function for the Ig deletion mutant.

Finally, misexpression of Vn:ΔIg with *T80-GAL4* resulted in bloated larvae (Figure 4D). The brains and imaginal discs in these larvae were smaller than those of wild type and showed reduced levels of BrdU incorporation (Figure 4E), indicating a defect in cell proliferation in these tissues. Misexpression of native Vn caused mild larval bloating but did not result in either of these disc or brain phenotypes. Together these data show that the Ig domain is not required for Vn to function as an activator, but nevertheless indicate a role for the Ig domain in modulating Vn activity because expression of Vn:ΔIg is toxic early in development.

The dominant negative effect of DN-Vn is not mediated by the Ig domain: The Ig domain is a known protein-protein interaction domain and therefore a candi-

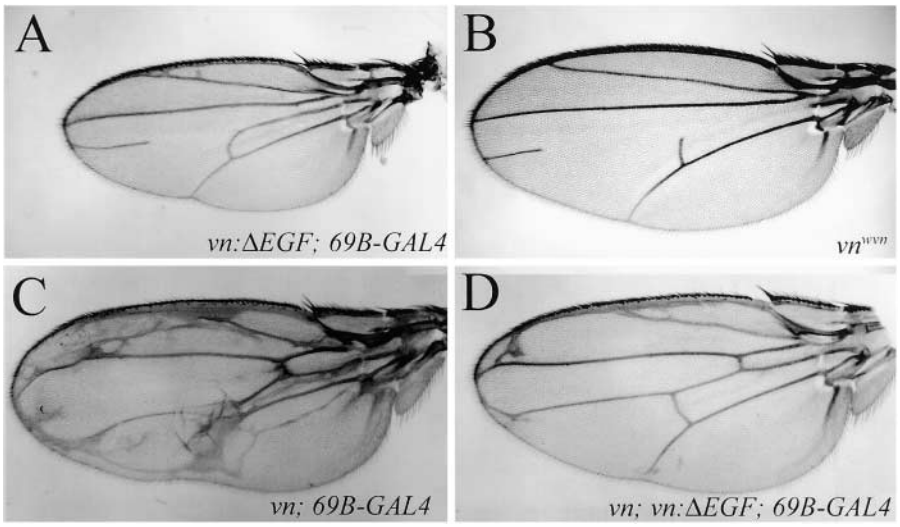

FIGURE 3.—Vn:ΔEGF functions as a dominant negative inhibitor of Vn/Egfr signaling. (A) Misexpression of *UAS-vn:ΔEGF* with *69B-GAL4* results in partial loss of vein L4 (29°), a phenotype that resembles the hypomorphic vn^{wvn} mutation (B). (C) Misexpression of *UAS-vn* in the wing results in an extra-vein phenotype. (D) Coexpression of *UAS-Vn:ΔEGF* suppresses the extra-vein phenotype.

date region for mediating the effect of the DN-Vn ligands such that the phenotypes caused by these ligands may result from sequestering a positive factor normally bound by the Ig domain of Vn. To test this, we generated flies expressing DN-Vn constructs in which the Ig domain was deleted (Vn::Aos-EGF-ΔIg and Vn:ΔEGF-ΔIg). These proteins were robustly expressed and localized in the ECM (not shown) but had no detectable effect on Egfr signaling. This suggests that either the Ig domain is required for the function of the DN-Vn inhibitors or the proteins are inactive due to a nonspecific effect. To distinguish between these possibilities, we tested for genetic interactions between DN-Vn and Vn:ΔIg. If the DN-Vn ligands were functioning through a mechanism involving the Ig domain, then DN-Vn would not be expected to alter the phenotype of Vn:ΔIg, as this molecule would necessarily function independently of the Ig domain.

We analyzed the phenotypes resulting from coexpression of Vn:ΔIg and DN-Vn (with *69B-GAL4*). We found that the wing phenotypes caused by expression of the weaker Vn:ΔIg line were rescued by coexpression of DN-Vn (Figure 4G). (This phenotype was also rescued by coexpression with UAS-Aos, which demonstrates that Vn:ΔIg is having its effects through the Egfr pathway Figure 4, H and I.) Furthermore, while expression of most Vn:ΔIg lines alone resulted in embryonic lethality (see above), we found that coexpression of DN-Vn rescued this lethality and produced viable adult progeny that exhibited a mild extra-vein phenotype (not shown). Thus DN-Vn ligands are effective suppressors of Vn molecules lacking an Ig domain, suggesting that some other region mediates the dominant negative effect. We suggest that this region is part of a highly conserved sequence (MCR) in the middle portion of Vn (see below).

Vn:ΔPEST is a stronger agonist than native Vn: PEST domains serve as signals for proteolytic degradation of proteins (RECHSTEINER and ROGERS 1996). Thus, if the PEST domain of Vn is functional, its removal would be expected to generate a more stable protein. Misexpression of Vn:ΔPEST with *69B-GAL4* and *71B-GAL4* caused lethality whereas misexpression with *1348-GAL4* resulted in a strong extra-vein phenotype (Figure 5B). This phenotype was more severe than that resulting from misexpression of wild-type Vn (Figure 5A), indicating that Vn:ΔPEST has enhanced signaling capacity, possibly through an increase in protein stability. Misexpression of Vn:ΔPEST resulted in a high level of Vn expression in embryos and the protein appeared to be more widely distributed and not limited primarily to the surface of cells (Figure 5C). This distribution could be a result of Vn persisting in the cells.

PEST domains are involved in targeting proteins to the 26S proteasome. In Drosophila, two mutants, *DTS5* and *DTS7*, affect the β6 and β2 proteasome subunit genes, respectively (MYKLES 1999). *DTS5* and *DTS7* heterozygous flies develop normally at the permissive temperature (25°), but die in the pupal stage when grown at the restrictive temperature (29°). Shifting to the restrictive temperature for 48 hr during the third larval instar allows the flies to survive to adulthood and these exhibit a mild extra-vein phenotype (Figure 5, D and E; both alleles have vein spurs around L5, not shown). The *UAS-*$Vn^{1.1}$ line also has a mild constitutive extra-vein phenotype caused by leaky expression of the transgene (Figure 5F). In combination with the *DTS5* and *DTS7* mutations and following a shift to the restrictive temperature, this extra-vein phenotype is dramatically enhanced (Figure 5, G and H), suggesting that impairing proteasome function enhances Vn activity, possibly by reducing Vn degradation.

Amino acids 177–476 of Vn are required for full activity whereas amino acids 93–177 negatively regulate Vn activity: The Vn middle region (amino acids 93–476) lacks sequences with homology to known functional

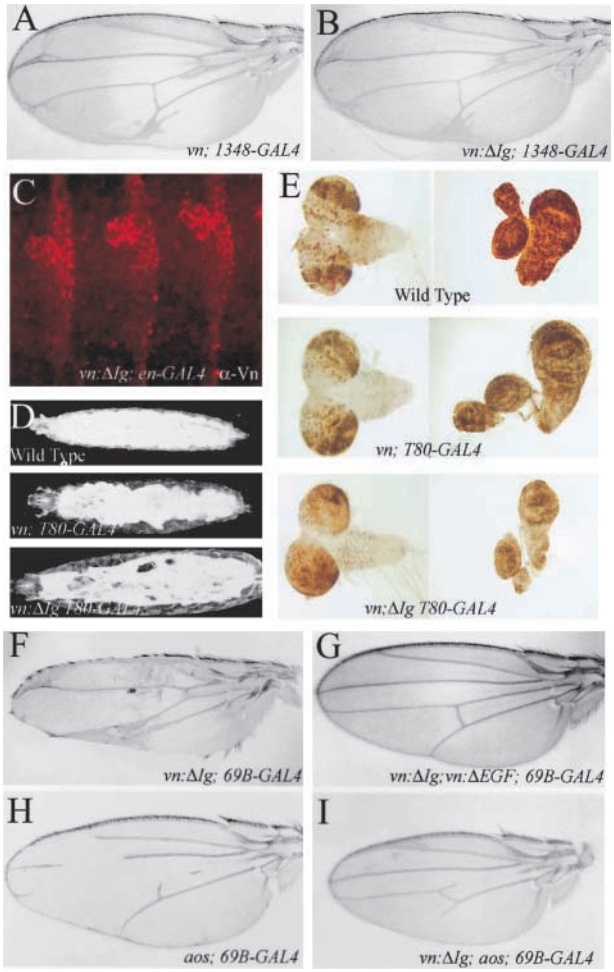

FIGURE 4.—Phenotypic effects of expression of Vn:ΔIg. (A and B) Similar extra-vein phenotypes result from expression of *UAS-vn* (A) or *UAS-vn:ΔIg* (B) in pupal intervein regions with *1348-GAL4*. (C) The Ig domain is not required for the localization of the Vn protein. *UAS-vn:ΔIg* was expressed in embryos using *en-GAL4*. Staining with a Vn antibody shows that the Vn:ΔIg protein accumulates around the periphery of cells in the *en* stripes. Anterior is to the left. (D) Expression of *UAS-vn:ΔIg* with *T80-GAL4* causes bloated larvae (bottom), whereas expression of Vn causes only a slight effect (middle) compared to wild type (top). (E) Vn:ΔIg causes a reduction in cell proliferation in imaginal tissues. (Top) Pattern of BrdU incorporation in wild-type brain (left) and wing, haltere, and third leg discs (right). Expression of *UAS-vn:ΔIg* causes a reduction of BrdU incorporation (bottom), whereas expression of *UAS-vn* does not (middle). Crosses in D and E were performed at 17°. (F and G) The effect of DN-Vn is not mediated through the Ig domain. Expression of *UAS-vn:ΔIg* in the wing (using a weakly expressed transgene because most are lethal; see text) causes severe notching of the wing margin (compare with Figure 3C) and an extra-vein phenotype (F). These phenotypes are rescued by coexpression of *UAS-vn:ΔEGF* (G). (H and I) The effect of Vn:ΔIg is mediated through the EGFR pathway. Expression of *UAS-aos* in the wing results in a vein-loss phenotype (H) and can rescue the extra-vein phenotype caused by *UAS-vn:ΔIg* expression (I).

domains, although stretches of the region are highly conserved with the mosquito (Figure 1B). Deletion mutants spanning the middle region show that this region is indeed required for Vn function and that some parts promote activity while another negatively regulates Vn activity.

Vn:ΔMR$^{93\text{-}213}$, which removes amino acids 93–213, functioned as a superstrong activator. Misexpression of Vn:ΔMR$^{93\text{-}213}$ with *69B-GAL4* and *71B-GAL4* caused embryonic and early pupal lethality, respectively. One line of Vn:ΔMR$^{93\text{-}213}$ appeared to be weaker than the others tested (presumably due to a position effect of the transgene insertion site). In this line, expression with *71B-GAL4* primarily caused lethality in late pupal/pharate adult stage but a few escapers survived that exhibited a strong extra-vein phenotype (Figure 6C). With expression induced by *1348-GAL4*, most of the Vn:ΔMR$^{93\text{-}213}$ lines produced a strong extra-vein phenotype and wing blisters (Figure 6B). These phenotypes are much stronger than those seen following misexpression of native Vn (Figures 4A and 6A). In contrast, the mutants Vn:ΔMR$^{177\text{-}395}$ and Vn:ΔMR$^{395\text{-}476}$, which remove amino acid residues 177–395 and 395–476, respectively, both functioned as weak activators when compared to native Vn, producing mild extra-vein phenotypes with a strong driver (*69B-GAL4*; Figure 6, E and G). Together these results suggest that amino acid residues 93–213 of the "middle region" negatively regulate Vn activity whereas the remainder of the middle region is required for full Vn activity.

Amino acids 213–395 may mediate the dominant negative effect of DN-Vn ligands: The DN-Vn ligands are presumed to function by competing with Vn for binding to a factor that promotes Vn/Egfr interaction. The region responsible for binding is therefore expected to have a positive effect on Vn activity. Two middle region deletion mutants, Vn:ΔMR$^{177\text{-}395}$ and Vn:ΔMR$^{395\text{-}476}$, reduced the activity of Vn and are thus candidates for this region. To test this possibility, we determined whether coexpression of DN-Vn could suppress the extra-vein phenotype induced by expression of these deletion mutants (we also tested Vn:ΔMR$^{93\text{-}213}$, although this was considered a less likely candidate as this deletion enhanced Vn activity). We analyzed the wing vein phenotypes resulting from coexpression of DN-Vn with each middle region deletion.

69B-GAL4; Vn:ΔMR$^{93\text{-}213}$ flies died as embryos and we observed no rescue of this lethal phase by coexpression of DN-Vn (not shown). However, when we coexpressed DN-Vn and the Vn:ΔMR$^{93\text{-}213}$ transgene that had the weakest effect with *71B-GAL4*, we found that more flies survived to adulthood (compared to Vn:ΔMR$^{93\text{-}213}$ expression alone) and that the extra-vein phenotype of these flies was reduced (Figure 6, C and D).

Coexpression of DN-Vn with *69B-GAL4* was also able to suppress the extra-vein phenotypes resulting from overexpression of Vn:ΔMR$^{395\text{-}476}$ (Figure 6H). Expression of Vn:ΔMR$^{395\text{-}476}$ alone resulted in deltas at the distal tips of L3 and L4 as well as a thickening of the distal portion of L2. Coexpression of DN-Vn eliminated these extra veins.

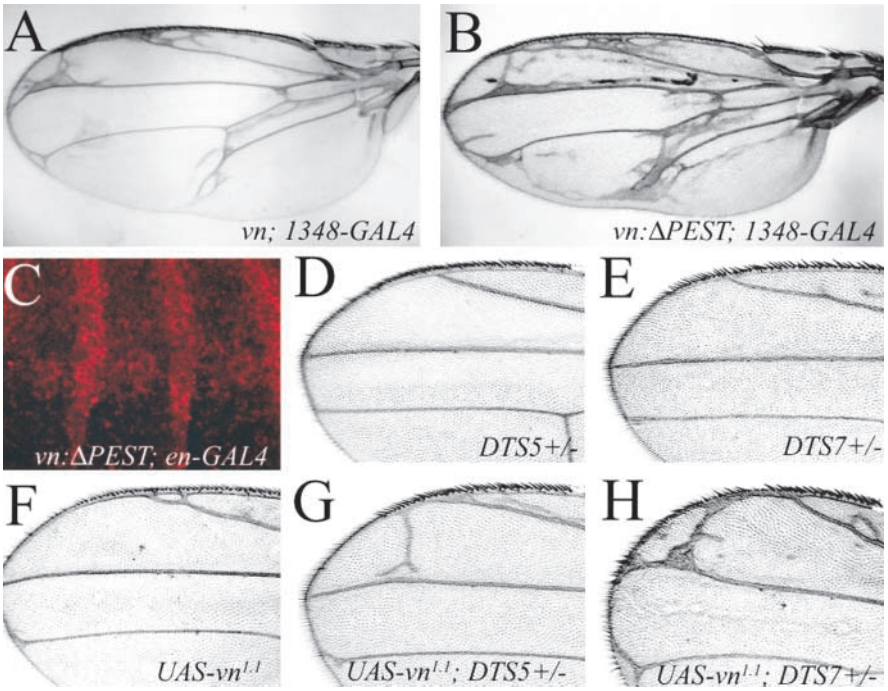

Figure 5.—Function of the PEST domain. (A–C) Deletion of the PEST domain enhances Vn activity. Expression of *UAS-vn:ΔPEST* (B) produces a stronger extra-vein phenotype than that of *UAS-vn* (A) does and results in increased intracellular Vn accumulation in the embryo (C; compare to Figure 4C). (D–H) The proteasome may play a role in Vn/Egfr signaling. Temperature-sensitive mutations in proteasome subunits β6 (D) and β2 (E) cause mild extra-vein phenotypes. The β6 (G) and β2 (H) mutations enhance the extra-vein phenotype of a leaky *UAS-vn* line (F). Animals in D–H were shifted to 29° for 48 hr during third larval instar.

In contrast, there was no suppression of the extra-vein phenotype resulting from coexpression of Vn:ΔMR$^{177\text{-}395}$ and DN-Vn (Figure 6F). The deltas and thickenings observed at the distal portions of the lateral veins caused by misexpresson of Vn:ΔMR$^{177\text{-}395}$ were unaffected by coexpression of DN-Vn. This result implicates the sequences between residues 213 and 395 in mediating the effect of the DN-Vn ligands and as the region in native Vn that is required for binding a factor that promotes Vn/Egfr interaction. This falls within a region we found to be highly conserved with the mosquito (MCR, Figure 1B).

Deletion mutants with enhanced activity in the wing do not influence embryonic cell fate: We found that each of the deletion mutants could at least partially rescue the wing disc phenotype of *vn* mutants (see supplemental Figure 2 at http://www.genetics.org/supplemental/). However, the observation that several of the Vn deletion mutants tested (Vn:ΔIg, Vn:ΔPEST, and Vn:ΔMR$^{93\text{-}213}$) differed from wild-type Vn in that they caused embryonic lethality suggested the possibility that these mutations were transforming Vn into a stronger agonist, more similar to Spi. Spi is more potent than Vn when misexpressed in embryos and results in an expansion of ventral cell fates, which can be monitored by examining the expression of *orthodenticle* (*otd*; Figure 7, A–C; Wieschaus *et al.* 1992; Schweitzer *et al.* 1995b; Golembo *et al.* 1996a; 1999; Schnepp *et al.* 1998). Misexpression of Vn:ΔIg, Vn:ΔPEST, or Vn:ΔMR$^{93\text{-}213}$ with *Kr-GAL4* caused no expansion of *otd* expression (Figure 7, D–F). This indicates that although two of these mutant forms, Vn:ΔPEST and Vn:ΔMR$^{93\text{-}213}$, have enhanced capacity to induce ectopic veins and that all three cause lethality when expressed in the embryo, they do not resemble Spi in their ability to induce ectopic ventral cell fates.

DISCUSSION

Mechanisms that govern production and presentation of an active ligand form the most fundamental levels of signaling control, presaging all other events in the pathway. Ligand activity can be controlled by both transcriptional and/or post-translational regulation. Transcriptional regulation is important for *vn*, which, unlike the other zygotically active ligands *spi* and *keren*, is expressed in a highly localized and dynamic pattern (Rutledge *et al.* 1992; Schnepp *et al.* 1996; Simcox *et al.* 1996; Yarnitzky *et al.* 1997; Golembo *et al.* 1999; Wessells *et al.* 1999; Reich and Shilo 2002). Vn is made as a soluble protein and thus does not require processing like the membrane spanning TGF-α ligands. However, its sequence predicts that Vn is a complex molecule and here we have shown that the activity of Vn is indeed regulated through multiple functional domains that mediate both negative and positive effects on Vn activity. The results reveal additional levels of complexity through which Egfr signaling is controlled and we discuss the potential conservation of these mechanisms.

Deletion of the Vn EGF domain creates an inhibitor: Remarkably, when the EGF domain is deleted, Vn becomes an inhibitor. The activity of this mutant molecule is similar to that of a chimeric ligand, Vn::Aos-EGF (Schnepp *et al.* 1998), that includes the EGF domain from Aos, the natural Egfr antagonist. We had previously ascribed the inhibitory function of Vn::Aos-EGF to pos-

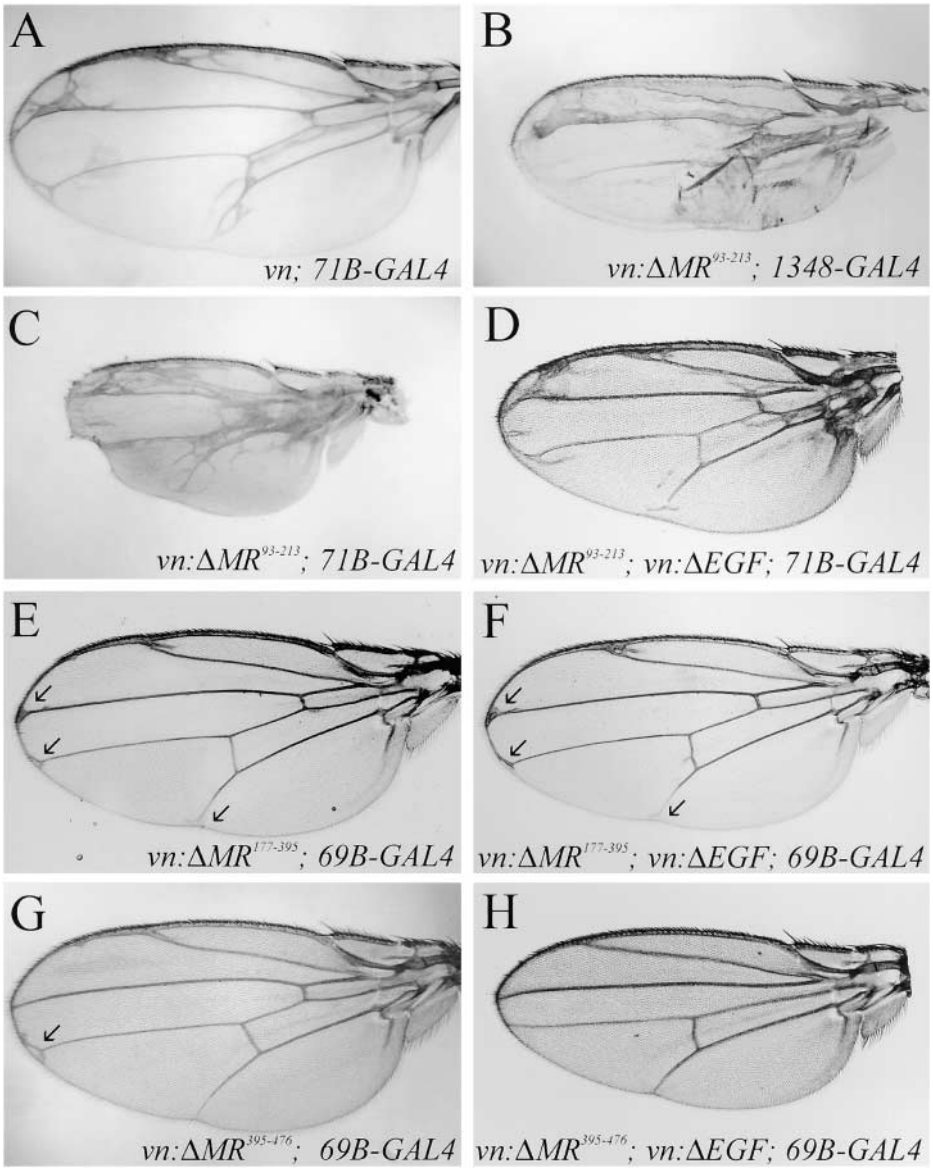

Figure 6.—The central region of the Vn protein contains sequences that both positively and negatively regulate Vn activity. (A–C) Amino acids 93–213 negatively regulate Vn activity. Compared to expression of *UAS-vn* with *71B-GAL4*, which causes only a slight extra-vein phenotype (A), expression of the weakest *UAS-vn:*Δ*MR*$^{93\text{-}213}$ (stronger lines were lethal; see text) results in a smaller wing with a strong extra-vein phenotype and notching (C). Expression of *UAS-vn:*Δ*MR*$^{93\text{-}213}$ in pupal interveins causes a strong extra-vein phenotype and blistering (B; compare to Figure 4A). (D) Amino acids 93–213 do not mediate the dominant negative effect of DN-Vn. Coexpression of *UAS-vn:*Δ*MR*$^{93\text{-}213}$ and *UAS-vn:*Δ*EGF* partially rescues the extra-vein phenotype of *UAS-vn:*Δ*MR*$^{93\text{-}213}$ expression. (E and G) Amino acids 177–476 are required for full Vn activity. Compared to expression of *UAS-vn* (Figure 3C), *UAS-vn:*Δ*MR*$^{177\text{-}395}$ (E) or *UAS-vn:*Δ*MR*$^{395\text{-}476}$ (G) with *69B-GAL4* causes very weak extra-vein phenotypes (arrows). (F and H) Amino acids 213–395 may mediate the dominant negative effect of the DN-Vn ligands. Coexpression of *UAS-vn:*Δ*EGF* rescues the extra-vein phenotype of *UAS-vn:*Δ*MR*$^{395\text{-}476}$ (H), but not *UAS-vn:*Δ*MR*$^{177\text{-}395}$ (F, arrows), suggesting that the latter region, which includes part of the MCR, is important for mediating the effect of DN-Vn.

session of the Aos sequence but in light of the findings described here, it is likely that both Vn::Aos-EGF and Vn:ΔEGF function by a dominant negative mechanism. This also allows us to reconcile the difference in the activities of Vn::Aos-EGF and Spi::Aos-EGF chimeras (Howes *et al.* 1998; Schnepp *et al.* 1998); both share the Aos EGF domain, but only the Vn::Aos-EGF chimera is able to function as an inhibitor of Egfr signaling through a dominant negative mechanism involving a critical domain found only in the Vn "backbone."

Several possible models could explain how these DN-Vn mutants are able to inhibit Egfr signaling. In the simplest model, Vn/Egfr signaling could involve dimerization of Vn. A dimer formed between Vn and DN-Vn would likely be inactive. Expression of DN-Vn would thus reduce the number of active Vn-Vn dimers and result in inhibition of Vn/Egfr signaling. However, the recent structure of Egfr in complex with its ligands excludes the possibility that a Vn dimer is part of the receptor-ligand complex because the two ligands are expected to be ~70–80 Å apart on opposite sides of the complex (Garrett *et al.* 2002; Ogiso *et al.* 2002). However, this does not rule out the possibility that Vn-Vn interactions have a role in subsequent multimerization of receptor dimers to form, for example, tetramers. In an alternative model, Vn/Egfr activation could depend on an interaction between Vn and another factor. In this case, overexpression of DN-Vn would compete for binding with this factor and abrogate Vn-mediated receptor activation.

Both models predict that there must be a region in Vn that mediates the effect of the inhibitors by competing for binding to this factor. The normal role for this region is therefore to potentiate Vn function and hence deletion of the region should lower Vn activity. Furthermore, the model predicts that a molecule lacking the key region would not be influenced by the DN-Vn li-

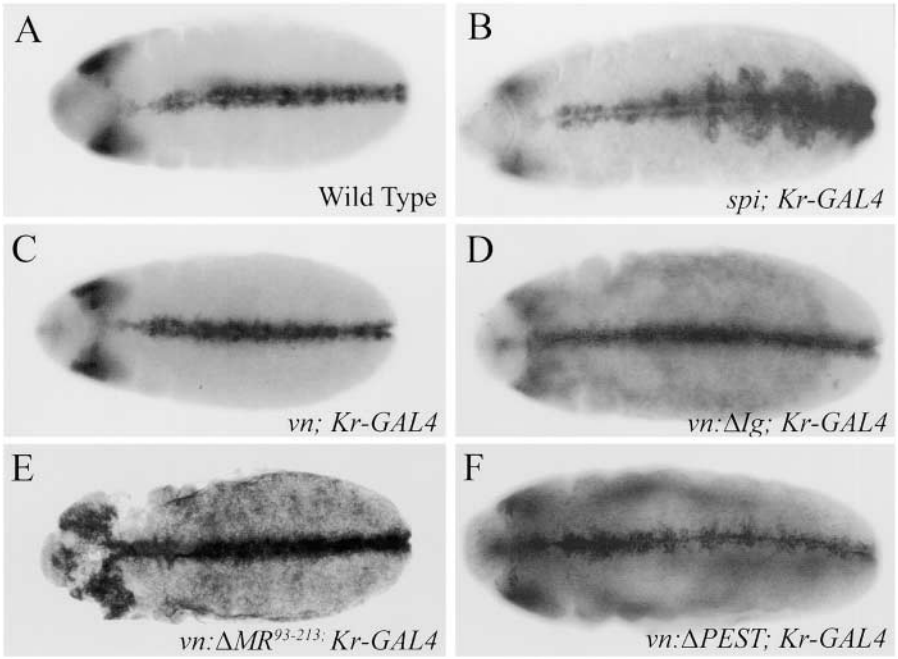

Figure 7.—Ectopic expression of native Vn or the deletion mutants does not affect ventral cell fate determination. (A–F) Expression of *UAS-vn* (C), *UAS-vn:ΔIg* (D), *UAS-vn:ΔMR*$^{93\text{-}213}$ (E), or *UAS-vn:ΔPEST* (F) with *Kr-GAL4* does not alter the pattern of *otd* expression compared to wild type (A), indicating that these factors are not like *UAS-sSpi* (B) in their ability to affect ventral cell fate determination.

gands. In our analysis of Vn deletion mutants we found two adjacent regions (MR$^{177\text{-}395}$ and MR$^{395\text{-}476}$) that reduced Vn function in an ectopic expression assay. While both deletions remove blocks of conserved sequences (MCR), only Vn:ΔMR$^{395\text{-}476}$ was able to be suppressed by DN-Vn. This suggests that residues 213–395 are important for mediating the dominant negative effect. It will be important to map the required region in more detail to determine if the MCR performs this role, but our data indicate that the C-terminal portion of the MCR (which is relatively less conserved) is not required.

One question that arises from this work is whether an inhibitory vertebrate ligand can be created. van de Poll *et al.* (1997) and Lohmeyer *et al.* (1997) generated EGF molecules with extended B loops in attempts to mimic Argos function. None of these factors had inhibitory properties. While we believe the inhibitory nature of Vn::Aos-EGF is primarily mediated through a dominant negative mechanism independent of the Aos sequence, we did note that the Vn::Aos-EGF chimera was a more potent inhibitor than Vn:ΔEGF because stronger induction of the transgene (elevating the rearing temperature to increase the activity of Gal4 and hence *UAS-transgene* expression) was required to produce an equivalent phenotype. This suggests that some intrinsic property of the Aos EGF domain also has an effect. But basing the design of an inhibitor on the Aos EGF region is unlikely to be a successful approach, given the results with vertebrate ligands and the lack of activity of Spi::Aos-EGF. Instead it may be efficacious to investigate vertebrate ligands that rely on binding with other factors to potentiate interaction with the receptor.

Vn activity may be regulated by protein degradation: Attenuation of signaling can be dependent on ligand destruction and structure-function analysis suggests that Vn may be regulated by degradation. Deletion of two regions in the N-terminal part of Vn produced mutant proteins with increased ability to activate Egfr as judged by their ability to produce ectopic veins. One of these regions (amino acids 58–96) is strongly predicted to contain a PEST sequence (Figure 1) by the PestFind algorithm (Rechsteiner and Rogers 1996; http://www.at.embnet.org/embnet/tools/bio/PESTFIND).

Our observation that the removal of the PEST domain of Vn results in a more potent activator suggests that Vn is subject to regulation by protein degradation. This would be a novel mechanism for regulation of an EGF ligand. In support of this idea we found a genetic interaction between a *vn* transgene and mutants for proteasome subunits. Analyzing this connection further not only will be important for understanding Vn regulation, but also may have broader implications, as PEST domains have been reported in two other EGF ligands, Gurken and Lin-3 (Hill and Sternberg 1992; Neuman-Silberberg and Schüpbach 1993), and can also be detected in the neuregulins (S.-H. Wang and A. Simcox, unpublished observations). Therefore, any such degradation mechanism may be conserved and involve multiple ligands.

Different roles for the Ig domain in Vn and the vertebrate neuregulins: Of the four vertebrate neuregulin (*NRG*) genes, both *NRG*-1 and *NRG*-2 are alternatively spliced to produce isoforms that possess an Ig domain (Falls 2003). The Ig domain in *NRG*-1 binds to heparin sulfate proteoglycans. This maintains a high local concentration of ligand that results in enhanced receptor activation and extends the duration of the response (Li and Loeb 2001). Although Vn resembles the Ig-

containing *NRG* isoforms, we show here that the Ig domain in Vn is unlikely to have a similar role. Deletion of the Ig domain apparently did not diminish the activity of Vn or prevent its association with the ECM. However, small changes in activity and binding may not be detectable in the assays. Instead, the Vn:ΔIg mutant appeared to have additional properties and caused a number of detrimental effects when ectopically expressed that were not observed with native Vn. The evolutionary relationship of the vertebrate and invertebrate ligands is not clear. Certain residues in the EGF domain are characteristic of the neuregulins (Buonanno and Fischbach 2001), but these are not conserved in Vn, suggesting that it may be no more related to the neuregulins than any other Drosophila EGF ligand. Furthermore, the Ig domains of Vn and the neuregulins appear to have at least some distinct functions.

Role of ligand regulation in cell signaling control: The analysis of the Drosophila TGF-α genes has highlighted the importance that processing of the membrane-bound ligand precursors plays in signaling regulation. Here we show that there is also a remarkable potential to regulate activity of the only secreted agonist, Vn. We found that Vn activity was altered by deleting each of three known conserved domains (PEST, Ig, and EGF) and also identified a novel domain that is required for activity. In subsequent analysis it will be important to define the mechanisms that govern these activities and to determine which, if any, are conserved in other animals.

We thank Talila Volk for the Vn antibody and the Bloomington Stock Center for fly lines. This work was supported by grants from the National Science Foundation and United States Army Medical Research and Materiel Command to A.S.

LITERATURE CITED

Bang, A. G., and C. Kintner, 2000 Rhomboid and Star facilitate presentation and processing of the Drosophila TGF-alpha homolog Spitz. Genes Dev. **14:** 177–186.

Brand, A., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Buonanno, A., and G. D. Fischbach, 2001 Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr. Opin. Neurobiol. **11:** 287–296.

Casci, T., and M. Freeman, 1999 Control of EGF receptor signalling: lessons from fruitflies. Cancer Metastasis Rev. **18:** 181–201.

Falls, D. L., 2003 Neuregulins: functions, forms, and signaling strategies. Exp. Cell Res. **284:** 14–30.

Ferguson, K. M., M. B. Berger, J. M. Mendrola, H. S. Cho, D. J. Leahy *et al.*, 2003 EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Mol. Cell **11:** 507–517.

Freeman, M., C. Klämbt, C. S. Goodman and G. M. Rubin, 1992 The *argos* gene encodes a diffusible factor that regulates cell fate in the Drosophila eye. Cell **69:** 963–975.

Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams *et al.*, 2002 Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. Cell **110:** 763–773.

Ghiglione, C., E. A. Bach, Y. Paraiso, K. L. Carraway, III, S. Noselli *et al.*, 2002 Mechanism of activation of the Drosophila EGF receptor by the TGFalpha ligand Gurken during oogenesis. Development **129:** 175–186.

Golembo, M., E. Raz and B.-Z. Shilo, 1996a The Drosophila embryonic midline is the site of Spitz processing, and induces activation of EGF receptor in the ventral ectoderm. Development **122:** 3363–3370.

Golembo, M., R. Schweitzer, M. Freeman and B.-Z. Shilo, 1996b *argos* transcription is induced by the Drosophila EGF receptor pathway to form an inhibitory feedback loop. Development **122:** 223–230.

Golembo, M., T. Yarnitzky, T. Volk and B. Z. Shilo, 1999 Vein expression is induced by the EGF receptor pathway to provide a positive feedback loop in patterning the Drosophila embryonic ventral ectoderm. Genes Dev. **13:** 158–162.

Harris, R. C., E. Chung and R. J. Coffey, 2003 EGF receptor ligands. Exp. Cell Res. **284:** 2–13.

Hartenstein, V., and J. W. Posakony, 1989 Development of adult sensilla on the wing and notum of Drosophila melanogaster. Development **107:** 389–405.

Hill, R. J., and P. W. Sternberg, 1992 The gene lin-3 encodes an inductive signal for vulval development in C. elegans. Nature **358:** 470–476.

Holbro, T., G. Civenni and N. E. Hynes, 2003 The ErbB receptors and their role in cancer progression. Exp. Cell Res. **284:** 99–110.

Holt, R. A., G. M. Subramanian, A. Halpern, G. G. Sutton, R. Charlab *et al.*, 2002 The genome sequence of the malaria mosquito Anopheles gambiae. Science **298:** 129–149.

Howes, R., J. D. Wasserman and M. Freeman, 1998 *In vivo* analysis of Argos structure-function: sequence requirement for inhibition of the Drosophila epidermal growth factor receptor. J. Biol. Chem. **273:** 4275–4281.

Jin, M. H., K. Sawamoto, M. Ito and H. Okano, 2000 The interaction between the Drosophila secreted protein argos and the epidermal growth factor receptor inhibits dimerization of the receptor and binding of secreted spitz to the receptor. Mol. Cell. Biol. **20:** 2098–2107.

Kramer, R., N. Bucay, D. J. Kane, L. E. Martin, J. E. Tarpley *et al.*, 1996 Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc. Natl. Acad. Sci. USA **93:** 4833–4838.

Lee, J. R., S. Urban, C. F. Garvey and M. Freeman, 2001 Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell **107:** 161–171.

Li, Q., and J. A. Loeb, 2001 Neuregulin-heparan-sulfate proteoglycan interactions produce sustained erbB receptor activation required for the induction of acetylcholine receptors in muscle. J. Biol. Chem. **276:** 38068–38075.

Loeb, J. A., and G. D. Fischbach, 1995 ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. J. Cell Biol. **130:** 127–135.

Loeb, J. A., T. S. Khurana, J. T. Robbins, A. G. Yee and G. D. Fischbach, 1999 Expression patterns of transmembrane and released forms of neuregulin during spinal cord and neuromuscular synapse development. Development **126:** 781–791.

Lohmeyer, M., S. Mason and W. J. Gullick, 1997 Characterization of chimeric proteins constructed from human epidermal growth factor (EGF) and the Drosophila EGF-receptor antagonist *argos*. Int. J. Oncol. **10:** 677–682.

Lu, H. S., D. Chang, J. S. Philo, K. Zhang, L. O. Narhi *et al.*, 1995 Studies on the structure and function of glycosylated and nonglycosylated neu differentiation factors. Similarities and differences of the alpha and beta isoforms. J. Biol. Chem. **270:** 4784–4791.

Meyer, D., and C. Birchmeier, 1995 Multiple essential functions of neuregulin in development. Nature **378:** 386–390.

Mount, S. M., C. Burks, G. Hertz, G. D. Stormo, O. White *et al.*, 1992 Splicing signals in Drosophila: intron size, information content, and consensus sequences. Nucleic Acids Res. **20:** 4255–4262.

Mykles, D. L., 1999 Structure and functions of arthropod proteasomes. Mol. Biol. Rep. **26:** 103–111.

Neuman-Silberberg, F., and T. Schüpbach, 1993 The Drosophila gene *gurken* produces a dorsally localized RNA and encodes a TGFα-like protein. Cell **75:** 165–174.

Ogiso, H., R. Ishitani, O. Nureki, S. Fukai, M. Yamanaka *et al.*, 2002 Crystal structure of the complex of human epidermal

growth factor and receptor extracellular domains. Cell **110:** 775–787.

Olayioye, M. A., R. M. Neve, H. A. Lane and N. E. Hynes, 2000 The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. **19:** 3159–3167.

Patel, N. H., 1994 Imaging neuronal subsets and other cell types in whole-mount Drosophila embryos and larvae using antibody probes. Methods Cell Biol. **44:** 445–487.

Puro, J., 1982 New mutants. Dros. Inf. Serv. **58:** 205–208.

Rechsteiner, M., and S. W. Rogers, 1996 PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21:** 267–271.

Reich, A., and B. Z. Shilo, 2002 Keren, a new ligand of the Drosophila epidermal growth factor receptor, undergoes two modes of cleavage. EMBO J. **21:** 4287–4296.

Rutledge, B. J., K. Zhang, E. Bier, Y. N. Jan and N. Perrimon, 1992 The Drosophila *spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. Genes Dev. **6:** 1503–1517.

Schnepp, B., G. Grumbling, T. Donaldson and A. Simcox, 1996 Vein is a novel component in the Drosophila epidermal growth factor receptor pathway with similarity to the neuregulins. Genes Dev. **10:** 2302–2313.

Schnepp, B., T. Donaldson, G. Grumbling, S. Ostrowski, R. Schweitzer *et al.*, 1998 EGF domain swap converts a Drosophila EGF-receptor activator into an inhibitor. Genes Dev. **12:** 908–913.

Schweitzer, R., R. Howes, R. Smith, B.-Z. Shilo and M. Freeman, 1995a Inhibition of Drosophila EGF receptor activation by the secreted protein Argos. Nature **376:** 699–702.

Schweitzer, R., M. Shaharabany, R. Seger and B.-Z. Shilo, 1995b Secreted *spitz* triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. Genes Dev. **9:** 1518–1529.

Shilo, B. Z., 2003 Signaling by the Drosophila epidermal growth factor receptor pathway during development. Exp. Cell Res. **284:** 140–149.

Simcox, A., G. Grumbling, B. Schnepp, C. Bennington-Mathias, E. Hersperger *et al.*, 1996 Molecular, phenotypic, and expression analysis of *vein*, a gene required for growth of the Drosophila wing disc. Dev. Biol. **177:** 475–489.

Stein, R. A., and J. V. Staros, 2000 Evolutionary analysis of the ErbB receptor and ligand families. J. Mol. Evol. **50:** 397–412.

Tautz, D., and C. Pfeiffle, 1989 A nonradioactive *in situ* hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98:** 81–85.

Tsruya, R., A. Schlesinger, A. Reich, L. Gabay, A. Sapir *et al.*, 2002 Intracellular trafficking by Star regulates cleavage of the Drosophila EGF receptor ligand Spitz. Genes Dev. **16:** 222–234.

Urban, S., J. R. Lee and M. Freeman, 2001 Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell **107:** 173–182.

Urban, S., J. R. Lee and M. Freeman, 2002 A family of Rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. EMBO J. **21:** 4277–4286.

van de Poll, M. L. M., M. J. H. van Vugt, A. E. G. Lenferink and E. J. J. van Zoelen, 1997 Insertion of Argos sequences into the B-loop of epidermal growth factor results in a low-affinity ligand with strong agonistic activity. Biochemistry **36:** 7425–7431.

Wang, S. H., A. Simcox and G. Campbell, 2000 Dual role for Drosophila epidermal growth factor receptor signaling in early wing disc development. Genes Dev. **14:** 2271–2276.

Wasserman, J. D., and M. Freeman, 1998 An autoregulatory cascade of EGF receptor signaling patterns the Drosophila egg. Cell **95:** 355–364.

Wessells, R. J., G. Grumbling, T. Donaldson, S. H. Wang and A. Simcox, 1999 Tissue-specific regulation of vein/EGF receptor signaling in Drosophila. Dev. Biol. **216:** 243–259.

Wieschaus, E., N. Perrimon and R. Finkelstein, 1992 orthodenticle activity is required for the development of medial structures in the larval and adult epidermis of Drosophila. Development **115:** 801–811.

Yarnitzky, T., L. Min and T. Volk, 1997 The Drosophila neuregulin homolog Vein mediates inductive interactions between myotubes and their epidermal attachment cells. Genes Dev. **11:** 2691–2700.

Zecca, M., and G. Struhl, 2002 Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling. Development **129:** 1357–1368.

Communicating editor: T. C. Kaufman