TGF-α ligands can substitute for the neuregulin Vein in Drosophila development

Christina L. Austin, Sathiya N. Manivannan and Amanda Simcox*

ABSTRACT
ErbB receptors, including the epidermal growth factor receptor (Egfr), are activated by EGF ligands to govern cell proliferation, survival, migration and differentiation. The different EGF-induced cell responses in development are regulated by deployment of multiple ligands. These inputs, however, engage only a limited number of intracellular pathways and are thought to elicit specific responses by regulating the amplitude or duration of the intracellular signal. The single Drosophila Egfr has four ligands: three of the TGF-α-type and a single neuregulin-like called vein (vn). Here, we used mutant combinations and gene replacement to determine the constraints of ligand specificity in development. Mutant analysis revealed extensive ligand redundancy in embryogenesis and wing development. Surprisingly, we found that the essential role of vn in development could be largely replaced by expression of any TGF-α ligand, including spitz (spi), in the endogenous vn pattern. vn mutants die as white undifferentiated pupae, but the rescued individuals showed global differentiation of adult body parts. Spi is more potent than Vn, and the best morphological rescue occurred when Spi expression was reduced to achieve an intracellular signaling level comparable to that produced by Vn. Our results show that the developmental repertoire of a strong ligand like Spi is flexible and at the appropriate level can emulate the activity of a weak ligand like Vn. These findings align with a model whereby cells respond similarly to an equivalent quantitative level of an intracellular signal generated by two distinct ligands regardless of ligand identity.

KEY WORDS: Vein, Spitz, Egfr, Neuregulin, Wing, Drosophila

INTRODUCTION
Drosophila Egfr is the sole homolog of the vertebrate family that includes Egfr/ErbB1, neu/ErbB2, ErbB3 and ErbB4. Perturbation of the pathways, which have key roles in proliferation and differentiation during development and homeostasis, results in major developmental defects and problems in human health, including heart disease, schizophrenia and cancer [reviewed by, for example, Mei and Xiong (2008); Sanchez-Soria and Camenisch (2010); Yarden and Pines (2012)]. In mammals there are 11 EGF-like ligand genes (Groenen et al., 1994; Yarden, 2001). Drosophila has four EGF ligands, representing two major classes: the TGF-α ligands spitz (spi), gurken (grk) and Keratin (Krn), and the neuregulin-like (NRG) ligand vein (vn) (Rutledge et al., 1992; Neuman-Silberberg and Schüpbach, 1993; Schnepp et al., 1996; Reich and Shilo, 2002; Urban et al., 2002).

Deployment of the four ligands, which operate both in distinct and in overlapping processes, contributes significantly to Drosophila development (Shilo, 2003). grk has a maternal role (Schüpbach, 1987), whereas spi has a major role in zygotic development and mutants die as embryos with ventral defects (Mayer and Nüsslein-Völlhard, 1988). vn mutants have milder ventral defects and die at the pupal stage (Simcox, 1997). spi; vn double mutants have more than an additive phenotype, consistent with redundant ligand function (Schnepp et al., 1996). Further redundancy is suggested by the finding that Krn single mutants are viable and fertile adults, but functions in the eye, gut, ovary and brain are revealed when other ligands are compromised, animal physiology is altered or when Krn is expressed ectopically (Yang and Baker, 2003; McDonald et al., 2006; Brown et al., 2007; Jiang and Edgar, 2012; Rahm et al., 2013). Ectopic Krn expression can restore MAPK signaling in a spi mutant, thereby further demonstrating functional redundancy of the TGF-α ligands (Reich and Shilo, 2002). Some roles, by contrast, are ligand specific; for example, vn has a major role in wing development, whereas spi or Krn are not required (Simcox et al., 1987; Simcox, 1997; McDonald et al., 2006).

Here, we investigated the requirement for ligands by examining mutants lacking combinations of genes, and for ligand specificity by determining the ability of one ligand to replace another. We found that Egfr signaling is fully ligand dependent in embryogenesis and that all zygotic ligands play a role in embryo and wing development. Surprisingly, each of the TGF-α ligands could largely replace the essential role of vn in development when the active ligand was expressed in the vn pattern. Our results support the idea that stimulating the pathway to a particular level, and not the specific ligand bound, is important.

RESULTS AND DISCUSSION
Egfr-mediated embryonic patterning is dependent on function of all three zygotically active ligands
As single mutants, only spi and vn have defects in embryonic ventral patterning, and the double mutant is more severe (Schnepp et al., 1996) (Fig. 1A-C). Here, we found that Krn plays a redundant role with spi in embryonic cuticle patterning. spi; Krn double mutants had a similar phenotype to spi; vn double mutants (Fig. 1C,D). The triple mutant spi; vn; Krn was much more extreme than either double mutant and was indistinguishable from an Egfr null mutant (Fig. 1E,F). Both mutants lack ventral denticle belts and head structures. As expected, zygotic removal of the maternal ligand grk (spi; grk; vn; Krn) had no additional effect (Fig. 1G).

The equivalent phenotypes of mutant embryos lacking the receptor or the ligands suggest that there is no signaling through Egfr in the absence of a ligand (Fig. 1E-G). Ligand-independent signaling has been reported for overexpression of Egfr in Drosophila and in mammalian cells, where the high levels of Egfr are thought to cause spontaneous dimerization and signaling (Schweitzer et al., 1995; Nagy et al., 2010; Endres et al., 2013). Our results suggest that, at the normal physiological level of the receptor in the fly embryo, all signaling is ligand dependent. The

Department of Molecular Genetics, Ohio State University, Columbus, OH 43210, USA.

*Author for correspondence (simcox.1@osu.edu)


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mutant combinations, however, suggest extensive redundancy between the ligands, consistent with roles that are only revealed when multiple ligands are compromised.

**Wing vein patterning requires the function of all three zygotic ligands.**

*vn* has a role in patterning of the wing blade into territories of veins and interveins (Sturtevant and Bier, 1995; Simcox et al., 1996). No role has been found for *spi* or *Krn* in wing patterning (Simcox, 1997; Guichard et al., 1999; Nagaraj et al., 1999; McDonald et al., 2006). Reducing activity of Egfr or the protease Rhomboid (Rho), which is required to process the TGF-α ligands, causes vein loss, and *rho* *vn* double mutants lack all veins (Sturtevant et al., 1993; Sturtevant and Bier, 1995; Guichard et al., 2000; Ghiglione et al., 2002; Urban et al., 2002). This suggests that either or both *spi* and *Krn* could have redundant roles in wing development. We found that eliminating *Krn* function exacerbated the vein-loss phenotypes in *vn* mutants (Fig. 1H-K). Reducing *spi* levels had no observable effect on *vn* phenotypes (Fig. 1L; supplementary material Fig. S1). The most extreme phenotype resulted from reduction of function in all three genes (Fig. 1M), thereby suggesting that all three contribute to wing vein patterning.

**The TGF-α ligands can replace the essential function of *vn* in development.**

The *vn*-GAL4 allele, generated as part of this study, has an amorphic phenotype and mutants die as white pupae with tiny wing disks (Fig. 2D-F; supplementary material Fig. S2). This allele, which is expressed in the endogenous *vn* pattern (supplementary material Fig. S3), allowed us to test whether ectopic activity of the TGF-α ligands rescued *vn* mutants.

Expression of *UAS-vn* with *vn*-Gal4 (*UAS-vn; *vn*Gal4) rescued ~30% of *vn* mutants to adult flies with grossly normal patterning of the wing (Fig. 2G-I). Some extra vein material was observed, consistent with mild Egfr overactivity (Fig. 2I). Expression of *rho* (*UAS-rho; *vn*Gal4) pharate adult, (K) wing disk and (L) wing with fused L3 and L4 veins (arrow) and extra vein material (*). Wing disks were stained for Tsh (green) and Wg (red). Scale bars: 50 μm.

![Fig. 2. Expression of rho rescues developmental abnormalities in vn mutants. rho encodes a protease that cleaves TGF-α ligands into an active form. (A) Wild-type adult fly, (B) wing disk and (C) wing. (D) *vn*Gal4 mutant pupa, (E) leg and wing disk and (F) higher magnification view of wing disk in E. *vn*Gal4 mutants die as pupae and have very small wing disks. (G) *UAS-vn; *vn*Gal4 adult, (H) wing disk and (I) wing with a mild extra-vein phenotype consistent with overactivity of Egfr signaling (*). (J) *UAS-rho; *vn*Gal4 pharate adult, (K) wing disk and (L) wing with fused L3 and L4 veins (arrow) and extra vein material (*). Wing disks were stained for Tsh (green) and Wg (red). Scale bars: 50 μm.](image_url)
Expression of any TGF-α ligand rescues developmental abnormalities in vn mutants. Expression of transgenes encoding the TGF-α ligands in the vn expression pattern rescues differentiation in vn mutant flies, including the body wall and wing. The flies are pharate adults that do not eclose. The wings have fused L3 and L4 veins and extra vein material (arrows). (A) UAS-sspi; Gal80TS/+, vnL6/vnGal4 pharate adult and (B) wing (27°C). (C) UAS-sKrn, vnL6/vn-Gal4 pharate adult and (D) wing (17°C). (E) UAS-sgrk, vnL6/vn-Gal4 pharate adult and (F) wing (17°C). Gal80TS is a repressor of Gal4 and reduces transgene expression (supplementary material Fig. S6).

Fig. 3. Expression of any TGF-α ligand rescues developmental abnormalities in vn mutants. Expression of transgenes encoding the TGF-α ligands in the vn expression pattern rescues differentiation in vn mutant flies, including the body wall and wing. The flies are pharate adults that do not eclose. The wings have fused L3 and L4 veins and extra vein material (arrows). (A) UAS-sspi; Gal80TS/+, vnL6/vnGal4 pharate adult and (B) wing (27°C). (C) UAS-sKrn, vnL6/vn-Gal4 pharate adult and (D) wing (17°C). (E) UAS-sgrk, vnL6/vn-Gal4 pharate adult and (F) wing (17°C). Gal80TS is a repressor of Gal4 and reduces transgene expression (supplementary material Fig. S6).

Rho can cleave all three TGF-α ligands, and to determine whether expression of a particular ligand can replace the function of vn, we tested each ligand individually. Secreted forms were expressed to detail the ability of secreted Spi (sSpi) to rescue mutants. To correlate rescue phenotypes with signaling, we examined in more detail the ability of secreted Spi (sSpi) to rescue vn mutants. Expression of sSpi at varying levels (supplementary material Fig. S6) produced different degrees of rescue in vn mutants (Fig. 4A). Adult head, leg and abdomen development were restored across a range of expression levels tested, suggesting that these body parts are relatively insensitive to the level of Egr signaling (activity (Fig. 4A); supplementary material Figs S4 and S5). The wings had regions of vein fusion and extra-vein material, characteristic of Egr overactivity (Fig. 3).

The level of TGF-α transgene expression had a significant impact on the extent of rescue (supplementary material Fig. S4). To correlate rescue phenotypes with signaling, we examined in more detail the ability of secreted Spi (sSpi) to rescue vn mutants. Expression of sSpi at varying levels (supplementary material Fig. S6) produced different degrees of rescue in vn mutants (Fig. 4A). Adult head, leg and abdomen development were restored across a range of expression levels tested, suggesting that these body parts are relatively insensitive to the level of Egr activity (Fig. 4A; supplementary material Figs S4 and S5). By contrast, a progression of rescue phenotypes was seen, as sSpi expression increased, from animals lacking all wing disk structures, to those with just wings and to optimal cases of animals with wings and a thoracic body wall (Fig. 4A I-III; supplementary material Fig. S7). At the highest Spi levels tested, the animals died as larvae (Fig. 4A IV). The Spi expression level that gave rise to optimal rescue correlated with a signaling output, as measured by MAPK phosphorylation (dpErk), which most closely matched to wild type (WT) (Fig. 4B,C). High levels of sSpi expression produced strongly elevated dpErk levels, which most likely accounted for the early death of these animals.

The intrinsic activity of an EGF ligand correlates with its biological effect; thus, at saturating concentrations, a ligand with high intrinsic activity has a higher maximum biologic effect than a ligand with low intrinsic activity (Wilson et al., 2012). Spi, therefore, appears to have a higher intrinsic activity than Vn, because at higher levels Spi exceeded the required biologic response and was toxic (Fig. 4; supplementary material Fig. S4). Spi also has a higher affinity than Vn (Alvarado et al., 2010). Low affinity might limit the access of Vn to the receptor through negative cooperativity, which proposes that, when the first ligand binds, a conformational change occurs that occludes the second site, rendering it inaccessible only to a high affinity ligand (Macdonald and Pike, 2008; Alvarado et al., 2010). The biological role of a low-affinity ligand like Vn might therefore be limited, because some cell responses require signaling levels that can be evoked only by high levels of a high-affinity ligand (Krall et al., 2011).

The Ig-domain is not required for the essential function of vn in development

Even in cases of the best morphological phenotypes, rescue of vn mutants by spi was incomplete because the flies did not eclose. This could reflect the artificial Gal4-UAS expression system. Alternatively, the Ig-domain found in Vn and the vertebrate NRGs could confer a unique function. In mice, Ig-containing NRG isoforms are essential (Kramer et al., 1996). A form of Vn lacking the Ig-domain (Vn-Dlg) rescued vn mutants to adults, thus demonstrating that Ig containing forms are not essential for viability (supplementary material Fig. S8A). The wings of these flies were abnormal and had notched margins (supplementary material Fig. S8B), a phenotype that was also seen when this transgene was expressed in a wild-type fly (Donaldson et al., 2004). The Ig-domain could mediate the role of Vn in another pathway independent of its role in Egfr signaling. The wing phenotype is reminiscent of Notch or wingless mutants (Cosson et al., 1994; Price et al., 1997; Wang et al., 2000), and the Vn Ig-domain physically
interacts with Hedgehog co-receptors (Özkcan et al., 2013), making any of these pathways candidates for interacting with Vn via the Ig domain.

We found a chimeric ligand, in which the Spi EGF-domain had been swapped with the Vn EGF-domain (Schnepp et al., 1998), rescued a vn mutant. These animals developed to pharate adults with pattern defects consistent with Egfr overactivity (supplementary material Fig. S8C,D). The phenotypes were similar to those seen following rescue by sSpi, showing that linking the Spi EGF-domain with an Ig-domain does not significantly alter its activity relative to the receptor.

Conclusions

In summary, our results show a high degree of functional redundancy among Egfr ligands and, moreover, that low levels of a strong TGF-α ligand like Spi can largely replace the developmental function of a weaker ligand like Vn. This is not reciprocal, however, because the lower biological activity of Vn limits its ability to replace or emulate the activity of Spi (Schnepp et al., 1998; Golembio et al., 1999; Donaldson et al., 2004; McDonald et al., 2006; Jiang and Edgar, 2009). The results support the idea that the appropriate cell response will occur regardless of ligand identity, provided a certain quantitative level of signaling is met. This conclusion is based on signaling resulting from activity of the EGF domain, which all Egfr ligands possess. Other unique domains, such as the Ig-domain found in Vn and the neuregulins, might facilitate engagement of other pathways and hence regulate different targets.

In the first example documenting distinct responses to different growth factors engaging MAPK signaling, the duration of signaling was shown to determine whether cells differentiated or proliferated (Marshall, 1995). The subsequent understanding of pathway architecture, including feedback loops, provides a deeper mechanistic view of how intracellular signaling through shared pathways produces different transcriptional responses (Nakakuki et al., 2010). Determining the amplitude and duration of signaling and specific transcriptional outputs induced by Spi and Vn will help shed light on potential differences between the ligands. These might account for the incomplete rescue of vn mutants by the TGF-α ligands and the patterning defects they induced in the wing. Nevertheless, the substantial rescue resulting from ligand substitution prompts the question of why multiple ligands have evolved in the fly when it seems that, in theory, fewer could suffice. Complex animals like the fly might have evolved a collection of high- and low-affinity ligands that are active in specific patterns and at different levels because this is more parsimonious than deploying a multitude of tissue-specific modulators to fine-tune the actions of fewer ligands.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used: spi^{ts4}, grk^{Hf}, vn^{1}, vn^{6}, Knr^{17}, Knr^{1}, Egfr^{APB} and Egfr^{F24}. The following transgenes were used: vn-Gal4, sd-Gal4, UAS-vmn^{1.1} (Schnepp et al., 1996); UAS-vmnAlp (Donaldon et al., 2004); UAS-rho, UAS-mspi·GFP (Tsruya et al., 2002); UAS-sspi (Schweitzer et al., 1995); UAS-askn (Urban et al., 2002); UAS-sygrk (Queenan et al., 1999); UAS-vn·SpiEGF (Schnepp et al., 1998); UAS-splhB{RNAi} (TRiP 34645 and 28387); UAS-vnAlbRNA (VDRC 50358 and 109437) and tubP-Gal80{Gal4} Stocks with combinations of alleles were generated from single mutants, with the exception of a spi^{ts4}, grk^{Hf}/CyO stock that was a gift from Trudy Schüpbach (Princeton University, NJ, USA). The vn-Gal4 allele was obtained using the method of Sepp and Auld (1999). The vn-lacZ element (P[PZ, ry^{+}] var^{F284}; F80t005059) was replaced with the P[GavR, w^{+}] element in the PG142/FM7 stock (a gift from Norbert Perrimon, Harvard Medical School, Boston, MA, USA). The Gal4 element in the vn-Gal4 allele is in the 5′-UTR, 590 bp upstream of the open reading frame. Knr recombinants were genotyped by PCR, as homozygotes have no phenotype. Primers were used to amplify across the deleted region (8 kb in WT) and gave a band of ~1 kb for Knr{ts} or ~1.2 kb for Knr{F} (F: AGTCGCGCGCCGTAATTCCG, R: TCTCGGCGCTCCT-TGGCGT).

Protein analysis

Immunoblotting and immunohistochemistry were conducted using standard protocols with the following antibodies: anti-Phospho-p44/p42 MAPK (Cell Signaling Technology, #9106; 1:1000), anti-ERK 2 (Santa Cruz Biotechnology, K-23; 1:2000), anti-Tshirt (a gift from Steven Kerridge, Developmental Biology Institute of Marseille, France; 1:500), anti-GFP (Sigma, #G 1544; 1:1000) and anti-Wingless (Developmental Studies Hybridoma Bank, 4D4; 1:166).

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors performed experiments, analyzed data, prepared the figures and wrote the manuscript.

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RESEARCH REPORT


Fig. S1. Reduction of spi expression does not enhance vn mutant wing phenotypes. (A) 69B-Gal4; UAS-spi<sup>dsRNA</sup>. The fly has small eyes indicating effective RNAi. (B) scalloped (sd)-Gal4; UAS-spi<sup>dsRNA</sup> (29°C). The wing is normal. (C) sd-Gal4; UAS-vn<sup>dsRNA</sup> (29°C) and (D) sd-Gal4; UAS-spi<sup>dsRNA</sup>; UAS-vn<sup>dsRNA</sup> (29°C). The wings have similar phenotypes with loss of parts of L4, L2 and the ACV. Two dsRNA lines for spi were tested (TRiP 28387 and 34645) with multiple drivers (29°C) that are expressed in the wing (71B-Gal4, 69B-Gal4, Act5C-Gal4, and sd-Gal4). None resulted in wing defects, but all gave a small eye phenotype (except 71B-Gal4). Two dsRNA lines for vn were tested (VDRC 109437 and 50358) and both gave a vein-loss phenotype with 69B-Gal4 and sd-Gal4. The vein loss with sd-Gal4 was more extreme and is shown here.
Fig. S2. *vn-Gal4* is an amorphic allele of *vn*. (A) Wild-type third instar wing disk (arrow) and leg disk (arrowhead). (B) Wild-type adult. (C) *vn^{L6/RG}* wing disk (arrow) and leg disk (arrowhead). (D) *vn^{L6}/vn-Gal4* wing disk (arrow) and leg disk (arrowhead). In both (C) and (D) the wing disk fails to grow beyond a rudimentary size. *vn^{L6}* and *vn^{RG}* are known molecular nulls (Donaldson et al., 2004) and the similar phenotype associated with the *vn-Gal4* allele shows it functions as a amorph. (E) Terminal *vn^{L6}/vnGal4* animal dissected from pupal case, lacking any wing disk derived structures and without pigmented adult cuticle. Partially everted leg structures are present (arrowheads).
Fig. S3. *vn-Gal4* is expressed in a similar pattern to *vn*. *vn* RNA detected using *in situ* hybridization (*vn* RNA) is expressed in a similar pattern as GFP (*UAS-CD8::GFP; vn-Gal4*) in early (second larval instar, L2) and late (third instar, L3) wing disks.
Fig. S4. Extent of rescue of vn mutants by expression of TGF-α ligands is dependent on expression level. Each UAS-ligand transgene was expressed using the vn-Gal4 allele. The animals were classified according to the extent of rescue using the color-coded key shown in the figure. Only adults and pharate adults (differentiated adults that fail to eclose) were
The classes range from pharate adults lacking any derivatives of the wing disk (wing and body wall) to normal flies that eclosed. Transgene expression was manipulated by temperature and/or co-expression of the Gal80TS inhibitor (Fig. S2). (A) The histogram shows the numbers of animals scored in each phenotypic class. Survival varied by genotype and so different total numbers were examined for each genotype. In optimal conditions, approximately 30% of the expected number of vn mutants survived to adults when rescued by UAS-vn. In optimal conditions, rescue by the TGF-α ligands allowed a fraction of the expected progeny to reach the pharate adult stage, approximately 10% with UAS-sspi, 7% with UAS-sKrn and 5% with UAS-sgrk. (B) The same data are shown as a percentage of total animals scored. The results suggest that expression of UAS-vn was relatively insensitive to dose and showed a similar extent of rescue across the range of conditions tested. In contrast, each of the transgenes encoding TGF-α ligands were sensitive to the level of expression and optimal rescue of wing-disk derived structures occurred within a narrow expression range. A single UAS-transgene was tested for grk and Krn. Five different UAS-vn transgenes were tested and all gave similar results (UAS-vn1.1, UAS-vn1.2, UAS-vn2.8, UAS-vnGFP^{58} and UAS-vnGFP^{12}). Four different UAS-spi transgenes were tested and gave similar results (UAS-sspi on X, UAS-sspi at 49A, UAS-sspiGFP^{M36} and UAS-sspiGFP^{F5N}).
Fig. S5. Expression of the TGF-α ligands in the correct expression domain rescues leg development in vn mutants. Tarsal segments of the second leg and the terminal claw of the indicated genotypes are shown. (A) wild type. (B) UAS-vn; vn^{L6}/vn-Gal4 (17°C). (C) UAS-sspi; Gal80^{TS}; vn^{L6}/vn-Gal4 (27°C). (D) UAS-sKrn, vn^{L6}/vn-Gal4 (17°C). (E) UAS-sgrk, vn^{L6}/vn-Gal4 (17°C). All genotypes have five tarsal segments and claws. The flies shown in (C-E) are pharate adults and have shorter legs because they are not fully extended.
Fig. S6. Production of a range of vn-Gal4-driven transgene expression levels. The level of transgene expression was manipulated by temperature (Gal4 is more active at higher temperature) and/or co-expression of the Gal80<sup>TS</sup> inhibitor and temperature (Gal80<sup>TS</sup> is less active at higher temperature). The figures show expression of UAS-GFP in the indicated genotypes at two different temperatures. (A) vn-Gal4; UAS-GFP at 29°C shows the strongest expression of GFP. Expression is most prominent in the salivary glands (arrow) and the muscles (lattice throughout the body). (B) vn-Gal4; UAS-GFP at 25°C shows expression of GFP in the salivary glands (arrow) and the muscles (lattice throughout the body) although the level of expression is lower. (C) Gal80; vn-Gal4; UAS-GFP at 29°C shows expression of GFP in the salivary gland (arrow). (D) Gal80; vn-Gal4; UAS-GFP at 25°C shows expression of GFP in the salivary gland (arrow). Expression in (D) appears weaker than in (C). All images were taken with the same exposure.
Fig. S7. Partial rescue of vn mutant wing disks. (A) UAS-vn; vn^{L6}/vn-Gal4 (25°C). (B) UAS-sspi; Gal80^{TS}; vn^{L6}/vn-Gal4 (25°C). (C) UAS-sKrn, vn^{L6}/vn-Gal4 (17°C). (D) UAS-sgrk, vn^{L6}/vn-Gal4 (17°C). All the wing disks have a round shape corresponding to the wing region only. These develop into the thorax of pharate adults with wings but no body wall (for example, fly shown in Fig. 5A, II). Wing disks were stained for Tsh (green), Wg (red) and DAPI (DNA, blue).
Fig. S8. An Ig-domain is not essential for rescuing vn mutants. (A)

Expression of vn transgene lacking the Ig-domain (UAS-vnΔIg; vn^{L6}/vn-Gal4) resuces vn mutants to adults. The wing (B) had deletions of margin bristles (arrowheads) and notches (arrow). (C) Expression of transgene encoding a chimera in which the Vn EGF is replaced by the Spi EGF domain (Gal80^{TS}/UAS-vn::spiEGF; vn^{L6}/vn-Gal4) rescues vn mutants to the pharate adult stage. The wing (D) had extra veins, characteristic of Egfr overactivity (arrow).