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Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins

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The activation signal from tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR), is relayed via a highly conserved intracellular pathway involving Ras, Raf, and MAPK. In *Drosophila*, the EGFR and components of the intracellular pathway are broadly expressed, yet receptor activation evokes tissue-specific cell responses. Extracellular events that lead to receptor activation are one mechanism by which signaling is modulated. Here we show molecular and genetic evidence that *Drosophila vein* (*vn*) encodes a candidate EGFR ligand and that *vn* expression is spatially restricted. Consequently, *vn* may promote tissue-specific receptor activation. Unlike two other ligands, Gurken (Grk) and Spitz (Spi), which are transforming growth factor α-like proteins, Vn has both an immunoglobulin-like and an EGF-like domain. This combination of domains mirrors those in the vertebrate neuregulins that bind EGFR relatives.

[*Key Words*: *vein*; *Drosophila*; EGF receptor; EGF; neuregulin]

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The *Drosophila* epidermal growth factor receptor (DER) is a receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) subfamily that currently also includes four vertebrate proteins (EGFR/erbB1, neu/erbB2, erbB3, and erbB4) and *Caenorhabditis elegans* Let-23. These receptors have important roles in development: EGFR mutant mice have epithelial defects that cause a number of phenotypes and early death (Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995); *neu* and *erbB4* mutant embryos die in utero with heart and neural defects (Gassmann et al. 1995; Lee et al. 1995); *let-23* is involved in determining vulval cell fate (Aroian et al. 1990; Hill and Sternberg 1992); and *DER* has multiple roles in *Drosophila* development involving cell survival, proliferation, and differentiation in embryos, imaginal discs, and the ovary (Shilo and Raz 1991; Clifford and Schüpbach 1992; Raz and Shilo 1992; Baumann and Skaer 1993; Raz and Shilo 1993; Xu and Rubin 1993; Clifford and Schüpbach 1994; Kuo et al. 1996). EGFR-class receptors are activated by ligands that have an EGF-like domain, and one known route of intracellular signal transduction from the activated receptors is through the highly conserved Ras/Raf/MAPK pathway, which is also common to a number of other receptor tyrosine kinases (Egan and Weinberg 1993).

The tissue specificity of signaling from EGFRs is achieved in part by spatially restricted extracellular factors such as ligands. For example, in the developing mouse heart, the ligand, neuregulin, limits the functional domain of neu and erbB4 to a subset of the cells that express these receptors (Gassmann et al. 1995; Lee et al. 1995; Meyer and Birchmeier 1995). In *C. elegans* and *Drosophila* there are two striking examples of ligand localization restricting receptor function. In *C. elegans* vulval development, *lin-3*, which encodes the Let-23 ligand, is expressed by the single anchor cell and produces a local source of ligand for activation of the receptor only in the overlying presumptive vulval cells (Hill and Sternberg 1992). *Drosophila* Grk is an oogenesis-specific EGF-like ligand involved in establishing egg polarity (Neuman-Silberberg and Schüpbach 1993; González-Reyes et al. 1995; Roth et al. 1995). *grk* transcripts are localized to the anterior-dorsal region of the oocyte and a local source of Grk is thought to activate DER only in the adjacent follicle cells (Neuman-Silberberg and Schüpbach 1993).

A structure and function analysis of DER suggests it is regulated by multiple ligands (Clifford and Schüpbach 1994), and two candidates in addition to Grk have been identified. These are the EGF-like proteins Spi, which activates DER function, and Argos, which inhibits DER function (Freeman et al. 1992; Rutledge et al. 1992; Neuman-Silberberg and Schüpbach 1993; Schweitzer et al. 1995a,b; Golembo et al. 1996). Spi and Argos are zygotically active in both the embryo and the adult. *spi* mu-

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tants are embryonic lethal and have a complex phenotype, involving cells derived from the ventral ectoderm, which includes a deletion of ventral cuticle (Mayer and Nüsslein-Volhard 1988; Rutledge et al. 1992). *DER*, *rhomboid (rho)*, *Star (S)*, and *pointed (pnt)* have mutant phenotypes similar to *spi* and are collectively called the spitz group (Mayer and Nüsslein-Volhard 1988; Bier et al. 1990; Clifford and Schüpbach 1992; Raz and Shilo 1992; Rutledge et al. 1992; Kim and Crews 1993; Klämbt 1993). *argos* mutants have the opposite phenotype, whereby there is an expansion of the ventral cuticle that is lost in spi-group mutants (Freeman et al. 1992; Golembo et al. 1996). Molecular characterization and genetic interactions of the spi-group suggest these genes are involved in potentiating *DER* signaling (Díaz-Benjumea and García-Bellido 1990; Rutledge et al. 1992; Klämbt 1993; Raz and Shilo 1993; Sturtevant et al. 1993). The biochemical roles of some of the genes are known. Pnt is a target of Rolled/MAPK (Rl) and thus may act at the end of the pathway (Biggs et al. 1994; Brunner et al. 1994a; O'Neill et al. 1994). The biochemical roles of Rho and S are not known, but both are membrane proteins, and may be involved in the processing of Spi into its active form, secreted Spi (Bier et al. 1990; Kolodkin et al. 1994; Schweitzer et al. 1995b). There is direct evidence Spi activates DER and that this activation can be competed by Argos (Schweitzer et al. 1995a). Thus, these two proteins act as ligands to modulate DER activation. DER is broadly expressed in embryos, including cells that are not affected in mutants, likewise, its ligand, Spi, is also broadly expressed (Schejter et al. 1986; Kammermeyer and Wadsworth 1987; Zak et al. 1990; Katzen et al. 1991; Rutledge et al. 1992; Sturtevant et al. 1994). Tissue-specific Spi/DER activation may be mediated by spatially localized cofactors encoded by other spi-group genes such as *S* and *rho* (Schweitzer et al. 1995b).

Here we show molecular and genetic data that strongly link *vn* to the DER pathway and suggest it encodes a novel ligand for DER. *vn* was discovered as a mutant with abnormal wing venation but additional alleles, originally called *defective dorsal discs* (*ddd*) suggest the gene has a more extensive role in wing development affecting the global proliferation of wing disc cells (Shearn et al. 1971; Puro 1982; Wurst et al. 1984; Simcox et al. 1987). We show *vn* also functions in larval patterning and, furthermore, acts synergistically with *spi. vn* expression is spatially and temporally restricted and, thus, it may encode another extracellular factor which regulates tissue-specific activation of DER.

Results

Molecular cloning of the vn *locus*

The P-element in the vn^{D4} allele was used as a cloning tag to isolate 52 kb of DNA in the *vn* region (Fig. 1A). Ten lesions associated with *vn* mutants map to this 52 kb region (Fig. 1A). Two of these lesions, $vn^{ddd RY}$ and $vn^{\gamma 4}$ are phenotypic and molecular nulls that disrupt the transcribed region (Fig. 1A). Northern analysis shows that there are two transcripts from the gene of 5 and 6.8 kb, which are expressed throughout development; the larger message is more abundant (data not shown). Ten cDNAs were isolated and their relationship to the genomic DNA and the intron–exon structure of the gene were determined by Southern hybridization and sequencing (Fig. 1A,B). These clones define two cDNAs of 5082 and 6864 bp, which correspond in size to the two transcripts seen in Northern analysis (Fig. 1A,B; data not shown). The two cDNAs differ by the presence or absence of a 1782-bp intron (intron 4; Fig. 1A) that is spliced out in the smaller cDNA. Northern analysis with intron 4 as a probe shows hybridization to the 6.8-kb message only (data not shown). These data show the gene makes two messages, a 6.8-kb message that includes intron 4 and a 5-kb message in which intron 4 is spliced out. Analysis of the 5′ and 3′ splice site sequences of the 4 introns shows that all have the *Drosophila* consensus sequence at the 3′ site. At the 5′ site, introns 1–3 match the consensus (GTA/GAGT) exactly (Mount et al. 1992), whereas intron 4 has the sequence GTACAG. This deviation from the consensus may explain why this intron is less efficiently spliced [only 25% of cDNAs show this splice (see Materials and Methods), and the spliced message is less abundant in Northern blots, data not shown].

We confirmed these transcripts correspond to the *vn* gene through transformation rescue of *vn* mutants with a cDNA corresponding to the 5-kb message. A *UAS–vn* construct activated by the *T80-GAL 4* driver, which is ubiquitously expressed in embryos and discs (data not shown; Wilder and Perrimon, 1995), partially rescued the pattern defects in *vn* larvae and the proliferation defect in *vn* wing discs (Fig. 2).

vn *encodes secreted EGF-like proteins*

The *vn* cDNAs encode two predicted proteins that differ only in their extreme carboxy-terminal region (Fig. 1B,C). The 5-kb cDNA encodes a predicted protein of 622 amino acids (type 1) and the 6.8-kb cDNA encodes a predicted protein of 621 amino acids (type 2). These proteins are similar up to residue 606 (the end of exon 4) where they diverge because of the inclusion of a 1782-bp region in the larger cDNA. The type 1 protein ends with 16 amino acids that differ from the terminal 15 amino acids in the type 2 protein (Fig. 1B,C). Both proteins are predicted to be ~71 kD. The function of this carboxy-terminal region and the significance, if any, of these protein types is not known.

In the region common to both proteins there are a number of features that suggest function. The predicted proteins begin at a likely translational start site (CGC<u>CATG</u>) (Cavener 1987). This methionine is preceded by stops in all three frames (Fig. 1B). There is a hydrophobic region at the amino terminus that has the characteristics of a signal sequence (Briggs and Gierasch 1986; von Heijne 1986, 1987) and we have shown the protein is indeed secreted (see below). The other features of the proteins are a PEST region that is found in proteins with

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A

B

C

--WPDSRYSSPESCKNYQGGYY*

Figure 1. Structure of the *vn* gene. (*A*) Restriction map of the *vn* region is shown at the *top*. *vn* mutations that have been mapped to the region and are shown above (inserts and break points) and below (deletions) the line. The mutations are described in detail elsewhere (Simcox et al. 1996). Boxes represent cDNAs that correspond to the two different messages encoded by *vn* (5 and 6.8 kb). In the larger cDNA (type 2), intron 4 (demarcated by arrows) is not spliced out. Open rectangles represent translated sequences. (B) *Bam*HI; (S) *Sal*I; (T) *Sst*I. (*B*) Sequence of the 5082-bp *vn* cDNA (type 1). The open reading frame starts at the first methionine that is preceded by stops in all three frames. There is a signal sequence (S; single underline), a PEST region (P; single underline), an *opa* (polyglutamine) region, an Ig-like domain (boxed), and an EGF-like domain (boxed). Circled residues indicate potential N-linked glycosylation sites and conserved cysteines involved in disulfide bonds are highlighted. Arrows mark intron-exon boundaries. A potential polyadenylation signal is underlined. The 6864-bp cDNA (type 2) includes an additional 1782 bp of sequence (not shown), which is spliced out in the 5082-bp cDNA (type 1). Inclusion of the intron gives rise to a predicted protein that is colinear with the type 1 except for the carboxyl terminus (shown *below*). (*C*) Carboxy-terminal region of predicted protein encoded by the type 2 cDNA. (Arrow) Exon 4/intron 4 boundary; (arrowhead) start of the region where the two proteins differ at the amino acid level.

short half-lives (Rogers et al. 1986), an *opa* (polyglutamine) region (Wharton et al. 1985), an Ig-like domain, and an EGF-like domain. There are a number of residues that are potential N-linked glycosylation sites.

The EGF-like domain in Vn is 43 amino acids long and has the six invariant cysteines and highly conserved glycine and arginine residues characteristic of the motif (Fig. 3A; Groenen et al. 1994). The cysteines are thought to form disulfide bonds and, hence, produce a looped structure. The EGF motif in Vn is between 30% and 44% identical to other EGF-like ligands (Fig. 3A). It shares 37% identity with Spi and 33% identity with Grk. Amino-terminal to the EGF domain is an Ig-like domain of the C2 type that include nonimmunological proteins (Fig. 3B; Williams and Barclay 1988). The Ig-like domain in Vn has the two invariant cysteine residues typical of the domain. The Ig region in Vn shares between 33 and 35% identity with the Ig region in the neuregulins and is 32% identical with the Ig region in NCAM (Fig. 3B).

Vn is secreted into the medium by S2 Drosophila *cells expressing a* vn *cDNA*

S2 cells were stably transformed with a *vn* cDNA under the control of the inducible metallothionein promoter (pMK33 vector). The conditioned media from induced transformed and control cells were analyzed by Western blotting with a rabbit polyclonal anti-Vn antibody. A ~70-kD protein, which reacts with the anti-Vn antibody, is secreted into the medium by *Drosophila* S2 tissue-culture cells expressing a *vn* cDNA (Fig. 4, lane 2), but not by untransfected control cells (Fig. 4, lane 1). The lower band in lane 2 is presumed to be a breakdown product.

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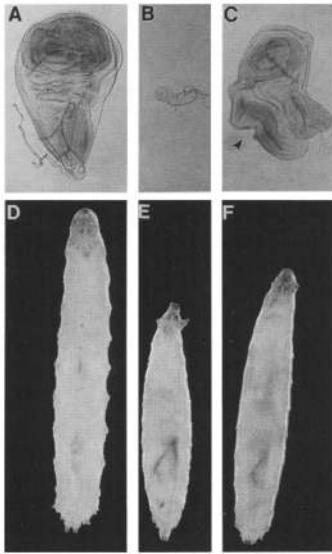

Figure 2. Rescue of *vn* mutants by a *vn* cDNA. A *UAS-vn* gene was expressed in *vn* mutants with the *T80-GAL4* driver, which is expressed ubiquitously in embryos and imaginal discs (data not shown; Wilder and Perrimon 1995). A partial rescue of the wing disc proliferation and larval pattern defects was observed. (*A*) Wild-type late third instar wing disc. (*B*) $vn^{dddL6/\gamma 3}$ late third instar wing disc. (*C*) *UAS-vn/T80-GAL4;* $vn^{dddL6/\gamma 3}$ late third instar wing disc. The disc is much larger than the *vn* mutant disc (*B*) but the phenotype is not completely wild type (*A*) as the wing pouch is duplicated (arrowhead). This is a phenotype seen in *vn* hypomorphs (Simcox et al. 1996). (*D*) Wild-type late third instar larva. (*E*) $vn^{dddL6/\gamma 3}$ late third instar larva. The larva is smaller and narrower than wild type. The reduction in the width of the larva is most pronounced at the posterior tip. (*F*) *UAS-vn/T80-GAL4;* $vn^{dddL6/\gamma 3}$ late third instar larva. The length and width of the larva are rescued compared with the *vn* mutant larva (*E*) but it is not fully wild type (*D*).

ventral cuticle defects in vn *mutants*

The spi-group mutants *spi*, *rho*, *S* and *pnt* are embryonic lethal and have similar cuticle phenotypes; they are shorter than wild type and have deletions of ventral cuticle (Mayer and Nüsslein-Volhard 1988; Fig. 5C). We examined the cuticle phenotype of *vn* null mutant embryos (Table 1; Fig. 5B). vn^{dddRY} embryos were shorter (91%, $P < .05$) and the Keilin's organs and ventral black dots were closer together than wild-type (92%, $P < .05$, 95%, $P < 0.05$, respectively). The measurements show it is ventral cuticle between the Keilin's organs that is deleted in vn^{dddRY} embryos. Cuticle measurements show the deletions occur in a similar region in spi-group mutants; *spi*, and *rho* have a larger portion of ventral cuticle deleted than *vn* mutants, but *pnt* embryos have similar deletions (Mayer and Nüsslein-Volhard 1988).

spi-group mutants have abnormal Keilin's organs with deleted or separated hairs (Mayer and Nüsslein-Volhard 1988) and Keilin's organs defects were also observed in *vn* embryos. In vn^{dddRY} embryos, the sensory hairs were surrounded by a pit structure (Fig. 5A,B) and in $vn^{\gamma 4/\gamma 3}$ embryos 50% of Keilin's organs were missing (data not shown). The head region is affected in *spi*-group mutants (Mayer and Nüsslein-Volhard 1988) but is apparently normal in *vn* mutants as judged by the structure of the head skeleton and the inventory of sense organs (data not shown).

Unlike the spi-group genes, *vn* is not critical for embryonic survival and null mutants can develop to the pupal stage. Null *vn* animals have a pleiotropic lethal phase. Most $vn^{\gamma 4/\gamma 3}$ individuals die as embryos or as larvae, but a small number pupariate (~5%). Individuals that survived to pupariate secreted a pupal case with pattern abnormalities; the pupal cases were shorter and slimmer than wild-type (Fig. 6E,F).

vn *and* spi *interact genetically*

vn and *spi* show a strong synergistic genetic interaction suggesting a molecular interdependence. Reducing *vn* dose in a *spi* null genotype dramatically worsened the phenotype to produce a collapsed embryo with an extruded head skeleton (Fig. 5C,D). *spi; vn* double mutants were much more severely affected than the predicted additive phenotype of the single mutants as they lacked almost all ventral denticles and head skeleton structures (Fig. 5E). However, the double mutants were not as severely affected as a *DER* null (Fig. 5F). Similar *vn-spi* genetic interactions were seen in combinations with the strong EMS-induced vn^{dddL6} and spi^{IIA14} alleles (data not shown), thus, these interactions are not allele specific.

vn *interacts with* DER *and* rolled/MAPK

Genetic interactions are also observable between *vn* and *DER*, and *vn* and *rolled/MAPK* (*rl*), a downstream component in the DER pathway (Biggs et al. 1994; Brunner et al. 1994b; O'Neill et al. 1994). Lowering *vn* dose increased the frequency of missing pattern elements in loss-of-function *DER* mutants (Table 2). The gain-of-function alleles, $DER^{Ellipse}$ (DER^{Elp}; Baker and Rubin 1989) and $rl^{Sevenmaker}$ (rl^{Sem}; Brunner et al. 1994b) rescued the viability of *vn* temperature-sensitive mutants (Table 2). These alleles also rescued proliferation defects in strong and null *vn* mutants (Fig. 6). *vn* mutants ($vn^{\gamma 4/\gamma 3}$, $n = 20$ wing discs) have tiny wing discs (Fig. 6B). $vn^{dddL6/\gamma 3}$ discs have a similar phenotype (Fig. 2B; $n = 44$ wing discs). The *vn* small wing disc phenotype is rescued by rl^{Sem} in most vn^- individuals ($rl^{Sem/+}$; $vn^{dddL6/\gamma 3}$, $n = 68$ wing discs) such that 10% of discs have the phenotype shown (Fig. 6C), and 70% show a lesser rescue, growing to about twice the size of a null disc (not shown). The *vn* small wing disc phenotype was rescued dramatically by DER^{Elp} ($DER^{Elp1/+}$; $vn^{\gamma 4/\gamma 3}$, $n = 31$ wing discs) such that 70% of these discs had a duplicated wing pouch (Fig. 6D), and 30% had a fan shaped wing pouch (not shown). These rescued wing discs were not fully wild-type but resembled those seen in hypomorphic *vn* mutants, suggesting the hyperactive receptor cannot fully compensate for *vn* loss (Simcox et al. 1996). The pupal cases secreted by *vn* mutants (Fig. 6F, $vn^{\gamma 4/\gamma 3}$, $n = 14$ pupae) are shorter and narrower than

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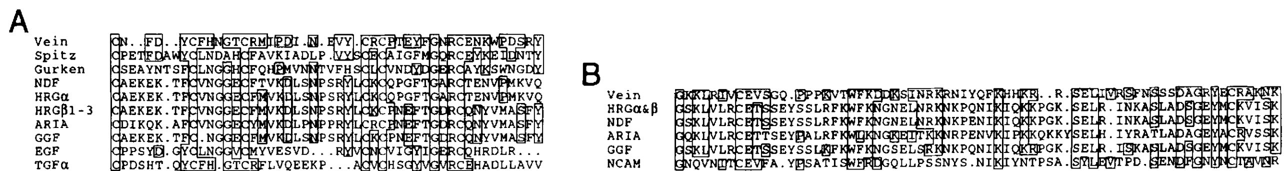

Figure 3. Alignment of the EGF and Ig domains of Vn with similar domains in EGF-like ligands. Comparisons are made relative to Vn with identical amino acids boxed. (*A*) The EGF-like domain in Vn has the six invariant cysteines and highly conserved glycine and arginine residues characteristic of the motif. (*B*) Alignment of the Ig-like domain of Vn. Vn, the neuregulins, and NCAM have a C2 type Ig-like domain that spans about 50–70 residues and is flanked by two invariant cysteine residues. Proteins listed are: the neuregulins [rat NDF, human heregulins α and β1–3, chicken ARIA and bovine GGF (Holmes et al. 1992; Wen et al. 1992; Falls et al. 1993; Marchionni et al. 1993; Yardin and Wen 1994)]; EGF/TGF-α proteins [*Drosophila* Spi (Rutledge et al. 1992) and Grk (Neuman-Silberberg and Schüpbach 1993), rat EGF (Simpson et al. 1985), and TGFα (Marquardt et al. 1984)], and mouse NCAM (Barthels et al. 1987).

wild-type (Fig. 6E). The size of the pupal case in *vn* animals was rescued toward wild-type by DER^{Elp} (Fig. 6G, $DER^{Elp1/+}$; $vn^{\gamma4/\gamma3}$, $n = 22$ pupae) but not by rl^{Sem}, although we did observe a change in the spacing of terminal pattern elements toward wild-type in $rl^{Sem/+}$; $vn^{-/-}$ larvae (not shown). Similar suppressed phenotypes were seen with the vn^{dddL6} and the hypermorphic DER^{ElpB1} alleles (data not shown), therefore, the observed interactions are not allele specific.

vn *expression in embryos*

Consistent with a tissue-specific function, *vn* transcripts are spatially localized (Fig. 7). *vn* is expressed in blastoderm embryos in two ventrolateral stripes that are brought to the midline as gastrulation proceeds (Fig. 7A). These cells include precursors of the ventrolateral epidermis that is affected in *vn* and *spi*; *vn* mutants (Fig. 5). Expression in midline cells persists but is progressively limited to single cells (Fig. 7B,E,F). In the germ-band retraction stage, cells in the CNS and epidermis express *vn* (Fig. 7G, insert). The significance of the CNS expression in *vn* mutants has not been examined. *vn* is expressed in the anlagen of the amnioserosa at late blastoderm and in the amnioserosa proper until the end of germ-band extension (Fig. 7B,C). Survival of the amnioserosa cells is dependent on *DER* (Clifford and Schüpbach 1992). In late germ-band extended embryos, there is expression in some PNS precursors (Fig. 7D) that, from their position at the end of germ-band retraction, include precursors of the Keilin's organs and a subset of the cells of the chordotonal organs (Fig. 7F,G). The Keilin's organs are often missing or abnormal in vn^- embryos (Fig. 5B), however, the chordotonal organs appear normal (data not shown). *vn* is expressed in the head throughout development; in the clypeolabrum, the maxillary and labial lobes, and around the stomodeum (Fig. 7F,G). There are no overt head defects in vn^- embryos, but the head is severely disrupted in *spi;vn* mutants (Fig. 5D,E), and *DER* is re-

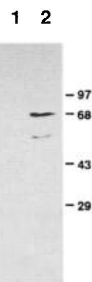

Figure 4. *vn* encodes a secreted protein. A Schneider's S2 cell line expressing a *vn* cDNA under the control of the metallothionein promoter was established. A ~70-kD band that is immunoreactive with anti-Vn antibodies is detected in media from S2-Vn cells (lane *2*) but not from untransfected control cells (lane *1*). The lower band in lane *2* is a presumed breakdown product.

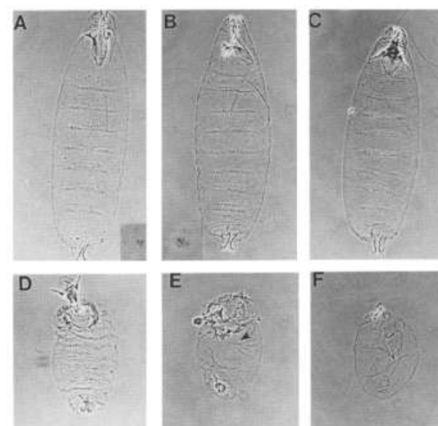

Figure 5. *vn* mutants have ventrolateral defects and interact genetically with *spi*. Cuticle preparations of (*A*) wild-type, (*B*) $vn^{dddRY/dddRY}$, (*C*) $spi^{OD12/VA17}$, (*D*) $spi^{OD12/VA17}$; $vn^{dddRY/+}$, (*E*) $spi^{OD12/VA17}$; $vn^{dddRY/dddRY}$, and (*F*) $DER^{3F18/3F18}$ embryos. The width of the denticle belt and distance between the Keilin's organs is reduced in *vn* (*B*) and *spi* embryos (*C*) (Table 1). *spi* embryos with reduced *vn* dose have an anterior-ventral cuticle hole that extends to the first abdominal segment, an extruded head skeleton and a collapsed body structure (*D*). *spi*; *vn* double mutants have between four and six ventral denticle belts comprised of a small number of denticles each (arrowhead), a large anterior-ventral cuticle hole and a severely reduced head skeleton (*E*). *DER* null embryos are more extreme than the *spi;vn* double mutants and lack all denticles (*F*).

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Table 1. vn *mutants have ventrolateral deletions*

	Wild-type	vn^{ddRY}	% WT
Length of embryo	13.0 ± 0.05 (n = 29)	11.9 ± 0.28 (n = 26)	91
Distance between Keilin's organs	4.8 ± 0.05 (n = 29)	4.4 ± 0.04 (n = 27)	92
Distance between ventral black dots	8.0 ± 0.05 (n = 29)	7.6 ± 0.08 (n = 25)	95

Dimensions were measured with a micrometer eye piece and are given in arbitrary units ± S.E. Embryo length was measured at 4× lower magnification. Distances between sense organs were measured in T3. By Student's t-test, these differences are statistically significant ($P < 0.05$).

quired for the survival of cells in the head (Clifford and Schüpbach 1992; Raz and Shilo 1992). In late embryos, *vn* expression decays in all ectodermal cells and appears in the segmental muscles and the gut wall (Fig. 7H).

Discussion

vn *acts in the* DER *signaling pathway*

vn shows genetic interactions with components of the *DER* signaling pathway (Table 2; Figs. 5 and 6). The most dramatic and informative of these is the rescue of *vn* null phenotypes by gain-of-function *DER* alleles. *vn* null wing discs are tiny, arresting growth at the equivalent of a late second/early third-instar-size disc (Simcox et al. 1996), however, *vn* null wing discs that are heterozygous for the DER^{Elp} allele are rescued and grow to a large size (Fig. 6A,B,D). These discs are not fully wild-type as they have a duplicated wing pouch, which is a phenotype characteristic of *vn* hypomorphs (Simcox et al. 1996). Thus, the hyperactive DER receptor encoded by the DER^{Elp} allele can override the wing proliferation defects and partially compensate for *vn* loss. The DER^{Elp} allele also rescued the larval patterning defects of *vn* nulls as reflected in the phenotype of the pupal case (Fig. 6G). These results have two major implications. First, the results show *DER* is epistatic to *vn*, which is consistent with Vn acting as a ligand for DER. Second, the results suggest that there is redundancy in the signaling system, whereby activation of a *hyperactive* receptor by another ligand(s) compensates for Vn loss. It is also possible the

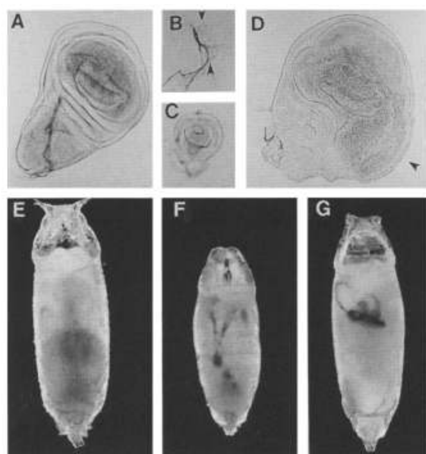

Figure 6. *vn* interacts genetically with *DER* and *rl*. (*A*) Wild-type wing disc. (*B*) Tiny wing disc from $vn^{\gamma4/\gamma3}$ larva (arrowheads demarcate disc). (*C*) Rescued wing disc in $rl^{Sem/+}$; $vn^{dddL6/\gamma3}$ larva. (*D*) Rescued wing disc in $DER^{Elp/+}$; $vn^{dddL6/\gamma3}$ larva. The disc has a duplicated wing pouch (arrowhead) and resembles a *vn* hypomorphic phenotype (Simcox et al. 1996). (*E*) Wild type pupal case. (*F*) Shorter and slimmer $vn^{\gamma4/\gamma3}$ pupal case. (*G*) Rescued $DER^{Elp1/+}$; $vn^{\gamma4/\gamma3}$ pupal case. The pupal case is close to wild-type size and shape.

Table 2. *Genetic interaction between* vn *and* DER *and* vn *and* rl

A. Reducing vn *dose enhances* DER *loss-of-function phenotypes*

	Missing wing vein		
Genotype[a]	acv[b]	L4[c]	*n* Wings
$vn^{ddL6}/TM1$	0.00	0.00	50
$vn^{dddRG}/TM3$	0.00	0.00	50
$DER^{top1/top1}$; $+/TM3$	0.17	0.01	90
$DER^{top1/top1}$; $vn^{dddL6}/TM3$	0.90	0.04	80
$DER^{top1/CA27}$; $+/TM3$	0.50	0.00	60
$DER^{top1/CA27}$; $vn^{dddL6/+}$	0.94	0.10	36
$DER^{top1/EC20}$; $+/TM3$	0.70	0.00	56
$DER^{top1/EC20}$; $vn^{dddL6/+}$	1.00	0.33	50
$DER^{top1/top1}$; $+TM8$	0.29	ND	62
$DER^{top1/top1}$; $vn^{dddRG}/TM8$	1.00	ND	52

B. DER *and* rl *gain-of-function alleles rescue* vn *temperature-sensitive mutants*

	Viability[d]	
Genotype[a]	17°C	25°C
$vn^{tsWB/\gamma3}$	0.39	0.01
$DER^{Elp1/+}$; $vn^{tsWB/\gamma3}$	0.66	0.50
$rl^{Sem/+}$; $vn^{tsWB/\gamma3}$	0.67	0.49

[a]vn^{dddL6} and vn^{dddRG} are strong EMS-induced alleles; vn^{tsWB} is an EMS-induced heat-sensitive allele (Simcox et al. 1987); and $vn^{\gamma3}$ is a deletion allele (Simcox et al. 1996). DER^{Elp1} is a gain-of-function allele (Baker and Rubin 1989). Other DER alleles used here are described in Clifford and Schüpbach (1989). rl^{Sem} is a gain-of-function *rl/MAPK* allele (Brunner et al. 1994b). *TM1*, *TM3*, and *TM8* are balancer chromosomes.
[b]acv, anterior crossvein.
[c]L4, longitudinal vein four, which was only partially deleted in the mutants.
[d]Viability is the fraction of flies expected based on the number of their heterozygous sibs (n = 100–300).

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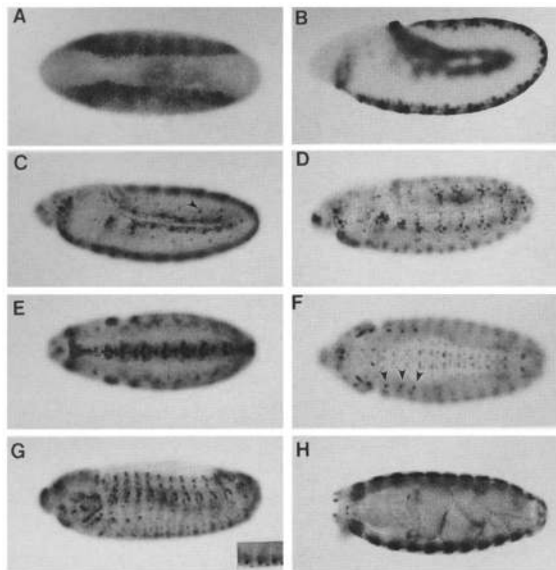

Figure 7. Expression pattern of *vn* transcripts in embryos. (*A*) Gastrula embryo (stage 5, ventral view). There are two ventrolateral stripes with pair-rule modulation and a central dorsal patch, the presumptive amnioserosa (out of plane of focus), of *vn* expressing cells. (*B*) Germ-band extended embryo (stage 10, lateral view). Cells of the ventral midline and the cells of the amnioserosa express *vn* at high levels. (*C*) Germ-band extended embryo (stage 10, lateral view). *vn* is expressed transiently in the presumptive tracheal pits (arrowhead). (*D*) Germ-band extended embryo (stage 11, lateral view). As epidermal segmentation becomes apparent, expression in the amnioserosa subsides and there is expression in lateral cells that are likely to be a subset of the precursors of the dorsal PNS. There is expression in the head and gnathal segments. (*E*) Germ-band extended embryo (stage 11, ventral view). Midline expression is restricted to fewer cells. (*F*) Germ-band retraction (stage 13, ventral view). There is expression in a small subset of midline cells. (*G*) Germ-band retraction (stage 13, lateral view). There is expression in a subset of cells of the PNS dorsal cluster, and cells likely to be precursors of the chordotonal and Keilin's organs. The insert shows CNS cells and epidermal cells at the ventral midline express *vn*. (*H*) Late embryo (stage 16). *vn* is expressed in the mesoderm in the segmental muscles and in the gut wall. Anterior is to the *left*; in lateral views dorsal is at the *top*.

hyperactive receptor no longer requires ligand activation; in this scenario some localized cofactor(s) would presumably be required for the spatial restriction of signaling.

vn *encodes an EGF-like protein with similarity to the neuregulins*

vn encodes secreted proteins with domains characteristic of two protein superfamilies; an EGF-like domain and an Ig-like domain (Figs. 1 and 3). The secreted nature of the proteins is consistent with the non cell-autonomous behavior of *vn* in clones (Wurst et al. 1984; Simcox et al. 1987; García-Bellido et al. 1994). The EGF domain, found in all EGFR ligands, is important for receptor binding and activation (Groenen et al. 1994). The Ig-like domain, known to be involved in protein–protein interaction (Williams and Barclay 1988), may facilitate Vn dimerization or interaction with other extracellular proteins. The only known ligands that have both EGF-like and Ig-like domains are the vertebrate neuregulins, also known as Neu differentiation factor, the heregulins, ARIA, and glial growth factors (Holmes et al. 1992; Wen et al. 1992; Falls et al. 1993; Marchionni et al. 1993; Yardin and Wen 1994). The neuregulins were described initially as direct ligands for neu but later shown to bind its relatives, erbB3 and erbB4, furthermore, signaling through the neuregulins can occur through receptor heterodimers (Caraway and Cantley 1994). In *Drosophila*, *DER* is the only *EGFR*-like gene that has been identified, therefore, it is likely Vn directly binds and activates DER. However, we cannot exclude the possibility that Vn activation of DER signaling is indirect and similar to the situation with the neuregulins that bind specific receptors but can activate others in receptor heterodimers or, indeed, occurs via a parallel pathway. The potential of Vn as a direct DER ligand can be tested by use of the tissue-culture system that was developed to show Spi activates DER (Schweitzer et al. 1995b).

vn *is a localized component in the* DER *signaling pathway*

DER is expressed broadly in embryos, but required in specific cells (Kammermeyer and Wadsworth 1987; Zak et al. 1990); for example, DER is expressed throughout the embryonic epidermis, but only the ventral epidermis is affected in mutants. The EGF-like ligands encoded by *vn* and *spi* activate DER signaling, and, thus, spatial restriction of these ligands may confer tissue-specificity to DER function in the developing zygote, analogous to the situation in the ovary where Grk restricts DER activation (Neuman-Silberberg and Schüpbach 1993; González-Reyes et al. 1995; Roth et al. 1995).

At least three mechanisms operate to control the spatial and temporal pattern of Spi/DER signaling. Spi is expressed broadly, and, therefore, its distribution cannot restrict receptor function directly; however, processing Spi into a secreted factor is apparently required for its activity (Rutledge et al. 1992; Schweitzer et al. 1995b). Thus, the first mechanism operates to restrict the spatial production of the active Spi ligand and may depend on the activity of cofactors that are localized. Spi, like most EGF proteins, has a predicted transmembrane domain carboxy-terminal to the EGF-like region that anchors the protein to the cell in a transmembrane pro-form (Rutledge et al. 1992). Proteolytic cleavage is thought to release the soluble form and tissue-culture experiments, and the activity of *spi* transgenes in embryos suggests it is the soluble form of Spi, secreted Spi (sSpi), that is active (Schweitzer et al. 1995b). Genetic experiments suggest that the localized production of sSpi may require Rho and S function: sSpi can rescue ventral fates in *rho* and *S* mutant embryos, suggesting it acts after these genes, consistent with a possible role for Rho and S in

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the processing of Spi into its active form (Schweitzer et al. 1995b). Thus, the generation of sSpi may be limited to cells in the ventrolateral domain that express Rho and S. Two mechanisms operate to limit the duration of Spi/DER signaling. The first of these involves *argos* which encodes an EGF-like protein with an unusual EGF domain in which the spacing between the third and fourth cysteines is much greater than in other EGF-like proteins (Freeman et al. 1992). Genetic and biochemical evidence suggests Argos is an inhibitor of the activation of DER by Spi (Schweitzer et al. 1995a). Interestingly, *argos* expression is up-regulated in cells that have undergone Spi/DER signaling, effecting a negative feedback loop to limit DER activation in neighboring cells (Golembo et al. 1996). In a second mechanism, the production of the receptor itself is affected, as *DER* transcripts were shown to be down-regulated in cells that recently have undergone DER signaling (Sturtevant et al. 1994).

The regulation of Vn/DER signaling is different from Spi/DER signaling. First, unlike *spi*, *vn* is expressed in a localized pattern and, thus, would limit Vn/DER signaling to cells in the Vn expression domain. Second, the Vn proteins we characterized lack a potential membrane-spanning domain to anchor them to the cell surface and would not require a processing step to release a soluble form. In the embryo, *vn* and *spi* are expressed in overlapping domains and our genetic data suggest they function together to achieve the required level of DER activation for normal development of ventrolateral cells. As Vn/DER signaling occurs in the same domain as Spi/DER signaling, it would be modulated by down-regulation of the receptor and may also be effected by Argos inhibition, although this will have to be tested directly. The situation is different in the wing, where Vn is expressed primarily in developing interveins, and the genes that modulate Spi/DER signaling (*rho*, *S*, and *argos*) are expressed in developing veins (Heberlein et al. 1993; Sturtevant et al. 1993; Sawamoto et al. 1994; Sturtevant and Bier 1995; Simcox et al. 1996). Mirroring the situation in the embryo, prevein cells that are presumed to have undergone high levels of DER activation down-regulate expression of the receptor, so that in pupal wings, DER expression colocalizes with *vn* expression in intervein regions (Sturtevant et al. 1994; Simcox et al. 1996). Thus, the development of intervein cells apparently requires Vn/DER signaling and is not subject to modulation by the factors that influence Spi/DER signaling in the developing veins.

vein *and* spi *interact genetically*

Unlike *spi* mutants which are embryonic lethal, *vn* mutants have a pleiotropic lethal phase and can survive to pupate. The ventral cuticle defects in vn^- embryos are correspondingly milder than those seen in spi^- embryos (Fig. 5B,C). There are strong genetic interactions between *vn* and *spi*, suggesting the two genes function in the same process. Lowering *vn* dose worsened the *spi* phenotype and the double mutants were much more severely affected than would be predicted from an additive effect (Fig. 5D,E). Both genes encode EGF-like proteins and interact genetically with *DER* and, therefore, are probable ligands for DER. This has been confirmed biochemically for Spi (Schweitzer et al. 1995b).

The *spi–vn* interaction and suppression of *vn* phenotypes by DER^{Elp} alleles reveals an interplay between the ligands that we interpret according to the following model (Fig. 8). In the wild-type situation, both Spi and Vn contribute to activating DER signaling to the threshold level required for normal ventral patterning. Eliminating *vn* or *spi* reduces ventral pattern elements and is more extreme in *spi* mutants. A hyperactive receptor (DER^{Elp}) can return signaling to near wild-type levels in a *vn* mutant by augmenting Spi/DER signaling. Reducing the *vn* dose in a *spi* mutant further reduces signaling compared with the *spi* mutant with wild-type levels of *vn* and removal of both *vn* and *spi* in the double mutant severely affects ventral patterning. However, this phenotype is not as severe as that seen in a DER null mutant; these

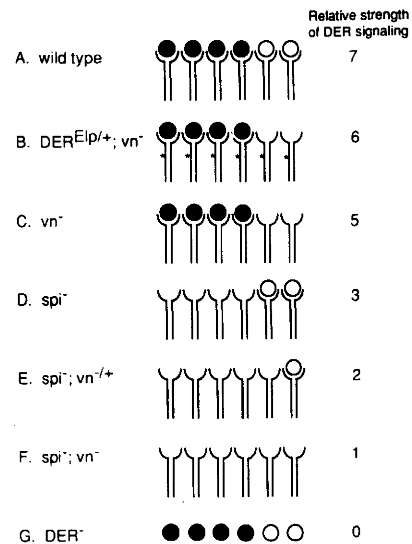

Figure 8. Model of Spi/DER and Vn/DER signaling in the ventrolateral cells in the embryo. (*A*) Wild-type. Spi and Vn activate DER signaling to the threshold for normal development. The contribution of Spi to the overall level of signaling is greater and represented here as more receptor dimers binding Spi. We show Vn binding DER directly, however, the effect on DER could be indirect via binding in a receptor heterodimer or via a parallel pathway. (*B*) $DER^{Elp/+}$; *vn*. The absence of Vn is compensated for by an enhancement of Spi/DER signaling by the hyperactive DER^{Elp} receptor (*) and the ventral cuticle is almost wild-type. (*C*) *vn*. In the absence of Vn, the level of DER activation is decreased and there are subtle ventral defects. (*D*) *spi*. In the absence of Spi, DER signaling is substantially reduced and there are ventral defects. (*E*) *spi*; *vn*/+. Lowering Vn dose in the absence of Spi increases the severity of the ventral defects over those seen in a *spi* mutant. (*F*) *spi*; *vn*. In the absence of Spi and Vn, DER is active at a very low level and there are severe ventral deletions. The remaining DER signal is most likely due to basal activity of the receptor or activity of another ligand or cofactor. (*G*) *DER*. In the absence of DER there is no signaling and the ventral cuticle is completely deleted. (●) Spi; (○) Vn; (Y) DER; (Y) DER^{Elp}.

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embryos have no ventral cuticle. There are at least three possibilities that explain this observation. First, maternal spi^+ or vn^+ products may mask the severity of the *spi*; *vn* zygotic phenotype, but germ cell and ovary transplants (respectively) suggest *spi* and *vn* do not have maternal effects (T. Schüpbach, pers. comm.; A. Simcox and E. Hersperger, unpubl.). More likely, the difference could be caused by basal activity of the receptor or activation by another (unknown) ligand or cofactor.

The vertebrate EGFR family and its ligands are implicated in human cancer, and understanding the extracellular events that lead to receptor activation will be important for the development of therapies targeted at ligand–receptor interaction (Jeschke et al. 1995; Levitzky and Gazit 1995). The intriguing similarity between Vn and the neuregulins and the emerging complexity of EGFR signaling in *Drosophila*, including the discovery of the inhibitor Argos (Schweitzer et al. 1995a; Golembo et al. 1996), suggests the fly will be an important model for understanding these extracellular events.

Materials and methods

Drosophila *stocks*

The *vn* alleles vn^{dddRY} and $vn^{\gamma4}$ are null as they disrupt the transcribed region (Fig. 1A). $vn^{\gamma3}$, is a gross deletion, vn^{dddRG} and vn^{dddL6} are strong EMS-induced alleles and vn^{tsWB} is a temperature-sensitive, EMS-induced allele (Simcox et al. 1996). spi^{OD12} and spi^{VA17} are overlapping deletions and the combination is spi^- (Rutledge et al. 1992). The DER^{3F18} allele is a deficiency, DER^{top1}, DER^{CA27}, and DER^{EC20} are described in Clifford and Schüpbach (1989).

Embryo cuticle preparation

Embryos were manually dechorionated, devitellinized, and cooked in lactic acid and 70% ethanol (9:1) at 45°C overnight. Cleared embryos were mounted in Hoyer's medium.

Lethal phase determination

The hatch rate of *red* $vn^{\gamma4}$/*red* $vn^{\gamma3}$ individuals was determined by counting of total and unhatched eggs from a cross of *red* $vn^{\gamma4}$/*TM3 X red* $vn^{\gamma3}$/ +. Of 412 eggs, 73 failed to hatch, these should be mainly the *red* $vn^{\gamma4}$/*red* $vn^{\gamma3}$ embryos as the three other genotypes are fully viable, thus the embryonic lethality rate for *red* $vn^{\gamma4}$/*red* $vn^{\gamma3}$ is ~70%. (We did not check what portion of the unhatched eggs were unfertilized, thus, this number may be slightly over estimated.) Eighty-three hatched *red* $vn^{\gamma4}$/*red* $vn^{\gamma3}$individuals were separated in the second and early third instars and allowed to develop without competition from their more vigorous sibs, 18 of these pupariated, none developed to the pharate or adult stage.

Cloning the vn *genomic region*

Cloning of the *vn* region by transposon tagging was initiated from a P element at 64F that is associated with the vn^{D4} allele (kindly provided by R. Karess, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). This P element is responsible for the *vn* phenotype because the allele reverted when the P element excised. Sequences flanking the P DNA were used to isolate a set of overlapping clones from a λ Charon 4 wild-type *Drosophila* genomic library (Maniatus et al. 1978). A total of 52 kb around the P-element insert was cloned.

Isolation of vn *cDNAs*

A 214-bp genomic fragment (corresponding to nucleotides 2580–2794; Fig. 1B) was used as the initial probe to isolate three phage from a disc cDNA library (A. Cowman and G. Rubin, University of California, Berkeley). A cDNA fragment derived from these clones (corresponding to nucleotides 3424–3980; Fig. 1B) was then used as a probe to isolate seven more cDNAs (three from an embryo library (T. Kornberg, University of California, San Francisco) and four from the disc library (A. Cowman and G. Rubin)). All contained a single *Eco*RI fragment and were subcloned into pBSKS$^+$ for sequencing. The combination of two of these cDNAs gives a 6864-bp sequence that corresponds in size to the larger message (6.8 kb) seen in Northern analysis. Sequencing of the cDNA clones revealed two forms that differed by the presence or absence of a 1782-bp intron (6 of 8 cDNAs that cover this region have the 1782-bp sequence). cDNAs that splice out the intron are 5082 bp in length and correspond to the smaller transcript seen in Northern analysis (5 kb).

DNA sequencing and analysis

The *vn* cDNA clones and some genomic clones were sequenced on both strands by use of a USB Sequenase 2.0 kit with single-stranded templates. Gene-specific primers were designed to extend the sequence on a given strand. Initial searches of the data base for similarities were performed with BLAST at NCBI. Sequence alignments were done with the Genetics Computer Group Wisconsin Package with the PileUp program.

Generation of Vn antibody

Anti-Vn antibodies were generated by cloning a partial *vn* cDNA (nucleotides 1282–2705, Fig. 1B) into the *Eco*RI site of the pRSET expression vector (Invitrogen). Polyclonal rabbit antibodies were generated against the resulting purified fusion protein (Cocalico Biologicals, Inc.).

Activation of UAS-vn

The complete open reading frame of a *vn* cDNA (1679–3980, Fig. 1B) was cloned into the *Xba*I/*Xho*I site in pUAST (Brand and Perrimon 1993) and introduced into flies by germ-line transformation. The *UAS-vn* gene and the *T80-GAL4* driver were crossed into a *vn* mutant background ($vn^{dddL6/\gamma3}$). The *vn* mutants (*UAS-vn/T80-gal 4*; $vn^{dddL6/\gamma3}$ and *UAS-vn/CyO*; $vn^{dddL6/\gamma3}$) were recognized by the *red* Malphigian tubule marker and examined for their larval and wing disc phenotypes. Approximately 50% of these larvae showed a partially rescued phenotype when compared with $vn^{dddL6/\gamma3}$ controls; they were bigger, more vigorous and had large wing discs. The $vn^{dddL6/\gamma3}$ wing disc phenotype is 100% penetrant (Simcox et al. 1996), and, thus, we assume these rescued animals were *UAS-vn/T80-gal 4*; $vn^{dddL6/\gamma3}$.

Tissue culture

Schneider's S2 cells were grown in *Drosophila* Schneider medium (GIBCO), supplemented with 10% fetal bovine serum. Ten micrograms of a Vn-expression construct, which contained nucleotides 1679–3980 inserted into pMK33, was used for transfection with Lipofectin (GIBCO) in serum-free medium following the manufacturer's protocol. A stable cell line was estab-

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lished following selection in 200 μg/ml hygromycin B (Calbiochem). S2-Vn cells were induced for 36 hr in serum-free medium containing 0.7 mM $CuSO_4$.

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