Functional Requirement and Redundancy of Egfr Ligands in Drosophila Development

Dissertation

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Abstract

In both vertebrates and the fruit fly Drosophila melanogaster, the Epidermal growth factor receptor (Egfr) pathway is required for development and homeostasis. Dysfunction of the pathway is implicated in many human pathologies including heart disease, schizophrenia and multiple types of cancer.

The Egfr receptor is a member of the ErbB family. In vertebrates there are four genes that encode ErbB receptors. The receptors function as homodimers or heterodimers leading to many different active configurations. There are also multiple EGF ligands, which bind and activate the receptors. In vertebrates there are eleven ligand genes, some of which encode multiple isoforms.

To understand the pathway and its specific biological roles it is necessary to examine the function of individual components. The many ligands and many possible receptor combinations make this type of analysis prohibitively complex in vertebrates.
In contrast, the *Drosophila* pathway is much simpler—there are four *Drosophila* EGF ligands and a sole Egfr receptor. This makes *Drosophila* a very attractive system for asking questions of ligand requirement and specificity *in vivo*.

*Drosophila* has four ligands representing two major classes of vertebrate ligands: three TGFα-like ligands spitz (*spi*), *gurken* (*grk*), and *Keren* (*Krn*) and one neuregulin-like ligand, *vein* (*vn*). To investigate the requirement for ligands in *Drosophila* Egfr signaling, as well as the functional redundancy of the *Drosophila* Egfr ligands, I employed two strategies. In the first, I removed ligands to reveal possible redundant roles in development. In the second, I tested ligand specificity by determining if the TGFα ligands were able to replace the unique role of the neuregulin ligand *vn* in development.

For ligand removal, I generated fly stocks bearing all possible combinations of single and multiple ligand mutants, examined phenotypes in the embryonic cuticle and compared these to the cuticles of Egfr receptor mutants. If there is no ligand independent signaling in early development, mutants lacking all zygotic ligands should have a comparable phenotype to the receptor mutant, which is indeed what I observed. I found no evidence of ligand independent signaling in embryogenesis. I did find that all zygotic ligands play a role, although some were only revealed in mutant combinations. These data revealed a previously undescribed redundant role for the ligand Keren in early development. To further investigate ligand redundancy in development, I examined the wing patterning phenotypes of flies with reduced expression of TGFα ligands in a *vn* hypomorphic
mutant background. Neither spi nor Krn have required functions in wing development individually; however, I demonstrate that they have redundant roles in wing patterning.

To investigate ligand specificity, I expressed each TGFα ligand in the endogenous vein pattern during development. Surprisingly, I found that each of the TGFα ligands could largely replace the essential role of vn in development. My results support the idea that the Egfr ligands are largely functionally redundant, and that stimulating the pathway to a particular level, not the intrinsic signaling of the specific ligand bound, is important.
Dedication

to all the dancers.
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Chapter 1: Introduction

1.1 Egfr/ErbB signaling in normal and disease processes

Egfr is the founding member of a family of receptor tyrosine kinases that in mammals includes Egfr/ErbB1, ErbB2/HER2/neu, ErbB3 and ErbB4. The receptors mediate diverse biological processes by communicating both proliferation and differentiation cues via a complex array of intercellular routes (Fig 1.1). ErbB family members are critical for normal development, including cardiac (Citri and Yarden 2006), mammary (Stern 2000), and neural (Riese and Stern 1998; Citri and Yarden 2006). The pathways are involved in diverse cellular processes including cell morphogenesis, migration, differentiation, and proliferation (Yarden and Sliwkowski 2001). Perturbation of the pathways results in major developmental defects and problems in human health, including heart disease, schizophrenia, and cancer (reviewed in, for example, (Mei and Xiong 2008; Sanchez-Soria and Camenisch 2010; Yarden and Pines 2012). ErbB family members are widely implicated in human cancers including lung, breast, and prostate cancers (Yarden and Sliwkowski 2001). ErbB2/HER2/neu is amplified or overexpressed in the particularly aggressive “HER2-positive” cancers, which comprise 20-30% of breast cancers and correlate with poor prognosis (Slamon et al. 1987; Spector and Blackwell 2009; Slamon
et al. 2011). The pathways are under intense investigation because of their involvement in normal biology and human health.

### 1.2 ErbB receptors

The Egfr/ErbB family receptor tyrosine kinases function by dimerizing following ligand binding, upon which the receptors’ intracellular kinase domains autophosphorylate, triggering downstream signaling of growth and survival. Egfr and the other ErbB family members form both homo- and heterodimers, and become catalytically active upon ligand binding to at least one receptor in a dimer (Yarden and Sliwkowski 2001). Upon ligand binding, the kinase domains of both receptors of the dimer are activated by trans-autophosphorylation (Jura et al. 2009).

Each of the four family members can signal in conjunction with any one of the others (Yotsumoto et al. 2009), though there are subtle differences among the receptors. The monomer conformation of ErbB2 is distinct from that of the other family members and resembles that of ligand-bound Egfr (Alvarado et al. 2009) (Fig 1.2), though ErbB2 has no known ligands. And though ErbB3 can participate in potent signaling dimers, it has a defective kinase domain and has generally been classified as a pseudokinase (Guy et al. 1994; Sierke et al. 1997; Citri et al. 2003). Recent evidence, however, suggests that ErbB3 retains a low level of kinase function (~1000 fold less active than Egfr) (Shi et al. 2010).
Each of the four receptors can dimerize and signal with any other, though
ErbB2/HER2/neu is the preferred binding partner of each of the other ErbB receptors
(Groenen et al. 1994; Tzahar et al. 1996; Yarden 2001), and neither ErbB2 nor ErbB3 can
signal as homodimers.

1.3 ErbB ligands

In mammals there are eleven EGF-like ligand genes that activate signaling through the
ErbB receptors (Groenen et al. 1994; Yarden 2001). The ligands fall into two classes. The
first class of ligands, which can bind and signal through Egfr/ErbB1, are amphiregulin
(AREG), betacellulin (BTC), epidermal growth factor (EGF), epigen (EPGN), epiregulin
(EREG), heparin-binding EGF-like growth factor (HBEGF) and transforming growth
factor-α (TGFα). The second class of ErbB ligands, which signal exclusively through
ErbB3 and ErbB4, are the Neuregulins1-4 (NRG1, NRG2, NRG3, NRG4) (Yarden and
Sliwkowski 2001; Schneider et al. 2009; Schneider and Wolf 2009) (Fig 1.1). Adding to
the diversity of ErbB ligands, NRG1, for example, is spliced into 15 isoforms (Falls
2003).

1.4 Why are there so many ErbB ligands?

A major open question in cell signaling is why there are so many ErbB family ligands.
Individual ligands have specific roles in normal development, yet the mutant phenotypes
of knockout mice lacking a single ligand are mostly mild, indicating functional redundancy among ligands in development (Schneider et al. 2008). It is also possible that ligand-independent signaling occurs. This has been reported in mammalian cells in vitro, where overexpression of Egfr/ErbB receptors causes dimerization and activation of the pathway (Nagy et al. 2010; Endres et al. 2013).

*In vitro* systems can be designed to analyze the function of single ligands and single receptors. Yet despite all that can be learned through the use of *in vitro* techniques, such approaches have limitations for asking questions about development and normal function in Egfr signaling. In particular, the strong overexpression of a receptor(s) and the exogenous supply of ligands at saturating concentrations likely do not represent physiological conditions in development and homeostasis *in vivo*, when Egfr pathway components are under strict regulation. Saturating concentrations of receptor or ligand may reveal functions that are not present at physiological concentrations. This provides valuable information for research into pathologies such as in the many cancers in which ErbB components are implicated and often overactive, but may obscure the normal function of the pathway and interactions between pathway components. Knowledge of normal physiological Egfr signaling and its native regulation is also of value in the development of technologies applicable to the amelioration of human pathologies, in addition to being of general scientific interest.

The complexity of the vertebrate system—four receptors forming 14 active homo- and hetero-dimers (neither ErbB2 nor ErbB3 signal as homodimers) and a multitude of
possible ligands (7 Egfr ligands, 15 isomers of NRG1, NRG2-4), prohibits the analysis of individual ligands and receptors. For this reason, my thesis is focused on probing questions of ligand redundancy and specificity in *Drosophila*; the *Drosophila* Egfr pathway is described in the next sections.

1.5 Drosophila as a model for EGF ligand biology

*Drosophila* Egfr is the sole ErbB family member in the fly, removing confounding effects of receptor heterodimerization. There are only four EGF ligands: the TGFα-like ligands *spitz* (*spi*), *gurken* (*grk*), and *Keren* (*Krn*) and the neuregulin-like ligand, *vein* (*vn*) (Rutledge et al. 1992; Neuman-Silberberg and Schupbach 1993; Schnepp et al. 1996; Reich and Shilo 2002; Urban et al. 2002) (Fig 1.3, Fig 1.4). Furthermore, two of the four ligands, Grk and Krn, are dispensable for zygotic development. The two essential ligands are the TGFα-like ligand, Spitz, and the neuregulin-like ligand, Vein, representing two major classes of mammalian ligands (Egfr/ErbB1 ligands, including TGFα, and exclusive ErbB3/4 ligands, the neuregulins).

The relative simplicity of the *Drosophila* system makes it better suited to answer questions of ligand specificity and redundancy *in vivo* than any mammalian system. This simplicity, in addition to sophisticated genetics tools available, makes *Drosophila* a very attractive model for systematically removing ligands to observe combinatorial phenotypes and interrogate ligand redundancy or indeed to remove all of the ligands and investigate the possibility of ligand-independent signaling *in vivo*. *Drosophila* also
presents an attractive system for discerning the molecular basis of ligand specificity \textit{in vivo} by testing whether one ligand can replace the function of another if expressed in the correct endogenous pattern. Seeking answers to the major questions of ligand redundancy (Chapters 3 and 4) and ligand specificity (Chapter 4) is the rationale for my thesis work. In the next sections I describe what is known about the Egfr pathway in \textit{Drosophila} with a focus on the receptor and the ligands.

\textit{1.6 The Drosophila Egfr pathway}

The Egfr pathway in \textit{Drosophila} functions in multiple roles, including cell fate specification, migration, differentiation, and proliferation. Despite relative simplicity, the \textit{Drosophila} pathway generates sufficient complexity for development by deployment of the four ligands, which operate in both distinct and overlapping processes (Shilo 2003). Once the ligands activate Egfr, the signal is transmitted through the cannonical Ras/MAPK pathway to trigger induction of sets of target genes (Shilo 2003) (Fig 1.3).

\textit{1.7 Drosophila Egfr}

\textit{Drosophila} Egfr is most similar to ErbB2 among the mammalian receptors; \textit{Drosophila} Egfr and ErbB2 share a structural configuration distinct from vertebrate Egfr and ErbB3/4 (Alvarado et al. 2009) (Fig 1.2). It was previously reported that this “extended” configuration facilitated ErbB2 dimerization in the absence of ligand binding (Ferguson 2008), allowing receptor activation in the absence of a growth signal. ErbB2 is of
particular interest given that it is the preferred binding partner of other ErbB receptors, and for its involvement in human cancers—it is also known as the neu oncogene. ErbB2/HER2/neu is implicated in many different human cancers; in particular it is overexpressed in about 20-25% of breast cancers (Spector and Blackwell 2009). It has recently been shown, however, that the extended configuration common to Drosophila Egfr and ErbB2 is not a conformation that renders the receptor more potent because of its so-called ‘poised to dimerize’ structure, but contains many auto-inhibitory interactions that would make ErbB2 as dependent upon ligand binding as any of the other family members (Alvarado et al. 2009). This idea is supported by the body of Drosophila research that indicates that the structurally similar Drosophila Egfr is tightly dependent upon ligand binding (Alvarado et al. 2009). In this thesis I will directly show that all signaling by Drosophila Egfr is strictly ligand dependent in embryogenesis, providing further evidence for this argument (Chapter 3).

1.8 Drosophila Egfr ligands

Drosophila Egfr has four activating ligands representing two major classes of vertebrate ligands: the TGFα-like ligands spitz (spi), gurken (grk), and Keren (Krn) and the neuregulin-like ligand, vein (vn) (Rutledge et al. 1992; Neuman-Silberberg and Schupbach 1993; Schnepp et al. 1996; Reich and Shilo 2002; Urban et al. 2002) (Fig 1.3, Fig 1.4). The developmental consequences of removing any one of these four ligands are different, reflecting their diverse roles in normal development, yet all activate signaling through the sole receptor, Drosophila Egfr.
The three TGFα ligands are translated in inactive forms that require proteolytic cleavage by members of the Rhomboid family for activation (Urban et al. 2002) (Fig 1.3). Thus, the domain of Spitz activity is delineated by the expression of its processing factors while Vein, in contrast, is translated in active form, requires no processing prior to secretion (Schnepp et al. 1996; Shilo 2003), and the domain of activation of Vein is deliniated by its domain of expression (Wessells et al. 1999).

Vein is further differentiated from the TGFα-like ligands because it contains an Ig domain in addition to its EGF domain; the vertebrate neuregulins are the only other ligands shown to contain both domains (Schnepp et al. 1996) (Fig 1.5). Vein also contains a PEST domain, which affects protein degradation (Rechsteiner and Rogers 1996), suggesting that the rate of protein turnover and perdurance of the protein is regulated. Vein is commonly reffered to in the literature as the ‘weak’ ligand and Spi as the ‘strong’ ligand (Shilo 2003). This raises the interesting possiblility of comparing the ligands and designing experiments to determine whether the ‘weak’ ligand Vein induces only a subset of the downstream targets induced by the ‘strong’ ligand Spi, or if there is a qualitative difference between the ligands so that each induces some unique targets. In this thesis (Chapter 4), I present evidence that support the idea that the difference between Vn and Spi in Egfr signaling is primarily a quantitative difference.
1.9 The role of Egfr ligands in embryonic patterning

Of the four ligands, only *spi* and *vn* are required in embryonic development to pattern the embryonic cuticle. *spi* mutants die as embryos with ventral defects (Mayer and Nüsslein-Volhard 1988). *vn* mutants have milder ventral defects apparent in the embryo, but these are not severe enough to cause death at this stage and *vn* null mutants die at the pupal stage (Simcox 1997). *grk* has a strictly maternal role (Schupbach 1987). *Krn* null mutants are viable fertile adults suggesting that the gene has no critical function (McDonald et al. 2006).

Previous work from our lab has shown *spi* and *vn* function redundantly in embryogenesis because the double mutant (*spi; vn*) has a more than additive phenotype (Schnepp et al. 1996). This phenotype, however, does not recapitulate the phenotype of *Drosophila Egfr* mutants. The phenotype of the receptor mutant embryos is significantly more severe than that of the *spi; vn* double ligand mutants (Nusslein-Volhard et al. 1984; Schejter and Shilo 1989).

The phenotypic discrepancy between the zygotically required *Drosophila* Egfr ligand mutants and the receptor mutant suggests that either ligand-independent signaling occurs, which has been reported for *Drosophila* cells *in vitro* (Schweitzer et al. 1995), or that there is a redundant function provided by an additional ligand or ligands. As mentioned above, there is no known requirement for Keren, but it is a candidate ligand for a redundant function (McDonald et al. 2006).
Specifically, I hypothesized that the ligand Keren functions redundantly with Vn and Spi in embryonic development, and that there is no ligand-independent signaling through *Drosophila* Egfr. I investigated the requirement for ligand-mediated signaling and a possible redundant function of *Krn* in early development by examining mutants lacking all possible combinations of ligands and comparing the combinatorial mutant phenotypes with that of the receptor. If *Krn* functions redundantly with Spi and Vn in early development, removal of *Krn* should exacerbate the *spi* and the *vn* mutant phenotypes, as well as exacerbating the phenotype of the *spi; vn* double mutant. If there is no ligand-independent signaling through the receptor in early development, removal of all zygotically active ligands should result in a phenocopy of the *Egfr* mutants. Here I show that there is indeed a role for *Krn* in embryogenesis, but it is only revealed in mutant combinations, and that there is no evidence for ligand independent signaling in embryogenesis (Chapter 3).

1.10 The role of *Egfr* ligands in wing development

The *Drosophila* wing has long been a model for the role of signaling pathways in development. The Egfr pathway plays a major role in wing development and our lab has shown that Vn is a key ligand. *vn* has a role in both early wing development to initiate signaling that results in the proliferation of the cells of the wing imaginal disc, as well as in wing patterning in later development when the wing blade differentiates into vein and
intervein territories. No role for *spi* and *Krn* has been reported in wing development (Simcox et al. 1987; Simcox 1997; Guichard et al. 1999; Nagaraj et al. 1999).

Spi and Krn require processing by the Rhomboid protease to become active (Sturtevant et al. 1993; Guichard et al. 2000; Ghiglione et al. 2002; Urban et al. 2002); therefore, *rho* mutants are expected to lack the function of both *spi* and *Krn*. Unlike either *spi* or *Krn* single ligand mutants, *rho* mutants have a vein loss phenotype, and *rho, vn* double mutants lack all veins (Sturtevant et al. 1993; Sturtevant and Bier 1995). This requirement for *rho*, the upstream regulator of *spi* and *Krn*, but neither of the individual ligands, suggests as yet undescribed and redundant roles for TGFα ligands in wing development.

Specifically, I hypothesized that *spi* and *Krn* act redundantly in wing vein patterning. By taking a genetic approach and examining mutant combinations, I found Spi and Krn do indeed contribute to wing vein patterning in a redundant fashion (Chapter 3).

1.11 Drosophila EGF ligand specificity

The *Drosophila* EGF ligands represent two major classes, TGFα and neuregulin. This offers the possibility of testing ligand specificity by determining if a ligand in one class can replace the function of another in development. Previous attempts to rescue *vn* mutants with broad expression of *vn* itself or with activated forms of the receptor, Rho (the protease involved in Spi production), or activated MAPK were unsuccessful.
Ubiquitous expression of \( vn \) in the embryo and imaginal discs only partially rescues \( vn \) mutants. Proliferation in the wing disc is restored, but patterning is abnormal; the discs are larger than wild type and have a duplication of the wing pouch. Gain of function alleles of the receptor (\( Egfr^{Elp} \)) and MAPK (\( rolled^{Sevenmaker} \)) also partially rescue \( vn \) mutants, but as with broad overexpression of Vn itself, the wing discs had patterning abnormalities (Schnepp et al. 1996; Zecca and Struhl 2002).

In an ideal experiment, the ligand to be tested would be expressed in the pattern of the ligand it is replacing. This is analogous to knock-in technology in mice—in which one ligand gene can be inserted in the locus of another ligand. This simultaneously makes a mutation in the ‘host’ gene and causes expression of the other in the ‘host’ pattern. In \textit{Drosophila}, a similar experimental design can be accomplished using the Gal4-UAS system (Brand and Perrimon 1993). I created the required reagents to test the ability of each of the TGF\( \alpha \) ligands to replace the function of \( vn \) in development. The results presented in Chapter 4 show that, surprisingly, each of the TGF\( \alpha \) ligands rescued \( vn \) mutants. The results support a model in which a ‘strong’ ligand and a ‘weak’ ligand differ in their quantitative effects on signaling.
Figure 1.1 Vertebrate Egfr pathway components. The vertebrate Egfr ligands are initially synthesized as membrane bound precursors and are cleaved by specific proteases to release soluble ligand. On ligand binding, the kinase domains of the receptors in the dimer are activated. Trans-autophosphorylation of the kinase domains results in the recruitment of adaptor molecules and the activation of downstream signaling. There are three additional receptors in the vertebrate Egfr/ErbB family: ErbB2/HER2/neu (which has no known ligand), ErbB3 (which has a defective kinase domain), and ErbB4. Four neuregulin ligands signal exclusively through binding ErbB3 and ErbB4. Reproduced with permission (Schneider et al. 2009).
Figure 1.1

Adapted from Schneider et al. 2009
Figure 1.2 The monomer structure of ErbB2 resembles ligand-bound Egfr/ErbB1 as well as Drosophila Egfr. (a) The inactive monomer conformation of human Egfr is “tethered” by autoinhibitory interactions between domains of the protein. Upon ligand binding, the conformation changes to the “extended” conformation. (b) Space filling model of ligand-bound human Egfr. (c) Space filling model of ErbB2 with no ligand bound, mimicking the “extended” conformation. (d) The monomer conformation of Drosophila Egfr with no ligand bound also resembles the “extended” receptor conformation. Reproduced with permission (Alvarado et al. 2009).
Figure 1.2

Alvarado et al. 2009
**Figure 1.3 Drosophila Egfr pathway components.** The TGFα ligands (yellow) are synthesized as membrane-bound precursors which require the trafficking factor Star (maroon) and cleavage by a Rhomboid protease (orange) to be secreted as active ligands. The neuregulin ligand Vn (blue) is synthesized as an active ligand and is secreted upon translation. Ligand binding of Egfr triggers a downstream signaling cascade involving Ras and MAPK, ultimately resulting in the activation of transcription factors and transcription of pathway targets.
Figure 1.3

Neuregulin
Vein

Spitz
Keren
Gurken

Egfr

Ras

Map Kinase

Transcription Factors
transcriptional targets

Rhomboid: Protease
Star: Trafficking Factor
Figure 1.4 Activating ligands of the *Drosophila* Epidermal Growth Factor Receptor.

*Drosophila* Egfr has four ligands. Three resemble TGFα and are synthesized as membrane-bound precursors, requiring processing prior to secretion. The fourth ligand Vn resembles a neuregulin, contains an Ig domain in addition to the EGF domain, and is secreted upon translation. Reproduced with permission (Shilo 2003).
Figure 1.4

Adapted from Shilo 2003
Figure 1.5 Vein shares an Ig domain and a PEST domain with some vertebrate neuregulins. *Drosophila* TGFα ligands (*spi, grk*, and *Krn*) contain neither PEST nor Ig domains; however, some vertebrate neuregulins contain one or both, as does the *Drosophila* ligand Vn.
Figure 1.5

Vein

Neuregulin 1
Chapter 2: Methods

2.1 Fly culture conditions

Flies were grown on standard cornmeal-molasses media, according to the recipe published by the Bloomington Stock Center (“Current Bloomington Recipe” 2013). While vials cooled, they were covered in cheesecloth. Once cool, the cheesecloth was removed and vials were covered tightly with Press-n-Seal cling wrap (Glad), and stored at 4°C in large Ziplock bags until used. Food was supplemented with live yeast.

Flies were grown at 25°C unless otherwise specified. Weak or low fecundity stocks sometimes required the addition of a square of tissue with the center poked into the food with a spatula (to provide flies a dry place to stand) or the addition of small amounts of ddH2O to rehydrate food.

2.2 Generation of multiple mutant ligand stocks

The following mutant alleles were used: spi^{2A}, grk^{HF}, vn^{1}, vn^{16}, Krn^{27}, Krn^{9}, Egfr^{3F18} and Egfr^{F24}. Stocks with combinations of alleles were generated from existing single mutants,
with the exception of a $sp^{2A}$, $grk^{HF}/CyO$ stock that was a gift from Trudy Schüpbach. Oregon R flies were used as wild type.

2.3 vn and Kn recombinant genotyping

$Vn$ recombinants were tested for presence of the vn allele by crossing to a $vn^{RG}/Bal$ stock and examining the phenotypes of the progeny. $vn^{l}$ recombinants were identified by loss of the anterior crossvein and partial loss of L4, and $vn^{L6}$ recombinants were identified by lethality.

$Kn$ recombinants were genotyped by PCR, as homozygotes are viable and have no easily scored phenotype. Primers were used to amplify across the deleted region and gave a band of ~1 kb for $Kn^{27}$ or ~1.2 kb for $Kn^{9}$. These primers span over 8 kb of wild type sequence; wild type amplification would be easily distinguished, but in my hands did not occur. (”kn del” F: $AGTCGGCGGCCGTCAATTCG$, R: $TCCTGGGGCTCCTTGCGTGT$). A 423 bp wild type sequence within the deletion region was amplified as a control for the quality of template DNA, to ensure that the absence of a deletion band was not simply due to poor DNA isolation. (”kn wt” F: $TGCCACAGCCGCTTGGGTTC$, R: $GCCTGTTCCTCGTGCCAT$).
2.4 Embryo cuticle preparation and determination of embryo phenotype

Embryos were manually dechorionated on double-sided tape, devitalinized in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$) with a tungsten needle, cleared in lactic acid/ethanol (9:1) overnight, and mounted in Hoyer’s Medium (50 mL distilled water, 30 g Gum Arabic, 200 g Choral Hydrate, 20 mL glycerin) (Anderson 1954). Embryos were photographed, and were examined for size, formation of head skeleton, the presence or absence of an anterior hole, and denticle size, shape, and number (or absence).

2.5 Wing preparation and analysis

Adult flies were dissected in ethanol. Wings were mounted in Berlese’s Solution (chloral hydrate/gum Arabic solution), photographed, and scored for the presence or absence of defects in a given vein.

Wing area was measured by tracing wing margins, using the brush selection tool and Analyze/Measure function in ImageJ version 1.47t. The margins were traced from landmark 1 around to landmark 2 (Fig 2.1), and then closed between the landmarks to define the wing blade area.
2.6 Statistics

A two sample Z test was employed to test whether the fraction of embryos with denticles was significantly different between the quadruple ligand mutant embryos and receptor mutant embryos.

A two sample T-test (ANOVA) was employed to test the significance of the difference in group means for wing blade area.

2.7 vn-Gal4 Generation

The \textit{vn-Gal4} allele was generated in using the method of Sepp and Auld (Sepp and Auld 1999). The \textit{vn-lacZ} element (\textit{P}[\textit{PZ}, \textit{ry}^+] \textit{vn}^{rF264}, FBti0005059) was replaced with the \textit{P}[\textit{GawB}, w^+] element from the male-lethal \textit{PG142/FM7} stock (FBti0022312, a gift from Norbert Perrimon).

A stock containing both a male-lethal Gal4 element (\textit{w}+) and the \textit{vn-lacZ} (\textit{ry}+) element, in a \textit{white} background, was crossed to a stock bearing the \textit{P}[\Delta2-3] transposase on a Sb marked chromosome (FBst0003629) to mobilize the P-elements. The female progeny of this cross, which contained all three elements (\textit{Gal4}, \textit{lacZ}, and transposase), were crossed to \textit{TM3, Ser/TM6} males. All non-Sb male progeny with colored eyes were individually crossed with \textit{TM3, Sb/TM6} females to generate balanced stocks (Fig 4.1A). The stocks were tested to determine the expression pattern of the new Gal4 insertions by crossing to
*UAS-GFP* (visualized directly with UV light) and *UAS-lacZ* (visualized by staining as described in section 2.8).

Of 4197 male progeny examined, 33 had colored eyes indicating a transposition event and 3 matched the *vn* expression pattern. Male #24 was the first identified and the resultant stock was used for all further applications.

2.8 *lacZ* staining

Late third instar larvae were dissected in PBS, divided into anterior and posterior halves, and the anterior half was inverted. Carcasses were transferred to a 24-well plate in PBS, rinsed with PBS, and fixed in 1% glutaraldehyde for 20 minutes. Following fixation, samples were rinsed 3 times with PBS and once with staining solution (3.1 mM K3Fe(CN)6, 3.1 mM K4Fe(CN)6, 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1 mM MgCl2). Samples were then stained in staining solution with 0.2% X-gal for 30 minutes to overnight. Staining was halted by rinsing several times with PBS.

2.9 *vn-Gal4* mapping

The *P[GawB]* element insertion into the *vn* 5’ UTR was mapped by sequencing PCR amplicons spanning the boundaries of the insert. Internal primers were designed pointing outward, and a variety of potential primers oriented toward the center of the 5’ UTR were
tested in a pairwise fashion to locate the insert ends. Successful amplicons were sequenced to confirm the location of the insert ends.

Primer pairs: 5’ end forward “vg4 fish3F” AGAGCCGACTGTTGCGACGAC; 5’ end reverse “pGawB mapZ” TGCACGTTTTGCTTGTTGAGAGG; 3’ end forward “pGawB mapD” AGCTAGAGCTTTGCGTGACTCGC; 3’ end reverse “vnL6 seq R” ACGCCTGGGCGGGATCT.

2.10 Determination of vn-Gal4 phenotype

vn-Gal4 flies were crossed to vnL6 flies and the progeny were examined. The phenotype of vn-Gal4/vnL6 flies in third instar wing discs, phenotype at death, and timing of lethality was compared to that of flies heterozygous for known molecular null alleles, vnL6/vnRG.

To examine third instar wing discs, larvae were dissected in PBS and the size of the wing discs was examined. vn mutant wing discs fail to proliferate and are very small. vn mutants die shortly after forming their pupal case and prior to forming any pigmented adult cuticle; they do not form any wing disc-derived structures. Dead mutants were dissected out of their pupal cases in 95% ethanol and examined for the presence or absence of pigmented cuticle and wing disc-derived structures.
2.11 Visualization of Gal80 inhibition of vn-Gal4 activated transgene expression

A tub-Gal80<sup>TS</sup>; vn-Gal4/TM6, Tb stock was generated using a temperature-sensitive Gal80 stock (P[tubP-GAL80<sup>ts</sup>], FBti0027796). tub-Gal80<sup>TS</sup>; vn-Gal4/TM6, Tb and vn-Gal4/TM6, Tb flies were crossed to UAS-GFP flies; progeny were examined for GFP expression by detection by Western blotting and by visual examination of the expression pattern.

Protein samples from late third instar larvae raised at 25°C and lacking the Tb marker (ensuring selection of larvae carrying vn-Gal4) were prepared as described in section 2.12. Western blots were performed as described in section 2.13. 15 µg of total protein was loaded in lanes marked 100% (Fig 4.4A). Different fractions (by volume) of uninhibited (vn-Gal4/UAS-GFP) larvae were analyzed for comparison with the signal from inhibited (tub-Gal80<sup>TS</sup>; vn-Gal4/UAS-GFP) larvae. The following antibodies were used: anti-GFP (G10362, Invitrogen, 1:2000), and Cy3-conjugated goat anti-Rabbit IgG (111-165-045, Jackson ImmunoResearch, 1:4000).

GFP fluorescence in live larvae raised at both 25°C and 29°C was visualized directly.

2.12 Preparation of protein samples from larvae

Larvae were rinsed in PBS and homogenized in Tn1 buffer (125 mM NaCl, 50 mM Tris, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton-X, 1x Roche
“Complete, mini” Protease inhibitor cocktail), 2 μL buffer/mg larvae. The homogenate was incubated on ice for 30 minutes. The homogenate was then centrifuged at 4°C for 15 minutes, and supernatant from below the lipid layer was removed to a clean tube. 4X Laemmli buffer with urea (4X: 0.8% w/v SDS, 40% v/v glycerol, 24% v/v 1M Tris pH 6.8, 0.04% w/v Bromophenol Blue, 2.5 M urea) was added to a final concentration of 1X. Samples were boiled for 10 minutes, incubated on ice for 2 minutes, and centrifuged at 14k RPM (~18,000 x g) for 10 minutes. Samples were used immediately or stored at -80°C overnight. Samples were not usable after prolonged storage (approximately one week or more), due to protein degradation.

2.13 Western Blot Protocol

All steps were performed at room temperature unless otherwise noted. Samples were run on 5x8 cm, 0.75 mm acrylamide gels (Stacking gel: 5% final concentration of acrylamide from 40% liquid stock solution, 10% v/v 1M Tris pH 6.8, 0.1% w/v sodium dodecyl sulfate (SDS), 0.1% w/v ammonium persulfate, 0.1% v/v TEMED. Running gel: 12% final concentration of acrylamide from 40% liquid stock solution, 25% v/v 1.5 M Tris pH 8.8, 0.1% w/v sodium dodecyl sulfate (SDS), 0.1% w/v ammonium persulfate, 0.1% v/v TEMED). Gels were run at 100V in SDS Running Buffer (25 mM Tris-HCl pH 7.6, 192 mM Glycine, 0.03% SDS) until the blue dye front reached the bottom of the gel. Protein from the gel was transferred to PVFD membrane in a BioRad Mini-Protean apparatus with ice insert, in 1X Running Buffer with 10% methanol at 15V overnight at 4°C, with stirring to eliminate a temperature gradient in the buffer.
Membranes were rinsed once with 5% acetic acid, followed by two 5 minute washes with 5% acetic acid. All incubations and washes for Western blot analysis were conducted on a rotary platform with medium agitation. Following the acetic acid wash, membranes were washed in Ponceau S protein stain (0.1% w/v Ponceau S in 5% v/v Acetic Acid), to verify efficacy of transfer, for 5 minutes, then rinsed repeatedly with distilled water until the water remained clear. Membranes were rinsed twice in TBST (19 mM Tris base, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween-20) prior to blocking. Blocking was in 5% dry milk powder in TBST for one hour. Following blocking, membranes were incubated in primary antibody diluted in TBST with 5% dry milk for one hour, washed 3 times for 5 minutes in TBST, followed by incubation in secondary antibody for one hour. Membranes were washed 3 times for 20 minutes before development with the SuperSignal West Pico Chemiluminescent Substrate kit from Thermo Fisher, following manufacturers’ instructions, and detection on X-ray film.

2.14 Rescue of vn mutants with UAS-vn

UAS-vn1.1; vnL6/TM6, Tb flies were crossed to vn-Gal4/TM6, Tb and Gal80TS; vn-Gal4/TM6, Tb flies and progeny were raised at 17° C, 25° C, and 29° C. Second to early third instar mutant larvae were sorted by absence of the Tb marker into new food vials of no more than 50 larvae. Sorting and counting larvae allowed calculation of the fraction of total larvae achieving each developmental stage. Animals were counted as pharate adults
if they exhibited pigmented cuticle and had formed legs. Animals were counted as eclosed adults if they had partially or fully emerged from their pupal case.

2.15 Rescue of vn mutants with TGFα ligands at 17°C

Stocks were generated homozygous for UAS-ligand transgenes, in combination with $vn^{L6}/TM6$, $Tb$ and crossed to $vn-Gal4/TM6$, $Tb$. $vn^{L6}/vn-Gal4$ larvae or pharate adult progeny were identified by lack of the $Tb$ phenotype. The following transgenic alleles were used: $UAS-vn1.1$ (Schnepp et al. 1996), $UAS-sspi$ (Schweitzer et al. 1995), $UAS-sKrn$ (Urban et al. 2002), and $UAS-sgrk$ (Queenan et al. 1999). Oregon R flies were used as wild type.

Second to early third instar larvae were sorted, by lack of the $Tb$ marker, into new food vials of no more than 50 larvae to allow weak genotypes to survive in the absence of competition from their more vigorous siblings. Counting and sorting larvae also allowed calculation of the fraction of total larvae achieving each developmental stage. Animals were counted as pharate adults if they exhibited pigmented cuticle and had formed legs. Animals were counted as eclosed adults if they had partially or fully emerged from their pupal case.
2.16 Optimizing conditions for rescue of vn mutants

*UAS-\textit{vn}1.1* (Schnepp et al. 1996), *UAS-\textit{sspi}* (Schweitzer et al. 1995), *UAS-\textit{sKrn}* (Urban et al. 2002), and *UAS-\textit{sgrk}* (Queenan et al. 1999) were used as above. Oregon R flies were used as wild type.

*UAS-ligand; vn\textit{L6}/TM6, Tb* stocks were crossed to *vn-Gal4/TM6, Tb* and *Gal80\textit{TS}; vn-Gal4/TM6, Tb*. Flies from both crosses, using each ligand, were raised at 17° C, 20° C, 25° C, and 29° C. Rescued larvae or pharate adults were identified by lack of the Tb phenotype.

2.17 Analysis of pharate adult phenotypes

*UAS-ligand; vn\textit{L6}/vn-Gal4* pharate adults were identified by the lack of the Tb maker. Pupal cases were removed from pharate adults in 95% ethanol. Animals were counted as pharate adults if they exhibited pigmented cuticle and had formed legs. Pharate adults were photographed and scored on the presence or absence of wing(s) and notum.

2.18 Wing and notum mounting

Pharate adults were dissected out of their pupal case in 95% ethanol. Eclosed adults were preserved in 95% ethanol.
Adult wings were removed in ethanol and mounted in Berlese’s solution. To prepare the nota of adults and pharate adults and wings of pharate adults, which are not expanded, the flies were processed in KOH. Adults (lacking wings) and pharate adults were rehydrated in sequential incubations of 70%, 50%, 30% ethanol, PBS, rinsed 3 times in PBS, then incubated in a covered dish of 10% KOH overnight on a slide warmer to remove soft tissue and inflate the wings of the pharate adults. Flies were then dehydrated through incubations of 30%, 50%, 70%, 100% ethanol, and rinsed twice more in 100% ethanol. In 100% ethanol, the notum of each fly and the wings of the pharate adults were removed; wings and nota were both mounted in Berlese’s solution.

2.19 Western blot analysis of ERK signaling in larvae

Flies were raised at 25°C. The following genotypes were use: Oregon R, vn\textsuperscript{L6}/vn-Gal4, UAS-sspi; vn\textsuperscript{L6}/vn-Gal4, UAS-sspi; Gal80\textsuperscript{TS}; vn\textsuperscript{L6}/vn-Gal4, UAS-vm1.1; vn\textsuperscript{L6}/vn-Gal4, UAS-vm1.1; Gal80\textsuperscript{TS}; vn\textsuperscript{L6}/vn-Gal4.

UAS-ligand; vn\textsuperscript{L6}/vn-Gal4 and UAS-ligand; Gal80\textsuperscript{TS}; vn\textsuperscript{L6}/vn-Gal4 second to early third instar larvae were sorted, by lack of the Tb marker, into new food vials of no more than 50 larvae. This was to allow weak genotypes to progress to late third instar in the absence of competition from their more vigorous siblings (vn\textsuperscript{L6}/TM6, Tb and vn-Gal4/TM6, Tb larvae). Protein samples from late third instar larvae were prepared as described in section 2.12.
Western blots were performed as described in section 2.13, using the following antibodies: anti-Phospho-p44/p42 MAPK (#9106, Cell Signaling Technology, 1:1000 dilution), anti-ERK 2 (K-23, Santa Cruz Biotechnology, Inc., 1:500), and Cy3-conjugated goat anti-Rabbit IgG (111-165-045, Jackson ImmunoResearch, 1:4000).

2.20 Quantitation of ERK signal from Western blots

Three biological replicates were analyzed. Quantitation of Western blots was carried out using the gel analyzer tools in ImageJ version 1.47t. For each blot, intensity measurements were normalized to the wild type sample for that blot. The mean of the normalized values for each genotype are displayed in the histogram (Fig 4.9). Error bars represent the highest and lowest (normalized) intensity measurement observed for each genotype.

2.21 Rescue of vn mutant flies with additional sSpi constructs

Stocks were generated with UAS-ligand transgenes in combination with vnL6/TM6, Tb and crossed to vn-Gal4/TM6, Tb or Gal80TS; vn-Gal4/TM6, Tb. Rescued larvae or pharate adults were identified by lack of the Tb phenotype. The following transgenic alleles were used: UAS-sspi^{49A} (A gift from Norbert Perrimon), UAS-sspiGFP^{M36}, and UAS-sspiGFP^{F5N}.
2.22 Rescue of vn mutant flies with vn mutant constructs

Stocks were generated with \textit{UAS-ligand} transgenes in combination with \textit{vn}^{L6}/\textit{TM6}, \textit{Tb} and crossed to \textit{vn-Gal4}/\textit{TM6}, \textit{Tb} or \textit{Gal80}^{TS}; \textit{vn-Gal4}/\textit{TM6}, \textit{Tb}. Rescued larvae or pharate adults were identified by lack of the \textit{Tb} phenotype. The following transgenic alleles were used: UAS-vn1.2, UAS-vn2.8, UAS-vnGFP^{58}, UAS-vnGFP^{12}, UAS-\textit{sspi}^{49A}, UAS-\textit{sspiGFP}^{M36}, UAS-\textit{sspiGFP}^{F5N}, UAS-vn\textit{AMR3.3}, UAS-vn\textit{AMR2.12}, UAS-vn::Aos\textit{EGF2.1}, UAS-vn\textit{AMR4.1}, UAS-vn\textit{AMR1.1}, UAS-vn\textit{AMR3.39}, UAS-vn3\textit{AMR3.64}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn\textit{AMR4.3}, UAS-vn::spi\textit{EGF2.1}, UAS-vn::vn\textit{EGF1.1}. 

Figure 2.1 Landmarks for measuring wing area. A wild type wing is shown. (A) Two landmarks were chosen to ensure consistency in the measurement of wing blade area. (B) Beginning at Landmark 1, the wing margin was traced as closely as possible until reaching Landmark 2. A straight line was drawn between Landmark 2 and Landmark 2. The wing blade area was taken as the measurement of the area of the enclosed region.
Figure 2.1
Chapter 3: Requisite and Redundant Functions of Egfr Ligands in Embryo and Wing Development

*Drosophila* has four Egfr ligands, but only two (*spi* and *vn*) have zygotic defects in embryonic ventral patterning as single mutants. *grk* mutants have a strictly maternal role and phenotype (Neuman-Silberberg and Schupbach 1993; Shilo 2003) and *Krn* mutants are known to be viable and fertile (McDonald et al. 2006).

*spi* mutants are embryonic lethal (Mayer and Nüsslein-Volhard 1988). They have narrow head structures and defects in their ventral denticle belts; they successfully pattern 8 abdominal denticle belts, but the belts are abnormally shaped and have partial fusions (Rutledge et al. 1992) (Fig 3.1B). *vn* mutant embryos survive embryogenesis, but have subtle ventral patterning defects, and imaginal disc defects later in development (Simcox et al. 1987; Schnepp et al. 1996). *vn* mutant embryos have all 8 abdominal denticle belts.

The *spi; vn* double mutant is more severe than either single mutant, with a more than additive phenotype that is indicative of ligand redundancy. The *spi; vn* double mutant embryos have disrupted head skeleton structures and an anterior-ventral hole. They have a reduced number of abdominal denticles belts (4-6), with some partial fusions similar to those seen in the *spi* single mutants (Schnepp et al. 1996) (Fig 3.1C).
However, *Egfr* mutant embryos have a phenotype that is significantly more severe than that of the *spi; vn* double mutant. The phenotype of the receptor mutant embryos—severely disrupted and reduced head structures, a large anterior hole, reduced cuticle, and near-complete dorsalization of the embryo—originally garnered the gene name *faint little ball* (Nusslein-Volhard et al. 1984; Schejter and Shilo 1989) (Fig 3.1D, Fig 3.2).

The discrepancy between the phenotype of embryos lacking the receptor and the phenotype of embryos lacking both zygotically required ligands (*spi* and *vn*) suggested three possibilities. First, it is possible that an additional undescribed ligand (or ligands) is functioning in early development. Second, it is possible that either *Krn* or *grk*, or both, have redundant function during early development. Third, it is possible that ligand-independent signaling through Egfr functions in early development, a mechanism that had been previously described in *Drosophila* cell culture (Schweitzer et al. 1995).

### 3.1 Egfr-mediated embryonic patterning is dependent on function of all three zygotically active ligands

I hypothesized that the additional functional ligand is *Krn*, as recent evidence detailed redundant functions for *Krn* in other developmental processes, including functions in the eye, gut, ovary and brain which are revealed when other ligands are compromised, 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; Rahn et al. 2013).
To investigate, I generated mutant stocks in all combinations of the four ligands and examined the single and combinatorial embryonic phenotypes. If grk has no zygotic function, removal of grk should have no phenotypic effect on either single or combinatorial ligand mutant genotypes. Given no zygotic role for grk, if removal of Knr has no phenotypic effect or if the spi; vn, Knr mutant failed to phenocopy the Egfr mutant, it would indicate the action of either additional ligand(s) or ligand-independent signaling in early embryonic development. If the spi; vn, Knr triple mutant and the spi, grk; vn, Knr quadruple mutant are comparable and both phenocopy the Egfr mutant, it may be concluded that Knr is the missing ligand and has a redundant role in early embryonic development. The phenocopy of the receptor mutant by embryos lacking all zygotic ligand function would also not support a model including ligand-independent signaling in early development.

The ligand grk is known to have a solely maternal effect and be expressed only in the female germ line, in the ovary (Neuman-Silberberg and Schupbach 1993; Shilo 2003). Therefore, it was not expected that removal of the maternally active ligand grk would have any phenotypic effect on other ligand mutant genotypes. As expected, removal of grk had no additional effect on the spi or vn or Knr, or on the spi; vn or spi; Knr or vn, Knr phenotypes (not shown).

While Knr mutants are viable and can be fertile, they have a very low hatch rate (<5%, Knr<sup>27/27</sup>).
I also found that *Krn* does play a redundant role in embryonic cuticle patterning. *spi; Krn* double mutants (Fig 3.1E) had a similar phenotype to *spi; vn* double mutants (Fig 3.1C), with disrupted head skeleton, an anterior hole, reduced number of abnormally shaped denticles belts, and denticles belts with partial fusions. *vn, Krn* double mutants survived embryonic development with grossly normal cuticle patterning (*vn* mutants have reduced distance between their Keilin’s sensory organs (Schnepp et al. 1996)). *vn* mutants survive the three larval instar stages and die after pupation, but *vn, Krn* double mutants survived only into the second larval instar stage. The *spi; Krn* and *vn, Krn* double mutant phenotypes support the model in which *Krn* has redundant function with both *spi* and *vn* in development.

The triple mutant, *spi; vn, Krn* was much more extreme than either double mutant (*spi; vn* or *spi; Krn*) and was indistinguishable from an *Egfr* null mutant (Fig 3.1F, D). Both the triple ligand mutants and the receptor mutants lack head structures and show near-total dorsalization of the embryo. Removal of *grk*, in the quadruple mutant (*spi, grk; vn, Krn*), had no additional phenotypic effect (Fig 3.1G, Fig 3.3), supporting the long-standing model that *grk* has only maternal effect.

Denticle belts mark ventral cell fates in the embryo. While no fully-formed denticles belts were observed in either the *Egfr* mutant embryos or the quadruple ligand mutant embryos, in both, approximately half of the embryos had a small number of denticles in up to two very small patches per embryo (Fig 3.4).
The phenotypic equivalency between embryos lacking all ligands and those lacking the receptor itself suggest there is no signaling through Egfr in the absence of the known ligands (Fig 3.1D, F-G). It is unlikely that there are yet unknown Drosophila Egfr ligands as the Drosophila genome is completely sequenced. Krm itself was identified following the completion of the Drosophila genome sequence (Urban et al. 2002), but neither that study nor functional screens have identified any additional ligands.

Ligand-independent signaling has been reported for overexpression of Egfr in Drosophila S2 tissue-culture cells where the high levels of Egfr are thought to cause spontaneous dimerization and signaling (Schweitzer et al. 1995). This is also the case in mammalian cells, where overexpression of Egfr/ErbB receptors causes dimerization and activation of the pathway (Nagy et al. 2010; Endres et al. 2013). My results suggest that at the normal physiological level of the receptor in the fly embryo, all signaling is ligand dependent. The double and triple ligand mutant combinations, however, suggest extensive redundancy between the ligands consistent with flexible roles that are only revealed when multiple ligands are compromised.

3.2 Wing vein patterning requires the function of all three zygotic ligands

I next wished to determine if the ligands also had redundant and flexible roles in another context and examined wing development.
vn is known to play a role in both early wing disc growth and the later patterning of the wing blade into territories of veins and interveins (Shearn et al. 1971; Simcox et al. 1987; Paul et al. 2013). In contrast, no required role has been found for spi in either growth or patterning of the wing. spi mutant wing discs cultured in vivo differentiate morphologically normal wing tissue. Further, spi mutant clones have no effect on wing vein patterning even in a vn hypomorphic background (Simcox 1997; Guichard et al. 1999; Nagaraj et al. 1999). Likewise, flies homozygous for a complete deletion of the Krn locus are viable and have normally patterned wings (McDonald et al. 2006) (Fig 3.5B).

The TGFα ligands, including spi and Krn, require processing by the Rhomboid protease to become active (Sturtevant et al. 1993; Guichard et al. 2000; Ghiglione et al. 2002; Urban et al. 2002); therefore, rho mutants are expected to lack the function of both spi and Krn. Unlike either spi or Krn single ligand mutants, rho mutants have a vein loss phenotype, and rho, vn double mutants lack all veins (Sturtevant et al. 1993; Sturtevant and Bier 1995). This requirement for rho, the upstream regulator of spi and Krn, but neither of the individual ligands, suggests redundant roles for TGFα ligands in wing development.

In contrast to the normally patterned wings of Krn null mutants (Fig 3.5B), vn hypomorphs exhibit loss of the anterior crossvein (ACV) and portions of the posterior crossvein (PCV), longitudinal vein four (L4), as well as a reduced overall wing size (Fig 3.5C, Fig 3.6C-D, Fig 3.7). Rarely (7%, n=55), vn hypomorphic flies lack parts of
longitudinal vein three (L3). This phenotype was exacerbated in vn, Krn double mutants, of which about half lack portions of L3 (52%, n=64, Fig 3.5D, 3.6B). This suggests that Krn has a redundant role in wing vein patterning.

Reducing the dose of spi did not produce additional vein loss in vn hypomorphic flies (Fig 3.5E, Fig 3.6B), but when Krn was also absent in these animals, the most extreme phenotype was seen (Fig 3.5F, Fig 3.6B). In these mutants 90% had partial deletions of L3 (n=69), and the deletions were more extensive. This further suggests that in the absence of Krn, in vn hypomorphs, there is still compensatory redundant function of spi.

Of the three zygotically active ligands (spi, vn, and Krn), only vn mutants have a wing phenotype as single mutants. Removing the function of both TGFα ligands (spi and Krn) by removing their processing factor rho greatly increases the severity of the vein loss phenotype for which vn is named, to an extent far greater than individually removing either spi (which has no effect on vein loss) or Krn (which has a mild effect on vein loss). Reducing the dose of spi in the absence of Krn, however, has a much stronger effect on the vn hypomorphic vein loss phenotype. Together the data show that all three zygotic ligands play a role in wing patterning, with redundant roles for spi and Krn, but that only the function of vn is non-redundant.
3.3 Effect of ligands on wing size

In addition to the patterning effects, the multiple ligand mutant combinations, exhibit a phenotype regarding wing size. Reducing the dose of \( vn \), as in \( vn \) hypomorphic flies, reduces total wing area. Reducing the dose of either TGF\( \alpha \) ligand (\( spi \) or \( Kn \)) by half, in contrast, results in an increase in total wing area (Fig 3.7, compare the first two columns in each grouping, as well as the first versus second group and the third versus fourth group). The effect of reducing the dose of \( spi \) is more marked in flies that are wild type at the \( vn \) locus (Fig 3.7, Groups 1 and 2). The effect of reducing the dose of \( Kn \) by half is more marked in the \( vn \) hypomorphic flies. Removal of a second dose of \( Kn \) erased this \( Kn \)-influenced increase in wing size. This suggests that there may be a balance between proliferation and differentiation instructive signaling in the developing wing that is at least partially managed by which ligands are signaling. The weaker ligand \( vn \) may instruct primarily proliferation while the stronger TGF\( \alpha \) ligands \( spi \) and \( Kn \) may signal both proliferation and differentiation.

3.4 Concluding remarks

It has long been known from studies utilizing mammalian PC12 cells that the duration of MAPK signaling induced by NGF versus EGF can mean the difference between proliferative signaling and differentiation. Treatment of PC12 cells with NGF results in several hours of elevated MAPK activation and cessation of proliferation and outgrowth of neurites. Treatment of PC12 cells with EGF results in transient MAPK activation and
proliferative cell growth (Marshall 1995; Vaudry et al. 2002). If the Drosophila wing, as a system, is dependent upon a competitive balance of Egfr activity from two types of signals: Vn primarily instructing proliferative signaling (inferred from the unique role of vn in early wing development where it induces target genes that lead to proliferation) and the stronger TGFα ligands Spi and Krn instructing both growth and differentiation, it then follows that perturbation of the system by removing one dose of TGFα ligand may result in more cell proliferation and thus larger wings. Removal of an additional dose of TGFα ligand may reduce total Egfr signaling such that no excess proliferation occurs.
Figure 3.1 Egfr signaling is strictly ligand-dependent in embryogenesis. (A) Wild-type embryo, with eight denticle belts (open circles). (B) spi^{2A} mutant embryo, with eight narrow denticle belts (open circles), some of which are fused (*). (C) spi^{2A}; vn^{L6} double mutant embryo, with a reduced number of denticle belts (open circles), fusions (*), and an anterior hole (arrow). (D) Egfr^{3F18/F24} receptor null embryo, which is very short with no denticle belts and a large anterior hole (arrow). (E) spi^{2A}; Krn^{27} double mutant embryo, with a reduced number of denticle belts (open circles), fusions (*), and an anterior hole (arrow). (F) spi^{2A}, vn^{L6}, Krn^{27} triple mutant embryo, has similar phenotype to the receptor mutant and is very short with no denticle belts and a large anterior hole (arrow). (G) spi^{2A}, grk^{HF}, vn^{L6}, Krn^{27} quadruple mutant embryo, which also lacks zygotic function of the maternally active grk gene, is similar to the triple ligand mutant and lacks denticle belts and has a large anterior hole (arrow).
Figure 3.1
Figure 3.2 Plate of Egfr null embryos. Egfr mutant (Egfr\textsuperscript{F24/F18}) embryos displayed a very consistent phenotype. Embryos were short, with reduced cuticle, no denticle belts, few or no individual denticles, dorsal hairs over majority of the cuticle, severely reduced head structures, and a large anterior hole.
Figure 3.2
Figure 3.3 Plate of ligand null embryos. \(spi^{2A}, grk^{HF}, vn^{16}, Knr^{27}\) quadruple mutant embryos also displayed a very consistent phenotype that was similar to the Egfr mutants (Fig 3.2). Embryos were short, with reduced cuticle, no denticle belts, few or no individual denticles, dorsal hairs over majority of the cuticle, severely reduced head structures, and a large anterior hole.
Figure 3.4 Percent of ligand null and receptor null embryos with denticles. A portion of both the $Egfr$ mutant embryos ($Egfr^{F24/3F18}$) and the quadruple ligand mutant embryos ($sp^{2A}, gr^{HF}, vn^{L6}, Kn^{27}$) retained some denticles in small patches. Both $Egfr$ mutant embryos (n = 28) and quadruple ligand mutant embryos (n = 30) were examined for the presence of denticles. Approximately half of the embryos of each genotype exhibited some denticles. There was no significant difference between the proportion of quadruple ligand mutant embryos versus the proportion of $Egfr$ mutant embryos, which had denticles.
Figure 3.4

Ligand null

- 53% No Denticles
- 47% Denticles

n=30

Receptor null

- 54% Denticles
- 46% No Denticles

n=28

\( p = 0.70 \) (no significant difference)
Figure 3.5 Redundant roles for EGF ligands in wing vein patterning. (A) wild-type wing, with five longitudinal veins (L2-4 are marked) and two crossveins—the anterior crossvein (ACV) and posterior crossvein (PCV). (B) $Krn^{27/9}$ wing, with a normal vein pattern. (C) $vn^{1/6}$ hypomorphic mutant wing, with loss of part of L4, the ACV and part of the PCV. (D) $vn^{1/6}, Knr^{27/9}$ wing, with loss of parts of L3 in addition to the L4, ACV, and PCV defects seen in $vn$ mutants. (E) $spt^{2A/+}, vn^{1/6}$ wing, with only the characteristic L4, ACV, PCV, defects seen in $vn$ hypomorphs. (F) $spt^{2A/+}, vn^{1/6}, Knr^{27/9}$ wing, with more substantial loss of L3 than the $vn, Knr$ mutant (compare with (D)).
Figure 3.5
Figure 3.6 Frequency of wing vein defects in single mutants and combinations of ligand mutants. Flies were scored for defects in longitudinal veins L3, L4, and the anterior crossvein. (A, E) Some defects were observed in L2 and L5, but these were rare and not included in the analysis. (B-D) Neither Krn single mutants (3) nor Krn mutants with a reduced dose of spi (6) showed any defects in L4 or the anterior crossvein. All vn hypomorphic flies have loss of L4 and the anterior crossvein (7-12). 7.3% of vn hypomorphic flies have defects in L3 (7). This is not significantly affected in vn hypomorphs heterozygous for a deletion of the Krn locus (8). 51.6% of vn hypomorphs homozygous for a deletion of the Krn locus have defects in L3 (9). 89.9% of vn hypomorphs homozygous for a deletion of the Krn locus that also have a reduced dose of spi have defects in L3 (12).
Figure 3.6

A

L2

Files with L2 defects

B

L3

Files with L3 defects

C

Anterior Crossvein

Files with AC defects

D

L4

Files with L4 defects

E

L5

Files with L5 defects
Figure 3.7 Reduction of Spi dose increases wing size. Flies mutant for *Krn* and/or *vn* were examined in an otherwise wild type background or a background heterozygous for a strong *spi* mutation. *Krn* heterozygous or homozygous mutant wings were similar in size to wild type. *vn* hypomorphic mutants had wings that were significantly smaller than wild type. Flies heterozygous for a strong *spi* mutation were significantly larger than wild type in a wild type background, a *Krn* mutant background, a *vn* mutant background, and a *vn*, *Krn* double mutant background.
Chapter 4: Developmental rescue of \textit{vn} mutants by TGF\(\alpha\) ligands and Vn deletion constructs

In light of the functional redundancy of the ligands in embryonic development and wing patterning, and because I was particularly interested in the functional capacity of \textit{Krn}, I decided to test the ability of the TGF\(\alpha\) ligands to replace the exclusive role of the neuregulin \textit{vn} in the development of wings and wing imaginal disc derived structures (the wing imaginal disc also gives rise to the notum of the adult fly). At least two models are possible. If the intrinsic activity of a ligand matters more to the outcome than the level of signaling through the receptor, then the TGF\(\alpha\) ligands should not be able to rescue \textit{vn} mutants, due to inability to reproduce unique intrinsic functions of Vn. Alternately, if the level of signaling is the primary instructive principle, it should be possible to rescue mutants of the weak ligand \textit{vn} with appropriate doses of the stronger TGF\(\alpha\) ligands, by restoring overall Egfr signaling to appropriate levels, regardless of ligand.

In the first larval instar stage of development, wing imaginal discs are comprised of approximately 20 cells. Over the course of about 6 days at 25\(^\circ\) C, these 20 cells proliferate rapidly, and at the end of the third larval instar the wing imaginal disc is comprised of approximately 50,000 cells (Fig 4.3A). In \textit{vn} mutants, that proliferation does not occur, and though the animals survive through the third larval instar and pupate,
they die shortly thereafter with no differentiated adult structures (Simcox et al. 1987) (Fig 4.3C-E).

The requisite pulse of Egfr signaling that promotes wing disc proliferation in normal development comes from Vn alone. Though vn mutants have wild type levels of both spi and Krn, the wing discs do not proliferate. Proliferation of the wing disc can be restored in vn mutants though expression of an activated form of Egfr, however, that fails to rescue development to pharate adults or proper patterning of wings and body wall. This could be due to either excess of signaling or failure of the activated Egfr mutant to recapitulate unique signaling through Egfr generated by binding the Vn ligand.

vn has an extremely dynamic expression pattern throughout development. Ubiquitous expression of vn in the embryo and imaginal discs only partially rescues vn mutants. Proliferation in the wing disc is restored, but patterning is abnormal; the discs are larger than wild type and have a duplication of the wing pouch. Gain of function alleles of the receptor (Egfr<sup>Elp</sup>) and MAPK (rolled<sup>sevenmaker</sup>) also partially rescue vn mutants, but as in broad overexpression of Vn itself, the wing discs had patterning abnormalities (Schnepp et al. 1996). Tight regulation of EGF signaling by vn in the wing disc is required for normal wing development. These types of experiments, therefore, cannot inform us of the capacity of the TGFα ligands to substitute for the neuregulin vn in development. To answer that question, I required a tool to faithfully recapitulate the intricate pattern of vn activity.
4.1 Generation of vn-Gal4

Unlike the TGFα ligands, Vn does not require proteolytic processing prior to secretion as an active ligand; Vn is secreted as an active ligand upon translation (Schnepp et al. 1996; Shilo 2003). Therefore, vn is regulated primarily at the transcriptional level.

To recapitulate the native vn expression pattern, I generated a transgenic stock with an insertion of the $P[GawB]$ element encoding the Gal4 transcriptional activator (Brand and Perrimon 1993) in the 5’ UTR of the vn locus. I accomplished this utilizing a previously published technique (Sepp and Auld 1999) to convert existing lacZ enhancer trap lines that bypasses the necessity of generating and validating new insertions.

Briefly, $P[PZ, ry^+]$ elements are more mobile than the newer $P[GawB, w^+]$ elements. In the presence of transposase, the lacZ elements are more likely to hop, leaving short inverted repeats footprints flanking a double-stranded break, which are repaired via homology-directed double-stranded break repair mechanisms. In flies bearing all three elements (lacZ, Gal4, and transposase), the homology in the P-element footprints can guide repair of the break at the locus of the former lacZ element from a stationary Gal4 element (Sepp and Auld).

A stock containing both a male-lethal Gal4 element ($w^+$) and the vn-lacZ ($ry^+$) element in a white background was crossed to a stock bearing the $P[\Delta2-3]$ transposase on a Sb marked chromosome to mobilize the P-elements. The female progeny of this cross which
contained all three elements (Gal4, lacZ, and transposase) were selected and crossed to TM3, Ser/TM6 males. All non-Sb male progeny with colored eyes were individually crossed with TM3, Sb/TM6 females to generate balanced stocks (Fig 4.1A).

Of 4197 male progeny examined, 33 had colored eyes indicating a transposition event and 3 matched the vn expression pattern. Male #24 of 33 was the first identified to recapitulate the vn expression pattern and the resultant stock was used for all further applications. The Gal4 element in the new vn-Gal4 allele is in the 5’UTR, 590 bp upstream of the open reading frame at the vn locus (Fig 4.2). vn-Gal4/vnL6 flies phenocopy vnL6/vnRG flies; both vnL6 and vnRG are known molecular null alleles. In vn-Gal4/vnL6 flies, the wing discs fail to proliferate and the animals die as white pupae with no differentiated adult structures (Fig 4.3). vn-Gal4 is expressed in the native vn pattern (Butchar et al. 2012).

Generation of the vn-Gal4 flies provided the possibility of creating flies in which expression of the neuregulin-like vn and each of the TGFα ligands (spi, grk, and Krn) can be regulated by the Gal4 transgene under the control of the endogenous vn regulatory elements. Thus temporally and spatially restricted to the expression domain of native vn, each ligand can be tested for the ability to restore development in vn mutant animals.
4.2 Co-expression of Gal80 inhibits the activity of the Gal4 protein, allowing calibration of transgene expression level

In addition to the pattern of expression, I anticipated a need to titrate the dose of the ligands, due to the fact that the active form of Spi, secreted Spi (sSpi), is known to have an approximately 12-fold higher affinity for the receptor than does Vn (Alvarado et al. 2010). Thus, expression of sSpi in the endogenous vn pattern is predicted to lead to higher than wild type physiological levels of Egfr signaling. To control for this, I utilized a temperature-sensitive construct of the Gal80 yeast protein, which is an inhibitor of the yeast Gal4 transcriptional activator (McGuire et al. 2003). Expression of Gal80 is predicted to negatively regulate Egfr signaling when co-expressed with Gal4-responsive \textit{UAS-ligand} transgenes, via inhibition of the Gal4 transcriptional activator responsible for Gal4-responsive transgene regulation. Gal80 is more functional, and hence effects greater inhibition of Gal4, at lower temperatures, whereas the Gal4 protein is more functional and drives higher expression of the target genes at higher temperatures (McGuire et al. 2003) (Fig 4.4A). By utilizing Gal80, Gal4, and several different growth temperatures, we could thus control the level of TGF\(\alpha\) ligand being expressed from the Gal4-responsive transgenes.

At 25\(^{\circ}\) C, Gal80 inhibition reduced \textit{vn-Gal4} driven expression of \textit{UAS-GFP} to 20-25\% of the uninhibited dose (Fig 4.4B-D). The increase in target (GFP) expression from 25\(^{\circ}\) C to 29\(^{\circ}\) C was also dramatic (Fig 4.4C,E). The published literature indicates that the \textit{tub-Gal80}\textit{TS} construct is inactive at 29\(^{\circ}\) C (McGuire et al. 2003; Berdnik et al. 2006),
however, in my hands substantial inhibition was observed at this temperature (Fig 4.4E-F).

4.3 Proof of principle: UAS-vn expression can rescue vn mutants

Expression of UAS-vn in the endogenous vn pattern (UAS-vn; vn^{L6}/vn-Gal4) produced flies with grossly normal patterning in the thoracic body wall and wing (Fig 4.5E-G). Efficacy of rescue was relatively insensitive to dose, and some adult flies were produced at each condition tested (Fig 4.6). Some extra wing vein material was observed, consistent with mild Egfr overactivity (Fig 4.5G). The extent of extra wing vein material in the rescued flies was commensurate with the level of transgene expression; at those conditions which promote lower doses of vn expression, the wings had little to no extra vein material. At the highest dose, all eclosed adults had wings held out and a large amount of excess wing vein material. The lowest dose of vn expression was produced by utilizing Gal80 inhibition at 17° C, while for the highest dose of vn expression, the flies were raised at 29° C.

The UAS-vn1.1 insertion is on the X chromosome, and rescued adult flies in the F1 generation were all hemizygous males (UASvn1.1/Y; vn^{L6}/vn-Gal4). The inability to rescue lethality in all animals likely reflects the artificial Gal4-UAS system used to govern vn expression in the mutants; in particular, the duration of transcriptional activation may be affected by the perdurance of the Gal4 protein. The poorest rescues were female (UASvn1.1/w-; vn^{L6}/vn-Gal4). The F1 males, however, were fertile, and
homozygous female adult progeny (UASvn1.1/UAS-avn1.1; vn^{L6}/vn-Gal4) were obtained. These females laid eggs, but were not fertile.

4.4 The TGFα ligands can replace the essential function of vn in development, though dosage is critical.

Expression of transgenes encoding secreted forms of each of the TGFα ligands also rescued development and wing disc derivatives in the vn mutants (Fig 4.5H-P). Secreted forms were expressed to bypass the requirement for cleavage by Rho family proteases (Urban et al. 2002).

Initial studies were conducted at 17°C. At this temperature, expression of either Vn or sGrk increased the proportion of larvae which survived to pupation, relative to vn mutants with no ligand supplied. Expression of sSpi or sKrm, however, was detrimental to achieving that developmental milestone, with expression of sSpi being extremely detrimental (Fig 4.7). Expression of the sSpi-encoding transgene at 17°C failed to yield appreciable rescue of vn mutants; no pharate adults were recovered. Expression of sKrm yielded very few pharate adults relative to expression of Vn or sGrk.

Three possibilities existed for the failure of the Spi to rescue vn under these conditions. First, Spi may lack intrinsic activity required to substitute for Vn in development. Second, there may be insufficient Egfr signaling, which I thought unlikely due to the
higher affinity of Spi for Egfr than Vn. Third, there may be an excess of signaling, reaching a level of toxicity.

Increasing the dosage of the TGFα ligands by raising the flies at 25° C rather than 17° C did not improve the rescue of vn mutants. Expression of the TGFα ligands in the vn pattern at 25° C produced poorer results than expression at 17° C. Expression of sKrn in the vn domain at 25° C caused embryonic lethality. Expression of sSpi rescued the proliferation of the wing imaginal disc, but killed the animals prior to pupation. Expression of sGrk yielded few pharate adults, all of which were more poorly developed than the vn mutants rescued by expression of sGrk raised at 17° C. vn mutants in which sGrk was expressed in the vn pattern raised at 29° C died shortly after pupation.

After observing the clear inverse relationship between efficacy of rescue and transgene dose in the vn mutants in which sGrk was expressed, as determined by temperature of rearing which affects the activity of the Gal4 transcriptional activator, I hypothesized that an excess of Egfr signaling was causing the premature lethality of the vn mutants in which the TGFα ligands were being expressed. I therefore empirically tested the optimal conditions for rescue of vn mutants for each ligand. Optimal conditions were different for each ligand (Fig 4.8).

In contrast to expression of UAS-vn, the level of TGFα transgene expression had a significant impact on the extent of rescue. From the lowest dosage condition tested
(Gal80 inhibition at 17°C) to the highest (uninhibited expression with \textit{vn-Gal4} at 29°C), a spectrum of rescue was observed (Fig 4.8). But for each of the TGF\(\alpha\) ligands, an optimal level of signaling was found such that development and body patterning, as well as wing disc proliferation, and wing and notum patterning, were rescued, and the animals grew into well-formed pharate adults (Fig 4.5).

\textit{vn} null mutants show no differentiation of any adult body parts; expression of TGF\(\alpha\) ligands at varying levels produced different degrees of rescue in these animals. Head, leg, and abdomen development were restored across a range of expression levels tested, suggesting these body parts are relatively insensitive to the level of Egfr activity (Fig 4.8, Fig 4.9A). \textit{vn} is required for distal leg patterning including the terminal claws (Campbell 2002; Galindo et al. 2005). The rescued animals had claws (Fig 4.10), suggesting that the TGF\(\alpha\) ligands also can also fulfill this specific role of \textit{vn}. In contrast, differentiation of derivatives of the wing disc was variable and dependent on TGF\(\alpha\) ligand dose. A progression of rescue phenotypes was seen as expression increased, from animals lacking all wing disc structures, those with just wings, to optimal cases of animals with wings and a thoracic body wall (Fig 4.5E,H,K,N, Fig 4.9 A3).

At the highest Spi doses tested, the animals died as larvae (Fig 4.8, Fig 4.9A). At the highest Krn doses tested, the animals died as embryos, and at the highest Grk dose tested, the animals died as pupae without differentiated adult structures (Fig 4.8).
4.5 Multiple transgenes were tested for rescue of vn mutants by Vn and sSpi

The rescue of vn mutants by expression of UAS-vn1.1 was relatively insensitive to dose; rescued flies eclosed at every condition tested. This was true for multiple additional transgenes tested (UAS-vn1.2, UAS-vn2.8) (Fig 4.11) and is therefore unlikely to be an artifact of rescue with the vn1.1 construct or its insertion site.

The rescue of vn mutants by sSpi was optimal at 27° C with Gal80 inhibition. Three spi transgenes were tested for rescue of vn mutants (UAS-sSpi49A, UAS-sSpiGFP\textsuperscript{M36} and UAS-sSpiGFP\textsuperscript{F5N}) in addition to an X chromosome insertion. All were pre-pupal lethal when expression was driven with the vn-Gal4 and flies were raised at 25° C. However, when expression was driven by the vn-Gal4, but inhibited with Gal80 expression, all of the UAS-sSpi transgenes rescued vn mutants to pharate adults. The consistency of this effect across multiple transgene insertions suggests that the failure of UAS-sSpi\textsuperscript{X} to rescue vn mutants when expressed at higher levels is indeed due to toxicity of sSpi at higher levels, rather than insufficient or qualitatively incorrect signaling, or an artifact of the particular transgene first used.

4.6 Optimal rescue of vn mutants occurs when transgenic spi expression restores signaling close to wild-type levels.

Expression of sSpi was able to rescue vn mutants only in a narrow range of expression level. I examined the level of Egfr signaling generated by sSpi under conditions in which
the expression of sSpi is able to rescue \( vn \) mutants and conditions in which it fails to rescue \( vn \) mutants, as well as the level of Egfr signaling generated by the expression of Vn under the same conditions. To analyze this, the signaling output was assessed by MAP Kinase (MAPK) phosphorylation in late third instar larvae, due to the lethality of uninhibited expression of sSpi prior to the formation of pharate adults.

The Spi expression level that gave rise to flies with both wings and a body wall correlated with a signaling output which most closely matched to wild type, as measured by MAPK phosphorylation (dpERK). Expression of Vn, both uninhibited and under Gal80 inhibition) resulted in signaling output that closely matched wild type (Fig 4.9B-C).

4.7 Rescue of \( vn \) mutants with \( vn \) mutant constructs

The neuregulin ligand Vn has significant structural dissimilarities compared to the TGF\( \alpha \) ligands (Shilo 2003). The Vn protein has several conserved domains, including a PEST domain, the Mosquito Conserved Region (MCR), and an Ig domain, in addition to the EGF domain. Previous work identified the ability of \( vn \) transgenes with partial coding sequence deletions to effect wing patterning in a wild type background (extra veins are a classic Egfr pathway hyperactivity phenotype), or to rescue the wing disc failure to proliferate phenotype (Donaldson et al. 2004). I tested the ability of these \( vn \) mutant constructs and a small number of additional GFP fusion transgenes to rescue \( vn \) mutant animals to further investigate which features of the protein were essential to the developmental function of the ligand.
All Egfr ligands have an EGF domain which is required for binding the receptor and generating signaling. I expected a mutant protein with a deletion of the EGF domain of the vn protein to fail to rescue vn mutants. In light of the large degree of functional redundancy I observed between the native ligands, I expected a mutant protein bearing the functional EGF domain of any of the activating ligands to have the ability to rescue vn mutants. Due to the function of the PEST domain in regulating protein degradation, I would expect that removal of this domain from the Vn protein would increase the level of signaling generated due to increased perdurance of the resulting protein. The ability of expression of any of the TGFα ligands, which lack the Ig domain, to rescue vn mutants demonstrates that this domain is not absolutely required to rescue development in vn mutants to the pharate adult stage. I expected that the removal of the Ig domain may compromise the ability of the protein to rescue eclosure of vn mutants, but I expected expression of a vnΔIg construct to be able to rescue vn mutants to at least the pharate adult stage.

Previous work had identified some domains of Vn that, when deleted, alter the protein to be more potent activators of Egfr signaling than native Vn (VnΔPEST, VnΔIg, some MCR deletions) (Donaldson et al. 2004), and I expected expression of those proteins to be able to rescue vn mutants, however, I anticipated that they may require Gal80 inhibition to reduce Egfr signaling levels.
Other constructs were identified as inhibitors of Egfr signaling (Vn::AosEGF, VnΔEGF, some MCR deletions). I expected that expression of those proteins would fail to rescue vn mutants.

Two Vn-GFP fusion constructs were tested. Both were able to rescue vn mutants to adults (Fig 4.11A, C), though both were stronger ligands that the native Vn, judged by their efficacy of rescue at the various temperature conditions, with and without Gal80 inhibition. This may be due to the effect of GFP peptide on the perdurance of Vn. In normal development, Vn is an extremely labile protein with a dynamic expression pattern. If the GFP fusion constructs are impaired in their ability to be properly degraded, it could increase their biological effect.

A swap of the vn EGF domain for that of spi rescued animals to pharate adults (Fig 4.11I). This further supports the functional redundancy of the native Drosophila Egfr ligands. That Gal80 inhibition was not required to effect rescue of the vn mutants at 25°C, however, suggests that aspects of the ligands outside of the sequence of the EGF domain itself and its binding affinity for the receptor contribute to the intrinsic activity of the ligands.

A construct with a deletion of the PEST domain, which affects protein degradation (Rechsteiner and Rogers 1996) was able to rescue to pharate adults, but not eclosed adults, similar to the stronger TGFα ligands (Fig 4.11D). This supports the model in
which rapid turnover of the Vn protein is necessary for proper regulation of Egfr signaling.

A construct with a deletion of the Vn Ig domain—which is unique to \( vn \) among the \textit{Drosophila} Egfr ligands—was able to rescue \( vn \) mutants to adults, though many of the pharate adults obtained from that rescue had notal duplications in place of one of their wings (Fig 4.11G). In flies, ectopic expression of Vn lacking the Ig domain in otherwise wild-type flies is more toxic than expression of native Vn, and survivors have wings with notched margins (Donaldson et al. 2004). Here we found that expression of a form of Vn lacking the Ig domain (Vn\( \Delta \)Ig) rescued \( vn \) mutants to adults, demonstrating that Ig-containing forms are not essential for viability (Fig 4.11G, Fig 4.13A). The wings of these flies, however, were abnormal and had bristles missing from the margin, deleted regions and fused veins (Fig 4.13B-C). These are similar to the abnormalities which were seen from ectopic expression of Vn\( \Delta \)Ig in a wild-type background (Donaldson et al. 2004). The Ig domain could mediate the role of Vn in another pathway independent of its role in Egfr signaling via the EGF domain. The abnormal wing phenotype is reminiscent of \textit{Notch} or \textit{wingless} mutants, both of which interact with \( vn \) genetically (Price et al. 1997; Wang et al. 2000), and the Vn Ig-domain physically interacts with Hedgehog coreceptors (Ozkan et al. 2013), making any of these pathways candidates.

The ability of Vn\( \Delta \)Ig to rescue \( vn \) mutants to eclosed adults suggests that the lack of an Ig domain is not responsible for the failure of the TGF\( \alpha \) ligands to rescue eclosure in \( vn \) mutants. The notal duplication phenotype, however, which was not observed in any other
rescue of \( vn \) mutants by any of the proteins that I tested, suggests that the Ig domain does indeed mediate some unique functions of \( vn \) in patterning.

Constructs that did not rescue included some deletions of the MCR, a swap of the \( vn \) EGF domain for that of \textit{Argos} (an EGF domain bearing negative regulator of Egfr signaling which does not bind the receptor) and a deletion of the \( vn \) EGF domain (Fig 4.12). This was as expected. Argos is a negative regulator of the Egfr pathway, so it follows that the Argos EGF domain would not positively regulate Egfr signaling. It also follows that the absence of an EGF domain, and therefore no ability to bind the receptor, would obviate the functionality of the Vn protein.

\textit{4.8 Vn alone was able to rescue vn mutants to adults.}

Even in cases of the best morphological phenotypes, rescue of \( vn \) mutants by \( spi \) was incomplete because the flies did not eclose (Fig 4.7). \( vn \) is required in addition to \( spi \) for the differentiation of specific muscle precursors, and \( vn \) has a critical role in muscle-tendon attachment in development (Yarnitzky et al. 1997; Yarnitzky et al. 1998). Expression of GFP under the control of the \( vn\text{-Gal4} \) in the body wall muscles is clearly visible (Fig 4.4C-D). In the \( vn \) mutants rescued by expression of Vn, in addition to the adults which eclosed there were many pharate adults which did not eclose (Fig 4.8). In a subset of these flies, I observed that they had excreted meconium into their pupal cases. This class of flies, pharate adults which had excreted their meconium, was also observed in the \( vn \) mutants rescued by the expression of sSpi and sKrn. The failure of these flies to
eclose despite patterning complete adult cuticle and apparently completing development may be due to malformation of the adult muscles. Paralyzed flies would be unable to eclose.

As discussed above, this could be due to the artificial Gal4-UAS expression system. Vn is an extremely labile protein, and contains a PEST domain which mediates protein degradation (Rechsteiner and Rogers 1996). Even a small increase in the duration of signaling due to the longer perdurance of the Gal4 protein relative to the Vn protein itself may affect signaling. 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). In flies, ectopic expression of Vn lacking the Ig domain, though rescuing viability, produced novel phenotypes and was more toxic than native Vn (Donaldson et al. 2004). The Vn Ig domain interacts with Hedgehog co-receptors (Ozkan et al. 2013), implying it could have a unique role in another pathway independent of its role in Egfr signaling mediated by the EGF domain.

4.9 Concluding remarks

The intrinsic activity of an EGF ligand correlates with its biological effect—so a ligand with high intrinsic activity has a higher maximum biologic effect than a ligand with low intrinsic activity when both are at saturating concentration (Wilson et al. 2012a). Spi appears to have a higher intrinsic activity than Vn because at higher levels Spi exceeded the required biologic response and was toxic (Fig 4.7, Fig 4.9A). The ligands also have
different affinities; Spi is a high affinity ligand and Vn is a low affinity ligand (Alvarado et al. 2010). Low affinity may limit access of Vn to the receptor due to the negative cooperativity of ligand binding—so that when the first ligand binds, a conformational change occurs that occludes the second site rendering it accessible only to a high affinity ligand (Macdonald and Pike 2008; Alvarado et al. 2010). The biological role of a low affinity ligand like Vn may be limited because some cell responses require signaling levels that can be evoked only by high doses of a high affinity ligand (Krall et al. 2011).

The TGFα ligands, when constrained to both the appropriate pattern and absolute level of signaling, were able to rescue most but not all of the functions of Vn in development. In particular, they did not rescue eclosure. The data do not exclude unique intrinsic activities of individual ligands. However my data support a model in which both the pattern and absolute level of Egfr signaling generated by a given ligand are the primary determinants of ligand functionality, as demonstrated by the ability of any of the TGFα ligands to effect excellent rescue of development and patterning in vn mutants.
Figure 4.1 Generation of vn-Gal4. (A) The vn-lacZ element was replaced with a $P[GawB, w^+]$ element (recessive lethal) on the X chromosome. A stock containing both the $P[GawB, w^+]$ element ($w^+$) and the $vn$-lacZ ($ry^+$) element in a white' background was crossed to a stock bearing the $P[\Delta2-3]$ transposase on a Sb marked chromosome. The transposase was used to mobilize the P-elements. The female progeny of this cross which contained all three elements ($Gal4$, lacZ, and transposase) were selected and crossed to TM3, Ser/TM6 males. All non-Sb male progeny with colored eyes were individually crossed with TM3, Sb/TM6 females to generate balanced stocks. (B) To visualize the expression pattern of the new Gal4 insertions, flies from the newly created stocks were crossed to UAS-GFP and UAS-lacZ.
Figure 4.1

A

\[ P\{GawB, w^*\}, w^* \times P\{PZ, ry^*\} \]

BL# 3629, transposase

\[ w^* ; P\{PZ, ry^*\} \]

\[ w^* ; S_{CyO}\ ; P\{\Delta2-3, Sb\} \]

\[ w^* ; TM3, Ser \]

\[ w^* ; TM6b \]

all non-Sb males with colored eyes

\[ w^* ; P\{GawB, w^*\} \]

\[ w^* ; TM3, Ser \]

\[ w^* ; TM6 \]

\[ w^* ; P\{GawB, w^*\} \]

\[ w^* ; TM3, Sb \]

\[ w^* ; TM6 \]

B

\[ w^* ; P\{GawB, w^*\} \]

\[ w^* ; UAS-GFP \]

\[ w^* ; UAS-GFP \]

\[ w^* ; P\{GawB, w^*\} \]

\[ w^* ; UAS-lacZ \]

\[ w^* ; UAS-lacZ \]
Figure 4.2 *vn-Gal4* is inserted 590 bp upstream of the start of the *vn* coding region.

Sequences spanning both ends of the *P[GawB, w^+]* insertion in the *vn* locus were amplified by PCR and sequenced both to determine insertion location and to ensure that the excision of the *lacZ* and transposition of the *GawB* had not generated novel deletions. No deletions or additional insertions were found.
Figure 4.2
**Figure 4.3 vn-Gal4 is an amorphic allele of vn.** (A) wild-type third instar wing disc (arrow) and leg disc (arrowhead). (B) wild-type adult. (C) $vn^{L6/RG}$ wing disc (arrow) and leg disc (arrowhead). (D) $vn^{L6}/vn$-Gal4 wing disc (arrow) and leg disc (arrowhead). In both (C) and (D) the wing disc 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 an amorph. (E) Terminal $vn^{L6}/vn$-Gal4 animal dissected from pupal case, lacking any wing disc derived structures and without pigmented adult cuticle. Partially everted leg structures are present (arrowheads).
Figure 4.3
Figure 4.4 Co-expression of Gal80 reduces the expression of UAS-GFP induced by Gal4 in a temperature-dependent manner. (A) Gal4 activity increases with temperature, whereas activity of a heat-sensitive allele of Gal80 function decreases with temperature. This means that Gal4 drives a higher level of UAS-GFP at higher temperatures and Gal80 inhibits Gal4 function most effectively at lower temperatures. This allows titration of transgene expression level from lower doses at low temperature with Gal80 inhibition to higher doses at higher temperatures without Gal80 inhibition. (B) At 25°C, Gal80 inhibition reduced vn-Gal4 driven expression of UAS-GFP to 20-25% of the uninhibited dose. (C-F) vn-Gal4 driven expression of UAS-GFP is more strongly expressed at 29°C than 25°C, and is substantially inhibited by the co-expression of Gal80TS at both temperatures. Expression is very strong in the salivary glands, and clearly visible in the larval muscles.
Figure 4.4

A

Gal80<sup>TS</sup> activity

17°

29°

B

vn-Gal4<sup>+</sup>GFP at 25°

C

uninhibited

loading volume

100% 80% 60% 40% 20% 10% 0%

100% inhibited with Gal80

α-GFP

C

vn-Gal4/UAS-GFP at 25°

D

vn-Gal4/UAS-GFP at 25°

E

vn-Gal4/UAS-GFP at 29°

F

vn-Gal4/UAS-GFP at 29°
Figure 4.5 Expression of the TGFα ligands in the correct expression domain rescues developmental abnormalities in mutants of the neuregulin \textit{vn}. (A) Wild-type adult fly (B) notum and (C) wing. (D) \textit{vn}^{L6}/\textit{vn-Gal4} mutant. The mutants show an amorphic phenotype and arrest development at the white pupal stage with no differentiated adult structures. (E) \textit{UAS-vn; vn}^{L6}/\textit{vn-Gal4} adult (F) notum and (G) wing (17°C). When a \textit{vn} transgene is expressed in \textit{vn} mutants in the correct endogenous pattern with the \textit{vn-Gal4} driver, some flies are viable. The wings had a mild extra-vein phenotype consistent with overactivity of Egfr signaling (G). (H-P) Expression of transgenes encoding the TGFα ligands in the \textit{vn} expression pattern rescues differentiation in \textit{vn} mutant flies including the body wall and wing. The flies do not eclose, however. The wings have bristle duplications and extra veins, characteristic of Egfr overactivity (J, M, P). (H) \textit{UAS-sspi; Gal80^{TS}; vn}^{L6}/\textit{vn-Gal4} pharate adult (I) notum and (J) wing (27°C). (K) \textit{UAS-sKrn, vn}^{L6}/\textit{vn-Gal4} pharate adult (L) notum and (M) wing (17°C). (N) \textit{UAS-sgrk, vn}^{L6}/\textit{vn-Gal4} pharate adult (O) notum and (P) wing (17°C).
Figure 4.5

wild type

\textit{vn}^r

\textit{Vn} rescuing \textit{vn}^r

\textit{Spi} rescuing \textit{vn}^r

\textit{Km} rescuing \textit{vn}^r

\textit{Grk} rescuing \textit{vn}^r

\textit{vn}^r\textit{Gnl-Gal4} animals die shortly after pupation.
Figure 4.6 Rescue of vn mutants by expression of UAS-vn. UAS-vn1.1 was expressed in a vn mutant background, with (UAS-vn1.1; Gal80TS; vnL6/vn-Gal4) and without (UAS-vn1.1; vnL6/vn-Gal4) Gal80 inhibition at a range of temperatures. Larvae of the desired genotypes were sorted into separate vials. Percentages in each category represent the proportion of total larvae that reached each developmental milestone. Substantial rescue, including adult flies, was observed at each condition, though the highest proportion of both pharate adults and eclosed adults was observed at 25° C.
Figure 4.6
Figure 4.7 Rescue of vn mutants by TGFα ligands at 17° C. vn mutants (vn<sup>L6</sup>/vn-Gal4), those expressing vn (UAS-vn1.1; vn<sup>L6</sup>/vn-Gal4), secreted Spi (UAS-sSpi<sup>X</sup>; vn<sup>L6</sup>/vn-Gal4), secreted Krn (UAS-sKrn; vn<sup>L6</sup>/vn-Gal4), or secreted Grk (UAS-sGrk; vn<sup>L6</sup>/vn-Gal4) were sorted into separate vials as larvae and raised at 17° C. Percentages in each category represent the proportion of total larvae that reached each developmental milestone. Vn and sGrk both increased the percent of animals that pupated; sSpi and sKrn were apparently toxic and decreased the proportion of animals surviving to this stage. The effect of sSpi was particularly strong. Expression of Vn, sKrn, and sGrk produced rescue of some vn mutants to pharate adults or in the case of Vn, to adults.
Figure 4.7
**Figure 4.8 Extent of rescue of vn mutants by expression of TGFα ligands is dependent on dose.** Each *UAS-ligand* transgene was expressed using the *vn-Gal4* allele. The level of transgene expression was manipulated by temperature (Gal4 is more active at higher temperature) or co-expression of the Gal80<sup>TS</sup> inhibitor and temperature (Gal80 is less active at higher temperature). 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 scored; animals that died prior to the pharate adult stage were not scored. Animals were counted as pharate adults if they exhibited pigmented cuticle and had formed legs. Animals were counted as eclosed adults if they had partially or fully emerged from their pupal case. The classes range from pharate adults lacking any derivatives of the wing disc (wing and body wall) to normal flies that eclosed. 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. 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 TGFα ligands were sensitive to the level of expression and optimal rescue of wing-disc derived structures occurred within a narrow dose range. Results shown for *vn* are utilizing *UAS-vn1.1* and results for sSpi are utilizing *UAS-sSpi<sup>Y</sup>*. Four additional *UAS-vn* transgenes were tested and all gave similar results (*UAS-vn1.2, UAS-vn2.8, UAS-vnGFP<sup>58</sup> and UAS-vnGFP<sup>12</sup>).* Three additional *UAS-spi* transgenes were tested and gave similar results (*UAS-sspi at 49A, UAS-sspiGFP<sup>M36</sup> and UAS-sspiGFP<sup>F5N</sup>).* A single UAS-transgene was tested for *grk* and *Krn.*
Figure 4.8
Figure 4.9 Optimal rescue of \(vn\) mutants occurs when transgenic sSpi expression restores ERK signaling close to wild type levels. (A) \(vn\) mutants die as pupae with no adult structures (left). \(vn\) mutant flies rescued with increasing doses of sSpi (\(UAS\)-sspi; \(Gal80\)\(^{TS}/+;vn^{L6}/vn\)-Gal4) are shown as raised at (1) 17°C, (2) 25°C and (3); 25-29°C. Rescue of adult structures show differing levels of dose sensitivity. The head, legs and abdomen are restored over a broad range starting at the lowest dose. The wing and notum form in a narrower range. (4) At high dose sSpi is toxic and individuals died as larvae (\(UAS\)-sspi; \(vn^{L6}/vn\)-Gal4, 17°C). (B-C) Western blot analysis and quantitation of dpErk (activated MAPK) levels in the indicated genotypes (25°C). \(wt\): wild type. (-): \(vn^{L6}/vn\)-Gal4. \(spi\): \(UAS\)-sspi; \(vn^{L6}/vn\)-Gal4. \(G80\)\(spi\): \(UAS\)-sspi; \(Gal80\)\(^{TS}/+;vn^{L6}/vn\)-Gal4. \(vn\): \(UAS\)-vn1.1; \(vn^{L6}/vn\)-Gal4. \(G80\)\(vn\): \(UAS\)-vn1.1; \(Gal80\)\(^{TS}/+;vn^{L6}/vn\)-Gal4. Quantitation in (C) is the average of three experiments. dpErk levels and phenotypic rescue were sensitive to the expression of \(spi\) and only lower levels of \(spi\) expression (\(Gal80\)-inhibited) and correspondingly lower dpErk levels rescued body structures including the wing and thorax. Expression of \(vn\) transgenes at high (non-inhibited) and low (\(Gal80\)-inhibited) levels resulted in a similar amount of dpErk and produced fully rescued flies with wings and a thoracic body wall. (D) Cartoon showing \(spi\) rescuing development in a \(vn\) mutant. The wild-type fly results from activity of \(vn\) (purple) and \(spi\) (green) in many parts of the body (grey). The wing and notum (derivatives of the wing disc) are shown in purple to indicate the essential role for \(vn\) in these structures. The \(vn\) mutant (in which \(spi\) is active) dies as an undifferentiated white pupa. The \(vn\) mutant in which \(spi\) is expressed in its normal pattern as well as the \(vn\) pattern is rescued to a fully formed pharate adult. (The wing is small to indicate that the animals do not eclose and expand their wings.)
Figure 4.9

A. *vn* null with increasing *vnG4>spi* dose

<table>
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<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>no wings</td>
<td>wings</td>
<td>wings</td>
<td>pre-pupal</td>
</tr>
<tr>
<td>no body wall</td>
<td>body wall</td>
<td>lethal</td>
<td>lethal</td>
</tr>
</tbody>
</table>

B. 

- *vn1.6/vn-Gal4>*
- *wt*, *spi*, *G80*, *spi*, *vn*, *vn*
- *dpErk* and *Erk* expression levels

C. Fold change in *dpERK* to *total ERK*

D. 

- *wt*, *spi*; *vn*[^+]
- *vn*, *spi*; *vn*[^-]
- *vn rescued by spi*
Figure 4.10 Expression of TGFα ligands rescues distal leg patterning in vn mutants.

(A) Distal portion of a wild type leg, with claws. (B) Ventral view of a vn<sup>L6</sup>/vn-Gal4 animal. vn mutants die without forming adult cuticle. (C-F) Expression of any of the *Drosophila* EGF ligands effects substantial rescue of leg patterning, including claw formation. (C) UAS-vn<sup>1.1</sup>; vn<sup>L6</sup>/vn-Gal4. (D) UAS-sSpi; Gal80<sup>TS</sup> /+; vn<sup>L6</sup>/vn-Gal4. (E) Gal80<sup>TS</sup> /+; vn<sup>L6</sup>, UAS-sKrn/vn-Gal4 leg. (E) vn<sup>L6</sup>, UAS-sGrk/vn-Gal4.
Figure 4.10

A. wild type

B. vnt<sup>6</sup>/vn-Gal4

C. UAS-vnt<sub>1.1</sub>; vnt<sup>20</sup>/vn-Gal4

D. UAS-Spi; Gal80<sup>T5</sup> /+; vnt<sup>6</sup>/vn-Gal4

E. Gal80<sup>T5</sup> /+; vnt<sup>20</sup>, UAS-sKrn/
vnt-Gal4

F. vnt<sup>6</sup>, UAS-sKrn/vn-Gal4
Figure 4.11 Expression of transgenes encoding chimeric and deletion Vn proteins rescues developmental abnormalities in vn mutants. (A) UAS-vnGFP12; vnL6/vn-Gal4, a Vn-GFP fusion construct (B) UAS-vn2.8; vnL6/vn-Gal4, an alternate isoform of Vn. (C) UAS-vnGFP58; vnL6/vn-Gal4, a Vn-GFP fusion construct. (D) UAS-vnΔPEST4; vnL6/vn-Gal4, a construct in which vn lacks the PEST domain which mediates protein degradation. (E) UAS-vnΔMR3.39; vnL6/vn-Gal4, missing a portion of the Mosquito Conserved Region (MCR). (F) UAS-vnΔMR1.1; vnL6/vn-Gal4, missing a portion of the MCR. (G) UAS-vnΔIg14; vnL6/vn-Gal4, missing the Ig domain, which is unique to Vn among Drosophila EGF ligands and in common with some vertebrate neueregulins. (Fig 4.13 for additional photographs.) (H) UAS-vn::spiEGF2.1; vnL6/vn-Gal4, in which the native EGF domain of Vn has been replaced by that of Spi. (I) UAS-spi::vnEGF; vnL6/vn-Gal4, in which the native EGF domain of Spi has been replaced by that of Vn. (J) UAS-vn::vnEGF1.1; vnL6/vn-Gal4, in which the native EGF domain of Vn has been replaced by that of Vn, by the same method used to replace the EGF domain of Spi in (I).
Figure 4.12 Expression of some vn constructs in the correct expression domain fails to rescue developmental abnormalities in mutants of the neuregulin vn. Expression of additional native vn transgenes and Vn-GFP fusion constructs was able to rescue vn mutants to adults. Expression of some additional constructs, as seen in Fig 4.11, was able to rescue vn mutants to pharate adults. Certain regions of Vn, however, were critical for rescue, and expression of transgenes lacking those regions either failed to rescue eclosure, or resulted in lethality prior to the pupal stage, including some (but not all) deletions in the Mosquito Conserved Region, the substitution of the EGF domain of vn for that of Argos, and removing the EGF domain of vn entirely. vn mutants expressing VnΔIg exhibited notum duplication in place of one of the wings of approximately a third of the pharate adults recovered (5 of 17).
<table>
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<th>Genotype</th>
<th>Rescue?</th>
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<tbody>
<tr>
<td>UAS-vnGFP&lt;sup&gt;58&lt;/sup&gt;; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>adults</td>
</tr>
<tr>
<td>UAS-vn1.2; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>adults</td>
</tr>
<tr>
<td>UAS-vn2.8; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>adults</td>
</tr>
<tr>
<td>UAS-vnGFP&lt;sup&gt;12&lt;/sup&gt;; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>adults</td>
</tr>
<tr>
<td>UAS-vnDelta_PEST4; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pharate adults</td>
</tr>
<tr>
<td>UAS-vnDelta_MR1.1; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pharate adults, with wing(s) but no nota</td>
</tr>
<tr>
<td>UAS-vnDelta_MR2.12; vnL6/vn-Gal4</td>
<td>no</td>
</tr>
<tr>
<td>UAS-vnDelta_MR3.3; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pharate adults</td>
</tr>
<tr>
<td>UAS-vnDelta_MR34.3; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pupal lethal</td>
</tr>
<tr>
<td>UAS-vnDelta_MR3.64; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pharate adults</td>
</tr>
<tr>
<td>UAS-vnDelta_Ig14; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>adults, some pharates with notum duplication in place of wing</td>
</tr>
<tr>
<td>UAS-vn::spiEGF2.1; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pharate adults</td>
</tr>
<tr>
<td>UAS-vn::AosEGF2.1; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
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<td>pharate adults</td>
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<tr>
<td>UAS-vn::vnDelta_EGF; vn&lt;sup&gt;L6&lt;/sup&gt;/vn-Gal4</td>
<td>pupal lethal</td>
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</table>
Figure 4.13 Expression of the Vn lacking the Ig domain rescues viability in vn mutants. (A) UAS-vnΔIg; vn^{L6}/vn-Gal4 adult and (B) wing (25°C). Expression of Vn lacking the Ig domain was not essential for viability and patterning most of the body, but the wings were abnormal and had missing bristles on the wing margin (arrowheads) and reduction of intervein regions between the margin and vein 2 and veins 4-5 (arrows).
Figure 4.13
Chapter 5: Conclusions

The reason for multiple Egfr ligands, their specific effects, or indeed if a ligand is always required to initiate signaling through ErbB family receptors, are problems of broad interest not only for developmental biology and Drosophila research, but to the cell signaling field at large. Basic research investigating pathway regulation and targets, including the differential effects of signaling following activation by specific ligands, has the potential to reveal new therapeutic targets in human cancers (potentially, the ligands themselves) while elucidating answers to fundamental questions in biology. Basic research is of vital importance in the development of therapeutic strategies; as we increase our understanding of the systems involved in tumorigenesis and cancer progression, we have more targets available, and a more nuanced understanding of therapeutic effects.

5.1 Ligand dependence

Ligand independent Egfr/ErbB signaling has been reported in both Drosophila (Schweitzer et al. 1995) and mammalian (Nagy et al. 2010; Endres et al. 2013) cells in vitro, where overexpression of Egfr/ErbB receptors causes dimerization and activation of the pathway. In the case of the Drosophila study, high levels of expression of Drosophila
Egfr induced spontaneous autophosphorylation of the receptor, while lower levels of expression were sufficient for assays testing induction of the pathway by the sSpi ligand and did not induce spontaneous autophosphorylation.

The mammalian studies, likewise, were conducted with very high levels of receptor overexpression, resulting in cell surfaces densely packed with receptor at very high concentrations—up to $3 \times 10^6$ ErbB2 receptors per cell (Nagy et al. 2010) compared to the ~$1.2 \times 10^6$ total ErbB2 receptors per cell recently measured in the strongly-overexpressing, ErbB2-amplified BT474 breast cancer cell line (Ward et al. 2013), and more than 500,000 ErbB1 receptors per cell, compared to the endogenous ~50,000 ErbB1 receptors per cell observed in human HeLa cells. ErbB1 receptors did not spontaneously dimerize at concentrations below 200,000 receptors per cell (Nagy et al. 2010). EGF binding was sufficient to trigger phosphorylation of ErbB1 at the lowest densities studied, only ~30,000 receptors per cell, while spontaneous ErbB1 phosphorylation approaching half of the level observed when stimulated with ligand required ~800,000 receptors per cell (Endres et al. 2013). These studies, then, may not reflect signaling that occurs at physiological concentrations of the receptors, even in most human cancers.

The results of my study demonstrate the requirement for ligands for Egfr signaling in vivo, and do not provide evidence of ligand-independent signaling in early Drosophila development. That the Drosophila Egfr is dependent in vivo upon ligands to signal, and that human ErbB2 so closely resembles the Drosophila Egfr and shares many autoinhibitory features with Drosophila Egfr that act to prevent signaling in the absence
of ligand (Alvarado et al. 2009), suggests that ErbB2, though it itself has no known ligands, may yet be dependent upon ligands to signal. ErbB2 may be dependent upon ligands to signal through the ligand binding capacity of its heterodimerization partners (Egfr/ErbB1, ErbB3, ErbB4).

Egfr has long been known to have two populations of ligand binding sites—low affinity sites and high affinity sites (Shoyab et al. 1979; Magun et al. 1980). Recent structural data have elucidated that the low and high affinity ligand binding sites on the receptor correspond to the order of occupancy of the two sites on a receptor dimer. The first site to be occupied on a dimer exists as a high affinity site for ligand binding, and can be occupied by a high or low affinity ligand. The conformation of the second ligand binding site on the receptor dimer, however, is altered by the binding of the first ligand, and thus the second ligand binding site on the receptor dimer site exists as a low affinity site that can be occupied by only a high affinity ligand (Alvarado et al. 2010). A singly-occupied receptor dimer can generate signal, offering a mechanism by which ErbB2 may be unable to bind ligand directly and yet be dependent upon ligand binding, to its heterodimerization partner, to signal.

If ErbB2 is indeed dependent upon ligands to signal despite its monomeric resemblance to the ligand-bound forms of ErbB family members, then its action can be regulated by therapeutic interventions directed at the ligands.
ErbB2/HER2/neu is overexpressed in around 20-25% of breast cancers, specifically, the particularly-aggressive “HER2-positive” breast cancers (Spector and Blackwell 2009). Because of this, HER2 has been the focus of much research, resulting in the development of trastuzumab, a monoclonal antibody therapy for HER2-positive cancers. Trastuzumab and Egfr/HER1-directed therapies have not, however, achieved the hoped for level of clinical efficacy (Morgillo and Lee 2005). One explanation for these observations is the fact that the receptor-targeted therapies do not block receptor dimerization, and potent signals can still be transmitted through heterodimerization with family members; ErbB2/HER2 is the preferred heterodimerization partner of each of the other three ErbB family members (Tzahar et al. 1996). Ligand-directed therapies have been proposed as a result (Yotsumoto et al. 2009), and my research supports the model in which ligand-directed therapies may be effective in cancers with overexpression of ErbB family members, including HER2, utilizing an innate regulatory mechanism—ligand dependence.

5.2 Ligand functional redundancy

I have also shown that there is a high degree of functional redundancy between all the Drosophila Egfr ligands, and that a low dose of a strong TGFα ligand like Spi can largely replace the developmental function of a weaker ligand like Vn. This is consistent with a signaling model in which the signal generated upon binding of a specific ligand can be characterized by the “strength” of the ligand; stronger ligands induce a set of targets, and weaker ligands induce a subset of those targets (Fig 5.1A).
Theoretically, flies could dispense with *vn* and *Krn* for zygotic development and rely on expression of *spi* in its normal pattern and at low levels in tissues where *vn* is required. *spi* alone could suffice because *Krn* mutants are phenotypically normal and *grk* has a strictly maternal role. Indeed, *C. elegans* with its relatively simple body plan has only one EGF ligand (Hill and Sternberg 1992). Yet flies are more complex and have evolved to depend on four ligands with different intrinsic activities, affinities, and patterns of expression rather than on mechanisms that modulate signaling levels with one or a smaller number of ligands. Nevertheless, it appears that replicating the quantitative level of signaling is largely sufficient for appropriate cell responses.

Individual ligands have specific roles in normal development, yet the mutant phenotypes of knockout mice lacking a single ligand are mostly mild, indicating functional redundancy between ligands in development (Schneider et al. 2008).

5.3 Ligand functional specificity

Despite the surprisingly extensive rescue of *vn* mutants effected by the TGFα ligands, and in contrast to the capacity of Spi to replace the function of Vn, the lower biological activity of Vn limits its ability to replace or emulate the activity of Spi (Schnepp et al. 1998; Golembo et al. 1999; Donaldson et al. 2004; McDonald et al. 2006; Jiang and Edgar 2009). Vn, as a low affinity ligand, cannot occupy the second ligand-binding site on a receptor dimer (Alvarado et al. 2010), and may never, at any dose, be able to induce
some targets of Spi or a high affinity ligand. The striking yet incomplete rescue of vn mutants by TGFα ligands does not exclude a model of signaling whereby EGF ligands induce primarily targets that are part of a set common to all EGF ligands, but also “weak” ligands induce unique targets dependent upon their intrinsic activity (Fig 5.1B). Furthermore, the unique phenotypes seen in the rescue of the vn mutants with expression of VnΔIg, a synthetic ligand that differs from native Vn outside of the EFG domain, demonstrates that properties of a ligand other than the binding affinity of the receptor interaction domain can alter the signaling outcome of the ligand.

Recently it has been demonstrated that two ligands of the vertebrate Egfr can elicit binary differences in signaling- on or off for a given target- despite utilizing many shared core components of the pathway (Nakakuki et al. 2010), and difference in intrinsic signaling between ligands have been demonstrated which arise from differences in the ligands which lie outside of the EGF domain involved in ligand-receptor binding (Wilson et al. 2009; Wilson et al. 2012a; Wilson et al. 2012b).

In addition to the in vitro data, which demonstrate the effect of individual ligands at saturating concentrations that likely do not reflect physiological levels, there are also data demonstrating that ligands elicit functionally specific responses in vivo; for example, expression of amphiregulin under the K14 promoter in mice has a more severe phenotype than TGFα expression from the same promoter (Vassar and Fuchs 1991; Dominey et al. 1993; Cook et al. 1997; Cook et al. 2004; Wilson et al. 2009).
In both breast and lung cancer, EGF expression correlates with improved prognosis whereas TGFα or amphiregulin expression is associated with tumor aggressiveness and resistance to gefitinib. EGF, with a lower intrinsic activity for stimulating tumor aggressiveness and chemoresistance, may in fact competitively inhibit TGFα and amphiregulin (Kakiuchi et al. 2004; Lee et al. 2007; Lemos-Gonzalez et al. 2007; Revillion et al. 2008; Wilson et al. 2009).

I have presented evidence here that such competitive inhibition of growth is observed in vivo, in the developing fly wing. Reducing the dose of the ligand Spi increased the size of the fly wing, as did reducing (but not eliminating) the dose of Krn.

5.4 Implications of ligand dependence paired with ligand functional specificity

Ligand dependence of the ErbB family receptors, together with ligand functional specificity and the ability of some ligands to competitively inhibit the adverse effects of others, would suggest the possibility of designing small molecule ErbB kinase inhibitors that mimic EGF ligands. The mimics would be designed to have comparable binding affinities to the native ligands but lack the ability to stimulate tumor aggressiveness.

These small molecule ligand-mimics could be used to competitively inhibit the negative effects of the native ligands, and, coupled with the innate regulatory mechanism of ligand dependence, could have a powerful effect on cancers with ErbB misregulation, including cancers with resistance to our current receptor-targeted therapies (monoclonal antibodies, kinase inhibitors).
Figure 5.1 Quantitative versus qualitative signaling models. Two hypothetical arrangements of target gene sets induced by EGF ligands. Shown are quantitative (A) versus qualitative (B) differences in the target gene sets. An increase in the strength of a ligand as pictured in the quantitative model (A) is indicated by an increase in the radius of the circle denoting the subset.


Golembo M, Yarnitzky T, Volk T, Shilo BZ. 1999. Vein expression is induced by the EGF receptor pathway to provide a positive feedback loop in patterning the Drosophila embryonic ventral ectoderm. Genes & development 13: 158-162.


