Development 134, 3011-3020 (2007) doi:10.1242/dev.006445

Control of *Drosophila* wing growth by the *vestigial* quadrant enhancer

Myriam Zecca and Gary Struhl*

Following segregation of the *Drosophila* wing imaginal disc into dorsal (D) and ventral (V) compartments, the wing primordium is specified by activity of the selector gene *vestigial* (*vg*). In the accompanying paper, we present evidence that *vg* expression is itself driven by three distinct inputs: (1) short-range DSL (Delta/Serrate/LAG-2)-Notch signaling across the D-V compartment boundary; (2) long-range Wg signaling from cells abutting the D-V compartment boundary; and (3) a short-range signal sent by *vg*-expressing cells that entrains neighboring cells to upregulate *vg* in response to Wg. Furthermore, we showed that these inputs define a feed-forward mechanism of *vg* autoregulation that initiates in D-V border cells and propagates from cell to cell by reiterative cycles of *vg* upregulation. Here, we provide evidence that this feed-forward mechanism is required for normal wing growth and is mediated by two distinct enhancers in the *vg* gene. The first is a newly defined 'priming' enhancer (*PE*), that provides cryptic, low levels of Vg in most or all cells of the wing disc. The second is the previously defined quadrant enhancer (*QE*), which we show is activated by the combined action of Wg and the short-range *vg*-dependent entraining signal, but only if the responding cells are already primed by low-level Vg activity. Thus, entrainment and priming constitute distinct signaling and responding events in the Wg-dependent feed-forward circuit of *vg* autoregulation mediated by the *QE*. We posit that Wg controls the expansion of the wing primordium following D-V segregation by fueling this autoregulatory mechanism.

KEY WORDS: *Drosophila* wing, Morphogen, Organ growth, Selector gene, Vestigial

INTRODUCTION

The *Drosophila* wing is a discrete organ of stereotyped pattern, size and shape specified by a selector gene, *vestigial* (*vg*) (Williams et al., 1991; Williams et al., 1993; Kim et al., 1996; Halder et al., 1998; Liu et al., 2000). Shortly after the wing primordium is first apparent as a cluster of ~40 *vg*-expressing cells (Wu and Cohen, 2002), it is subdivided into dorsal (D) and ventral (V) compartments by the heritable activation of the selector gene *apterous* (*ap*) in the D compartment (Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994). Short-range Delta/Serrate/LAG-2 (DSL)-Notch signaling across the D-V boundary then initiates a dramatic 200-fold expansion of the wing primordium to a population of ~8000 *vg*-expressing cells under the control of the long-range morphogens Wingless (Wg) and Decapentaplegic (Dpp) (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Zecca et al., 1995; de Celis et al., 1996; Doherty et al., 1996; Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997). Here, we examine how morphogens and selector genes control organ growth, using the Wg-driven expansion of the population of *vg*-expressing cells – the wing primordium – as a paradigm.

In the accompanying study (Zecca and Struhl, 2007), we focused on how Wg signaling controls *vg* expression and wing growth, taking advantage of *ap* mutant discs. Normally, short-range DSL-Notch signaling across the D-V boundary induces 'border' cells flanking the boundary to express both *wg* and *vg* (Williams et al., 1994; Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; de Celis et al., 1996; Kim et al., 1996; Neumann and Cohen, 1996; Rulifson et al., 1996). However, in *ap* mutant discs, border cells are not specified, the early expression of *vg* that normally precedes the D-V segregation dissipates, and the presumptive wing primordium fails to develop (Williams et al., 1993). By generating clones of cells that ectopically express Vg, Wg or both, we showed that cells within *ap* mutant discs could be recruited to express *vg* in response to Wg, but only if they were located near or next to cells that already express Vg. These results defined a previously unknown feed-forward mechanism of *vg* autoregulation, and led us to propose that D-V border cells normally control the expansion of the wing primordium by providing both a long-range morphogen, Wg, as well as the initial Vg-dependent feed-forward signal that entrains neighboring cells to express *vg* in response to Wg.

Here, we extend our results in *ap* mutant discs by testing whether this autoregulatory circuit is required for normal wing growth in wild-type discs. We first demonstrate that the previously identified quadrant enhancer (*QE*) of the *vg* gene mediates *vg* autoregulation in response to Wg, the feed-forward signal, and a newly defined third input: 'priming' of the *vg* locus by pre-existing low levels of Vg. We then present evidence that *QE*-driven expression of *vg* is necessary and sufficient for the expansion of the wing primordium organized by D-V border cells. These findings support our hypothesis that wing growth normally depends on a non-autonomous autoregulatory circuit of *vg* gene expression triggered by short-range DSL-Notch signaling and fueled by long-range Wg signaling.

MATERIALS AND METHODS

Genetic materials

Most mutant alleles, transgenes and protocols employed are described in the accompanying study (Zecca and Struhl, 2007). Transgenes and protocols unique to this study are as follows.

5XQE>Tubα1-flu-GFP>vg and *5XQE>CD2,y2>vg* transgenes were assembled using DNAs described in Zecca and Struhl (Zecca and Struhl, 2007). *5XQE>vg* derivatives of these transgenes generated by germ-line

Howard Hughes Medical Institute, Department of Genetics and Development, Columbia University College of Physicians and Surgeons, 701 W 168th Street, New York, NY 10032, USA.

*Author for correspondence (e-mail: gs20@columbia.edu)

Accepted 12 June 2007

excision of the intervening Flp-out cassette resulted in dominant larval lethality. Similarly, induction of *5XQE>vg* clones in first instar larvae carrying such transgenes resulted in late larval or pupal lethality. Hence, our analysis of *5XQE>vg* transgene activity was restricted to the behavior of Flp-out clones in the wing disc.

The *rp49>CD2,y2>vg* transgene was assembled using the *rp49* promoter, as well as the destabilizing *Hsp70* 3′ UTR (Greenwood and Struhl, 1997; Casali and Struhl, 2004). *rp49>vg* derivatives (referred to subsequently as *rp49-vg*) of each of several *rp49>CD2,y2>vg* transgene insertions were generated by germ-line excision of the *>CD2,y2>* cassette. All but one of these resulted in pupal lethality when present in one copy. However, the exceptional *rp49-vg* derivative was viable and fertile in one copy, albeit pupal lethal when homozygous, indicating that this transgene expresses a lower level of exogenous Vg than the others, and on this basis we selected it for use in all subsequent experiments.

Generation of clones of cells that ectopically express, or lack, gene activity

Clones of *vg* or *arrow* (*arr*) mutant cells were generated by Flp-mediated mitotic recombination (Golic, 1991). The *Minute* technique (Morata and Ripoll, 1975) was used to generate *vg83b27* (vg^b) clones with a growth advantage (Fig. 2E). Clones expressing exogenous Vg were generated using the Flp-out technique (Struhl and Basler, 1993). In some cases, two types of clones were generated in the same disc to yield either (1) adjacent clones of different type (e.g. *5XQE>vg* clones next to *Tubα1>vg* clones; e.g. Fig. 4); (2) coincident clones of different type [e.g. *vg83b27R* (vg^0) *5XQE>vg* clones; e.g. Fig. 3C]; or (3) 'clones within clones' (e.g. arr^0 clones inside *5XQE>vg* clones; Fig. 3D,E). Unless otherwise stated, clones were induced by heat shocking first instar larvae [24-48 hours after egg laying (AEL)] at 36°C for 30 minutes; for 'clones within clones', larvae were heat shocked, as above, during the first instar, and then given a second heat shock of the same length and temperature at 60-84 hours AEL (late second to early third instar), or 48-72 hours AEL (second instar). In all of the experiments in this study, mature wing discs were dissected from late third instar larvae, and fixed and analyzed as previously described (Zecca and Struhl, 2002).

Twin spot analysis of vg^b and vg^0 clones

Larvae were heat shocked (35°C 10 minutes) at the times indicated in Fig. 2. Mutant clones were marked by loss of GFP expression, whereas their wild-type sibling (twin) clones were marked by strong GFP expression (owing to homozygosity of the *Hsp70-flu-GFP* transgene). All, and only those, wild-type clones that contributed to the presumptive wing pouch area (marked by *1XQE-lacZ* expression) were scored for the presence and contribution of their associated mutant twins. For further details, see Fig. 2.

Genotypes

Genotypes are listed by figure panel; except where stated otherwise, the X chromosome was *y w Hsp70-flp*.
1B: *1XQE-lacZ* vg^{83b27}/vg^{83b27}.
1C: *1XQE-lacZ* vg^{83b27R}/vg^{83b27R}.
1D: *1XQE-lacZ* vg^{83b27}/vg^{83b27}*; rp49-vg/rp49-vg*.
1E: *1XQE-lacZ* vg^{83b27R}/vg^{83b27R}*; rp49-vg/rp49-vg*.
1F: *1XQE-lacZ* vg^{83b27}/vg^{83b27R}*; Tubα1>flu-GFP,* y^+*>vg/+*.
1G: *1XQE-lacZ* vg^{83b27R}/vg^{83b27R}*; Tubα1>flu-GFP,* y^+*>vg/+*.
2A,B,F: *FRT42D* vg^{83b27}*/1XQE-lacZ FRT42D Hsp70-flu-GFP.*
2C: *y w 5XQE-DsRed/y w Hsp70-flp; FRT42D* vg^{83b27R}*/1XQE-lacZ FRT42D Hsp70-flu-GFP; rn-lacZ/+*.
2D,F: *FRT42D* vg^{83b27}*/1XQE-lacZ FRT42D Hsp70-flu-GFP; rp49-vg/rp49-vg*.
2E: *FRT42D Minute(2)IK Hsp70-flu-GFP/FRT42D* vg^{83b27}*; 1XQE-lacZ/+*.
2F: *1XQE-lacZ FRT42D Hsp70-flu-GFP/FRT42D Tubα1-DsRed* (for vg^+ clones).
3B,C: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; FRT42D* vg^{83b27R}*/1XQE-lacZ FRT42D Hsp70-flu-GFP.*
3D: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; FRT42D* arr^2*/FRT42D Tubα1-DsRed; 1XQE-lacZ/+*.
3E: *y w 5XQE-DsRed/y w Hsp70-flp; FRT42D* arr^2*/FRT42D Hsp70-CD2; Tubα1>flu-GFP,* y^+*>vg/+*.
4A: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; Tubα1>flu-GFP,* y^+*>vg/+*.
4B,C: *1XQE-lacZ* ap^{56f} vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; Tubα1>DsRed, y2>vg/5XQE>Tubα1-flu-GFP>vg*.
5A: *FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; BE-*vg^{GFP}*/+*.
5B,C: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; FRT42D* vg^{83b27R}*/1XQE-lacZ* ap^{56f} vg^{83b27R}*; BE-*vg^{GFP}*/+*.
5D: *FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; BE-*vg^{GFP}*/5XQE>Tubα1-flu-GFP>vg*.
5E: *FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; BE-*vg^{GFP} *rp49-vg/+*.
5F,G: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; BE-*vg^{GFP} *rp49-vg/+*.
5H: *FRT42D* vg^{83b27R}*/1XQE-lacZ* vg^{83b27R}*; BE-*vg^{GFP} *rp49>vg/5XQE>Tubα1-flu-GFP>vg*.
6A,C: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp.*
6B: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; 1XQE-lacZ FRT42D Hsp70-flu-GFP/1XQE-lacZ* ap^{56f} vg^{83b27R}.
6D: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; Dll-lacZ/+; C765-Gal4/+*.
6E: *y w 5XQE>CD2,y2>vg/y w Hsp70-flp; Dll-lacZ/5XQE-DsRed* vg^{83b27R}*; UAS-wg rp49-vg/C765-Gal4.*

RESULTS

Following the segregation of the wing imaginal disc into D and V compartments, *vg* expression is induced in D-V border cells and then extends into the rapidly expanding 'pouch' of the disc, defining the growing wing primordium (Williams et al., 1993; Williams et al., 1994; Kim et al., 1995; Kim et al., 1996). Border cell and pouch expression are associated, respectively, with the activity of distinct boundary enhancer (*BE*) and quadrant enhancer (*QE*) elements (Williams et al., 1994; Kim et al., 1996). Here, we have sought to determine whether *QE* elements are responsible for mediating the feed-forward propagation of *vg* in response to Wg, and if so, whether operation of this autoregulatory circuit is necessary and sufficient for the normal expansion of the wing primordium that occurs following the D-V segregation.

Our main approach has been to generate a *vg* transgene that is expressed under *QE* control, validate that it is activated in response to the combined inputs of Wg and the *vg*-dependent feed-forward signal, and then test whether it is necessary and sufficient to mediate *vg* expression and wing growth away from the D-V compartment boundary. Crucial to the success of this approach has been our discovery of a third input necessary for the *vg* autoregulatory response: priming of the *vg* gene by pre-existing low levels of Vg. We begin by describing our evidence for priming, which arose unexpectedly from experiments designed to test the capacity of a *BE*-deficient allele of *vg* to mediate feed-forward autoregulation.

vg feed-forward autoregulation requires 'priming' by cryptic, low levels of Vg

vg^{83b27} (henceforth vg^b) is an internal deletion of the intron 2 segment of *vg* that removes the previously defined *BE* as well as adjoining sequences, but leaves intact the rest of the gene, including the *QE* (Williams et al., 1993; Kim et al., 1996). Mature vg^b mutant discs, like *vg*-null (henceforth vg^0) discs, lack the wing primordium (Fig. 1A-C) (Williams et al., 1993), as expected if D-V border cells require *BE* activity to express *vg* and to initiate propagation of *vg* expression into neighboring tissue. However, clones of vg^b cells should retain the capacity to propagate *vg* expression in response to wild-type border cells and, hence, to contribute normally to the developing wing. In testing this prediction, we obtained evidence that the vg^b mutation deletes a previously unknown 'priming' enhancer (*PE*), in addition to the

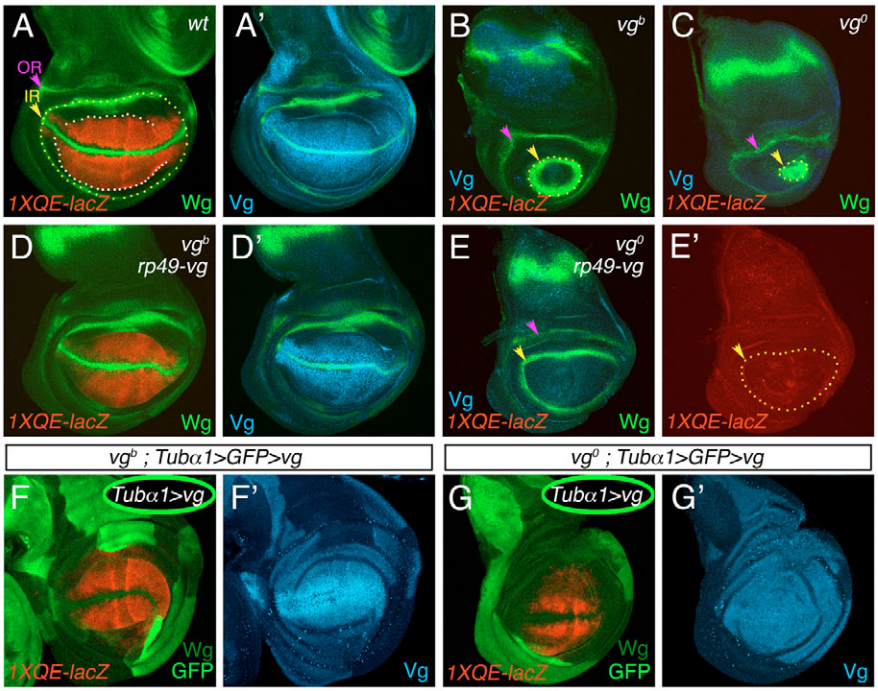

Fig. 1. Vg expression, *QE* activity and wing development in wild-type, vg^b and vg^0 wing discs. (**A**,**A'**) *1XQE-lacZ* (A, β-gal, red) and Vg (A′, blue) expression define the wing primordium of the mature *Drosophila* wing disc (also delimited by a characteristic fold, white dots). The surrounding *rotund* (*rn*)-only domain (unlabeled) is circumscribed by the inner of two rings of Wg expression [green; inner ring (IR), yellow arrowhead and yellow dots in A-C,E; outer ring (OR), purple arrowhead]. (**B**,**C**) Vg and *1XQE-lacZ* expression and the wing primordium are absent, and the *rn*-only domain is reduced or eliminated in vg^b (B) and vg^0 (C) discs. (**D-E'**) Vg and *1XQE-lacZ* expression and wing development are rescued in *rp49-vg* vg^b discs (D,D′), but not in *rp49-vg* vg^0 discs (E,E′; except for a few patches of weak *1XQE-lacZ* expression visible in the overexposed image in E′). The *rn*-only domain appears rescued in both *rp49-vg* vg^b and *rp49-vg* vg^0 discs. Vg expression is also restored in D-V border cells of *rp49-vg* vg^b discs (albeit only in the wing pouch), indicating additional *BE*(s) in the vg^b gene. (**F-G'**) *Tubα1>vg* clones (black by the absence of GFP, green) autonomously rescue *1XQE-lacZ* expression and wing development in vg^b (F,F′) and vg^0 (G,G′) discs. *1XQE-lacZ* is expressed in a quadrant pattern in both cases, presumably in response to Wg-expressing (faint green) D-V border cells. Vg expression (bright blue) appears normal in the wing pouch of the vg^b disc (F′), indicating a normal *QE* response by the vg^b allele superimposed on the uniform, moderate level of exogenous Vg (dull blue, compare F′,G′). Here, and in the remaining figures, clones were induced during the first larval instar (unless otherwise stated), discs are from mature third instar larvae, anterior is left, dorsal is up, protein or reporter gene stains are indicated by color, and relevant genotypes are indicated either above the images or, in the case of clones, indicated by outlined ovals filled in red, blue or black, as marked in the experiment.

BE, and that cryptic, low levels of Vg, expressed under the control of this enhancer, are a prerequisite for feed-forward autoregulation.

Initially, we generated vg^b clones in otherwise wild-type discs carrying the standard *1XQE-lacZ* reporter. As expected, vg^b clones induced after D-V segregation (mid- to late second instar) were able to contribute normally to the wing pouch and express *1XQE-lacZ* (Fig. 2B,F; data not shown). However, vg^b clones induced at earlier times showed a progressive decrease in their ability to do so (Fig. 2A,F). Indeed, vg^b clones induced during the first instar and given a competitive growth advantage using the *Minute* technique (Morata and Ripoll, 1975) failed to express *1XQE-lacZ* and appeared to be excluded from the wing pouch, forming non-wing tissue at its expense (Fig. 2E). Thus, early-induced vg^b clones appear compromised for the ability to activate the *QE* and develop as wing tissue, even when they abut wild-type Vg-expressing cells and are in a position to receive Wg as well as Dpp.

One explanation for this unexpected result is that vg^b cells lack an additional component of the proposed *vg* autoregulatory circuit. The *QE* contains binding sites for Scalloped (Sd), the DNA-binding protein that combines with Vg to form a composite transcriptional activator (Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Guss et al., 2001; Halder and Carroll, 2001). Moreover, Sd and the presence of its binding sites are necessary for *QE* activity (Halder et al., 1998; Guss et al., 2001). Hence, the *QE* might need to be 'primed' by cryptic, low-level Vg to mediate feed-forward autoregulation, and the presence of such pre-existing Vg might depend on a distinct 'priming' enhancer (*PE*) deleted in the vg^b allele. According to this hypothesis, sufficient Vg would perdure in vg^b cells induced after the D-V segregation to supply the requisite priming function, but not in the descendents of vg^b cells induced before D-V segregation.

To test this, we generated an *rp49-vg* transgene in which the *vg* coding sequence is expressed at exceptionally low level, under the control of the uniformly active, but weak, *ribosomal protein 49* (*rp49*; also known as *RpL32* – Flybase) promoter (Greenwood and Struhl, 1997; Casali and Struhl, 2004), and asked whether such low-level expression is sufficient to rescue normal wing development and endogenous *vg* expression in early-induced vg^b clones.

Wing discs homozygous for the *rp49-vg* transgene express so little Vg protein that we were unable to detect it by antibody staining in wild-type or vg^0 discs. In addition, homozygosity for the transgene failed to rescue wing development in vg^0 discs. Instead, vg^0*; rp49-vg* discs formed abnormally small wing pouches composed of cells that appeared to correspond to the periphery of the normal pouch, where neither Vg nor *1XQE-lacZ* expression were readily detectable (Fig. 1E). Nevertheless, homozygosity for the *rp49-vg* transgene almost completely rescued the capacity of early-induced vg^b clones to express endogenous Vg, as well as the *1XQE-lacZ* reporter, and to contribute to the wing pouch (Fig. 2D,F). Indeed, it restored normal *vg* expression and wing development in the wing pouch of entirely mutant vg^b discs, including in D-V border cells, despite the absence of the well-defined and evolutionarily conserved *BE* (Fig. 1D).

Thus, the capacity of vg^b cells to express *vg* and to develop as normal wing tissue appears to depend on cryptic, low-level Vg activity, defining a third input, 'priming', that is required together with Wg and the feed-forward signal, for upregulation of *vg* away from the D-V boundary. These findings also indicate that the vg^b allele retains

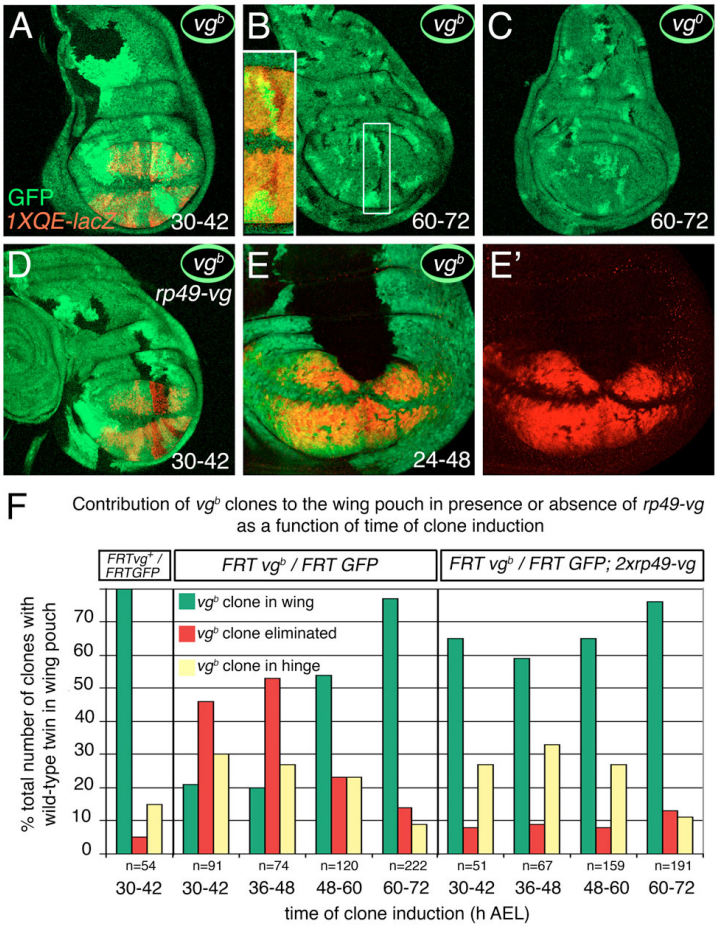

Fig. 2. Vg expression and wing development in vg^b clones; evidence that *QE* activity requires 'priming' by Vg. (**A-C**,**E**,**E'**) vg^b (A,B,E,E') and vg^0 (C) clones (black by the absence of GFP, green) induced before (A,E,E') or after (B,C) D-V segregation. Both vg^b and vg^0 clones contribute normally to the proximal hinge and notum primordia. However, vg^b clones induced before the D-V segregation often fail to contribute to the wing pouch (A,F) or to express *1XQE-lacZ* (E', red), whereas clones induced afterwards succeed (B, inset; see F). vg^0 clones invariably fail to contribute to the wing pouch (C). For A-D, the sibling 'twin' clones are marked by doubled GFP expression, bright green. For E, the *Minute* technique was used to give the vg^b clone a growth advantage. Numbers correspond to time of clone induction in hours after egg laying (h AEL); the D-V segregation occurs at ~60 h AEL. (**D**) Early-induced vg^b clones generated in homozygous *rp49-vg* discs contribute to the wing pouch and express *1XQE-lacZ* (red). (**F**) Bar charts showing the survival of vg^b or control (vg^+) clones in the wing pouch, relative to that of their wild-type twin clones, depending on the time of induction (h AEL) and the absence or presence of *rp49-vg*. Bars represent the percentage of wild-type twin clones that contribute to the pouch (1) with an associated vg^b clone that contributes to the pouch (green), (2) without an associated vg^b clone (red), or (3) with an associated vg^b clone that contributes only to the hinge primordium (yellow). *n*, total number of clones scored for each experimental condition. In the absence of the *rp49-vg* transgene, early-induced vg^b clones contribute only rarely to the pouch, and appear instead to sort into the hinge primordium or to be lost. The ratio of vg^b clones that contribute to the pouch (type 1, green) increases at the expense of the other two types as a function of time of induction, reaching the wild-type distribution after the D-V segregation. In *rp49-vg* discs, both early- and late-induced vg^b clones contribute almost normally to the pouch.

at least one additional *BE*, and that activity of this *BE* depends on priming. Hence, the primary cause of the vg^b 'no wing' phenotype appears to be the deletion of the *PE*, not the *BE*, in intron 2. In subsequent experiments, we used the *rp49-vg* transgene to satisfy the requirement for priming in the absence of the endogenous *vg* gene.

Generation of a *vg* transgene that expresses Vg under the control of quadrant enhancer sequences

The *QE* is active in all cells in which we posit that feed-forward autoregulation occurs, consistent with the *QE* being responsible for mediating this autoregulatory circuit as well as normal wing growth. To test this, we sought to assay the behavior of cells in which the endogenous *vg* gene is replaced with a transgene that expresses Vg under the control of *QE* sequences.

To express Vg under the control of *QE* sequences, we generated transformants of a *5XQE>CD2>vg* Flp-out transgene in which five copies of the *QE* drive the expression of either rat CD2 or Vg. In the absence of Flp recombinase, the *5XQE>CD2>vg* transgene insertions behaved like the previously described *1XQE-lacZ* and *5XQE-DsRed* reporter transgenes, showing expression of CD2 that was tightly restricted to the wing pouch but excluded from the D-V border cells within the pouch (Fig. 3A) (Kim et al., 1996; Zecca and Struhl, 2007). Upon heat shock-induced expression of Flp, the *>CD2>* cassette is excised in single cells, generating clones of *5XQE>vg* cells marked in the prospective wing pouch by the absence of CD2 and the expression of exogenous Vg. Different transgene insertions showed some variation in the level and extent of Vg expression following excision of the *>CD2>* cassette. For the most strongly active insertions, clones of *5XQE>vg* cells that were also vg^0 developed as wing tissue and expressed levels of exogenous Vg protein similar to those of endogenous Vg expressed in surrounding wild-type cells (Fig. 3B,C). By contrast, clones of *5XQE>vg* vg^0 cells generated using the less active insertions showed weaker, patchy expression of Vg and rescued wing development less well (data not shown). We therefore focused our analysis on one such strongly active *5XQE>CD2>vg* insertion, and performed the experiments described below to validate that its activity depends on Wg and the Vg-dependent feed-forward signal.

Requirement for Wg input

To test whether expression of the chosen *5XQE>CD2>vg* transgene requires Wg input, we heat shocked first instar larvae to generate large clones of *5XQE>vg* cells in otherwise *5XQE>CD2>vg* wing discs, and then heat shocked them again during the late second to early third larval instar to generate smaller clones of cells mutant for *arr* (arr^0), which encodes a co-receptor essential for transducing Wg (Wehrli et al., 2000). We observed that surviving arr^0 clones showed greatly reduced or no expression of both CD2 and Vg, irrespective of whether the clones were located within the *5XQE>vg* territories (Fig. 3D) or in surrounding *5XQE>CD2>vg* tissue (data not shown). These results indicate that expression of both the excised and intact forms of the transgene require Wg input.

Because arr^0 clones are associated with the loss of endogenous *vg* expression (data not shown), it is possible that activity of the *5XQE* element depends only on the presence of Vg protein, and hence might 'report' Wg input indirectly, via activation of other, as

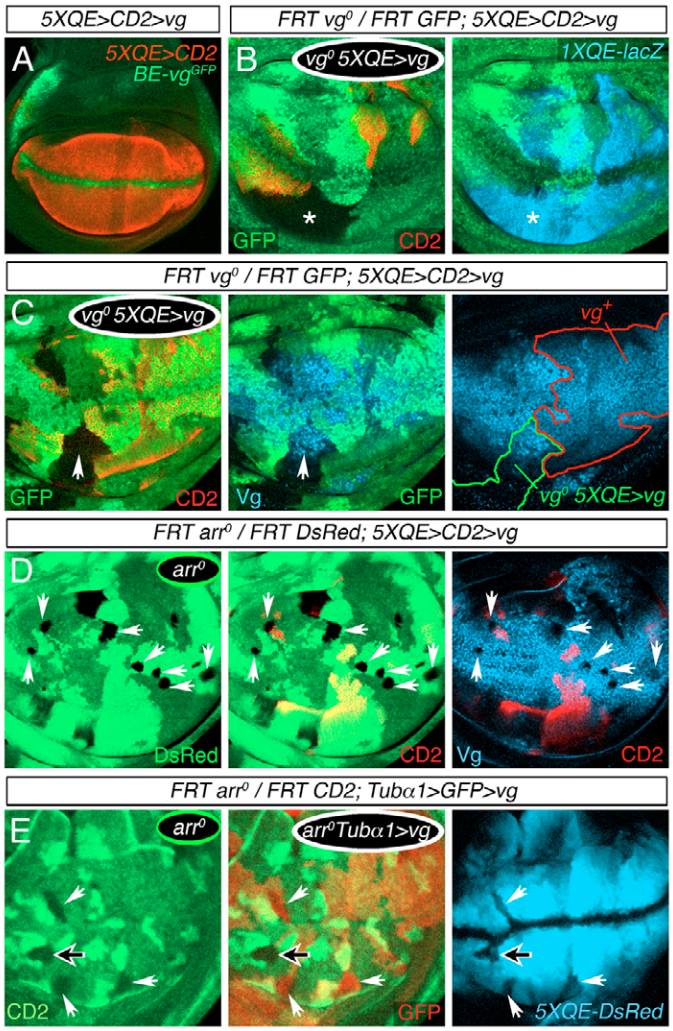

Fig. 3. *5XQE* transgene expression requires Wg input. (**A**) *5XQE>CD2>vg* expression (CD2, red) in a wild-type wing disc (the D-V boundary is marked by *BE-vg^{GFP}* expression, green). (**B**,**C**) Rescue of wing development in *vg^{0} 5XQE>vg* clones [black by the absence of both GFP and CD2, and blue by the expression of *1XQE-lacZ* (B) or Vg (C); *vg^{+} 5XQE>CD2>vg* tissue appears yellow]. Note in C that the level of Vg expressed within the *vg^{0} 5XQE>vg* clone (outlined in green) is similar to that in neighboring wild-type tissue (*vg^{+} 5XQE>CD2>vg*, outlined in red). (**D**) Small, late-induced *arr^{0}* clones (black by absence of DsRed, green; white arrows) in a *5XQE>CD2>vg* wing disc composed almost entirely of large, early-induced *5XQE>vg* clones (remaining patches of *arr^{+} 5XQE>CD2>vg*-expressing tissue are marked by CD2 expression, red). *5XQE>vg* (Vg, blue) is reduced or absent in the *arr^{0}* clones. (**E**) *5XQE-DsRed* expression (blue) is lost in an *arr^{0}* clone located inside a *Tubα1>vg* clone (black arrow; black by the absence of both CD2, green, and GFP, red), and in *arr^{0}* clones in the surrounding *Tubα1>GFP>vg* tissue (white arrows; appears red).

yet unidentified, Wg-responsive enhancers in the endogenous *vg* gene. To assess this, we replaced the *5XQE>CD2>vg* transgene with a *Tubα1>GFP>vg* transgene (Fig. 1G) (*Tubα1* is also known as *αTub84B* – Flybase) (Zecca and Struhl, 2007) to create clones of *Tubα1>vg* cells that continuously express moderate levels of exogenous Vg, irrespective of Wg input. We then generated clones of *arr^{0}* cells within such *Tubα1>vg* clones and asked whether activity of the *5XQE* element still requires Wg input, using expression of a *5XQE-DsRed* reporter to monitor *5XQE* activity. *Tubα1>vg* clones make sufficient Vg protein to rescue expression of both the *5XQE-DsRed* and *5XQE>CD2>vg* transgenes, as well as wing development, in *vg^{0}* discs (Fig. 1G; data not shown). Nevertheless, *arr^{0}* clones generated within *Tubα1>vg* clones ceased to express the *5XQE-DsRed* transgene (Fig. 3E). We conclude that *5XQE* transgene activity does not merely reflect the presence of Vg protein, but instead depends on Wg input even when cells are supplied continuously with exogenous Vg. Significantly, such *arr^{0}* clones are subsequently lost from the wing pouch, within ~12 hours after they cease to express the *5XQE-DsRed* reporter, despite being independently and continuously supplied with exogenous Vg (data not shown). Hence, cells within the wing primordium still require continuous Wg input to survive and grow, even when they are provided with Vg protein by other means (see Discussion).

Requirement for feed-forward input

To determine whether activity of the *5XQE>CD2>vg* transgene requires feed-forward input, we asked whether expression of either the intact or excised form of the transgene depends on the presence of neighboring Vg-expressing cells. *vg^{0}* wing discs carrying *5XQE>vg* clones in a background of *5XQE>CD2>vg* cells do not express either Vg or CD2 (data not shown). To test whether they fail to do so because both forms of the transgene require feed-forward input, we generated *Tubα1>vg* clones concomitantly in these same discs (by excision of a *Tubα1>GFP>vg* transgene) and asked if such exogenous Vg-expressing clones could act non-autonomously to induce either *5XQE>vg* or *5XQE>CD2>vg* expression.

Activity of the intact *5XQE>CD2>vg* transgene in this experiment was monitored by CD2 expression and that of the excised *5XQE>vg* transgene was monitored by either Vg (data not shown) or *1XQE-lacZ* expression (which is robustly expressed in all cells that express the *5XQE>vg* transgene, e.g. Fig. 3B, Fig. 4A, Fig. 6B). Accordingly, the presence of *5XQE>vg* clones can only be visualized if the experiment gives a positive result: namely, that *Tubα1>vg* clones (marked by the absence of GFP) can indeed induce *5XQE>vg* expression in neighboring clones of *5XQE>vg* cells (as monitored by Vg or *1XQE-lacZ* expression). Nevertheless, we identified many such positively responding clones (lavender-colored clone in diagram in Fig. 4A; data not shown). Importantly, in all cases, these clones were adjacent to *Tubα1>vg* clones (green clone in diagram, Fig. 4A). Thus, it appears that clones of *Tubα1>vg* cells can induce *5XQE>vg* expression in neighboring *5XQE>vg* clones. Moreover, induction appears to depend on contact between the two clones.

Significantly, *5XQE>vg* expression was not restricted to those *5XQE>vg* cells that abut the neighboring *Tubα1>vg* clone. Instead, *5XQE>vg* expression appeared to spread many cell diameters into the *5XQE>vg* clone, away from the abutting *Tubα1>vg* clone, and was associated with an expansion of the rescued wing primordium. In addition, such *5XQE>vg*-expressing cells were also able to induce neighboring *5XQE>CD2>vg* cells far from the abutting *Tubα1>vg* clone to express CD2 (yellow cells in Fig. 4A). Thus, the *5XQE>vg* transgene appears to have the capacity not only to respond to the feed-forward signal, but also to propagate feed-forward signaling from one cell to the next. As evident in Fig. 4A, the range over which *5XQE>vg* cells can induce *5XQE>CD2>vg* expression across the clone border is tightly restricted to only a few cell diameters, consistent with the feed-forward signal being dependent on cell contact.

In principle, activation of the *5XQE>vg* transgene by feed-forward signaling should require priming by low levels of Vg protein in the responding cells, and hence is unexpected in *vg^{0}* discs. However, the *5XQE>vg* cells in this experiment carry the *5XQE>vg* as well as the *Tubα1>GFP>vg* transgene, either one of which could

provide cryptic Vg expression and hence the requisite priming activity. The same explanation also applies to activation of the *5XQE>CD2>vg* transgene by adjacent *5XQE>vg*-expressing cells, as even the intact *5XQE>CD2>vg* transgene was able to provide a cryptic, priming activity in other experiments (Fig. 5A,B,E).

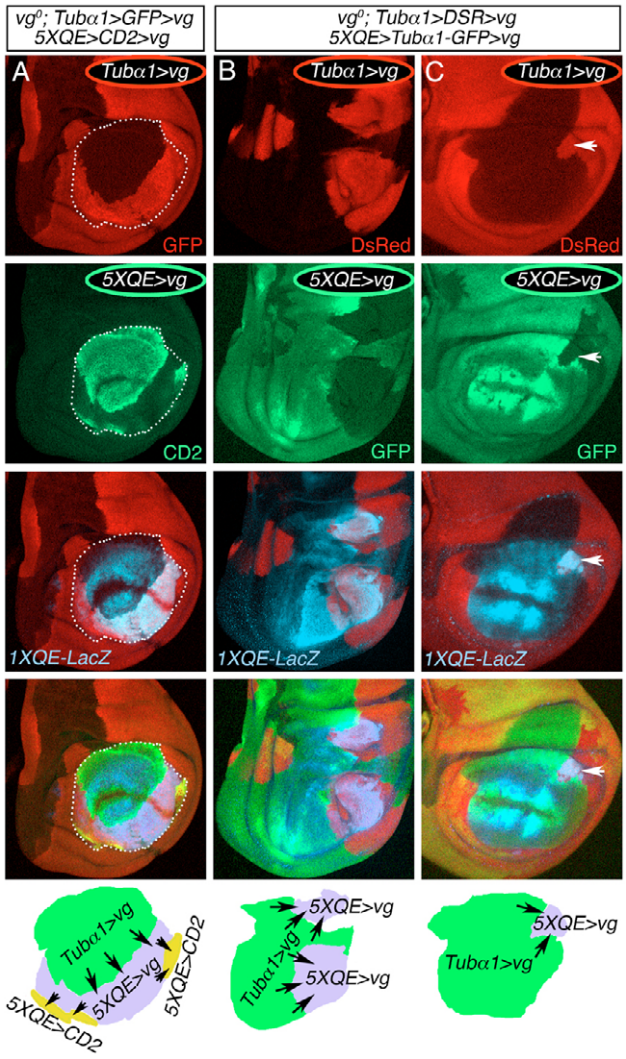

Fig. 4. *5XQE* transgene expression requires the *vg*-dependent feed-forward signal. (**A**) vg^0 disc carrying two types of clones: *Tubα1>vg* clones, black by the absence of GFP (red) and *5XQE>vg* clones, black within the prospective *Drosophila* wing pouch (outlined by dotted line) by the absence of CD2 expression (green). Here (and in B,C), expression of the *5XQE>vg* transgene is monitored indirectly in *Tubα1>GFP>vg* tissue by robust expression of *1XQE-lacZ* (appears lavender). As illustrated at the bottom, the *Tubα1>vg* clone (green) has induced adjacent cells in the abutting *5XQE>vg* clone (lavender) to express the *5XQE>vg* transgene, and induction of the *5XQE>vg* transgene has propagated through the clone and induced *5XQE>CD2>vg* expression (yellow) in neighboring cells on the other side. Expression of both the *1XQE-lacZ* and *5XQE>CD2>vg* transgenes are rescued within the *Tubα1>vg* clone (as in Fig. 1F,G). (**B**,**C**) vg^0 discs that carry abutting *Tubα1>vg* and *5XQE>vg* clones, as in A, except that the *5XQE>vg* and *Tubα1>vg* clones are black by, respectively, absence of GFP (green, owing to excision of a *>Tubα1-GFP>* Flp-out cassette) and absence of DsRed (red). As in A, the *Tubα1>vg* clones in both discs (green in the diagram) have induced *5XQE>vg* expression (lavender) that propagates into the abutting *5XQE>vg* clones, rescuing wing development.

We note that the response of *5XQE>vg* and *5XQE>CD2>vg* cells appeared to depend on their capacity to express Vg, as expression of the *5XQE>vg* transgene was strongly induced by adjacent *Tubα1>vg* cells (Fig. 4), whereas that of the *5XQE>CD2>vg* transgene was not (data not shown). We infer that both the *5XQE>vg* and *5XQE>CD2>vg* transgenes initially respond only weakly to feed-forward input from abutting *Tubα1>vg* cells, but that the initial weak response of the *5XQE>vg* transgene raises the level of Vg protein in these cells (and hence the strength of the priming input), thereby initiating an autoregulatory amplification of *5XQE>vg* expression induced by the feed-forward signal. By contrast, *5XQE>CD2>vg* cells would lack the capacity to autoregulate in this way, preventing them from mounting a robust response to *Tubα1>vg* cells. It is also notable that the response of *5XQE>CD2>vg* cells depended on the level of Vg expressed in the inducing cells. *Tubα1>vg* cells express only moderate levels of Vg, well below peak endogenous levels (Fig. 1F′,G′), and were ineffective. However, *5XQE>vg*-expressing cells make much higher levels (Fig. 3C) and were able to strongly activate *5XQE>CD2>vg* expression in abutting cells (yellow cells in Fig. 4A). Hence, we infer that *5XQE>vg*-expressing cells are more potent inducers of *5XQE>CD2>vg* expression because they provide a correspondingly stronger feed-forward signal.

Although the experimental design of using the *5XQE>CD2>vg* transgene to generate *5XQE>vg* clones has the virtue that it allows the *5XQE>CD2* response to be assayed in cells outside of the clone, it suffers from the fact that cells within such clones can only be identified if they respond positively, by expressing the *5XQE>vg* transgene. We therefore repeated the experiment using an equivalent transgene, *5XQE>Tubα1-GFP>vg*, which allows all of the *5XQE>vg* cells to be scored independently by the loss of a *Tubα1-GFP* transgene within the Flp-out cassette (see Materials and methods). We again found that the resulting *5XQE>vg* transgene was only activated in *5XQE>vg* clones that abut *Tubα1>vg* clones (the latter being marked independently in this experiment by excision of a *>DsRed>* cassette; Fig. 4B,C), confirming the requirement for the Vg-dependent feed-forward signal.

Control of wing growth by the quadrant enhancer

The experiments described above establish that activation of the *5XQE>vg* transgene requires both Wg and the Vg-dependent feed-forward signal. In the following experiments, we use this transgene to test our hypothesis that feed-forward autoregulation mediated by the *QE* is necessary and sufficient for the dramatic expansion of the wing primordium organized by D-V border cells. To do so, we asked whether the presence of the *5XQE>vg* transgene can rescue wing growth in discs in which Vg expression is otherwise driven only by the *BE*.

To generate such '*BE-vg*-only' wing discs, we used a *BE-*vg^{GFP} transgene that expresses a functional Vg-GFP chimeric protein under the control of a minimal form of the intron 2 *BE* (Zecca and Struhl, 2007). In otherwise wild-type discs, this transgene behaves like the standard *BE-lacZ* reporter gene (Williams et al., 1994; Kim et al., 1996), being expressed in a thin stripe of border cells flanking the D-V compartment boundary within the wing pouch, and in a broader stripe in the surrounding hinge and notum primordia (Fig. 3A). In vg^0 discs, the *BE-*vg^{GFP} transgene was expressed only weakly and sporadically in D-V border cells within the pouch, affording detectable, but very limited, rescue of wing development (Fig. 5A). This minimal response appeared to reflect a requirement for priming for efficient activation of the *BE-*vg^{GFP} transgene, as

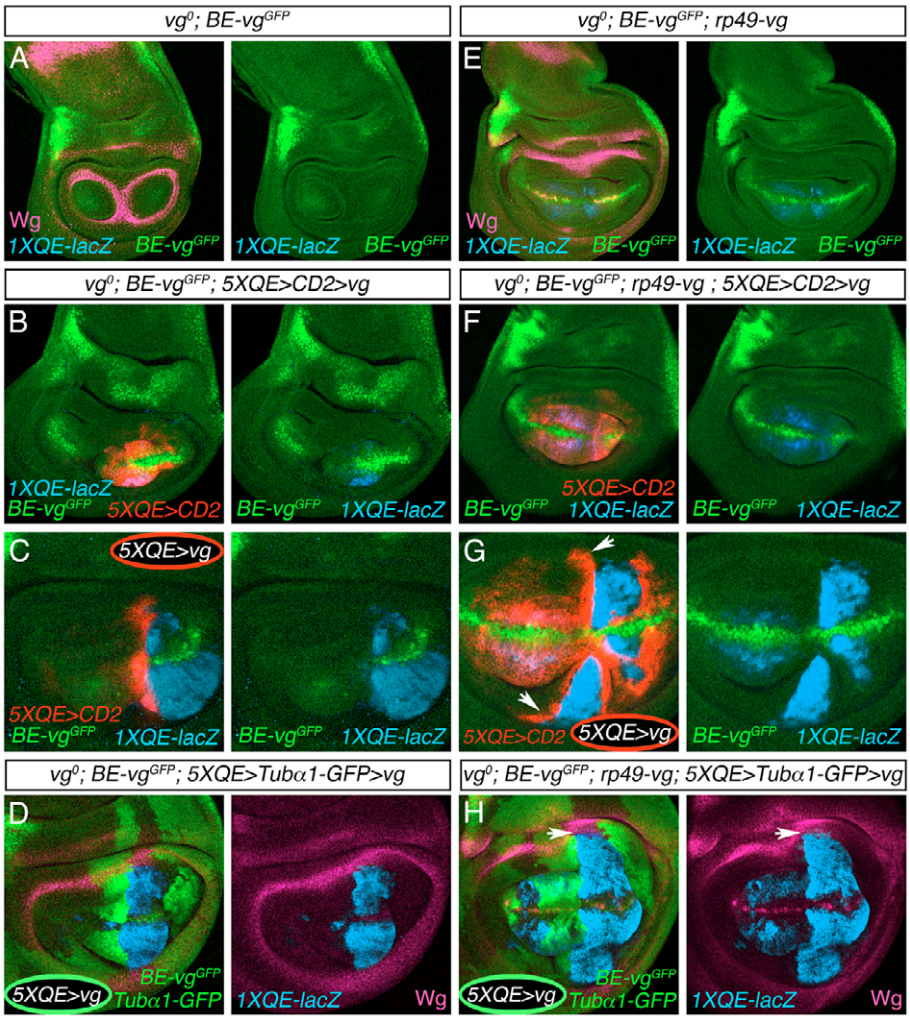

Fig. 5. Control of *Drosophila* wing growth mediated by the *5XQE* element. (**A-D**) *vg⁰ BE-vg^GFP 1XQE-lacZ* discs, either lacking (A) or carrying the *5XQE>CD2>vg* (B,C) or *5XQE>Tubα1-GFP>vg* (D) transgene, and either lacking (A,B) or bearing (C,D) early-induced *5XQE>vg* clones. *5XQE>vg* clones derived from the *5XQE>CD2>vg* transgene (C) are black within the prospective wing pouch by absence of CD2 (red) coupled with *1XQE-lacZ* expression (blue), which is strongly upregulated in cells in which the *5XQE>vg* transgene is active. *5XQE>vg* clones derived from the *5XQE>Tubα1-GFP>vg* transgene (D) are black by the absence of GFP (green); they express *1XQE-lacZ*, as in C, when located within the rescued wing pouch. *BE-vg^GFP* expression (green) is only barely and sporadically detectable along the D-V boundary of *vg⁰ BE-vg^GFP 1XQE-lacZ* discs (A), typically yielding small, anterior and posterior patches of wing tissue, encircled by rings of Wg expression (purple) in the hinge primordium. Addition of the intact *5XQE>CD2>vg* transgene (B) yields detectably stronger *BE-vg^GFP* expression and is associated with weak, local expression of *1XQE-lacZ*, possibly owing to the contribution of cryptic, low-level Vg derived from the added transgene. *5XQE>vg* clones generated in this background (C,D) show significant rescue of wing growth, as visualized by the expanded domains of *1XQE-lacZ*-expressing cells. They also induce neighboring cells outside the clone to activate the *5XQE>CD2>vg* transgene (red). (**E-H**) Same as in A-D, except for the added presence of one copy of the *rp49-vg* transgene, which largely rescues *BE-vg^GFP* expression along the D-V boundary (E) and augments the weak, local activity of both the *1XQE-lacZ* and *5XQE>CD2>vg* transgenes in the absence of *5XQE>vg* clones (E,F, compare with A,B). *5XQE>vg* clones in this background cause dramatic expansions in wing growth (G,H), approximating to or exceeding that normally observed in wild-type discs, and are associated with local induction of the intact *5XQE>CD2>vg* transgene in neighboring cells outside the clone (arrows). Note that *1XQE-lacZ* expression is confined to the prospective wing pouch, demarcated by the inner ring of Wg (purple, arrow in H), even though the clone extends into the proximal hinge territory, indicating an independent limit to the propagation of *5XQE>vg* expression.

adding the *rp49-vg* transgene significantly enhanced border cell expression of Vg^GFP, as well as local rescue of wing development along the D-V boundary (Fig. 5E; data not shown).

Despite the limited response of the *BE-vg^GFP* transgene in *vg⁰* discs, these discs still provide a context in which Vg expression in the pouch depends primarily on the *BE*. Hence, we asked whether clones of *5XQE>vg* cells could rescue wing growth away from the D-V boundary in this context, and found that this was indeed the case. Clones of *5XQE>vg* cells induced in *vg⁰ BE-vg^GFP* discs were associated with activation of both the *5XQE>vg* and *1XQE-lacZ* transgenes, as well as with the expansion of wing tissue away from *BE-vg^GFP*-expressing border cells (Fig. 5B,C). In addition, they induced adjacent *5XQE>CD2>vg* cells to express *CD2*, indicating propagation of the feed-forward signal across the clone border.

These results indicate that the *5XQE>vg* transgene is both necessary and sufficient to restore wing growth in discs in which *vg* expression is otherwise dependent only on *BE* and cryptic priming activity. However, it is apparent that growth is not fully rescued, as the expansion of wing tissue associated with *5XQE>vg* clones was significantly less than the expansion that normally occurs following D-V segregation in wild-type discs. One explanation is that both *BE-* and *QE*-driven expression of Vg are compromised by inadequate Vg priming that derives from the *BE-vg^GFP* and *5XQE>vg* transgenes (the only possible sources of pre-existing Vg activity in the *5XQE>vg* clones). We therefore repeated the experiment in the presence of the *rp49-vg* transgene, to ensure adequate priming in all cells.

In the absence of *5XQE>vg* clones, *vg⁰ BE-vg^GFP* discs carrying the *rp49-vg* transgene, as well as the *5XQE>CD2>vg* and *1XQE-lacZ* transgenes, showed robust expression of the *BE-vg^GFP* transgene in a narrow stripe of D-V border cells, and this was accompanied by weak expression of both the *5XQE>CD2>vg* and *1XQE-lacZ* transgenes in flanking cells (Fig. 5F). We note that in these discs, as well as in otherwise wild-type discs, both the *BE-vg^GFP* and standard *BE-lacZ* transgenes were expressed at a low level in cells up to several cell diameters away from the D-V boundary (data not shown). Hence, the weak *5XQE>CD2>vg* and *1XQE-lacZ* expression detected in cells flanking the D-V boundary might reflect a response to this low-level Vg^GFP activity.

Strikingly, when clones of *5XQE>vg* cells were generated in this background, near or next to the D-V compartment boundary, they were associated with an autonomous upregulation of the *1XQE-lacZ* transgene and a dramatic expansion of prospective

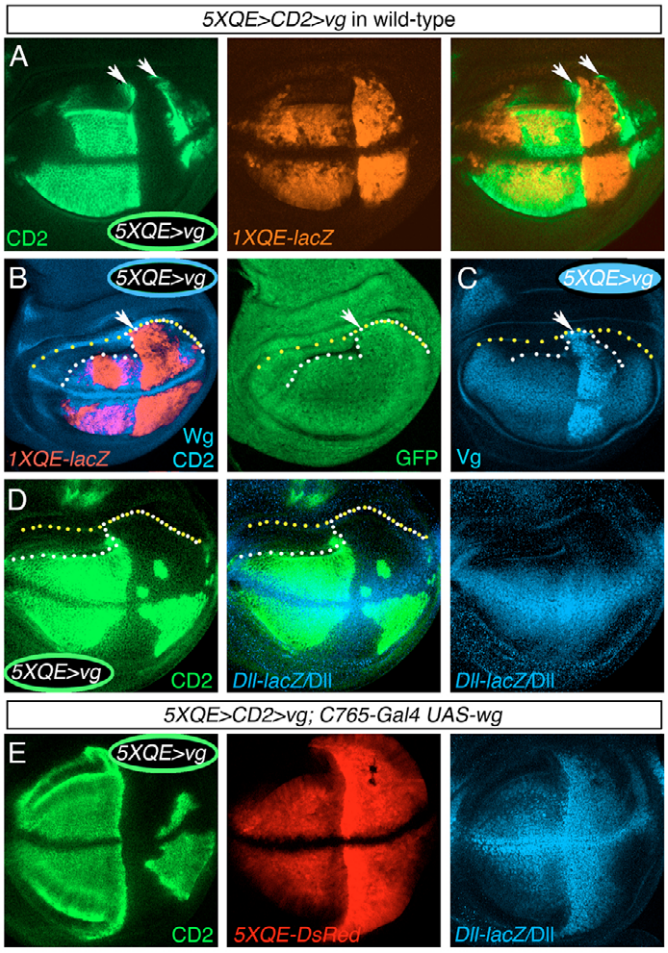

Fig. 6. Enhanced potency of the *QE* response increases wing growth. (**A-D**) *5XQE>vg* clones in wild-type *Drosophila* discs, black by the absence of CD2 (green, A,D), and by robust expression of *1XQE-lacZ* (red, A,B) or Vg (C, blue). The clones are associated with expansion of the *1XQE-lacZ*-expressing (wing) tissue, apparently at the expense of the *rn*-only domain, normally circumscribed by an inner and outer fold (dotted white and yellow lines, B-D, as indicated by the arrows). The disc in B is counterstained with Wg (blue) and uniform expression of *Hsp70-GFP* (green) to visualize the folds; the outer fold correlates with the inner ring of Wg expression. (D) *Dll* expression (summed staining for Dll and a *Dll-lacZ* transgene, blue) is not expanded within such *5XQE>vg* clones. (**E**) Same as in D, except for the added presence of ubiquitous, ectopic Wg, driven under *C765-Gal4/UAS* control. *Dll* expression now extends into the expanded domain of wing tissue (marked by *5XQE-DsRed* expression, red).

wing tissue (Fig. 5G). Furthermore, they appeared to induce an equally dramatic, albeit short-range, induction of CD2 expression in neighboring *5XQE>CD2>vg* cells (arrows in Fig. 5G). Corresponding experiments using the *5XQE>Tubα1-GFP>vg* transgene instead of *5XQE>CD2>vg* confirmed the rescue of wing growth and also showed that it is an autonomous property of the *5XQE>vg* clones (marked independently by the absence of GFP; Fig. 5D,H).

Thus, in the added presence of exogenous Vg priming activity provided by the *rp49-vg* transgene, the *5XQE>vg* transgene appears both necessary and sufficient to greatly expand the domain of prospective wing tissue, mimicking the normal growth of wing tissue organized by D-V border cells.

Regulation of wing growth by the quadrant enhancer

If wing growth is governed by the capacity of *QE* sequences to mediate *vg* feed-forward autoregulation, the size of the wing primordium should depend on the strength of the *QE* response. To test this, we assayed the effects of *5XQE>vg* clones on wing growth in otherwise wild-type discs, where they appear to generate a more sensitive and potent upregulation of Vg expression driven by the combined *QE*-dependent activities of the transgene and endogenous *vg*.

Early-induced *5XQE>vg* clones were associated with an abnormal, cell-autonomous expansion of prospective wing tissue, extending beyond the normal limit of detectable Vg expression into the surrounding '*rotund* (*rn*)-only' territory of the wing pouch delimited by the inner ring of Wg expression (Fig. 6A-C) (see Zecca and Struhl, 2007). These clones also induced adjacent *5XQE>CD2>vg* cells that abutted the abnormally expanded wing primordium to ectopically express CD2 (Fig. 6A,B). Note that these CD2-expressing cells did not appear to express either Vg or *1XQE-lacZ*, suggesting that the *5XQE* transgene has a greater capacity to respond to one or more of its normal inputs than either endogenous *vg* or the *1XQE-lacZ* transgene. Taken together, these results suggest that strengthening and/or sensitizing the *QE* response by introducing the *5XQE>vg* transgene causes an enhanced expansion of wing tissue.

We note that even though *5XQE>vg* clones formed abnormally enlarged domains of wing tissue, other elements of wing pattern were not similarly expanded within the clones. In particular, the domain of *Distal-less* (*Dll*) expression, which normally depends on Wg but is less broad than that of *vg*, remained unaltered in such clones (Fig. 6D). However, the *Dll* domain could be expanded in response to ectopic Wg (Fig. 6E). It follows that the effects of Wg on wing size (via control of *vg* expression) can be dissociated from its effect on wing pattern (via control of other target genes such as *Dll*).

DISCUSSION

The dramatic expansion of the *Drosophila* wing primordium following the D-V compartmental segregation provides a valuable paradigm of organ growth. Growth in this context is manifest as a rapid ~200-fold expansion of the population of cells expressing the wing selector gene *vg*, under the control of the long-range morphogens Wg and Dpp. This system thus poses the fundamental question of how morphogens organize the increase in the mass and number of cells that express a given selector gene, to yield an adult appendage of appropriate size and shape.

In the accompanying paper, we defined a novel autoregulatory property of *vg* that appears crucial for this process. We presented evidence that *vg*-expressing cells send a short-range feed-forward signal that neighboring cells must receive in order to express *vg* in response to Wg. This led us to hypothesize that Wg controls wing development by fueling this non-autonomous autoregulatory mechanism. Here, we establish that the *vg* quadrant enhancer (*QE*) can mediate *vg* autoregulation in response to Wg and then use a transgene that expresses Vg under *QE* control to provide a proof-in-principle that wing growth normally depends on the operation of the autoregulatory circuit.

vg autoregulation and expansion of the wing primordium in response to Wg

As illustrated in Fig. 7, we envisage wing growth following D-V segregation as an outcome of *vg* autoregulation, primed by cryptic, low-level Vg in all cells that is seeded by DSL-Notch-mediated

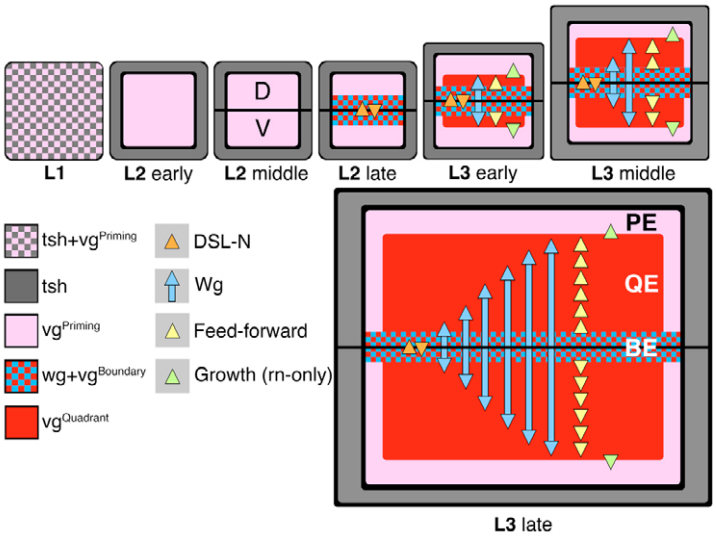

Fig. 7. Model for the control of *Drosophila* wing growth by feed-forward autoregulation of *vg* mediated by the *QE* and fueled by Wg. During the first larval instar (L1), *tsh* and *vg* are coexpressed in the nascent wing disc, the latter driven at least in part by the *vg* priming enhancer (*PE*). Wg signaling in early L2 represses *tsh* in the distal portion of the disc, segregating the disc into heritably distinct hinge/notum and pre-blade primordia. Both primordia are then subdivided into dorsal (D) and ventral (V) compartments in mid-L2. After the D-V segregation, *vg* expression becomes dependent on DSL-Notch signaling (orange) across the D-V boundary, which activates both *wg* and *vg* expression in D-V border cells, the latter driven by *vg* boundary enhancer (*BE*) elements. By early L3, Wg (blue) and the Vg-dependent feed-forward signal (yellow) sent by border cells activate *vg* quadrant enhancer (*QE*) elements in neighboring cells, upregulating *vg* expression (red). During the mid- and late L3, the domain of *vg*-expressing cells expands dramatically by reiterative cycles of short-range feed-forward signaling, fueled by long-range Wg and Dpp signaling from D-V border and A-P border cells (for simplicity, Dpp and the A-P compartment boundary are not shown). Feed-forward autoregulation is required both to recruit new cells to express *vg*, as well as to maintain *vg* expression in cells already recruited; Wg and Dpp are also required for the survival and proliferation of cells already recruited. Lastly, Vg-expressing cells produce a signal (green) that stimulates proliferation in the surrounding, '*rn*-only' cells, sustaining the cell population from which wing cells are recruited. Short-range (DSL-N, Feed-forward and Growth) and long-range (Wg) signals are indicated, respectively, by arrowheads and arrows.

induction of specialized D-V border cells that express high levels of *vg* and *wg*, and then propagated by the capacity of *vg*-expressing cells to induce and sustain *vg* expression in neighboring cells in response to Wg. In support, we have been able to restore wing growth in vg^0 discs in a step-wise manner by the sequential addition of transgenes that provide, first priming (*rp49-vg*), then initiation ($BE\text{-}vg^{GFP}$), and finally feed-forward propagation (*5XQE>vg*). As we observe (Fig. 1E, Fig. 5), priming is necessary but not sufficient for wing development, initiation provides local rescue of wing tissue, and propagation is responsible for the dramatic expansion in the size of the prospective wing.

Importantly, priming and feed-forward signaling are linked in a self-reinforcing autoregulatory circuit in which a gain in either input leads to an amplification of both. We envisage that the *QE* normally integrates both the priming and feed-forward inputs together with Wg in a way that is sensitive to the initial strength of each input and subject to autoamplification. For example, in the 'resting' state, cells have a low level of priming that falls beneath the minimal threshold necessary to specify the wing state or generate appreciable feed-forward signal. Upon receipt of sufficient Wg and feed-forward signal, the level of Vg expression rises, crossing the threshold defining the wing state and enhancing the capacity of the responding cell both to send and to receive the feed-forward signal. Amplification of this circuit then leads to the maximum output of Vg expression and feed-forward signaling that can be supported by the strength of the Wg signal received.

The self-reinforcing nature of this autoregulatory circuit, both between and within cells, helps explain how Wg spreading from D-V border cells normally fuels the expansion of the population of *vg*-expressing cells. It also helps account for the unexpected responses we observed in experiments using the *rp49-vg*, $BE\text{-}vg^{GFP}$ and *5XQE>vg* transgenes to mimic the normal priming, initiation and feed-forward inputs (Figs 4,5). All of these transgenes depend on heterologous promoters and potentially complex enhancer elements operating outside of their normal genomic contexts. Consequently, weak, inappropriate activities of any of these transgenes (e.g. cryptic priming by $BE\text{-}vg^{GFP}$ and *5XQE>vg* transgenes, or faint *QE* activity of the $BE\text{-}vg^{GFP}$ transgene) could be amplified by the autoregulatory circuitry, yielding spatially inappropriate responses. Nevertheless, despite these experimental limitations, our results indicate that the major factor governing the expansion of the wing primordium is feed-forward autoregulation mediated by the *QE*.

As discussed in the accompanying paper (Zecca and Struhl, 2007), wing growth does not depend solely on the capacity of Wg to recruit and maintain cells in the wing primordium by fueling *vg* autoregulation. Instead, we show here that even when wing pouch cells are supplied constitutively with exogenous Vg (thus bypassing the requirement for *vg* autoregulation), they still depend on continuous Wg input to survive and grow within the context of the wing primordium (Fig. 3) (see also Johnston and Sanders, 2003). This is in contrast to cells in the more proximal hinge and notum primordia, which survive and grow without Wg input (Chen and Struhl, 1999; Giraldez and Cohen, 2003). Thus, Wg appears to promote wing growth via two distinct mechanisms: by continuously 'selecting' which cells enter and remain within the wing primordium, and by allowing the survival and growth of cells so selected. We cannot, at present, distinguish the relative contributions of these two mechanisms. However, as we show here, both appear essential, as cells fail to enter, or stay, within the wing primordium when either one is eliminated.

Dpp and feed-forward autoregulation of Vg

Wing growth depends not only on Wg emanating from D-V border cells, but also on Dpp secreted by A compartment cells along the A-P compartment boundary (Zecca et al., 1995; Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997), suggesting that the *QE* might mediate feed-forward autoregulation in response to Dpp, as well as Wg. In support, the *QE* contains binding sites for the Dpp transducer Mad, and there is evidence that these sites, as well as Mad itself, contribute to *QE* activity (Kim et al., 1997; Halder et al., 1998; Guss et al., 2001). Moreover, clones of cells that cannot transduce Dpp behave like those that cannot transduce Wg: they cease to express Vg and are lost specifically from the wing primordium, in contrast to clones located in the more proximal hinge and notum primordia (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002; Moreno et al.,

2002; Gibson and Perrimon, 2005; Shen and Dahmann, 2005). Hence, we think it likely that Dpp and Wg act together to fuel the feed-forward autoregulatory circuit, and by so doing, regulate the size and shape of the developing wing.

Morphogen gradients and organ growth

The ability of Wg, and potentially Dpp, to promote wing growth by fueling a non-autonomous autoregulatory circuit of *vg* expression is, to our knowledge, novel, and has implications for the control of organ growth by morphogens. As epitomized by the developing wing, a long-standing enigma is that gradient morphogens drive relatively uniform growth and proliferation across a tissue at the same time that they function in a concentration-dependent manner to organize complex patterns of gene expression and cell differentiation (Garcia-Bellido and Merriam, 1971; Milan et al., 1996; Resino et al., 2002). We suggest that a minimum threshold level of morphogen might be sufficient to fuel both feed-forward autoregulation of organ selector genes and the growth and proliferation of cells so selected. Accordingly, as illustrated in Fig. 7, organ growth would be governed primarily by the progressive expansion in the range of morphogen (a process that might itself depend on the ability of morphogen to regulate expression of its receptors and other binding proteins) and by any boundary conditions that limit the availability and capacity of surrounding cells to respond.

We thank X.-J. Qiu, A. Adachi and C. Bonin for technical assistance, and K. Irvine, L. Johnston, P. A. Lawrence, R. Mann and A. Tomlinson for advice and comments on the manuscript. M.Z. is a Research Associate and G.S. an Investigator of the Howard Hughes Medical Institute.

References

Blair, S. S., Brower, D. L., Thomas, J. B. and Zavortink, M. (1994). The role of apterous in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of Drosophila. *Development* **120**, 1805-1815.
Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing Drosophila wing. *Development* **122**, 2261-2269.
Casali, A. and Struhl, G. (2004). Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. *Nature* **431**, 76-80.
Chen, C. M. and Struhl, G. (1999). Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila. *Development* **126**, 5441-5452.
Couso, J. P., Knust, E. and Martinez Arias, A. (1995). Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. *Curr. Biol.* **5**, 1437-1448.
de Celis, J. F., Garcia-Bellido, A. and Bray, S. J. (1996). Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc. *Development* **122**, 359-369.
Diaz-Benjumea, F. J. and Cohen, S. M. (1993). Interaction between dorsal and ventral cells in the imaginal disc directs wing development in Drosophila. *Cell* **75**, 741-752.
Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-4225.
Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in Drosophila wing formation. *Genes Dev.* **10**, 421-434.
Garcia-Bellido, A. and Merriam, J. R. (1971). Parameters of the wing imaginal disc development of Drosophila melanogaster. *Dev. Biol.* **24**, 61-87.
Gibson, M. C. and Perrimon, N. (2005). Extrusion and death of DPP/BMP-compromised epithelial cells in the developing Drosophila wing. *Science* **307**, 1785-1789.
Giraldez, A. J. and Cohen, S. M. (2003). Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**, 6533-6543.
Golic, K. G. (1991). Site-specific recombination between homologous chromosomes in Drosophila. *Science* **252**, 958-961.
Greenwood, S. and Struhl, G. (1997). Different levels of Ras activity can specify distinct transcriptional and morphological consequences in early Drosophila embryos. *Development* **124**, 4879-4886.
Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. and Carroll, S. B. (2001). Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164-1167.
Halder, G. and Carroll, S. B. (2001). Binding of the Vestigial co-factor switches the DNA-target selectivity of the Scalloped selector protein. *Development* **128**, 3295-3305.
Halder, G., Polaczyk, P., Kraus, M. E., Hudson, A., Kim, J., Laughon, A. and Carroll, S. (1998). The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in Drosophila. *Genes Dev.* **12**, 3900-3909.
Johnston, L. A. and Sanders, A. L. (2003). Wingless promotes cell survival but constrains growth during Drosophila wing development. *Nat. Cell Biol.* **5**, 827-833.
Kim, J., Irvine, K. D. and Carroll, S. B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. *Cell* **82**, 795-802.
Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature* **382**, 133-138.
Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A. (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-308.
Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.
Liu, X., Grammont, M. and Irvine, K. D. (2000). Roles for scalloped and vestigial in regulating cell affinity and interactions between the wing blade and the wing hinge. *Dev. Biol.* **228**, 287-303.
Martin-Castellanos, C. and Edgar, B. A. (2002). A characterization of the effects of Dpp signaling on cell growth and proliferation in the Drosophila wing. *Development* **129**, 1003-1013.
Milan, M., Campuzano, S. and Garcia-Bellido, A. (1996). Cell cycling and patterned cell proliferation in the Drosophila wing during metamorphosis. *Proc. Natl. Acad. Sci. USA* **93**, 11687-11692.
Morata, G. and Ripoll, P. (1975). Minutes: mutants of Drosophila autonomously affecting cell division rate. *Dev. Biol.* **42**, 211-221.
Moreno, E., Basler, K. and Morata, G. (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. *Nature* **416**, 755-759.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.
Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**, 3477-3485.
Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**, 871-880.
Paumard-Rigal, S., Zider, A., Vaudin, P. and Silber, J. (1998). Specific interactions between vestigial and scalloped are required to promote wing tissue proliferation in Drosophila melanogaster. *Dev. Genes Evol.* **208**, 440-446.
Resino, J., Salama-Cohen, P. and Garcia-Bellido, A. (2002). Determining the role of patterned cell proliferation in the shape and size of the Drosophila wing. *Proc. Natl. Acad. Sci. USA* **99**, 7502-7507.
Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**, 72-74.
Shen, J. and Dahmann, C. (2005). Extrusion of cells with inappropriate Dpp signaling from Drosophila wing disc epithelia. *Science* **307**, 1789-1790.
Simmonds, A. J., Liu, X., Soanes, K. H., Krause, H. M., Irvine, K. D. and Bell, J. B. (1998). Molecular interactions between Vestigial and Scalloped promote wing formation in Drosophila. *Genes Dev.* **12**, 3815-3820.
Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-540.
Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A. and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407**, 527-530.
Williams, J. A., Bell, J. B. and Carroll, S. B. (1991). Control of Drosophila wing and haltere development by the nuclear vestigial gene product. *Genes Dev.* **5**, 2481-2495.
Williams, J. A., Paddock, S. W. and Carroll, S. B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions. *Development* **117**, 571-584.
Williams, J. A., Paddock, S. W., Vorwerk, K. and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299-305.
Wu, J. and Cohen, S. M. (2002). Repression of Teashirt marks the initiation of wing development. *Development* **129**, 2411-2418.
Zecca, M. and Struhl, G. (2002). Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling. *Development* **129**, 1357-1368.
Zecca, M. and Struhl, G. (2007). Recruitment of cells into the Drosophila wing primordium by a feed-forward circuit of vestigial autoregulation. *Development* **134**, 3001-3010.
Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. *Development* **121**, 2265-2278.
Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.