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Long-range action of Wingless organizes the dorsal-ventral axis of the *Drosophila* wing

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SUMMARY

Short-range interaction between dorsal and ventral (D and V) cells establishes an organizing center at the DV compartment boundary that controls growth and specifies cell fate along the dorsal-ventral axis of the *Drosophila* wing. The secreted signaling molecule Wingless (Wg) is expressed by cells at the DV compartment boundary and has been implicated in mediating its long-range patterning activities. Here we show that Wg acts directly, at long range, to define the expression domains of its target genes, *Distal-less* and *vestigial.* Expression of the *Achaete-scute* genes, *Distal-less* and *vestigial* at different distances from the DV boundary is controlled by Wg in a concentration-dependent manner. We propose that Wg acts as a morphogen in patterning the D/V axis of the wing.

Key words: axis formation, *Drosophila*, Wingless, wing disc, cell fate

INTRODUCTION

During development of multicellular organisms the growth and patterning of groups of cells are coordinated in a manner that suggests that cell fate is being controlled as a function of a cell's position within the population (Wolpert, 1969, 1989). Secreted signaling molecules of the Hedgehog, Wnt and TGF-β families have been implicated in providing this positional information. It has been suggested that these secreted proteins may form concentration gradients which provide cells with information about their position based on their distance from the source of the signal (Green and Smith, 1992; Struhl and Basler, 1993; Basler et al., 1993; Riddle et al., 1993; Gurdon et al., 1994, 1995; Heemskerk and DiNardo, 1994; Roelink et al., 1995; Fan et al., 1995; Ingham and Fietz, 1995; Nellen et al., 1996; Lecuit et al., 1996). Consequently, localized expression of one of these signaling molecules in a particular group of cells can function as an organizing center that influences the growth and pattern of the surrounding tissue (Struhl and Basler, 1993; Basler and Struhl, 1994; Diaz-Benjumea and Cohen, 1993, 1995; Diaz-Benjumea et al., 1994; Tabata and Kornberg, 1994; Zecca et al., 1995; Hoppler and Bienz, 1995; Nellen et al., 1996; Lecuit et al., 1996; Kim et al., 1996; Burke and Basler, 1996).

In the developing limbs of Drosophila, organizing centers are generated by short-range interaction between distinctly specified cells in adjacent compartments. Subdivision of the limb primordia into anterior and posterior compartments is inherited from the embryonic ectoderm at the time that the disc primordia are formed (Cohen et al., 1993). Posterior compartment identity is specified by expression of the homeobox genes engrailed and invected which function as selector genes to assign cells their posterior identity (reviewed by Lawrence and Struhl, 1996). The secreted Hedgehog (Hh) protein transmits a signal from posterior to anterior cells that induces Decapentaplegic (Dpp) and Wingless (Wg) expression in nearby anterior cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994). In the wing disc Dpp is the primary target for Hh activity and serves to relay a long-range signal that instructs cells about their prospective fate and controls growth of the disc with respect to the anterior-posterior axis (Capdevila and Guerrero, 1994; Zecca et al., 1995; Ingham and Fietz, 1995; Nellen et al., 1996; Lecuit et al., 1996; Burke and Basler, 1996). Dpp is thought to exert its organizing activity by regulating downstream genes in a concentration-dependent manner, suggesting that Dpp may function as a morphogen (Nellen et al., 1996; Lecuit et al., 1996).

A second organizing center is established at the dorsal-ventral (DV) compartment boundary of the wing disc. The subdivision into dorsal and ventral compartments is controlled by localized expression of the LIM-homeodomain protein Apterous in dorsal cells during the second larval instar (Diaz-Benjumea and Cohen, 1993; Blair, 1993). Interaction between dorsal and ventral cells induces an organizing center at the compartment boundary that controls growth and patterning of the wing (Diaz-Benjumea and Cohen, 1993; Williams et al., 1994). Apterous directs expression of Fringe and Serrate in dorsal cells (Irvine and Wieschaus, 1994; Kim et al., 1995). Serrate serves as a short-range signal to induce expression of the nuclear protein, Vestigial (Vg) and Wingless in cells at the DV boundary (Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995; Kim et al., 1995; Couso et al., 1995). Wg and Vg are direct targets for activation by the Notch pathway (Kim et al., 1996; Neumann and Cohen, 1996b); both are required for

growth and patterning of the wing along the DV axis (Williams et al., 1993, 1994; Phillips and Whittle, 1993; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995).

We have previously suggested that Wg mediates the growth and patterning activity of the D/V organizer (Diaz-Benjumea and Cohen, 1995), and that the growth-promoting function of Wg is at least partially mediated by the activation of *vestigial* in the wing pouch (Neumann and Cohen, 1996b). Here we present evidence that localized expression of Wg in cells at the DV boundary can act directly at long-range to activate expression of target genes such as *Distal-less* (*Dll*) and *vestigial,* and to control growth of the wing. We propose that the local concentration of Wg protein instructs cells about their prospective fate as a function of their distance from the source of the Wg signal, suggesting that Wg acts as a morphogen in patterning the DV axis of the wing.

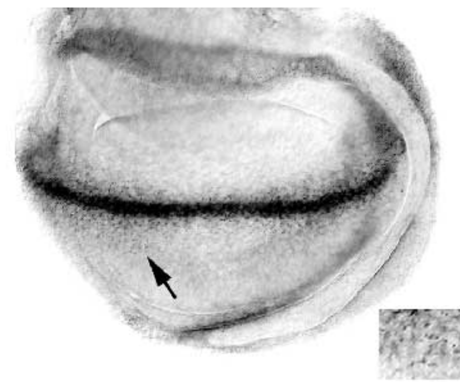

Fig. 1. Wg protein expression in a third instar wing imaginal disc. The *wg* gene is expressed in a narrow stripe of cells straddling the DV compartment boundary. High levels of Wg protein can be visualized in these cells by antibody staining. Lower levels of Wg protein can be seen in nearby cells, mainly in small punctate spots. In the embryo these spots have been shown to represent Wg protein in vesicular structures in responding cells. Wg-containing vesicles can be observed at a considerable distance from the source of the protein, at least 10 cells away (arrow). The inset shows Wg-containing vesicles from the region near the tip of the arrow. We observe Wg expression at a greater distance from the DV boundary than has previously been reported (Couso et al., 1994), perhaps due to the greater sensitivity of the monoclonal antibody to Wg (Brook and Cohen, 1996).

MATERIALS AND METHODS

Drosophila stocks

*spade^flag^* (*spd^fg^*), *Df(2L)spd^hL2^* and UAS*dsh* are described by Neumann and Cohen (1996a). *spd^fg^* is a small deletion in the 5′ regulatory region of *wg* that removes an enhancer element driving expression at the DV boundary (and also in the hinge). *Sternopleural* (*Sp*) is described by Neumann and Cohen (1996c). When *Sp* is crossed to *Df(2L)spd^hL2^*, a deficiency that removes the *wg* locus, the resulting larvae show a loss of Wg expression in both the anterior notum and in the outer ring surrounding the wing pouch, and a reduction of Wg expression at the DV boundary (not shown). This suggests that enhancer sequences driving Wg expression at the DV boundary are affected by *Sp*. *Sp* maps genetically to the 3′ regulatory region of *wg*. *spd^fg^ Sp* is a recombinant chromosome that carries both mutations. The *wg* null allele, wg^{CX4}, and the temperature sensitive allele wg^{IL114} are described by van den Heuvel et al. (1993). *A101/neuralized-lacZ* is described by Ghysen and O'Kane (1989). UASwg^{+} is described by Lawrence et al. (1995). *dpp*GAL4 is described by Wilder and Perrimon (1995). All GAL4-UAS crosses were performed at 22°C. dsh^{VA153} is described by Perrimon and Mahowald (1987), dsh^{75} by Noordermeer et al. (1994) and $arm^{H8.6}$ by Orsulic and Peifer (1996).

Clonal analyses

dsh and *arm* mutant clones were induced in larvae of genotype *dsh*$^{-}$ *FRT101* (or *arm*$^{-}$ *FRT101)/N-myc FRT101; SbHSFLP3/+*. *dsh* clones were induced at 36±12 hours, while *arm* clones were induced at 60±12 hours. *arm* clones shown in Fig. 6 were kept at the permissive temperature (17.5°C) until mid-third instar, and then shifted to 25°C for 18 hours before fixation. The *arm* clones described in Table 1 were shifted to 25°C in early third instar for 48 hours before fixation.

Antibodies

Anti-Dll is described by Diaz-Benjumea and Cohen (1995), anti-Vg in Williams et al. (1993), monoclonal anti-Wg in Brook and Cohen (1996), anti-Dsh in Yanagawa et al. (1995). Rabbit anti-Engrailed was produced by Charles Girdham and Pat O'Farrell. Mouse monoclonal anti-Myc (9E10) was used to mark clones in discs.

RESULTS

Wg protein is secreted and can be detected in vesicular structures in the cytoplasm of nearby cells in the embryo (van den Heuvel et al., 1989). In 3rd instar wing discs Wg is produced in a stripe of cells 3-4 wide straddling the DV boundary and can be detected in vesicles in nearby cells (Fig. 1). The number of Wg-containing vesicles decreases with distance from the source, suggesting that the protein may form a concentration gradient centered on the DV boundary. Wg expression in cells along the DV boundary is thought to control growth of the wing, because clones of Wg-expressing cells produce outgrowths from the wing surface that resemble the axis bifurcations caused by producing an ectopic DV boundary (Diaz-Benjumea and Cohen, 1995), and because removing Wg activity (after the wing has been specified in second instar) results in loss of tissue from the outer edge of the wing (Couso et al., 1995; Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996b).

Direct action of Wg at a distance

To ask whether Wg acts directly at a long-range to specify cell fates in the wing we examined the requirement for Wg signaling activity for expression of two Wg-dependent target genes. Vestigial (Vg) and Distal-less (Dll) proteins are expressed in broad domains centered on the DV boundary (Williams et al., 1994; Diaz-Benjumea and Cohen, 1995; see also Fig. 6A). Vestigial fills the wing pouch (Figs 2A, 6A). Except at the DV boundary, Vg expression in the wing pouch depends on Wg activity (Neumann and Cohen, 1996b). At the boundary Vg is controlled by Notch, not Wg (Kim et al., 1996; Neumann and Cohen, 1996b). Dll expression is highest at the DV boundary and decreases in a graded manner toward the proximal regions of the wing pouch (Diaz-Benjumea and Cohen, 1995). Although the maximal extent of the Dll domain is similar to that of Vg, Dll expression is more graded, and decreases to rather low levels near the edge of the wing pouch (Fig. 6A).

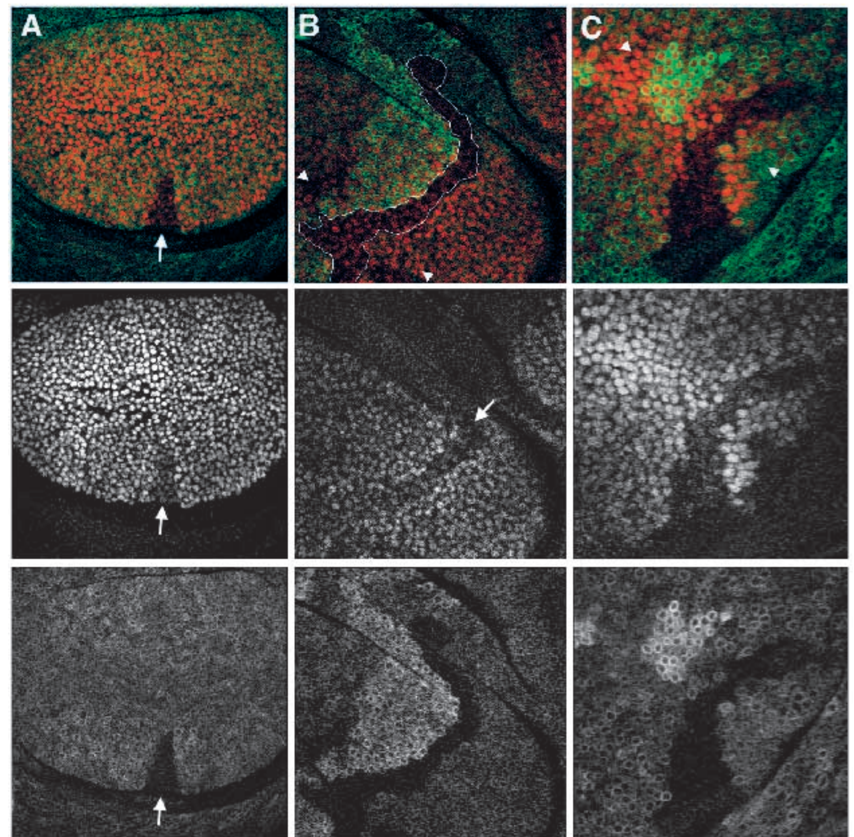

Fig. 2. Cell-autonomous reduction of Vestigial and Distal-less expression in *dsh* mutant clones. (A) Clone of cells homozygous for dsh^{VA153} (arrow). The clone is marked by the absence of N-myc expression (green in A, single channel below; bottom row). Note the reduction of Vg expression (red; middle row) within the clone. (B) Clone of cells homozygous for dsh^{75}. The clone is marked as in A. Note that reduction of Vg within the clone is strongest in the cells of the clone that are far away from the DV boundary. The arrowheads indicate the DV boundary. (C) Clone of cells homozygous for dsh^{VA153}. The clone is marked as in A. Note the reduction of *Dll-lacZ* expression (red) in the clone and the narrowness of the expression domain. The arrowheads indicate the DV boundary.

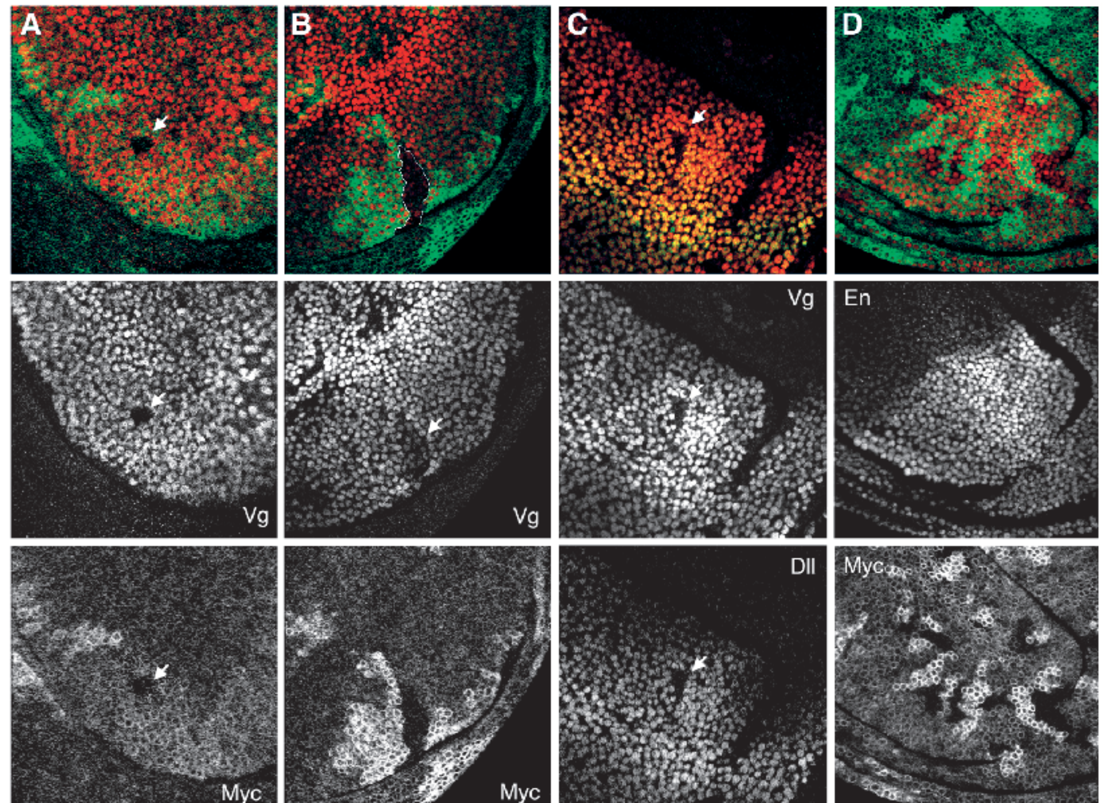

Fig. 3. Cell-autonomous loss of Vestigial and Distal-less expression in *arm* mutant clones. (A) Clone of cells homozygous for $arm^{H8.6}$ shifted to the non-permissive temperature in mid third instar, 18 hours before staining. The clone is marked by the absence of N-myc expression (green in A,B,D, single channels below). Note the absence of Vg expression in the clone (red in A, single channel below middle). (B) Clone of cells homozygous for $arm^{H8.6}$, treated and stained as in A. Note that in this case Vg expression is only partially lost. The clone is outlined in B. (C) Clone of cells homozygous for $arm^{H8.6}$, treated as in A and double labeled for Vg and Dll expression (Dll in green). In this case, absence of Vg expression was used to mark the clone. Note that the cells in which Vg is reduced, lack Dll expression. (D) Clones of cells homozygous for $arm^{H8.6}$, treated as in A and double labeled for N-myc (green) and Engrailed (red). Engrailed expression is not affected in the clones.

To ask whether Wg signaling directly defines the spatial domains of Vg and Dll expression we examined clones of cells mutant for two components of the Wg signal transduction pathway, *dishevelled* and *armadillo* (Peifer et al., 1991; Siegfried et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Klingensmith et al., 1994). Cells mutant for *dsh* show reduced levels of Dll expression (Fig. 2C). Dll levels are reduced near the DV boundary, where Wg levels are expected to be high (Fig. 1), but are lost further away from the DV boundary, where Wg levels are expected to be lower, with the result that Dll expression is restricted to a narrower region of the wing within the clone than in the adjacent wild-type cells. The Dll levels in the center of the clone are similar to those located at a greater distance from the wing margin in the neighboring wild-type cells. Vg expression is also reduced in *dsh* mutant cells near the edge of the wing pouch (Fig. 2A,B). However, unlike Dll, Vg expression does not completely disappear. The finding that Dll expression is more strongly reduced than Vg, and is restricted to a narrower domain in the mutant clones, suggests that a higher level of Wg signaling activity is required to activate Dll than is needed to activate Vg. The effects on Vg and Dll expression are autonomous to mutant cells.

The observation that Dll and Vg expression are reduced more strongly in *dsh* mutant cells at a distance from the source of Wg, than in cells near the source, suggests that the level of Wg activity is graded across the wing pouch. The *dsh* alleles used here (VA153 and 75) have been characterized genetically as null alleles (FlyBase). The maternally produced *dsh* product is sufficient to support development until early pupal stages in the absence of any zygotic *dsh* activity (Perrimon and Mahowald, 1987). Larvae homozygous mutant for dsh^{V26}, another null allele, develop wing discs with a discrete wing pouch despite the absence of zygotic *dsh* activity (Couso et al., 1994). This indicates that the maternal *dsh* product persists at least until the mid-second larval instar when Wg activity is required to specify the wing pouch (Ng et al., 1996). The apparent stability of the *dsh* product raises the possibility that *dsh* null mutant clones retain some Dsh activity. Clones of cells mutant for dsh^{VA153} or dsh^{75} show reduced, but detectable, levels of Dsh protein (data not shown) suggesting that these clones behave as though Dsh activity were reduced, but not completely removed, which may explain why Dll and Vg expression is reduced at a distance from the wing margin where Wg activity should be lowest, but not in regions where Wg activity is high.

Couso et al. (1994) have shown that dsh^{-} clones autonomously lose bristle cell fates at the wing margin, and that AS-C expression at the DV boundary is completely lost in zygotic *dsh* mutants. This suggests that a partial reduction of the activity of the Wg pathway in dsh^{-} clones is sufficient to cause complete loss of AS-C expression, while Dll and Vg expression are only reduced. This suggests that a very high level of Wg signal is required to activate AS-C expression, while lower levels are sufficient to activate Dll and Vg (although expression is weakened in dsh^{-} clones).

Armadillo is the homologue of β-catenin, a component of adherens junctions (Peifer, 1993). Large clones of cells mutant for strong *armadillo* (*arm*) alleles cannot be recovered, especially in regions where Wg signaling is required (Peifer et al., 1991). To circumvent this problem we have made clones mutant for the temperature sensitive hypomorphic allele $arm^{H8.6}$, which impairs Wg signaling but does not compromise the cell adhesion function of Arm (Orsulic and Peifer, 1996). $arm^{H8.6}$ clones were induced and larvae were raised at the permissive temperature (17.5°C) to allow Arm to function while the clones grow. Larvae were then shifted to 25°C, to reduce Arm protein activity, at different times before the end of third instar. Mutant clones could be recovered at reasonable frequency in the wing pouch of discs shifted to the restrictive temperature 18 hours before the end of 3rd instar (Fig. 3), but not in discs shifted 48 hours before the end of third instar (Table 1). Mutant clones were recovered in the portion of the disc that gives rise to the dorsal thorax, or notum, under both conditions. After 18 hours at 25°C, clones in the wing pouch have either lost Vg expression (Fig. 3A) or show reduced levels of Vg expression (Fig. 3B,C). Dll expression is also lost in *arm* mutant clones (Fig. 3C). Both effects are cell autonomous. Note that Dll is lost from cells that still retain a reduced level of Vg expression, again consistent with the proposal that different levels of Wg signaling activity are required for Dll and Vg expression. To be certain that the effect on Vg and Dll expression is specific, and does not reflect a general loss of gene expression, we verified that Engrailed expression is unaffected in $arm^{H8.6}$ mutant clones produced under the same conditions (Fig. 3D).

Table 1. Differential recovery of *armadillo* mutant clones in wing and body wall

	Mutant clones	Wild-type twins
Wing pouch	3*	84
Notum	76	91

$arm^{H8.6}$ clones were induced in early second instar and allowed to grow at 17.5°C until early third instar. Larvae were then shifted to 25°C to reduce Arm protein activity for 48 hours. Mutant clones and wild-type twin spots were counted in the wing pouch and notum in 34 imaginal discs.

*The three clones recovered in the wing pouch were very small.

By using the temperature sensitive allele $arm^{H8.6}$ to reduce Arm activity late in development and by examining clones soon after Arm function was reduced, we were able to recover clones with either reduced or no Vg expression. Clones of cells mutant for a null allele of *vestigial* die in the wing pouch, but survive in the notum, where the gene is not expressed (Kim et al., 1996). Given that very few *arm* mutant clones could be recovered in the wing pouch of discs kept at 25°C for 48 hours (Table 1), while many clones were observed in the notum, it seems likely that *arm* mutant clones die in the wing pouch because they lose Vestigial expression. Loss of Vg expression would be sufficient to explain the differential survival of $arm^{H8.6}$ clones in the wing pouch versus the notum (Table 1).

Taken together, the reduction of Vg and Dll expression in *dsh* mutant clones and the loss or reduction of Vg and Dll expression in *arm* mutant clones suggest that the ability to receive the Wg signal is required directly in all cells of the wing pouch to activate these genes. Because the pattern of Wg expression in the wing disc is dynamic it is necessary to ask whether the localized expression of Wg in cells at the DV boundary is directly responsible for this long-range activity. At this point it is important to note that *wg* is transiently expressed at low levels in all cells of the wing pouch in the early third

instar larva (Phillips and Whittle, 1993; Ng et al., 1996), so it is formally possible that this domain of Wg expression could be responsible for activating Vg and Dll. However, clones of cells mutant for a null allele of *wg* (wg^{CX4}) that meet the DV boundary from either side or that occupy large regions of the wing pouch without meeting the DV boundary have no effect on either Dll or Vg expression (data not shown) and do not cause loss of the wing margin or non-autonomous loss of wing blade tissue (Diaz-Benjumea and Cohen, 1995). Non-autonomous loss of wing tissue was only observed when *wg* mutant clones eliminate *wg* in cells on both sides of the DV boundary (Diaz-Benjumea and Cohen, 1995). We observe loss of Vg and Dll expression associated only with very large clones that remove *wg* from an extensive region on both sides of the DV boundary (data not shown). These observations effectively rule out the possibility that the transient domain of *wg* expression throughout the wing pouch in early third instar discs could be responsible for the broad expression of Vg and Dll. We conclude that Wg expression at the DV boundary is directly responsible for activation of Vg and Dll throughout the wing pouch.

Ectopic Wg causes ectopic target gene expression and overgrowth of the wing pouch

The results presented above indicate that Dll and Vg expression are controlled by the localized stripe of Wg expression at the DV boundary. Misexpression of Wg under control of *dppGAL4* generates a second source of Wg in cells along the AP compartment boundary, along a line perpendicular to the endogen-ous source at the DV boundary. Under these conditions Vg and Dll expression are elevated in broad domains centered on the AP boundary to levels comparable to those seen at the DV boundary (Fig. 4). Although Wg is expressed only in anterior cells, ectopic expression of both target genes is also seen in the posterior compartment. This is evident when compared to the effects of cell-autonomous activation of the Wg pathway by over-expression of *dishevelled* using *dppGAL4*, which results in increased expression of Dll and Vg only in anterior cells (Fig. 4B,C). Double labelling for Dll and Vg shows that both proteins are over-expressed in the same cells (Fig. 4B). Double labeling for Dll and Ci protein (a marker for the anterior compartment) shows that Dll is not misexpressed in posterior cells under these conditions (Fig. 4C). The domain of Dll expression has a sharp posterior border, unlike the case when Wg is over-expressed (Fig. 4A). Although over-expression of *dsh* directs over-expression of Vg, it has a much more limited effect on the growth of the wing pouch than over-expression of Wg. These observations confirm and extend previous reports that Wg can act non-autonomously to control gene expression and growth of the wing, and that this long-range effect is not mediated by a signal relay mechanism (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996b).

Discrete thresholds for Wg activity

Wg acts directly at a distance to regulate Dll and Vg expression. This suggests that the spatial domains of target gene expression might be defined by the level of Wg activity generated at the DV boundary. To address this issue we made use of two regulatory mutants, *spade^{flag}* (*spd^{fg}*) and *Sternopleural* (*Sp*), that reduce Wg activity in the wing margin (Materials and Methods; Neumann and Cohen, 1996a,c) and a temperature sensitive allele of *wg* (wg^{ts}). In combination with wg^{CX4} (a null mutant) the *spd^{fg} Sp* double mutant causes a substantial loss of wing tissue (Fig. 5B) which correlates with a reduced level of Wg protein at the DV boundary (Fig. 5D, arrow; the mutant disc in D was stained together with the wild-type disc in C to permit a direct comparison of Wg levels). A similar phenotype can be generated by reducing Wg activity using wg^{ts} (Fig. 6F; Couso et al., 1995). Reducing the level of Wg in the *spd^{fg} Sp/wg^{cx4}* disc reduces both the maximum level of Dll expression near the DV boundary and the distance from the DV boundary at which Dll can be activated (Fig. 5E,F). Note the proximity of the Dll-expressing sense organs to the DV boundary in the mutant disc (arrows). In wild-type discs these cells are normally found near the base of the wing pouch at a considerable distance from the DV boundary (arrows, Fig. 5E). These observations illustrate that reducing Wg activity leads to loss of cell fates that normally occur close to the DV boundary. Cell types normally found at a distance from the boundary, where Wg activity is expected to be low, are still specified in the mutant discs, but in a position much closer to the DV boundary.

In double label experiments, we compared the effect of partially reducing Wg activity using wg^{ts} on the domains of Dll and Vg expression. Vestigial (Vg) is normally expressed throughout wing pouch; the level of Vg is higher at the DV boundary and relatively even in the rest of the pouch (Fig. 6A). The two domains of Vg expression are controlled by different enhancers (Williams et al., 1994; Kim et al., 1996). Except at the DV boundary, Vg expression in the wing pouch depends on Wg activity (Neumann and Cohen, 1996b). The high level of Vg expression at the DV boundary is controlled by Notch (Kim et al., 1996). Dll is highest at the DV boundary, and decreases across the wing pouch. Low levels of Dll can be detected toward the edge of the Vg domain (Fig. 6A). In wg^{ts} mutant discs shifted to 22°C (a temperature that reduces Wg activity to intermediate levels) the wing pouch is reduced in size (Fig. 6B). Vg expression still fills the wing pouch (arrows), but the domain of Dll is narrowed relative to that of Vg (note the absence of Dll expression near the arrows). This result is comparable to the situation in *dsh* mutant clones where the Dll domain is narrowed, whereas the Vg domain, though reduced, extents to the edge of the wing pouch in the clones (Fig. 2). At 24°C (a temperature that strongly reduces, but does not eliminate Wg activity) Dll expression is completely lost from the wing pouch, but a low level of Vg expression can be seen away from the boundary (Fig. 6C, arrow). This result suggests that the level of Wg activity minimally required to activate Dll is higher than that required to activate Vg.

Wg expression at the DV boundary is also required to specify wing margin cell fates in adjacent cells through induction of Achaete-Scute expression (Skeath and Carroll, 1991; Blair, 1992; Phillips and Whittle, 1993; Couso et al., 1994). This reflects a third expression domain restricted to a region of high Wg activity. The intermediate level of Wg activity produced in discs shifted to 22°C is not sufficient to support the specification of wing margin bristles (Fig. 6E,F), suggesting that it has fallen below a critical threshold for activation of AS-C gene expression, while remaining above the thresholds for activation of Dll and Vg. Taken together, these results suggest that the level of Wg protein produced at the DV

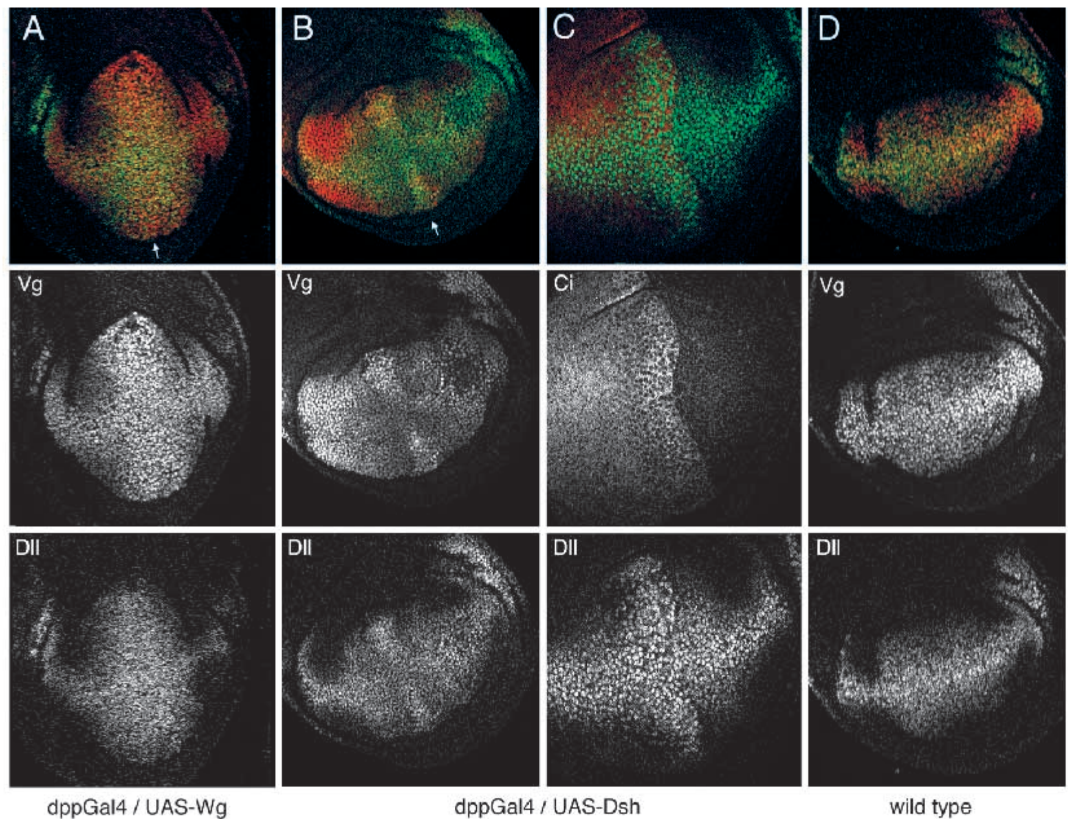

Fig. 4. Non-autonomous activation of Vestigial and Distal-less by Wg, but not by Dsh. (A) *dppGAL4:UASwg* wing disc. Vg in red, Dll in green (single channels below). *dppGAL4* is expressed along the anterior side of the AP boundary. Note high levels of Vg and Dll expression along the A/P compartment boundary (arrow), and the graded expression to either side. Note also the overgrowth of the disc along the AP axis. (B) *dppGAL4:UASdsh* wing disc. Channels as in A. Note elevation of Vg and Dll in the same cells along the AP boundary (arrow). Note also the lack of overgrowth. (C) *dppGAL4:UASdsh* wing disc, double labeled for Ci (red) and Dll (green). Ci is expressed only in anterior cells. Note that Dll is only elevated in a stripe of cells to the anterior of the compartment boundary, corresponding to the cells in which the *dppGAL4* driver is expressed. The image in C is magnified approx. 1.6 fold compared to those in A, B and D). (D) Wild-type disc. Staining as in A and B.

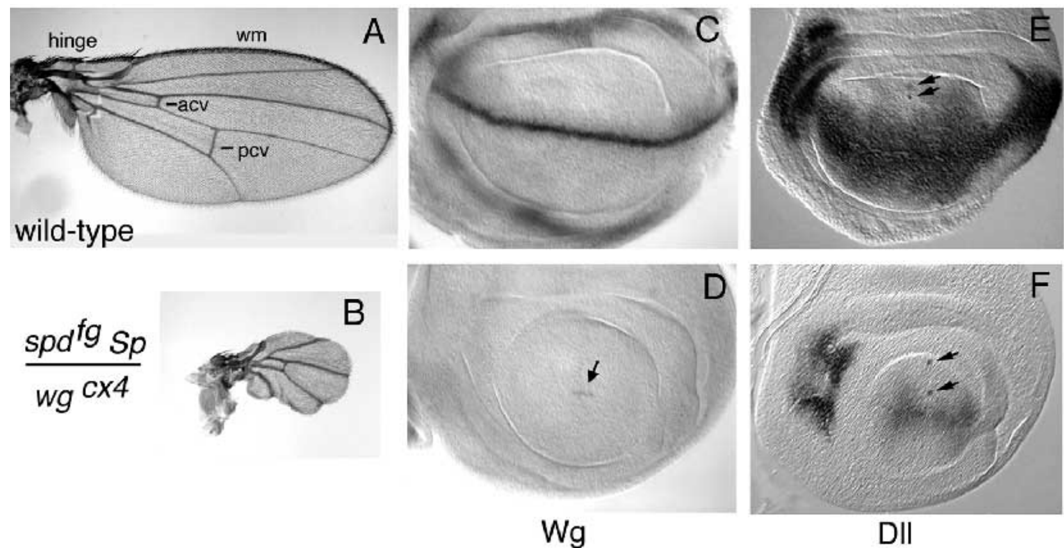

Fig. 5. Loss of wing tissue and alteration of target gene expression in *wg* mutant discs. (A) Wild-type adult wing. The wing margin (wm) consists of rows of bristles along the dorsal and ventral edges of the wing. The margin is produced by cells adjacent to the Wg-expressing cells at the DV compartment boundary. Hinge indicates the hinge region at the base of the wing; acv, anterior crossvein; pcv, posterior crossvein. (B) *spd^{fg} Sp/wg^{cx4}* mutant wing. Structures distal to the pcv are missing. (C) Wg protein expression in wild-type and (D) *spd^{fg} Sp/wg^{cx4}* mutant wing discs. The level of Wg protein is reduced in the mutant, and can only be seen as a faint patch of expression at the center (arrow). (E) Distal-less protein expression in wild-type and (F) *spd^{fg} Sp/wg^{cx4}* mutant discs. In wild-type, Dll expression is highest at the D/V boundary and decreases in intensity away from the wing margin. The arrows in E and F indicate sense organs near the base of the wing pouch that label with Dll antibody. (F) In the mutant disc Dll is expressed at a much lower level at the D/V boundary and in a much narrower domain. Note the proximity of the distal-most sense organ to the DV boundary. Note also that the separation between the sense organs is increased. This could be due to a shallower gradient of Wg protein (and hence positional information) emanating from the DV boundary in the mutant disc.

boundary regulates the size of the wing pouch and controls the spatial domains of three different target genes in a concentration-dependent manner.

DISCUSSION

Wg acts directly on all cells of the developing wing blade

Ectopic expression of Wg in the developing wing blade can induce formation of an ectopic wing margin as well as outgrowths from either the dorsal or ventral surface of the wing (Fig. 4A; Diaz-Benjumea and Cohen, 1995). Specification of wing margin fate occurs in cells immediately adjacent to the source of the Wg signal, reflecting a requirement for high levels of Wg activity (Blair, 1992, 1994; Phillips and Whittle, 1993; Couso et al., 1994). The long-range effect of Wg-expressing clones on growth of non-adjacent cells suggests that Wg can also act at a distance to influence cell behavior. We have shown here that Wg, expressed at the DV boundary, acts over long distances to control the expression of Dll and Vestigial in the wing pouch. We can consider two models for how Wg might act at a distance to direct target gene expression. Wg protein might act directly to induce expression of its target genes in cells distant from the source. Alternatively Wg might act locally to induce a second signal that relays information to more distant cells.

Two lines of evidence argue against a signal relay mechanism for Wg. First, we have shown here that clones of cells reduced in their ability to transduce the Wg signal lose expression of Dll and Vg. This indicates that the ability to receive the Wg signal is required directly by all cells of the wing pouch, independent of their position. Second, if there were a signal relay mechanism we would expect cells in which the Wg signal transduction pathway is activated to exert a non-autonomous influence on surrounding cells. This has been examined in two ways. Removing activity of *zw3* is equivalent to activating the pathway (Siegfried et al., 1992). Cells mutant for *zw3* in the wing blade express three different targets for Wg activity: the proneural proteins of the Achaete-Scute complex, Dll, and Vg (Blair, 1992, 1994; Couso et al., 1994; Diaz-Benjumea and Cohen, 1995). Activation of these target genes

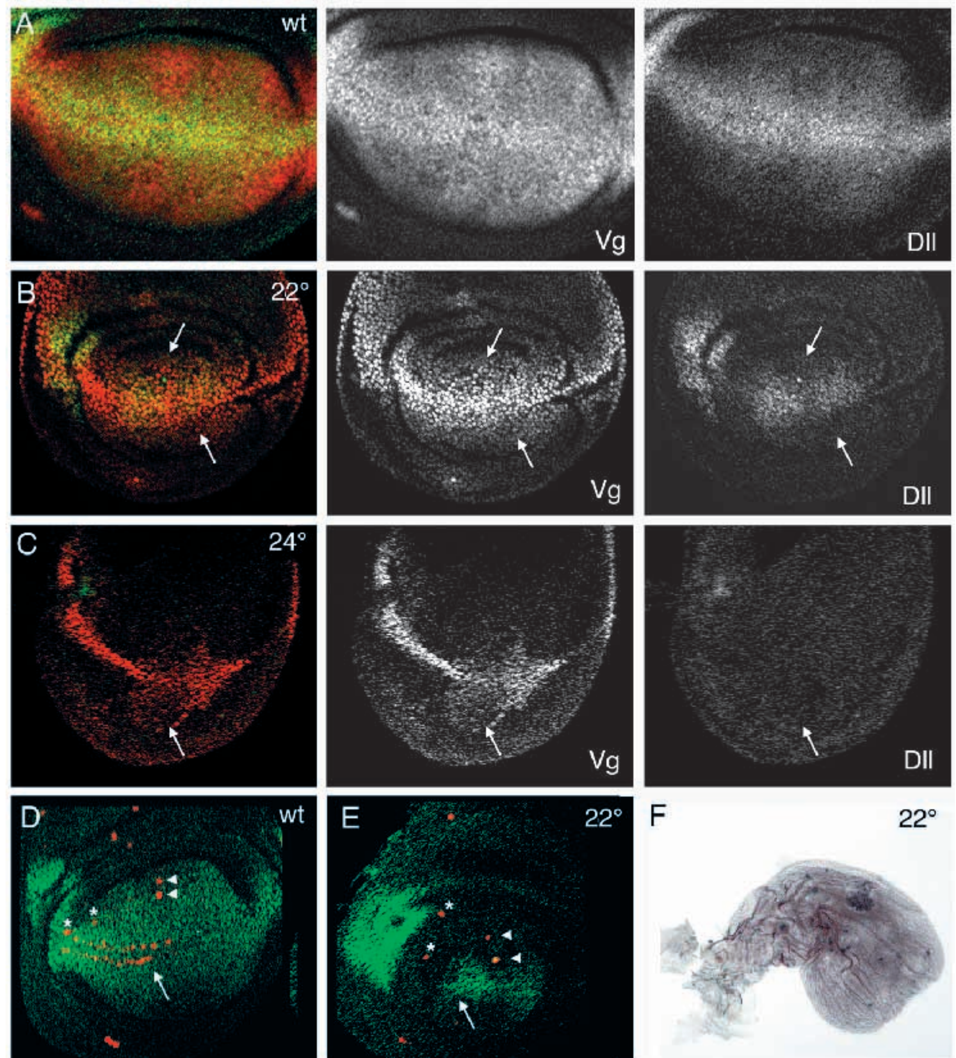

Fig. 6. Two concentration thresholds for Wg signaling in the wing pouch. (A) Wild-type. Dll expression is shown in green, Vg in red. The single channels are shown to the right. Vg expression extends throughout the wing pouch, and is slightly graded (see Williams et al., 1994). Dll expression is strongest near the DV boundary and can be detected at lower levels towards the edges of the wing pouch. The gradient of Dll expression appears to be steeper than that of Vg expression. (B) wg^{IL114} disc shifted to 22°C in early third instar to reduce Wg activity to an intermediate level. Dll expression (green) is lost from the base of the wing pouch, but is still present in the center, although it is weaker and narrower than in wild type. Vg expression (red) is also reduced and contracted in its spatial extent, but still occupies more territory than Dll (compare the expression of the two genes between the arrows). Vg expression at the DV boundary is unaffected. Single channels are shown to the right, as in A. (C) wg^{IL114} disc shifted to 24°C in early third instar to strongly reduce, but not eliminate Wg activity. Vg expression (red) can still be detected at low levels in the reduced wing pouch (arrow), but Dll expression (green) is not detectable. The single channels are shown to the right, as in A. (D) Wild type. *A101/neuralized-lacZ* expression (red) marks the rows of neuronal cells that give rise to the anterior wing margin bristles (arrow). Dll expression in green. Asterisks and arrowheads mark sense organs located away from the DV boundary. (E) wg^{IL114} disc shifted to 22°C in early third instar. *A101*-expressing cells at the D/V boundary are completely lost (arrow), while there is still some Dll expression (green). Note that Dll expression in this disc is reduced somewhat more than in the disc in B. (F) Wing from a wg^{IL114} individual shifted to 22°C in early third instar. The wing was dissected out of the pupal case and inflated, resulting in a somewhat damaged appearance (these larvae don't survive to adulthood). The wing margin is absent. These wings are of similar size to the one shown in Fig. 2B.

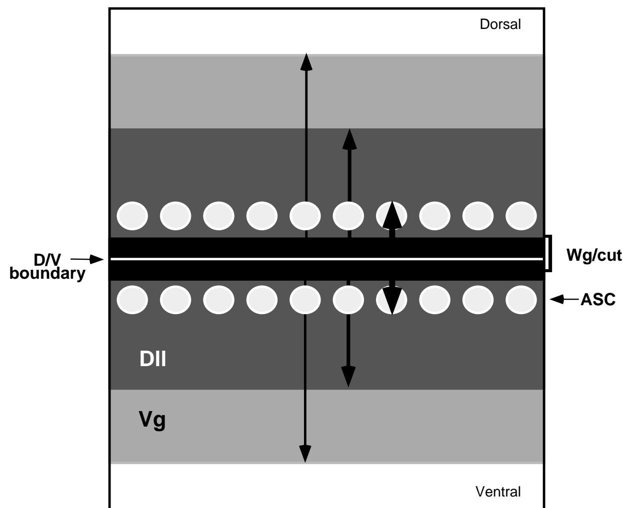

Fig. 7. Model for long-range organizing activity of Wg in the developing wing. The wing disc is depicted by a box. The disc is divided into dorsal and ventral compartments by expression of Apterous in dorsal cells (not indicated). The compartment boundary is indicated by the white line. Wg is expressed by cells adjacent to the compartment boundary in both compartments (black band). This is also the region where *cut* is expressed and from which expression of AS-C genes is excluded. Dll expression is depicted by medium gray shading and Vg expression by light gray shading. Dll expression is graded within its domain, and low levels of Dll can be seen close to the edge of the Vg domain. For simplicity the drawing does not attempt to show this. We propose that the local concentration of the secreted Wg protein defines the spatial domains of AS-C, Dll and Vg expression. Thus Wg may act as a morphogen to provide positional information that patterns the DV axis of the wing. The effects of Wg are symmetric, but dorsal cells are programmed to interpret the positional information differently, because of the presence of Apterous. Hence the dorsal wing margin differs from the ventral wing margin. Other differences in dorsal and ventral compartments include positions of sense organs.

is strictly autonomous to the *zw3* mutant cells and is not seen in the surrounding wild-type cells. Furthermore, although *zw3* mutant cells differentiate as wing margin cells they do not exert a non-autonomous influence on the growth of surrounding cells, in contrast to the effects of Wg expression, which does both (Diaz-Benjumea and Cohen, 1995). The Wg pathway can also be activated by over-expressing *dsh* (Yanagawa et al., 1995; Axelrod et al., 1996; Neumann and Cohen, 1996a). Over-expressing *dsh* using the GAL4 system leads to ectopic specification of wing margin bristles (Axelrod et al., 1996; Neumann and Cohen, 1996a), and to cell autonomous activation of Dll and Vg (Fig. 4B,C). Taken together, these observations effectively rule out the possibility that the long-range action of Wg might be mediated by relay of another downstream signal and strongly suggest that Wg acts directly on all cells of the wing pouch.

Multiple thresholds for Wg activity along the D/V axis of the wing

Wg acts non-autonomously to define the expression domains of at least 3 different target genes in the wing pouch: *AS-C*, *Dll* and *vg*. Because *AS-C* expression is restricted to cells in very close proximity to the source of Wg expression at the DV boundary this can be considered to reflect a requirement for high levels of Wg activity. Dll is expressed in a much broader domain and in a graded manner with high levels centered on the domain of Wg expression. Vg is expressed across the entire wing pouch. In all three cases Wg appears to act directly to control expression, because activity of the Wg signal transduction pathway is directly required for target gene expression in responding cells, even at late stages of development, and (in the case of Vg and Dll) at a distance from the DV boundary. This suggests that different thresholds of Wg activity are required to define the spatial domains of expression of these three target genes (Fig. 7). Consistent with this proposal, reducing the level of Wg activity at the DV boundary to an intermediate level leads first to the loss of wing margin cell fates, while still allowing the activation of Dll and Vg (Fig. 6B,E,F, although the domains of Dll and Vg become narrower and weaker). A further reduction of Wg activity leads to the loss of Dll expression, while Vg expression is still activated at low levels (Fig. 6C). It should be noted that Dll is expressed at low levels in cells near the edge of the Vg domain, so the difference between the minimal levels of Wg activity needed to activate Dll and Vg is not very different. Wg is also required in conjunction with Notch to define another domain: the narrow band of cells between the rows of sense organs at the wing margin. *cut* is expressed in these cells and depends on both Wg and Notch activity (Couso et al., 1994; Neumann and Cohen, 1996b). Taken together, these results indicate that distinct activity thresholds can be defined by Wg protein, leading to the generation of several different cell states (Fig. 7).

It is interesting to note that a very strong activation of the *wg* pathway in $zw3^-$ clones does not lead to overgrowth within the clone. Likewise, ubiquitous strong expression of Wg throughout the wing pouch does not lead to overgrowth (Neumann and Cohen, 1996a). However, expressing high levels of Wg from a localized source does cause overgrowth of surrounding tissue (Fig. 4A; Diaz-Benjumea and Cohen, 1995). This suggests that only an intermediate level of activation of the *wg* pathway can stimulate growth in the wing pouch. This would correspond to the cells which receive enough Wg to activate Vg and Dll, but not enough to activate the Achaete-Scute complex.

In conclusion, our results suggest that the secreted Wg protein acts at different threshold concentrations to define distinct regions along the DV axis of the wing (Fig. 7). This suggests that localized production of Wg protein at the DV compartment boundary generates a concentration gradient across the entire wing pouch and that cells are instructed about their prospective fate as a function of the local concentration of the Wg protein. We propose that Wg acts as a morphogen to pattern the DV axis of the developing wing.

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