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# Developmental territories created by mutual antagonism between Wingless and Decapentaplegic

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## SUMMARY

***Drosophila*** **appendages develop from imaginal discs which become subdivided into distinct regions during normal patterning. At least 3 axes of asymmetry are required to produce a chiral appendage such as a leg. The A/P compartments provide one axis of asymmetry in all discs. In leg and antennal discs, the anterior compartment becomes asymmetric in the D/V axis with *decapentaplegic (dpp)* expression defining dorsal anterior leg, and *wingless (wg)* expression defining ventral anterior leg. However, unlike wing discs, no D/V compartment has been demonstrated in legs or antennae. How are the dorsal anterior and ventral anterior territories defined and maintained? Here we show that *wg* inhibits *dpp* expression and *dpp* inhibits *wg* expression in leg and eye/antennal discs. This mutual repression provides a mechanism for maintaining separate regions of *wg* and *dpp* expression in a developing field. We propose the term 'territory' to describe regions of cells that are under the domineering influence of a particular morphogen. Territories differ from compartments in that they are not defined by lineage but are dynamically maintained by continuous morphogen signaling. We propose that the anterior compartment of the leg disc is divided into dorsal and ventral territories by the mutual antagonism between WG and DPP signaling.**

Key words: *Drosophila* development, imaginal disc, *wingless*, *decapentaplegic*, *dishevelled*, *punt*, *thick veins*, *wingless* regulation, *dpp* regulation, pattern formation

## INTRODUCTION

The generation of at least three axes of asymmetry in appendage primordia is an essential element of patterning (see Postlethwait, 1978; Cohen, 1993). The A/P axes in *Drosophila* appendages are established by the A/P compartments (strict lineage restrictions) (Garcia-Bellido et al., 1973, 1976; Garcia-Bellido, 1975; Steiner, 1976; Wieschaus and Gehring, 1976; Lawrence and Morata, 1977) which are defined by non-overlapping expression of *cubitus interruptus(ci)* in the anterior compartment (Eaton and Kornberg, 1990; Dominguez et al., 1996) and *engrailed (en)* in the posterior compartment (Kornberg et al., 1985; Sanicola et al., 1995; Zecca et al., 1995). The apical basal polarity of the epithelium may provide another axis. After the establishment of A/P asymmetry, the wing disc is further subdivided into dorsal and ventral compartments (Bryant, 1970; Garcia-Bellido et al., 1976; Blair, 1993) by the activity of the *apterous (ap)* gene (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Lawrence and Morata, 1993; Williams et al., 1993; Blair et al., 1994) but it has not been possible to demonstrate a D/V lineage restriction in the leg (Steiner, 1976). Is it possible that the establishment of D/V asymmetry in the leg utilizes a mechanism different from a lineage restriction?

In third instar leg discs, *wingless (wg)* is expressed in a ventral anterior wedge (Baker, 1988b; Couso et al., 1993) while *decapentaplegic (dpp)* is expressed strongly in an anterior stripe that abuts the A/P compartment boundary, and weakly in a ventral domain that overlaps *wg* expression (Masucci et al., 1990; Raftery et al., 1991). Several observations suggest that WG and DPP can interact in particular tissues (Jackson and Hoffmann, 1994; Kaphingst and Kunes, 1994; Staehling-Hampton and Hoffmann, 1994; Tabata and Kornberg, 1994; Ma and Moses, 1995; Treisman and Rubin, 1995; Penton and Hoffmann, 1996; Wiersdorff et al., 1996; Yu et al., 1996). We have compared the effect of WG and DPP signaling on each other's transcription and on cell fate specification in discs. We find that WG signaling inhibits *dpp* expression, and DPP signaling inhibits *wg* expression, in leg and eye/antennal discs but not in dorsal discs (i.e. wing and haltere). This mutual repression provides a mechanism for maintaining separate regions of gene expression in developing leg discs. The D/V restriction of *wg* and *dpp* divides the anterior compartment into dorsal and ventral territories, thus creating a D/V axis of asymmetry that is necessary for generating a chiral appendage. We define a territory as a group of cells that are under the domineering influence of a particular morphogen. Neighboring territories are not separated by lineage restriction nor are cells within a territory or their descendants 'determined' (Lawrence and Struhl, 1996) to remain as part of that territory. Descendants of cells from one territory that, by growth displacement or injury, find themselves closer to the source of another morphogen will become part of the territory defined by the second morphogen. In the

leg disc, cells primarily within the range of influence of DPP will acquire dorsal/dorsolateral positional values, while cells that lie within the range of influence of WG will acquire ventral/ventrolateral positional values regardless of their lineage. Cells in the zone between the two territories will receive varying levels of both signals. The mutual repression will serve to maintain separate territories of morphogen expression.

## MATERIALS AND METHODS

### Drosophila stocks

A *y w sn* stock was used as a control for the temperature upshifts, the mRNA in situ hybridizations and the immunostaining reactions because this allowed controls and mutants to be stained in the same tubes and then separated by color of mouthparts, thus, permitting comparisons of the levels of staining.

Three alleles of *punt* were tested as homozygotes and in heteroallelic combinations with each other for temperature sensitivity. All heteroallelic combinations of $punt^{135\text{-}22}$, $punt^{\Delta 61}$ and $punt^{P1}$ exhibited temperature sensitivity (Letsou et al., 1995). The $punt^{135\text{-}22}$ allele was a kind gift from A. Letsou, University of Utah. The *punt* mutant stocks were balanced with a TM6 balancer chromosome that carries the dominant marker *Tubby (Tb)*. Heteroallelic *punt* larvae were identified by their non-*Tb* phenotype. The temperature sensitive *wg* allele, $wg^{IL114}$ (Nüsslein-Volhard et al., 1984), was balanced with the compound balancer TSTL which has a translocation between the *CyO* and TM6B,*Tb* balancers. Homozygous mutant larvae were identified by their non-*Tb* phenotype.

$dpp^{blk}/dpp^{blk}$; $dpp^{blk}$>Gal4 [39B2]/TM6, *Tb* flies were mated to flies homozygous for one of the following transgenes: UAS>*dpp-myc4*, UAS>*dpp-myc7* (kind gifts from R. Nichols and W. M. Gelbart, Harvard University) and UAS>*wg* (a kind gift from I. Livne-Bar and H. Kraus, University of Toronto) to generate larvae expressing either *dpp* or *wg* along the A/P boundary. Larvae that received the $dpp^{blk}$>Gal4 driver were identified by their non-*Tb* phenotype. The $dpp^{blk}$>Gal4; UAS>*dpp-myc4* and $dpp^{blk}$>Gal4; UAS>*dpp-myc7* combinations gave similar results and are referred to as $dpp^{blk}$>*dpp* in this manuscript. Clones of *tkv* were generated by heat shocking *y w* P[hs-FLP1]; $tkv^{IIB09}$ P[FRT40A]/P[*y*+25A]P[FRT40A] larvae.

### In situ hybridizations

*wg* and *dpp* expression were monitored by whole-mount in situ hybridization using digoxigenin-labeled antisense RNA probes. Plasmids used as templates for probes were wg651 (a kind gift from B. Cohen), a 3 kb *wg* cDNA, dppE55, a 4 kb *dpp* cDNA (Padgett et al., 1987), and hhC11, a 2.3 kb cDNA (Lee et al., 1992), all in bluescript. The probes were prepared according to the protocol accompanying the digoxigenin RNA labeling mix (Boehringer Mannheim 1277 073). Unincorporated nucleotides were removed by LiCl precipitation and the RNA from 1 µg of template was resuspended in 100 µl DEPC water; 20 µl of each probe were hydrolyzed, LiCl precipitated, resuspended in 100 µl hybridization solution and diluted 1:50 µl for the hybridization reaction.

The prehybridization procedure and hybridization conditions used are based on the protocol of Tautz and Pfeifle (1989) with the following modifications. Late third instar larvae and white prepupae were dissected in PBS (pH7), stored for less than 20 minutes on ice, and fixed for 20 minutes in 4% formaldehyde in PBT (PBS + 0.1% Tween 20). Discs were stored at –20°C in ethanol, progressively rehydrated, and digested with 5 µg/ml proteinase K in PBT for 5 minutes. After rinsing, the discs were fixed in 4% formaldehyde in PBT for another 20 minutes. The hybridization was carried out at 55°C for 2 days and the discs were washed for 3 days at 55°C after hybridization. After one 20-minute 50:50 PBT:hyb solution rinse and five 25-minute PBT rinses, discs were incubated in polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim 1093 274) diluted 1/2000 in PBT overnight at 4°C. The AP color reaction was developed according to the protocol accompanying the antibody.

### Immunohistochemistry

Discs were fixed as above and incubated overnight at 4°C with anti-EN monoclonal antibody diluted 1:1 with PBT (PBS + 0.1% Triton X-100) + 3% BSA. An AP-conjugated secondary antibody (Jackson Immunological Laboratory) was used at a1/1000 dilution and the AP color reaction was developed as described for in situs.

## RESULTS

### Loss of DPP signaling leads to ectopic *wg* gene expression

The DPP receptor is a heterodimer composed of two distantly related transmembrane serine/threonine kinases called type I and type II receptors (reviewed by Massaguè, 1996). The *punt* gene encodes a type II receptor that is essential for reception of the DPP signal (Letsou et al., 1995; Ruberte et al., 1995). We used temperature sensitive *punt* mutants to determine the consequences of loss of DPP signaling on *wg* expression. Mutant larvae were transferred from 18°C to the restrictive temperature, 25°C, for different lengths of time during larval

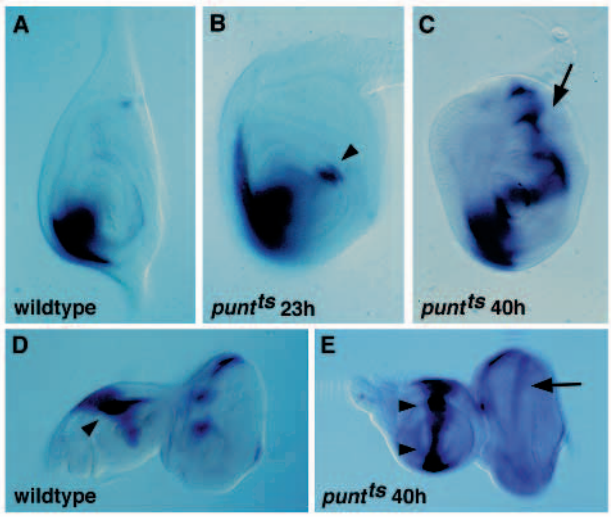


**Fig. 1**. Loss of DPP signaling leads to ectopic *wg* expression. In situs to *wg* mRNA. Anterior is to the left and dorsal is up. (A) Control *y w sn* leg disc. (B) $punt^{P1}/punt^{135\text{-}22}$ leg disc from a larva that was transferred from 18°C to the non permissive temperature (25°C) for 23 hours BPF. Note the ectopic dorsal *wg* expression which extends well into the femur (arrowhead). (C) $punt^{P1}/punt^{135\text{-}22}$ leg disc from a larva that was transferred from 18°C to 25°C for 40 hours BPF. In this disc there is an ectopic dorsal stripe of *wg* expression (arrow). (D) Control *y w sn* eye-antennal disc showing the dorsal wedge of *wg* expression in the antenna (arrowhead) and around the periphery of the eye. (E) $punt^{P1}/punt^{135\text{-}22}$ eye-antennal disc from a larva that was transferred from 18°C to 25°C for 40 hours BPF. A continuous stripe of *wg* expression now extends ventrally across the antenna (arrowheads). Ectopic *wg* also extends from the periphery into the morphogenetic furrow (arrow). Elevated *wg* was seen in 70 of 76 $pnt^{ts}$ discs examined.

development (measured retrospectively from the time of puparium formation) and stained for *wg* mRNA.

Loss of *punt* function results in the ectopic activation of *wg* expression along the dorsal A/P boundary of the leg disc (Fig. 1). By 23 hours after upshifting $punt^{P1}/punt^{135\text{-}22}$ larvae, *wg* expression has expanded into the dorsal region (Fig. 1B), and it continues to expand until reaching a maximum after about 40 hours at the restrictive temperature (Fig. 1C). Similar results are also observed in the eye-antennal disc. The antennal portion of the eye-antennal disc is analogous to a leg disc, but inverted in the D/V and A/P axes (Struhl, 1981). Thus *wg*, which is expressed in the ventral region of the leg disc, is expressed in the dorsal region of the wild-type antennal disc (Baker, 1988a) (Fig. 1D). *wg* is also expressed around the periphery of the eye portion of the disc (Baker, 1988a; Ma and Moses, 1995) (Fig. 1D). When temperature sensitive *punt* mutants are upshifted to 25°C, *wg* expression expands into the ventral domain of the antenna to form a continuous stripe along the A/P boundary from dorsal to ventral (Fig. 1E). In addition, *wg* expression expands from its normal location at the periphery of the eye anlage into the morphogenetic furrow (Fig. 1E). In wing discs, the pattern of *wg* expression and the morphology of the discs is normal in $pnt^{ts}$ animals even with a 70-hour upshift. In legs, the maximal ectopic *wg* expression is seen after only 40 hours at restrictive temperature (Fig. 1C).

In summary, when DPP signaling is blocked by transferring temperature-sensitive *punt* mutants from 18°C to 25°C, ectopic *wg* expression appears in regions of the leg and eye-antennal discs that coincide with regions of *dpp* expression in the wild type.

## Increased DPP signaling represses *wg* transcription

The previous experiments show that loss of DPP signaling causes ectopic *wg* expression. To perform the reciprocal test, namely whether increased DPP signaling represses *wg* expression, we expressed increased levels of *dpp* in its normal domain and monitored *wg* expression. In leg discs, *dpp* is strongly expressed in a dorsal stripe that abuts the A/P boundary and is weakly expressed in the ventral region of the disc, where its expression overlaps the *wg* expression domain (Masucci et al., 1990; Raftery et al., 1991). A $dpp^{blink}$>Gal4 transgene (Staehling-Hampton et al., 1994) was used to drive expression of a UAS>*dpp* transgene. In the leg disc, this combination drives *dpp* expression in a two wedges that abut the anterior side of the A/P boundary (Fig. 2C). The increase in *dpp* expression in the ventral region of the leg disc causes a reduction of *wg* expression (Fig. 2B). The normal anterior ventral wedge of *wg* expression (Fig. 2A) is lost and *wg* expression is restricted to a narrow D/V stripe across the tip of the tarsus (Fig. 2B). This region of *wg* expression coincides

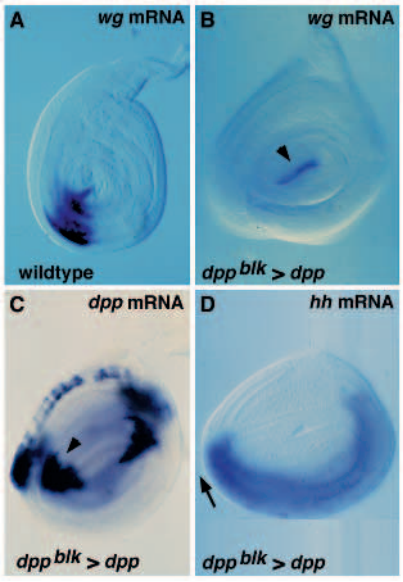


**Fig. 2**. Increased *dpp* signaling represses *wg* transcription. Anterior is to left and dorsal is up. (A) Control *y w sn* leg disc. (B-D) Lleg discs where the $dpp^{blink}$>Gal4 driver is driving UAS:*dpp* expression. (A) In situ to *wg* mRNA. (B) An in situ to *wg* mRNA reveals that *wg* expression is reduced to a dorsoventral stripe at the tip of the tarsus (arrowhead) when ventral *dpp* expression is increased ($n$ = 18 of 18 discs). Compare to wild type in A. (C) An in situ to *dpp* mRNA shows that expression of UAS:*dpp* under $dpp^{blink}$>Gal4 control drives increased levels of ventral *dpp* expression (arrowhead) in the leg. In addition, the shape of the *dpp* D/V stripe is altered to form a reversed 'C' shape. (D) An in situ to *hh* mRNA shows that the posterior compartment expands anteriorly (arrow) in the ventral region of leg discs when *dpp* is over expressed by the $dpp^{blink}$>Gal4 driver.

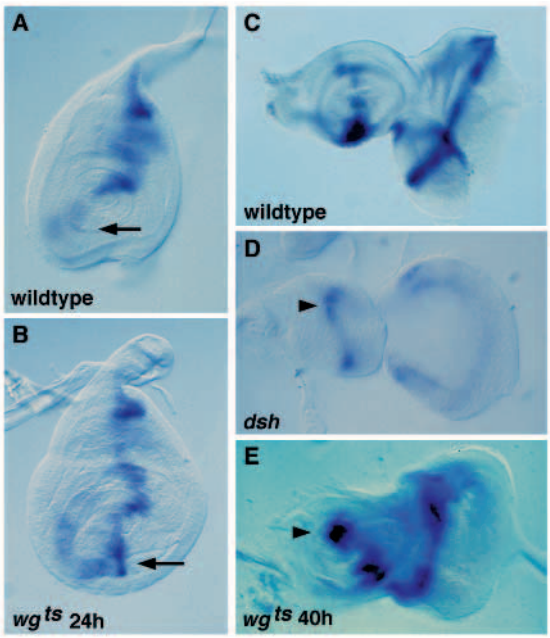


**Fig. 3**. Loss of WG signaling leads to ectopic *dpp* expression. (A-E) In situs to *dpp* mRNA. Anterior is to the left and dorsal is up. (A) Control *y w sn* leg disc showing normal *dpp* expression along the A/P boundary. Note the weak ventral expression (arrow). (B) $wg^{IL114}/wg^{IL114}$ leg disc from a larva that was transferred from 18°C to the non-permissive temperature (25°C) for 24 hours BPF. Ventral *dpp* expression is increased (12 of 12 discs) compared to wild type shown in A (arrows). (C) Control *y w sn* eye-antennal disc showing normal *dpp* expression restricted to a ventral stripe in the antenna. (D) $dsh^{VA153}/dsh^{VA153}$ eye-antennal disc. *dpp* is ectopically expressed in the dorsal region of the antenna (arrowhead). Compare to C. (E) $wg^{IL114}/wg^{IL114}$ eye-antennal disc from a larva that was transferred from 18°C to 25°C for 40 hours BPF. Like the *dsh* mutant shown in C, *dpp* is ectopically expressed in the dorsal region of the antenna (arrowhead).

with the only region along the A/P boundary that lacks *dpp* expression in this genetic background (Fig. 2C and Fig. 6A). Similar reductions in *wg* expression were also observed by expressing a ligand-independent, constitutively active type I DPP receptor, *thick veins (tkv)*, using a $dpp^{blk}$>Gal4 driver in leg discs.

Unlike the *punt* temperature-shift experiments described previously, where the transcription response of *wg* occurs within 23 hours, expression of *dpp* driven by $dpp^{blk}$>Gal4 is continuous during the approximately 4.5 days of disc development. This provides time for regulative growth to occur in response to ectopic *dpp* expression. This pattern regulation produces leg discs that are wider in the anterior-posterior axis. To better understand the basis of the morphological changes, we monitored *hedgehog* (*hh*) expression to demarcate posterior from anterior compartments. The wide discs exhibit an expansion of the posterior compartment of the disc as evidenced by expanded *hh* expression (Fig. 2D and Fig. 6A). Thus, continuous elevated *dpp* expression in the ventral region of the leg disc suppresses *wg* expression and causes expansion of the posterior compartment in this region.

Expression of either *dpp* or ligand independent *tkv* in wing discs using the $dpp^{blk}$>Gal4 driver produced extremely wide wing discs but the pattern of *wg* expression, even in the face of extensive ectopic growth, was essentially normal. Since expression driven by the $dpp^{blk}$>Gal4 driver intersects the bands of *wg* expressesion only along the A/P boundary, we used the A9>Gal4 driver to express both activated *tkv* and *dpp* in a broad band that overlaps the band of *wg* expression along the wing margin. Again, no reduction in *wg* expression was observed, indicating that the mutual antagonism between WG and DPP does not operate in the wing.

The loss of *wg* expression in the $dpp^{blk}$>*dpp* discs and the expanded *wg* expression in the *punt* mutants demonstrates that DPP signaling restricts the *wg* expression domain in the leg disc.

## Loss of WG signaling leads to ectopic activation of *dpp* transcription

To examine the effect of WG signaling on *dpp* expression, we used a temperature-sensitive allele of *wg*. Larvae, mutant for $wg^{IL114}$ (Nüsslein-Volhard et al., 1984), were raised at 18°C, transferred to 25°C for 24 hours and 40 hours BPF and the expression of *dpp* in discs of white prepupae was examined. As controls, *y w sn* larvae were upshifted on the same schedule and stained in the same vials. Within 24 hours, loss of *wg* function causes *dpp* in the ventral region of the leg to increase and expand (Fig. 3B). In the eye-antennal disc, *dpp* expression also expands into the dorsal region of the antenna within 40 hours at 25°C (Fig. 3E). The changes in *dpp* expression caused by loss of *wg* function provide an explanation for the phenotypes seen in $wg^{ts}$ pharate adults, which exhibit a duplication of dorsal in place of ventral structures in the legs (Couso et al., 1993).

If WG signaling represses *dpp* expression, then *dpp* expression should also expand when transduction of the *wg* signal is blocked, such as in *dsh* mutants (Klingensmith et al., 1994; Theisen et al., 1994). Maternal and zygotic loss of *dsh* causes embryonic lethality with a cuticular phenotype indistinguishable from that of *wg* mutants (Perrimon and Mahowald, 1987). However, when *dsh* is maternally supplied, homozygous mutant larvae survive to early third instar with very small leg discs but reasonably developed eye-antennal discs. In the antennal discs of these mutants, *dpp* (which is normally confined to the ventral region (Masucci et al., 1990), Fig. 3C) is ectopically expressed in the dorsal region of the antenna (Fig. 3D). In wing discs, the pattern of *dpp* expression and the disc morphology is normal in $wg^{ts}$ animals upshifted for 49 hours, whereas a 24-hour upshift is sufficient to achieve maximal ectopic *dpp* expression in the leg discs (Fig. 3B). Thus, loss of WG signaling, causes *dpp* expression to expand in leg and eye-antennal discs but not in wing discs.

## Ectopic expression of *wg* represses *dpp* expression

Blocking WG signaling causes an expansion of *dpp* expression. To test whether ectopic WG signaling inhibits *dpp* expression, we used the $dpp^{blk}$>Gal4 (Staehling-Hampton et al., 1994) driver to activate a UAS>*wg* transgene in a stripe along the A/P boundary of the leg disc (Fig. 4B). The ectopic expression of *wg* in the dorsal region of the disc suppresses expression of *dpp* to the point that the level of expression appears similar to or lower than the weak ventral expression of *dpp* seen in normal discs (compare Fig. 4A with 4C). Note also that the weak ventral expression of *dpp* which coexists with *wg* in normal discs is unaffected by ectopic expression of *wg* (Fig. 4C). Thus, ectopic expression of *wg* reduces *dpp* expression in leg discs. These discs become long and narrow in contrast to the short wide shape of discs with increased *dpp* signaling (Figs 4B-D compared with 2B-D and 6A). The expansion of *en* expression reveals that the dorsal posterior compartment is enlarged in these leg discs (Figs 4D and 6A). Wing discs with $dpp^{blk}$ driving *wg,* exhibit extensive pattern regulation but strong expression of *dpp* in a D/V stripe remains, indicating that ectopic WG does not suppress *dpp* in wing discs.

## Cell fate changes corelate with altered *dpp* and *wg* expression patterns

To determine whether alterations in gene expression have predictable developmental consequences, we examined pharate adults from upshifted $punt^{ts}$ animals and adults with *tkv* clones for altered cuticular patterns. $punt^{ts}$ pharate adults produced by late first/early second-instar upshifts have legs in which dorsal structures are absent and ventral structures are duplicated (Fig. 5A,B). The duplication of ventral leg elements is consistent with ectopic *wg* expression since *wg* is normally expressed ventrally (Baker, 1988b; Couso et al., 1993) and specifies ventrolateral fates when ectopically expressed in the leg disc (Struhl and Basler, 1993; Wilder and Perrimon, 1995). Interestingly, the ectopic dorsal expression of *wg* in $punt^{ts}$ animals is correlated with specification of extreme ventral fates in the dorsal region. For example, on the distal tibia of the second leg shown in Fig. 5B there are two apical bristles which are indicators of the ventral-most cell fate in leg discs. In addition, $punt^{ts}$ pharate adults exhibit reduced or missing eyes (Fig. 5C) and duplicated antennae (Fig. 5D). The loss of eye tissue is consistent with the ectopic expression of *wg* in the eye field antagonizing DPP and consequently restricting the morphogenetic furrow (Ma and Moses, 1995; Treisman and Rubin, 1995). The duplicated antennae branch from the ventral side as would be expected from regulatory growth if *wg* were ectopically expressed on this side.

The *tkv* gene encodes a type I receptor that is essential for reception of the DPP signal (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994). We induced *tkv* clones in second instar larvae. In the tarsus, dorsal *tkv* clones convert dorsal cells to ventral or ventrolateral cell fates. These clones often produce outgrowths that include normal neighbors, some of which have also been respecified to ventral cell fates (not shown). This is consistent with DPP specifying dorsal cell fates and its loss converting cells to ventral identity followed by an intercalary response as expected when ventral cells confront dorsal cells (French et al., 1976). Unexpectedly, *tkv* clones in the ventral tarsus produce excess ventral-most structures (e.g. the peg-like bristles characteristic of *wg*-expressing cells on the tarsus, Fig. 5E). These clones often initiate outgrowths consisting only of mutant cells (Fig. 5E). This observation demonstrates that DPP signaling also plays a role in patterning the ventral territory of leg discs. Tarsal *tkv* clones also cause polarity disruptions in neighboring normal cells (e.g. reversed bristle-bract vectors; reversed bristles, Fig. 5E,F) that show a positional bias (Fig. 5G) with all ventral *tkv* clones associated with polarity defects (9 of 9) while less than half of the dorsal clones (4 of 10) show polarity disruptions. The polarity effects are not solely the result of extensive regulative growth since they occur even when the patterning response is negligible (Fig. 5F). Clones of *tkv* in the tarsal region, where WG and DPP signaling normally overlap, contain many marked cells while clones in the tibia, femur and more proximal segments or clones induced in first instar contain only one or two marked cells, suggesting that growth of cells in the tarsus is less sensitive to the loss of DPP signaling than other regions of the disc. The cuticle effects of $punt^{ts}$ and *tkv* clones are consistent with the ectopic expression of *wg* affecting cell fate and with WG and DPP signals being antagonistic both in terms of domain of gene expression and in terms of effects on cell fate.

## DISCUSSION

### Mutual repression by WG and DPP maintains territories in the leg and eye/antennal imaginal discs

We find that the D/V asymmetry of *wg* and *dpp* expression in the anterior compartment of leg and antennal discs is maintained by inhibition of *wg* expression by DPP signaling, and inhibition of *dpp* expression by WG signaling (Fig. 6B). Blocking response to DPP signaling in $punt^{ts}$ mutants leads to expansion of *wg* expression, but only along the A/P boundary (Fig. 1C,E). Conversely, ventrally boosting DPP signaling inhibits *wg* expression in the leg (Fig. 2B). Similarly, blocking WG signaling with temperature sensitive *wg* mutants or *dsh* mutants, leads to expanded *dpp* expression, but again only along the A/P boundary (Fig. 3B,D), while ectopic expression of *wg* dorsally reduces *dpp* expression (Fig. 4C).

The restriction of *wg* and *dpp* expression to the region along the A/P boundary is governed by negative inputs. Repression by EN excludes *wg* and *dpp* from the posterior compartment (Sanicola et al., 1995). HH protein diffusing anteriorly across the A/P boundary allows *wg* and *dpp* to be expressed in domains that abut the A/P compartment boundary (Fig. 6B) (Basler and Struhl, 1994; Tabata and Kornberg, 1994). The positive effect of HH may be mediated by it antagonizing a repressive activity of Patched (PTC) or protein kinase A on *dpp* and/or *wg* expression in the anterior compartment (Phillips et al., 1990; Basler and Struhl, 1994; Capdevila et al., 1994; Jiang and Struhl, 1995; Johnson et al., 1995; Li et al., 1995).

The leg disc is specified in the embryo at a point were ventral *wg* and dorsal *dpp*-expressing cells abut (reviewed by Williams and Carroll, 1993). During the first and second instar, *wg* and *dpp* expression become largely overlapping and separate again during the third laval instar (Masucci et al., 1990; Couso et al., 1993). Our data suggest that the changes in the domains of *dpp* and *wg* expression during the third laval instar is due at least in part to a mutual repression that operates throughout development. The temperature shift experiments demonstrate that the mutual repression operates until late in larval development while the clonal analysis and *dsh* mutants suggest it operates early. It is unclear whether the initial D/V bias of *dpp* and *wg* seen in the embryo is partially maintained through first and second instar or whether other mechanisms are responsible for reinitiating the restriction of *wg* and *dpp* to the ventral and dorsal regions respectively. Nevertheless, once separate domains of expression are established, the mutually repressive interactions between WG and DPP signaling can then maintain the expression of these genes in different regions of the leg disc as growth and patterning proceed. This mutual repression can also provide a mechanism to regenerate patterning domains in the event of injury to the disc.

### Compartments and territories in generating chiral appendages

Based largely on studies of the wing imaginal disc, a model of pattern formation involving stepwise delineation of compartments has been proposed (Garcia-Bellido, 1975; Lawrence and Struhl, 1996). Compartments are defined by strict lineage restriction which once defined, do not change. Each compartment acquires a genetic address that is defined by the expression of selector genes (e.g. *en*, *ci*, *ap*) which once turned on or off become fixed in the founder cells and their descendants (Lawrence and Struhl, 1996). The demarcation of anterior and posterior compartments (Blair, 1993), follows directly from embryonic segmentation which generates adjacent stripes of *ci* and *en* expressing cells (Kornberg et al., 1985; Eaton and Kornberg, 1990) that are preserved as lineage restrictions (compartments) in both larval segments and discs (Garcia-Bellido et al., 1973; Garcia-Bellido, 1975; Steiner, 1976; Wieschaus and Gehring, 1976; Lawrence and Morata, 1977; Szabad et al., 1979; Lawrence and Struhl, 1982). While it has been possible to demonstrate a D/V compartment restriction in wing discs (Bryant, 1970; Garcia-Bellido et al., 1976; Blair, 1993; Diaz-Benjumea and Cohen, 1993; Lawrence and Morata, 1993; Williams et al., 1993; Blair et al., 1994), demonstration of D/V compartments or proximal/distal compartments in other discs has remained elusive (Steiner, 1976). As an alternative, we use the term ‘territory’ to describe a region of cells that are under the dominating influence of a particular morphogen. For example, the ventral cells in the leg disc that are responding to the predominant influence of WG, even though they are integrating high WG and low DPP input, constitute a ventral territory (Fig. 6B). Territories differ from compartments in that they are not defined by lineage or by sharp boundaries of irreversibly committed selector gene expression (e.g. *en*, *ci*, *ap*). If, as a result of growth displacement, the descendants of territory-founding cells find themselves closer

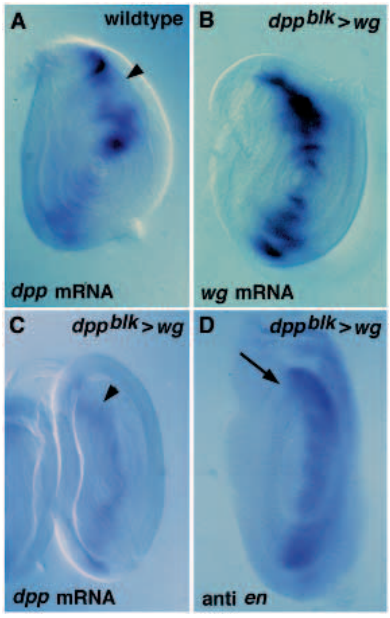


**Fig. 4.** Ectopic WG signaling inhibits *dpp* expression. (A, C) In situs to *dpp* mRNA. Anterior is to the left and dorsal is up. (A) Control *y w sn* leg disc. (B) A control in situ to *wg* mRNA reveals the expression pattern of *wg* when UAS:*wg* is driven by $dpp^{blink}$>Gal4. (C) Dorsal *dpp* expression (arrowhead) is lost in leg discs (15 of 15 discs) where expression of a UAS:*wg* transgene is driven by $dpp^{blk}$>Gal4. Compare to A. (D) An anti-EN antibody stain shows a symmetric posterior compartment with slight expansion of the dorsal posterior compartment into the anterior region of a $dpp^{blk}$>*wg* leg disc (arrow).

to a different territory-defining morphogen, they will acquire the properties of cells in that territory regardless of lineage origin. Thus, territory borders are less sharp than compartment boundaries and they are more dynamic. We propose that the anterior compartments of the leg and antennal discs are divided into dorsal and ventral territories by the mutual antagonism between WG and DPP signaling (Fig. 6B).

The effect of $punt^{ts}$ on *wg* expression in the eye is consistent with the observation that WG inhibits the ability of DPP to propagate the morphogenetic furrow (Ma and Moses, 1995; Treisman and Rubin, 1995). The reduced eyes seen in $punt^{ts}$ animals (Fig. 5C) suggest that the ectopic expression of *wg* that extends from the periphery into the eye field (Fig. 1E) restricts the domain of DPP influence in specifying eye tissue. Loss of eye tissue accompanied by ectopic *wg* expression is also seen in *Mad* clones which provide a downstream block to DPP signaling (Wiersdorff et al., 1996). Thus, the regulatory interactions between WG and DPP are similar in the eye and leg discs.

## Regulatory interactions between WG and DPP are tissue-specific

We find that the regulatory interactions between WG and DPP seen in leg and eye/antennal discs do not hold in dorsal discs (wing and haltere). In wing discs, the pattern of *wg* expression and the morphology of the discs is normal in $pnt^{ts}$ animals even

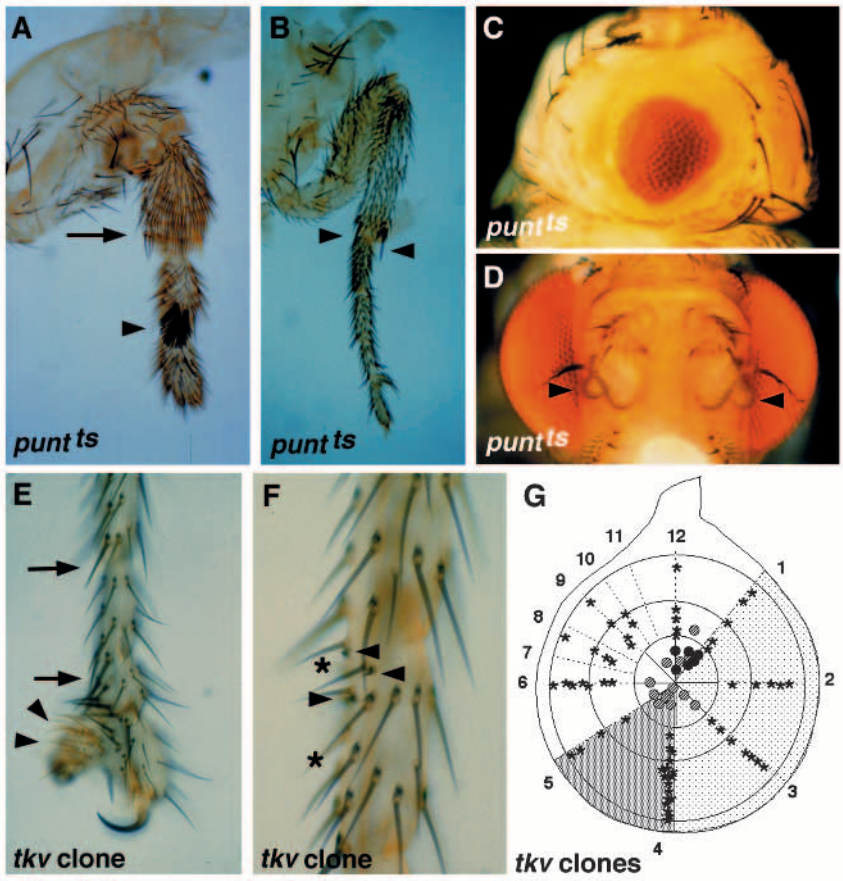


**Fig. 5.** The patterning consequences of loss of DPP signaling are consistent with the changes in *wg* expression. (A-D) Pharate adults from $punt^{\Delta 61}/punt^{135\text{-}22}$ larvae that were transferred from 18°C to 25°C at late first/early second instar. (E,F) *tkv* clones induced in second instar. In A-C; E-G ventral is to the left. (A) In this $punt^{ts}$ pharate first leg, both ventral (e.g. transverse row; arrow) and ventrolateral (e.g. sex combs; arrowhead) structures are duplicated. (B) $punt^{\Delta 61}/punt^{135\text{-}22}$ pharate second leg. Note the duplication of ventral-most structures such as the apical bristle (arrowheads). This leg also has a row of ventral-most peg-like bristles running down both sides of the tarsus. (C) In a $punt^{ts}$ pharate the head is normal but the eye is reduced. (D) $punt^{ts}$ pharate head with duplicated antennae. The ectopic antennal branches (arrowheads) are ventral as expected of ectopic ventral *wg* expression. (E) Ventral tarsal *tkv* clone that results in a small bifurcation. The mutant yellow cells (arrowheads) produce the stout bristles characteristic of the ventral *wg*-expressing cells (arrows) and they exhibit planar polarity disruptions. (F) A ventral *tkv* clone that comprises only a few mutant bristles (out of plane of focus marked by **). This clone does not significantly alter pattern but still causes planar polarity disruptions as evidenced by the distal location of bracts in neighboring wild-type tissue (arrowheads). (G) Summary map of *tkv* clones in the leg. ⊘ indicates the location of clones that affect both pattern and polarity; ● indicates clones affecting pattern but not polarity and * indicates the location of clones that marked only 1 or 2 bristles. Note the positional bias of clones affecting polarity to the ventral tarsus.

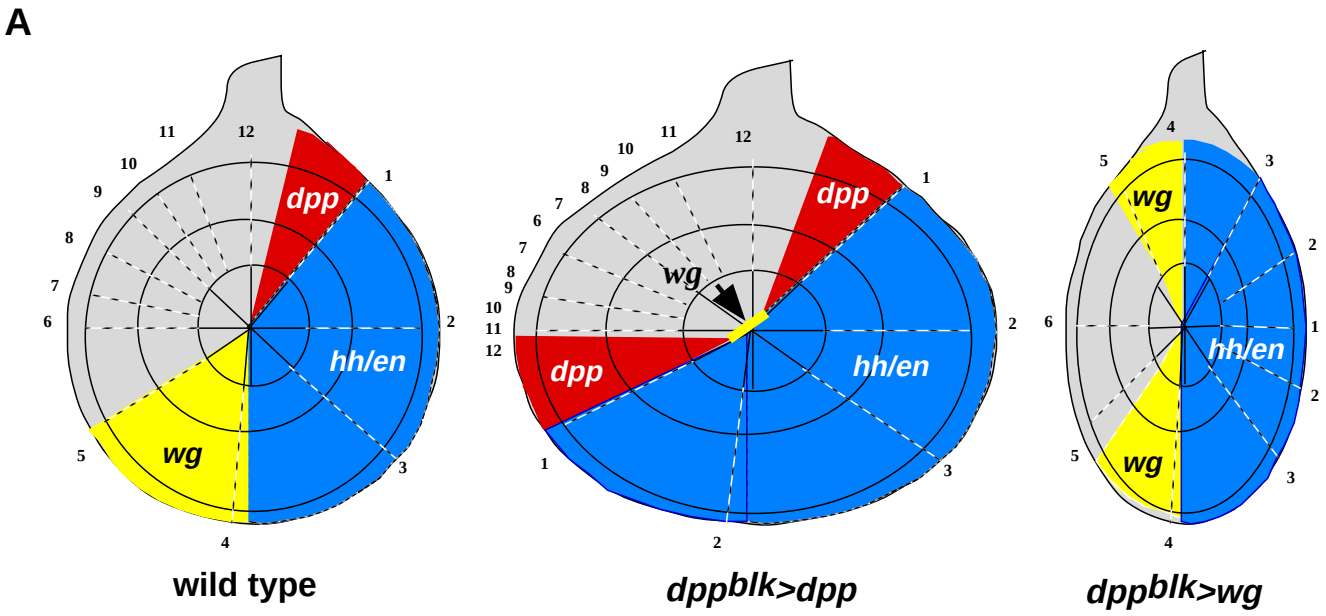


**Fig. 6.** (A) The shape changes in *dpp*$^{blk}$>*dpp* and *dpp*$^{blk}$>*wg* are consistent with formal models of pattern formation. A map of the leg disc with arbitrary positional values (1-12) is drawn to reflect the concentration of positional values in the dorsal anterior quadrant of the leg disc (Schubiger, 1971; Blair, 1995). The ectopic ventral wedge of *dpp* expression driven by the *dpp*$^{blk}$ promoter can be considered a transplant of values between 12-1 to the ventral region with subsequent intercalation of intermediate values by the shortest distance. Since DPP inhibits *wg*, this produces a symmetrical duplication of the condensed dorsal anterior territory with deletion of the ventral anterior quadrant. In contrast, ectopic *wg* driven by *dpp*$^{blk}$ >GAL4 is equivalent to placing positional value 4 between 12; 1 with subsequent intercalation. Since WG suppresses *dpp*, the dense cluster of values in the dorsal anterior quadrant is eliminated and a thin symmetrical disc results. (B) Summary of regulatory interactions serving to define territories and compartments in leg discs. In leg and eye/antennal imaginal discs, the expression domains of *wg* and *dpp* become mutually exclusive. Posterior compartment cells continuously express *engrailed* which suppresses *wg* and *dpp* expression and they secrete Hedgehog protein which permits *wg* and/or *dpp* expression in adjacent anterior cells perhaps by inhibiting a repressive influence of Patched or PKA on these genes (see text). In the anterior compartment, WG signaling represses *dpp* expression and DPP signaling represses *wg* expression. This maintains a D/V asymmetry in the leg disc, and divides the anterior compartment of the leg disc into two dynamic territories, thereby dividing the leg into three regions: the two anterior territories plus the posterior compartment.

with an extented temperature upshift of 30 hours past the time needed for maximal ectopic *wg* expression in legs (i.e. 40 hours, Fig. 1C). Similarly, the pattern of *dpp* expression and the morphology is normal in *wg*$^{ts}$ animals upshifted for twice the time (49 hours) that is sufficient (24 hours) to achieve maximal ectopic *dpp* expression in leg discs (Fig. 3B). The difference may lie in the different developmental histories of legs and wings. During arthropod evolution legs appeared first followed later by wings (Kukalova-Peck, 1978; Birket-Smith, 1984; Williams and Carroll, 1993; Williams et al., 1994). In *Drosophila*, a colony of cells migrates away from the leg disc anlage to become the wing disc anlage (Cohen et al., 1993) after the A/P compartments have been established (Steiner, 1976; Wieschaus and Gehring, 1976; Lawrence and Morata, 1977). Once separated, the wing and leg discs take distinct developmental paths. In wing discs, *wg* expression is absent during first instar but reappears in the second instar in a ventral patch of expression which overlaps *dpp* (Couso et al., 1993). A D/V compartment boundary is established by a series of genes whose functions in D/V compartment specification are unique to wing and haltere (e.g. *vg*, *ap*; Blair, 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994; Williams et al., 1994). Concurrently, the expression of *wg* and *dpp* change rapidly so that by mid third instar, *dpp* and *hh* expression are uniform along the D/V axis (Masucci et al., 1990; Lee et al., 1992; Tabata et al., 1992) while bands of *wg*

expression run perpendicular to the stripe of *dpp* expression in several locations (Baker, 1988b; Couso et al., 1993). No candidate genes that might specify D/V compartments in the legs have emerged, suggesting different developmental strategies may be operative in wings and legs. The reason for expression of ectopic *wg* in clones of *punt* or *tkv* that fall near the distal crossover point of *dpp* and *wg* expression in the wing blade (Penton and Hoffmann, 1996) while temperature shift experiments do not alter *wg* expression, may be due to clones eliciting an intercalary regenerative response due to sharp positional discontinuities while temperature shifts cause a general depression of the signaling response. These considerations and the failure of either WG or DPP to affect the other's expression in wing discs, either when signaling is compromised or enhanced, support the view that the molecular basis for generation of D/V asymmetry in legs and wings may be different.

In the gut, WG and DPP signaling positively affect each others expression in parasegments 7 and 8 (Staehling-Hampton and Hoffmann, 1994; Yu et al., 1996) and it has been suggested that WG promotes *dpp* expression in the germ band retracting embryo (Jackson and Hoffmann, 1994) providing two other examples where interactions between WG and DPP differ among tissues.

### Changes in disc morphology in response to ectopic *wg* or *dpp* expression are consistent with formal models of pattern regulation

The patterning responses of adult cuticle elements to manipulations of *wg* or *dpp* are consistent with the hypothesis that *wg* specifies ventral positional values and *dpp* specifies dorsal positional values. Changes in shape and size of discs suggest that pattern regulation also occurs in response to the ectopic expression of either *wg* or *dpp* (Fig. 6A). Since *dpp* signaling inhibits *wg* expression, strong *dpp* expression along the A/P compartment boundary should replace the anterior ventral territory with the anterior dorsal one. The Polar Coordinate (French et al., 1976; Bryant et al., 1981) and Boundary models (Meinhardt, 1983) would predict a discontinuity in positional values at the ventral A/P compartment boundary followed by intercalation leading to an expansion of the posterior compartment. In situ hybridization to *hh* in discs with $dpp^{blk}$>*dpp* confirms that the posterior compartment has expanded, resulting in a pear shaped disc (Fig. 6A). In contrast, ectopic *wg* expression dorsally causes a mirror image duplication of the anterior ventral territory while repressing formation of the anterior dorsal territory producing an expansion of posterior and suppression of anterior values leading to a long thin disc (Fig. 6A). The pattern of *en* expression in $dpp^{blk}$>*wg* discs confirms the expansion of the posterior compartment (Fig. 4D).

### WG and DPP exert antagonistic influences on cell fate

Ectopic expression of *wg* in the dorsal region of the leg, even at high levels, failed to specify the extreme ventral fates that normally arise from the ventral *wg*-expressing cells (Struhl and Basler, 1993; Wilder and Perrimon, 1995). Thus, it is surprising that ectopic expression of *wg* seen in $punt^{ts}$ mutants is able to specify extreme ventral cell fates (Fig. 5A,B). The key difference between these experiments may be that, in $punt^{ts}$ animals, ectopic *wg* is accompanied by blocking DPP signaling by $punt^{ts}$ (Letsou et al., 1995; Ruberte et al., 1995). This suggests that the potential of an ectopically expressed morphogen to affect cell fate may be fully realized only when competing antagonistic signals are removed (e.g. DPP in this case). We conclude that WG and DPP are mutually antagonistic at the level of cell fate specification as well as transcription.

Previously, it was unclear whether the weak *dpp* expression, which overlaps *wg* in the ventral region of leg discs (Fig. 3A), had a functional role in patterning of the leg. The fact that ventral *tkv* clones in tarsi overproduce the ventral peg-like bristles (Fig. 5E) suggests that the weak *dpp* expression is functioning to antagonize and modulate WG signaling during normal patterning. The genetic insertion of these 'hyper *wg*-like' cells by *tkv* clones accounts for fatter legs and the outgrowths that do not incorporate neighboring cells. The fact that ventral *tkv* clones also cause polarity disruptions could be explained if excess WG signaling reduced the amount of *dsh* available for establishing polarity. The effect of loss of *tkv* in the ventral region of leg discs where WG signaling is operative, suggests that cells may integrate input from both signaling pathways to determine cell fate. Such integration of competing signals could provide a mechanism for specification of intermediate cell fates.

In the leg, both *punt* and *tkv* clones located dorsally cause bifurcations (Penton and Hoffmann, 1996; our observations). However, ventral *punt* clones are reported to cause no abnormalities (Penton and Hoffmann, 1996) while we see that ventral *tkv* clones cause excess ventral cells and bifurcations. One interpretation is that *punt* alleles may not be nulls. An alternative explanation is that ventral DPP signaling requires *tkv* but not *punt*. A recently isolated second BMP type II receptor that is expressed in all discs might provide an alternative to signaling through *punt* in the ventral leg disc (Marques and O'Connor, unpublished observations).

A number of synergistic interactions between growth factors have been described (e.g. Kimelman et al., 1992; Rothbacher et al., 1995; Watabe et al., 1995) but the results reported here add antagonistic interactions to the repertoire of regulatory mechanisms available during patterning. Negative interactions may play an important role in integrating multiple positional cues during the specification of cell fate. The mutually negative effect of WG and DPP on each other's expression and the antagonistic influence of each on cell fate choice adds a new dimension to the role these factors play in patterning of discs. It also raises the possibility of such negative feedback loops playing a general role during patterning in other systems such as vertebrate limb specification.

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## Note added in proof

A recent communication (Jiang, J. and Struhl, G. *Cell* **86**, 401; 1996) examines the mutually antagonistic effects of WG and DPP at the level of protein accumulation reaching similar conclusions to our studies examining mRNA levels. Thus, the conclusion that regulation of transcript levels is the major mechanism of regulation is supported.