Proximal–distal axis formation in the *Drosophila* leg

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Limb development requires the formation of a proximal–distal axis perpendicular to the main anterior–posterior and dorsal–ventral body axes. The secreted signalling proteins Decapentaplegic and Wingless act in a concentration-dependent manner to organize the proximal–distal axis. Discrete domains of proximal–distal gene expression are defined by different thresholds of Decapentaplegic and Wingless activities. Subsequent modulation of the relative sizes of these domains by growth of the leg is required to form the mature pattern.

Short-range interactions between cells in adjacent compartments trigger the patterning of the legs and wings of *Drosophila*^{1,2}. These interactions direct expression of the secreted signalling molecules Wingless (Wg) and Decapentaplegic (Dpp) in cells adjacent to the dorsal–ventral (D–V) and anterior–posterior (A–P) compartment boundaries of the wing^{3–9}. The secreted Dpp protein is thought to form a concentration gradient that organizes the A–P axis of the wing by directly specifying distinct domains of gene expression^{10–13}. Similarly, localized expression of Wg in cells adjacent to the D–V boundary is thought to generate a gradient of Wg activity that directly specifies distinct domains along the D–V axis of the wing^{9,14–16}.

In the developing leg, Wg and Dpp have been implicated in both D–V and proximal–distal (P–D) axis formation. In contrast to the wing, Wg and Dpp are both expressed in the leg along the A–P boundary under the control of Hedgehog (Hh)⁴. Wg is expressed ventrally, whereas Dpp is expressed at high levels dorsally and at low levels ventrally (Fig. 1). The asymmetry in Wg and Dpp expression is maintained by mutual repression^{17–19}. Wg is required for specification of ventral cell fates, and Dpp is required for specification of dorsal cell fates^{17,18,20–23}.

The central region of the disc where cells expressing Wg and Dpp

meet corresponds to the most distal tip of the leg. It has been shown that producing an ectopic intersection point between cells expressing Wg and Dpp can induce a supernumerary P–D axis^{17–19,21,22,24}. Here we present evidence that Wg and Dpp act directly to provide positional information along the P–D axis of the leg. The combined activity of both signals is required in a spatially graded manner to define the distinct domains of gene expression along the P–D axis. We also show that the time at which these expression domains are stably defined plays an important role in the patterning of the leg.

Wg- and Dpp-dependent target genes in the leg

The Dachshund (Dac) and Distal-less (Dll) proteins are expressed in distinct domains along the P–D axis of the leg (Fig. 1). *Dll* encodes a homeodomain protein that is required for limb development²⁵. In the mature leg disc, *Dll* is expressed in a central domain that corresponds to the presumptive tarsal segments and distal tibia²². The *dac* gene encodes a nuclear protein required for development of the femur and tibia²⁶. Dac is expressed in a ring corresponding to the presumptive femur, tibia and first tarsal segment, but is absent from the more distal tarsal segments of the leg disc (Fig. 1b–e). Although there is little or no overlap between the *Dll* and *Dac* domains at early stages (Fig. 1b), by mid third instar

Figure 1 Subdivision of the P–D axis by expression of *Dll* and *Dac*. **a**, Wg protein (blue) and *dpp-lacZ* (red) expression in a third instar leg imaginal disc. A, P, D and V denote anterior, posterior, dorsal and ventral, respectively. The white line indicates the A–P compartment boundary. Wg is expressed by ventral anterior cells; secreted Wg protein can also be seen in the posterior compartment; *dpp-lacZ* expression in dorsal anterior cells is visualized with anti- β -galactosidase; *dpp-lacZ* is also expressed at lower levels ventrally (not visible here). **b**, *Dll* (red) and *Dac* (green) expression in a late second- or early third-instar leg disc (~72 h at 25°C). *Dll* is expressed in a central domain, *Dac* is expressed in a ring. The *Dac* domain overlaps the edge of the *Dll* domain by at most one or two cells. **c**, **d**, Mid and late third-instar discs. Note the overlap of the *Dll* and *Dac* domains (yellow). Scale bars indicate the relative magnification. By mid–late third instar, *Dll* is activated in a secondary domain forming a thin ring at the level of the trochanter (not visible in these optical sections; see Fig. 2d). **e**, Partly everted pupal leg disc showing overlapping *Dll* and *Dac* expression. **f**, Schematic representation of the relationship between the third instar disc and the adult leg. Distal structures derive from the centre of the disc, progressively more proximal structures from a series of concentric rings. The concentric nature of these expression domains is partly obscured by the folding of the disc epithelium.

the combination of Dac and Dll expression defines three regions along the P–D axis (Fig. 1c,d). The relationship between the Dll and Dac expression domains and the segments of the adult leg can be seen in the partly everted pupal leg (Fig. 1e,f).

Dll and Dac are expressed in circular domains centred on the point at which the Wg and Dpp expression domains meet in the leg disc. Two lines of evidence suggest that Dll expression in the centre of the disc depends on the combined activity of *wg* and *dpp*: (1) Dll is not expressed in *wg* or *dpp* mutant discs²²; and (2) misexpressing Wg directs ectopic Dll expression only on the dorsal side of the disc where Dpp is expressed, whereas misexpressing Dpp or ectopically activating the Dpp signal transduction pathway directs ectopic Dll expression only on the ventral side of the disc where Wg is expressed²² (Fig. 2).

Wg and Dpp act directly to induce Dll

We can consider two mechanisms by which Wg and Dpp could induce Dll. Wg and Dpp might act locally in the centre of the disc to induce expression of a third signalling molecule that works at a distance. In this case, cells in which the Wg and Dpp signal transduction pathways are activated would be expected to generate a signal that induces Dll expression non-autonomously in nearby cells. Alternatively, the two signals might both act directly at a distance, in which case Dll expression would be restricted to cells in which both signal transduction pathways are activated. To distinguish between these possibilities, we compared the effects of Dpp-expressing clones with clones in which the Dpp signal transduction

pathway is constitutively activated by expressing a ligand-independent form of the Dpp receptor, Thick-veins (*Tkv**; the asterisk denotes the activated receptor)^{11,12}. Dpp-expressing cells act non-autonomously to induce Dll expression in cells surrounding the clone (Fig. 2a). In contrast, Dll is induced only in cells expressing *Tkv**, but not in neighbouring cells where the pathway has not been activated (Fig. 2b,c). Similarly, although Wg-expressing cells act non-autonomously to induce Dll (Fig. 2d), ectopic expression of Dll and Dac are restricted to *zw3* mutant cells in which the Wg signal transduction pathway is activated cell autonomously (Fig. 2e,f). These results indicate that the long-range effects of Wg and Dpp cannot be due to induction of another secreted signal in cells in which both pathways have been activated. Rather, Wg and Dpp seem to act directly and at long range to induce Dll. Evidence that Dac is regulated similarly is presented below.

Transient requirement for Wg and Dpp signals

Although Wg and Dpp signalling seems to be required to induce Dll and Dac expression, we found that both target genes are expressed normally in clones of cells mutant for a null allele of *dishevelled* (*dsh*), a component of the Wg signal transduction pathway (Fig. 3a,c). This contrasts with the wing, in which *dsh* clones cause a strong reduction in Dll expression^{15,16} (Fig. 3b). The *dsh* clones were induced after the onset of Dll and Dac expression in these experiments, raising the possibility that Dll and Dac do not require continuous input from Wg once expression has been induced in the leg. To test this proposal we examined discs in which Dll is

Figure 2 Wg and Dpp do not act by relay of another signal to induce Dll. **a–c**, Comparison of clones expressing Dpp and *Tkv** 24 h after clone induction. **a**, UAS $\gg$ Dpp expressing clone (arrow). The clone is marked by the absence of CD2 expression (green). Dll (red) is expressed both inside and outside the clone (outlined in the higher-magnification image, right). **b**, Leg disc with multiple clones expressing UAS $\gg$ *Tkv**. V, ventral; D, dorsal. **c**, Detail of the boxed region in **b**. Note that the panel was rotated so that ventral is at the bottom. Arrow, ventrally located clone showing Dll expression exclusively inside the clone; open arrowhead, lateral clone in which the Dll level decreases from ventral to dorsal; filled arrowhead, dorsal clone with little or no Dll expression. **d**, Leg disc with multiple Wg-expressing clones (green, visualized by expression of the flu epitope tag)

examined 36 h after clone induction. Arrow, dorsally located clone showing non-autonomous activation of Dll; open arrowheads, overgrowths induced in the Dll expressing region on the dorsolateral sides of the disc which distort the shape of the disc. Ventral and lateral clones do not induce ectopic Dll. **e, f**, Dorsal regions of leg discs with *zw3* mutant clones induced 24–40 h before staining (marked by the absence of lacZ expression, green). Dll (**e**) and Dac (**f**) are expressed autonomously in mutant cells where the Wg pathway is activated; *zw3* mutant clones induced at this stage do not express Wg (data not shown), although clones induced before the start of third instar can induce Wg in the anterior compartment and act non-autonomously¹⁸.

induced *de novo* outside its endogenous expression domain, and investigated whether Dll could be induced in *dsh* mutant cells. We made use of the fact that Hh directs expression of both Wg and Dpp in anterior leg cells, and that Wg signalling interferes with the ability of Hh to induce Dpp in ventral cells^{17,18}. Consequently, Wg-dependent repression of Dpp cannot occur in ventral–anterior clones of *dsh* mutant cells¹⁸. Thus *dsh* clones serve as a source of Dpp and induce a secondary P–D axis, but are themselves unresponsive to Wg. A large *dsh* mutant clone located outside the endogenous Dll domain that has caused an axis duplication is shown in Fig. 3d. Dll is induced in cells adjacent to the clone (arrow) but not in the *dsh* mutant cells (asterisk). Taken together these results indicate that cells need to receive the Wg signal to induce Dll expression, but do not require continuous signalling to maintain Dll expression after its initial induction.

Figure 3 Wg signalling is required for induction, but not maintenance, of Dll and Dac. **a, b**, *dsh*^{VA153} mutant clones induced in second instar larvae (60 ± 12 h; marked by the absence of β-gal expression, green). **a**, Leg disc with a *dsh* clone in the Dll domain (asterisk). Dll expression (red) is normal in the clone. **b**, Dll expression is strongly reduced in a comparable clone in the wing disc. **c, d**, Axis bifurcations associated with *dsh*^{VA153} mutant clones induced in second instar (60 ± 12 h). *dsh* mutant clones in the ventral–anterior region of the disc alleviate Wg-dependent repression of Dpp and induce secondary P–D axes. Axis duplications were identified as morphologically visible outgrowths associated with *dsh* mutant clones on the ventral side of the disc. Clones were marked by the absence of β-gal expression (green). **c**, *dsh* clone (asterisk) located in the Dac expression domain on the ventral side of the disc. Dac expression (purple) is normal in the clone, but is expanded in the induced outgrowth (arrow; compare with wild-type disc in Fig. 1). **d**, *dsh* clone located outside the endogenous Dll expression domain. Dll is not expressed in the *dsh* mutant cells (asterisk) but is induced in adjacent wild-type cells (arrow). A higher magnification of the duplication is shown on the right. The ectopic Dll domain appears red where it overlaps the heterozygous *dsh*^{+/dsh} cells, and yellow where it overlaps the bright green cells carrying two copies of the β-gal marker and of the *dsh*⁺ chromosome. Ectopic expression of Dll or Dac is always associated with obvious axis bifurcation. Comparable results were obtained for Dac expression associated with *dsh* clones in the antenna (not shown).

A similar distinction between induction and maintenance can be made for Dpp signalling. Clones of cells mutant for *Mad* (a component of the Dpp signal transduction pathway^{27,28}) or the Dpp receptor gene *tkv* express normal levels of Dll (Fig. 4a,b) and Dac (Fig. 4c). These results indicate that continuous Dpp signalling is not required to maintain Dll or Dac expression in the leg. We examined axis bifurcations induced by *Mad* mutant clones to look at *de novo* induction of Dll and Dac. *Mad* mutant clones block transduction of the Dpp signal and so allow Hh to induce Wg in dorsal cells (as reported for the Dpp receptors *punt* or *tkv*^{18,19,29}). *Mad* clones induced near the A–P boundary cause axis bifurcation. However, to increase the frequency of axis bifurcation we made clones simultaneously mutant for *Mad* and *pka*. The *pka* mutant cells behave as though the Hh pathway is activated^{30–33}, thereby allowing *Mad pka* mutant clones to express Wg, regardless of

Figure 4 Dpp signalling is required for induction, but not maintenance, of Dll and Dac. **a, b**, *Mad* mutant clones in the leg disc (arrows, marked by the absence of β-gal, green). Dll expression (red) is unaffected in *Mad* clones induced in early third **(a)** or second **(b)** instar larvae (60 ± 12 h). **c**, Dac expression (purple) is unaffected in a *tkv* mutant clone (arrow, induced 60 ± 12 h). **d–f**, *Mad pka* double-mutant clones in the antenna disc (induced 60 ± 12 h; marked by the absence of β-gal, green). Dorsal and anterior are indicated. Clones located outside the Dll and Dac domains are easier to find in the antennal disc, because it is less folded than the leg disc. Identical results were obtained in leg discs, but fewer examples were found. **d**, Wg expression (blue) induced in a *Mad pka* clone far from the A–P boundary (asterisk). **e**, Dll expression (red) is induced in cells adjacent to a comparable clone (arrow), but not in the *Mad pka* mutant cells. **f**, Dac expression (purple) is induced in cells adjacent to a *Mad pka* clone (arrows), but not in the mutant cells. The arrowhead indicates a ventral *Mad pka* clone in the endogenous Dac domain near the A–P boundary. Dac is expressed normally in the clone. No axis duplication results because the clone is located in the region where Wg is endogenously expressed. The Dac domain is narrower than the Dll domain in the antenna, so that Dll expression extends to a more proximal region of the antenna, the reverse of the situation in the leg. Dac is expressed on the side of the clone close to the source of Dpp, whereas Dll is expressed on both sides of the clone.

whether they are within range of the Hh signal (Fig. 4d, asterisk). *Mad pka* mutant clones located outside the endogenous Dll or Dac domains can induce axis bifurcations and expression of Dll and Dac. Dll and Dac are induced only in wild-type cells adjacent to the clone (Fig. 4e,f, arrow), but not in the *Mad pka* mutant cells, which serve as a source of Wg, but are unresponsive to Dpp. These results indicate that, like Wg, Dpp signalling is required for the initial induction of Dll and Dac expression but not for their subsequent maintenance. Thus the requirement for these signals seems to be transient.

Distinct thresholds of Wg and Dpp activity

The spatial domains of Dac and Dll differ along the P–D axis of the leg. Dll is expressed in a distal cap that has its proximal limit in the tibia; Dac is expressed in a ring that has its proximal limit in the femur. We have excluded the possibility that Wg and Dpp induce a new signal that radiates outward from the centre of the disc, and

have shown that both signals are required directly for the induction of Dac and Dll. This raises the possibility that the domains of Dll and Dac expression are defined in response to different threshold levels of Wg and Dpp activity. Evidence has been presented for a graded activity of Wg in the specification of cell fate along the ventral-to-dorsal axis of the leg^{15,17,21}. To assess the effective range of the Wg signal, we examined Dll expression in discs carrying many small *Tkv*^{*}-expressing clones (in which the level of Dpp signalling activity should be uniform). Dll is expressed in *Tkv*^{*} clones located far from the source of Wg (Fig. 2b, double labels with Wg not shown). Dll is induced in all cells of ventrally located *Tkv*^{*} clones (arrow in Fig. 2c). In a ventrolaterally located clone (open arrowhead), Dll is found at a higher level in cells close to the source of Wg than in the cells on the far side of the clone. Clones located more dorsally express little or no Dll. The decrease in Dll expression with increasing distance from the source of Wg suggests that the level of Wg activity is limiting (when there is sufficient Dpp activity). We

Figure 5 Distinct thresholds for activation of Dll and Dac by Wg and Dpp. **a, b**, Leg discs double-labelled for Dac (green) and Dll (red). **a**, Ventral clones expressing the activated Dpp receptor *Tkv*^{*} express Dll, but not Dac (arrowhead). Dll is shown separately to the left. Dac is activated in more dorsally located clones (arrow). The image is focused on the proximal region of the disc which is overgrown. Only the tarsal region of the normal Dll domain is in the plane of focus; most of the normal Dac domain is not. **b**, Clones expressing an activated form of the Wg pathway component Armadillo. Left, side view of a leg disc showing the approximate plane of the optical section on the disc shown in the right panel. The arrow shows a dorsal clone expressing Dac. Right, Dac and Dll are coexpressed in activated Armadillo clones located near the source of Dpp on the dorsal side of the disc (arrowhead). Dac is expressed alone in clones located more laterally (arrows). The endogenous Dll and Dac domains in the centre of the disc cannot be seen. **c**, Wild-type disc (from Fig. 1d). The asterisk indicates the approximate position of the Wg-expressing clone shown in **d** relative to the endogenous Dll and Dac domains. The box indicates the portion of the disc shown. **d**, Dorsal Act >> Wg-expressing clone located outside the endogenous Dll and Dac domains (arrow). Dac (green) is misexpressed in a larger region than Dll (red). The ectopic Dac expression domain may have merged proximally with the endogenous domain (these domains are separated by a deep fold of the disc epithelium). **e**, Wild-type disc. The asterisk indicates the approximate positions of the Dpp-expressing clones in the discs in **f** and **g**. **f, g**, Tub >> Dpp-expressing clones outside the endogenous Dll and Dac domains (arrows). **f**, Clone examined 24 h after induction, in which Dac is induced, but not Dll. The expression of Dll near the edge of the Dac domain is the endogenous proximal ring of Dll at the border between femur and trochanter²². **g**, Clone examined 40 h after induction, in which Dll is induced in a subset of Dac-expressing cells and Dac is repressed in the centre of the Dll-expressing region (arrows). Dac is shown separately on the right. The Wg- and Dpp-expressing clones were not directly marked in these experiments because antibodies to Dac and the clone markers are both of mouse origin. Based on the relationship between Dll expression and marked clones in Fig. 2, the Dll expression domain shows the approximate position of the clone, to allow comparison with Dac expression.

propose that a spatial gradient of Wg activity determines both the range over which Dll can be activated and the level of Dll activation. It is possible that the graded Wg activity reflects a gradient of Wg protein, although this cannot be demonstrated directly because biologically active levels of Wg are below the limits of antibody detection^{15–18}.

To investigate whether spatially graded Wg activity can generate different thresholds for activation of Dac and Dll, discs carrying multiple *Tkv** clones were double labelled (Fig. 5a). Ventrally located clones express Dll (arrowhead); more dorsally located clones express Dac but not Dll (arrow). Thus Dac can be induced at a greater distance from Wg than Dll, which suggests that Dac can be induced at levels of Wg activity that are not sufficient to induce Dll in the presence of a constant level of Dpp signalling activity. Consistent with this interpretation, Dac is induced in a larger region than Dll by a clone of Wg-expressing cells (Fig. 5c,d). The clone is located proximal to the normal Dac domain on the dorsal side of the disc (Fig. 5c). Clones expressing low levels of Wg (*act* $\gg$ Wg; Fig. 5d) induce Dll over a shorter range than clones expressing high levels of Wg (*C765/UAS* $\gg$ Wg; Fig. 2d). In both examples the position of the clone is equivalent. The *C765/UAS* $\gg$ Wg clones were examined 36 h after clone induction, whereas the *Act* $\gg$ Wg clones were examined 40 h after induction, so the apparent difference in range cannot be attributed to the time allowed for diffusion of Wg protein or expansion of the domains by growth. These observations support the proposal that the local concentration of Wg protein defines the spatial limits of Dll and Dac expression.

To compare the range over which Dpp can activate Dll and Dac, we examined discs carrying multiple clones in which the Wg pathway is constitutively activated by expression of the activated form of Armadillo¹⁵. Dorsally located clones express both Dac and Dll (Fig. 5b, right, arrowhead), whereas laterally located clones

express Dac but not Dll (arrows), suggesting that Dac can be activated at levels of Dpp that are not sufficient to induce Dll. Clones of cells expressing Dpp were induced to compare directly the range over which Dpp can induce Dll and Dac. The clones shown in Fig. 5f,g are located in a lateral region of the disc outside the endogenous Dll and Dac domains (as indicated in Fig. 5e). The pattern of Dac and Dll expression depends on the time allowed for Dpp expression after clone induction. After 24 h, Dac is expressed but Dll is not (Fig. 5f). By 40 h, Dll is induced and a gap appears in the centre of the Dac domain, producing an overlapping pattern of Dll and Dac expression (Fig. 5g, arrow). This pattern suggests that low levels of Dpp can induce Dac but not Dll, and that higher levels can induce Dll and repress Dac. The change in the expression patterns may reflect the time needed to accumulate higher levels of Dpp after induction of the clones.

Taken together, these results suggest that the spatial domains of Dac and Dll expression are defined by different threshold levels of both Wg and Dpp activities.

Wg and Dpp actively maintain Dac repression

Consistent with the proposal that increased levels of Dpp may repress Dac, we found that Dac can be expressed in *Mad* mutant clones located in the tarsal region of the leg disc (Fig. 6a). Similarly, Dac is derepressed in *dsh* mutant clones in the tarsal region (Fig. 6b). Derepression of Dac was observed in clones induced as late as mid third instar. These observations indicate that Wg and Dpp act directly to repress Dac in the centre of the disc. Thus Dpp and Wg define both the proximal and distal limits of the Dac domain, one by induction at a low level of combined activity, the other by repression at higher levels of combined activity. Further, we note that Dac repression is actively maintained by Wg and Dpp signalling long after Dac and Dll have been induced and are stably expressed in the absence of further signalling. These observations are reminiscent of the concentration-dependent effects of Activin, which can define both on and off thresholds for *Xbra* expression in *Xenopus* explants^{34,35}. However, although activation of Dac is non-reversible, as would be predicted by Gurdon's ratchet model for production of threshold responses to a concentration gradient³⁴, repression is reversible for some time. This reversibility is important for the generation of the final pattern (see below).

Importance of growth in P-D axis patterning

Although the Dll and Dac domains overlap considerably by the mid third instar, there is little or no overlap between them at earlier stages (Fig. 1). This suggests that the threshold levels of Wg and Dpp activity required to activate Dll are similar to those required to repress Dac in the centre of the disc in late second instar. As indicated above, Dac repression continues to require both Wg and Dpp signalling well into the third instar, whereas Dll expression depends only transiently on Wg and Dpp and, once activated, Dll does not require continued signalling. It is therefore possible that the Dll domain could expand beyond the effective range of the Wg and Dpp signals as the disc grows. If this is the case, cells near the edge of the Dll domain might be unable to maintain repression of Dac as they are displaced out of range of Wg and Dpp, leading to coexpression of the genes in a ring surrounding the Dac repression domain.

To test this possibility, we produced clones of cells capable of expressing the activated Dpp receptor *Tkv** under the control of a *Dll-Gal4* driver (*DllGal4/UAS* $\gg$ *Tkv**). The clones were induced in second instar, when the Dll and Dac domains are non-overlapping. Only clones in the Dll-expression domain can express *Tkv**, so expression of *Tkv** begins in the region where Dac is already repressed. This links expansion of the region in which the Dpp signal transduction pathway is activated to growth of the Dll domain, in a manner independent of the actual range of the Dpp signal. As the Dll domain expands, the clones carry constitutive

Figure 6 Repression of Dac by high levels of Wg and Dpp. **a**, *Mad* mutant clone in the centre of the leg disc (induced in mid third instar, ~84 h). **b**, *dsh* mutant clone induced in late second instar (~60 h). Clones are marked by the absence of β -gal (blue). Dac (green) is expressed in *Mad* or *dsh* mutant clones, suggesting that Dac is actively repressed by Wg and Dpp signals in the centre of the disc. Because Dac cannot be induced *de novo* in *Mad* or *dsh* mutant clones outside the endogenous domain, expression here must be due to alleviation of repression in cells already instructed to express Dac. Wg and Dpp may act together to induce repression of Dac in the centre of the disc. **c**, Leg disc double-labelled for Dac (green) and Dll (red). The disc carries clones of cells expressing the activated Dpp receptor under control of *Dll Gal4* (*DllGal4/UAS* $\gg$ *Tkv**, arrows). Clones were induced at 60 $\pm$ 12 h.

Figure 7 Roles of Wg and Dpp in the D-V and P-D axes. **a**, Wg and Dpp expression domains (blue and green, respectively). Blue and green lines represent the threshold levels of Wg and Dpp activities required for Dll induction (red). We propose that higher levels of one signal may compensate for lower levels of the second signal in the formation of circular expression domains in the centre of the disc. Note that these domains are specified in second instar, when

the disc is quite small. **b**, Expression pattern of a hypothetical target gene activated by Wg alone at the same threshold level as Dll (purple). Note that expression is both dorsal and ventral. **c**, Expression pattern of H15 (orange) relative to Wg and Dpp. H15 is activated by Wg and repressed by Dpp¹⁷. Dpp activity restricts H15 expression to the ventral side of the leg disc.

activation of the Dpp pathway into the region of overlap and maintain repression of Dac (Fig. 6c). Because Wg activity is also required, repression is observed on the ventral side of the disc where Wg is expressed. This observation shows how the Dll and Dac domains can make the transition from non-overlapping to significantly overlapping as a function of growth. The model is dependent on the finding that the signals initially used to specify Dll are only transiently required, whereas continued signalling is required to repress Dac. This is in contrast to the situation in the developing wing disc, where continuous signalling by both Wg and Dpp is required to maintain target gene expression along the A-P and D-V axes^{11,15,16}.

Wg and Dpp organize D-V and P-D axes

Two criteria have been applied to define Wg and Dpp as concentration-dependent morphogens in wing development: they act directly and at long distance to regulate expression of their target genes; and they specify distinct spatial domains of gene expression at different threshold activity levels^{11–13,15,16}. We have demonstrated that the combined action of Wg and Dpp in patterning the P-D axis fulfils both of these criteria. Our results suggest that Wg and Dpp act together in a manner dependent on the levels of both activities to specify discrete cell fates along the P-D axis of the leg. This graded activity is likely to reflect gradients of the proteins, but this cannot be demonstrated directly.

Wg and Dpp also pattern the D-V axis of the leg. The two signals act antagonistically to repress each other's expression and to specify dorsal and ventral fates^{17–19,29}. Wg specifies ventral cell fate and directs expression of its target gene *H15* in a stripe along the entire P-D axis of the leg on the ventral side¹⁷. As well as repressing Wg expression, Dpp blocks the ability of Wg to activate *H15*, suggesting that Wg and Dpp act antagonistically to define dorsal and ventral fates¹⁷. Thus at least one gene expressed in a specific D-V domain is regulated positively by one signal and negatively by the other. It remains to be seen whether other targets of Wg and Dpp are also regulated by antagonistic action of the two signals to define other D-V domains. In contrast, genes with expression dependent on the positive action of both signals will be expressed in domains defined by the overlap of the two signals in the centre of the disc (Fig. 7). These domains will form circles of different diameter that are centred on the distalmost tip of the leg, defining domains along the P-D axis (Fig. 7). Although induction of Dll and Dac in these domains occurs quite early, when the disc is small, the circular domain in which Dac is repressed provides a direct measure of the range of the combined signals at one activity threshold in mid third-

instar larvae. Thus Wg and Dpp simultaneously organize both D-V and P-D axes of the leg. A similar synergy between Wg and Dpp has been reported in the regulation of Ubx expression in the *Drosophila* embryonic midgut³⁶.

Our results show that the A-P, D-V and P-D patterning systems of the fly leg are coupled: Hedgehog regulates Dpp and Wg expression along the A-P axis, and Wg and Dpp work antagonistically to organize the D-V axis, and work cooperatively to organize the P-D axis. A-P, D-V and P-D patterning systems are also coupled in vertebrate limb development, although the mechanisms are rather different. Sonic hedgehog, Wnt7a, FGF4 and FGF8 signalling are interdependent in that they are required to maintain each other's activity to promote limb outgrowth^{37–40}. Although Wnt7a is involved in dorsal fate specification in the ectoderm⁴¹, there is no evidence for a comparable role for bone morphogenetic proteins (BMPs) in ventral fate specification. Perhaps the most striking difference is the use of fibroblast growth factor (FGF) signalling proteins for P-D outgrowth of the vertebrate limb. FGFs have been implicated in both the initial specification of the limb bud and in directing subsequent outgrowth^{38,40,42,43}. There is no evidence suggesting that FGFs are involved in P-D axis formation in the fly leg; rather, our results suggest that Wg and Dpp work together in the fly to perform functions analogous to those of FGFs in the vertebrate limb.

In conclusion, our findings highlight the importance of growth as a means of generating new positional information. We propose that spatial gradients in the activity of secreted signalling molecules and the time at which the signals are interpreted are both important determinants of P-D pattern in the developing leg. □

Methods

Clones of genetically marked cells expressing high levels of Dpp, Tkv* or Wg were made using the combined GAL4, flip-out system^{11,15}. Clones were induced in larvae of the following genotypes:

Dpp: ywHSFlp1; UAS >> CD2y⁺ >> dpp; C765Gal4;
Wg: ywHSFlp1; UAS >> CD2 >> fluWg; C765Gal4;
Tkv*: ywHSFlp1; UAS >> CD2 >> Tkv*; C765Gal4 and ywHSFlp1; UAS >> CD2 >> Tkv*/DlIGal4;
activated armadillo: ywHSFlp1; UAS >> CD2 >> fluΔArm; C765Gal4.
Clones expressing low levels of Dpp were made using tub >> f⁺ >> dpp¹⁰. Clones expression low levels of Wg were made using act >> y⁺ >> wg²¹.

Lack-of-function mutant clones were induced in larvae of the following genotypes:

wHSFlp1; Mad^{1,2} FRT40A/armadillo-lacZ FRT40A (Mad^{1,2} is a strong hypomorphic allele^{12,28});

w HSF1p1; Mad^{1,2} pka^{89/18} FRT40A/armadillo-lacZ FRT40A (pka^{89/18} is a strong hypomorphic allele³¹)
w HSF1p1; tkv^{a12} FRT40A/armadillo-lacZ FRT40A (tkv^{a12} is a null allele¹¹)
w zw3^{D127} FRT18A/armadillo-lacZ FRT18A; HSF1p38/+ (on chromosome 2). sgg-zw3^{D127} is a null allele⁴⁴;
dsh^{VA153} FRT18A/armadillo-lacZ FRT18A; HSF1p38/+. dsh^{VA153} is a null allele (Flybase).

Antibodies used were: mouse anti-Wg¹⁷; mouse anti-Dac²⁶; polyclonal mouse anti-Dll⁴⁵; rabbit anti-Dll⁴⁶; mouse anti-CD2 (Serotec); rabbit anti-β-gal (Cappel); mouse anti-flu (BabCo).

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