Development 135, 627-636 (2008) doi:10.1242/dev.014670

Logic of Wg and Dpp induction of distal and medial fates in the *Drosophila* leg

Carlos Estella and Richard S. Mann*

Drosophila leg development requires the cooperation of two secreted signals, Decapentaplegic (Dpp) and Wingless (Wg), to form the proximodistal (PD) axis. Wg and Dpp are also required to pattern the dorsoventral (DV) axis of the leg. Here, we show that *Distalless* (*Dll*) and *dachshund* (*dac*), genes expressed at different positions along the PD axis, are activated by Wg signaling and repressed by Brinker (Brk), a transcriptional repressor in the Dpp pathway. The levels of both Brk and Wg determine which of these PD genes is activated. Surprisingly, Brk does not play a role in DV axis specification in the leg, suggesting that Dpp uses two distinct mechanisms for generating the PD and DV axes. Based on these results, we present a model for how Dpp and Wg, which are present as dorsal and ventral gradients, respectively, induce nearly circular domains of gene expression along the PD axis.

KEY WORDS: Brinker, Decapentaplegic, Wingless, Leg development, Morphogens, Proximodistal axis

INTRODUCTION

Animal body plans require the specification of unique cell fates along two primary axes: anteroposterior (AP) and dorsoventral (DV). In addition, for those animals bearing appendages, a third, proximodistal (PD), axis must be generated orthogonal to the primary body axes. Studies in *Drosophila* have revealed many of the signals and pathways that control the formation of these three axes (reviewed by Morata, 2001). However, as many of the same pathways are used for forming all three of these axes, it remains unclear how they are uniquely specified. Moreover, once established, it is not understood how positional information is specified along an the PD axis of an appendage.

In *Drosophila*, the appendages are derived from imaginal discs, sheets of epithelial cells that are patterned during larval development. Imaginal discs are divided into anterior (A) and posterior (P) compartments, groups of cells that are segregated from each other early in development (Lawrence and Morata, 1977). Compartment boundaries are sources of signaling molecules, morphogens, that provide positional information to the cells in the developing discs (Tabata and Takei, 2004). In the leg disc, Hedgehog (Hh) is expressed and secreted by cells of the P compartment, and induces the expression of two long-range signaling molecules, Decapentaplegic (Dpp) and Wingless (Wg), in A compartment cells that are adjacent to the AP compartment boundary. Hh activates *dpp* in the dorsal half of the leg disc and *wg* in the ventral half (Basler and Struhl, 1994). Once activated, the *wg* and *dpp* expression domains are maintained by a mutually antagonistic repression between them (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). *dpp* is required to pattern the dorsal half of the leg (Morimura et al., 1996; Theisen et al., 1996), whereas *wg* is required to specify ventral leg fates (Couso et al., 1993; Johnston and Schubiger, 1996; Struhl and Basler, 1993; Wilder and Perrimon, 1995) (Fig. 1A). Thus, the DV axis of the leg is specified by these two opposing morphogens, probably by regulating unique sets of target genes in a concentration-dependent manner (Abu-Shaar and Mann, 1998; Brook and Cohen, 1996; Hays et al., 1999).

In contrast to the DV axis, Dpp and Wg act combinatorially to generate the proximodistal (PD) axis of the leg by inducing a different set of target genes, including *Distalless* (*Dll*) and *dachshund* (*dac*) (Campbell et al., 1993; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997; Mardon et al., 1994) (Fig. 1A). Unlike Wg and Dpp, which are expressed in ventral and dorsal sectors of the leg disc, respectively, *Dll* and *dac* are expressed in approximately circular domains whose centers are located where the Wg and Dpp sectors meet in the middle of the disc (Fig. 1A). *Dll* is expressed in a large central domain of the leg disc that gives rise to the distalmost positions of the adult leg (tarsus and distal tibia), whereas *dac* is expressed in more medial PD positions. It has been proposed that the PD identity of a cell and, consequently, whether it activates *Dll* or *dac*, is determined by integrating the levels of both Wg and Dpp: high levels of Wg and Dpp activate *Dll* and repress *dac*, whereas intermediate levels of Wg and Dpp activate *dac* but are insufficient to activate *Dll* (Lecuit and Cohen, 1997). However, an important and unresolved question stemming from this model is how do the sector-like expression domains of *wg* and *dpp*, which give rise to ventral and dorsal gradients of Wg and Dpp, respectively, generate nearly circular and concentric *Dll* and *dac* expression domains?

After the expression of *Dll* and *dac* is initiated, these genes become independent of Wg and Dpp, and maintain their expression by an unknown mechanism (Galindo et al., 2002; Lecuit and Cohen, 1997). By the end of larval development, the PD axis is divided into at least four domains based on the expression of these two genes: a distal Dll-only domain, a domain that expresses both genes (Dll+Dac), a Dac-only domain, which is expanded dorsally, and a proximal domain that expresses neither gene (Fig. 1A). In addition, the distalmost (tarsal) segments of the leg are more finely patterned by the graded activity of the Epidermal Growth Factor Receptor (EGFR) pathway, that controls another set of target genes, including *aristaless* (*al*), *Drosophila Lim1* (*Lim1*), *Bar* and *apterous* (*ap*) (Campbell, 2002; Galindo et al., 2002). The activation of this pathway also depends on Wg and Dpp, which trigger the expression and secretion of EGFR ligands from the center of the leg disc.

Department of Biochemistry and Molecular Biophysics, Columbia University, 701 West 168th Street, HHSC 1104, New York, NY 10032, USA.

*Author for correspondence (e-mail: rsm10@columbia.edu)

Accepted 29 November 2007

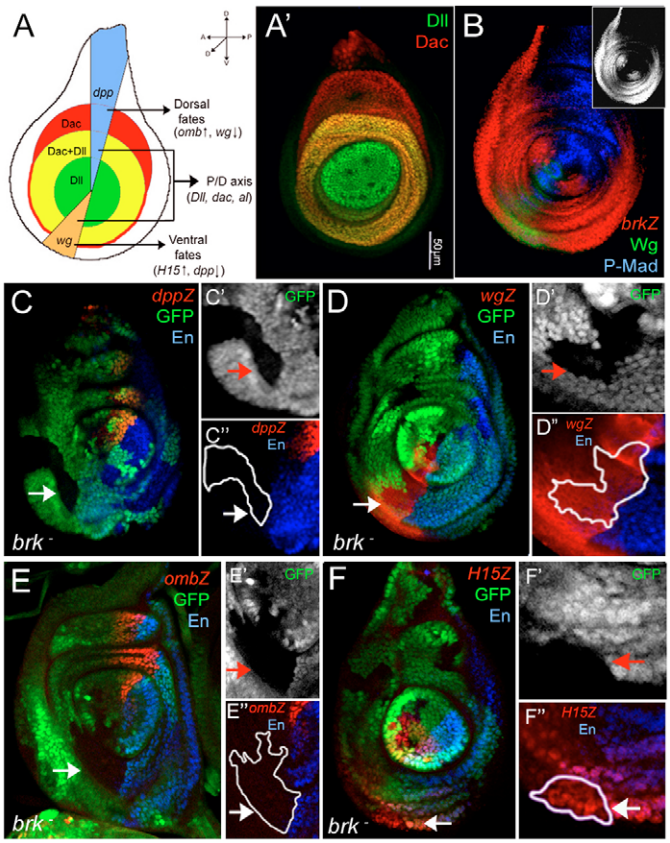

Fig. 1. *brk* is not required for DV axis specification. (**A**) Schematic representation of a third instar leg imaginal disc, summarizing the functions of Dpp and Wg in DV and PD axis development. The Wg (orange), Dpp (blue), Dll (green), Dll+Dac (yellow) and Dac-only (red) domains are indicated for a third instar leg disc. The Dac-only domain is larger in the dorsal disc. While Dpp promotes dorsal fates and Wg promotes ventral fates, Dpp and Wg act combinatorially to establish the PD axis. (**A′**) Expression of *Dll* (green) and *dac* (red) in a third instar leg disc. All images of leg discs are oriented anterior leftwards and dorsal upwards. (**B**) A wild-type third instar leg disc stained for *brk-lacZ* (red), Wg (green) and P-Mad (blue). *brk-lacZ* expression is reciprocal to P-Mad, which is highest dorsally but is also weakly observed ventrally. The inset shows *brk* expression, which is weakly downregulated in ventral cells at this stage. (**C-F″**) *brk*⁻ clones (absence of GFP, arrows) have no effect on *dpp-lacZ* (C-C″), *wg-lacZ* (D-D″), *omb-lacZ* (E-E″) or *H15-lacZ* (F-F″). Engrailed (En, blue) marks the posterior compartment. The ′ and ″ panels show enlargements of the clones as indicated.

In this study, we investigate how Wg and Dpp specify the DV and PD axes of the leg. How do these same morphogens regulate different target genes when specifying these two axes? How are Wg and Dpp inputs integrated during the activation of *Dll* and *dac*? How do Wg and Dpp create an asymmetric DV axis such that dorsal fates are expanded relative to ventral fates (Fig. 1A)? We provide answers to these questions in part by analyzing the role of *brinker* (*brk*) in leg development. *brk* encodes a transcriptional repressor of Dpp target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). In the wing, where it has been best studied, *brk* is itself repressed by Dpp signaling and is therefore expressed as a reciprocal gradient to the Dpp activity gradient. The expression domains of two Dpp target genes in the wing, *optimotor blind* (*omb*) and *spalt* (*sal*), are established by different sensitivities to Brk repression (Moser and Campbell, 2005; Muller et al., 2003). By extension, different levels of Brk are thought to pattern other aspects of the AP axis of the wing. Dpp signaling represses *brk* by triggering the assembly of a transcriptional repressor complex that is composed of at least three proteins: Medea, Schnurri and Mothers against Dpp (Mad), the nuclear localization of which is induced by phosphorylation upon Dpp signaling (Marty et al., 2000; Pyrowolakis et al., 2004). In addition to repressing *brk*, Dpp signaling is likely to control the transcription of other target genes via Mad, Medea, and, in some cases, Schnurri. Thus, in principle, Dpp signaling has the potential to regulate gene expression in at least two ways: (1) via Mad/Medea and (2) by silencing Brk (reviewed by Affolter et al., 2001).

In the leg, we show, surprisingly, that *brk* is not required for DV axis specification but that it plays an essential role in PD axis formation. Thus, Dpp uses distinct mechanisms for specifying these two axes in the leg. In PD axis formation, we show that *Dll* and *dac* are activated by Wg signaling and repressed by Brk. Moreover, *Dll* and *dac* have different sensitivities to both Brk levels and Wg signaling. The different sensitivities to these two inputs establish the *Dll* and *dac* expression domains at different PD positions. By demonstrating that Brk is a repressor and Wg is an activator of PD axis genes, our data explain the requirement for both Wg and Dpp inputs for their activation. When considered together with the expression pattern of *brk*, our findings also suggest a mechanism for the generation of asymmetry in the DV axis of the leg.

MATERIALS AND METHODS

Fly stocks

brk^{XA} is a P (*lacZ*) insertion and is larva lethal (Campbell and Tomlinson, 1999). Mad^{1-2} is a strong hypomorph (Wiersdorff et al., 1996), whereas the rest are considered nulls: tkv^{a12} (Nellen et al., 1994), brk^{M68} (Jazwinska et al., 1999), gro^{E48} and $CtBP^{l(3)87De-10}$. dpp^{discs} mutant larvae are heterozygous for In(2L)dpp^{d12} and Df(2L)dpp^{d14} (St Johnston et al., 1990), and $wg^{CX3/CX4}$ are hypomorph (Brook and Cohen, 1996). Other stocks used were: *omb-Z* (Grimm and Pflugfelder, 1996), *dpp-Z* (Blackman et al., 1991), *H15-Z* (Brook and Cohen, 1996), *wg-Z* (Ingham, 1991), UAS-*brk* (Jazwinska et al., 1999; Muller et al., 2003), UAS-*axin* (Willert et al., 1999), UAS-*gro* (Hasson et al., 2005), UAS-tkv^{QD} (Nellen et al., 1996) and UAS-TCF-RNAi (gift by Barry Dickson and Daniel McKay).

Clonal analysis

Clonal analysis was performed using the FRT/Flp technique (Xu and Rubin, 1993) using the following stocks: gain-of-function clones, *y w hs FLP122; tub>y+>Gal4 UAS-GFP*. Flip-out clones were induced by heat-shocking the larvae for 10 minutes at 37°C. Loss-of-function clones were generated using the following genotypes: $y\ f^{36a}\ brk^{M68}$ FRT 18A/FRT18A; hsFlp, *w hsFlp GFP* FRT 19A/*y* brk^{M68} FRT 19A, gro^{E48} or $CtBP^{l(3)87De-10}$ 82B/FRT82B *ubiGFP*, Mad^{1-2} or tkv^{a12} FRT 40A/*arm-Z* or *ubiGFP* FRT 40A. Double mutant clones for *brk* and *tkv* or *Mad* were generated as described previously (Campbell and Tomlinson, 1999; Jazwinska et al., 1999). Clones were induced by heat-shocking the larvae for 1 hour at 37°C.

More than 10 clones were analyzed for each genotype, except for the double *brk; tkv* or *brk; Mad* clones, where, owing to their low frequency, more than five clones were analyzed for each genotype. Except where indicated, all clones were generated 48-72 hours AEL.

MARCM experiments

We used the MARCM technique (Lee and Luo, 1999) to express *brk*+ or *axin*+ in *brk*⁻ cells using: *yw tubGal80* FRT19A/brk^{M68} FRT 19A; *tubGal4*-UAS *lacZ, hsFlp* (from G. Struhl)/UAS-*axin* or UAS-*brk*. To vary the amount of Axin or Brk, the larvae were grown at different temperatures, 17°C versus 29°C (Brand et al., 1994).

More than 10 clones were analyzed for each genotype. Except when indicated, all clones were generated 48-72 hours AEL.

Histochemical methods

Imaginal discs were fixed and stained using standard procedures. The primary antibodies used were: rabbit and mouse anti-β-Gal (Capell and Promega), mouse anti-Dachsund, mouse anti-Engrailed, mouse anti-Wingless (Hybridoma bank), guinea pig anti-P-Mad (Ed Laufer), guinea pig anti-Distal-less, rabbit anti-Homothorax, rat anti-Aristaless (Andrew Tomlinson) and rat anti-Lim (Gerard Campbell).

RESULTS

brk is repressed by Dpp signaling in the leg

In the leg imaginal disc, the expression of *dpp* and *wg* is generally restricted to the dorsal and ventral halves, respectively, owing to a mutually antagonistic repression that exists between these two pathways (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). As in wing discs, throughout development, *brk* expression was reciprocal to Dpp signaling in leg discs, as visualized with an antibody directed against P-Mad (Fig. 1B; see Fig. S1A,B in the supplementary material). In addition, at all stages, *brk* was expressed laterally and ventrally, where it overlapped with *wg* expression (Fig. 1B; see Fig. S1B in the supplementary material). In late third instar discs, *brk* expression was partially downregulated in ventral cells, which also have weak P-Mad staining at this stage (Fig. 1B). Such a pattern suggests that Dpp signaling represses *brk* in the leg. To test this, we generated clones expressing a constitutively activated form of the Dpp receptor *thickveins* (tkv^{QD}) and also examined *Mad* loss-of-function clones, in which the Dpp pathway was inactive. Expression of Tkv^{QD} repressed *brk* cell autonomously, whereas *brk* was derepressed in Mad^{-} clones (see Fig. S2A,B in the supplementary material). Thus, as in the wing, *brk* is negatively regulated by Dpp signaling in the leg.

brk is not required for DV axis specification in the leg

To specify the DV axis of the leg, Dpp and Wg regulate distinct sets of target genes that pattern the dorsal and ventral halves of the disc, respectively. For example, Dpp signaling represses *wg* and activates *omb*, whereas Wg signaling represses *dpp* and activates *H15* (Fig. 1A) (Brook and Cohen, 1996; Couso et al., 1993; Hays et al., 1999; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996; Wilder and Perrimon, 1995). As described above, Dpp also negatively regulates *brk* in dorsal cells. As Brk is a transcriptional repressor in the Dpp pathway, we tested the possibility that *brk* contributes to ventral fate specification by repressing dorsal genes such as *dpp* and *omb*. To address this issue, we generated mitotic clones of a null allele of *brk* and examined the expression of *dpp*, *wg*, *omb* and *H15*. Surprisingly, all four of these genes were expressed normally in brk^{-} clones, no matter where they arose in the disc (Fig. 1C-F). Most importantly, ventral brk^{-} clones still expressed *wg* and *H15*, and did not derepress *dpp* or *omb*. By contrast, Tkv^{QD}-expressing clones, in which the Dpp pathway was fully active, repressed both *H15* and *wg* (see Fig. S4A,B in the supplementary material). Leg discs entirely mutant for a hypomorphic allele of *brk* also did not show any ventral expansion of P-Mad staining or *wg* repression (see Fig. S3C,D in the supplementary material). These results strongly suggest that *brk* is not playing a role in DV axis specification in the leg. Notably, these findings contrast with those of Theisen et al. (Theisen et al., 2007) who suggested that *brk* is a repressor of *dpp* in the ventral leg disc. However, our genetic tests of this model, which were not carried out by Theisen et al., suggest that this model cannot be correct, at least in its most simple form.

The conclusion that *brk* does not play a role in DV axis specification in the leg is further supported by the phenotype of brk^{-} clones in the adult appendage. Although brk^{-} clones can be recovered anywhere in the adult leg, when they arose in the distal leg (tarsus and distal tibia) they either had no phenotype (see Fig. S4E in the supplementary material) or only subtly changed the bristle pattern (proximal tibia and distal femur, see below). By contrast, when they arose in the ventral or lateral proximal leg (proximal femur, trochanter or coxa) these clones generated leg-like outgrowths (Fig. 2A). These outgrowths (n=15) were strictly cell-autonomous and had no leg joints or distal leg structures, such as tarsi or claws. An analysis of the bristle pattern in these outgrowths suggests that they were

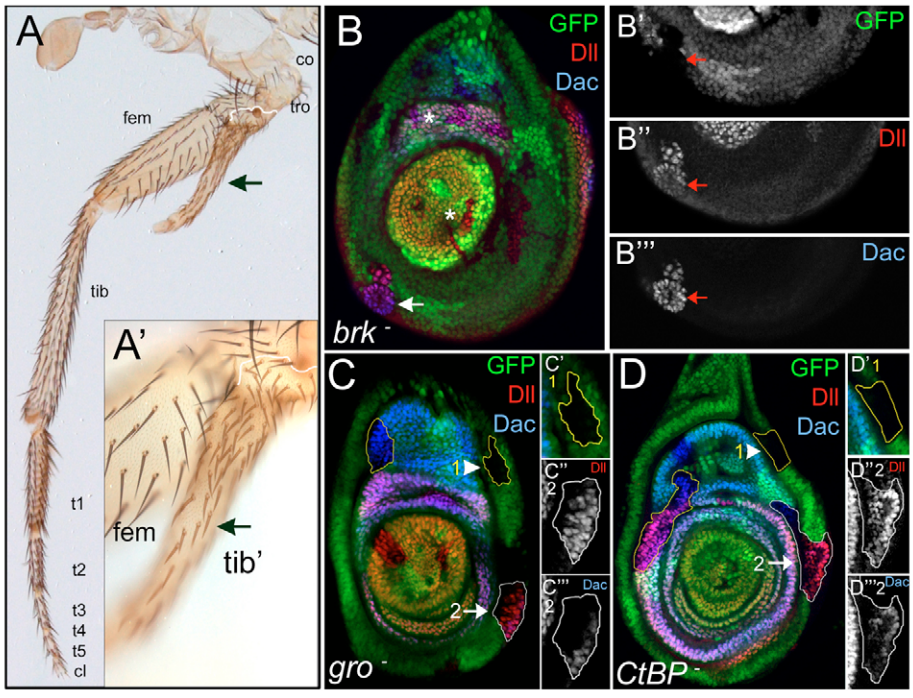

Fig. 2. *brk* is required for PD axis specification. (**A**) A brk^{-} clone originating in the ventral proximal femur marked by *yellow* and *forked* (arrow, outlined in white) creating a cell-autonomous outgrowth. (**A'**) The entire outgrowth appears to have a single, tibia-like DV identity. co, coxa; tro, trochanter; fem, femur; tibia, tib; tib', tibia-like outgrowth; t, tarsal segments 1-5; cl, tarsal claw. (**B**) brk^{-} clone proximal to the Dll and Dac domains (absence of GFP, arrow) showing cell-autonomous derepression of *Dll* and *dac*. No effects on *Dll* or *dac* are observed in brk^{-} clones within their normal domains (asterisks). (**C-D'''**) gro^{-} (C) or $CtBP^{-}$ (D) clones (absence of GFP, green) derepress *Dll* and *dac* in proximoventral regions of the disc (arrows and clones labeled '2'; isolated clones are outlined in white and shown in C'', C''', D'' and D'''). No derepression is observed in dorsal gro^{-} or $CtBP^{-}$ clones (arrowheads and clones labeled '1'; isolated clones are outlined in yellow and shown in C' and D'). Clones within the Dac or Dll domains (outlined, but not numbered) have no effect on Dac or Dll expression.

composed of a single DV identity that is most similar to the ventral or lateral tibia. Consistent with the expression pattern of *brk*, clones in the dorsal proximal leg were wild type. Moreover, consistent with the marker analysis in leg discs (see above), *brk*⁻ clones behave very differently from clones expressing Tkv^{QD}, which cause a clear transformation to a dorsal identity in both proximal and distal regions of the leg (see Fig. S4C,D in the supplementary material). Thus, based on these molecular and morphological readouts, we conclude that *brk* does not play a role in DV axis specification. However, as explored below, these results suggest that *brk* is playing an important role in PD axis specification.

brk is required for forming the PD axis in the leg

The PD axis duplications that result from removing *brk* suggest that Brk normally acts to repress *Dll*, *dac* and perhaps other PD genes. To address this issue we analyzed the expression of *Dll* and *dac* in *brk*⁻ clones. *brk*⁻ clones generated anywhere in the Dll or Dll+Dac domains did not affect the expression of these genes (Fig. 2B), suggesting that *brk* does not regulate these genes once their expression domains are established. This conclusion is consistent with the fact that by the third instar, both the Dll and Dac domains partially overlap with *brk* expression in the ventral disc.

In contrast to distal clones, all ventral or lateral clones generated outside (i.e. proximal to; *n*=25) the Dll and Dac domains activated both genes cell autonomously (Fig. 2B). As expected from its expression pattern, dorsal *brk*⁻ clones failed to derepress *Dll* or *dac*. In *brk*⁻ clones that derepressed these genes, the pattern of *Dll* and *dac* expression showed significant organization. Cells that had high levels of Dll generally had low levels of Dac and vice versa. However, despite this relationship, 100% of the clones (*n*=25) had cells that co-expressed both genes. By contrast, 52% of these clones had cells that expressed Dac, but not Dll, whereas only 4% had cells that expressed Dll, but not Dac. This pattern of Dll and Dac derepression is consistent with the observation that *brk*⁻ clones give rise to tibia-like outgrowths in the adult (Fig. 2A), as the tibia is derived from cells in the leg disc that express both Dll and Dac (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997). *brk*⁻ clones in the Dac-only expression domain derepress *Dll*, accounting for the subtle change in bristle pattern observed in distal tibia and femur clones (see above).

Previous results suggest that repression by Brk uses two co-repressors, C-terminal binding protein (CtBP) and Groucho (Gro) (Hasson et al., 2001). Consistent with a role for these co-repressors in *Dll* and *dac* repression in the leg, clones mutant for *gro* or *CtBP* derepressed both genes in the same region of the leg disc (proximal ventral and lateral) as *brk*⁻ clones (Fig. 2C,D). In addition, like *brk*⁻ clones, *gro*⁻ or *CtBP*⁻ clones had no affect on *Dll* and *dac* expression in their normal domains and did not derepress these genes in dorsal clones. These results suggest that, as elsewhere in development, Brk uses these two co-repressors to repress *Dll* and *dac* in the leg disc. Interestingly, they also illustrate an inherent asymmetry to the way in which *Dll* and *dac* are regulated during leg development: while *Dll* and *dac* are repressed ventrally and laterally by Brk/Gro/CtBP, our data suggest that there is no Gro- or CtBP-dependent repressor that functions to keep these genes switched off in the dorsal leg disc. We will present additional evidence to support this idea.

Dpp signaling is not required for *Dll* and *dac* activation in the absence of *brk*

Our finding that *brk* loss-of-function clones derepress *Dll* and *dac* in the ventral half of the leg disc (Fig. 2B) suggest two models for the role of Dpp signaling in PD gene activation. The first model suggests that Dpp signaling is only required to eliminate Brk repression. Alternatively, Dpp signal transduction may still be required to activate *Dll* and *dac* even in the absence of *brk*. This model follows from the observation that Brk can repress some Dpp target genes by competing with Mad for DNA binding (Kirkpatrick et al., 2001), raising the possibility that in the absence of Brk, *Dll* and *dac* may respond to lower levels of Dpp signaling. We carried out two experiments that both support the first model, namely that, in the absence of Brk, Dpp signaling is not essential for *Dll* and *dac* expression.

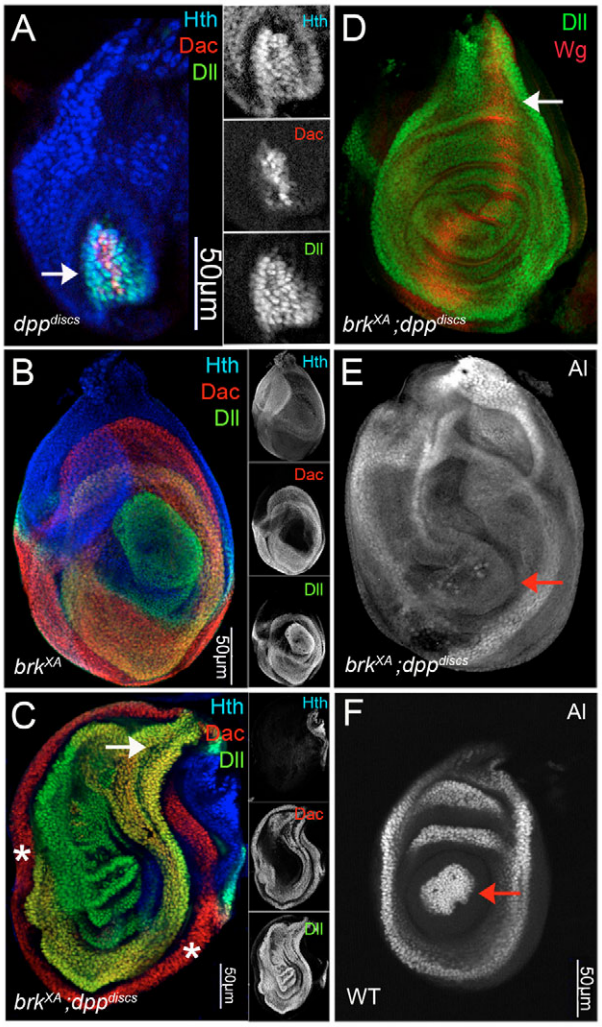

Fig. 3. A PD axis does not require Dpp signaling in the absence of Brk. (**A**) dpp^{discs} mutants have very small discs and the normal distal and medial *Dll* and *dac* domains are absent. The *Dll* and *dac* expression that remains corresponds to the Dpp- and Wg-independent trochanter region (arrow), which also expresses Homothorax (Hth, blue). (**B**) brk^{XA} discs are overgrown, and the *Dll* and *dac* expression domains are expanded in the ventral and lateral parts of the disc. (**C**) brk^{XA}; dpp^{discs} discs are overgrown and Dll, Dll+Dac and Dac expression domains are all present. *Dll* expression is expanded dorsally and the dorsal Dac-only domain is not present (arrow). Dac expression is expanded laterally compared with wild type (asterisks; compare with Fig. 1A′). (**D**) In brk^{XA}; dpp^{discs} discs, *wg* (red) is expressed along the entire DV axis. Dll is also expanded dorsally, following the dorsal Wg expression (arrow). (**E**) In brk^{XA}; dpp^{discs} discs, the distal domain of *al* is not present (arrow), whereas other, Dpp- and Wg-independent domains of *al* are still present (compare with wild type, F). (**F**) Expression of *al* in a wild-type disc. The distal *al* domain is indicated by the arrow.

In the first approach, we examined leg discs mutant for hypomorphic alleles of *dpp* and *brk*. In $dpp^{d12/d14}$ (dpp^{discs}) leg discs, very little *Dll* and *dac* expression remained and the discs were very small (Diaz-Benjumea et al., 1994) (Fig. 3A). The remaining *Dll* and *dac* expression was derived from the trochanter region of the leg that is formed without Dpp input (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994). Conversely, brk^{XA} hemizygotes had overgrown leg discs (Campbell and Tomlinson, 1999). Notably, the expression of *Dll* and *dac* was expanded ventrally and laterally in brk^{XA} discs compared with wild type (Fig. 3B). In addition, and consistent with the brk^{-} clonal analysis, *wg* expression was still limited to the ventral leg disc in brk^{XA} discs, as it is in wild-type discs (see Fig. S3B,D in the supplementary material). In brk^{XA}; dpp^{discs} double mutants, the shape and size of the leg discs resembled brk^{XA} leg discs. In this double mutant, no P-Mad staining was detected (see Fig. S3E in the supplementary material) and, consistently, *wg* was expressed along the entire AP compartment boundary, creating a leg disc with ventral-ventral symmetry (Fig. 3D). Surprisingly, although these discs exhibited no evidence of Dpp signaling, *Dll* and *dac* were still expressed, and their relative spatial domains were similar to wild type: *Dll* was expressed in the center of the disc, *dac* in a medial domain and there was a region in between where both genes were co-expressed (Fig. 3C, compare with Fig. 1A′). However, despite the overall integrity of the Dll and Dac domains, brk^{XA}; dpp^{discs} leg discs no longer had the normally large Dac-only domain in the dorsal region of the disc, and *dac* expression was expanded laterally (Fig. 3C). The *Dll* domain was also expanded dorsally compared with wild type. These phenotypes are consistent with *wg* derepression in the dorsal leg disc, creating a ventral-ventral symmetric appendage.

Other genes required for PD axis formation are the EGFR pathway target genes *al* and *Lim*, which are both expressed, among other places, in the center of the leg disc (the future pretarsus, Fig. 3F) (Campbell, 2002; Galindo et al., 2002). In brk^{XA}; dpp^{discs} leg discs, the distal expression of *al* and *Lim* was absent whereas the other, more proximal, expression domains remained (Fig. 3E and data not shown). Thus, despite the expression of *Dll* and *dac*, the PD axis formed in brk^{XA}; dpp^{discs} leg discs was incomplete.

A potential caveat in the brk^{XA}; dpp^{discs} experiments is that both of these alleles are hypomorphs, raising the possibility that sufficient Dpp activity exists to activate *Dll* and *dac* when Brk levels are compromised. To address this possibility, we generated clones doubly mutant for a null allele of *brk* and a null allele of *tkv* (Fig. 4A-D) or a strong hypomorphic allele of *Mad* (data not shown). *Dll* and *dac* were derepressed in both brk^{-}; tkv^{-}-null mutant clones and brk^{-} Mad^{-} clones (Fig. 4A-D and data not shown). As brk^{-}; tkv^{-} clones cannot transduce the Dpp signal, these findings demonstrate that in the absence of Brk, Dpp signal transduction is not essential for *Dll* and *dac* expression. As with brk^{-} clones, when brk^{-}; tkv^{-} clones have had enough time to grow there is significant organization to *Dll* and *dac* expression. This organization cannot be dependent on Dpp signaling but, instead, as shown below, is probably due to Wg input.

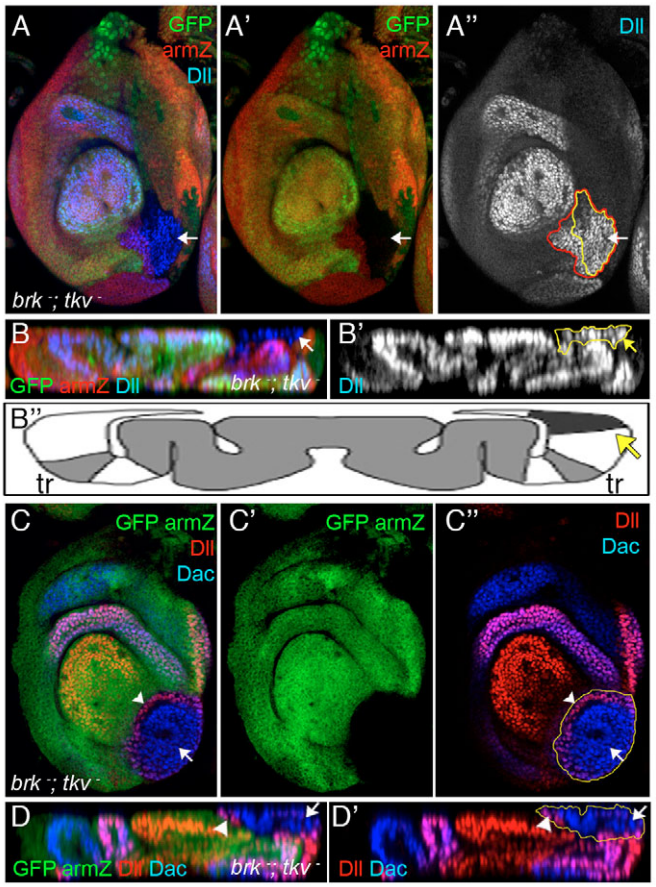

Fig. 4. *Dll* and *dac* expression in the absence of Dpp signaling and *brk*. (**A-A″**) Mitotic clones mutant for *brk* (arrow; marked by the absence of GFP, green, and outlined in red in A″) and *tkv* [arrow; marked by the absence of *arm-lacZ* (red) and outlined in yellow in A″] derepress *Dll*. These clones were induced separately and therefore result in independent clonal events. Cells mutant for both genes show no GFP (green) and no β-gal staining (red) (A′). (A″) *Dll* is derepressed cell autonomously throughout the brk^{-} clone (marked by the red line), including cells that are also tkv^{-} (marked by the yellow line). (**B-B″**) Cross-section of the same disc in A showing that the brk^{-}; tkv^{-} clone is proximal to the Dll expression domain (arrow, outlined in yellow). (B″) Schematic representation of the cross-section shown in B. *Dll* expression is shown in gray and the yellow arrow marks the region where *Dll* is derepressed (dark gray). tr, trocanter. (**C-C″**) A proximal clone doubly mutant for *brk* (absence of GFP, green) and *tkv* (absence of *arm-lacZ*, green) showing derepression of *Dll* (arrowhead, red) and *dac* (arrow, blue). These clones were induced at the same time, and are therefore congruent. (**D-D′**) Cross-section of the same disc as in C showing that the brk^{-}; tkv^{-} clone (outlined in yellow) is proximal to the *Dll* and *dac* expression domains.

Different Wg levels establish the Dll and Dac domains

We have shown that in the absence of Brk, *Dll* and *dac* become derepressed, even in the absence of Dpp signaling. These findings raise the issue of what activates *Dll* and *dac* in the brk^{-}; tkv^{-} clones, a situation where Dpp signal transduction cannot occur. One likely candidate is Wg, which is required for the initial activation of *Dll* and *dac* (Diaz-Benjumea et al., 1994). Consistent with this idea, only a subset of brk^{-} (or brk^{-}; tkv^{-}) clones derepress *Dll* and *dac*; dorsal or dorsolateral clones, which are far form the Wg source, fail to activate *Dll* or *dac*.

We carried out two experiments that provide more direct evidence that Wg signaling is required to activate these two genes. In the first approach, we examined the consequences of reducing both Wg signaling and Brk levels. As reported above, brk^{XA} larvae had overgrown leg discs with large Dll and Dac domains (Fig. 3B). If in this background we also reduced Wg signaling (i.e. in brk^{XA}; $wg^{CX3/CX4}$ larvae), the Dll and Dac domains were reduced in size (compared with brk^{XA} discs), suggesting that Wg signaling is

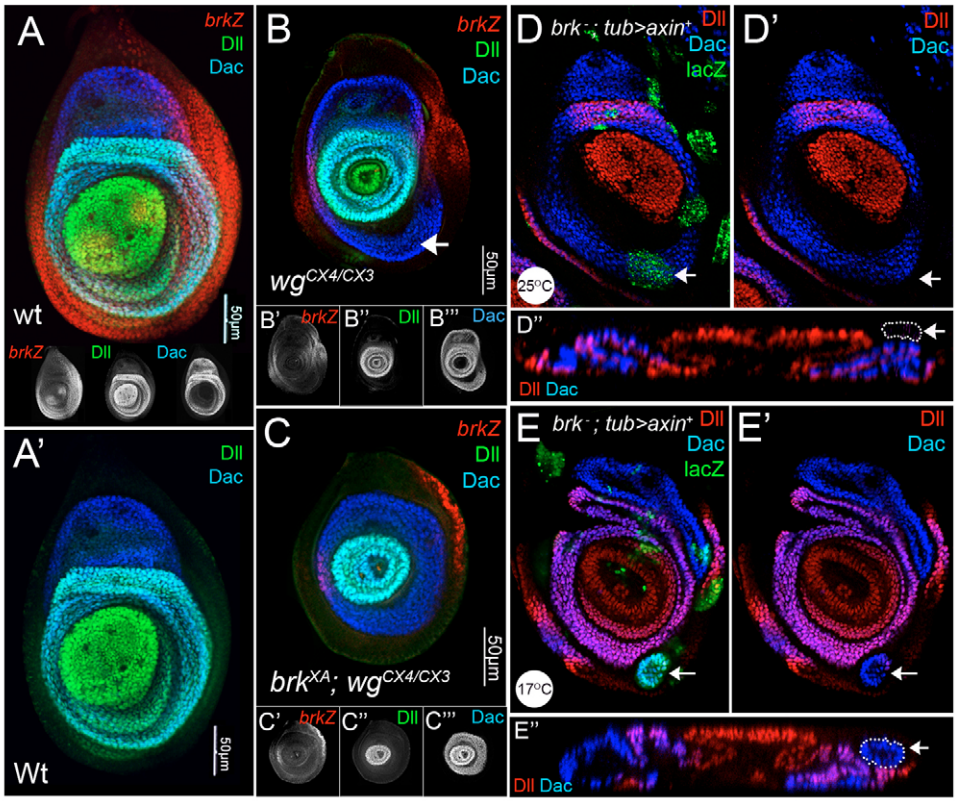

Fig. 5. Different levels of Wg signaling activate *Dll* and *dac*. (**A**) Expression of *Dll* (green), *dac* (blue) and *brk* (red) in a wild-type third instar leg disc. The individual channels are shown below. (**A′**) Expression of Dll (green) and Dac (blue) in a wild-type third instar leg disc. (**B-B‴**) In $wg^{CX4/CX3}$ discs, the Dll (green) domain is reduced in size compared with wild type (compare with A) and the Dac (blue) domain is expanded ventrally (arrow). *brk* is repressed in the ventral domain because of *dpp* derepression (data not shown). (**C-C‴**) In brk^{XA}; $wg^{CX4/CX3}$ discs, the Dll domain is reduced in size compared with wild type and the Dac domain is nearly circular. Compare discs in B and C with a wild-type disc in A and A′. The Dll-only domains in leg discs of this genotype and in $wg^{CX4/CX3}$ discs vary from small (as in the example in B) to its complete absence (as in the example in C). (**D-E″**) *brk*⁻; *tub>axin+* clones marked positively by *lacZ* (green) grown at different temperatures. (D) At 25°C, axin levels are sufficient to block both *Dll* and *dac* derepression (arrow). Compare with the *brk*⁻ clone in Fig. 2B, showing both *Dll* and *dac* derepression. (D″) Cross-section of the same disc as in D showing the location of the clone (broken line, arrow). (E) Leg disc of the same genotype in D grown at 17°C. The Wg signaling that remains permits *dac* but not *Dll* derepression (arrow). Approximately one-third of these clones show *dac* only derepression like the clone shown here, a phenotype that is never observed in *brk*⁻ clones. Approximately two thirds of these clones show derepression of both genes similar to the clone shown in Fig. 2B. (E″) Cross-section of the same disc in E showing the location of the clone (broken line, arrow).

playing a crucial role in the activation of these two genes (Fig. 5C; compare with Fig. 3B). Remarkably, brk^{XA}; $wg^{CX3/CX4}$ leg discs, as well as their Dll and Dac domains, were nearly circular; the Dac domain no longer had a dorsal bias that normally exists in wild-type leg discs (compare with Fig. 5A). In $wg^{CX3/CX4}$ hypomorphic larvae, in which Wg signaling was compromised but Brk levels were normal, the leg discs had a dorsal-dorsal symmetry, as evidenced both by the *dac* and *brk* expression patterns (Fig. 5B). This dorsal-dorsal symmetry probably occurs because *dpp* is derepressed ventrally in $wg^{CX3/CX4}$ leg discs (Brook and Cohen, 1996; Hays et al., 1999). In addition to providing evidence that Wg signaling is an activator of *Dll* and *dac*, these results suggest that the ratio of Wg signaling to Brk levels is important for controlling the shape and size of the Dll and Dac domains and, ultimately, the leg disc (see Discussion).

In a second experiment, we blocked Wg signaling in *brk*⁻ clones by expressing the Wg pathway inhibitor, Axin, using the MARCM method (Lee and Luo, 1999; Willert et al., 1999). In all *brk*⁻; *tub>axin+* clones, neither *Dll* or *dac* were activated, even in ventral positions of the leg disc (Fig. 5D). Taken together, these results strongly support the idea that Wg signaling is an essential activator of *Dll* and *dac*.

Because Wg is a diffusible morphogen, we also tested the idea that high levels of Wg signaling would be required to activate *Dll*, whereas lower levels would be sufficient for *dac* activation. Our previous experiments demonstrate that both genes are activated in ventral or ventrolateral *brk*⁻ clones (Fig. 2B) and that neither is activated in *brk*⁻; *tub>axin+* clones (Fig. 5D). We tested whether intermediate levels of Wg signaling were sufficient for *dac* (but not *Dll*) activation by expressing lower levels of Axin in *brk*⁻ clones, by growing the larvae at 17°C instead of 25°C (see Materials and methods). Strikingly, *brk*⁻; *tub>axin+* clones generated in larvae grown at 17°C often derepressed *dac*, but not *Dll* (Fig. 5E), a phenotype that was never observed in *brk*⁻ clones. These results suggest that during normal leg development, different levels of Wg signaling help to pattern the PD axis by activating different target genes; high Wg levels activate *Dll*, moderate levels activate *dac* but are insufficient for *Dll* activation, and low or no Wg signaling fails to activate either target.

Different Brk levels establish the Dll and Dac domains

Like Wg, Dpp is a secreted morphogen that can produce different effects depending on its concentration. Although our previous results show that Dpp signaling is not required for *Dll* and *dac* activation in the absence of Brk, they do not address the possibility that different levels of Dpp signaling contribute to target gene choice during wild-type development. According to this idea, *Dll* and *dac* may be differentially sensitive to different levels of Dpp and, consequently, Brk. To test this idea, we resupplied different levels of Brk in *brk*⁻ null clones and examined *Dll* and *dac* expression (see Materials and methods). As shown previously, ventral *brk*⁻ clones derepressed both *Dll* and *dac* (Fig. 2B). When grown at 25°C, *brk*⁻; *tub>brk+* clones failed to derepress either gene (Fig. 6A). However, when grown at 17°C, ventral *brk*⁻; *tub>brk+* clones derepressed *dac* but not *Dll* (Fig. 6B). These

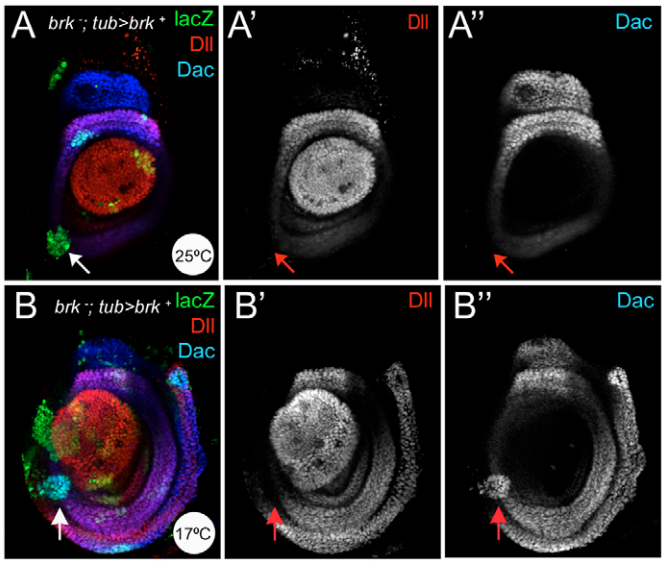

Fig. 6. Different levels of Brk activate *Dll* and *dac*. (**A-B″**) *brk⁻; tub>brk+* clones (*lacZ*, green) grown at different temperatures. (A) At 25°C, the levels of Brk are sufficient to prevent both *Dll* and *dac* derepression (arrow). Compare with the *brk⁻* clone in Fig. 2B, where both *Dll* and *dac* are derepressed. (B) At 17°C the levels of Brk are sufficient to prevent *Dll* derepression but not *dac* derepression (arrow). Approximately half of the clones examined with this genotype showed *dac*, but not *Dll*, derepression like the clone shown here, a phenotype that was never observed in *brk⁻* clones. The other (approximately) half of these clones failed to derepress either *Dll* or *dac*. The ′ and ″ panels show the single Dll or Dac expression patterns, respectively.

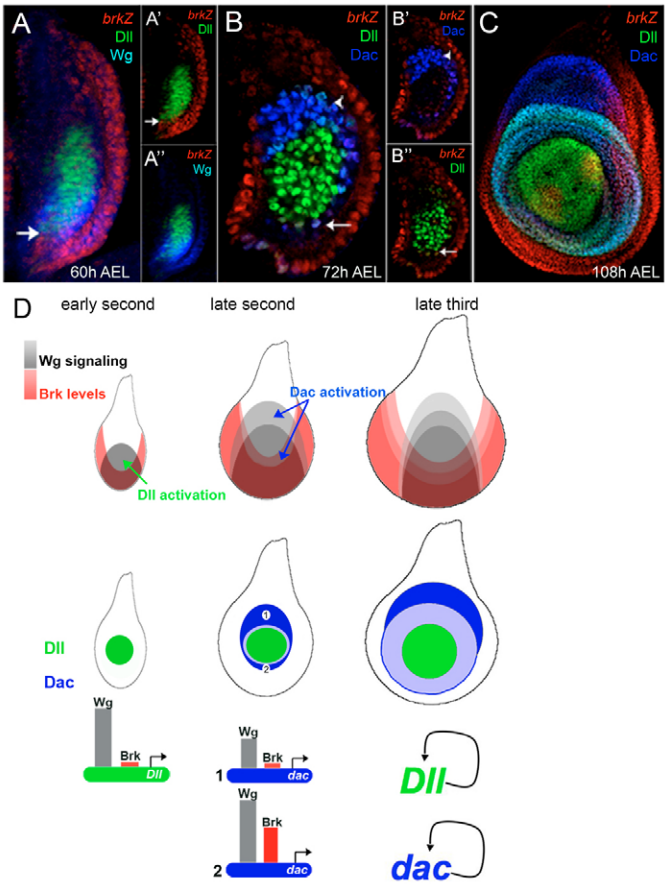

Fig. 7. Temporal progression of *Dll* and *dac* expression during leg development. (**A-C**) Expression of *brk*, *Dll* and *dac* during leg development. The discs are not shown to scale. (A) At ~60 hours AEL Dll (green) is present in the center of the disc where there is no detectable Brk (red). In the ventral and lateral regions of the disc (arrow), there is a sharp border that separates *Dll* and *brk* expression. Wg (blue) is highest in the ventral region of the disc but is also observed in *Dll*-expressing cells. (B) At about 72 hours AEL, *dac* (blue) is broadly expressed in the dorsal part of the leg disc where there is no *brk* (arrowhead) and in the lateral and ventral regions where there are low levels of Brk (arrow). In the cells where *dac* is activated, *Dll* is also expressed at low levels. The ′ and ″ panels show subsets of these expression patterns as indicated. (C) At about 108 hours AEL, the expression of *Dll* and *dac* overlaps with *brk*. At this time these genes are refractory to Brk repression. (**D**) In the early second instar (left), *Dll* is activated in the center of the disc where there is no Brk and high Wg levels. In a late second instar disc (middle), *dac* is activated: (1) in the dorsal region of the disc where there is no Brk and low levels of Wg signaling (region 1); and (2) in lateral and ventral cells, where there is high Wg signaling and low Brk (region 2). In late third instar (right), *Dll* and *dac* expression is maintained and become refractory to Brk repression. The bottom set of schematics illustrate the unique Brk:Wg ratios required to activate *Dll* and *dac*. For *dac* activation, two ratios are suggested.

results suggest that, as with Wg, Brk levels, which are governed by Dpp levels, help to establish the *Dll* and *dac* expression domains along the PD axis of the leg.

Expression of *brk*, *Dll* and *dac* during leg development

The above genetic manipulations demonstrate that the levels of both Brk and Wg signaling determine whether *dac* or *Dll* are activated in the leg disc. To place these observations in the context of wild-type leg development, we examined the temporal sequence of *Dll* and *dac* activation during larval development, and compared it with the Wg, Brk and P-Mad patterns. In the mid-second instar (~60 hours AEL), P-Mad, a readout of Dpp signaling, was broadly observed throughout the dorsal half of leg discs (see Fig. S1A in the supplementary material). At this same stage, *brk* was expressed ventrally, where there is no detectable P-Mad, and Wg staining was also limited to ventral cells (Fig. 7A; see Fig. S1A in the supplementary material). At this stage, *Dll* was expressed in the center of the disc, in cells that (1) had no detectable Brk, (2) stained for P-Mad and (3) had detectable Wg staining. *dac* was not expressed at this stage (Abu-Shaar and Mann, 1998). Slightly later in development, in early third instar discs (~72h AEL), the *brk* expression domain was larger and P-Mad staining was more restricted to a subset of dorsal cells (see Fig. S1B in the supplementary material). At about this time, *dac* was first observed in dorsal cells where there was no detectable Brk and in a small number of lateral and ventral cells that had low Brk levels (Fig. 7B). These early Dac+ cells also had low levels of Dll. In older leg discs (~108 hours AEL) the expression domains of *Dll* and *dac* overlapped with *brk* (Fig. 7C), consistent with our results showing that Brk does not play a role in *Dll* or *dac* expression once their domains have been established.

DISCUSSION

Although the evolutionary history of chordate and arthropod appendages is distinct, numerous similarities exist in the underlying genetic pathways that control appendage formation in these two

phyla, raising the possibility that a common ancestor had appendage-like outgrowths that used many of the same mechanisms that currently operate in both phyla (reviewed by Shubin et al., 1997). As all appendages share the property of having PD axis, an understanding of the mechanisms governing the formation of this ancient axis may reveal additional common themes that operate in all animal appendages. Moreover, as dorsal appendages, such as wings, are thought to have been derived from ventral appendages (i.e. legs), the analysis of leg development is more likely to reveal such commonalities (reviewed by Shubin et al., 1997).

In *Drosophila*, central to the development of this appendage are two signaling pathways, Wg and Dpp, that are used repeatedly in animal development. In the leg, these signaling pathways serve at least two purposes: to specify positional values along the DV axis and to establish the PD axis. Our experiments reveal that the logic by which Dpp input is used to generate these two axes is distinct: for *Dll* and *dac* activation, the primary targets of the PD axis of the leg, Dpp input is mediated via Brk. By contrast, Dpp uses Brk-independent mechanisms to specify the DV axis. Also unclear from previous studies is how Wg and Dpp induce *Dll* and *dac* at distinct positions along the PD axis. In particular, how do Wg and Dpp, which are present in ventral and dorsal gradients, respectively, create concentric rings of PD fates? Finally, how are Wg and Dpp inputs integrated by *Dll* and *dac*? As discussed below, our results provide answers to these questions and allow us to present a revised model for PD axis formation in the *Drosophila* leg.

DV versus PD axis specification

In the leg, as in other tissues, *brk* is expressed in a pattern that is complementary to the pattern of Dpp pathway activation, as revealed by P-Mad levels. Brk, a transcriptional repressor, could in principle be responsible for repressing Dpp targets relevant to both the DV and PD axes. Surprisingly, our experiments do not support a role for *brk* in DV axis specification. For all four DV readouts examined (*dpp*, *wg*, *omb* and *H15*), expression was normal in *brk*$^{-}$ clones. By contrast, two of the primary PD markers, *dac* and *Dll*, are repressed by Brk early in leg development. Thus, Dpp signaling controls gene expression in the DV and PD pathways in different ways: Dpp signaling both activates (e.g. *omb*) and represses (e.g. *wg*) DV genes independently of *brk*, perhaps via the Mad-Med and Schnurri transcription factors (Muller et al., 2003). By contrast, Dpp regulates *Dll* and *dac* in a *brk*-dependent manner. We suggest that these different modes of gene regulation by Dpp signaling are necessary for Dpp to execute these two distinct functions during leg development.

In contrast to the Dpp pathway, our evidence suggests that Wg signaling does not go through a transcriptional repressor to control *Dll* and *dac* expression. From previous results, the most likely candidate for such a Wg pathway repressor is Tcf, the downstream transcription factor in the Wg pathway. In the absence of Wg, Tcf has been shown to repress Wg target genes in a *gro*- and *CtBP*-dependent manner (Brannon et al., 1999; Brantjes et al., 2001; Cavallo et al., 1998; Lawrence et al., 2000; Roose et al., 1998; Valenta et al., 2003). In the leg, such a Wg pathway repressor would be predicted to be active in the dorsal leg disc, away from the source of Wg. An argument against this scenario, however, is our observation that *gro*$^{-}$ or *CtBP*$^{-}$ clones fail to derepress *Dll* or *dac* in the dorsal leg disc, but derepress these genes in the ventral leg disc. We also examined the consequences of knocking down *Tcf* function in clones by RNAi. Although *Tcf-RNAi*-expressing clones were able to block Wg repression of *dpp* in the ventral leg disc, no derepression of *Dll* or *dac* was observed in dorsal regions of the disc (data not shown). Taken together, these results suggest that whereas Dpp signaling controls *Dll* and *dac* by repressing the Brk repressor, Wg signaling controls *Dll* and *dac* positively, without going through an intermediate repressor.

Different levels of Brk and Wg signaling control the choice between activating *Dll* or *dac*

During leg development, *Dll* is activated in the center of the disc whereas *dac* is activated in medial regions of the disc. Previous genetic studies suggested that the activation of these two outputs depends on different levels of Wg and Dpp (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Our results support this view, and provide additional insights into how *Dll* and *dac* are activated by these two secreted signals during leg development. By independently manipulating the levels of Brk or Wg signaling, we found that both of these regulators impact whether *Dll* or *dac* is activated. Specifically, we created situations in which both genes have the potential to be expressed (i.e. in ventral *brk*$^{-}$ clones), but at the same time varied the amount of Wg signal transduction or Brk. We found that *Dll* can be activated only when the Wg pathway is fully active. By contrast, *dac* can be activated when the Wg pathway is only partially active. Analogously, when we expressed different levels of Brk in *brk*$^{-}$ clones, we found that *Dll* could only be activated in the absence of Brk, but that *dac* could be activated in the presence of intermediate Brk levels. As Brk levels normally vary in response to Dpp activity, these data demonstrate that the levels of both Wg and Dpp inputs play a role in choosing which PD target gene is activated during wild-type leg development (Fig. 7D).

Signal integration into *Dll* and *dac*

Although, the requirement for multiple inputs into gene regulation is typical in transcriptional regulation, for most cases where multiple positive inputs are required, the underlying mechanism is not well understood (reviewed by Arnosti, 2003; Barolo and Posakony, 2002; Guss et al., 2001; Mann and Carroll, 2002; Merika and Thanos, 2001). By contrast, there are many examples of genes that integrate both positive and negative inputs to be activated in a spatially or temporally restricted manner (Arnosti, 2003). The enhancers mediating this control can be thought of as 'logic integrators' that are active only in the presence of the correct activators and in the absence of repressors (Istrail and Davidson, 2005). We suggest that such a mechanism can account for why Dpp and Wg are both required for the activation of *Dll* and *dac* during leg development. Although both pathways are required for activating these genes, our finding that Dpp functions by repressing a repressor creates a situation in which positive and negative inputs ultimately determine where *Dll* and *dac* are activated. For *Dll*, we suggest that activation occurs only in cells that satisfy two conditions: (1) they have little or no *brk* expression owing to high Dpp signaling and (2) they are experiencing a strong Wg input. These conditions are satisfied in the center of the young leg disc, where Wg and Dpp signals coincide, precisely where *Dll* is expressed (Fig. 7A,D). Importantly, Brk plays a crucial role in this model because it keeps *Dll* switched off in a large part of the disc, thus allowing Wg-mediated activation of *Dll* to occur in only a small subset of the disc. Consistently, a *Dll* enhancer that activates expression in the young leg disc directly integrates Wg and Dpp inputs by binding Tcf, Mad and Brk (Estella et al., 2008). In addition to these inputs, there must be additional factors that limit *Dll* activation to ventral, but not dorsal (e.g. wing), imaginal discs. Ventral specificity may be mediated by the expression of *Dll*, itself, via an early embryonic enhancer or by the

activity of other ventral-specific factors such as *buttonhead* (*btd*), which is required for *Dll* expression (Castelli-Gair and Akam, 1995; Estella et al., 2003; Vachon et al., 1992).

The activation of *dac* appears to be more complex, but we speculate works using similar logic as that used for *Dll* activation. When *dac* is first activated in the wild-type leg disc, it is turned on in a relatively large number of cells dorsal to the *Dll* domain, but also in a smaller number cells lateral and ventral to the *Dll* domain. Based on their position in the leg disc, it is likely that cells in each of these regions have different levels of Brk and Wg signaling at the time when *dac* is first activated. Cells in the dorsal *dac* domain have no (or very low levels of) Brk and intermediate levels of Wg signaling, whereas cells ventral to the *Dll* domain have intermediate levels of Brk and high levels of Wg signaling (Fig. 7B,D). We suggest that both of these ratios of Brk to Wg (Brk:Wg) inputs are competent to activate *dac*. Furthermore, we speculate that these Brk:Wg inputs are not sufficient to activate *Dll*. We suggest that an analogy can be made between how *Dll* and *dac* are activated in the leg disc, to how *even-skipped* (*eve*) is activated during embryogenesis. As we suggest for *Dll* and *dac*, the activation of *eve* stripe 2 integrates both repressors and activators (Small et al., 1992). Moreover, for *eve* stripe 2 activation, the ratio of activation:repression is more important than the absolute amount of activation (Arnosti et al., 1996). By analogy, *dac* may be able to be activated by a wide range of Wg levels, as long as there is a compensatory change in the levels of Brk.

A model for asymmetric *dac* expression and leg disc development

Another previously unexplained aspect to leg development is that *dac* expression, both when it is first activated and throughout all of development, is asymmetric: there are many more cells expressing *dac* dorsal to the *Dll* domain than there are lateral or ventral to the *Dll* domain. If *dac* were simply activated by lower levels of Dpp and Wg, it is difficult to account for this asymmetry. We suggest that the activation of *dac* by specific ratios of Brk:Wg, together with the asymmetric expression of *brk* in the leg, accounts for this asymmetry. The absence of *brk* expression in the dorsal leg disc allows those cells to be more sensitive to activation by Wg signaling. By contrast, lateral or ventral cells express *brk*, making them less responsive to Wg input. This idea is supported by our experiments in which we varied the Brk:Wg ratio. Most informative are the brk^{XA}; $wg^{CX3/CX4}$ leg discs, which have nearly symmetrical and circular *dac* and *Dll* expression domains (Fig. 5C). We suggest that these phenotypes result from a reduction in both Wg signaling and Brk levels. Less Wg results in less *dac* activation in dorsal cells, but the Wg that remains is more effective at activating *dac* ventrally and laterally owing to lower Brk levels. Thus, we suggest that the shape of the *dac* expression domain, and ultimately the shape of the adult appendage, is governed by the ratio of Brk:Wg and the responses to these inputs by key genes such as *Dll* and *dac*.

Revised model for PD axis formation

Prior to this study, the model for PD axis formation in the leg failed to explain how the activities of Wg and Dpp, present in ventral and dorsal gradients, respectively, are integrated to create the PD axis. In light of our data, we suggest the following steps in PD axis formation (Fig. 7D). First, we suggest that *Dll* is activated only in cells that are receiving high Dpp and Wg signals, because it is kept off in most of the disc due to repression by *brk*. Based on previous results (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997), we also posit that high levels of both signals repress *dac*, keeping this gene switched off in cells that activate *Dll*. As the disc grows, we suggest that *dac* is activated in cells that have the appropriate ratios of Brk:Wg inputs, as outlined above, and that *Dll* cannot be activated by these input ratios.

Once the initial *dac* and *Dll* domains are initially defined, we further suggest that they are locked into place and maintained by a mechanism that no longer requires Wg and Dpp inputs. This idea is consistent with previous genetic studies showing that the requirement of *Dll* and *dac* for Wg and Dpp inputs is transient (Galindo et al., 2002; Lecuit and Cohen, 1997). Once activated, we suggest that an autoregulatory and/or Polycomb-dependent mechanism can create the three main domains that exist along the mature PD axis: a distal Dll-only domain, a medial Dll+Dac domain and more proximal Dac-only domain. The Dll-only and Dac-only domains are simplest to understand, as they are derived from cells that were only able to activate one of these two genes because of the Brk:Wg input they received. We suggest that the domain that co-expresses *Dll* and *dac* is derived from cells present at the interface between the initial *dac* and *Dll* activation domains, which have, by virtue of their position in the leg disc, the potential to activate both genes. Once both *Dll* and *dac* are activated, even in the same cell, we imagine that they, too, become locked into an expressed state by an autoregulatory and/or Polycomb-dependent mechanism. A recent analysis of the cis-regulatory elements controlling *Dll* expression in the *Drosophila* leg is consistent with this model (Estella et al., 2008). In particular, *Dll* expression in the leg disc uses two *cis*-regulatory elements. One directly receives input from Wg and Dpp and, consequently, is only active in the center of the leg disc where both signals meet. The second element maintains this initial expression at least in part by via an autoregulatory mechanism, by directly binding Dll (Estella et al., 2008). In the future, it will be important to further test the model proposed here by analyzing the regulatory elements controlling *dac* during leg development.

We thank Gerarld Campbell, Gary Struhl, Konrad Basler, Miriam Zecca, Ze'ev Paroush, Daniel McKay and Andrew Tomlinson for reagents, and Gary Struhl, Andrew Tomlinson, Laura Johnston, Gines Morata, Daniel McKay, Matt Giorganni, Francisco A. Martin and Hector Herranz for comments on the manuscript. This work was supported by NIH and March of Dimes grants to R.S.M., and by funding from the Spanish Ministry to C.E.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/4/627/DC1

References

Abu-Shaar, M. and Mann, R. S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* **125**, 3821-3830.

Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. (2001). Nuclear interpretation of Dpp signaling in Drosophila. *EMBO J.* **20**, 3298-3305.

Arnosti, D. N. (2003). Analysis and function of transcriptional regulatory elements: insights from Drosophila. *Annu. Rev. Entomol.* **48**, 579-602.

Arnosti, D. N., Barolo, S., Levine, M. and Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development* **122**, 205-214.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev.* **16**, 1167-1181.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein *Nature* **368**, 208-214.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. and Gelbart, W. M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. *Development* **111**, 657-666.

Brand, A. H., Manoukian, A. S. and Perrimon, N. (1994). Ectopic expression in Drosophila. *Methods Cell Biol.* **44**, 635-654.

Brannon, M., Brown, J. D., Bates, R., Kimelman, D. and Moon, R. T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development* **126**, 3159-3170.

Brantjes, H., Roose, J., van De Wetering, M. and Clevers, H. (2001). All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res.* **29**, 1410-1419.
Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg. *Science* **273**, 1373-1377.
Campbell, G. (2002). Distalization of the *Drosophila* leg by graded EGF-receptor activity. *Nature* **418**, 781-785.
Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-562.
Campbell, G., Weaver, T. and Tomlinson, A. (1993). Axis specification in the developing Drosophila appendage: the role of wingless, decapentaplegic, and the homeobox gene aristaless. *Cell* **74**, 1113-1123.
Castelli-Gair, J. and Akam, M. (1995). How the Hox gene Ultrabithorax specifies two different segments: the significance of spatial and temporal regulation within metameres. *Development* **121**, 2973-2982.
Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-608.
Couso, J. P., Bate, M. and Martinez-Arias, A. (1993). A wingless-dependent polar coordinate system in Drosophila imaginal discs. *Science* **259**, 484-489.
Diaz-Benjumea, F. J., Cohen, B. and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. *Nature* **372**, 175-179.
Estella, C., Rieckhof, G., Calleja, M. and Morata, G. (2003). The role of buttonhead and Sp1 in the development of the ventral imaginal discs of Drosophila. *Development* **130**, 5929-5941.
Estella, C., McKay, D. and Mann, R. (2008). Molecular integration of Wingless, Decapentaplegic, and autoregulatory inputs into *Distalless* during *Drosophila* leg development. *Dev. Cell* **14**, 86-96.
Galindo, M. I., Bishop, S. A., Greig, S. and Couso, J. P. (2002). Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science* **297**, 256-259.
Grimm, S. and Pflugfelder, G. O. (1996). Control of the gene optomotor-blind in Drosophila wing development by decapentaplegic and wingless. *Science* **271**, 1601-1604.
Guss, K. A., Nelson, C. E., Hudson, A., Kraus, M. E. and Carroll, S. B. (2001). Control of a genetic regulatory network by a selector gene. *Science* **292**, 1164-1167.
Hasson, P., Muller, B., Basler, K. and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *EMBO J.* **20**, 5725-5736.
Hasson, P., Egoz, N., Winkler, C., Volohonsky, G., Jia, S., Dinur, T., Volk, T., Courey, A. J. and Paroush, Z. (2005). EGFR signaling attenuates Groucho-dependent repression to antagonize Notch transcriptional output. *Nat. Genet.* **37**, 101-105.
Hays, R., Buchanan, K. T., Neff, C. and Orenic, T. V. (1999). Patterning of Drosophila leg sensory organs through combinatorial signaling by hedgehog, decapentaplegic and wingless. *Development* **126**, 2891-2899.
Ingham, P. W. (1991). Segment polarity genes and cell patterning within the Drosophila body segment. *Curr. Opin. Genet. Dev.* **1**, 261-267.
Istrail, S. and Davidson, E. H. (2005). Logic functions of the genomic cis-regulatory code. *Proc. Natl. Acad. Sci. USA* **102**, 4954-4959.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.
Jiang, J. and Struhl, G. (1996). Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during Drosophila leg development. *Cell* **86**, 401-409.
Johnston, L. A. and Schubiger, G. (1996). Ectopic expression of wingless in imaginal discs interferes with decapentaplegic expression and alters cell determination. *Development* **122**, 3519-3529.
Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. *J. Biol. Chem.* **276**, 18216-18222.
Lawrence, N., Dearden, P., Hartley, D., Roose, J., Clevers, H. and Arias, A. M. (2000). dTcf antagonises Wingless signalling during the development and patterning of the wing in Drosophila. *Int. J. Dev. Biol.* **44**, 749-756.
Lawrence, P. A. and Morata, G. (1977). The early development of mesothoracic compartments in Drosophila. An analysis of cell lineage and fate mapping and an assessment of methods. *Dev. Biol.* **56**, 40-51.
Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the Drosophila leg. *Nature* **388**, 139-145.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible neurotechnique cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Mann, R. S. and Carroll, S. B. (2002). Molecular mechanisms of selector gene function and evolution. *Curr. Opin. Genet. Dev.* **12**, 592-600.
Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. *Development* **120**, 3473-3486.
Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* **2**, 745-749.
Merika, M. and Thanos, D. (2001). Enhanceosomes. *Curr. Opin. Genet. Dev.* **11**, 205-208.
Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.
Morata, G. (2001). How Drosophila appendages develop. *Nat. Rev. Mol. Cell Biol.* **2**, 89-97.
Morimura, S., Maves, L., Chen, Y. and Hoffmann, F. M. (1996). decapentaplegic overexpression affects Drosophila wing and leg imaginal disc development and wingless expression. *Dev. Biol.* **177**, 136-151.
Moser, M. and Campbell, G. (2005). Generating and interpreting the Brinker gradient in the Drosophila wing. *Dev. Biol.* **286**, 647-658.
Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.
Nellen, D., Affolter, M. and Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by *decapentaplegic*. *Cell* **78**, 225-237.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.
Penton, A. and Hoffmann, F. M. (1996). Decapentaplegic restricts the domain of wingless during Drosophila limb patterning. *Nature* **382**, 162-164.
Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K. and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during Drosophila development. *Dev. Cell* **7**, 229-240.
Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608-612.
Shubin, N., Tabin, C. and Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. *Nature* **388**, 639-648.
Small, S., Blair, A. and Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the *Drosophila* embryo. *EMBO J.* **11**, 4047-4057.
St Johnston, R. D., Hoffmann, F. M., Blackman, R. K., Segal, D., Grimaila, R., Padgett, R. W., Irick, H. A. and Gelbart, W. M. (1990). Molecular organization of the decapentaplegic gene in Drosophila melanogaster. *Genes Dev.* **4**, 1114-1127.
Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-540.
Tabata, T. and Takei, Y. (2004). Morphogens, their identification and regulation. *Development* **131**, 703-712.
Theisen, H., Haerry, T. E., O'Connor, M. B. and Marsh, J. L. (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development* **122**, 3939-3948.
Theisen, H., Syed, A., Nguyen, B. T., Lukacsovich, T., Purcell, J., Srivastava, G. P., Iron, D., Gaudenz, K., Nie, Q., Wan, F. Y. et al. (2007). Wingless directly represses DPP morphogen expression via an armadillo/TCF/Brinker complex. *PLoS ONE* **2**, e142.
Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J. and Cohen, S. (1992). Homeotic genes of the bithorax complex repress limb development in the abdomen of the drosophila embryo through the target gene *Distal-less*. *Cell* **71**, 437-450.
Valenta, T., Lukas, J. and Korinek, V. (2003). HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells. *Nucleic Acids Res.* **31**, 2369-2380.
Wiersdorff, V., Lecuit, T., Cohen, S. M. and Mlodzik, M. (1996). Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the Drosophila eye. *Development* **122**, 2153-2162.
Wilder, E. L. and Perrimon, N. (1995). Dual functions of wingless in the Drosophila leg imaginal disc. *Development* **121**, 477-488.
Willert, K., Logan, C. Y., Arora, A., Fish, M. and Nusse, R. (1999). A Drosophila Axin homolog, Daxin, inhibits Wnt signaling. *Development* **126**, 4165-4173.
Xu, T. and Rubin, G. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.