# Quantitative effects of *hedgehog* and *decapentaplegic* activity on the patterning of the *Drosophila* wing

## P.W. Ingham and M.J. Fietz*

Molecular Embryology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

**Background:** Members of the *hedgehog* (*hh*) gene family encode a novel class of proteins implicated in positional signalling in both invertebrates and vertebrates. In *Drosophila*, the *hh* gene has been shown to regulate patterning of the imaginal discs, the precursors of the insect limbs. In a remarkably similar fashion, the function and expression of the *sonic hedgehog* (*shh*) gene is closely associated with the 'zone of polarizing activity' (ZPA) that controls antero-posterior patterning of the vertebrate limb. Both of these functions suggest a role for hedgehog family proteins as morphogens. An alternative possibility, however, is that *hh* and its homologues act to control the expression of other instructive signalling molecules.
**Results:** We have explored this issue by examining the effects on *Drosophila* wing patterning of ectopically expressing varying levels of *hh* and *shh*, as well as of the

putative *hh* target gene, *decapentaplegic* (*dpp*), a member of the transforming growth factor-β family of signalling molecules. We find that different levels of *hh* activity can induce graded changes in the patterning of the wing, and that zebrafish *shh* acts in a similar though attenuated fashion. Varying levels of ectopic *hh* and *shh* activity can differentially activate transcription of the *patched* and *dpp* genes. Furthermore, ectopic expression of *dpp* alone is sufficient to induce the pattern alterations caused by ectopic *hh* or *shh* activity.
**Conclusion:** Thus, *hh* family proteins can elicit different responses in a dose-dependent manner in the imaginal disc. The principal function of *hh*, however, is to activate transcription of *dpp* at the compartment boundary, thereby establishing a source of *dpp* activity that is the primary determinant of antero-posterior patterning.

## Background

The segment polarity gene *hedgehog* (*hh*) plays a central role in the development of *Drosophila*, its protein product controlling the specification of positional identity in both the larval and adult body segments [1]. The discovery of a number of *hh* homologues in various vertebrate species [2–5] has established *hh* as a member of a family of highly conserved putative secreted proteins of novel structure; the most notable vertebrate member of the family to date is *sonic hedgehog* (*shh*), the function of which has been implicated in both midline signalling [2–4] and limb patterning [5].

In the *Drosophila* embryo, there is compelling evidence that the Hh protein acts as a short-range signal which regulates the transcription of genes in neighbouring cells. In particular, *hh* activity is required for the maintenance of transcription of *wingless* (*wg*) in cells immediately adjacent to the *hh* expression domain [6,7]. As *wg* itself encodes a signalling molecule [8,9] that regulates the patterning of each larval segment [10,11], the role of *hh* can be seen as maintaining a signalling centre in each parasegment [12].

The involvement of *Drosophila hh* in the patterning of imaginal discs presents some striking parallels with the presumed role of its vertebrate homologue in limb patterning [5]. Although expression of *hh* is restricted to the posterior portion of each disc, coinciding precisely with the posterior lineage compartment [13–15], its

activity is required for the normal patterning of the entire disc [1,16]. Ectopic activation of *hh* in the anterior compartments of imaginal discs can induce the duplication of anterior compartment structures [16–18]. While this finding could suggest a role for Hh as a morphogen, it seems more likely that, as in the embryo, it acts to regulate the expression of some other signalling molecule.

In the case of the wing imaginal disc, the best candidate for such a signal is the product of the *decapentaplegic* (*dpp*) gene, a member of the transforming growth factor-β family of secreted signalling molecules. Although *dpp* activity is required for the development of the entire wing imaginal disc [19], its transcription is limited to a narrow band of cells adjacent to the *hh* expression domain at the anterior–posterior compartment boundary [20]. Expression of *dpp* along the compartment boundary requires *hh* expression [16]; and ectopic expression of *hh* results in the ectopic activation of *dpp* [15–18]. Thus, *hh* seems to act in the imaginal disc to maintain the source of a signalling molecule at the compartment boundary, just as in the embryo it maintains the expression of *wg* at the parasegment boundary [6,7].

In this study, we have explored further the relationship between the activity of *hh* and *dpp*, using the GAL4/UAS system developed by Brand and Perrimon [21]. In particular, we have examined the effects of varying levels of ectopic *hh* and *dpp* activity on wing patterning, either by manipulating the levels of transcriptional activation of each gene or, in the case of *hh*, by substituting its expression

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*Present address: Department of Biochemistry, University of Tasmania, Hobart, Tasmania, Australia. Correspondence to: P.W. Ingham.

with that of the zebrafish *shh* gene. Our results demonstrate that different levels of *hh* activity can elicit different responses at the level of transcriptional activation, but suggest that, in normal development, the control of growth and patterning of the imaginal disc by *hh* is mediated principally through its regulation of *dpp* transcription.

## Results

To investigate the postulated functional relationship between *hh* and *dpp* activity in imaginal discs, we used the GAL4 expression system [21] to activate transcription

of either gene inappropriately in the same cell populations in developing imaginal discs. For this purpose, we constructed UAS*hh* transgenic fly lines, in which a cDNA fragment including the entire *hh* open reading frame is cloned downstream of the GAL4-dependent upstream activating sequence (UAS). Similar lines carrying the *dpp* open reading frame downstream of UAS [22] were kindly provided by M. Hoffman. A number of GAL4 enhancer trap lines (kindly provided by A. Brand and N. Perrimon) were screened for their ability to activate UAS*hh* in imaginal discs without early development being compromised. Experiments using two of these lines, 30A [21] and 34B, are described in this study.
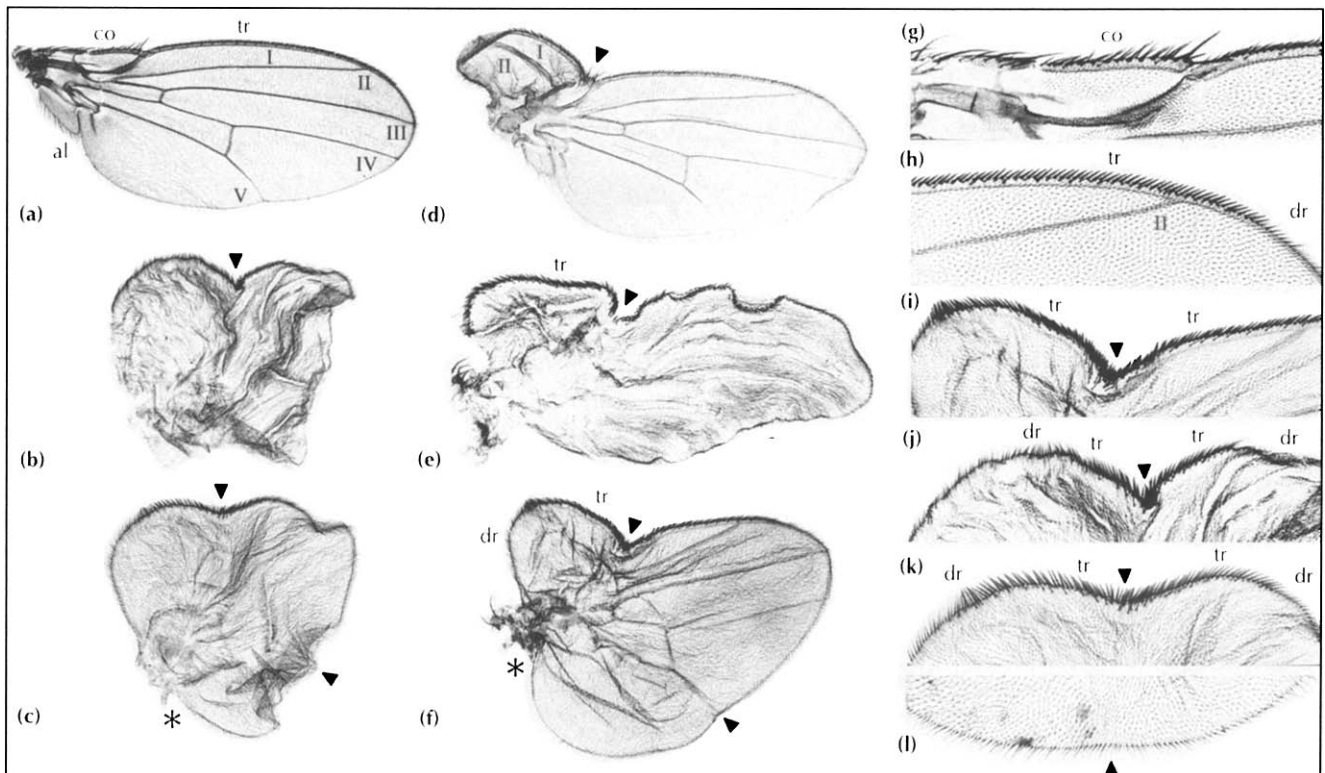


**Fig. 1.** Reorganization of wing patterning by ectopic expression of *hh*, *shh* and *dpp* in the GAL4 enhancer trap line 30A. Wings are arranged with their distal tips to the right. **(a)** Wild-type wing; veins I–III are in the anterior compartment, whereas veins IV and V are in the posterior compartment. Note the anterior proximal structure, the costa (co, shown in detail in **(g)**), and the posterior proximal structure, the alula (al). The anterior margin is characterized by triple row bristles (tr) proximally, and by double row (dr) bristles distally (shown in detail in **(h)**). **(b)** Wing blade dissected from a 30A*hh* pharate adult cultured at 25 °C. The proximal region of the anterior compartment is eliminated and replaced by more distal structures duplicated with reversed polarity; the axis of duplication, indicated by the arrowhead, lies quite distally, and the duplicated structure includes correspondingly few triple row bristles (seen more clearly at higher magnification in **(j)**). **(c)** Wing dissected from a 30A*dpp* pharate adult grown at 25 °C. The proximal regions of both compartments are replaced by more distal structures, the arrowheads marking the duplication axes. These are quite distally located, as evidenced by the reduction of triple row bristles along the anterior margin (shown in detail in **(k)**); in the anterior compartment, the effect is very similar to that caused by ectopic *hh* expression (compare (j) and (k)). Note the absence of the alula in the posterior compartment (∗). **(d)** Wing blade of a 30A*shh* fly. The proximal and medial segments of the costa are eliminated and replaced by a mirror-image duplication of more distal wing blade material, including veins I and II and marginal triple row bristles. The duplication axis is much more proximal than in a 30A*hh* wing, lying in the distal costa, and is indicated by the arrowhead. **(e)** Wing blade dissected from a 30A*hh* pharate adult cultured at 18 °C. The proximal region of the anterior compartment is eliminated and replaced by more distal structures duplicated with reversed polarity (for instance, triple row bristles replace the more proximal costa). The arrowhead marks the axis of duplication; note that this lies much more proximally than in flies of the same genotype raised at 25 °C (compare with (b)). **(f)** Wing of a 30A*dpp* fly cultured at 18 °C. In the anterior compartment, the costa is eliminated and replaced by more distal wing blade bounded by triple row and double row marginal bristles (shown in detail in **(i)**). The axis of duplication (arrowhead) lies just distal to the costa, much more proximally than in flies of the same genotype raised at 25°C. In the posterior compartment there is an analogous replacement of proximal structures by distal structures. This is most obviously manifest in the dramatic enlargement of the posterior wing blade and by the absence of the alula (∗). The arrowhead indicates the location of the duplication axis, revealed by the reversal of polarity of the marginal hairs (see **(l)**).

## Respecification of the wing anterior compartment by ectopic *hh*

The enhancer trap line 30A [21] expresses GAL4 in a broad ring of cells corresponding to the proximal region of the presumptive wing blade (see below, Fig. 2d). Expression of *hh* driven by this GAL4 line results in flies (designated 30A*hh*) that die as uneclosed pupae and exhibit a dramatic respecification of the anterior compartments of their wings (Fig. 1b). The proximal anterior wing structure, the costa (Fig. 1a,g), is completely eliminated; in addition, much of the triple row of bristles present on most of the anterior wing margin (Fig. 1a,h) is replaced by double row bristles, typical of the most distal part of the anterior margin. These structures are duplicated with reversed polarity, the axis of duplication lying close to the region where vein II normally meets the anterior margin. As well as effects on anterior wing structures, 30A*hh* flies also show a consistent duplication of notal structures on either side of the notum (data not shown). In contrast to the effects on the anterior wing, however, the posterior compartment is unaffected, with proximal structures, such as the axilliary cord and alula, differentiating normally.

To investigate the effect of lower levels of ectopically expressed *hh*, we took advantage of the temperature sensitivity of the GAL4 protein. 30A*hh* flies raised at 18 °C are also pupal-lethal and exhibit similar types of duplication of the anterior wing (Fig. 1e). However, the axis of duplication in these flies is located more proximally than in their siblings raised at the higher temperature, and fewer structures are eliminated from the original wing; in addition, there is no duplication of notal structures (data not shown).

## Ectopic expression of the zebrafish *shh* gene has a similar though attenuated effect on imaginal disc patterning

Previous studies have shown that the signalling activity of *hh* in the *Drosophila* embryo has been conserved during
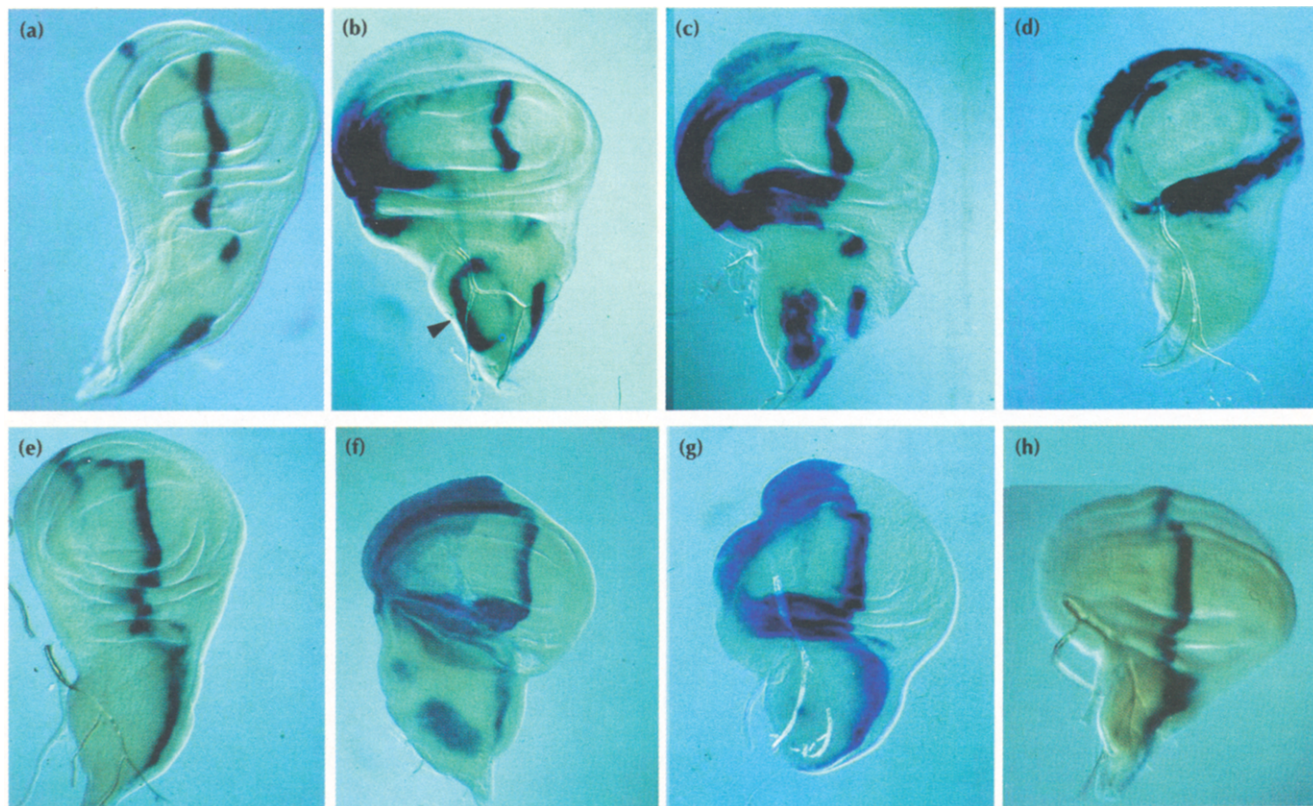


**Fig. 2.** Reporter gene expression patterns in mutant and wild-type mesothoracic (wing) imaginal discs. Anterior is to the left in all cases. **(a)** Expression of a *dpp–lacZ* reporter gene in a wild-type mesothoracic disc, showing the expression domain of the endogenous *dpp* gene along the anterior–posterior compartment boundary. **(b)** Pattern of *dpp–lacZ* reporter gene expression in a 30A*hh* mesothoracic disc. Note the ectopic activation of the reporter gene at the anterior margin of the disc and, in addition, in the presumptive notum (arrowhead). **(c)** *dpp–lacZ* reporter gene expression in a 30A*shh* mesothoracic disc. In contrast to 30A*hh* discs (b) expression of the reporter gene is more widespread and nearly co-extensive with the 30A expression domain (d). **(d)** Expression of a UAS–*lacZ* reporter gene driven by GAL4 in the 30A enhancer trap line. This shows that GAL4 is expressed in a ring of cells corresponding to the proximal region of the wing blade. **(e)** Expression of a *ptc–lacZ* reporter (from the H84 enhancer trap line) in a wild-type disc. Although endogenous *ptc* is expressed throughout the anterior compartment, this line reveals only the domain where transcription is enhanced in response to *hh* activity along the antero–posterior compartment boundary. **(f)** Ectopic *ptc–lacZ* expression in a 30A*hh* disc. Note the ectopic expression in the anterior wing blade (compare with the wild-type pattern in (e)); this is very similar to, though slightly more extensive than, the *dpp–lacZ* pattern in 30A*shh* discs (c). **(g)** Ectopic *ptc–lacZ* expression in a 30A*shh* disc. The pattern of ectopic expression is very similar to that induced by *hh* driven by the same GAL4 line (f). **(h)** Expression of the *ptc–lacZ* reporter gene in a 30A*dpp* wing disc. Despite the enlargement of both the anterior and posterior compartments, the size and relative position of the *ptc* expression domain is unchanged compared to wild type.

vertebrate evolution, the zebrafish *shh* gene being capable of activating *wg* expression when overexpressed during *Drosophila* embryogenesis [3]. To determine whether *hh* activity in imaginal discs has been similarly conserved, we cloned a cDNA fragment containing the entire open reading frame of the zebrafish *shh* gene [3] downstream of the UAS sequences in the vector pUAST, and generated transgenic flies carrying this construct (see Materials and methods).

In contrast to their 30A*hh* counterparts, most 30A*shh* flies eclose, but like 30A*hh* flies, they exhibit an invariant effect on the patterning of the anterior wing (Fig. 1d). In this case, the axis of duplication is located very proximally, in the distal costa, the rest of the costa being eliminated and replaced by a mirror-image duplication of anterior wing blade, bounded by triple row marginal bristles and including veins I and II. In contrast to 30A*hh* flies, there is no duplication of notal structures.

### Reorganization of the anterior wing by *hh* and *shh* is presaged by ectopic expression of *dpp* and *ptc*

To analyze the effects of ectopic *hh* and *shh* activity on imaginal disc cells prior to their differentiation, we monitored the transcription of *dpp* using a *dpp–lacZ* reporter construct that accurately reflects the wild-type *dpp* transcription pattern [20] (see Fig. 2a). Wing discs of 30A*hh* flies show a significant enlargement of their anterior compartments compared to wild type. The *dpp* reporter gene is activated ectopically in an arc of cells at the anterior margin of the enlarged disc and, in addition, in a patch of cells in the presumptive notum (Fig. 2b). Notably, the ectopic *dpp* expression domain in the anterior wing blade is not co-extensive with the 30A expression domain, as revealed by a UAS–*lacZ* reporter gene (compare Fig. 2b and d). Indeed, it corresponds to a region of the disc where the 30A enhancer appears relatively inactive, suggesting that *dpp* transcription is activated only by low levels of *hh* activity.

To investigate this possibility further, we analyzed Hh protein accumulation and *dpp–lacZ* reporter activity simultaneously, using antibodies directed against *Drosophila* Hh and *Escherichia coli* β-galactosidase. Hh protein is localized to cells within the 30A expression domain, as expected if the protein does not diffuse significantly, and
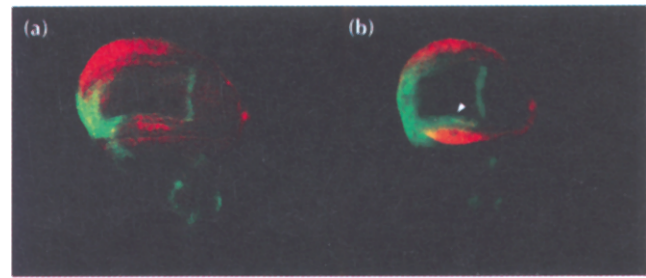


**Fig. 3.** Expression of *dpp–lacZ* relative to the Hh or Shh protein distribution in 30A*hh* and 30A*shh* wing discs. **(a)** 30A*hh* wing disc showing the distribution of Hh (red) and β-galactosidase (green) proteins. Any overlap between the two proteins appears as orange or yellow. Note that the distribution of the two proteins appears almost mutually exclusive, the *dpp–lacZ* reporter being activated in cells where Hh levels are below the level of detection. Note also the absence of *dpp–lacZ* induction in cells adjacent to those expressing Hh at high levels. The levels of ectopic Hh driven by the 30A line are well above those of the endogenous protein, which is restricted to the posterior compartment and is barely visible under these conditions. **(b)** 30A*shh* wing disc, showing the distribution of Shh protein and the activation of the *dpp–lacZ* reporter. In this case, the expression of the *dpp–lacZ* reporter is much more widespread (see also Fig. 2c) and there is significant overlap with cells expressing Shh. In addition, the reporter construct is activated in cells adjacent to those expressing Shh (arrowhead).

reaches its highest levels in cells in which the 30A enhancer appears maximally active (Fig. 3). As expected, expression of the *dpp–lacZ* reporter is limited to those regions where the levels of ectopic Hh protein are lowest. In wing discs from 30A*shh* larvae, by contrast, activation of the *dpp–lacZ* reporter appears much more widespread, occurring in most of the cells of the anterior compartment in which the 30A enhancer is active. Simultaneous visualization of β-galactosidase and Shh proteins shows that the *dpp* reporter is activated both within and adjacent to cells expressing the Shh protein.

We also examined the effects of ectopic *hh* and *shh* expression on another target of *hh* activity, the segment polarity gene *patched* (*ptc*), using the *ptc–lacZ* enhancer trap line H84 [23]. In normal development, *ptc* is transcribed at low levels throughout the anterior compartment of each imaginal disc, but the levels of expression are significantly enhanced at the anterior–posterior compartment boundary [24,25] (see Fig. 2e); this localized
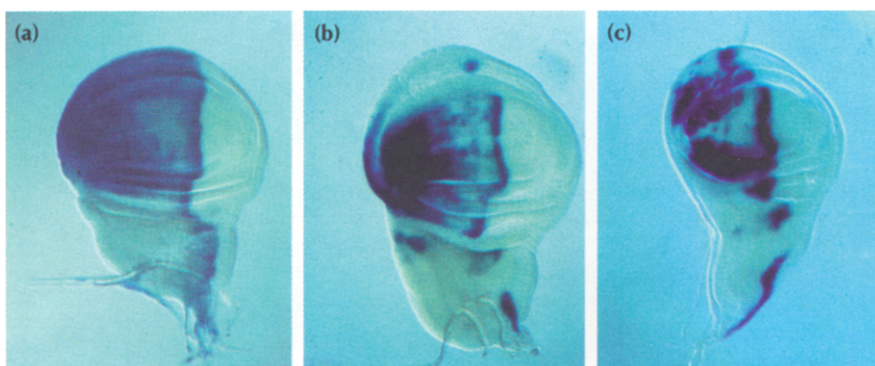


**Fig. 4.** Altered patterns of *dpp* and *ptc* expression in 34B*hh* and 34B*shh* wing imaginal discs. **(a)** Expression of a *ptc–lacZ* reporter gene in a 34B*hh* disc. Expression is activated almost uniformly throughout the entire anterior compartment of the presumptive wing blade. **(b)** Expression of a *dpp–lacZ* reporter gene in a 34B*hh* disc. Ectopic activation of *dpp* is more widespread than in 30A discs, extending through the presumptive anterior wing blade almost up to the compartment boundary, but is not as extensive as that of *ptc* (compare with (a)). **(c)** Expression of a *dpp–lacZ* reporter gene in a 34B*shh* disc.

enhancement of transcription depends upon *hh* activity [26] and mirrors the regulatory relationship between *hh* and *ptc* in the embryo [23]. In contrast to the differential response of *dpp* to varying levels of *hh* activity, *ptc* transcription is activated throughout the 30A expression domain in the anterior compartments of both 30A*hh* and 30A*shh* wing discs (Fig. 2f,g; compare with Fig. 2b,c). The finding that neither *dpp* nor *ptc* expression is activated by 30A-driven *hh* or *shh* expression in the posterior compartment is not surprising: *hh* is normally expressed throughout the posterior compartment but does not activate *dpp* or *ptc* transcription there. This is most likely due to the specific repression of both genes by the activity of *engrailed*, which is known to repress *ptc* transcription in the embryo [6].

### Ectopic activation of *dpp* in 30A flies respecifies both the anterior and the posterior compartment

To investigate whether the ectopic *dpp* expression observed in the wing discs of 30A*hh* and 30A*shh* larvae is sufficient to account for the pattern duplications induced by both, we used the same GAL4 line to activate *dpp* itself in the identical region of the developing wing imaginal disc. Most such 30A*dpp* flies die as pharate adults when raised at 25 °C and exhibit gross pattern alterations in their wings (Fig. 1c). Contrary to the recent paper of Capdevila and Guerrero [17], we find that, in the anterior compartment, these alterations are indistinguishable from those seen in 30A*hh* flies raised at the same temperature (compare Fig. 1c and d). Proximal structures (the costa and the proximal half of the wing margin) are eliminated and replaced by more distal structures with reversed polarity. Strikingly, and in contrast to 30A*hh* wings, an analogous duplication is also induced in the posterior compartment. This is most clearly revealed by the elimination of the alula and its replacement by marginal hairs that show reversed polarity (Fig. 1l). Unlike 30A*hh* flies, there is no duplication of notal structures in 30A*dpp* flies (data not shown).

When raised at 18 °C, most 30A*dpp* flies eclose; the wings show the same kinds of pattern abnormalities described above, but the axes of duplication are shifted proximally (Fig. 1f). Thus, fewer proximal structures are eliminated from the original wing, while the duplicated structures include correspondingly more proximal structures. In the anterior compartment, the duplicated structure is similar to that induced in 30A*shh* wings, consisting of a region of the wing blade including veins I and II, and bounded by anterior marginal triple row bristles.

The similarities between the effects of ectopic *hh*, *shh* and *dpp* when driven by the same GAL4 line, together with the ectopic activation of *dpp* by ectopic *hh* or *shh* activity, strongly suggest that the Hh family proteins act via induced *dpp* activity. That ectopic *dpp* also effects the patterning of the posterior compartment suggests that it is normally responsible for patterning both compartments in the wild-type wing. Using the *ptc–lacZ* reporter gene, we analyzed the expression of *ptc* in 30A*dpp* wing discs.

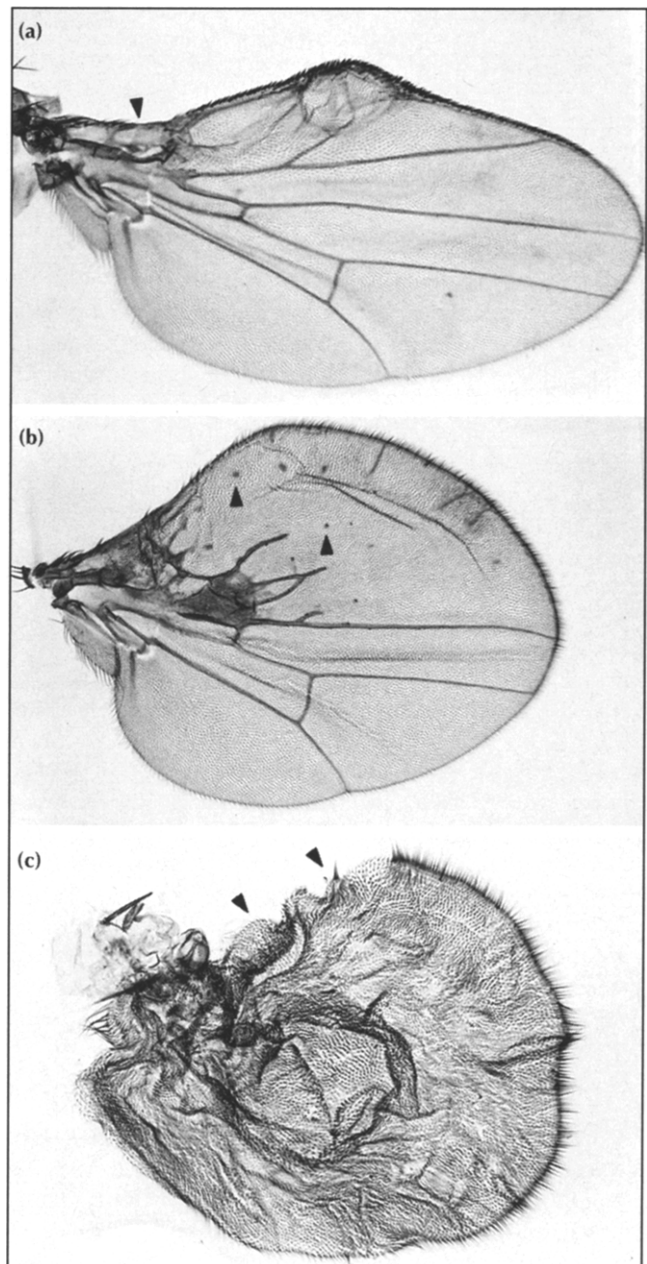Although such discs exhibit considerable overgrowth of both anterior and posterior compartments, the position



**Fig. 5.** Varying effects of ectopic *hh* and *shh* expression in the GAL4 line 34B. **(a)** Wing of a 34B*hh* fly raised at 18 °C. Note the suppression of the costa (arrowhead) and the overgrowth in the anterior compartment resulting in a bulge in the anterior margin (associated with a partial duplication of vein II) and an increase in the distance between veins II and III (compare with Fig. 1a). **(b)** Wing blade from a 34B*shh* fly. Note the replacement of triple row bristles along the anterior margin by double row bristles. In addition, veins I and II are eliminated and replaced with multiple truncated vein III tissue bearing the characteristic sensilla campaniformia; the latter also form in isolation in the anterior wing blade (arrowheads). **(c)** The wing blade from an uneclosed 34B*hh* pupa reveals a dramatic respecification of positional identity of cells in the anterior compartment. This is most easily seen along the wing margin, where all of the triple row bristles are eliminated and replaced by double row bristles distally and naked margin proximally (arrowhead).

and size of the domain of elevated *ptc* expression remains the same as in wild-type discs (Fig. 2h). Thus, the regulation of *ptc* transcription is independent of *dpp* and is a function of the juxtaposition of anterior and posterior cells rather than of the positional identity of cells within the disc.

### Altered positional identity correlates with ectopic *dpp* expression

The pattern duplications induced by the establishment of a second localized source of *dpp* in the presumptive proximal wing of 30A*hh* flies are consistent with *dpp* acting in a graded manner to specify different positional values. To investigate this interpretation further, we looked for lines in which UAS target genes are more homogeneously expressed. One such line, 34B, was identified on the basis of its phenotype when expressing a UAS*hh* target gene.

Low levels of Hh protein are detectable throughout most of the anterior compartment of the prospective wing blade of 34B*hh* imaginal discs (data not shown) and, concomitantly, expression of *ptc* is activated almost uniformly throughout this region (Fig. 4a), while expression of *dpp* is widespread, extending from the anterior edge of the disc almost to the compartment boundary (Fig. 4b). On differentiation of the wing, all triple row bristles are eliminated from the anterior margin, such that it is devoid of bristles proximally and bears only double row bristles distally (Fig. 5c). Within the wing blade there are multiple campaniform sensillae, characteristic of vein III, indicating a shift in the positional specification of cells towards identities typical of the centre of the normal wing, where *dpp* is normally transcribed. Thus, there appears to be a close correlation between the expression of *dpp* and the positional identity of cells revealed by the structures into which they differentiate. At 18 °C, 34B*hh* flies are fully viable and show only minor disruption of patterning of the venation in the anterior compartment (Fig. 5a); ectopic Hh protein is barely detectable under these conditions (data not shown).

Ectopic *shh* expression in 34B flies similarly results in a stronger phenotype than in 30A flies, though again the effects are attenuated compared to those of the *Drosophila hh* gene. As in 34B*hh* flies, in 34B*shh* flies the triple row bristles of the anterior margin are replaced by double row bristles. As many 34B*shh* flies survive to adulthood, it is possible to analyze the venation patterns. Veins I and II are both eliminated and replaced by a plexate structure with many supernumary campaniform sensillae, indicative of vein III character (Fig. 5b).

## Discussion

One of the central questions in the analysis of the function of Hh family proteins in both vertebrates and invertebrates concerns the dichotomy between short-range versus long-range modes of signalling. Whilst there is compelling evidence that *hh* acts as a short-range signal

to maintain the transcription of *wg* in the *Drosophila* embryo, the effects of *hh* mutations on the patterning of the dorsal larval cuticle have been taken as evidence for a long-range, morphogen-like activity of the protein at later stages of embryogenesis [27]. In vertebrates, *Shh* has been implicated in the induction of the floor plate, a classic example of a short-range, contact-dependent inductive interaction. However, *in vitro* assays that demonstrate this activity, in which neural plate explants are combined with *Shh*-expressing cells, also reveal the induction of motor neuron differentiation [4].

Whether or not this latter effect represents an indirect consequence of the induction of the floor plate [28], or an additional direct effect of *Shh* activity on neural plate cells, remains an open question. In the latter case, however, this would entail a dual mode of action for *Shh*, as motor neuron differentiation, in contrast to that of the floor plate, is known to be induced by a diffusible notochord-derived signal [29]. The recent finding that COS cells transfected with *Shh* can induce *Pax1* expression in somitic mesoderm in a long-range, contact-independent manner [30], provides strong support for such a role for *Shh*. And the association of the 'zone of polarizing activity' (ZPA), classically regarded as the source of a long-range morphogen, with *Shh* activity adds further weight to this interpretation.

Here, we have shown that varying levels of ectopic *hh* activity can induce graded effects on the patterning of the *Drosophila* wing, effects that are consistent with Hh family proteins acting as long-range morphogens. In this view, different levels of *hh* activity would be responsible for eliciting different positional identities within the developing imaginal discs. An alternative interpretation, however, is suggested by the finding that ectopic *hh* expression results in the ectopic transcriptional activation of *dpp* [15–18], a finding that we have confirmed and extended in this analysis. This interaction suggests that, as in the embryo, where *hh* acts by controlling the transcription of the signal-encoding gene *wg*, the principal role of *hh* in the imaginal discs may be to regulate the transcription of *dpp*, the secreted product of which would in turn specify positional identity within the disc. Compelling support for this interpretation is provided by our demonstration that ectopic *dpp* expression alone is sufficient to induce pattern duplications similar to those generated by ectopic *hh* expression, as also recently reported by Capdevila and Guerrero [17]. In addition, we have shown that varying the levels of *dpp* activity results in graded effects on wing patterning that parallel the variable effects induced by differing levels of *hh* activity. Thus, the simplest explanation for the graded effects of varying *hh* activity is that they in turn lead to varying levels of *dpp* transcription.

We therefore favour a model in which *hh* acts to establish a source of *dpp* activity in the centre of the developing imaginal disc, the activity of *dpp* emanating from this source acting as the primary determinant of positional

identity along the antero-posterior axis of the wing. Such an instructive role for *dpp* is suggested by the close correlation between the levels of *dpp* activity and the positional identity of cells within the wing. Thus, while lowering the level of *dpp* expression in 30A flies through the temperature sensitivity of GAL4 results in a shift towards more antero-proximal identity within the duplicated structure, the widespread expression of *dpp* induced in the anterior compartments of 34B*hh* wing discs results in most cells adopting identities more appropriate to cells close to the compartment boundary, where the levels of *dpp* are normally at their highest. That *dpp* acts to pattern both the anterior and posterior compartments of the wing is indicated by our finding that ectopic *dpp* expression induces pattern duplications in both.

One unexpected and paradoxical finding of our analysis is the differential response of cells to ectopic *hh* activity. Thus, while *ptc* transcription is activated wherever *hh* is ectopically expressed in 30A flies, only a subset of these cells also activate the *dpp* reporter gene. A similar restriction in the activation of *dpp* was also noted by Capdevila and Guerrero [17], who interpreted it in terms of a restriction in the competence of cells to activate *dpp* in response to *hh* activity. However, our finding that *dpp* reporter activation is essentially co-extensive with the distribution of Shh protein driven by 30A argues against such an explanation. Instead, we suggest that transcriptional activation of *dpp* is sensitive to the levels of *hh* activity: this would explain why *dpp* is activated only where the levels of ectopic *hh* are at their lowest, whereas *shh*, which we presume to have an intrinsically lower activity in the fly than the endogenous gene, activates *dpp* essentially wherever it is expressed.

This still leaves us with the paradoxical situation that less extreme effects on the patterning of the wing are associated with more extensive ectopic expression of *dpp*. One explanation could be that *hh* activity contributes to the pattern respecification independently of its effects on *dpp*; however, as ectopic expression of *dpp* alone is sufficient to induce precisely the same pattern respecification as that induced by ectopic *hh* expression, we consider this to be unlikely. Rather, we favour the notion that, although more spatially restricted, the levels of *dpp* transcription induced by *hh* are higher than those induced by *shh*. Thus, increasing levels of *hh* activity would lead to increasing levels of *dpp* transcription up to a certain threshold level, above which such activation would not occur, perhaps due to saturation of the Hh receptor by its ligand.

That *shh* can elicit responses similar to *hh* in the imaginal disc as well as in the embryo [3] indicates that these two aspects of *hh* function are most likely mediated by the amino-terminal portion of the protein, where most of the homology between Hh and Shh resides [2,3]. As in the embryo, both proteins appear to act by antagonizing the activity of the transmembrane protein Ptc, their ectopic activity causing the up-regulation of *ptc* transcription,

presumably by blocking the auto-repression of *ptc* transcription [6]. By contrast, we show here that ectopic *dpp* activity has no effect on *ptc* transcription, confirming that it acts downstream of *ptc* and *hh*.

Our findings underline the remarkable parallels between the roles of *hh* family genes in the patterning of invertebrate and vertebrate limbs. Whether the effectors of *hh* family activity are also conserved remains to be seen. However, the finding that the gene encoding BMP2, the vertebrate homologue of *dpp*, is transcribed in a domain that overlaps that of *shh* and can be induced ectopically both by ZPA grafts [31] and by ectopic *shh* expression [32], suggests that this may indeed be the case. Despite these analogies, we note that the development of the *Drosophila* wing differs significantly from the vertebrate limb in one major respect: in the latter, the source of polarizing activity is located at the posterior margin of the bud, and grafts of this source result in the duplication of the entire set of digits. This effect contrasts with the duplications induced in the *Drosophila* wing by ectopic *hh*, which are limited to anterior compartment structures.

This difference in behaviour of the two systems reflects the compartmental organization of the *Drosophila* appendages. In effect, each *Drosophila* imaginal disc can be seen as two limb buds juxtaposed in reverse orientation. Thus, while the mechanism that specifies positional identity in each system may be similar, the way in which this mechanism is regulated must be different. In *Drosophila*, the spatial regulation of *hh* is achieved by a lineage-based mechanism that restricts its expression to the posterior compartment. In the vertebrate limb, no such lineage restrictions exist and another mechanism must operate to restrict the spatial expression of *shh* [33].

## Conclusions

The antero-posterior patterning of the wing seems to depend critically on the levels of *dpp* activity to which cells are exposed. In normal development, the source of *dpp* activity is restricted to a population of cells close to the antero-posterior compartment boundary. Confronting non-expressing cells with a second discrete source of *dpp* activity stimulates proliferation and results in the establishment of a second axis in both the anterior and posterior compartments. The induction of uniform levels of *dpp* activity throughout a compartment, by contrast, results in all cells adopting a similar identity. The transcription of *dpp* is controlled by the activity of *hh*, and it is the restricted range of the Hh protein that is responsible for defining the limits of the *dpp* domain. The levels of *dpp* activity appear to be directly proportional to those of *hh*, though above a certain threshold *dpp* is no longer activated by Hh. Thus, while there is no evidence for Hh acting as a long-range signal in the developing imaginal disc, varying levels of ectopic *hh* activity can induce variable effects on patterning typical of those expected of a classically defined morphogen.

## Materials and methods

### UAShh and UASshh construction and germ-line transformation

A 1.9 kb cDNA fragment containing the complete coding region of the *Drosophila hh* gene [34] was cloned into the $w^+$ P-element vector pUAST[21]. The fragment was inserted behind a minimal promoter consisting of five GAL4 binding sites (UASs), which are followed by the *hsp70* gene TATA box, thus allowing tissue-specific activation of the *hh* cDNA when crossed to enhancer trap lines expressing GAL4. The construct was used to transform *Drosophila* embryos using standard microinjection procedures [35], and transgenic lines were selected by eye colour. One line was initially established. The construct was then 'jumped' onto other chromosomes using the Δ2-3 gene [36], and five further lines were established. Three different lines were used in the described experiments to ensure that the observed phenotype was not dependent on insertion site.

A 1.6 kb *EcoRI* fragment containing the entire open reading frame of the zebrafish *shh* gene [3] was also cloned in the desired orientation into pUAST. Transgenic lines were produced using the method described above. Twenty independent lines were obtained, of which two were used to cross to the GAL4 lines.

### Ectopic expression in Drosophila imaginal discs

For the ectopic expression of *hh*, *shh* or *dpp*, flies homozygous for the respective UAS transgenes were crossed to the desired GAL4 lines (provided by A. Brand and N. Perrimon). Flies were cultured at either 18 °C or 25 °C in order to examine the effect of different levels of ectopic transcription of the target genes.

### Preparation of adult tissues

Adult flies and pharate larvae were dissected in 70% ethanol, cleared by incubation in 10 % NaOH at 80 °C for 5 min, dehydrated and mounted in Euparal for examination with the compound microscope.

### Detection of β-galactosidase activity

To detect β-galactosidase activity in imaginal discs, mature third instar larvae were cut in half in *Drosophila* Ringer's. The anterior halves were inverted and fixed and stained as described [37]. Stained discs were then dissected from the carcass in phosphate-buffered saline and mounted in 80 % glycerol for microscopic analysis.

### Analysis of protein distribution in imaginal discs

β-galactosidase protein was detected using a mouse monoclonal antibody (Sigma). Rabbit anti-Hh (A.M. Taylor, unpublished), and anti-Shh [38] antibodies were used at 1:2000 and 1:500 respectively. Fluorescein coupled anti-mouse IgG and Texas red-coupled anti-rabbit IgG were used to detect the primary antibodies and imaged on a BioRad MRC confocal microscope.

## References

1. Mohler J: **Requirements for *hedgehog*, a segment polarity gene, in patterning larval and adult cuticle of *Drosophila*.** *Genetics* 1988, **120**:1061–1072.
2. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, et al.: ***Sonic hedgehog*, a member of a family of putative signaling molecules is implicated in the regulation of CNS and limb polarity.** *Cell* 1993, **75**:1417–1430.
3. Krauss S, Concordet J-P, Ingham PW: **A functionally conserved homolog of the *Drosophila* segment polarity gene *hedgehog* is expressed in tissues with polarising activity in zebrafish embryos.** *Cell* 1993, **75**:1431–1444.
4. Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz i Altaba A, et al.: **Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord.** *Cell* 1994, **76**:761–775.
5. Riddle R, Johnson RL, Laufer E, Tabin C: **Sonic hedgehog mediates the polarizing activity of the ZPA.** *Cell* 1993, **75**:1401–1416.
6. Hidalgo A, Ingham P: **Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene *patched*.** *Development* 1990, **110**:291–302.
7. Ingham PW, Hidalgo A: **Regulation of *wingless* transcription in the *Drosophila* embryo.** *Development* 1993, **117**:283–291.
8. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, Nusse R: **The *Drosophila* homolog of the mouse mammary oncogene *int-1* is identical to the segment polarity gene *wingless*.** *Cell* 1987, **50**:649–657.
9. van den Heuvel M, Nusse R, Johnston P, Lawrence PA: **Distribution of the *wingless* gene product: a protein involved in cell–cell communication.** *Cell* 1989, **59**:739–749.
10. Baker N: **Embryonic and imaginal requirements for *wingless*, a segment polarity gene in *Drosophila*.** *Dev Biol* 1988, **125**:96–108.
11. Bejsovec A, Martinez Arias A: **Roles of *wingless* in patterning the larval epidermis of *Drosophila*.** *Development* 1991, **113**:471–485.
12. Ingham PW: **Segment polarity genes and cell patterning within the *Drosophila* body segment.** *Curr Opin Genet Dev* 1991, **1**:261–267.
13. Lee JJ, von Keller DP, Parks S, Beachy PA: **Secretion and localised transcription suggests a role in positional signalling for products of the segmentation gene *hedgehog*.** *Cell* 1992, **70**:777–789.
14. Tabata T, Eaton S, Kornberg TB: **The *Drosophila hedgehog* gene is expressed specifically in posterior compartment cells and is a target of *engrailed* regulation.** *Genes Dev* 1992, **6**:2635–2645.
15. Tabata T, Kornberg TB: **Hedgehog is a signalling protein with a key role in patterning *Drosophila* imaginal discs.** *Cell* 1994, **76**:89–102.
16. Basler K, Struhl G: **Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein.** *Nature* 1994, **368**:208–214.
17. Capdevila J, Guerrero I: **Targetted expression of the signalling molecule *decapentaplegic* induces pattern duplications and growth alterations in *Drosophila* wings.** *EMBO J* 1994, **13**:4459–4468.
18. Fietz M, Concordet J-P, Barbosa R, Johnston R, Krauss S, McMahon AP, et al.: **The *hedgehog* gene family in *Drosophila* and vertebrate development.** *Development* 1994, **Suppl**:43–51.
19. Posakony LG, Raftery LA, Gelbart WM: **Wing formation in *Drosophila melanogaster* requires *decapentaplegic* gene function along the antero-posterior compartment boundary.** *Mech Dev* 1991, **33**:69–82.
20. Blackman RK, Sanicola M, Raferty LA, Gillevet T, Gelbart WM: **An extensive 3′ *cis*-regulatory region directs the imaginal disc expression of *decapentaplegic*, a member of the TGF-β family in *Drosophila*.** *Development* 1991, **111**:657–665.
21. Brand AH, Perrimon N: **Targeted gene expression as a means of altering cell fates and generating dominant phenotypes.** *Development* 1993, **118**:401–415.
22. Staehlinghampton K, Hoffmann FM, Baylies MK, Rushton E, Bate M: ***dpp* induces mesodermal gene-expression in *Drosophila*.** *Nature* 1994, **372**:783–786.
23. Ingham PW, Taylor AM, Nakano Y: **Role of the *Drosophila patched* gene in positional signalling.** *Nature* 1991, **353**:184–187.
24. Phillips R, Roberts I, Ingham PW, Whittle JRS: **The *Drosophila* segment polarity gene *patched* is involved in a position-signalling mechanism in imaginal discs.** *Development* 1990, **110**:105–114.
25. Forbes AJ: **Segment polarity genes in *Drosophila* development.** D.Phil Thesis: University of Oxford; 1992.
26. Capdevila J, Estrada MP, Sanchez-Herrero E, Guerrero I: **The *Drosophila* segment polarity gene *patched* interacts with *decapentaplegic* in wing development.** *EMBO J* 1994, **13**:71–82.
27. Heemskerk J, Dinardo S: ***Drosophila* hedgehog acts as a morphogen in cellular patterning.** *Cell* 1994, **76**:449–460.

28. Placzek M, Yamada T, Tessier-Lavigne M, Jessell TM, Dodd J: **Control of dorsoventral pattern in vertebrate neural development: induction and polarising properties of the floor plate.** *Development* 1991, **Suppl**:105–122.

29. Yamada T, Pfaff SL, Edlund T, Jessell TM: **Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate.** *Cell* 1993, **73:**673–686.

30. Fan C-M, Tessier-Lavigne M: **Patterning of mammalian somites by the surface ectoderm and the notochord: evidence for scleretome induction by *Sonic hedgehog/Vhh-1.*** *Cell* 1994, **79:**1175–1186.

31. Francis PH, Richardson MK, Brickell PM, Tickle C: **Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb.** *Development* 1994, **120:**209–218.

32. Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C: ***Sonic hedgehog* and Fgf-4 act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud.** *Cell* 1994, **79:**993–1003.

33. Charite J, Degraaff W, Shen SB, Deschamps J: **Ectopic expression of *Hoxb-8* causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures.** *Cell* 1994, **78:**589–601.

34. Tashiro S, Michiue T, Higashijima S, Zenno S, Ishimaru S, Takahashi F, *et al.*: **Structure and expression of *hedgehog*, a *Drosophila* segment-polarity gene required for cell–cell communication.** *Gene* 1993, **124:**183–189.

35. Ingham PW, Forbes Z: **Microinjection into *Drosophila* embryos.** In *Essential Developmental Biology: A Practical Approach.* Edited by Stern CD, Holland PWH. Oxford: IRL Press; 1993.

36. Robertson HM, Preston CR, Phillis RW, Johnston-Schlitz D, Benz WK, Engels WR: **A stable genomic source of P element transposase in *Drosophila melanogaster.*** *Genetics* 1988, **118:**461–470.

37. Ashburner M: *Drosophila — a laboratory manual.* New York: Cold Spring Harbor Laboratory Press; 1989.

38. Bumcrot DA, Takada R, McMahon AP: **Proteolytic processing yields two secreted forms of Sonic hedgehog.** *Mol Cell Biol* 1995, **15:**2294–2303.