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Hedgehog signaling in vertebrate and invertebrate limb patterning

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Abstract. Invertebrate and vertebrate limbs have very different anatomies and modes of development. Despite these differences, recent studies demonstrate that a significant overlap exists in the signals used to pattern invertebrate and vertebrate limbs. One of these signaling molecules is Hedgehog, a secreted protein that functions to coordinate growth and proliferation along the anterior-posterior axis of developing limbs. Recent studies indicate that the mechanism of action, regulation and function of Hedgehog signaling in *Drosophila* and vertebrate limb development are often quite similar, yet at other times are distinct. Here we highlight the similarities and differences between the use of Hedgehog signaling in these two systems.

Key words. chick; *Drosophila*; hedgehog; limb development; mouse.

Introduction

The study of appendage formation in invertebrates and vertebrates has contributed substantially to our current understanding of mechanisms underlying pattern formation during animal development. In particular, studies in *Drosophila* have been instrumental in identifying genes and pathways involved in the specification of limb axes during wing and leg development [1]. In vertebrates, especially in the chick, embryological studies have defined tissue interactions and organizing regions that are essential for proper limb growth and patterning [2]. More recently, many of the signaling molecules that mediate the patterning activities of these organizing regions have been identified.

Of particular importance during limb development is the activity of several *hedgehog* (*hh*) genes, which encode secreted factors homologous to the product of the *Drosophila* segment polarity gene *hedgehog*, involved in many patterning processes in the embryo and imaginal discs. Specifically, in both *Drosophila* and vertebrates, Hedgehog (Hh) proteins are expressed in posterior regions of developing limb tissues (fig. 1) and play pivotal roles in anterior-posterior and proximal-distal specification of these tissues. However, while many aspects of Hh function appear to be conserved between fly and vertebrate appendages, important differences exist in the regulation of *hh* expression and in its cellular and molecular effects. Here we review these common and divergent functions of Hh in the context of invertebrate and vertebrate developmental limb anatomy and embryology.

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Hedgehog and *Drosophila* wing development

The *Drosophila* wing develops from a specialized larval structure called the wing imaginal disc, which consist of a small epithelial sac set aside during embryonic development [1]. During the first instar stage, the wing imaginal disc is divided into anterior and posterior compartments consisting of cells with independent lineages and fates [1, 3, 4]. Cells of the anterior compartment only contribute to anterior tissues of the adult

wing and likewise, cells of the posterior compartment only contribute to posterior wing tissues. During the second instar, the wing disc also becomes divided into dorsal and ventral compartments. The function of compartments in appendage development remained mysterious for some time after their discovery in the 1970s. However, recent experiments indicate that the primary function of compartmentalization is to govern the fates of cells within each compartment and to restrict the expression of important secreted factors to discrete locations within the developing imaginal disc.

One such secreted factor is the product of the *hh* gene, expressed in cells of the posterior compartment of the wing imaginal disc [5–8]. The function of *hh* in wing development (fig. 2) has been studied by both gain- and loss-of-function experiments [9–11]. Ectopic expression of *hh* results in dramatic mirror-symmetric pattern duplications. Importantly, these duplications only occur when *hh* is expressed in the anterior compartment, where it is normally absent. The homeodomain protein Engrailed (En) [12, 13], expressed in the posterior compartment, is responsible for this difference. In the posterior compartment, En represses the expression of *Cubitus interruptus* (*Ci*) [14], a gene encoding a zinc-finger transcription factor that mediates Hh signaling (reviewed in [15]), thereby making posterior cells refractory to the Hh signal [16]. In contrast to the patterning duplications seen from ectopic expression of *hh*, abrogation of *hh* function results in wing truncations [9]. Taken together, these studies suggest that the function of *hh* is to send a signal to cells of the anterior compartment. This signal is then necessary and sufficient for proliferation and patterning of the imaginal disc.

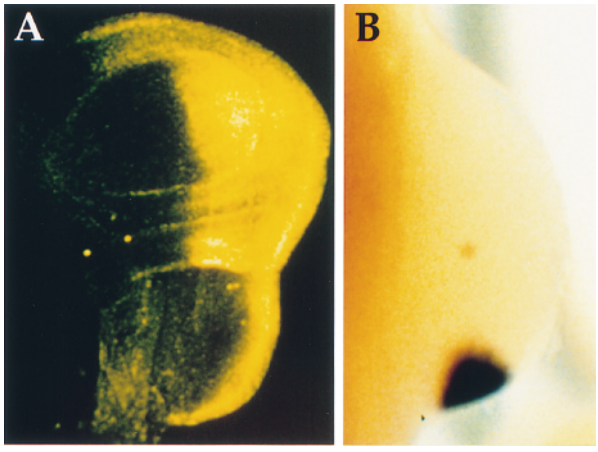

Figure 1. Expression of *hh* and *Shh* during wing development. (*A*) *Drosophila* third instar wing imaginal disc showing expression of Hh protein in the posterior compartment. Anterior is to the left, posterior to the right. (*B*) Chick wing bud at 3 days of development showing *Shh* expression in the posterior mesenchyme. Anterior is to the top and posterior to the bottom.

In principle, Hh could function as a long-range morphogen that patterns the anterior compartment. Alternatively, Hh could act as a short-range signal which through a signal relay mechanism induces the expression of other long-range signaling molecules [17]. Current evidence supports the latter. First, clones of cells which lack Smoothened (Smo), an essential component of the Hh reception complex, only affect wing pattern when located in the anterior compartment near the anterior-posterior compartment boundary [18–20]. This result indicates that Hh signaling is only required in anterior compartment cells near to the anterior-posterior compartment boundary. In the wing disc, *hh* induces the expression of *decapentaplegic* (*dpp*) (reviewed in [21]), a gene encoding a transforming growth factor-β (TGF-β) family member, specifically in anterior cells at the anterior-posterior compartment boundary. Ectopic expression of *hh* in the anterior compartment induces ectopic expression of *dpp*, and elimination of hh signaling at the anterior-posterior compartment boundary results in the loss of endogenous *dpp* expression [9, 11, 22]. Ectopic anterior compartment expression of *dpp* results in the formation of mirror-image duplications similar to those seen with ectopic *hh* expression [11, 22, 23]. Hence, the long-range proliferation and patterning effects of *hh* in the wing appear to be mediated by *dpp*. However, unlike *hh*, *dpp* is capable of inducing posterior duplications when expressed in the posterior wing disc, indicating that both posterior and anterior cells are able to respond to *dpp* signaling. This result suggests that the Dpp protein serves as a bidirectional signal, emanating from the compartment boundary and patterning both the anterior and posterior compartments (fig. 3). Interestingly, Hh appears to modulate a gradient of Dpp activity in the wing imaginal disc by controlling the expression of the Dpp receptor [24].

The obtention of mirror-image duplications of wing structures requires both patterning and proliferation, for which the interaction of Dpp with additional factors appears to be required. Distal outgrowth of the wing with mirror-image duplications occurs when clones of ectopic expression of *hh* or *dpp* intersect with the dorsal-ventral compartment boundary [9, 10, 25]. Hence, induction of *dpp* by *hh* alone is not sufficient to induce distal outgrowth accompanied by mirror-image duplications: additional *hh*-independent input from the dorsal-ventral compartment boundary is necessary.

A similar situation exists in the developing *Drosophila* leg. The six paired legs develop from leg imaginal discs in a manner analogous to that of the wing. The leg discs are divided into anterior-posterior compartments with *hh* expressed by posterior compartment cells. However, unlike in the wing disc, the leg disc is not divided into

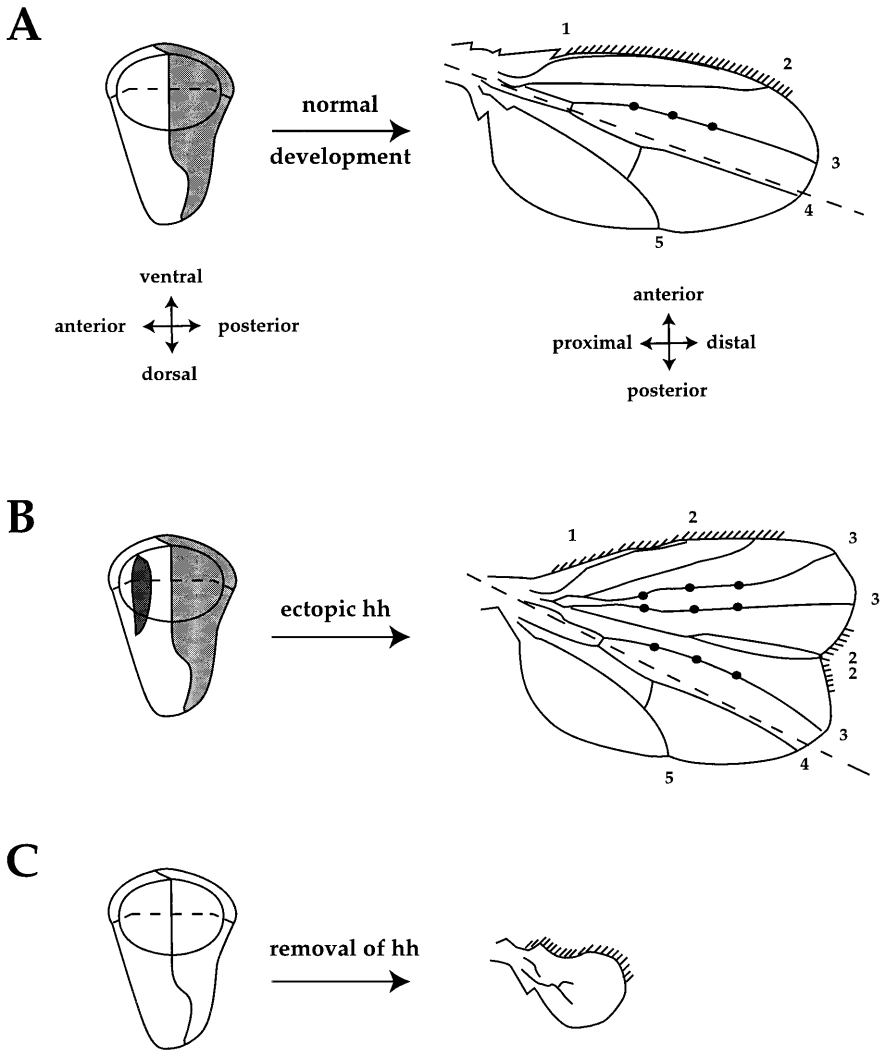

Figure 2. Effect of gain and loss of *hh* activity on *Drosophila* wing pattern. (*A*) Normal wing development. The third instar wing imaginal disc is divided into anterior-posterior and dorsal-ventral compartments. The posterior compartment expresses *hh* (dark shading), and the location of the dorsal-ventral compartment boundary is indicated by the dashed line. After eversion the wing imaginal disc is transformed into the adult appendage, schematized on the right. Only the wing blade is shown. The wing blade is composed of two closely apposed epidermal surfaces, dorsal and ventral, each containing a distinctive arrangement of veins, sensory organs and bristles. Each of the five longitudinal veins is uniquely identified by its position along the anterior-posterior axis (with vein 1 being the most anterior, and vein 5 being the most posterior), the location of cross veins (proximally between veins 3 and 4 and distally between veins 4 and 5), and the presence of three campaniform sensilae (black dots on vein 3). Located along the wing margin are sensory bristles, consisting of a triple row running from the base of the wing to a point in between veins 2 and 3, and a slender double row for the remainder of the wing. (*B*) Ectopic *hh* expression in the anterior compartment results in the duplication of wing tissues. When a clone of *hh*-expressing cells is generated in the anterior compartment at the dorsal-ventral compartment boundary, a dramatic reorganization of wing pattern results. In this example (taken from [9]) instead of the normal 12345 pattern of veins, a 123322345 pattern is observed. The supernumerary veins can be identified by the presence of campaniform sensilae on vein 3 and by the morphology of bristles along the wing margin. (*C*) Reduction of *hh* expression in the posterior compartment results in wing truncations. Generation of posterior clones that lack *hh* expression in the imaginal disc leads to the formation of small wings which lack most anterior-posterior and proximal-distal patterning elements [9]. The proximal-distal truncations are an indirect consequence of anterior-posterior patterning defects.

dorsal and ventral compartments. Despite this lack of dorsal-ventral compartmentalization, dorsal and ventral leg disc cells respond differently to *hh* [9, 26]. Anterior-dorsal cells at the anterior-posterior compartment boundary are induced to express *dpp* by *hh*, whereas anterior-ventral cells express *wg*. Combinatorial signaling by *wg* and *dpp* instructs distal outgrowth and proximal-distal patterning. Hence, the function of *hh* in leg

pattern is similar to that of its role in specifying wing pattern, serving as a signal from posterior cells which induces the expression of signaling molecules at the anterior-posterior compartment boundary.

In addition to indirect long-range effects on wing patterning mediated by *dpp*, *hh* appears to be directly responsible for specifying cell fates in the central region of the wing [27, 28]. Although ectopic expression of both *hh* and *dpp* in the anterior compartment give similar phenotypes, ectopic *hh* expression can result in the duplication of vein 3, whereas ectopic *dpp* cannot. Moreover, whereas *dpp* is able to compensate for most effects of loss of *hh* signaling, it is unable to restore vein 3 and the vein 3/4 intervening tissues. Hence, the central region of the wing blade appears to be directly specified by *hh* in a *dpp*-independent manner. The direct effects of *hh* are mediated by the transcription factor collier (*col*), which is induced by high levels of Hh signaling in anterior cells near the anterior-posterior compartment border [29, 30] (fig. 3). Hh signaling also induces expression of *en* in this region, where it controls the identity of marginal bristles [31, 32].

Although studies of *hh* function in wing patterning have focused on its role in anterior-posterior patterning and cell fate determination, recent studies have suggested that *hh* plays an important role in preventing cell mixing between the anterior and posterior compartments, a function originally attributed to *en* [33, 34]. Clones of cells that lack *en* do not respect compartment boundaries, leading to the suggestion that *en* might cell-autonomously regulate adhesive properties of posterior cells, thereby preventing them from intermixing with anterior cells. According to this model, all cells of the posterior compartment would preferentially adhere to posterior compartment cells and all cells of the anterior compartment would adhere to anterior compartment cells, thereby preventing mixing between compartments. An alternative model that does not invoke a cell autonomous difference in adhesion suggests that anterior-posterior compartment boundary cells have special properties that prevent mixing between compart-

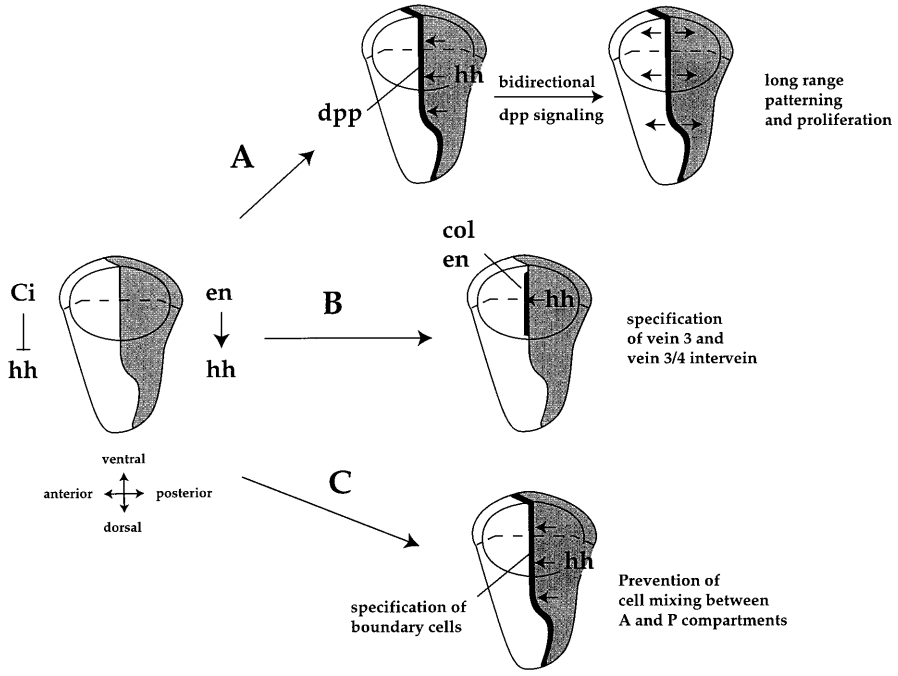

Figure 3. *hh* regulation and function in the wing disc. *hh* expression is positively regulated by *en* expression in the posterior compartment and negatively regulated by Ci in the anterior compartment. (*A*) Indirect long-range patterning by *hh*. Diffusion of Hh into the anterior compartment results in the induction of *dpp* expression at the anterior-posterior compartment boundary in an 8–10-cell-wide band. Posterior cells do not express *dpp*, owing to *en* expression. In turn, the Dpp protein diffuses bidirectionally to affect pattern and proliferation in both compartments. Anterior and posterior compartment cells adopt different fates in response to the Dpp signal as a result of *en* and/or *Ci* expression. (*B*) Direct short-range patterning by Hh. Diffusion of Hh into the anterior compartment also results in the induction of *collier* and *en* expression in a narrow (2–4 cells) band at the anterior-posterior compartment border, leading to specification of the central region of the wing blade. (*C*) Control of compartment boundaries by *hh*. Diffusion of Hh into the anterior compartment is required for anterior-type cell sorting (in the posterior compartment, En specifies posterior-type sorting).

ments. The behavior of clones of cells which lack the ability to respond to Hh signaling support this latter model and point towards a role for *hh* in maintenance of the anterior-posterior compartment boundary. Anterior compartment clones of cells mutant for *smo* cannot respond to *hh* and do not express *en*. When these clones are formed near the anterior-posterior compartment boundary, they mix with posterior cells. Recently, it has been shown that an activator form of Ci is necessary and sufficient to define anterior compartment-type cell sorting, and that En specifies posterior-type cell sorting [35]. Thus, Ci and En could control cell segregation at the anterior-posterior boundary by regulating a single cell adhesion molecule.

Sonic hedgehog and vertebrate limb development

The vertebrate limb exhibits a mode of development very different from that seen in the *Drosophila* leg or wing. In vertebrates [2], limbs form from contributions of the lateral plate mesoderm, somitic mesoderm and flank ectoderm, which contribute to the connective and skeletal tissues, the muscle and the skin, respectively. The first visible structure is the limb bud, a thickening of the mesenchyme covered by a uniform ectodermal sheath which forms through a process involving differential proliferation of the presumptive limb regions versus the interlimb regions. Around this time, migratory myoblasts derived from the lateral dermamyotome invade the limb where they proliferate and eventually differentiate and fuse to form muscle fibers. Following limb bud formation, the first morphological sign of differentiation is a thickening of the ectoderm along the distal tip of the limb bud to form the apical ectodermal ridge (AER), a structure which is essential for continued outgrowth of the limb [36]. The AER also maintains the underlying mesenchyme in a proliferative and undifferentiated state. This region is called the progress zone (PZ; [37]). It is thought that cells in the PZ receive positional information with respect to all three axes and when cells leave the PZ, that positional information is set.

In vertebrates, several homologues of *Drosophila hh* have been identified [38–41], including *Sonic* (*Shh*), *Desert* (*Dhh*) and *Indian hedgehog* (*Ihh*). Of these three *hh* homologues, only *Shh* is expressed during early limb development, and its expression is confined to a discrete region of the posterior mesenchyme in a region known as the zone of polarizing activity or ZPA (fig. 1). The ZPA plays an important role in proliferation and anterior-posterior limb patterning as evidenced by its ability to induce mirror-image duplications in the host tissue [42]. Based on this observation, it was proposed that the normal function of the ZPA during limb development is to act as the source of a morphogen whose activity induced proliferation of limb mesenchyme and, at the same time, instructed the naive tissue to adopt positional fates along the anterior-posterior axis [43].

The colocalization of *Shh* transcripts with ZPA activity raised the exciting possibility that the Shh protein might be responsible for mediating the effects of the ZPA. This possibility was tested by ectopic expression and extirpation studies in the chick and loss-of-function studies in the mouse (fig. 4). Implantation of cells expressing Shh or beads containing recombinant Shh protein both induce mirror-image duplications of pattern elements very similar to those induced by ZPA grafts [40, 44]. Conversely, surgical removal of the *Shh*-expressing region leads to limb truncations [45]. This latter experiment does not prove that Shh is the signal responsible for polarizing activity; however, mice lacking *Shh* also lack polarizing activity in the limb buds and have severe limb truncations [46]. As in *Drosophila*, these gain- and loss-of-function studies point towards a dual role for *Shh* in anterior-posterior patterning and proliferation. However, as described below, the mechanisms by which these activities are implemented (fig. 5) appear to be different in *Drosophila* and vertebrates.

Although the vertebrate limb is not divided into compartments, the mechanism of action of *Shh* might be similar to that of *hh* during *Drosophila* limb development. Indeed, a homologue of *dpp*, *bmp-2*, is expressed in a domain of cells slightly larger than the *Shh* domain, and it can be induced in anterior limb mesenchyme by ectopic *Shh* expression [47]. However, unlike in *Drosophila*, ectopic *bmp-2* expression does not induce mirror-image duplications in vertebrate limbs, although it may generate an extra digit in certain experimental settings [48]. In fact, in some assays, BMPs inhibit proliferation, induce cell death and repress the AER (reviewed in [49, 50]). Since BMPs act as heterodimers, and several *bmp* genes are expressed in the limb [51], it is possible that specific heterodimer combinations may have significant polarizing activity, but evidence for this is lacking. Moreover, it is unlikely that *bmp-2* is a direct target of *Shh* signaling since several hours are required for *Shh* to induce *bmp-2*. Also, several other BMPs are expressed in the limb in patterns not consistent with regulation by *Shh*. Thus, BMPs are unlikely to be a secondary signal that mediates the bulk of patterning activities of *Shh*. However, BMPs have been recently proposed to specify digit identity through a *Shh*-triggered mechanism (see [52]).

Other targets of *Shh* signaling in the limb mesenchyme include members of the *hoxd* cluster [53], which are known to be essential for limb patterning. Several genes of the *hoxd* cluster are expressed in the posterior limb mesenchyme and can be induced in anterior mesenchyme by *Shh* [40, 47]. This induced expression also

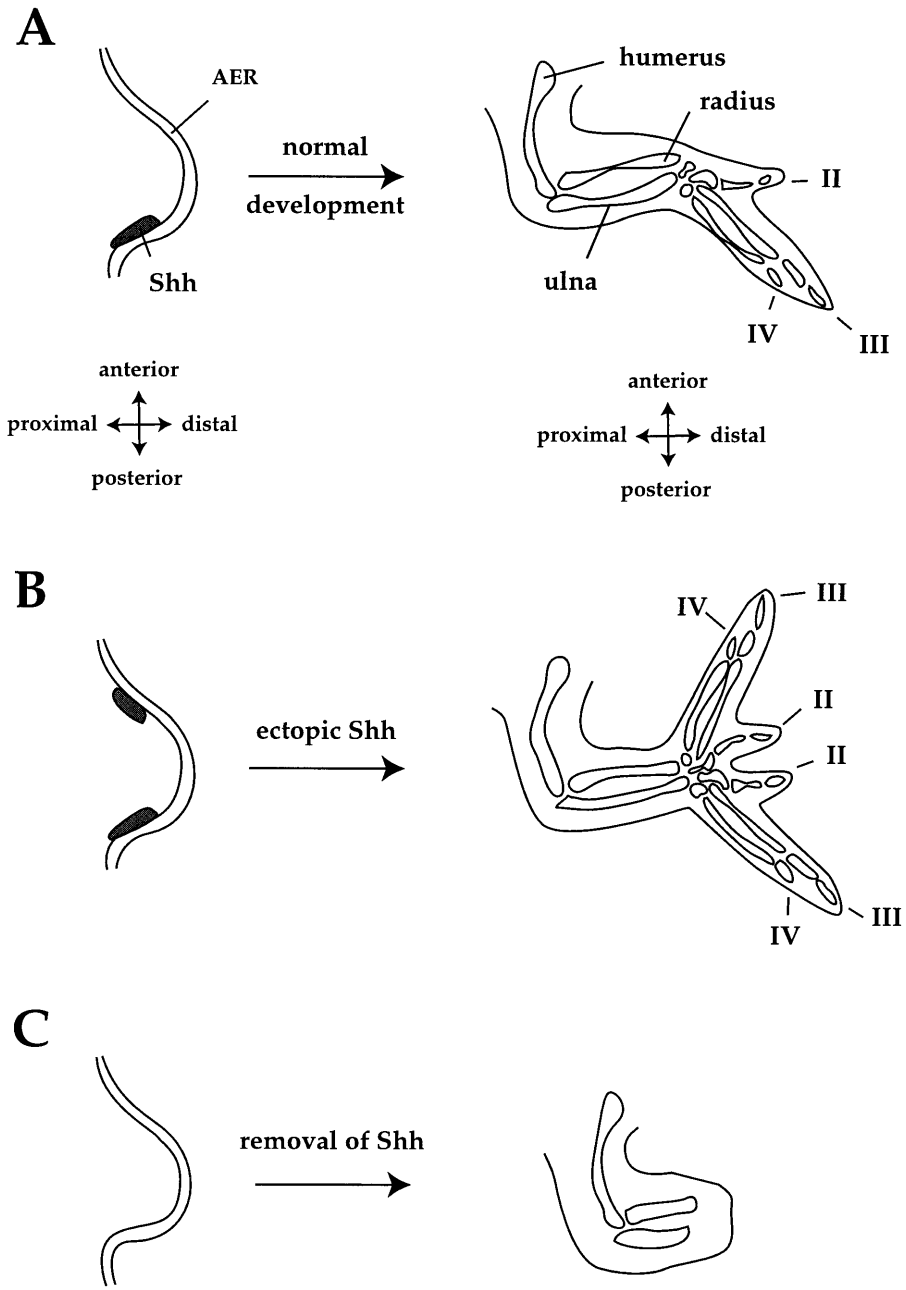

Figure 4. Effect of gain and loss of *Shh* activity on chick wing pattern. (*A*) Normal chick wing development. At 3 days of development, the chick limb bud is composed of a mesenchyme surrounded by an ectodermal sheath. Running along the anterior-posterior axis at the dorsal-ventral interface (dorsal is out of the plane of the paper and ventral is into the plane of the paper) is a thickened epithelium, the AER. *Shh* (dark shading) is expressed in the posterior mesenchyme in the region of the ZPA. By 10 days of development, the characteristic adult morphology of the wing is apparent. At the proximal region of the wing is the humerus, followed by the radius and ulna, and carpal bones of the wrist. At the distal end of the limb are the digits, named II, III and IV from anterior to posterior. Each digit is uniquely identified by its morphology. (*B*) Effect of ectopic anterior expression of *Shh* on chick wing patterning. When cells expressing *Shh* or a bead soaked in Shh protein is implanted along the anterior margin of the limb, a dramatic reorganization of limb pattern results. Instead of the normal II-III-IV digit pattern, the anterior limb tissues are induced to proliferate and patterned to form a mirror-symmetric IV-III-II-II-III-IV arrangement. (*C*) Effect of removal of the *Shh*-expressing mesenchyme on chick wing pattern. Removal of the ZPA region that expresses *Shh* results in the truncation of the limb. In most cases, only the humerus forms. Similar effects are seen when *Shh* activity is removed from mouse limb buds by gene targeting.

requires input from the AER, since AER removal abolishes the response of limb mesenchyme to *Shh* [40, 47]. However, *Shh* is unlikely to be responsible for the induction of *hoxd* gene expression since some expression of *hoxd* genes can be detected in the absence of *Shh* expression [54].

In addition to its role in modulating patterning along the anterior-posterior axis, *Shh* influences proliferation within the limb mesenchyme. One mechanism by which *Shh* can cause this effect is through the alteration of the properties of the AER. *Shh* (expressed in the posterior mesenchyme) and *fibroblast growth factor-4* (*fgf-4*); (expressed in the posterior AER) are involved in a reciprocal feedback loop by virtue of which their expression becomes mutually dependent [47, 55]. In this manner *Shh* indirectly promotes limb outgrowth through an effect on the AER. This mechanism of growth control employed by *Shh* contrasts with that used by *hh* in *Drosophila*. In vertebrates *Shh* mediates this effect through mesenchymal-to-ectodermal signaling, resulting in increased *fgf-4* expression in the ectoderm. Recent evidence suggests that upregulation of *fgf-4* by *Shh* is indirect and mediated through the actions of the *gremlin* and *formin* gene products [56–58]. *Gremlin* encodes a BMP antagonist expressed in the posterior limb mesenchyme, and *Shh* is required to maintain its expression. Blocking BMP activity by *gremlin* or by other BMP antagonists results in upregulation of *fgf-4* in the AER. Maintenance of *gremlin* expression in turn depends on mesenchymal expression of formin, the product of the limb deformity gene [56]. In *limb deformity* mutants, limb bud gremlin expression is lost and *fgf-4* is not maintained in the AER. FGF-4 in turn signals back to the mesenchyme, promoting its proliferation and directly or indirectly maintaining *Shh* expression. This reciprocal feedback loop seen in the vertebrate limb bud contrasts with the mechanism of *hh* action in the wing imaginal disc. As detailed above, *hh* signals between anterior and posterior compartment cells, both located within a single epithelial monolayer (the imaginal disc), and its function is to induce the expression of *dpp*, a *bmp* homologue, which in turn mediates most of the patterning activities of *hh*.

Recently, a role for *Shh* in muscle patterning was identified [59, 60]. The muscle of the limb derives wholly from migrating myoblasts of somitic origin. To ensure sufficient numbers of myoblasts to form all limb muscles, a balance between myoblast proliferation and differentiation is required. *Shh* influences this balance by promoting proliferation and inhibiting differentiation. This effect can be mimicked by appropriate doses of BMPs [60], and since *bmp* expression in the limb can be induced by *Shh*, this suggests that BMPs may mediate effects of *Shh* on muscle patterning.

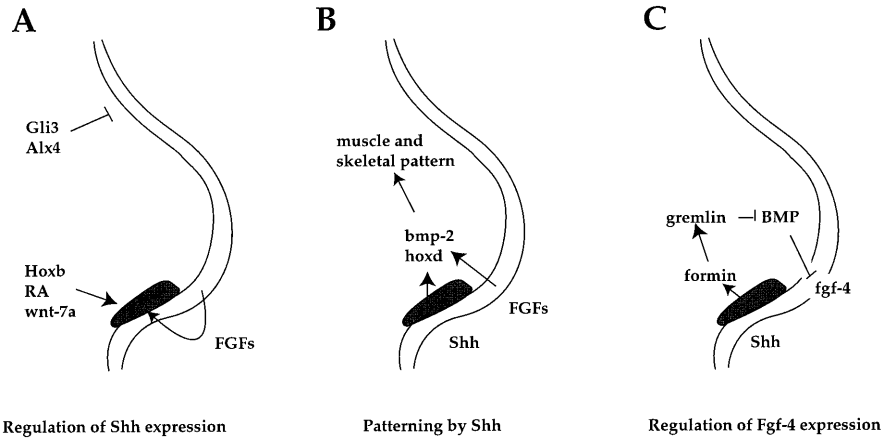

Figure 5. Complex regulation and multiple functions of *Shh* during vertebrate limb development. (*A*) Induction of *Shh* in the posterior mesenchyme is thought to require input from *hox* genes (e.g. *hoxb8*) and retinoic acid (RA), although other factors are also believed to be required to position *Shh* expression. Maintenance of *Shh* expression depends on factors from the AER, including the FGF-4 protein. *Shh* is repressed by multiple genes in the anterior limb bud, including *Gli3* and *Alx4*. Mutations in additional genes result in ectopic *Shh* expression in the anterior limb bud, suggesting that other genes also function to repress *Shh* expression. (*B*) Expression of downstream targets of *Shh* in limb mesenchyme, such as *bmp-2* and *hoxd* genes, requires input from the AER in the form of FGFs. Modulation of *bmp* expression may affect muscle pattern, and effects on *hoxd* gene expression are thought to play an important role in skeletal patterning. Additional mesenchymal targets of *Shh* are likely, since neither *hoxd* nor *bmps* appear to mediate all the effects of *Shh*. (*C*) *Shh*-dependent maintenance of *fgf-4* expression in the AER. *Shh* maintains *fgf-4* expression in the AER via formin expression in the posterior mesenchyme. Formin in turn is responsible for expression of the BMP antagonist gremlin in the posterior limb mesenchyme, which antagonizes a repressive effect of BMPs on *fgf-4* expression and AER maintenance.

Short-range versus long-range signaling

An important question is whether Hh proteins function as short-range or long-range signaling molecules during limb patterning [17]. One possibility is that Hh proteins function solely to induce the local expression of secondary long-range signaling molecules. Alternatively (or additionally) Hh proteins could function as long-range signaling molecules, perhaps even as morphogens, specifying distinct cell fates depending on their concentration. Evidence in *Drosophila* suggests that Hh functions mainly as a short-range signal, on the range of 1–10 cell diameters. Hh protein cannot be detected more than a few cells away from the posterior compartment in wing imaginal discs [10], and the direct target genes *dpp* and *patched (ptc)* are only induced in close proximity to the compartment boundary [9, 19]. Further evidence supporting a short-range signaling function for Hh in *Drosophila* limb patterning derives from cell-autonomous manipulation of the *hh* signaling pathway. Using these methods, Hh signaling has been shown to be required only near the anterior-posterior compartment border and cell-autonomous activation of the *hh* signaling pathway leads to non-cell-autonomous effects [61–64]. These long-range non-cell-autonomous effects are largely mediated through Dpp, which, unlike Hh, apparently functions at some distance from its site of synthesis.

Although Hh functions via short-range signaling in the wing disc, some cells do appear to be able to sense different concentrations of Hh. In a row of two to three anterior cells at the anterior-posterior compartment boundary, high levels of Hh induce the expression of *collier*, whereas lower levels of Hh are able to induce *dpp* expression at a distance of 8–10 cells [9, 19, 29, 30]. A nondiffusible membrane-tethered form of Hh has been used to examine the role of Hh diffusion in wing patterning. Membrane-tethered Hh is able to induce both *dpp* and *en* expression in anterior cells, but only in directly adjacent cells [27]. Membrane-tethered Hh is also able to rescue most of wild-type *hh* functions when expressed in wing discs that lack endogenous *hh* function. However, the central region of the wing is missing in the absence of diffusible Hh, indicating that short-range diffusion of Hh is necessary for patterning of this region of the wing. The differential responsiveness of anterior compartment cells to Hh signaling is thought to involve differential responses of Hh signaling pathway components including Ci and the serine-threonine kinase fused [29, 30].

In vertebrates, the question of whether Shh functions as a short-range or long-range signal is controversial. Shh protein colocalizes with cells that transcribe the *Shh* message [44, 65], indicating that little (if any) Shh protein diffuses away from its site of synthesis, although it is possible that available antibody reagents cannot detect an alternative, diffusible form of Shh. However, elevated levels of vertebrate *ptc* expression, a proposed direct target for *Shh* signaling, are detected at a distance from the *Shh*-expressing cells [66]. Short- versus long-range signaling by Shh has also been addressed in chicks by using membrane-tethered forms of the protein. When expressed in the anterior of the developing chick limb, a membrane-tethered form of Shh is still able to elicit dose-dependent pattern duplications [67], which seems to indicate that Shh patterns the limbs through induction of a secondary signal. If Shh is primarily or exclusively a short-range signal, then how can one account for the dose dependence of the ZPA? Perhaps Shh induces the expression of secondary signaling molecules in a dose-dependent manner. While BMPs are not likely to be this signal (or not exclusively), other as yet unidentified signaling molecules may carry out this function. Alternatively, a model that incorporates proliferation, dose and time of exposure to Shh has been proposed [67]. According to this model, cells that receive low doses or a short exposure to Shh are specified as anterior cells. As the limb grows, some of these cells will move away from the source of Shh signal while others will remain close to the *Shh*-expressing ZPA cells. The cells that move away from the ZPA no longer receive a Shh signal and their anterior-posterior positional values become fixed. In contrast, cells remaining close to the ZPA will continue to receive a Shh signal and could be 'promoted' to a more posterior fate. In this manner, a constant level, short-range Shh signal could pattern the anterior-posterior limb axis. A definition of the direct and indirect targets for Shh signaling in the limb mesenchyme and a more fundamental understanding of how those targets regulate anterior-posterior limb patterning will be required to address these questions.

Mechanisms regulating Hedgehog diffusion

Since Hh and Shh are potent signaling molecules, restriction of their activities to only a few cell diameters from their source is important for proper limb patterning. This restricted activity is achieved by at least two distinct mechanisms. The first mechanism is mediated by the Hedgehog receptor Ptc. In the absence of Hh, Ptc represses *hh* target genes, and binding of Hh to Ptc relieves this repression (reviewed in [68]). Low levels of Ptc are present throughout the anterior compartment to ensure that the *hh* signaling pathway is repressed where it is not needed. Only at the anterior-posterior compartment boundary is there sufficient *hh* expressed to overcome this repression. Hh diffusion is actually restricted by Ptc because, paradoxically, *ptc* transcription is up-

regulated by Hh signaling. Elevated levels of Ptc eventually shut down *hh* signaling entirely. When unliganded Ptc protein levels exceed liganded Ptc protein levels, no Hh signal is transduced since the repressing unliganded Ptc protein is in excess. Hence, in this context, Ptc functions to sequester Hh in a nonproductive complex [19]. The second mechanism involves a novel Hh-interacting protein (Hip) that has been recently identified in vertebrates [69]. The *Hip* gene encodes a membrane glycoprotein that has been shown to bind mammalian Hh proteins, thus attenuating their signaling activities. *Hip* is transcribed next to *hh*-expressing cells, in response to Hh signaling, acting as a *hh*-dependent mechanism that limits the range of activity of Hh proteins. So far, no *Hip* gene has been identified in *Drosophila*.

Conversely, membrane proteins encoded by the *EXT* tumor suppressor gene family appear to facilitate diffusion of Hh proteins. *EXT* genes are implicated in the multiple exostoses syndrome in humans [70, 71]. The *tout-velu* (*ttv*) gene was the first *EXT* gene described in *Drosophila* [72], where it was found to play an interesting role. Clonal analysis in the wing imaginal disc demonstrates that cells that lack *ttv* function cannot respond to Hh protein, with the notable exception of the cells that directly face *hh*-expressing cells, which indicates that the protein encoded by *ttv* is somehow required for Hh diffusion from the *hh*-expressing cells. Subcellular localization studies have detected EXT proteins in the endoplasmic reticulum [73, 74], where they are involved in the synthesis of cell-surface heparan sulfate glycosaminoglycans (or GAGs; [73, 75]). Different GAGs function in the reception of several signaling factors in a variety of organisms. In *Drosophila*, lack of a type of GAG (heparan sulfate proteoglycan, or HSPG), due to *ttv* inactivation, has been recently shown to impair the movement of Hh protein, although the exact mechanism of action of HSPG in this process is still unknown [76].

Regulation of *hedgehog* expression in limbs

As indicated above, in *Drosophila* the homeotic selector gene *en* is a positive regulator of *hh* expression in the posterior compartment of the imaginal discs. However, additional negative regulation is required to repress *hh* in the anterior compartment. Interestingly, Ci (a transducer of *hh* activity) is required for this repression, since in the absence of *Ci* function, *hh* is activated ectopically in the anterior compartment [16]. Likewise, *Shh* expression in the ZPA of the vertebrate limb is subject to both positive and negative regulation. For example, a number of polydactylous mouse mutants exhibit ectopic expression of *Shh* in the anterior portion of the limb [77–82], which indicates that several genes are required to repress *Shh* in the anterior margin of the limb bud, including *ptc* [83]. Interestingly, another one of these mutants in a vertebrate homologue of *Ci: Gli3*. In the chick limb, *Gli3* is repressed by *Shh*, so that a loop of mutual transcriptional repression between *Shh* and *Gli3* is established that contributes to restrict *Shh* transcription to the posterior margin [84, 85]. Additionally, Shh antagonizes the generation of the repressor form of Gli3, thus creating a gradient of repressor Gli3 protein along the anterior-posterior axis of the limb bud [86]. Although some parallels exist between *Ci* and *Gli* genes in *hh* and *Shh* regulation, it is clear that en homologs are not involved in *Shh* regulation in the vertebrate limb, rather they expressed in the ectoderm and are involved in dorsal-ventral patterning processes [87].

Several positively acting factors have been implicated in directing expression of *Shh* to the posterior limb mesenchyme, including retinoic acid, *hox* genes and the secreted glycoprotein Wnt-7a, a homolog of Wingless (Wg). Implantation of beads soaked in retinoic acid into the anterior margin of the limb bud leads to the delayed induction of *Shh* distal to the bead [40]. Since this induction requires at least 16 h, several intermediate steps would seem to be required. Blocking endogenous retinoic acid activities inhibits *Shh* expression, suggesting a requirement for retinoic acid signaling in activating *Shh* expression [88, 89]. In addition to retinoic acid, several *hox* genes appear to be involved in delimiting the region of the limb bud mesenchyme where the *Shh* gene is transcribed. The distribution of *hoxb8* in the chick flank and early forelimb mirrors the distribution of polarizing activity, which suggests that *hoxb8* could act as a regulator of *Shh*. Specifically, it has been proposed that *hoxb8* is required for the initiation of *Shh* expression in the posterior mesenchyme of the forelimb bud [90], although it is not required for *Shh* maintenance [91]. Besides, ectopic *hoxb8* in the anterior margin of the mouse limb bud can induce ectopic *Shh*, resulting in pattern duplications [91]. *hoxb8*, however, is clearly not the only regulator of *Shh* expression, since *Shh* is only activated in the most distal cells that express *hoxb8*. The dependence of *Shh* expression on AER signals [47, 55] also contributes to restrict *Shh* to the more distal region of the posterior mesenchyme of the limb bud. Recently, it has been shown that mice deficient in the *hoxb8* gene have normal limbs [92], which confirms that *hoxb8* cooperates with other genes (other *hox* genes among them [93–95]), in order to position the *Shh* domain. Finally, *wnt-7a*, expressed in the dorsal ectoderm is required to maintain proper levels of *Shh* expression in the posterior mesenchyme. Either removal of the dorsal ectoderm [96], or targeted deletion of *wnt-7a* [97] results in downregulation of *Shh* transcription in the posterior mesenchyme. Taken together, these data suggest that multiple inputs are required to

regulate *Shh* expression in the limb, and the identification of these factors remains a major challenge in vertebrate limb patterning. Indeed, control of *Shh* expression in the limb is likely to be complex. Analysis of cis-acting regulatory sequences within 20 kb of the *Shh* gene has failed to reveal any limb enhancer elements [98], and there is some evidence to suggest that these elements may be located a significant distance away from the *Shh* promoter, perhaps even up to 0.5–1 Mb [99].

Summary and conclusions

In both *Drosophila* and vertebrate limb development, Hh proteins function to regulate anterior-posterior patterning, distal outgrowth and proliferation. The mechanism by which Hh achieves this effect in *Drosophila* is predominantly through local induction of the long-range signaling molecule Dpp. In vertebrates, current evidence suggests that a similar mechanism may be operating, through short-range induction of a secondary signal or signals, but the number and nature of these signals in the vertebrate limb is not known. Although *Shh* regulates the expression of several secreted factors (BMP-2, FGF-4), neither individually nor collectively can these factors mimic the effect of Shh alone. Other intriguing parallels between *hh* function in vertebrate and invertebrate limb patterning include the role of *Ci/Gli3* in repression of *hedgehog* expression. Although it has been suggested that these parallels may reflect a common ancestry of appendages in vertebrates and invertebrates, it is more likely that they reflect a conserved fundamental role of Hh proteins in regulation of pattern and proliferation through cell-cell communication.

The significant differences in regulation of *hh* transcription and in the downstream effectors of Hh signaling in vertebrate and invertebrate limbs suggest that a common ancestry is unlikely. These differences are likely a reflection of the use of Hh signaling for multiple purposes, some of which (e.g. compartment boundary maintenance, regulation of muscle proliferation) are processes specific to either *Drosophila* or vertebrate limb development. Indeed, the Hh signaling pathway is used in many different contexts. For example, during limb development, there are examples of redeployment of the Hh signaling pathway in later morphogenetic events. In butterflies, the *hh* pathway is used to pattern wing eyespots [100] and in vertebrates another *hh* homologue, *Ihh*, appears to coordinate several aspects of skeletal morphogenesis, including chondrocyte proliferation and maturation, and osteoblast development [101–103]. In these cases, the signaling pathway appears to be conserved, but the effector molecules and mode of regulation appear to be distinct.

Many questions remain concerning *hh* function during vertebrate and invertebrate limb development. How are different responses to Hh signaling carried out? How does Hh signaling control digit identity and anterior-posterior patterning in the vertebrate limb? How is *Shh* transcription regulated? These difficult and intriguing questions will surely continue to offer new surprises and tantalizing insights into limb patterning in both vertebrate and invertebrate limb development.

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