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Evidence for an Expansion-Based Temporal Shh Gradient in Specifying Vertebrate Digit Identities

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Summary

The zone of polarizing activity (ZPA) in the posterior limb bud produces Sonic Hedgehog (Shh) protein, which plays a critical role in establishing distinct fates along the anterior-posterior axis. This activity has been modeled as a concentration-dependent response to a diffusible morphogen. Using recombinase base mapping in the mouse, we determine the ultimate fate of the Shh-producing cells. Strikingly, the descendants of the Shh-producing cells encompass all cells in the two most posterior digits and also contribute to the middle digit. Our analysis suggests that, while specification of the anterior digits depends upon differential concentrations of Shh, the length of time of exposure to Shh is critical in the specification of the differences between the most posterior digits. Genetic studies of the effects of limiting accessibility of Shh within the limb support this model, in which the effect of the Shh morphogen is dictated by a temporal as well as a spatial gradient.

Introduction

During embryogenesis, differential fates must be established in precise locations. One mechanism by which this is achieved, first proposed as a theoretical model by Wolpert (1969), is the use of a morphogen. A morphogen is defined as a graded signal that triggers distinct threshold responses, thereby specifying a series of different cell fates. This concept provided a hypothesis to explain a variety of developmental phenomena. As molecular details have been elucidated, the idea has held up fairly well in explaining a number of important embryonic events, from the initiation of anterior-posterior polarity in the *Drosophila* egg by a gradient of bicoid protein (Driever and Nusslein-Volhard, 1988) to the patterning of the ventral neural tube by different concentrations of Sonic hedgehog (reviewed in McMahon et al. [2003]). Interestingly, however, the developmental system that first inspired the theoretical idea of a morphogen, the anterior-posterior axis of the developing limb bud, is a setting in which the mechanism of morphogenic patterning has remained elusive or at least controversial.

The first clue into the mechanism of anterior-posterior limb patterning came from a classic experiment by Saunders and Gasseling (1968). They grafted tissue from the posterior of one chick limb bud to the anterior distal margin of a second limb bud, which resulted in remarkable mirror image duplications of the digits and (if the graft was performed at an early enough stage) the ulna. The ectopic structures included extra digits (polydactyly), but, strikingly, the polarity of the limb near to the graft was reversed such that the most anterior ectopic digits were the most posterior in character. Thus, chick wings, which normally have three digits, defined from anterior to posterior as digits 2, 3, and 4 were transformed to a pattern of 4, 3, 2, 2, 3, 4. The cells of the limb bud that were capable of causing such a polarizing effect were limited to a small domain at the posterior margin of the limb bud. This operationally defined population of limb mesenchyme was named the zone of polarizing activity (ZPA).

Subsequent studies soon showed that some aspect of ZPA activity had to involve a concentration-dependent response. Grafting of fewer ZPA cells resulted in the induction of fewer ectopic digits, but these grafts also displayed weaker polarizing activity such that the extra digits were less posterior in character, giving rise to patterns such as 3, 2, 2, 3, 4 or 2, 2, 3, 4 (Tickle, 1981). A concentration-dependent response to the ZPA was also indicated by the more complex patterns of digits that result from grafting ZPA tissue in central locations rather than at the anterior margin (Tickle et al., 1975). A major breakthrough came from the discovery that retinoic acid could recapitulate these mirror-image duplications (Tickle et al., 1982; Summerbell 1983) by inducing the formation of a second source of the morphogen (Riddle et al., 1993; Helms et al., 1994). However, an understanding of how the concentration effect of the ZPA worked awaited the discovery of the gene encoding the morphogen.

Sonic hedgehog (*Shh*) encodes a secreted factor that is expressed precisely in those cells experimentally identified as the ZPA (Riddle et al., 1993). Grafting *Shh*-expressing cells (Riddle et al., 1993) or implanting a bead soaked in Shh protein (Lopez-Martinez et al., 1995; Yang et al., 1997) results in the same types of mirror image duplications as a ZPA graft. Although *Shh* RNA is tightly localized to a small domain at the posterior margin, based on the induced expression of targets of Shh signaling such as *Patched-1 (Ptc1)*, Shh-responding cells encompass most if not all of the digit-forming field in the limb (Marigo et al., 1996a). Indeed, Shh protein can be detected in a gradient across a similar domain (Lewis et al., 2001). Consistent with Shh acting as a

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graded morphogen, Shh elicits a concentration-dependent response similar to grafting different numbers of ZPA cells (Yang et al., 1997). Any tissue expressing one of the three known vertebrate hedgehog family members can produce this effect when grafted into a limb bud (Pathi et al., 2001), and no known tissue or factor other than Shh and its relatives has this activity. Taken together, it is now quite clear that the original ZPA grafts were, in effect, an assay for cells producing Shh. While it remains possible that the threshold responses to Shh are indirect effects of a second factor, a true morphogen, induced by Shh, the prevailing model is one of a spatial concentration gradient across the limb bud. The spatial gradient, either of Shh itself or induced by Shh, leads to distinct morphogenic fates based on threshold concentrations. Such a mechanism would be analogous to the establishment of differential cell fates by a spatial gradient of Shh in the ventral neural tube, where the evidence for Shh's role as a morphogen is strongest (reviewed in McMahon et al. [2003]). Here, we report the results of studies, originally designed to map the fate of the ZPA cells, that lead to the unexpected conclusion that digits are specified not just by level of exposure to Shh but also by differences in time of exposure: a temporal gradient.

The ultimate fate of some key embryonic signaling centers, including the apical ectodermal ridge (AER) in the limb (Guo et al., 2003), is simply to undergo programmed cell death and not to contribute to any mature tissues. While it has not thus far been possible to follow the fate of ZPA cells after they cease to express *Shh* mRNA, DiI labeling of cells in the region of the limb in which *Shh* is expressed suggests that at least some cells from this region give rise to structures along the entire posterior proximal-distal axis (Vargesson et al., 1997). However, it remained unclear if these were derived from the *Shh*-expressing cells themselves. Moreover, in the chick limb bud, there is a region of cell death proximal to and partially overlapping that of the *Shh* expression domain (the posterior necrotic zone) (Saunders and Fallon, 1967). It is clear, at least in the chick limb, that some cells expressing *Shh* undergo cell death in the posterior necrotic zone (Sanz-Ezquerro and Tickle, 2000). This leads to the hypothesis that the survival of ZPA cells depends on factors (Fgf family members) expressed at the tip of the limb bud during early patterning, but later, when this source of survival-promoting factors was lost, the ZPA cells themselves would concomitantly undergo apoptosis.

To directly identify the fates of *Shh*-expressing cells in the mouse limb, we constructed two mouse lines that expressed CRE recombinase in all cells in which *Shh* is normally expressed. We then used a CRE-inducible reporter to irreversibly mark and fate map *Shh*-expressing cells. In this report, we present evidence that *Shh* descendants directly contribute to digits 3–5 of the mouse limb. In addition, we demonstrate that digits 3–5 are composed of cells that have been exposed to maximal concentrations of Shh protein for differing amounts of time during development. Our genetic studies support the view that this temporal gradient of Shh exposure is critical for specifying distinct digit morphogenesis in the posterior half of the limb.

Results

Insertion of a gfpcre Fusion Cassette into the Shh Locus

The *Shh* gene is expressed in a dynamic manner during the development of the mouse limb. Expression of *Shh* mRNA is first observed in the posterior of E9.75 limb buds. Over the course of the next 2.5 days, *Shh* mRNA is expressed in the mesenchyme at the posterior border of the distal limb bud. No expression has been observed in the limb mesoderm after E12. The fates of cells that have at one time expressed *Shh* in the developing limb are unknown, since it is difficult to follow the fates of *Shh*-expressing cells after *Shh* ceases to be transcribed. In an effort to fate map cells that have expressed *Shh* in the mouse limb, we used gene targeting to insert a gene that encodes a *gfpcre* fusion protein into the *Shh* locus (see Experimental Procedures). The *gfpcre* cassette contained a nuclear localization signal and was inserted at the ATG of *Shh*. In addition, during the construction of the *Shhgfpcre* allele, the first 12 amino acids of *Shh* were removed to create a *Shh* null allele. ES cells in which the *gfpcre* cassette was correctly targeted were used to make mice, and these animals were then analyzed. Mice heterozygous for the *Shhgfpcre* allele exhibited no noticeable phenotypes. *Shhgfpcre* heterozygous animals were viable and mated and produced offspring in expected Mendelian ratios.

Insertion of the *gfpcre* cassette at the ATG of *Shh* resulted in the production of GFP in cells that normally express *Shh* mRNA. In the limb, GFP was observed in the distal posterior region of E10–E12 embryos (Figure 1 and data not shown). GFP expression was observed to colocalize with *Shh* mRNA in the limb. No GFP expression was observed in parts of the limb that did not normally express *Shh*. *Shh* is expressed in numerous other locations throughout development, and GFP expression was also observed in all previously identified *Shh* cells outside the limb. A full description of the fate of *Shh*-expressing cells outside the limb will be published separately.

Fate Mapping of Cells that Have Expressed Shh in the Mouse Limb

In mice containing the *Shhgfpcre* allele, GFP expression mimics endogenous *Shh* mRNA expression, indicating that the transgene is expressed appropriately. However, since *Shh* mRNA is not detected after E12 in the mouse limb mesenchyme, the GFP marker cannot be used to follow the fates of *Shh* descendants. The *gfpcre* cassette is a translational fusion between *gfp* and the site-specific recombinase *cre*. In mice containing the *Shhgfpcre* allele, CRE should thus be expressed in all locations in which GFP (and *Shh* mRNA) is observed. Consequent CRE-mediated recombination in *Shhgfpcre* cells expressing an R26R reporter allele indelibly marks the cells and their descendents by the production of β-galactosidase (Mao et al., 1999). Thus, using this system, *Shh*-expressing cells and their descendants can be irreversibly marked and followed throughout development.

In the limb, β-galactosidase protein is first detected at E10 in *Shhgfpcre/+;R26R/+* embryos, just after *Shh* mRNA is first observed (data not shown). Similarly, by

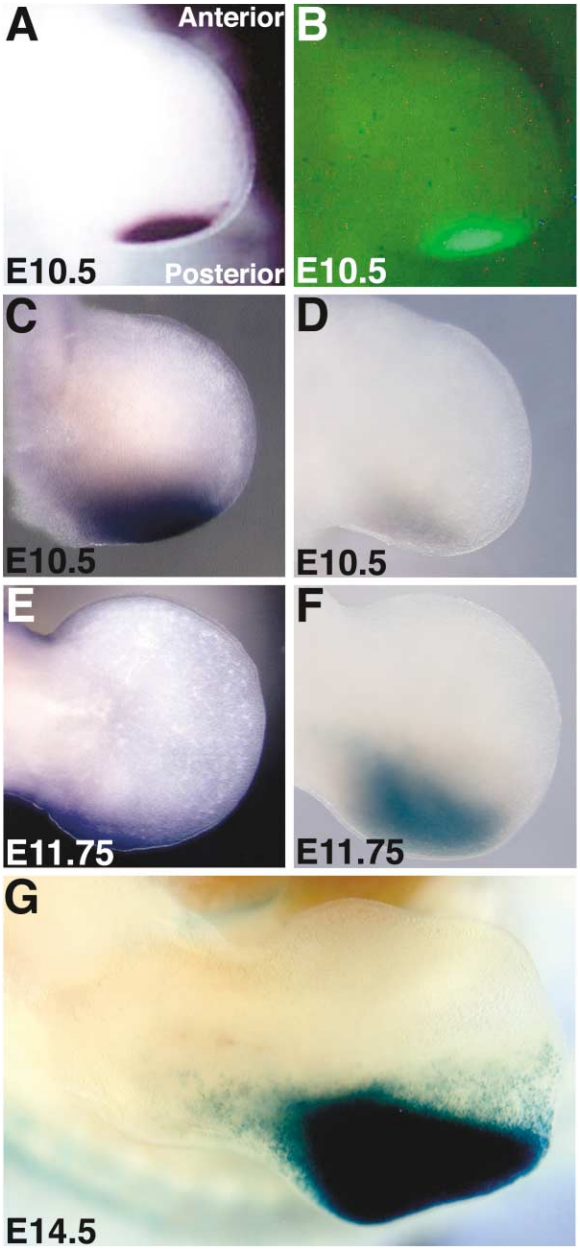

Figure 1. Shh Descendants Expand over Time and Survive the End of *Shh* Expression

(A and B) *Shh* expression by whole-mount in situ hybridization on E10.5 forelimbs (A) corresponds to the location of GFPCre (B) as shown by epifluorescence of GFP in *Shh::GFPCre/+* animals.

(C–F) The extent of Shh protein in the limb bud is independent of the expansion of the posterior cells. At E10.5, Shh protein extends in a gradient as detected by whole-mount immunohistochemistry on a forelimb (C). The domain of *Shh* descendants as detected by β-galactosidase in forelimbs of *ShhGFPCre/+, R26R/+* animals (D) is smaller than the region positive for Shh protein, indicating that Shh protein diffusion is not solely a consequence of expansion of the Shh domain. At E11.75, as *Shh* expression is downregulated, Shh descendants (F) extend beyond the gradient of Shh protein (E), as shown in this forelimb.

(G) At E14.5, Shh descendants encompass a broad posterior domain including digits 4 and 5 and contributing to the posterior of digit 3 as well as the posterior zeugopod, as shown in this *ShhGFPCre/+, R26R/+* hindlimb stained for β-galactosidase.

E10.5, *LacZ*-positive cells are observed in the distal posterior portion of the limb in a domain that overlaps with that of endogenous *Shh* mRNA (Figures 1A and 1D). However, at subsequent stages, the *Lacz*-positive cells form a domain that is markedly larger than the *Shh*-expressing domain, suggesting that the population of cells which at one time expressed *Shh* and hence *gfpcre* had expanded but that all except the most posterior of these had ceased to express *Shh*. By E14.5, whole-mount analysis of β-galactosidase staining shows that the posterior third of the distal limb bud appears to be largely made up from former Shh-expressing cells, while less uniform staining is visible through the posterior half of the hand plate. Some marked cells are also observed proximal to the hand plate in the presumptive forearm (Figure 1G). To get a clearer idea of the structures within the limb descended from ZPA cells, β-galactosidase staining was examined in histological sections. As in whole-mount, it was clear that, by E11, the marked former *Shh*-expressing cells encompassed a much larger domain than the cells actively expressing *Shh* (Figures 2A and 2B). By E12.5, continuing through E14.5, marked cells constitute the entirety of the interdigital mesenchyme posterior to digit 5 and between digits 4 and 5 in addition to within the digit primordia themselves, with a smattering of cells anterior and proximal to these digits also labeled (Figures 2C and 2D). By E17.5, it can be determined that all the cells of digits 5 and 4 are descended from ZPA cells, as are a subset of the cells in digit 3. Approximately one-third of the cells of the ulna are also descended from ZPA cells, along with most of the adjacent mesenchyme and perichondrium (Figures 2E and 2F). The radius and humerus are not stained (Figure 2F and data not shown).

In *Shh* mutant embryos, limbs develop a limited skeletal structure consisting of a humerus, radius, and single digit 1 (Chiang et al., 2001; Lewis et al., 2001; Kraus et al., 2001), a phenotype recapitulated in embryos homozygous for the *Shhgfpcre* allele (data not shown). This leads to the striking conclusion that the only skeletal element in the limb that is dependent upon Shh for its specification (i.e., missing in the *Shh*-homozygous mutant) but that does not at least partially derive from *Shh* expressing cells is digit 2. This changes the scope of the problem of anterior-posterior patterning in the limb bud, as the only digit that, at least in principle, needs to be explained by diffusion or transport of Shh protein is digit 2.

Expansion of Former ZPA Cells Does Not Determine the Movement of Shh Protein across the Limb

The expansion of the former *Shh*-expressing cells is so dramatic, we wondered what its functional significance might be for limb patterning. This domain does not appear to correlate with the spatial extent of expression of genes induced by Shh (*Gli1*, *Ptc1*, *Bmp2*) or the limit of expression of those repressed by Shh (*Gli2*, *Gli3*) (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/118/4/517/DC1). Another possibility was that, if Shh protein degrades slowly, Shh protein could be passively transported anteriorly as the former ZPA cells expand, and, hence, the expansion could actually

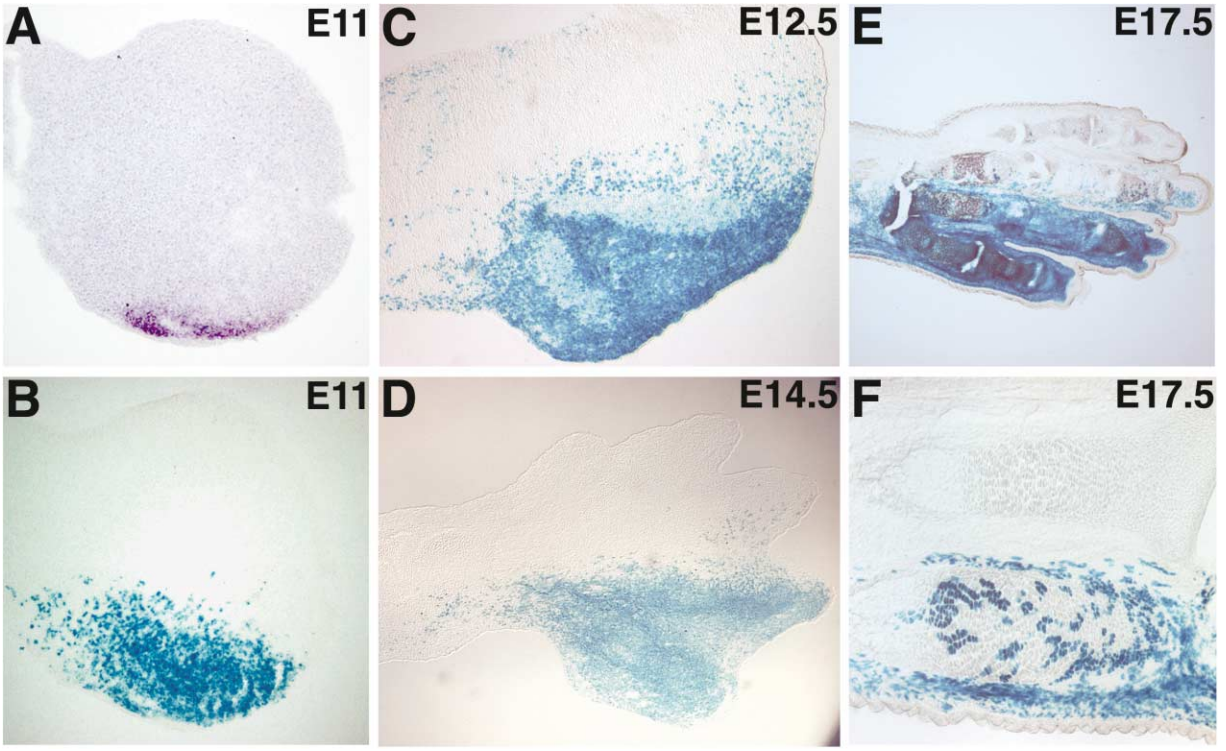

Figure 2. Shh Descendants Give Rise to Posterior Limb Elements

(A and B) By E11, Shh descendants, as detected by β-galactosidase in the forelimb (B), extend beyond the expression domain of *Shh* (A), as shown by section in situ hybridization in forelimbs of *Shh::GFPCre/+; R26R/+* animals.

(C–F) β-galactosidase staining in sections of *Shh::GFPCre/+; R26R/+* forelimbs shows that Shh descendants contribute to digits 3, 4, and 5 at E12.5 (C), E14.5 (D), and E17.5 (E) as well as the posterior zeugopod at E17.5 (F).

create or contribute to the observed graded distribution of Shh protein across the limb bud. However, comparison of anti-Shh antibody staining with β-galactosidase-marked former ZPA cells revealed that the Shh protein gradient is significantly broader at E10.5 than the expanded population (Figures 1C and 1D), indicating that the protein gradient can not be explained simply by the anterior expansion of former *Shh*-expressing cells. While at E11.75, the expansion encompasses a much wider domain than the detectable Shh protein (Figures 1E and 1F; note that the distribution of Shh protein is similar in width at E10.5 and E11.75 [Figures 1C and 1E], and the limb buds shown at these two stages are not at the same scale).

Dynamics of Gli3 Processing in the Limb Bud

A second possible functional role for the expansion of the posterior limb bud cells would be to affect the distribution of signaling components downstream of Shh protein. In particular, Gli3 is a zinc finger protein that mediates the downstream transcriptional effects of Shh. In the absence of Shh signaling, Gli3 protein is cleaved constitutively to produce a potent transcriptional repressor (GLI3R). Shh activity results in a block in Gli3 processing, yielding a full-length activator form (GLI3A). It has been shown that the repressor GLI3R is present at high levels in the anterior part of the limb and at low levels in the posterior (Wang et al., 2000).

While GLI3R distribution has been reported at a particular limb bud stage, the dynamics of this signaling gradient have never been explored. To determine when GLI3R is present in wild-type chick limbs, we dissected stage 20, 25, and 27 chick limb buds into anterior and posterior thirds and performed Western blots on protein from these dissociated limbs using an antibody that specifically recognizes GLI3R. In all Western blots, equal amounts of protein were loaded in all lanes (see Experimental Procedures). As early as stage 20, we observed a higher level of GLI3R in the anterior than in the posterior region of chick limb buds. This difference is maximal from stages 22–25 and is still detectable at stage 27 (Figure 3A and data not shown). To determine how fast the GLI3R repressor form disappears from cells exposed to Shh, we placed beads soaked with Shh in the anterior of stage 20 chick limbs and then harvested these limbs 1, 2, 4, or 8 hr later. A prominent decrease in anterior GLI3R protein was observed 4 hr after anterior placement of the Shh-soaked bead. By 8 hr, most anterior GLI3R had disappeared compared to GLI3R levels in an untreated limb (Figure 3B).

To determine how quickly GLI3R levels can accumulate once cells cease to be exposed to Shh signaling, we placed a Shh-soaked bead in the anterior of HH stage 20 chick limbs and removed the source of Shh 16 hr later. We assayed GLI3R levels at the time of removal and 1, 2, and 4 hr later. Sixteen hours after placement of a Shh-soaked bead in the anterior of the chick limb, the level of GLI3R was greatly decreased compared to

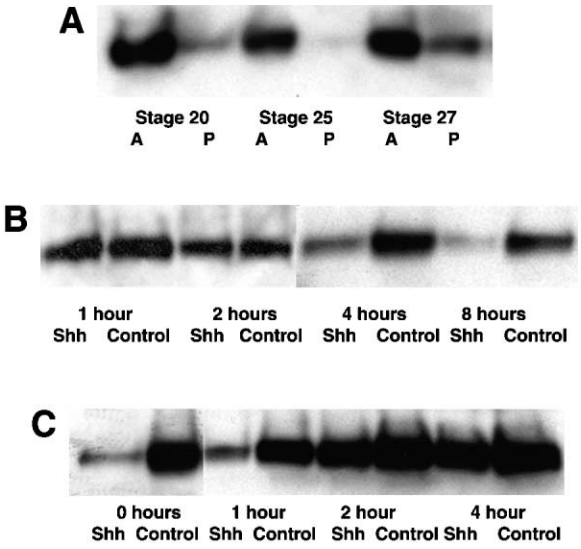

Figure 3. Gli3 Processing Rapidly Responds to Changes in Shh Signaling

(A) Western blot for Gli3R in protein samples collected from the anterior and posterior of chick forelimbs at stages 20, 25, and 27. Gli3R is reduced in the posterior of the limb during the time of *Shh* expression, with this reduction diminishing as *Shh* expression ceases at stage 27.

(B) Western blot for Gli3R in protein samples collected from the anterior of chick forelimbs following implantation of a 10 mg/mL Shh bead at stage 20, and contralateral control limbs. Gli3R levels decrease across the anterior of the limb by 4 hr after bead implantation.

(C) Western blot for Gli3R in protein samples collected from the anterior of chick forelimbs following implantation of a 10 mg/mL Shh bead at stage 20 and removal of the bead 16 hr later, and contralateral control limbs. By 2 hr after the removal of the bead, the levels of Gli3R return to control levels.

untreated limbs. One hour after removal of the Shh-soaked bead, GLI3R levels were significantly increased, and, 2 hr after the removal of the Shh-soaked bead, GLI3R levels in the anterior limb bud returned to normal (Figure 3C). Shh signal transduction, reflected in the cellular concentration of GLI3R, thus equilibrates too quickly to be significantly modulated by the expansion of the former *Shh*-expressing cells.

Insertion of a creER^{T2} Fusion Protein into the Shh Locus to Fate Map Populations of Shh-Positive Cells that Contribute to Individual Digits

The *Shhgfpcre* allele allowed us to determine that *Shh* descendants contribute to digits 3–5 of the limb. Since *Shh* is expressed from E9.75–E12 in the mouse limb, we could not determine using this allele whether early- or late-expressing *Shh* cells contributed to different digits. To identify which population of *Shh*-positive cells contributed to each digit, we knocked a tamoxifen-inducible *cre* reporter cassette into the *Shh* locus to create an *ShhcreER^{T2}* allele (see Experimental Procedures). Mice containing the *ShhcreER^{T2}* allele produced CRE in all cells in which *Shh* mRNA was normally expressed, but cytoplasmically sequestered CREER^{T2} protein was incapable of instigating a recombination event at the *R26R* reporter locus until the injection of tamoxifen (Figure 4B).

Using the *ShhcreER^{T2}* allele allowed us to activate the *R26R* reporter in populations of *Shh*-expressing cells at discrete developmental time points. All cells that expressed *Shh* prior to the introduction of tamoxifen were unlabeled (*LacZ* negative) in these experiments. Limbs in all experiments were analyzed for *LacZ*-positive cells at E14.5. When embryos were exposed to tamoxifen at E9.5, when *Shh* is first expressed, the recombined LacZ allele labeled cells throughout digits 4 and 5, as well as a subset of cells in digit 3, in a pattern indistinguishable from the labeling of cells by the *Shhgfpcre* transgene (Figure 4A). In contrast, the introduction of tamoxifen at E10.5 resulted in the expression of *LacZ* from the *R26R* locus in a smaller subpopulation of *Shh*-expressing cells in the limb. We found that the introduction of tamoxifen at E10.5 resulted in the labeling of *LacZ*-positive cells that contributed to digit 5 and the posterior half of digit 4 in E14.5 limbs (Figure 4C). Injection of tamoxifen at E11.5 resulted in the labeling of cells only in digit 5 (Figure 4D). Thus, cells that contribute to more anterior digits cease to express *Shh* at an earlier stage of limb development.

Digit Patterns under Conditions of Limited Shh Diffusion Support the Importance of Posterior-Anterior Expansion of the ZPA for Specification of Digits 5–3

Our data suggest a model (Figure 5) in which expansion of the posterior limb bud cell population affects the length of time a digit primordium is within the *Shh*-expressing domain and hence is exposed to maximal Shh signaling. We propose that this mechanism may be critical for differential specification of digits 5 and 4, which are entirely derived from former *Shh*-expressing cells, and that this mechanism may also contribute to the specification of digit 3, which is partially derived from such cells (digit 5 primordium sees maximal Shh levels for the largest time, while a subset of digit 3 sees the same levels for the shortest time). In contrast, the digit 2 primordium, which is Shh dependent but never makes Shh, therefore must require Shh diffusion or transport for its specification. Digit 1, based on the *Shh* null phenotype, is specified independent of Shh signaling.

We decided to test this model by genetically modifying the availability of Shh in non-Shh-producing limb bud cells. For this purpose, we made use of a targeted mutation in the gene *Dispatched 1* (*Disp1*), which has been shown to be required for movement of cholesterol-modified hedgehog protein into its target field in *Drosophila* (Burke et al., 1999). Previous studies have demonstrated that Disp1 is required for Hedgehog signaling in non-Hedgehog-producing cells, while those cells that both produce and respond to Hedgehog are Disp1 independent (Ma et al., 2002; Caspary et al., 2002; Tian et al., 2004). Limbs of mice that carry a *Disp1* null allele (*Disp1$^{\Delta 2}$*) (Caspary et al., 2002) and *Disp1* hypomorphic allele (*Disp1^{C829F}*) are normal (data not shown), although the animals die at birth due to defects in Shh-related facial patterning. However, further limiting the availability of Shh by severely reducing the level of Dispatched1

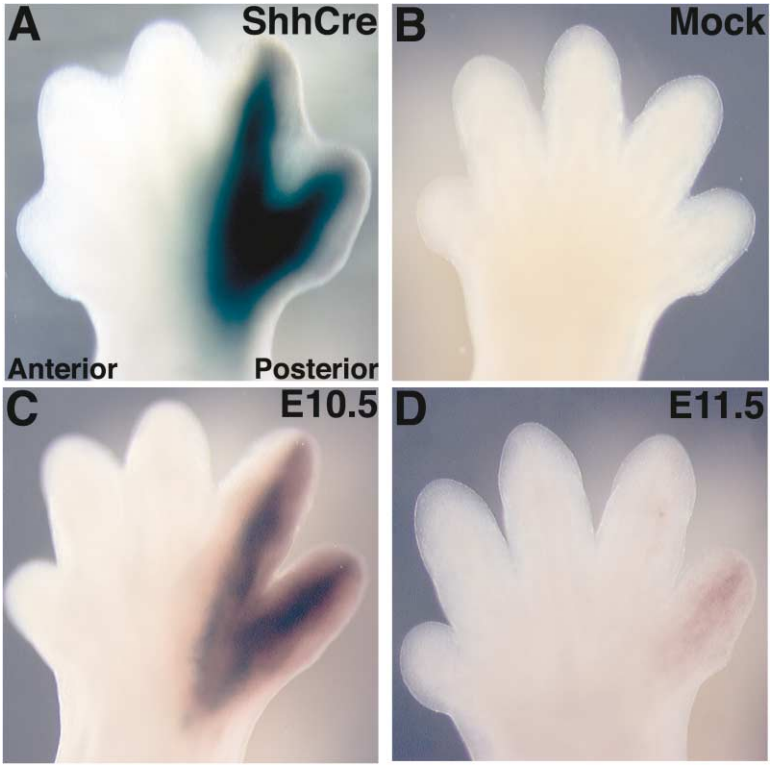

Figure 4. Cells that Form More Posterior Digits Express *Shh* Longer

(A) Whole-mount β-galactosidase staining of an E14.5 *Shh::GFPCre/+;R26R/+* forelimb.

(B) Whole-mount β-galactosidase staining of an E14.5 *Shh::CreER^{T2}/+;R26R/+* forelimb whose mother had been injected with corn oil at E9.5. There is very little ectopic activation of the Cre.

(C and D) Whole-mount β-galactosidase staining of forelimbs from E14.5 *Shh::CreERT/+; R26R/+* embryos whose mothers were injected with 6 mg of Tamoxifen at E10.5 (C) and E11.5 (D). Cells expressing *Shh* at E10.5 contribute to the posterior part of digits 4 and 5, while cells expressing *Shh* at E11.5 only contribute to digit 5.

and removing one copy of *Shh* (*Disp1$^{\Delta 2/C829F}$; Shh$^{+/-}$*) resulted in greater patterning defects.

We reasoned that, if a classical spatial gradient of Shh defines digit identities 5–2, then limiting the availability of Shh and hence producing a shallower gradient of Shh across the anterior-posterior axis of the limb should preferentially affect digit identities that require the highest level of Shh. Importantly, in the ventral neural tube, which does indeed appear to be specified by a classic spatial gradient, this is exactly what is observed when *Disp1* and *Shh* dosage are altered: the ventral-most cell fates requiring highest levels of Shh signaling are lost and replaced by cell fates requiring lower Shh thresholds. Our temporal gradient model, in contrast, predicts a very different outcome in the limb. In this model, the cells that form digits 5, 4, and part of 3 were all in the ZPA, expressed *Shh*, and may therefore depend on time of exposure and not require the diffusion of Shh protein to correctly pattern these digits. In contrast, digit 2 is not composed of any cells that expressed *Shh* and would be predicted to be most sensitive to decreasing Shh availability to target cells, as it is the only digit relying exclusively on diffusion/transport of the Shh protein and therefore the most vulnerable to being misformed in this model.

When we examined E10.5 limb buds from *Disp1$^{\Delta 2/C829F}$*; *Shh$^{+/-}$* embryos, we found that *Shh* mRNA was expressed normally (Figures 6C and 6D). However, as expected, the gradient of Shh protein extended across a significantly smaller domain within the limb bud (Figures 6E and 6F). This resulted in a smaller domain of Shh signaling, reflected in the decreased domain of expression of the target gene *Ptc1* (Figures 6G and 6H) and a more posterior restriction in the domain of expression of *Fgf4* in the AER, a signal known to be maintained by a feedback loop with *Shh* (Laufer et al., 1994; Niswander et al., 1994). The decrease in extent of Fgf production by the AER might be expected to cause a narrowing of limb bud and hence a loss of digits. However, if anterior truncation mediated by decrease in Fgf production was the only cause of digit loss, then we would expect to see a loss of the anterior-most digit 1. In contrast, when we examined skeletal preparations of such limbs, digit 1 was clearly present, as were digits 3–5. Rather strikingly, digit 2 (as judged by the relative lengths of the digits, their articulation with proximal elements, and their order of ossification) was specifically lost (Figures 6A and 6B). All other digits appeared to be specified correctly. Digit 2 is therefore most vulnerable to decrease in diffusible Shh protein. While there are other possible explanations for this result, it is exactly what we would expect if cellular expansion and time of Shh exposure were the more important parameters in the specification of the posterior digit identities, while digit 2 was more dependent on Shh diffusion.

Limb Bud Cells Maintain a Memory of Prior Exposure to Shh

If the time of Shh exposure matters, this implies that limb bud cells respond differently to a given concentration of Shh, depending on whether or not they have had prior exposure to it; in other words, the cells must acquire some "memory" of previous exposure, which affects their subsequent specification. To test this, we implanted beads containing either a high concentration of Shh (1 mg/ml) or buffer into the anterior of stage 20 chick limb buds. Ten hours later, the beads were removed. Consistent with a large number of previous studies, ex-

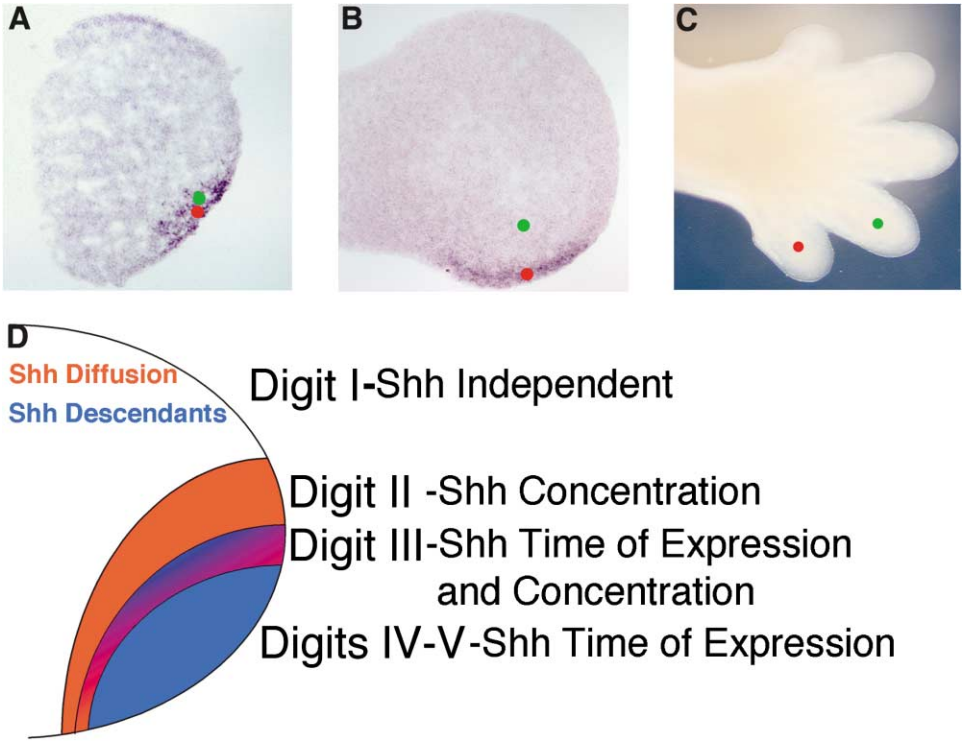

Figure 5. There Is a Temporal Gradient of Shh Exposure in the Posterior Limb Bud

Cells transcribing *Shh* themselves see the highest levels of Shh signaling in an autocrine manner. Early in limb development, the progenitors of both digit 4 (green dot) and digit 5 (red dot) are within the *Shh*-expressing ZPA domain (A), since both those digits are identified as Shh descendants in our lineage-tracing experiments. At later stages, as demonstrated by our tamoxifen-activated lineage-tracing experiments, the primordium of digit 5 still expresses *Shh*, but the primordium of digit 4 does not (B). Thus, the digits form (C) from cells that have seen maximal levels of Shh signaling for different lengths of time (D). Digit 3 contains some cells that have seen maximal Shh signaling for an even shorter period of time than digit 4 but also depends on low levels of Shh signaling from diffusion, since this digit is lost in mice producing only an altered form of Shh (N-Shh), which does not signal outside of the cells that produce it. Digit 2 is completely dependent upon Shh diffusion for its specification, as it is lost in *Shh* null animals but does not contain any cells descended from the *Shh*-expressing domain. See text for details.

posure for such a short period of time, even to the high concentration contained in the Shh beads, was insufficient to induce any change in digit pattern, and normal limbs developed from both the Shh-treated and buffer-treated control limbs (Figures 7A and 7B). A second set of limb buds was similarly treated with either Shh or buffer for 10 hr, but, when the beads in these limbs were removed (at approximately stage 22), they were immediately replaced by a second bead containing 1 mg/ml Shh. These second beads were left in place for 16 hr and then removed. Limb buds were allowed to develop, and digit patterns were assessed. Limbs which only saw buffer in the first bead and hence were exposed to Shh at a concentration of 1 mg/ml for 16 hr formed an ectopic digit 2 (5/8) but never exhibited more posterior digits (Figure 7C). In contrast, those that received Shh in both sequential beads and were hence exposed to 1 mg/ml Shh for a total of 26 hr formed ectopic digits 3 (3/6) or 4(2/6) (Figure 7D). This result verifies that the limb mesenchyme cells form a memory of exposure to Shh even when the duration of exposure is too short to alter their fate and that differential digit specification can be achieved by differences in the length of exposure to Shh even when they receive the same maximal concentration of Shh.

Discussion

A Temporal Gradient of Shh Signaling in the Limb Bud

Cells that express *Shh* comprise the classically defined ZPA in the vertebrate limb. Numerous in vivo experiments have demonstrated that differential levels of signaling by Shh along the anterior-posterior axis are critical for determining what type of digit is formed. Classically, the differential levels of signaling have been hypothesized to be established by a spatial morphogen gradient. Cells exposed to low amounts of Shh become anterior digits, while exposure to high levels of Shh results in the formation of posterior digits. Our data suggest that a second mechanism is also important in digit specification by Shh, a temporal gradient established by the expansion of descendants of *Shh*-expressing cells.

Persistence of Active Shh Signaling during Limb Development

It is implicit in the temporal gradient model that the cells within the ZPA continue to see high levels of Shh signaling over an extended period of time. Several genes believed to be Shh targets are expressed in a nested pattern overlapping with the Shh-expressing cells in the

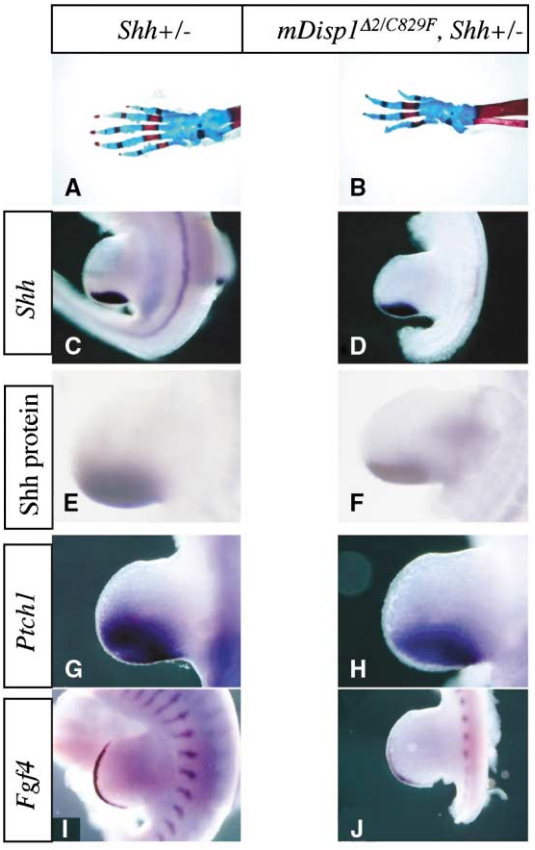

Figure 6. Digit 2 Is Most Sensitive to Decreased Shh Diffusion

(A and B) In a $Shh^{+/-}$ background (A), loss of Dispatched activity in $mDisp1^{\Delta 2/C829F}$, $Shh^{+/-}$ mice results in loss of digit 2 (B), as shown in this skeletal preparation of a hindlimb.

(C–J) *Shh* expression is not affected by loss of Dispatched activity (C and D); however, the range of diffusion of Shh protein is reduced, as shown by whole-mount immunohistochemistry on mouse hindlimbs (E and F). Nonetheless, low levels of Shh protein are observed in a gradient toward the anterior, resulting in a reduction in the anterior extent but a maintenance of graded expression of direct targets of Shh signaling such as *Ptch1* (G and H) and *Fgf4* (I and J), as shown by whole-mount in situ hybridization on hindlimbs.

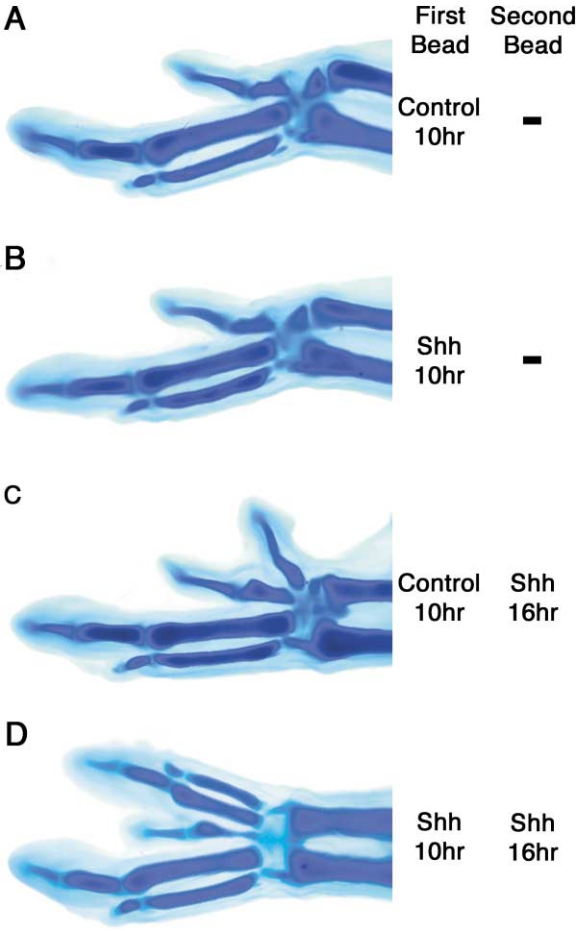

Figure 7. Priming with Shh Protein Leads to a Greater Duplication

(A and B) In the chick forelimb, exposure to a control bead (A) or a (1 mg/ml) Shh bead (B) for 10 hr, starting at stage 20, does not give rise to a duplication.

(C) Exposure to a control bead for 10 hr followed by a Shh bead (1 mg/ml) for 16 hr leads to a digit 2 duplication.

(D) Forelimbs that are primed with 10 hr of exposure to a Shh bead followed by 16 hr of exposure to another Shh bead have a duplication of digits 3 and 4. Thus, cells remember prior exposure to Shh.

early limb bud, including *Ptc1* (Marigo et al., 1996a; Goodrich et al., 1996), *Ptc2* (Pearse et al., 2001), *Bmp2* (Francis et al., 1994; Laufer et al., 1994), and *Gli1* (Marigo et al., 1996b). Subsequently, one of these, *Gli1*, is markedly downregulated in the ZPA region (Marigo et al. 1996b). However, the expression of the other putative Shh targets persists in the posterior limb bud with their strongest expression at the posterior margin as long as *Shh* is expressed (Marigo et al., 1996a, Pearse et al., 2001, Laufer et al., 1994). It is likely that *Gli1* shows a differential expression pattern, because it is only responding to the presence of Gli activators, while the activity of the other genes may be more attributable to the absence of Gli repressors. Late in limb development, *Gli2* and *Gli3* are transcriptionally downregulated in the ZPA region (Marigo et al., 1996b). As a consequence, one would expect Gli activator activity to be greatly diminished, while the lack of Gli repressors would, if anything, be enhanced. Regardless, as *Gli1* is irrelevant for normal limb morphogenesis (Park et al., 2000), the functional significance of its downregulation in the posterior limb is unclear. More importantly, the strong, persistent posteriorly graded expression of *Ptc1* and other targets indicates that there is continued active response to Shh within the ZPA region throughout the limb-patterning phase.

Fate Map of the ZPA

The chick limb bud has been very carefully fate mapped with DiI by Cheryll Tickle and colleagues (Vargesson et al., 1997). If one refers back to the fate maps they derived, when cells that would be expected to express *Shh* were labeled at stage 20 (posterior-distal blue, red and green dots in Figures 1A and B of Vargesson et al. [1997]), it can be seen that they expand to encompass a domain very much like the one described here by stage 28. That the posterior digits are entirely descended from Shh-expressing cells was not appreciated, however, be-

cause of other experiments indicating that the former *Shh*-expressing cells might all undergo apoptosis within the late limb bud (Sanz-Ezquerro and Tickle, 2000), implying that the posterior cells labeled by DiI by Vargesson et al. might mark non-*Shh* expressing cells in the posterior that are intermingled with the ZPA cells. However, we have confirmed earlier suggestions (Saunders and Fallon, 1967) that posterior apoptosis is not a feature of limb development in the mouse (see Supplemental Figure S2 at *Cell* web site). In light of these findings and with reference to conserved mechanisms of limb patterning across species, we would suggest that the Vargesson map might be taken at face value and that similar expansion of the *Shh*-expressing cells likely occurs in the chick as well. The observed apoptosis in the posterior chick limb, in fact, is at all stages proximal to the hand plate (Saunders and Fallon, 1967). The observed apoptosis in the posterior chick limb bud may be relevant to eliminating late-stage Shh signaling in the proximal limb (Sanz-Ezquerro and Tickle, 2000), but we would suggest that it is not relevant to expansion of former ZPA cells in the distal limb bud.

Cellular Memory of Prior Exposure to Shh

Our model explicitly depends on the ability of cells to respond differentially based on their time of exposure to Shh. Exposing chick limb bud mesenchyme to a high concentration of Shh for 10 hr had no effect on limb pattern. However, the treated cells exhibited a memory of this exposure, revealed when they were exposed to the same concentration for 16 more hours. The digit primordia preexposed to Shh adopted a more posterior fate than digit primordia treated in parallel but without the preexposure. These experiments indicate that time of exposure to Shh can indeed determine digit identity. Previous studies (Yang et al., 1997) have also provided evidence that this is the case. Beads soaked in high concentrations of Shh were implanted into the anterior of chick limb buds. The cells adjacent to the bead were marked with DiI. The beads were then removed after different lengths of time. Depending on the length of exposure to Shh, the equivalent, marked cells developed into an ectopic digit 2 (with short exposure) or digit 3 or 4 (with longer exposure). These data supported a "promotion" model (Tickle, 1995) in which digit primordia are first specified to an anterior fate and are then promoted to more posterior cell fates with longer times of exposure. Their model also postulated that different digit primordia received different lengths of exposure to Shh because of the expansion of the posterior tissue (or, in their experimental case, expansion of anterior tissue from the point source of extremely high Shh signaling provided by the bead).

The view expressed here is not inconsistent with the promotion aspects of the model proposed by Tickle (1995). Both models invoke time of exposure, based on expansion of the limb field, as a key parameter. The differences are that the promotion model focuses on the sequential steps by which digit identities are specified in limb mesenchyme, and that, according to this model, all digit primordia depend on both time and concentration of exposure. In contrast, our model does not explicitly address whether or not posterior digits are first transiently specified as anterior digits. However, it much more concretely suggests that the two most posterior digits are distinguished only by the length of time they are exposed to high levels of Shh, while digit 2 specification depends exclusively on low concentrations only achieved by a spatial diffusion gradient.

Shh Directly Specifies Digit Identity

It is currently unclear whether Shh acts directly to pattern the anterior-posterior axis or acts via a secondary morphogen (most often proposed to be the secreted factor BMP2, which is indeed induced in a broader domain in the posterior limb bud by Shh [Laufer et al., 1994]). There has also been the proposal that Shh is required over a long distance in a concentration-independent fashion to "prime" the limb mesenchyme, while BMP2, induced by high concentrations of Shh in the posterior limb bud, acts as a true graded signal specifying distinct digit identities (Drossopoulou et al., 2000).

Our genetic experiments, decreasing the levels of diffusible Shh protein with *Dispatched-1* mutant alleles, in addition to supporting our model for a temporal gradient in the posterior limb bud also suggest that Shh may indeed be a direct morphogen in digit specification. If, instead, BMP2 were the true morphogen, then one would not expect to lose digit 2, as the diffusion of BMP2 is not impeded in the mutant, and, hence, the threshold concentration for specifying digit 2 should be achieved normally. Definitive resolution of this issue will have to await the conditional removal of BMP activity from the limb bud.

Our experiments in decreasing the availability of Shh protein resulted in a specific loss of digit 2, as expected in a model where more posterior digits are less dependent upon Shh diffusion for their specification. We therefore conclude that digit 2 in particular is established by the concentration gradient of Shh. For this to be true, the digit 2 primordium must actually respond to Shh during normal limb development. From the Shh null phenotype, it is clear that digits 2–5 all depend upon Shh activity, but this does not necessarily imply that Shh directly acts on digit 2 cells. Such evidence has now been obtained by fate mapping studies analogous to those described here, in which CRE recombinase driven by the promoter of a known Shh target, *Gli1*, was used to mark cells in reporter mice. β-galactosidase staining of limbs where CRE was activated within the Gli1 domain at the time of Shh signaling showed that, indeed, digits 2–5 all arise from precursor cells that have actively responded to Shh during limb development (Ahn and Joyner, 2004 [this issue of *Cell*]).

Temporal Signaling Explains Phenotypes that Are Difficult to Understand on the Basis of a Spatial Morphogenic Gradient

Importantly, the temporal gradient model proposed here also provides an explanation for an aspect of the phenotype of another mutant affecting Shh activity in the limb bud. Shh has two major domains: a catalytic C-terminal half and an N-terminal half that contains the signaling activity. When this N-terminal portion is expressed in mammalian cells, it is not properly modified, although it retains its signaling potency. When an allele expressing

only the N-terminal signaling portion of Shh was created in place of the normal *Shh* allele, it was found that while N-Shh was produced at normal levels, it was only able to signal in the cells that produced it (Lewis et al., 2001). Thus, instead of target genes such as *Ptc1* being expressed in a broad, graded domain in the posterior limb bud, they are expressed in a small, sharply defined posterior domain identical to the domain of *Shh* itself. The skeletal phenotype observed in these animals is a loss of digits 2 and 3. Retention of digit 1 is expected, as it is Shh independent, and retention of digits 4 and 5 makes sense, as we find that these two digits are formed entirely from *Shh*-expressing cells. However, since digits 4 and 5 have different morphologies, and they arise in the absence of functional transport of Shh protein, the differential specification of these two digits cannot be based on a classic diffusion gradient, and another mechanism is required. Our data showing a difference in exposure time of the two primordia to Shh provide an explanation: in this mutant, as in wild-type, the primordia of digits 4 and 5 see the maximal level of Shh signaling for different lengths of time, and the temporal gradient of Shh exposure is the key parameter for discriminating between the identities of these two most posterior digits.

Conclusions

Taken together, our results are consistent with the specifications of digits 5–2 being determined by the dose of Shh received by the limb bud mesenchyme. However, they argue that the dose is critically dependent upon time of exposure in addition to the concentration of Shh protein available in different parts of the limb bud. In particular, specification of digit 2 depends strictly on low threshold concentration of Shh achieved by a spatial gradient, while differential specification of digits 4 and 5 seems to be defined by the differential time of exposure achieved by a temporal gradient. Digit 3 may be specified by a combination of these two parameters of exposure in time and space.

Experimental Procedures

Mouse Construction and Genotyping

The *Shhgfpcre* targeting construct utilized a *gfpcre* cassette containing an in-frame fusion between *gfp* and *cre* (Le et al., 1999). The *gfpcre* cassette was placed at the ATG of *Shh*. Base pairs located at −1 and −5 relative to the *Shh* ATG were changed from a G/C to a C/G and an A/T to a T/A, respectively, to create a *Sal1* site used to clone a 1.2 kb 5′ targeting arm. The 3′ targeting arm was 8 kb and began 35 bp downstream of the *Shh* ATG. The only genomic sequences lacking in correctly targeted ES cells were the first 35 base pairs after the ATG of *Shh*. These base pairs were excised to create a *Shh* null allele. All genomic sequence involved in regulating expression from the *Shh* locus should be present in correctly targeted ES cells. The *ShhcreER^{T2}* allele was made in an identical manner, except that a *creER^{T2}* cassette (Feil et al., 1997; Leone et al., 2003) was inserted instead of *gfpcre*.

Males and females containing both the *Shhgfpcre* and *R26R* (Soriano, 1999) alleles were mated to generate embryos containing both alleles in some of our experiments. Embryos generated by this cross were indistinguishable from embryos derived from parents containing either the *Shhgfpcre* or the *R26R* alleles. The lack of production of entirely blue animals when both the *Shhgfpcre* and *R26R* alleles originated from the same parent suggests that Shh descendants do not contribute to germ cell formation.

β-Galactosidase Staining

Analysis of β-galactosidase activity was performed using standard methods. In brief, embryos or sections were fixed in 4% paraformaldehyde and then washed in concentrated rinse buffer (0.1 M sodium phosphate [pH 7.4], 0.1% sodium deoxycholate, 2 mM $MgCl_2$, 0.2% NP-40) for 3 × 10 min. Samples were then allowed to develop in the dark overnight either at room temperature or at 37°C in staining solution (1 mg/ml X-Gal in DMF, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, in concentrated rinse buffer). The staining reaction was stopped by placing samples in 4% paraformaldehyde for 2 hr.

Skeleton Preparations and In Situs

For mouse skeletal preparations, 18.5 dpc embryos were processed as described previously (Karp et al., 2000). Whole-mount chick Alcian blue staining was performed on stage 35 wings as previously described (Goff and Tabin, 1997). Shh immunohistochemistry was performed as described previously (Lewis et al., 2001). Whole-mount and section in situ hybridization using digoxigenin-labeled RNA probes was performed as described previously (Murtaugh et al., 1999; Wilkinson, 1992).

Placement of Shh-Soaked Beads and Western Blots

Heparin beads (Sigma) were soaked in 10 mg/mL Shh protein (Curis) on ice for 1 hr. Eggs were incubated to stage 20 and windowed, and a bead was implanted into the anterior limb mesenchyme underneath the AER. The embryos were reincubated for the specified length of time and then either the bead was removed and it was reincubated again or they were harvested and the protein purified. Staging was done according to Hamburger and Hamilton (1951). Control limbs were from the untreated, contralateral side of experimental embryos. In all experiments, five treated or untreated limbs were pooled per lane. The antibody used to detect GLI3R was a gift from S. Mackem. This antibody primarily recognizes the N-terminal region of GLI3. Protein (10 μg) was loaded in each lane. Anterior and posterior limb buds were prepared and analyzed on Western blots as previously described (Wang et al., 2000; Litingtung, et al. 2002). To confirm equal loading of protein in each lane, an identical amount of protein was loaded on a separate gel and stained with Coomassie blue.

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