

Making digit patterns in the vertebrate limb

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Abstract | The vertebrate limb has been a premier model for studying pattern formation — a striking digit pattern is formed in human hands, with a thumb forming at one edge and a little finger at the other. Classic embryological studies in different model organisms combined with new sophisticated techniques that integrate gene-expression patterns and cell behaviour have begun to shed light on the mechanisms that control digit patterning, and stimulate re-evaluation of the current models.

Mesenchyme

A loose meshwork of cells found in vertebrate embryos, which is usually derived from the mesoderm, the middle of the three germ layers.

Ectoderm

The epithelium that is derived from the outer of the three germ layers of the embryo and will give rise to the epidermis of the skin.

Positional information

The instructions that are interpreted by cells to determine their differentiation with respect to their position within the embryo.

Morphogen

A diffusible chemical substance that carries information in embryos, for example, cell position.

A fundamental biological question is how the body plan is laid down during embryonic development and how precise arrangements of specialized cells and tissues arise. The vertebrate limb has a complex anatomy and is an excellent model in which to address this question. Vertebrate limbs develop from small buds of apparently homogeneous unspecialized mesenchyme cells that are encased in the ectoderm. As the buds grow out from the body wall, these unspecialized cells begin to differentiate into various tissues of the limb — including the cartilage and, later in development, the bone — that make up the skeleton. The limb skeleton consists of a defined number of bones of characteristic size and shape that are arranged in a specific pattern. The anatomy of the limb can be described with respect to three orthogonal axes, proximo–distal, from shoulder to finger tips, antero–posterior, from thumb to little finger, and dorso–ventral, from the back of the hand to the palm (FIG. 1). The processes that regulate limb formation are highly coordinated; for example, each digit forms in a specific place. But how is this pattern of cell differentiation controlled?

For the last 40 years or so, this problem and, in particular, how digit pattern arises, has been tackled by applying the concepts of positional information^{1,2}. According to these concepts, cells are first informed of their position in the limb bud and, as a result, they acquire a positional value that encodes this information. In a second step, these values are interpreted, leading to the formation of the appropriate structure at that position. Positional information across the antero–posterior limb axis is provided by signalling of the polarizing region at the posterior margin of the early limb bud. Classical experiments on chick embryos^{3,4} (BOX 1) led to the identification of this region and the proposal that the polarizing region produces a morphogen which diffuses, over time, into

adjacent limb tissue to give a concentration gradient. Cells at different positions across the limb bud would be exposed to different concentrations of the morphogen — cells nearest the polarizing region, at the posterior of the limb, would be exposed to high concentrations of the morphogen, whereas cells further away, at the anterior of the limb bud, would be exposed to low concentrations. Therefore, the local concentration of the morphogen could provide information about position across the antero–posterior axis. These experiments also indicated that a ratchet-type mechanism is in operation, such that the anterior positional values can be promoted irreversibly to more posterior positional values, and the most posterior positional values are then remembered.

In the last 15 years, the molecular basis of limb development has begun to be unravelled through the identification of the signals that are generated in the polarizing region and the discovery of molecules that are produced in response to these signals. These molecular advances come mainly from genetic studies in mice (*Mus musculus*), although many originated in fruitflies (*Drosophila melanogaster*). Recent sophisticated analyses in mouse embryos, which link gene expression and cell fate in developing limbs, have stimulated the re-evaluation of the mechanisms of digit patterning and have highlighted the particular problem of providing positional information in a growing organ. In this article, I begin by outlining the embryological studies that revealed the signalling properties of the polarizing region and then discuss the molecular basis of polarizing signalling in the developing limb bud, with particular emphasis on the secreted molecule, sonic hedgehog (SHH). I conclude by discussing the outstanding challenges in identifying how antero–posterior values are encoded molecularly and how they are ultimately translated into digit anatomy.

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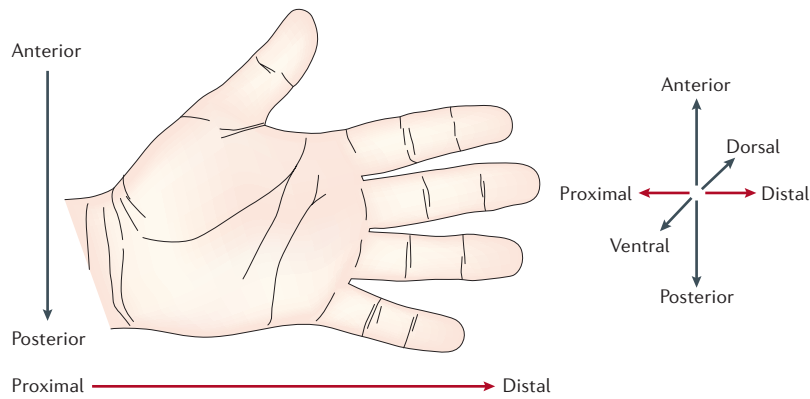


Figure 1 | The three main axes of the human hand. The diagram shows the three main axes, proximo–distal, antero–posterior and dorso–ventral, of a human hand. Tightly regulated processes during embryonic development ensure that the thumb arises at one edge of the hand, whereas the little finger arises at the other.

Embryology of digit patterning

Digits form at relatively late stages of vertebrate limb development. By this time, the small bud has grown substantially and changed shape so that the main regions of the future limb can be made out (BOX 2). However, experiments on chick embryos have shown that cells are specified to form limbs, long before any buds are visible, and that both the antero–posterior and the dorso–ventral polarity of the limb are already established (reviewed in REF. 5). Fibroblast growth factors (FGFs) and Wnt–signalling molecules are involved in limb initiation and budding and control the limb-specific expression of genes that encode members of the Tbx family of transcription factors⁶.

An early event in bud formation is the development of the apical ectodermal ridge. The apical ridge forms at a compartment boundary between the dorsal ectoderm and the ventral ectoderm — this ensures that the limb buds form at the sides of the body⁷. Continued outgrowth of the bud depends on FGF signalling by the apical ridge⁸. Another important event in limb-bud development is the formation of the polarizing region that controls the antero–posterior pattern of distal structures. Once equipped with an apical ectodermal ridge and a polarizing region, the limb bud can develop autonomously.

Insights from chick embryos. The normal chick wing has three digits known as 2, 3 and 4 (BOX 2), and it has been proposed that digit patterning involves signalling between the cells of the polarizing region and the adjacent limb-bud cells³. Grafting the polarizing region from one chick wing bud to the anterior (opposite) side of a second bud results in a dramatic change in digit pattern; six digits develop instead of three, with the extra set of digits in mirror-image symmetry with the normal set, giving rise to the pattern 4 3 2 2 3 4 (BOX 1). Grafts of the posterior margin of mammalian limb buds to chick wing buds have also been shown to have polarizing activity (for example, see REF. 9). The

extra digits induced in this case are, nevertheless, chick digits, which indicates that, although the signal is the same, the interpretation differs.

But how does the polarizing region produce such a pattern? The results of many chick embryological experiments are consistent with the idea that the polarizing region produces a long-range morphogen that specifies antero–posterior positional values. Polarizing activity can be detected in the posterior region of the chick wing bud from early stages until the stage that the digits start to form¹⁰. Long-range signalling by the polarizing region operates over a few hundred µm (about 10–30 cells; BOX 1). Therefore, it is likely that positional values are specified in the early limb bud. Indeed fate maps show that digits arise from the posterior region of the early limb bud, which then expands to fill the digital plate^{11,12}. Fate maps do not provide information about commitment, but, because there is experimental evidence for positional memory (BOX 1), the prevailing model has been that polarizing-region signalling sets up a morphogen gradient, which specifies antero–posterior positional values in the early bud (FIG. 2a). This set of initially tightly packed positional values then becomes distributed across the limb bud as the bud grows, and later dictates the development of each digit primordium.

Polarizing-region grafts change digit number in addition to pattern. One of the consequences of grafting a polarizing region to the anterior margin of the wing bud is an increase in the width of the bud to accommodate the extra digits⁴. This is accompanied by an increase in length of the apical ectodermal ridge. It was postulated a long time ago that the polarizing region regulates production of an apical-ridge maintenance factor by the mesenchyme cells in the posterior region of the bud¹³. It has also been suggested that signalling through the polarizing region might have a direct effect on cell proliferation¹⁴, because changes in cell proliferation have been detected prior to changes in ridge length.

Morphogen gradient mechanism

The vitamin-A derivative, retinoic acid, was the first defined signalling molecule to be identified as having the ability to induce mirror-image duplications in the chick wing¹⁵ (BOX 1). Although it was shown that retinoic acid is readily diffusible and functions in a concentration-dependent fashion¹⁵, the main role of endogenous retinoic acid in polarizing signalling is now thought to be the induction of the expression of the SHH gene¹⁶. It should be noted that retinoic acid also has other roles in limb-bud initiation¹⁷ and in patterning of the proximal part of the limb¹⁸.

Identification of candidate morphogens. SHH is expressed in the polarizing region of both the chick¹⁶ and the mouse limb buds, and immunohistochemical studies¹⁹ and a biological assay — based on the ability of SHH to induce differentiation in a cell line²⁰ — indicate that the SHH protein diffuses some distance away into the limb bud. Beads soaked in SHH protein cause concentration-dependent changes in digit pattern and the

Tbx family

Related transcription factors that contain a T-Box.

Apical ectodermal ridge

Thickening of the ectoderm rim at the tip of a developing limb bud in a vertebrate embryo. It is required for bud outgrowth.

Polarizing activity

The ability of cells, tissue or defined chemicals to induce the formation of extra digits from the anterior region of a chick limb bud.

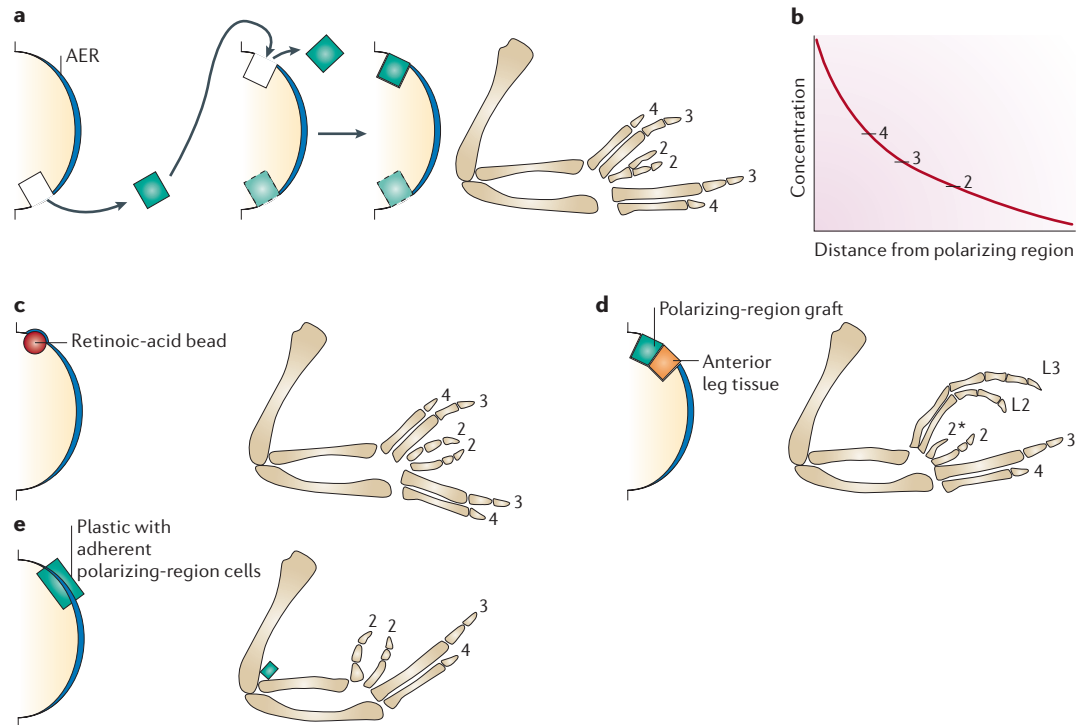
Fate map

A diagram that is obtained experimentally by tracing marked cells and shows the structures that derive from cells in different regions of an embryo.

Digital plate

Broad region that forms late during limb development at the distal end of the bud and contains the digit primordia.

Box 1 | Experimental evidence for the morphogen-gradient model.



The experiment that led to the identification of the polarizing region involved grafting tissue from the posterior margin of one chick wing bud to the anterior of a second wing bud (see figure, part a). This resulted in a mirror-image digit pattern 4 3 2 2 3 4 (given that the normal chick wing has three digits known as 2, 3 and 4; see BOX 2). In the figure, AER indicates the apical ectodermal ridge that rims the early limb bud. These experiments indicated that a morphogen gradient that is produced by the polarizing region could provide positional information (see figure, part b). Morphogen concentrations that might specify positional values for each of the three chick wing digits, 2, 3 and 4 are illustrated. A mirror-image duplication of the digit patterns can also be produced with beads soaked in retinoic acid (see figure, part c).

The experiments that indicated that the polarizing region specifies antero–posterior position in the early limb bud, which is then remembered, involved grafting a polarizing region to the anterior margin of a host wing bud, and then removing it at later time points^{40,64}. When grafts were removed earlier than 12 hours after implantation, extra digits did not form, whereas, when grafts were removed at 16–17 hours after implantation, just an extra digit 2 was formed. Grafts that were removed a few hours later produced extra digits 3 and 2. Sequential formation of digits can be explained by the progressive spread of a diffusible morphogen. No polarizing activity was detected in anterior host tissue after removal of polarizing-region grafts at 36 hours, which provides evidence for a positional memory.

The long-range nature of polarizing signalling was demonstrated by placing a graft of anterior-leg-bud tissue between the polarizing-region graft and the responding host wing bud (see figure, part d). Extra toes were induced in leg tissue (L3, L2) but also an extra digit, digit 2*, was induced in wing tissue, which had not been in contact with the polarizing-region graft (see REF. 65). The grafts of leg tissue were about 200 µm in diameter, showing that the polarizing-region signal can travel about 20 cell diameters.

Concentration dependency of polarizing-region signalling was demonstrated by grafting known numbers of polarizing-region cells onto small pieces of plastic film (see figure, part e). With small numbers of cells (9–80 cells), only an extra digit 2 is formed. The small piece of plastic film ended up at the elbow of the limb that developed (see REF. 66).

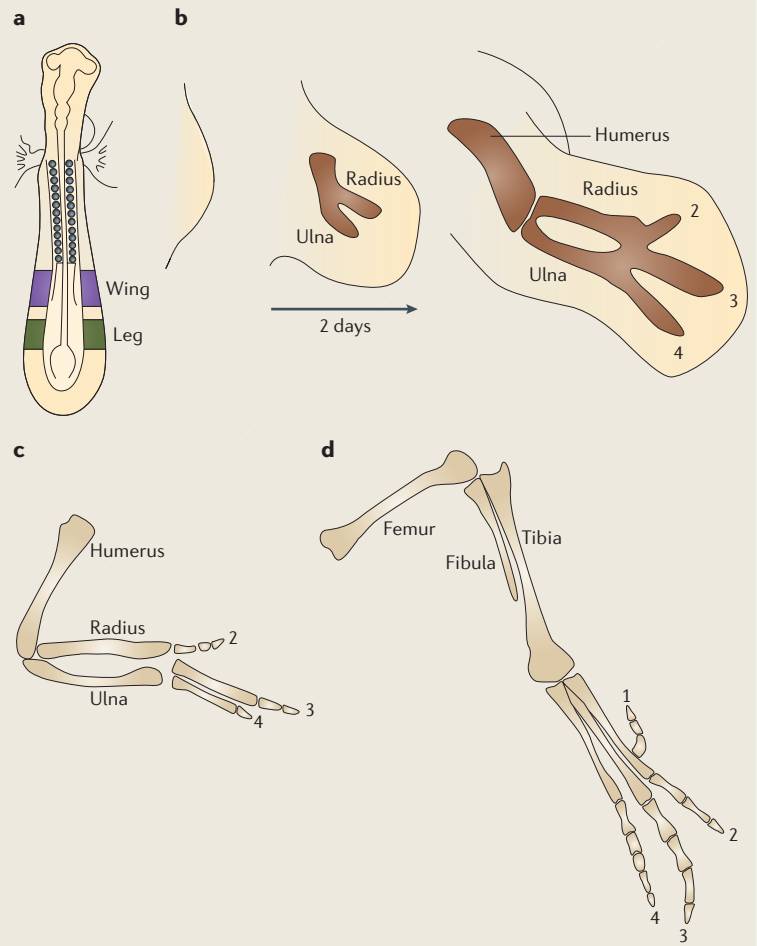
timing of digit induction in the chick wing bud matches that seen with polarizing-region grafts. A lag of approximately 14 hours occurs before any extra digits form, and is followed by sequential formation of the 3 digits between 16 and 24 hours²¹. Promotion of positional values over time was demonstrated directly by tracing the behaviour of cells at known distances from a SHH bead with a lipophilic dye that can be applied to the membranes of cells to mark them. When the SHH bead is removed at 16 hours, cells at a distance of 130 µm (about 13 cell diameters) from the bead participate in forming

an extra digit 2. When SHH treatment is extended, cells at 130 µm from the bead participate in forming more posterior digits. One interpretation of these data is that it takes about 24 hours to establish a stable SHH gradient across the anterior region of the chick wing bud. This provides an estimate of the time that is required to establish a SHH gradient in the posterior of the limb bud during normal development (FIG. 2a).

There is a positive-feedback loop involving FGF signalling from the apical ridge that maintains SHH expression in the polarizing region⁶; SHH, in turn, maintains

Box 2 | Description of chick limb development.

Studies in chick embryos have shown that cells receive signals to form limbs long before there are any visible signs of limb formation. Transplantation experiments indicated the regions that will give rise to wing or leg tissue when transplanted (see figure, part a). Cells in these regions give rise to swellings, which then become discrete buds. The buds elongate and grow out from the body and gradually form a limb-like shape. Development of the skeleton can be readily monitored by staining the developing limbs and making whole mounts. The skeleton is laid down in sequence, as the buds grow out, starting with structures nearest the body wall — for example, the humerus — and digits are the last structures to form (see figure, part b). It takes about 2 days to develop from the appearance of an early limb bud to a stage at which digit primordia have formed. The skeleton of the chick wing (see figure, part c) conforms to the general vertebrate plan, except that there are only three digits — 2, 3 and 4 — and each has a reduced number of phalanges. The chick leg (see figure, part d) has four toes — 1, 2, 3 and 4 — and each toe has a different number of phalanges.



expression of *FGF* genes, including *FGF4*, in the apical ridge (BOX 3). In *Shh*^{-/-} mouse embryos, the distal regions of the limbs are very reduced — at best, one digit develops²² — and this can be understood in terms of the failure to maintain the positive-feedback loop.

The role of *GLI* genes. Important components of the hedgehog (*Hh*) signalling pathway were first discovered through genetic studies in fruitflies, but it is now clear that these components have been largely conserved during evolution (reviewed in REF. 23). The three vertebrate *GLI* proteins, *GLI1*, *GLI2* and *GLI3*, are the transcriptional effectors of *SHH* signalling. In the absence of the *SHH* ligand, the *GLI2* and *GLI3* proteins are processed to short repressor forms that translocate to the nucleus and repress *SHH* target genes. By contrast, in the presence of the *SHH* ligand, full-length *GLI* proteins — in particular *GLI1* and *GLI2* — translocate to the nucleus where they function as activators to induce expression of *SHH* target genes, including the gene encoding *GLI1*.

Analysis of mouse embryos shows that *Gli3* has an important role in limb patterning. *Gli1*^{-/-} and *Gli2*^{-/-} mouse embryos have normal limbs, but *Gli3*^{-/-} mouse

limbs are polydactylous and have approximately eight unpatterned digits^{24,25}. In the normal limb, low levels of the short repressor form of *GLI3* (*GLI3R*) are present in the posterior region, from which the series of patterned digits develop, and high levels of *GLI3R* are present in the anterior region, which does not give rise to digits²⁶ (BOX 3). On the basis of these data, it has been suggested that the main role for *SHH* signalling is to relieve *GLI3* repression in the posterior region of the limb bud. In the absence of *SHH* — for example, in *Shh*^{-/-} embryos — the short *GLI3R* will prevail throughout the limb bud and therefore digit development will be severely reduced.

It is unclear whether the balance between the levels of the full-length activator form of *GLI3* (*GLI3A*) and *GLI3R* forms the basis for a graded response to *SHH* concentration, and subsequently leads to the specification of antero-posterior positional values, or if just the levels of *GLI3R* in different regions of the limb bud are important. Other mouse mutants with many unpatterned digits, in which novel vertebrate components of the *Hh* signalling pathway (such as the intraflagellar transport proteins) are affected, also

Humerus
The single bone in the upper arm, initially laid down in cartilage.

Polydactylous
Having more than the normal number of digits.

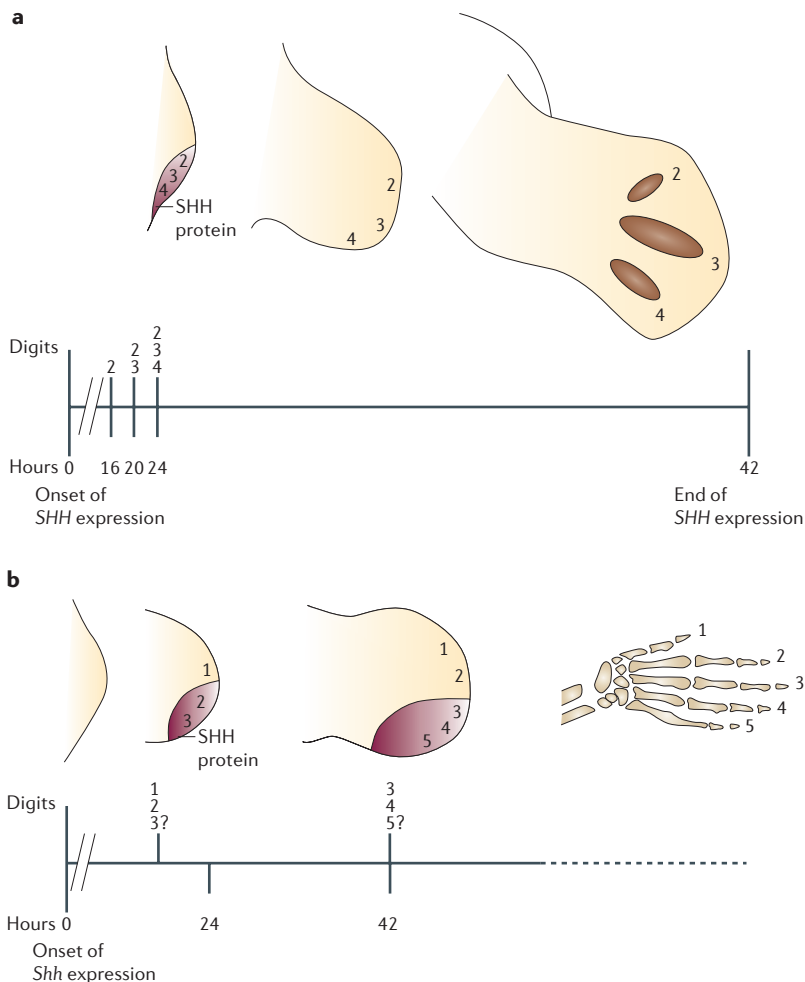


Figure 2 | Models for specifying antero-posterior positional values in the chick wing and the mouse limb. a | Morphogen-gradient model in the chick wing in which sonic hedgehog (SHH) concentration specifies antero-posterior positional values. The numbers represent the positional values for each chick wing digit. All positional values are specified in the early limb bud according to a morphogen gradient (pink shaded area) and the values are then remembered. The digit primordia develop approximately 24 hours after the positional values are fixed. The timeline illustrates the specification of positional values in the early limb bud from the onset of SHH expression on the basis of a time course of induction of extra digits by SHH beads²¹. Positional values are progressively promoted (between 16 and 24 hours) as the gradient of SHH becomes established. **b** | A new model for the specification of antero-posterior values in the mouse limb that involves both the concentration and the length of exposure to SHH³⁵. The numbers represent positional values for each mouse digit. *Shh* is expressed at embryonic day (E)9.75. By E10.5, the SHH concentration gradient (shaded area) that was established in the early limb bud has led to the specification of the positional value of digit 2, and contributes to the specification of digit 3. The development of digit 1 is not dependent on SHH signalling. By E11.5, digit 3 is specified according to both the concentration and the length of exposure to SHH, but digits 4 and 5 are specified according to differences in the length of exposure to SHH. The timeline shows that, according to this model, posterior digits will be specified at later stages of development than anterior digits.

The series of many unpatterned digits, which develop in the absence of GLI3 function in mouse mutants, is reminiscent of the digit pre-pattern that has been proposed to function in combination with the morphogen gradient². According to this proposal, a series of digit condensations, a pre-pattern, is specified by a wave-like distribution of a morphogen that is generated by a reaction-diffusion mechanism, with the peaks corresponding to the condensations. A gradient of another morphogen — for which SHH is a good candidate — then provides each peak with a positional value and a digit identity. The number of peaks that are generated by the reaction-diffusion mechanism depends on the width of the limb. Therefore, the fact that these polydactylous mouse mutants have broader limbs could explain why they have more digits; furthermore, that the balance between GLI3A and GLI3R, and/or the absolute levels of GLI3R, are abnormal could explain why the digits are unpatterned.

Does SHH function as a morphogen? A critical issue to elucidate is whether target genes, for which repression is alleviated by SHH, encode positional values, or if specification of positional values is indirect and involves the production of other signalling molecules. Genes encoding BMPs are expressed in early limb buds. *BMP2* is expressed at the posterior margin of the chick limb that overlaps with the polarizing region. Ectopic expression of *BMP2* can be induced at the anterior margin of a chick wing when SHH is applied²¹. BMPs could therefore be involved in the specification of positional values, and could function locally or diffuse across the limb to form a gradient. However, addition of *BMP2* to the anterior margin of the chick wing buds produces only small changes in digit pattern³⁰, which indicates that BMPs might only be able to specify positional values in cells already ‘primed’ by SHH. Experiments in chick wing buds further support this idea³¹, whereas other experiments indicate that BMP signalling might also operate at the anterior of the limbs during the early stages of limb development, possibly antagonizing SHH signalling³². In addition to these extracellular signalling molecules that are associated with the polarizing region, there is a gradient of gap-junctional communication across the antero-posterior axis of the chick limb³³. Interfering with gap-junctional communication in the cells within the polarizing region, and also in the responding cells, affects polarizing activity³⁴. It therefore seems that direct cell-cell interactions might also be involved in the specification of positional values.

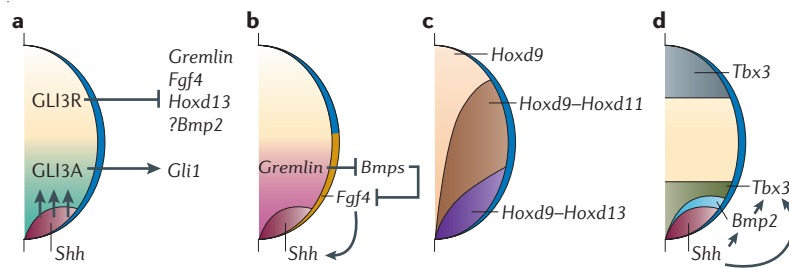
Timing mechanism

Another way of specifying positional values is through a timing mechanism — for example, through the length of exposure to a signal — and such a mechanism has been proposed for laying down the pattern along the long axis of the limb (see below). Recently, fate maps of cells in mouse embryos^{35,36} revealed that the length of time that cells are exposed to the highest concentrations of SHH might contribute to digit patterning.

have defective GLI3 processing^{27,28}. The increase in digit number in all these mutants is due to widespread expression of the *gremlin* gene, which is regulated by GLI3 repression²⁹, and is now known to encode a bone morphogenic protein (BMP) antagonist — the apical-ridge maintenance factor (BOX 3).

Intraflagellar transport proteins
Proteins that associate with, or in, a flagellum or cilium and the associated basal body.

Box 3 | SHH signalling and molecular interactions.



The diagrams illustrate the pivotal role of sonic hedgehog (SHH) signalling in controlling gene expression and function in digit patterning. SHH protein diffuses from the polarizing region, where *Shh* transcripts are located, into the posterior part of the limb bud (see figure, part a). This results in relatively high levels of the activator form of GLI3 (GLI3A) in the posterior of the limb; the repressor form of GLI3 (GLI3R) predominates in the anterior. *Gremlin*, *Fgf4* (*fibroblast growth factor-4*), *Hoxd13* (*homeobox D cluster-13*), *Bmp2* (*bone morphogenic protein-2*) and *Gli1* are all genes, which are expressed in the posterior part of the limb bud. Analysis of the expression patterns of these genes in *Gli3*^{-/-} mouse limbs^{24,25} indicates that this restricted pattern of expression is due to posterior activation of *Gli1* transcription, whereas transcription of the other genes is repressed anteriorly. However, the regulation of *Bmp2* is still unclear.

SHH signalling influences FGF signalling in the apical ridge (see figure, part b) by maintaining expression of *Gremlin*, the product of which antagonizes BMP signalling. This leads to the maintenance of *Fgf4* expression in the posterior part of the apical ectodermal ridge. FGF signalling (including signalling by FGF4) contributes to the maintenance of *Shh* expression in the polarizing region. SHH signalling maintains the nested set of expression domains of 5' *Hoxd* genes in the limb bud, including *Hoxd13* in the posterior region of the limb bud that will give rise to digits (see figure, part c) and maintains the posterior stripe of *Tbx3* expression (see figure, part d). The expression of *Tbx3* might be maintained through BMP2 signalling or through a direct effect of SHH³². For simplicity, only the key genes have been shown and interactions might be direct or indirect.

were found to contribute to digits 3–5. However, cells that expressed *Gli1* — these include *Shh*-expressing cells and a rim of cells anterior to the *Shh*-expression domain — contributed to digits 2–5 in a graded manner, with the highest number of marked cells being found in the posterior digits. These data confirm the results from the fate maps of early chick wing buds and indicate that, in the mouse as in the chick, there is considerable expansion of posterior tissue as the limb grows out. It is suggested, however, that, at this time, only the positional value that leads to formation of digit 2 has been specified by a low concentration of SHH (FIG. 2b). Cells that were marked in later mouse limb buds, between 11–12 days of development, were also found in the digits. Cells that had expressed *Shh* contributed to digits 3 and 4, but cells that had expressed *Gli1* contributed to digits 2–5. These data show that cells that contribute to digits 3, 4 and 5 have been exposed to high SHH concentrations for progressively longer periods of time than cells that give rise to digits 1 and 2. It has therefore been suggested that digits 3, 4, and possibly 5, will not have been specified until this time (FIG. 2b).

Experimental evidence also indicates that SHH concentration alone might not specify positional values. Alterations in the amount and spatial distribution of SHH in the mouse limb buds, through manipulation of a gene that affects SHH diffusion³⁵, resulted in the loss of a middle digit. If SHH concentration was responsible for the specification of the positional values, it would be expected that the most posterior digit would be lost.

Manipulation of the duration of SHH signalling would directly test the importance of time. Mouse mutants in which SHH signalling is curtailed are available — the naturally occurring mouse-limb-deformity mutant³⁷ and the *gremlin* knockout³⁸. In both of these mutants, digit number is reduced and posterior digits appear to be lost. Developing limbs of closely related Australian lizards that exhibit varying degrees of evolutionary limb reduction³⁹, are also characterized by shorter durations of *Shh* expression. Interestingly, this seems to be related to reductions in digit number rather than changes in pattern. In both examples, however — the mouse-limb-deformity mutants and the Australian lizards — levels of *Shh* expression are also reduced.

The importance of sustained SHH signalling for mouse digit patterning seems at odds with the chick embryological experiments that indicated that anterior-wing-bud cells remember their exposure to a polarizing signal⁴⁰, even when the signal has ceased (BOX 1). Whether addition of SHH to the anterior margin of a chick wing bud induces SHH expression or not is still unclear, as the results are conflicting. It is also unclear how this timing model could be applied to the digits of the chick wing. Recent work indicated that the most anterior digit in the chick wing, digit 2, arises in a SHH-independent fashion⁴¹, but the homologies between the individual digits in the chick and the mouse are still controversial⁴².

This has led to a detailed model for specification of positional values for each mouse digit that integrates both concentration and length of exposure to SHH³⁵. Specification of digit 1 is probably SHH-independent because the single digit that develops in the limbs of *Shh*^{-/-} mice most closely resembles digit 1 (REF. 22); low concentrations of SHH specify the positional value that leads to the formation of digit 2, whereas both time and concentration specify digit 3; cells are specified to form digits 4 and 5 according to the length of exposure to SHH (FIG. 2b).

The fate maps, which revealed the importance of timing in the mouse were made using activation of β-galactosidase to mark the cells that expressed either *Shh* (REF. 35) or *Gli1* (REF. 36). Staining for β-galactosidase can be undertaken at different stages of limb development and this procedure allows the contributions that are made by marked cells to the digits to be assessed. The gene encoding GLI1 is a known target of SHH signalling (BOX 3), therefore, marking of cells that express *Gli1* allows the fate of cells that have responded to SHH to be traced. Cells that expressed *Shh* between days 10 and 11 of development and were located at the very posterior of the early limb bud

Reaction–diffusion mechanism

Self-organizing system that consists of two or more interacting chemical substances and spontaneously generates spatial patterns.

Gap-junctional communication

Mechanism of direct cell–cell communication in which small molecules pass through aligned gap junctions on neighbouring cells and not extracellularly.

β-galactosidase

β-Galactosidase is a commonly used reporter molecule, which can be readily visualized.

Patterning and growth. The limb bud grows considerably during the patterning process and several models have been put forward to explain how patterning along the proximo–distal axis of the limb is generated as the limb bud grows out. According to the long-standing progress-zone model⁴³, the length of time that the cells spend at the distal tip of the limb bud, and their exposure to signals (such as FGFs) from the apical ectodermal ridge, determines the proximo–distal positional value. Therefore, in the early limb bud, only the most proximal positional values will have been specified, and progressively more distal positional values will be generated over time as the limb bud grows out. More recently, it has been suggested that proximo–distal pattern⁴⁴ is already specified in the early limb bud by an as-yet-unknown mechanism, and that the role of FGFs is to expand this pattern. Therefore according to this model, a complete set of positional values will already have been specified in the early limb bud. At later stages, the more proximal positional values have expanded, but the more distal positional values are still closely packed.

The progress-zone model⁴³ has recently been challenged and, instead, an early-specification model has been proposed⁴⁴, which is similar to the morphogen gradient for antero–posterior pattern. It is possible that the mechanisms that specify the positional values along the antero–posterior and proximo–distal axes of the limb are more similar than was previously appreciated. Alternatively, it is possible that both mechanisms — the morphogen gradient and timing — might operate in both axes. It is unclear whether either of these mechanisms operates along the dorso–ventral axis.

Antero–posterior positional values

One of the most important questions is how antero–posterior positional values are encoded molecularly. Early studies indicated that homeobox (Hox) genes (BOX 4) could be important, but orthologues of fruitfly wing-patterning genes are also probable candidates.

Hox genes. A striking nested set of 5′ Hoxd-gene-expression domains is established across the antero–posterior axis of the limb-forming region, with *Hoxd13* expression at the very posterior⁴⁵. Recently, these genes, together with 5′ Hoxa genes, have been shown to have a role in initiating *Shh* expression in the polarizing region⁴⁶. SHH signalling is then required to maintain this pattern of Hoxd gene expression, with cells at different positions across the antero–posterior axis expressing different combinations of Hoxd genes (BOX 3) as the limb bud grows out. When a polarizing region or a bead soaked in retinoic acid, SHH or BMP2 is grafted onto the anterior margin of a chick wing bud, 5′ Hoxd genes are ectopically expressed, which mirrors the pattern that is normally seen posteriorly (for example, see REF. 47). Furthermore, *HOXD11* misexpression leads to either an extra digit 2 in the wing, or changes in toe morphology that indicate posteriorization⁴⁸. These data are consistent with the idea that 5′ Hoxd genes

Box 4 | Hox genes.

Homeobox (Hox) genes are found in four clusters in vertebrates, each of which is related to the complex of homeotic selector (HOM) genes found in fruitflies (*Drosophila melanogaster*). Mutations in HOM genes in fruitflies are responsible for homeotic transformations — replacement of one part of the body by another — the most famous example being *attenapaedia*, in which the antenna is replaced by a leg. Hox genes contain a conserved 180-bp sequence, which is known as the homeobox and encodes a DNA-binding region. The clusters of vertebrate Hox genes have been highly conserved, which indicates that this gene organization must be important for their function. There are up to 13 Hox genes in each cluster, and individual genes are numbered according to their order in the cluster, with 1 being at the 3′ end and 13 being at the 5′ end of the cluster.

might encode position across the antero–posterior axis. However, the recent demonstration that anterior expression of *Hoxd12* (REF. 49) and other 5′ Hoxd genes⁵⁰ leads to ectopic *Shh* expression provides a more likely explanation for the induction of extra digits and pattern changes.

An enormous effort has been devoted to assessing the functions of 5′ Hoxd and 5′ Hoxa genes in the developing limb by making single, double and even triple mouse knockouts⁵¹. This has revealed that paralogous genes are required for the development of each of the main segments along the long axis of the limb, with *Hoxd13* and *Hoxa13* being responsible for digit development. Therefore, it seems likely that ectopic expression of Hoxd genes in the early stages of development after application of polarizing-region signals in chick wing buds simply reflects the establishment of a new limb axis that will develop digits.

Fruitfly wing-patterning genes. Insights into other genes that could encode antero–posterior positional values comes from the similarities between the signalling cascade in vertebrate limbs (where SHH regulates expression of *Bmp2*) and that in fruitfly wings (where Hh regulates expression of *decapentaplegic* (*dpp*), a relative of the *Bmp2* gene⁵²). In the fruitfly wing, gene targets of DPP signalling that could encode positional values have been identified and include the transcription factors Optomotor blind (**Omb**; also known as Bifid), **Spalt** and **Iroquois** (also known as Araucan). Particular combinations of these transcription factors contribute to the specification of individual wing veins⁵³. DPP signalling leads to overlapping domains of Spalt and Omb expression in the fruitfly wing. The *spalt* domain represents a response to the local DPP concentration, whereas the *omb* domain represents an expansion of a population of cells in which transcripts/proteins and memory of an earlier response to DPP persists⁵⁴. The observation that these two mechanisms appear to operate together in patterning the fruitfly wing is intriguing in light of the idea that both concentration and time operate in patterning the vertebrate limb.

Paralogous

A sequence, or gene, that originates from a common ancestral sequence, or gene, by a duplication event. For example, as seen in Hoxd gene clusters and designated by a number from 1–13.

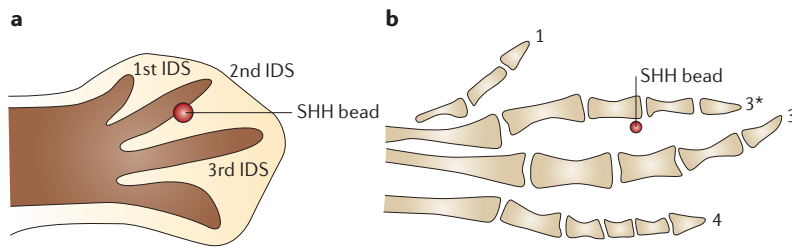


Figure 3 | Cell-cell signalling in the digital plate. a | The experimental manipulation that was described in REF. 62. A bead (red circle) soaked in sonic hedgehog (SHH) was placed in the second interdigital space (IDS) of a chick leg at a stage when the digital primordia that will form the four toes had developed. The digit primordia are still joined by soft tissue, which will later undergo programmed cell death to separate them. **b** | The skeleton of the foot that developed 5 days after the manipulation shown in **a**. One of the toes (indicated by 3*) adjacent to the bead, which would normally be toe digit 2, is elongated and has an extra phalanx. It resembles toe digit 3 and shows that the toe has been posteriorized. The other toe digits 1, 3 and 4 have normal morphology.

The vertebrate orthologues of the fruitfly genes *Tbx2*, *Tbx3* (*omb* orthologues), *Sall* (*spalt* orthologues) and *Irx* (*Iroquois* orthologues) are expressed in vertebrate limb buds (for example, see REFS 32,55,56) and there is evidence that *TBX2* and *TBX3* might encode posterior positional values in vertebrate limbs. *Tbx2* and *Tbx3* are expressed in anterior and posterior stripes in early limb buds and the posterior stripe of expression has been shown to depend on polarizing signalling^{32,57} (BOX 3). Overexpression of *TBX2* and *TBX3* has been reported to result in posteriorization of chick toes⁵⁷, although others report a shift in limb position⁵⁸. In addition, human patients with mammary-ulnar syndrome, caused by *TBX3* haploinsufficiency, have posterior limb defects⁵⁹. Although these studies on *Tbx* genes are an encouraging start, considerable efforts will be required to uncover the positional code for antero–posterior limb pattern to an extent that is comparable to the positional code that has been deciphered for other tissues, such as the dorso–ventral pattern of the neural tube (the forerunner of the central nervous system)⁶⁰.

Interpretation of positional values

The last step in pattern formation is the morphogenesis of the digits through interpretation of the positional values. Evidence from studies on chick legs indicates that the morphogenesis of each individual digit involves local interactions⁶¹. During this time, the digital plate is very broad and *SHH* expression is reduced and soon disappears. A change occurs from the global patterning system that operates in the limb bud, in which *SHH* has a pivotal role, to a series of patterning systems, which regulate the morphology of individual digits after the expression of *SHH* is extinguished. The idea of separate patterning systems for each digit is supported by the observation that manipulations at this late stage have local effects; the anatomy of a single digit changes independently from that of other digits^{61,62} (FIG. 3).

Grafting experiments showed that endogenous signals that control individual digit morphologies come

from interdigital tissue⁶¹; toe morphology can be both anteriorized (phalanx number reduced) or posteriorized (phalanx number increased). Although these effects are known to be mediated through BMPs (because co-implantation of beads soaked in BMP antagonists prevent toe elongation⁶¹) the gene targets of BMP signalling are currently unknown. *Hoxa13* and the four contiguous genes in the *Hoxd* gene cluster, *Hoxd10–Hoxd13*, are co-expressed throughout the digital plate, and a global regulatory region that controls digital expression has been identified⁶³. One possibility is that this late phase of *Hox* gene expression might be involved in interpreting positional values to give digit identity, but no changes in the expression of *Hoxd10–12* were detected following digit manipulations⁶¹.

SHH-soaked beads that are implanted between the digits also produce posteriorization (FIG. 3), possibly by mimicking the signalling of another vertebrate Hh, Indian hedgehog (*IHH*), which is expressed at this stage. *SHH*-soaked beads prolong FGF signalling in the apical ectodermal ridge. Interfering directly with FGF signalling by either enhancing it or abrogating it, can lead either to the formation of extra phalanges or to a reduction in phalanx number, respectively⁶². Interestingly, toes that are induced from these manipulations have normal tips, which indicates that there is a special programme that controls the formation of a limb tip when FGF signalling is switched off. This might explain why even the rudimentary digit of *Shh*^{-/-} mouse embryos is finished off by a claw²².

Conclusions

This review shows that a picture, albeit somewhat hazy, of the signalling cascades that are involved in digit patterning is beginning to emerge. The first cascade operates in the limb bud where *SHH* signalling has a pivotal role, and the second cascade operates in individual digit primordia and involves BMP signalling. Although a number of genes with potential roles in antero–posterior patterning have been recently identified, we are still facing the challenge to fill in the considerable gaps in our current understanding by identifying other genes that are involved in this process. Another challenge is to gain a deeper understanding of the cellular basis of limb development by finding new ways to visualize extracellular signalling molecules and measure their concentrations and the cellular responses to them. Finally, information about antero–posterior position must be coordinated with information about proximo–distal and dorso–ventral position, and fed into the interpretation process with information about whether the organ is a wing or a leg. A really detailed focus on the mechanisms of antero–posterior digit patterning might be relevant to understanding how positional information operates along the other two axes of the limb. The knowledge of how the limb develops should cast light on the basis of human congenital limb defects and the general principles will be relevant to tissue engineering and devising new approaches for tissue repair. In addition, many of the molecules that control cell activities during limb development have been also implicated in cancer.

Mammary-ulnar syndrome
Rare human congenital condition that is characterized by specific defects in both mammary glands and limbs.

Haploinsufficiency
Defines a genetic condition in which the defect is seen in heterozygous individuals.

Phalanx
(plural phalanges). One of the series of small bones that make up the fingers and toes in vertebrates.

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DATABASES

The following terms in this article are linked online to:

Entrez:

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>
 FGF4 | Hoxa13 | Hoxd10 | HOXD11 | Hoxd12 | Hoxd13 | Gli1 | Gli2 | Gli3

Flybase:

<http://flybase.bio.indiana.edu/>
 dpp | Hh | Iroquois | Omb | Spalt
 Swiss-Prot: <http://us.expasy.org/sprot/>
 BMP2 | GLI1 | GLI2 | GLI3 | SHH

FURTHER INFORMATION

Cheryll Tickle's homepage:

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