PATTERN FORMATION: OLD MODELS OUT ON A LIMB

Lee Niswander

The vertebrate limb is an excellent model for studying fundamental aspects of embryonic development. Cell proliferation, death and movement, and the assignment and interpretation of positional information, must be coordinated if an exquisitely patterned limb is to form. Recent results from gene targeting in mice and from experimental manipulation of the chick embryonic limb have significantly altered the way in which developmental biologists have conceptualized limb patterning.

MESENCHYME
Embryonic tissue that is composed of loosely organized, unpolarized cells of both mesodermal and ectodermal (for example, neural crest) origin, with a proteoglycan-rich extracellular matrix.

STYLOPOD
The proximal element of a limb that will give rise to the humerus in the forelimb and the femur in the hindlimb.

ZEUGOPOD
The intermediate elements of a limb that will give rise to the radius and ulna in the forelimb, and the tibia and fibula in the hindlimb.

AUTOPOD
The distal elements of a limb that will give rise to the wrist and the fingers in the forelimb, and the ankle and toes in the hindlimb.

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The developing limb has served as a model for studying many areas of genetics and cell biology that are related to pattern formation and signal transduction. Because the limbs are not necessary for embryonic survival, the embryonic limb can be experimentally and molecularly manipulated to define the important cellular and molecular interactions that regulate patterning and skeletal development. In addition, because the key genes that control limb formation are also used in many other developmental contexts, the knowledge gained from studies of the limb can be applied to understanding the development of other tissues and organs.

The embryonic limb is first visible as a small bud that protrudes from the body and contains morphologically homogenous MESENCHYME cells that are covered by a layer of ectoderm. Over time, the limb bud grows and lengthens along the proximal-to-distal (Pr–D) axis — from shoulder to finger tip. The overt manifestation of Pr–D pattern, the skeleton, is laid down progressively, starting from the proximal end and finishing at the distal end. First, the mesenchymal cells condense, then they differentiate into cartilage and, later, into bone (FIG. 1a). From the proximal condensation, the proximal element arises (STYLOPOD), followed by the intermediate elements (ZEUGOPOD), then the distal elements (AUTOPOD). Limb patterning is also established along two other axes: anterior–posterior (A–P: thumb to little finger, ulna to radius in the forelimb) and dorsal–ventral (D–V: back of hand to palm).

Many of the crucial genes that regulate growth and patterning of the limb in three dimensions are now well defined. What is less clear, and what lies at the heart of some recent publications, is how these molecules interact. This review highlights the embryological and genetic experiments that led to these studies, and contrasts the new ideas with earlier models on how limb patterning is established.

Limb patterning signals

FGFs — crucial signals for Pr–D growth. Pr–D limb development depends on a strip of specialized epithelium at the distal tip of the limb bud, called the apical ectodermal ridge (AER) (FIG. 1; BOX 1). Microsurgical removal of the AER from an early developing chick limb bud results in the loss of almost all limb structures, whereas its removal at progressively later stages results in progressively more distal loss, with proximal structures being unaffected[1,2] (BOX 1; FIG. 2). So, the AER is needed for Pr–D growth and for the realization of Pr–D patterning. Experiments in which an older AER was placed onto a young limb bud, or vice versa, showed that the signal from the AER is permissive, rather than instructive; in both cases, the skeleton was patterned normally[3], indicating that it is the limb-bud mesenchyme and not the AER that contains the information for Pr–D patterning.

The fibroblast growth factors (FGFs) are the key factors that are required for AER function, and four members of this family are specifically expressed in the AER (BOX 1). The first indications of the molecular nature of the AER signal were published in the 1990s,

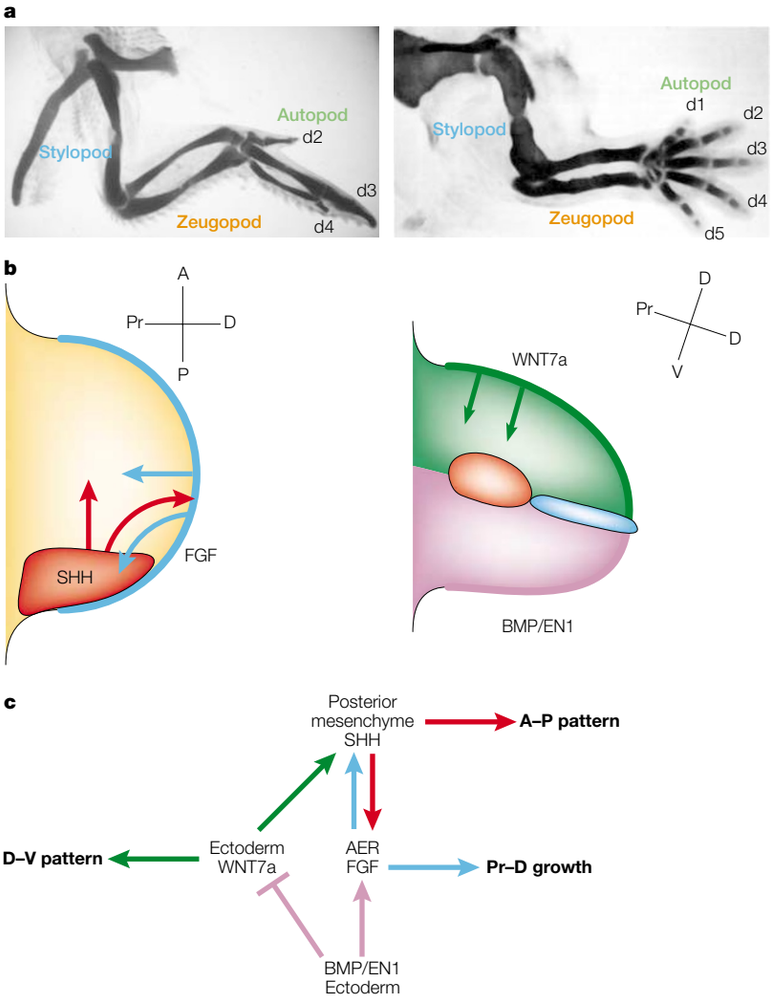


Figure 1 | **Signalling pathways in vertebrate limb development. a** | Chick and mouse skeletons are shown on the left and right, respectively. The stylopod is the proximal element that gives rise to the humerus in the forelimb and the femur in the hindlimb. The intermediate elements (zeugopod) give rise to the radius and ulna in the forelimb, and the tibia and fibula in the hindlimb, whereas distal elements (autopod) will give rise to the wrist and fingers in the forelimb, and ankle and toes in the hindlimb. Unlike the mouse, the chick 'hand' has three digits (d2–4). Image courtesy of C. Tickle, Dundee University. **b**,**c** | Molecular interactions that coordinate limb growth and patterning along the three limb axes: proximal–distal (Pr–D) axis is under the control of fibroblast growth factors (FGFs; blue) from the apical ectodermal ridge (AER), the anterior–posterior (A–P) axis is under the control of Sonic hedgehog (SHH; red) from the posterior mesenchyme, and the dorsal–ventral (D–V) axis is under the control of bone morphogenetic proteins (BMPs) and Engrailed1 (EN1; both in pink) from the ventral ectoderm and WNT7a (green) from the dorsal ectoderm. Panel **b** reproduced with permission from REF. 83 © (1990) University of the Basque Country Press.

when it was shown that substituting the AER with FGF-soaked beads (FGF2 or FGF4) could rescue Pr–D development of the chick limb[4,5]. Genetic evidence that FGFs are necessary for AER function came only recently when Sun *et al.*[6] used the CRE-LOX SYSTEM to create an AER-specific conditional mouse knockout of *Fgf8* and *Fgf4*. In the complete absence of these two gene products, the limbs fail to form (FIG. 2), conclusively showing that FGFs are necessary to perform the functions of the AER (see below for a discussion of how the AER-expressed FGFs influence Pr–D limb development).

CRE-LOX SYSTEM
A site-specific recombination system that is derived from the *Escherichia coli* bacteriophage P1. Two short DNA sequences (*lox*P sites) are engineered to flank the target DNA. Activation of the Cre recombinase enzyme catalyses recombination between the *lox*P sites, which leads to the excision of the intervening sequence.

SHH regulates A–P limb patterning. In 1968, Saunders discovered that the posterior region of the limb mesenchyme (zone of polarizing activity, ZPA) can repattern the A–P axis when grafted at the anterior of the developing limb bud[7] (FIG. 2): it causes anterior cells to form additional digits in a mirror-image duplication to the normal digits. For example, grafting 30 ZPA cells results in an ectopic digit 2 (normal chick digit pattern 2-3-4 changed to 2-2-3-4), whereas 130 cells are sufficient to induce a complete mirror duplication of the digits (4-3-2-2-3-4)[8]; the additional digits, however, are not derived from the transplanted cells, but from the host embryo, indicating that the ZPA is the source of a MORPHOGEN. The first molecule that was shown to mimic the ZPA was retinoic acid[9]. However, its role as the endogenous morphogen from the ZPA has fallen out of favour in recent years, although it might be required at early stages for limb growth and to establish the ZPA[10–14].

The molecular nature of the ZPA signal was established in the 1990s by the identification of *Sonic hedgehog* (*Shh*). *Shh* is a secreted molecule that is expressed in the posterior limb mesenchyme, and application of Shh-expressing cells or recombinant SHH protein to the anterior of the chick limb bud causes mirror-image digit duplications[15]. Conversely, genetic removal of *Shh* in mice results in the dramatic loss of skeletal elements along the A–P axis (the limb consists of stylopod, a single reduced zeugopod element and reduced digit 1[16,17] (FIG. 2). A similar phenotype is observed in a chick mutant *oligozeugodactyly* (*ozd*), which lacks Shh function in the limb[18]. Moreover, posterior mesenchyme from $Shh^{-/-}$ limbs lacks polarizing activity[16]. Collectively, these studies indicate that Shh is the key signal from the ZPA.

Molecular control of D–V patterning. The mesenchyme already contains the information for D–V limb patterning that occurs before limb-bud initiation. Just before the limb bud forms, the mesenchyme transfers this information to the overlying ectoderm[19]; the molecular nature of this early mesenchymal signal(s) is unknown. D–V patterning is subsequently regulated by the overlying ectoderm: when the limb ectoderm is rotated 180° relative to the mesenchyme, the mesenchymal structures (skeleton, muscle and tendons) become inverted such that they correspond to the polarity of the ectoderm[20].

The molecular network for D–V patterning (FIG. 1) is now well established[21]. The transcription factor Lmx1b is expressed in the dorsal mesenchyme of the limb and is required for cells to adopt a dorsal character. Although the early regulator of *Lmx1b* expression is unknown, as the limb bud forms Lmx1b is induced by Wnt7a, which is expressed in the dorsal limb ectoderm. In the absence of Wnt7a, the dorsal pattern of the distal structures (autopod) is not established and the limbs appear bi-ventral (FIG. 2). Expression of *Wnt7a* is restricted to the dorsal ectoderm because it is repressed in ventral ectoderm by the transcription factor

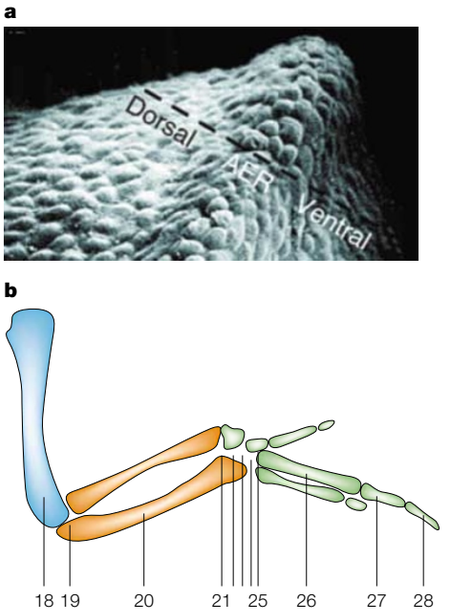
Engrailed1 (En1). In *En1*$^{-/-}$ limbs, *Wnt7a* is misexpressed in the ventral ectoderm, and the distal structures develop with bi-dorsal character (FIG. 2). En1 itself is induced in the ventral ectoderm by bone morphogenetic protein (BMP) signalling through the type I receptor, Bmpr1a. Loss of BMP signalling also leads to *Wnt7a* misexpression and bi-dorsal limbs[22,23] (FIG. 2).

Coordination of the limb organizing centres. Patterning and growth of the limb in three dimensions is coordinated through interactions between the limb organizing centres and the molecules that they produce (AER/FGFs, ZPA/Shh, dorsal ectoderm/Wnt7a)[24] (FIG. 1). For example, the AER is required for *Shh* expression — AER removal results in the rapid downregulation of

Box 1 | Formation and function of the apical ectodermal ridge

AER formation

The apical ectodermal ridge (AER) forms at the junction between the dorsal and ventral ectoderm and becomes morphologically visible as a ridge of epithelium (stratified in mouse, pseudostratified in chick) at the distal tip of the limb bud (**a**; chick AER). Three steps are involved in AER formation: induction of the AER precursors, migration of the precursors to the distal tip and compaction of these cells to form the tall morphological ridge. The key signals that mediate AER function are the fibroblast growth factors (FGFs). *Fgf8* is expressed throughout the AER, whereas *Fgf4*, *Fgf9* and *Fgf17* are restricted to the posterior and distal AER. Many other genes are expressed throughout the AER, including *Msx1* and *Msx2*, *Dlx5* and *Dlx6*, and *Bmp2*, *Bmp4*, and *Bmp7*, although only BMPs have so far been implicated in AER formation and function (see below).



Induction. The limb mesenchyme produces Fgf10, which signals to the limb ectoderm. Fgf10, and Wnt3a and BMP signalling, are required to induce *Fgf8* expression in the AER precursors in the ectoderm[22,23,35,37,38].

Migration. The AER precursors are initially spread over a relatively broad region of the ectoderm. In the chick, cell labelling studies indicate that AER precursors arise within both the dorsal and the ventral ectoderm[79]. In the mouse, the studies so far indicate that the AER precursors are located in the ventral ectoderm[80]. *Fgf8*-expressing AER precursors become concentrated at the distal tip, perhaps as a result of their migration. However, *Fgf8* might be downregulated in some cells — perhaps in cells that are not near the distal tip.

Compaction. *Fgf8*-expressing cells compact to form a columnar epithelium. Studies of the Engrailed 1(*En1*)$^{-/-}$ mutant limbs or limbs in which *En1* has been misexpressed, have indicated that compaction requires the formation of a dorsal, ventral and middle border in the distal ectoderm. In the *En1*$^{-/-}$ limbs, it seems that the ventral and middle borders are not properly formed, which results in a flattened AER that extends over a large portion of the ventral-limb ectoderm[80] (FIG. 2).

The compacted AER also serves a mechanical function to provide directed outgrowth and to maintain a dorsoventrally flattened shape of the limb.

AER function

The AER is necessary for Pr–D limb outgrowth. Surgical removal of the AER from the chick embryonic limb results in limb truncation. Removal of the AER at progressively later stages results in progressively more distal truncations (**b**; numbers indicate the limb stage at the time of AER removal and the lines point to the approximate level of truncation)[1,2]. Beads soaked in FGF2, 4 or 8 can compensate for the loss of AER function and rescue Pr–D development[4,5]. In the mouse, genetic loss of both *Fgf4* and *Fgf8* — two of the four FGFs that are expressed specifically in the AER — results in complete failure of limb formation[6]. Later inactivation of *Fgf4* and *Fgf8* results in the production of all Pr–D segments, but the distal elements are reduced in size and number (FIG. 2). Therefore, although many other genes are expressed in the AER, FGFs are the key signals from the AER that are necessary for Pr–D development. The AER provides a permissive signal to enable the realization of Pr–D pattern. The information for Pr–D patterning is intrinsic to the mesenchyme[3]. The recent studies on chick and mouse propose that the AER controls the initial size of the limb bud, and cell survival and proliferation, and generates sufficient numbers of mesenchyme cells to form appropriate size condensations[6,27].

AER regression

After the AER has completed its function, it regresses, returning to a flattened cubodial epithelium. This is accompanied by a downregulation of *Fgf* expression and a reduction in mesenchyme proliferation. Molecular experiments in the chick show that AER regression is mediated by BMP signalling[29]. When BMP signalling is blocked by misexpression of a BMP antagonist the AER does not regress, FGF expression is maintained and the mesenchyme continues to proliferate, resulting in additional soft-tissue outgrowth. Therefore, BMP negatively regulates the function of the AER and causes AER regression.

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MORPHOGEN
A diffusible signal that acts at a distance to regulate pattern formation in a dose-dependent manner.

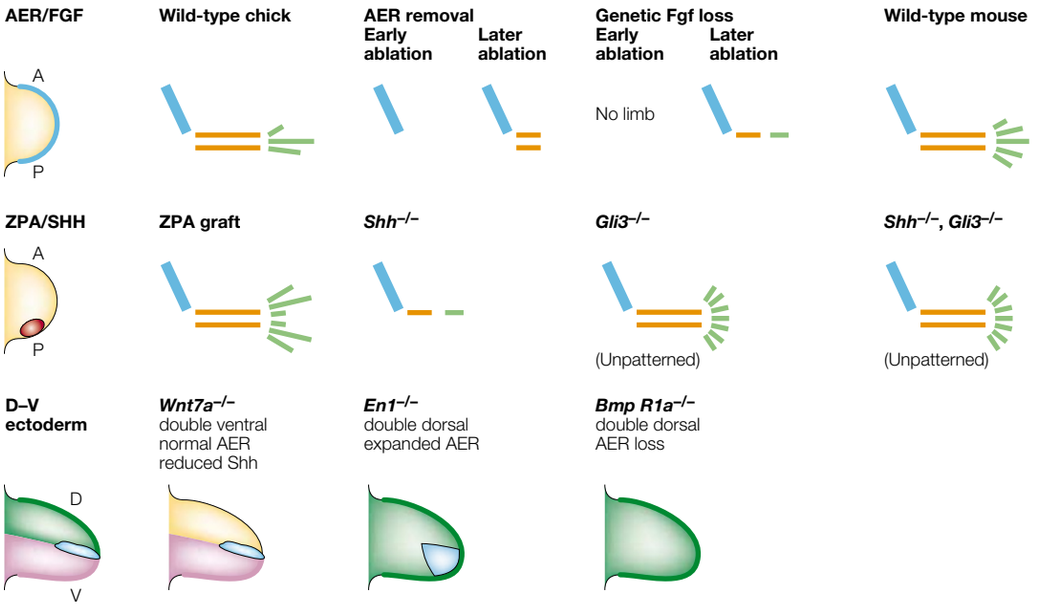


Figure 2 | **Experimental phenotypes in chick and mouse limb.** Phenotypes produced by experimental manipulations of the chick embryonic limb or by genetic manipulation in the mouse. Stylopod is shown in blue, zeugopod in orange and autopod in pale green. Dark green represents Wnt7a signalling from the dorsal ectoderm and the dorsal-limb fate; pink represents BMP/En1 signalling from the ventral-ectoderm and the ventral-limb phenotype. AER is represented as a pale blue disc. In the lower row, double ventral indicates the appearance of ventral characteristics, such as footpads and striated epidermis, and loss of dorsal characteristics, such as hair on the dorsal side. Double dorsal indicates the converse phenotype. AER, apical ectodermal ridge; Bmp r1a, bone morphogenetic protein receptor 1a; En1, engrailed; FGF, fibroblast growth factor; Shh, Sonic hedgehog; ZPA, zone of polarizing activity.

Shh, which can be rescued by an FGF-soaked bead[25,26]. However, in light of recent studies by Dudley *et al.*[27] (see below for further discussion), which indicate that AER removal causes massive death of the distal cells, it might be misleading to conclude, on the basis of AER removal experiments, that gene expression is AER dependent. More direct evidence for the role of FGF in the induction of *Shh* expression is provided by the *Fgf4/8* double-knockout mice[6]; in the absence of these AER signals, *Shh* expression is not induced.

Shh, in turn, maintains *Fgf4* expression in the AER by acting through the formin protein (encoded by the gene that is disrupted in the *limb deformity* mouse mutant) to maintain the expression of Gremlin, a BMP antagonist. Gremlin inhibits BMP signalling, and BMP inhibits *Fgf4* expression in the AER[28–30] (BOX 2). So, in this 'double-negative' situation, Shh positively regulates *Fgf4*. *Fgf4* is a useful marker of this interaction, and its expression is frequently perturbed in limbs in which the A–P pattern has been altered.

The removal of the dorsal ectoderm or the loss of *Wnt7a* by gene targeting causes a reduction or loss of *Shh* expression[31,32]. So, the dorsal ectoderm is also required, in conjunction with the AER, to maintain *Shh* expression. Therefore, these interactions between FGFs, Shh and Wnt7a might serve to coordinate signalling from the AER, ZPA and dorsal ectoderm.

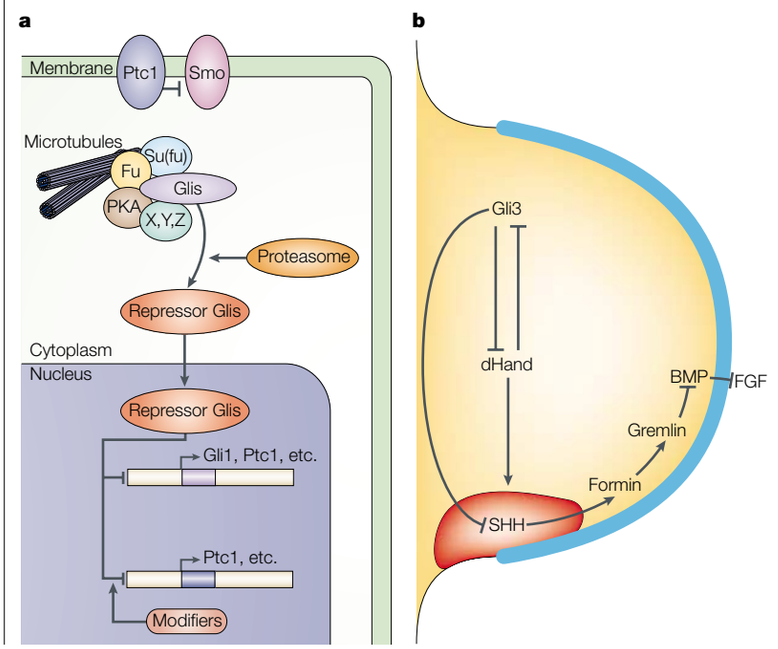
The formation of the AER and of the D–V axis is already linked during early limb development. Gain-and-loss studies of BMP function in chick[23], and conditional inactivation of *Bmpr1a* in mouse[22], revealed that, in the absence of BMP signalling, the AER is not formed and *Fgf8* expression is not induced. Moreover, the limbs are bi-dorsal due to the loss of *En1* and mis-expression of *Wnt7a* (FIG. 2). So, BMP signalling acts upstream of AER formation and of D–V patterning. Furthermore, BMP-mediated regulation of these two processes is independent of each other: it is the En1/Wnt7a/Lmx1b pathway that affects D–V patterning[22,23], whereas the regulation of AER formation might involve Msx transcription factors[23].

Embryological experiments in chick showed that the mesenchyme initially sends a signal to the ectoderm to regulate AER formation[33,34]. Genetic experiments in mouse and embryological manipulations in chick have defined the signal from the mesenchyme and have shown that members of the Wnt and FGF families control the position of limb-bud formation. *Wnt2b* and *Wnt8c* RNAs become localized to the presumptive forelimb mesenchyme and to the posterior of the embryo, to a region that overlaps the presumptive hindlimb region respectively[35]. Wnt signalling activates *Fgf10* expression in the limb mesenchyme, as shown by gain and loss of Wnt signalling in the chick embryo[35]. Subsequently, Fgf10 signals to the overlying ectoderm to induce *Fgf8* expression in the AER. *Fgf10*-knockout mice fail to form limbs, whereas application of an FGF bead to the flank results in the formation of ectopic AER and of an ectopic limb[36–39]. Fgf10 seems to act through Wnt3a in the ectoderm[35,40] to regulate the cascade outlined above, leading to AER formation.

Box 2 | **Shh and Gli3 in limb patterning**

The Sonic hedgehog (Shh) signal-transduction pathway is tightly regulated by a complex series of interactions (**a**). In the absence of Shh signalling, the Shh receptor Patched (Ptc1) represses the seven-transmembrane-domain protein Smoothened (Smo)[67], and the dual-function Gli transcription factors are proteolytically cleaved to generate a transcriptional repressor. The binding of Shh to Ptc relieves the repression of Smo and inhibits Gli proteolytic cleavage, generating potential transcriptional activator forms. In vertebrates, there are three Gli proteins — Gli1, Gli2 and Gli3. *Gli1* is expressed in and near the *Shh* domain, whereas *Gli2* and *Gli3* are expressed in a domain that is complementary to *Shh* in the limb bud. Moreover, Shh regulates *Gli1–3* expression. Mutations in *Gli1* or *Gli2*, or both, do not affect limb growth or patterning, indicating that these proposed positive mediators of the Shh signal are not required for its transduction, whereas *Gli3* mutants are POLYDACTYLOUS[69–72]. Shh-controlled Gli3 processing forms an anterior–posterior (A–P) gradient in the limb bud — more of the processed transcriptional repressor form is present in the anterior and much less in the posterior of the bud[64,81]. It has also been suggested that the unprocessed form of Gli3 acts as a transcriptional activator, with the highest ratio of activator to repressor being in the posterior of the limb bud[64], raising the possibility that the processing of Gli3 is a crucial aspect of Shh signalling and limb patterning.

The early molecular A–P network in the developing limb includes dHand and Gli3 transcription factors (**b**). *Gli3* is expressed in the anterior mesenchyme of the limb bud and *dHand* is expressed in the posterior mesenchyme, and they mutually restrict each other's expression[30,63]. The expression of dHand is thought to be regulated upstream of or parallel to retinoic-acid signalling because the initial pattern of dHand expression is normal in the forelimbs of mice that are mutant for an enzyme in the retinoic-acid biosynthetic pathway *Raldh2* (REF. 14). Together with the fibroblast growth factor (FGF) signals from the apical ectodermal ridge, dHand is required to activate *Shh* expression[6,82]. This Shh-independent network seems to pattern the stylopod and zeugopod, as the A–P pattern of the zeugopod elements is clearly identifiable in $Shh^{-/-}$ $Gli3^{-/-}$ limbs. This, and similar zeugopod asymmetry in $Gli3^{-/-}$ limbs, however, calls into question the requirement for Gli3 in early limb patterning. *Shh*-independent prepattern is confirmed by molecular analysis of $Shh^{-/-}$ and *Shh/Gli3* double-mutant limbs[63,64]. *Bmp2* and the Hox genes, which have been implicated as downstream targets of Shh signalling, are initially expressed normally in $Shh^{-/-}$ limbs, but this expression is not maintained; however, it can be largely restored in $Shh^{-/-}$ $Gli3^{-/-}$ limbs[63,64]. Fu, Fused; Su(fu), Suppressor of fused; PKA, protein kinase A. Panel **a** reproduced with permission from REF. 85 © (2001) Nature Publishing Group.



The studies that have been described so far have outlined the key molecular players that are involved in regulating the formation of the three limb axes. The question that lies at the heart of recent research is: how do FGFs and Shh regulate limb growth and patterning? The answers that have emerged recently from work on chick and mouse have been surprising and do not fit easily with previous models. The rest of this review discusses the new studies and compares them with older models of AER and ZPA signalling.

AER function

Progress zone model — progressive Pr–D specification. The progress zone model was proposed by Summerbell, Lewis and Wolpert[41] in the 1970s to explain the patterning of the limb along the Pr–D axis. The 'progress zone' was defined as the region of mesenchyme that underlies the AER and that is influenced by it. The model was based, in part, on experiments by Saunders and Summerbell, who showed that AER removal at progressive stages of development results in progressively more distal-limb truncation[1,2] (BOX 1). The model was also based originally on experiments in which the distal tips of two limbs of two different ages were exchanged — the old tip generated only distal structures, whereas the young tip generated all Pr–D structures. This was interpreted as evidence that cells undergo progressive changes in SPECIFICATION, from proximal to distal fate, as they remain under the influence of the AER — perhaps as a measure of time spent in the progress zone. As the limb grows, cells are pushed out of the progress zone and become fixed in the positional value that they acquired within it. Cells that are the first to leave the progress zone will generate proximal structures, whereas cells that leave later will form distal elements. So, according to the progress zone model, Pr–D fate is specified progressively.

The progress zone model was first introduced as a way of explaining the phenotype produced by AER removal. It proposes that when the AER is removed, cells at the distal tip cease progressing and do not acquire more distal fates. This could explain the progressively more distal loss at progressively later stages of AER removal. Moreover, the model espoused the idea that the extent of Pr–D truncation reflected the state of Pr–D specification at the time of AER removal.

The progress zone model has remained popular, despite the fact there were few experiments to prove or disprove the model. Below, I review the evidence used to support the model (AER removal, distal-tip exchange, and X-irradiation of limb buds) and put this in the context of the recent studies (see also REFS 42–44). Although the new data do not disprove the model, they do provide evidence that is difficult to reconcile with it and, instead, provide intriguing new ideas on how the AER might function to realize Pr–D pattern.

AER regulates cell survival and proliferation. The recent results of Dudley *et al.*[27] significantly extend our understanding of what happens after AER removal. It has been known that a transient but extensive cell

POLYDACTYLY
Having more than the normal number of digits.

SPECIFICATION
A cell or tissue is specified to become a particular structure if, when isolated and placed in a neutral medium, it develops autonomously into that structure. Specification might still be reversed or altered following exposure to a different environment. Specification of a region need not be the same as its fate in normal development.

FATE MAP
Shows how a cell or tissue moves and what it will become during normal development, although the commitment of the cell or tissue cannot be inferred from the fate map.

death occurs in a region of mesenchyme at the limb's distal tip[27,45,46]. Dudley *et al.* showed that removal of the AER between stages 18 and 22 results in a relatively constant 200 μm zone of cell death. After stage 24, AER removal does not affect cell survival, although cell proliferation is significantly reduced.

Combining cell labelling experiments with AER removal led Dudley *et al.*[27] to propose two new ideas. First, cells that will contribute to all Pr–D segments exist in stratified domains within the early limb bud, and second, the extent of the limb elements formed is a reflection of the population of Pr–D progenitors that lie outside the zone of cell loss and that differentiate according to their Pr–D fate, as specified early in limb-bud development.

Dudley *et al.*[27], similar to Vargesson *et al.*[47], used lipophilic dyes to label the surface membrane of a group of cells to determine cell fates at different positions in the limb. The results indicated that cells in different regions of the early limb bud contribute to a particular limb segment: at stage 19 the FATE MAP shows that cells that will form the stylopod lie 200–300 μm from the AER, whereas the zeugopod and autopod precursors are 100–200 μm and within 100 μm of the AER, respectively. So, Pr–D fates are stratified in the early limb bud. Fate maps change over time because of morphogenetic movements and growth. For example, cell labelling indicated that different Pr–D segments complete their expansion at different stages[27]. Dudley *et al.*[27] suggested that the population of Pr–D progenitors that reside in the zone of cell loss changes over time. From this and results showing that the zone of cell loss is relatively constant after early AER removal, Dudely *et al.* proposed the following. At early stages, essentially all Pr–D progenitors reside in the region of cell loss, hence the severe truncation of the limb following early AER removal. At later stages, the more proximal fates reside outside of the region of cell loss, therefore the truncations occur more distally. Because of the peculiarities of the pre-existing fate maps for older-stage limbs and the fact that late AER ablation mainly leads to reduced proliferation and not cell death, it has yet to be rigorously tested that the zone of cell loss correlates closely with the observed truncations at different stages. Although these data do not disprove the progress zone model, they provide a very plausible explanation of the AER removal experiments that need not involve progressive specification of limb-bud mesenchyme.

An important difference between the progress zone model and the model by Dudley *et al.* is the timing of Pr–D specification (FIG. 3). The progress zone model is based on progressive Pr–D specification that depends on the AER in which proximal fate is specified first, followed by progressively more distal specification. By contrast, Dudley *et al.* suggest that all Pr–D fates are specified within the early limb bud. Importantly, however, data of Dudley *et al.* indicate only the fate of the cells, not their state of specification. Their studies do not define when the cells 'know' what they are to form. Hence, it is still an open question as to when specification occurs. In this respect, it is interesting that pattern formation of the digits can be altered at a very late stage, even after the condensations have formed, and that the interdigital tissue seems to regulate this pattern[48].

Genetic removal of FGF from the AER is also informative in thinking about Pr–D patterning. In the absence of *Fgf8* in mice, the proximal skeletal element — the stylopod — is severely reduced or lost. Yet more distal elements are formed and they are relatively normal (except for the loss of digits 1-2) because the other FGFs, in particular *Fgf4*, are subsequently activated[49,50]. The loss of proximal but not distal fates is incompatible with the progress zone model, which proposes that proximal cells are specified first and distal cells last. It could be argued that the proximal structure was formed and subsequently lost, perhaps by cell death. Proximal mesenchyme cell death is observed in the $Fgf8^{-/-}$ hindlimb and this might represent loss of the stylopod precursors. However, this explanation is less convincing because a similar domain of proximal cell death is seen in forelimbs of $Fgf8^{-/-}$ or $Fgf4^{-/-}Fgf8^{-/-}$ mice (in which, initially, *Fgf8* and *Fgf4* are expressed transiently)[6], yet the stylopod forms normally. Transient activation and subsequent loss of *Fgf4* and *Fgf8* (REF. 6) should resemble AER removal shortly after the onset of FGF expression. Yet, instead of distal truncation, a very different pattern is observed that is not easily explained by the progress zone model. In the zeugopod, both elements are smaller than normal, but one of them (the radius) is more severely affected and is sometimes missing. In the autopod, one or two complete digits form, whereas the others are missing. One caveat of this study is that the AER is still present in the mouse double knockout and continues to express other AER genes, including *Fgf9* and *Fgf17*. It is possible that these FGFs (and perhaps other AER-derived molecules) are sufficient to maintain distal-cell viability and growth, although they are unable to rescue hindlimb development. Moreover, it is likely that the potential of the chick denuded mesenchyme to

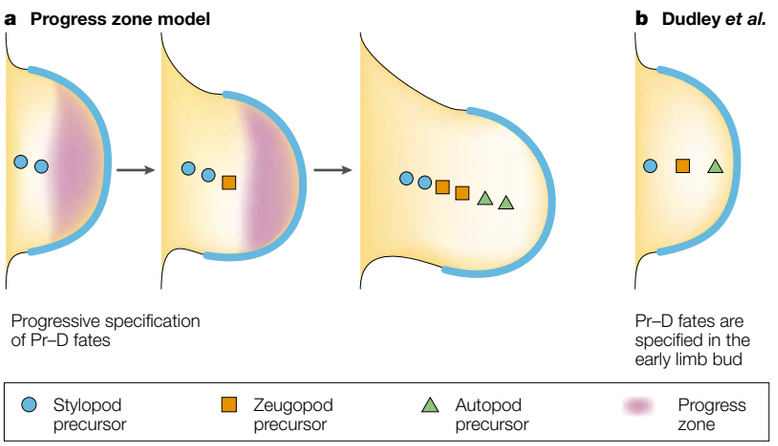


Figure 3 | **Graphical comparison of the progress zone and Dudley *et al.* models.** The progress zone model proposes progressive Pr–D specification that depends on the AER in which proximal fate is specified first, followed by progressively more distal specification. Dudley *et al.* propose that all Pr–D fates are specified within the early limb bud.

form distal structures is not evident because of the massive distal-cell death[27]. If this death was prevented, might distal condensations form? If so, would they be of normal size or smaller? Recombining normal chick mesenchyme with mouse *Fgf4*$^{-/-}$*Fgf8*$^{-/-}$ ectoderm might help to address these questions.

So, there are some inconsistencies between the results of the AER ablation and genetic removal of crucial FGFs. Whether this is because of the differences in experimental approaches or intrinsic differences between mouse and chick is unclear.

Interpretations of distal-tip exchange. The original progress zone model paper included experiments in which the distal tips from limbs of different stages were grafted onto the proximal stump of a limb at a different stage[41]. An old distal tip grafted onto a young stump formed an autopod and proximal stylopod — with deletion of the intervening zeugopod — whereas in the converse experiment a young distal tip (300 μm or equivalent to the whole limb bud at stage 19) formed all Pr–D segments. This was taken as evidence of progressive Pr–D specification and that once cells leave the progress zone their fate is determined. Dudley *et al.*[27] conducted a similar experiment in which a young distal tip (100 μm) gave rise only to autopod. Combined with the cell-labelling studies, these data were interpreted as evidence in support of the idea that Pr–D fates are specified and segregated within the early limb bud. So, in both cases, similar results were obtained (the differences might relate to the amount of tissue grafted), yet very different interpretations were provided depending on the chosen model.

Further relevant information was provided by Kieny and Pautou[51] and Hampé[52], whose experiments emphasized the regulatory nature of the limb bud — that is, its ability to make up for lost or added material. In similar old/young grafts between chick and quail that enabled cell contribution to be assessed, Kieny *et al.* found that stage 22 distal cells could intercalate and form the intermediate structures (the zeugopod) as well as distal parts, arguing against the idea that each limb part forms autonomously. This finding also indicates that cell fate can be respecified. The differences between the outcomes of these similar experiments[27,41,51,52] have not been adequately addressed and so it is not yet possible to assess how these data relate to the different models. Additional evidence that Pr–D fates can be respecified up to relatively late stages comes from studies that showed a striking ability of mesenchymal cells in the proximal part of the limb to restore Pr–D pattern[53–55]. When exposed to AER or FGF signals, the embryonic limb can completely restore Pr–D pattern after amputation of the distal 400–500 μm from forelimbs at stages 22–25 or after the removal of the whole forelimb bud at stage 22. If the amputations extend into regions undergoing condensation, the potential to regulate pattern declines, correlating with the extent of condensation and differentiation. By definition, this indicates that proximal fates are not DETERMINED at the time of the experiments.

DETERMINATION
Irreversible commitment of a cell or tissue. Pattern is fixed such that even if cells are exposed to different tissues or signals they will continue to develop according to their intrinsic pattern.

AER FGFs regulate mesenchyme cell number. Given the role of FGFs in the stimulation of cell proliferation, it is interesting and counterintuitive that decreased mesenchyme proliferation was not seen in *Fgf4/8*-mouse knockout limb bud[6]. This is consistent with earlier studies that indicated that AER removal at early stages does not differentially affect the rate of distal-cell division[45], and that bud proliferation is uniform along the Pr–D or A–P axes in the normal limb[56]. Sun *et al.*[6] proposed that FGFs act first to influence the initial size of the limb bud. They found that the early limb bud was smaller in the *Fgf8*$^{-/-}$ and in the *Fgf4/8* double-mutant hindlimb, and, because this effect was observed shortly after *Fgf8* expression would normally be detected, they thought it unlikely to be caused by altered cell proliferation or death. Instead, Fgf8 might affect morphogenetic movements or cell-adhesive processes that help to establish the initial number of cells in the early limb bud. This is consistent with other evidence that FGFs can influence cell migration in the established limb bud and morphogenetic movements during gastrulation[57–59].

Sun *et al.*[6] also proposed that the AER FGFs influence the number of cells in the limb bud by preventing their death. In the mutant limbs, there is significant death of proximal, but not distal, mesenchyme. The Pr–D pre-cartilage condensations form but they are smaller than in the wild type, in accordance with their ultimate size. So, Sun *et al.* suggested that the AER generates an adequate number of mesenchyme cells to enable correctly sized condensations to form. When the AER influence is disrupted, the number of skeletal progenitors is decreased so that small or no condensations are formed so, from this point of view, FGFs control mesenchymal cell number but not Pr–D patterning, which is a property of the mesenchyme[3].

The idea that sufficient mesenchymal mass is required to form normal-sized condensations might help to explain the third piece of evidence cited in favour of the progress zone model. X-irradiation ablation of mesenchymal cells before condensation results in skeletal loss and a reduction of the limb elements (hypoplasia)[60]. These results were originally interpreted within the framework of a progress zone and progressive Pr–D specification. Yet, they can fit easily with the idea that ablation causes loss of cells that are already patterned and segregated in the early limb and that a sufficient population of cells cannot be reestablished to form appropriate size condensations.

Over the years, much of the experimental data has been interpreted within the context of the progress zone model. However, recent data do not fit easily with this model. Moreover, older data can also be readily explained by other models of AER function, such as those proposed by Dudley *et al.*[27] and Sun *et al.*[6]. So, although the progress zone model is not yet 'dead'[43,44], attractive alternative models have been put forward that are supported by new experimental data and that can be further tested by additional experiments.

Multiple functions of the AER FGFs. The recent studies on chick and mouse show the multiple roles of the AER and FGFs. They include establishment of initial limb-bud size, regulation of cell viability and proliferation, and control of mesenchyme cell number to enable proper size condensations to form. In the chick AER ablation studies, the AER is present transiently, presumably enabling the initial limb-bud size to be established normally. On subsequent ablation of all AER signals, massive cell death eliminates the distal cells but leaves the proximal populations intact, resulting in a proximal element that is normal in size (although often distally truncated) but no distal elements. In the *Fgf4*$^{-/-}$ *Fgf8*$^{-/-}$ mouse hindlimb, no FGFs are expressed at the earliest stages, resulting in a small initial bud. Later, *Fgf9* and *Fgf17* expression — and possibly other AER activities — might prevent distal-cell death, but massive proximal-cell death occurs and insufficient numbers of mesenchymal cells are generated, resulting in the formation of a limited skeleton — only the pelvis and sometimes a small piece of unidentifiable cartilage. In the *Fgf4*$^{-/-}$ *Fgf8*$^{-/-}$ mouse forelimb, FGF signalling is transient, and initial limb-bud size is normal. *Fgf9* and *Fgf17* continue to be expressed in the AER. However, the AER signals are incapable of generating the appropriate numbers of mesenchymal cells, resulting in a skeleton in which all the Pr–D elements are formed but the distal elements are greatly reduced in number and in size. In the *Fgf8*$^{-/-}$ hindlimb, Fgf8 is never expressed and there is a delay before other FGFs — in particular, Fgf4 — are activated. In this case, the stylopod is severely affected, yet the zeugopod and autopod are essentially normal in both size and number, with the important exception that a single digit is absent. Reduction in the initial limb-bud size, little or no FGF signalling early in development and proximal-cell death might all contribute to the severe reduction or loss of the stylopod. In the *Fgf4* conditional knockout, the limb forms normally, showing that Fgf4 is not required[61,62]. The phenotype difference between the *Fgf8*$^{-/-}$ and the *Fgf4*$^{-/-}$ *Fgf8*$^{-/-}$ hindlimbs highlights the partial redundancy in FGF function. It is curious that there is a significant difference in the populations of mesenchyme cells that die following the genetic loss of AER signals or surgical removal of the AER. In the mouse double knockout, distal-cell death was not observed, although it does occur when the AER is completely removed[6], just as in the chick experiments. *Fgf9* and *Fgf17* are expressed normally in these mutant limbs and this expression might maintain distal-cell viability. Conversely, proximal-cell death is observed in the mouse double knockout but not in the chick AER removal experiments. In both cases, there is massive mesenchyme loss, but different populations are affected, and this might help to explain the differences in skeletal phenotypes; why proximal cell death is not observed in the chick, however, is unclear.

Despite recent advances, it still remains to be determined what regulates the stratification of Pr–D cell fates in the early limb bud, how Pr–D elements can complete their expansion at different times, and when Pr–D fates become specified and determined. Much remains to be learned about when and how the mesenchyme acquires Pr–D pattern and how the AER might influence this.

A–P patterning

Several reports in the past few years have provided important new information about the genes that are involved in A–P patterning of the limb. It is now known that Shh-independent events are involved in the initiation of limb development and that Shh-dependent events affect the progression of limb development. Moreover, Shh acts in an unexpected way by regulating Gli3 processing, but neither Shh nor Gli3 are required to make the limb skeleton; instead, they regulate digit number and identity.

Shh-independent A–P pattern. Early events, such as the initiation of limb-bud development and the induction of some mesenchymal and AER genes, occur before and independently of Shh signalling, as does significant prepatterning of the limb, including establishment of the A–P asymmetry. The early molecular A–P network includes dHand and Gli3 transcription factors (BOX 2). *Gli3* is expressed in the anterior mesenchyme of the limb bud and *dHand* is expressed in the posterior mesenchyme, and they mutually restrict each other's expression[30,63]. Although the restriction of dHand by Gli3 has recently been questioned[64], it has yet to be determined whether this relates to the time of analysis. Molecular details of early A–P patterning are not yet fully resolved; however, recent evidence indicates that Shh signalling is not required for stylopod or zeugopod patterning and growth.

Shh-dependent A–P pattern. Shh is involved primarily in autopod morphogenesis. Shh is thought to act as a morphogen[65,66]. Indeed, Shh can cause the formation of ectopic digits that are patterned in a dose-dependent way, where anterior and posterior digits form at low and high concentrations, respectively[67]. But does Shh act over the required distance of the limb bud, and, if so, how? Lewis *et al.*[68] altered the endogenous mouse *Shh* locus so that the Shh protein could not be cholesterol modified (*N-Shh*). Such modification is required for Shh protein localization. *N-Shh*/*Shh* null limbs have an intriguing digit pattern — most often, only three digits form. Although it is difficult to unequivocally identify mouse digits, they are classified as the two posterior digits 4 and 5 and the anterior digit 1, with the middle two digits missing. Lewis *et al.* concluded that the cholesterol-modified form of Shh is required for long-range signalling and for the formation of digits 2 and 3. Digits 4 and 5 form because they lie in the domain of Shh expression, whereas digit 1 formation is Shh independent (single digit 1 is formed in *Shh*$^{-/-}$ limbs).

Gli3 and Shh regulate digit number and identity. Recent studies of *Shh Gli3* double-mutant limbs have provided considerable insight and some surprising revelations about Shh function[63,64]. Although mutations in *Gli1* and/or *Gli2* do not affect limb growth or patterning, *Gli3* loss-of-function mutations cause polydactyly (*Gli3*$^{+/-}$, extra anterior digit; *Gli3*$^{-/-}$, multiple extra digits that are unpatterned)[69–71] (FIG. 2). The *Gli2*$^{-/-}$ mutation slightly enhances the polydactylous phenotype of *Gli3*$^{+/}$,

indicating that Gli2 has a redundant repressor activity in the limb[69–72]. As described above, *Shh* null mutant limbs have only a single digit 1 (REFS 16,17) (FIG. 2). Surprisingly, in the double-mutant *Gli3*$^{-/-}$ *Shh*$^{-/-}$ limbs, many digits are formed, although they do not have an obvious A–P pattern[63,64] and the zeugopod is restored (FIG. 2). It is suggested that the default state of the limb is to form many digits. Gli3 and Shh repress the digit-forming potential so that five digits are formed in a mouse limb. Also, Gli3 and Shh positively control digit identity. These data have, in many respects, turned the field on end as Shh and Gli3 are not needed for limb formation — instead, they regulate autopod morphology by constraining the number of digits and generating digit identity.

The limb phenotype of *N-Shh*/*Shh* null mutants could be explained if we speculate that, in the central region of the limb bud where digits 2 and 3 normally arise, Gli3 repressor activity was abnormally high and therefore represses the formation of these digits. One could then ask why digit 1 forms when digits 2 and 3 do not. Similarly, in *Shh*$^{-/-}$ mutant limbs, digit 1 forms, although Gli3 repressor is the predominant activity[64]. Moreover, digit 1 in the normal limb should be exposed to the highest level of the repressor form of Gli3, yet its formation is not repressed. It is possible that digit 1 responds to other signals that enable its formation. Other genes are differentially expressed in the anterior mesenchyme, but whether they have an active role in regulating digit 1 formation remains to be seen.

There is also an apparent limb-patterning paradox. Posterior pattern is not generated in the *Gli3*$^{-/-}$ limbs, despite the seemingly intact high level of Shh signalling in the posterior mesenchyme, as shown by the expression of Shh targets (*Gli1* and *Patched*) and posteriorly expressed genes (*Hoxd12, Hoxd13* and *Bmp2*)[63,64]. This result indicates that Gli3 activity (presumably the Gli3 activator function) is not required for the expression of these genes, which have been implicated in digit patterning, and yet, in combination, these genes are not sufficient to generate posterior digit identity. Perhaps another Gli family member or different transcription factor has an active role in transducing the Shh signal to regulate these targets.

Overall, Shh could still be considered as a morphogen, but it might serve this function through the regulation of Gli3 processing and perhaps by controlling the relative ratio of Gli3 repressor to Gli3 activator.

Given the crucial role of the AER in the regulation of mesenchymal mass, it is interesting that Gli3 represses the function of the AER, as the AER and the expression of *Fgf4* and *Fgf8* are greatly expanded in *Gli3*$^{-/-}$ limbs[63,64,73]. It seems that Gli3 represses *Gremlin,* which antagonizes BMP repressor function, and that Shh modulates the repressive activity of Gli3. In the absence of both *Shh* and *Gli3*, *Gremlin* and *Fgf4* are widely expressed in the mesenchyme and the AER, respectively[63,64], and this is likely to contribute to the expansion of the mesenchyme and the formation of additional digits. Conversely, in *Shh*$^{-/-}$ limbs, there is massive death of the anterior mesenchyme that is concomitant with degeneration and loss of the anterior AER FGFs[16,63]. So, the *Shh*$^{-/-}$ phenotype could, in part, be related to AER loss. Cell death and distal-limb development, but not the patterning, are rescued in a dose-dependent manner by reducing the dose of Gli3 (REF. 63). Abnormal cell death could explain why Shh signalling functions in the realization of zeugopod fate, based on analysis of *Shh*$^{-/-}$ limbs, whereas *Shh*$^{-/-}$ *Gli3*$^{-/-}$ limbs indicate that Shh signalling is required only for autopod morphology. The anterior- and distal-cell death detected in *Shh*$^{-/-}$ and *ozd* mutant limbs indicate the interesting possibility that the elements that do form arise from mesenchyme that was originally on the posterior side of the limb.

Pr–D and A–P axes are linked

Traditionally, it has been easier to think in terms of signals that regulate the Pr–D or A–P axis, but recent data have clearly shown that these signalling centres cannot be so simply defined. Moreover, their activities cannot be separated, as shown by molecular analysis of mutant limbs. The inability to separate the axes is evident in the *Fgf8*$^{-/-}$ mutant limb in which all Pr–D fates are realized (although the stylopod is reduced), and the most important defect is the loss of 1–2 digits — traditionally described as an A–P patterning defect. In the *Shh*$^{-/-}$ mutant limb, the distal structures (zeugopod and autopod) are severely reduced in size, in addition to digits being lost along the A–P axis. In the *Shh*$^{-/-}$ *Gli3*$^{-/-}$, the A–P axis is expanded — perhaps in direct relation to the expansion of the AER — yet Pr–D patterning is normal.

Therefore, perhaps it is more appropriate to consider a relationship between mesenchymal mass and progressive condensation of the mesenchyme cells in order to understand the reduction or expansion in number and size of skeletal elements observed in various limb mutants. Many mutations (such as loss of *Shh* and classical mutations like *limb deformity* and *Gli3* (*extra toes*) in the mouse or *talpid* and *diplopodia* in chick) produce the most severe defects in the autopod, leading to OLIGODACTYLY or polydactyly. When mesenchymal mass is reduced (for example, in *limb deformity* and in *Shh* mutants)[16,17,74,75], there are fewer digits. Conversely, when mesenchymal mass is increased, Pr–D pattern is normal but digit number is increased (as seen in *Gli3*, *Alx4* and *Obp* mutants)[71,76,77]. These results correlate with whether the A–P width of the autopod is reduced or extended, respectively. Moreover, the length of the AER varies in a way that is related to the size and form of the limb bud. Yet, Pr–D pattern is largely intact in the mutants mentioned above. The apparent autonomy of the Pr–D axis might be related to the segregation of Pr–D fates in the early limb bud[27]. Most often, the more anterior digits (digits 1 and 2) and/or the most posterior digit (digit 5) are affected, especially when mesenchymal mass is reduced, in agreement with data indicating that these structures are the last to condense[78]. Perhaps the number of digits that are ultimately formed is controlled by the amount of mesenchyme that is left at the time of digit condensation. Different mechanisms, such as altered cell death, A–P or AER signalling, can cause changes in mesenchymal mass. In terms of patterning, it is possible that

OLIGODACTYLY
Having fewer than normal digits.

mesenchyme cells might not yet have acquired an intrinsic pattern before and during condensation. Instead, the pattern that distinguishes a radius from a digit or one digit from another might be imparted onto these cells, depending on their position and on their neighbours, such as the interdigital tissue that can influence the pattern of the digits[48].

Conclusion

The recent research into limb development has given new insights into the mechanism by which the FGF proteins direct Pr–D growth. Yet, Pr–D segmentation and patterning are not directly affected by the loss of AER FGF. Moreover, Shh and Gli3 — which we thought were the two main regulators of A–P limb pattern — are not required for the formation of a complete limb with digits, although A–P pattern is lost in their absence. Despite recent revelations, the question remains as to what makes a humerus different from an ulna or a digit, or a thumb different from a little finger? Much remains to be discovered as to how the mesenchyme undergoes Pr–D specification, how cell fate is determined and what the downstream genes are that translate the signals from the three limb-organizing centres into skeletal pattern. For now, it is satisfying to have new experimental data that expand our thinking of how the Pr–D and A–P organizers and the molecules they produce control proper limb development from the shoulder to the hand.

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Shh

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