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REVIEW

# Next generation limb development and evolution: old questions, new perspectives

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## ABSTRACT

The molecular analysis of limb bud development in vertebrates continues to fuel our understanding of the gene regulatory networks that orchestrate the patterning, proliferation and differentiation of embryonic progenitor cells. In recent years, systems biology approaches have moved our understanding of the molecular control of limb organogenesis to the next level by incorporating next generation 'omics' approaches, analyses of chromatin architecture, enhancer-promoter interactions and gene network simulations based on quantitative datasets into experimental analyses. This Review focuses on the insights these studies have given into the gene regulatory networks that govern limb development and into the fin-to-limb transition and digit reductions that occurred during the evolutionary diversification of tetrapod limbs.

**KEY WORDS: SHH, WNT, BMP, FGF, HOXA, HOXD, Topological domains, Turing, Teleost**

## Introduction

For over 60 years, vertebrate limb bud development has been one of the most prominent experimental paradigms for studying study the fundamental processes underlying morphogenesis and organogenesis. Indeed, the manipulation and analysis of limb bud development has resulted in major advances in our understanding of the molecular mechanisms that govern diverse processes such as patterning and growth, epithelial-mesenchymal transitions and feedback signalling during embryogenesis (for a historical perspective, see Towers and Tickle, 2009). In particular, these studies have allowed us to gain insight into an evolutionary riddle that is fascinating scientists and non-scientists alike: namely, what are the molecular mechanisms and innovations underlying the so-called fin-to-limb transition, which ultimately enabled aquatic vertebrates to conquer land and colonise diverse terrestrial habitats? The continued success of limb bud development models, in particular in chick and mouse, is largely due to the fact that this external organ is amenable to experimental and genetic manipulation and that congenital malformations are in general viable, which allows for complex genetic and molecular analysis. Furthermore, rapid advances in *in vivo* imaging in combination with genome-wide analyses of transcriptional regulation, chromatin architecture, epigenetic modifications and chromosomal landscapes have resulted in a deep molecular understanding of limb bud development. These so-called 'next generation' molecular tools are increasingly being combined with mathematical modelling, enabling hypothetical models of limb development to be experimentally tested.

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Here, I review the basic mechanisms and molecular interactions underlying vertebrate limb bud development, focussing mostly on recent advances in the field. In particular, I discuss advances in our understanding of the cellular and molecular mechanisms that govern limb bud initiation and the current state of knowledge of how signalling and transcriptional networks control the growth and patterning of limb bud mesenchymal progenitors. With respect to evolutionary findings, recent discoveries of the molecular mechanisms, innovations and alterations underlying the fin-to-limb transition and tetrapod limb skeletal diversification, focusing on digit reductions, will be discussed.

## An overview of limb bud development

Limb bud development is initiated by the emergence of a small bud from the body wall (Fig. 1). Its mesenchyme is derived from the lateral plate mesoderm, which is enveloped by ectodermal cells. As the mouse forelimb bud emerges at embryonic day (E) 9.5, its antero-posterior (AP), proximo-distal (PD) and dorso-ventral (DV) axes can already be distinguished (Fig. 1). By around E10.0, the apical ectodermal ridge (AER), which acts as a signalling centre located at the DV interface of the limb bud ectoderm, has a morphologically distinct appearance (Saunders, 1948). The AP axis defines the direction from thumb to little finger, the PD axis from scapula (shoulder blade) to autopod (hands and feet) and the DV axis from the back to the palm of the hand. In mouse embryos (E9.5-E12.5), limb bud outgrowth and the patterning of mesenchymal progenitors are coordinately controlled by epithelial-mesenchymal interactions between the AER and mesenchyme (Fig. 1). The limb bud mesenchymal progenitors destined to form proximal skeletal elements condense first and undergo chondrocyte differentiation. Their cartilage primordia are already morphologically distinct by E11.5 (Fig. 1), whereas the more distal progenitors destined to form the digit rays are still being expanded by proliferation. During the formation and distal elongation of digit primordia, programmed cell death (apoptosis) eliminates the interdigital mesoderm from around E12.5-E13.5 onwards. This Review focuses mainly on mouse embryonic days E9.5-E12.5, i.e. the developmental period during which the mesenchymal progenitors that will give rise to the future skeletal elements are expanded and patterned, giving rise to the AP, PD and DV axes of the limb.

## Gene regulatory networks regulating the initial stages of limb bud development

Genetic studies in mouse have identified genes essential for limb bud positioning and initiation. For example, evidence indicates that the precise position at which the fore- and hindlimb buds emerge is controlled by a transcriptional network that includes Hox transcriptional regulators, which function in determining the AP body axis of the embryo (for more details see Kmita and Duboule, 2003). Genetic inactivation of *Hox8* paralogues alters the AP position at which the hindlimb bud emerges from the flank (van den Akker

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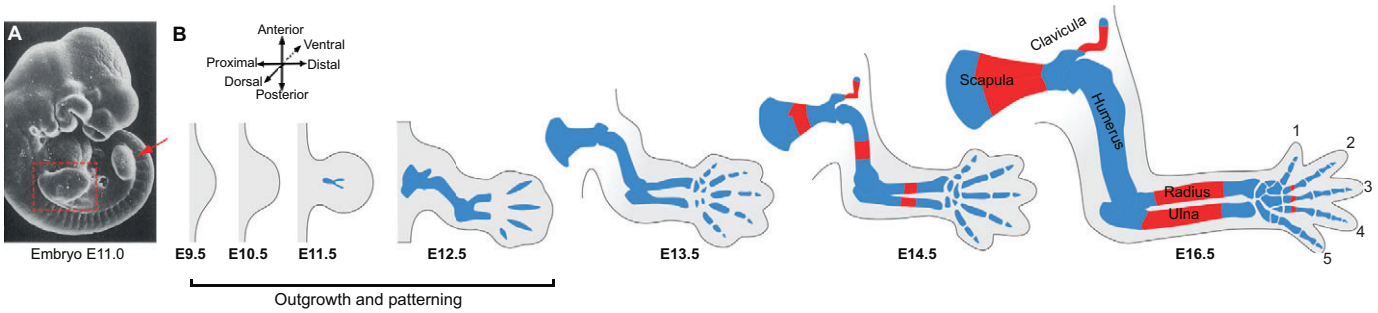


**Fig. 1. An overview of the stages of limb development.** (A) A scanning electron microscopy image of a whole mouse embryo at E11.0. The fore- and hindlimb buds are indicated with a red box and a red arrow, respectively. (B) Schematic representations of the morphological changes occurring during forelimb bud development (E9.5-E16.5). Hindlimb bud development (not shown) is delayed by half a day in comparison with the forelimb bud. Skeletal elements are depicted as they appear when stained with Alcian Blue (to highlight mature cartilage) and Alizarin Red (to highlight mineralised bone). Bone identities are indicated and digits are labelled 1 to 5 from anterior (thumb) to posterior (little finger). The proximal scapula (shoulder blade) and clavicle are located in the body. The three limb bud axes are also shown.

et al., 2001). A small set of other genes, including *Gdf11*, *Tbx3* and *Dicer1* is also involved in limb bud positioning (McPherron et al., 1999; Rallis et al., 2005; Zhang et al., 2011). In particular, microRNAs (miRNAs) seem to participate in positioning the hindlimb bud, as inactivation of the miRNA-processing enzyme *Dicer1* in mesodermal lineages shifts the hindlimb position (Zhang et al., 2011). This is in agreement with a previous observation that miRNA-196 (Mir196a-1) regulates the levels of *Hoxb8* and sonic hedgehog (*Shh*) transcripts, which are known to regulate hindlimb bud development (Hornstein et al., 2005).

Formation of the nascent limb bud also requires fibroblast growth factors (FGFs) and the transcription factor TBX5 (Fig. 2A). Experimental manipulation of FGF signalling in chicken embryos provided evidence that the limb bud forms as a consequence of continued local proliferation of the flank mesenchyme (Cohn et al., 1995; Ohuchi et al., 1997). Furthermore, in mice lacking *Fgf10*, limb buds form but are not maintained, and in mice lacking *Tbx5* complete forelimb bud agenesis is observed (Agarwal et al., 2003; Min et al., 1998; Rallis et al., 2003; Sekine et al., 1999). More recently, Gros and Tabin (2014) investigated the cellular events that underlie limb bud initiation in chicken embryos. Their study showed that, prior to limb bud emergence, limb bud mesenchymal progenitors originate from an epithelium formed by the somatopleure, which consists of lateral plate mesoderm and ectoderm. They provided experimental evidence that the mesenchymal progenitors of the nascent limb bud arise by localised epithelial-to-mesenchymal transition (EMT). In *Tbx5*- and *Fgf10*-deficient mouse embryos, the fraction of limb bud mesenchymal cells in the forelimb region was reduced, which, together with molecular analysis, pointed to partial disruption of the EMT process. The authors concluded that the limb bud mesenchymal progenitors arise by an EMT in the limb field that is to some extent regulated by*Tbx5* and *Fgf10* (Gros and Tabin, 2014). Furthermore, TBX5 and TBX4 are required to initiate fore- and hindlimb bud development, respectively (Agarwal et al., 2003; Naiche and Papaioannou, 2003; Rallis et al., 2003; ten Berge et al., 2008).

Limb bud initiation and the onset of distal outgrowth also depend on retinoic acid (RA)-mediated antagonism of FGF8 in the flank mesenchyme (Mic et al., 2004; Zhao et al., 2009) in concert with epithelial-mesenchymal feedback signalling between FGF8 from the AER and FGF10 in the mesenchyme (Ohuchi et al., 1997). In turn, the formation of a functional, FGF- and WNT-expressing AER depends on bone morphogenetic protein (BMP) signalling in the mesenchyme (Fig. 2A; Ahn et al., 2001; Benazet et al., 2009; Benazet and Zeller, 2013; Pajni-Underwood et al., 2007).

## AP axis formation and establishment of the SHH signalling centre

Mutual antagonism between the transcription factors HAND2 and GLI3 polarises the AP axis of the nascent limb bud. Initially, *Hand2* and *Gli3* are expressed throughout the presumptive limb field and, during the onset of limb bud outgrowth, direct cross-regulation results in posterior restriction of HAND2 and anterior restriction of GLI3. This mutually antagonistic interaction results in the activation and posterior restriction of *Shh* expression in the limb bud mesenchyme (Fig. 2B; Charité et al., 2000; Galli et al., 2010; te Welscher et al., 2002). Localised *Shh* expression also requires other regulators, such as HOX, PBX and ETS transcription factors (Capellini et al., 2006; Kmita et al., 2005; Kozhemyakina et al., 2014; Lettice et al., 2012; Mao et al., 2009; Zhang et al., 2010b, 2009). The posteriorly restricted *Shh* expression domain molecularly defines an essential signalling centre within the limb bud mesenchyme, namely the zone of polarising activity (ZPA; Fig. 2B; Riddle et al., 1993; Saunders and Gasseling, 1968).

SHH itself is a morphogen that acts over distances (see Box 1) and plays a key role in the AP patterning of the limb bud. The inactivation of *Shh* in mouse limb buds results in the loss of all digits with the exception of digit 1 (Chiang et al., 2001; Sagai et al., 2005). Experimental manipulation in chick and genetic analysis in the mouse have suggested that SHH participates in the early establishment AP axis polarity and controls the proliferative expansion of specified mesenchymal progenitors (Towers et al., 2008; Zhu et al., 2008). Furthermore, genetic fate-mapping studies have shown that digit 2 formation depends entirely on long-range SHH signalling (Ahn and Joyner, 2004; Harfe et al., 2004). However, SHH signalling may not be sufficient to determine digit identities on its own. Rather, evidence from experiments in chicken limb buds indicates that digit identities are only determined (i.e. fixed) during the post-SHH patterning phase (Dahn and Fallon, 2000; Suzuki et al., 2008). In parallel, upregulation of SHH signalling inhibits the processing of GLI3 to GLI3 repressor (GLI3R) such that the full-length GLI3 activator (GLI3A) accumulates in cell nuclei within the posterior limb bud mesenchyme (Ahn and Joyner, 2004; Osterwalder et al., 2014; Wang et al., 2000). During limb bud outgrowth, SHH propagates the expression of mesenchymal genes including *5′HoxD* and *HoxA13* (collectively called ‘distal Hox genes’; Sheth et al., 2012), which function in autopod patterning, i.e. specifying the correct number and identity of digits.

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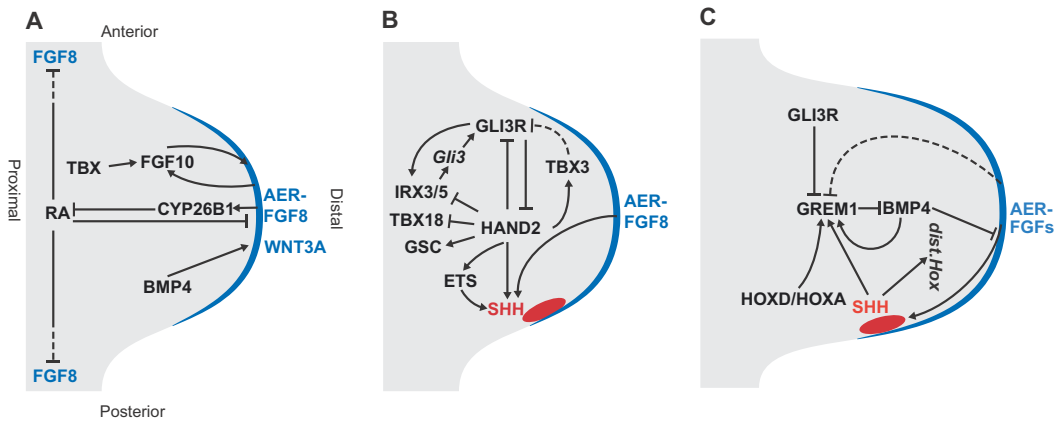


**Fig. 2. Gene networks controlling limb bud development.** (A-C) Scheme of a mouse limb bud showing the mesenchyme (grey), the apical ectodermal ridge (AER, blue) and the zone of polarising activity (ZPA, red). The key gene regulatory networks operating during initial limb bud positioning and the initiation of outgrowth (A), AP patterning (B) and PD patterning (C) are shown. (A) Antagonism between retinoic acid (RA) and FGF8 in the flank participates in positioning the limb field along the primary embryonic axis. Limb bud emergence from the flank is controlled by, among others, TBX genes, which upregulate *Fgf10* expression in the mesenchyme. FGF10 signalling from the mesenchyme and *Fgf8* activation by the ectodermal progenitors of the AER marker establishes an epithelial-mesenchymal feedback loop that participates in the initiation of limb bud outgrowth. Antagonistic interactions between RA (from the proximal mesenchyme), AER-derived FGF8 (AER-FGF8) and ectodermal WNT3A participate in PD patterning. CYP26B1 expression is upregulated by AER-FGF8 signalling and in turn degrades RA in the distal mesenchyme. (B) The gene networks that pre-pattern the limb bud mesenchyme upstream of SHH are shown. HAND2 controls the establishment of a proximal, anterior and posterior compartment in the early forelimb bud mesenchyme, by direct positive regulation of posterior and/or distal genes and repression of specific anterior and proximal genes. HAND2 (together with HOX transcription factors, not shown) also participates in activating *Shh* expression. Note that the inhibitory effect of TBX3 on GLI3R may be indirect (dashed line). (C) Distal limb bud outgrowth is propagated by an SHH/GREM1/AER-FGF epithelial-mesenchymal feedback signalling system. HOXD/HOXA transcriptional regulators positively regulate *Grem1* expression. SHH propagates the expression of *Grem1* and distal Hox genes. As BMPs repress AER-FGF gene expression, *Grem1* is essential to propagate these feedback signalling interactions. Ultimately, the SHH/GREM1/AER-FGF loop is terminated by downregulation of *Grem1* expression by GLI3R (anterior mesenchyme) and increasing inhibition by AER-FGFs (dashed line; the molecular mechanism is unclear).

## Models of PD patterning and limb bud outgrowth

Based mostly on experiments in chicken limb buds, two main models have been put forward to explain the establishment of the limb bud PD axis and the specification of proximal (e.g. humerus) and distal (e.g. hand plate) skeletal elements. The oldest one is the 'progress zone model', which proposes that the progressive specification of the PD skeletal elements depends on the time the progenitors have spent in the distal mesenchyme underlying the AER – the so-called progress zone (Summerbell, 1974). Progenitor cells in the progress zone remain undifferentiated and adopt different fates depending on the length of time they are exposed to AER-FGFs, which stimulate their proliferation and act as distalising factors (Niswander et al., 1993). More recently, the 'two-signal model' has been formulated, which proposes that two opposing signals, such as RA secreted from the flank and FGF secreted by the AER, pattern the skeletal elements along the PD axis. This model indicates that AER-FGFs have an instructive role in regulating the expression of specific genes and thereby identities along the PD axis (Mercader et al., 2000). In fact, extensive genetic analysis of the FGFs expressed by the AER (*Fgf4*, *Fgf8*, *Fgf9*, *Fgf17*) in mouse limb buds has led to the proposal that AER-FGFs indeed have instructive roles, i.e. they specify PD fates during limb bud outgrowth and patterning (Lewandoski et al., 2000; Mariani et al., 2008). This model differs from the simpler progress zone model in that cell-type specification depends on both the type and level of signals received by cells. Several studies have attempted to discriminate between the two models by experimental manipulation of chicken limb buds. Two studies show that at early stages, limb bud mesenchymal progenitors are specified as proximal, but they adopt distal fates upon exposure to distalising signals such as FGF8 (Cooper et al., 2011; Rosello-Diez et al., 2011). These authors propose that mesenchymal progenitors closest to the AER adopt progressively more distal fates owing to their continued exposure to FGF signalling. AER-FGFs maintain mesenchymal progenitors in an undifferentiated proliferative state together with WNT3A secreted by the limb bud ectoderm (Fig. 2A; Rosello-Diez et al., 2011). However, the two most recent studies suggest that external

### Box 1. Long-range signalling by SHH

Recent studies have provided key insights into the spatial control of signalling during limb development. The cellular and molecular mechanisms underlying long-range signalling has fascinated researchers for decades, and years ago Lewis Wolpert postulated the existence of a diffusible long-range morphogen that patterns digits according to thresholds (Wolpert, 1969). The search for this morphogen culminated in the identification of SHH as the signal expressed by the polarising region (Riddle et al., 1993), and subsequently it was shown that digits are probably patterned by a combination of temporal and spatial exposure to graded SHH signalling (Ahn and Joyner, 2004; Harfe et al., 2004). However, a recent study by Sanders et al. (2013) uncovers an interesting cellular mechanism that underlies long-range morphogenetic signalling by SHH in chicken limb buds. By optimising single-cell real-time imaging they were able to show that SHH accumulates as particles in actin-based filopodia of *Shh*-producing mesenchymal cells in the posterior limb bud. These cytoplasmic extensions span several cell diameters, which indicates that SHH ligands sequestered in particles in these filopodia might act at a distance without extracellular diffusion (Sanders et al., 2013). Furthermore, CDO and BOC are two cell-adhesion proteins that act as SHH co-receptors to positively regulate SHH signal transduction (Tenzen et al., 2006; Zhang et al., 2006). They are present in the filopodia of mesenchymal cells that respond to SHH signalling. These findings suggest that interactions are formed between the filopodia of SHH-producing and distant responding cells containing CDO and BOC proteins: this provides an alternative mechanism of SHH reception at a distance from producing cells (Sanders et al., 2013).

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signals are not sufficient to explain the patterning of PD limb bud axis and propose that intrinsic timing mechanisms are also involved (Rosello-Diez et al., 2014; Saiz-Lopez et al., 2015). Neither of the current models is able to integrate all experimental data on the analysis of PD patterning, which calls for an improved model that also includes intrinsic timers acting in a cell-autonomous manner in addition to signals.

### A self-regulatory signalling system coordinately controls limb bud outgrowth and patterning

In addition to its role in AP and PD patterning, SHH functions together with AER-FGFs in a positive-feedback loop that controls the epithelial-mesenchymal feedback signalling interactions that govern limb bud outgrowth along the PD axis (Fig. 2C; Laufer et al., 1994; Niswander et al., 1994). This feedback signalling system coordinately controls the proliferation and patterning of limb bud mesenchymal progenitors (Fallon et al., 1994; Lewandoski et al., 2000; Mariani et al., 2008; Towers et al., 2008; Zhu et al., 2008). SHH signalling from the posterior mesenchyme propagates FGF gene expression in the AER by upregulating the expression of the BMP antagonist *Grem1* in the posterior-distal mesenchyme (Fig. 2C; Zuniga et al., 1999). The transcriptional upregulation of *Grem1*, which increasingly antagonises BMP activity, is part of a self-regulatory SHH/GREM1/AER-FGF signalling system consisting of interlinked feedback loops (Benazet et al., 2009). GREM1-mediated BMP antagonism permits an increase in FGF gene expression in the AER, which in turn maintains *Shh* expression and cell proliferation in mesenchyme. This self-regulatory feedback signalling system, and thereby limb bud outgrowth, is terminated by increased spatial separation of the *Shh* and *Grem1* expression domains and progressive inhibition of *Grem1* expression by AER-FGFs and GLI3R (Fig. 2C; Lopez-Rios et al., 2012; Scherz et al., 2004; Verheyden and Sun, 2008). During the subsequent post-patterning phase, BMP activity increases again and participates sequentially in a variety of processes, such as inducing mesenchymal condensations, chondrogenesis and inter-digital cell death (Barna and Niswander, 2007; Benazet et al., 2012; Ganan et al., 1996).

### Coordinating the limb bud axes and signalling centres

As briefly introduced before, the AP and PD axes polarities are established upstream of *Shh* activation (Fig. 2) and the nascent limb bud mesenchyme is pre-patterned by mutually antagonistic interactions between the transcriptional regulators GLI3R and HAND2 (te Welscher et al., 2002). To identify the gene regulatory networks and hierarchies controlled by HAND2 and GLI3R, chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) has been used to determine the range of direct target genes in early mouse limb buds, providing insights into how AP and PD axis establishment is interconnected from the earliest limb bud stages onward (Osterwalder et al., 2014; Vokes et al., 2008). For example, the analysis of direct targets of endogenous HAND2 transcriptional complexes by Osterwalder and colleagues showed that they control the expression of both proximal genes (Li et al., 2014a) and posterior genes (Lettice et al., 2012). Interestingly, in addition to interacting directly with the distant enhancer that controls *Shh* expression in limb buds (Galli et al., 2010), HAND2 enforces *Shh* activation and expression by directly controlling the expression of ETS transcription factors in the posterior mesenchyme. In turn, the ETS transcription factors participate in upregulation of *Shh* expression (Lettice et al., 2012). Conversely, the HAND2-mediated anterior transcriptional repression of *Gli3* is reinforced via TBX3, which serves to define precisely the posterior boundary of the *Gli3* expression domain.

Another aspect of the tight coordination between the two axes is the regulation of *Cyp26b1*, which encodes an enzyme that catalyses RA inactivation, in the distal limb bud mesenchyme. Analysis of *Shh*-deficient mouse limb buds showed that *Cyp26b1* expression is downregulated, whereas the expression of proximal genes is distally expanded. Detailed analysis indicates that SHH upregulates *Cyp26b1* expression via AER-FGFs as part of the SHH/GREM1/AER-FGF signalling system, which in turn enhances the clearance of RA from the distal mesenchyme (Fig. 2A; Probst et al., 2011). Taken together, it is now clear from these and many other studies that, although the establishment of these axes was traditionally studied separately, the patterning and proliferative expansion of the AP and PD axes is tightly interconnected.

### The spatiotemporal dynamics and context-dependent functions of signalling pathways

Another important aspect, emerging mostly from genetic analyses in the mouse, is the temporal dynamics and context-dependent functions of particular pathways. As already mentioned, SHH specifies AP polarity during early limb bud development, but also controls the subsequent proliferative expansion of the early specified mesenchymal progenitors. Furthermore, BMP4 signalling from the mesenchyme and the BMP receptor 1 (BMPR1) in the ectoderm are first required for AER formation, as their early inactivation disrupts the AER and results in severe limb bud truncations (Ahn et al., 2001; Benazet et al., 2009; Pajni-Underwood et al., 2007). However, conditional inactivation of *Bmp4* after the AER has formed results in AER lengthening and digit polydactyly (Benazet et al., 2009), revealing a striking temporal change in BMP function during limb bud development. These results are corroborated by constitutive overexpression of *Grem1* in the limb bud mesenchyme, which shows that BMPs function in a dose- and time-dependent manner (Li et al., 2014b).

During the progression of distal limb bud outgrowth, BMP activity is modulated by the BMP antagonist GREM1 as part of the SHH/GREM1/FGF signalling system, and is necessary to restrict the autopod to pentadactyly. However, as the SHH/GREM1/AER-FGF feedback signalling system terminates, BMP activity is expected to increase again, as has been shown by data-driven simulations of the feedback signalling interactions (Fig. 2C; Benazet et al., 2009). Indeed, during this post-patterning phase, BMP signal transduction is required to induce the chondrogenic differentiation of SOX9-positive mesenchymal progenitors (Bandyopadhyay et al., 2006; Barna and Niswander, 2007; Benazet et al., 2009; Benazet et al., 2012). At this stage, GLI3R participates in the spatiotemporal restriction and termination *Grem1* expression, which promotes the spatiotemporally coordinated exit of proliferating progenitors towards BMP-dependent chondrogenic differentiation such that the developing autopod is restricted to pentadactyly (Fig. 2C; Lopez-Rios et al., 2012).

### Hox genes: landscapes and chromatin topology

The best-studied class of transcription factors involved in patterning and forming the limb skeletal elements are those encoded by the *HoxA* and *HoxD* gene clusters. The position of a particular Hox gene within its respective cluster determines whether it is expressed in limb buds and how its spatiotemporal expression domains will evolve during AP and the PD limb bud axis development (reviewed by Zakany and Duboule, 2007). The intricate expression domains of Hox genes are crucial for elaboration of the limb skeletal elements,

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and the *cis*-regulatory circuitry underlying their complex transcriptional regulation has recently been uncovered using complex genetics in mice in combination with transcriptional and epigenetic profiling and analyses of chromosomal architecture (reviewed by Noordermeer and Duboule, 2013).

The spatiotemporal expression of the *5′Hoxd* gene cluster in mouse limb buds is regulated by multiple enhancers embedded in a larger genomic landscape. For example, seven enhancers in the *HoxD* gene cluster control the expression of *5′Hoxd* genes in the presumptive digit area (referred to as 'distal limb' expression). For this reason they are referred to as 'distal enhancers', which are all located up to hundreds of kilobases 5′ of the gene cluster (Montavon et al., 2011). These upstream enhancers loop to the proximal promoters of the *5′Hoxd* genes, as revealed by circularised chromatin conformation capture (4C) analysis (Andrey et al., 2013). This technique also showed that the loops of the distal enhancers are part of a higher order chromatin structure that is called a topological chromatin domain (see Box 2). These topological domains are functionally important: the expression of *5′Hoxd* gene expression in the proximal and distal limb bud mesenchyme is regulated by two distinct domains, the so-called T-DOM and C-DOM encoding the clusters of telomeric and centromeric enhancers, respectively (Andrey and Duboule, 2014; Andrey et al., 2013). During early limb bud outgrowth, the telomeric enhancers of the T-DOM control proximal expression of the *Hoxd8* to *Hoxd11* genes (defined as 'early phase' Hox gene expression; Zakany and Duboule, 2007). During progression of limb bud outgrowth and hand plate formation, the centromeric enhancers of the C-DOM act on the later activated and more distally restricted *Hoxd12* and *Hoxd13* genes. Some of the early activated genes, such as *Hoxd11* and *Hoxd9*, shift their enhancer interactions from T-DOM to C-DOM, as T-DOM is no longer active during digit formation (Andrey et al., 2013). This topological organisation of the Hox gene cluster appears to predate the duplication of the gene clusters during evolution, as the enhancers regulating the *HoxA* gene cluster are organised in similar topological domains (Berlivet et al., 2013; Lonfat et al., 2014; Woltering et al., 2014). Genetic analysis of the limb enhancers regulating the *HoxA* cluster, together with the determination of their position and characterisation of their topological domains, showed that inactivation of specific enhancers does not alter their contacts with the respective *5′Hoxa* genes (Berlivet et al., 2013). For both Hox gene clusters, the same bimodal chromatin topology serves specific regulation of proximal and distal limb expression and identity, suggesting a common ancient *cis*-regulatory topology (Berlivet et al., 2013; Lonfat et al., 2014; Woltering et al., 2014). The functional importance of 3D chromatin architecture is corroborated by the fact that single isolated enhancers fail to recapitulate accurately the expression patterns of *5′Hoxd* and *5′Hoxa* genes in limb buds (for example, see Montavon et al., 2011). These studies are beginning to reveal how multiple enhancers act together to orchestrate the spatiotemporal dynamics of *5′Hox* gene expression.

More recent genome-wide approaches and studies of specific loci have identified a large number of other genomic landscapes and gene deserts that harbour multiple enhancers (e.g. Ghavi-Helm et al., 2014; *Fgf8*: Marinic et al., 2013; *Grem1* mesenchyme expression: Li et al., 2014b; Zuniga et al., 2012). Moreover, the distant enhancers controlling the expression of *Shh*, *Bmp7* and *Tfap2c* genes in limb buds are also embedded in large topological domains (Anderson et al., 2014; Lettice et al., 2003, 2002; Tsujimura et al., 2015). Detailed analysis of the 3D chromatin architecture of genes expressed in limb buds should reveal whether the principles underlying the regulation of *HoxA* and *HoxD* gene clusters are indicative of a more general mechanism of gene regulation by distant enhancers.

## Data-driven simulations of limb bud development and digit patterning

Recent studies using data-driven simulations of limb bud development are also providing new insights into the underlying growth-regulatory and patterning mechanisms. A classic example of the impact of modelling on our understanding of development relates to Lewis Wolpert's morphogen hypothesis (the 'French Flag' model; Wolpert, 1969). To explain the altered digit patterns resulting from numerous cutting and pasting experiments performed in chicken wing buds, Wolpert concluded that the polarising region must produce a diffusible morphogen that patterns responding cells and acts at a distinct threshold, providing them with so-called 'positional information' (reviewed in detail by Green and Sharpe, 2015). His model could not only explain these grafting experiments, but also inspired the search for the elusive morphogen – SHH (Riddle et al., 1993). Now that all of the major pathways controlling limb bud development appear to have been identified, we can use results from experimental studies to model and simulate limb bud development. As discussed before, the different signalling pathways are interconnected in space and time, and although we have sophisticated genetic and other tools to manipulate limb bud development, the sheer spatiotemporal complexity of interactions and networks is currently impossible to grasp by experimental and genetic manipulation alone. Therefore, data-driven mathematical simulations can complement the ongoing molecular and genetic analyses of limb development.

Turing's self-organising reaction-diffusion model, another model that has fascinated developmental biologists for decades (reviewed in detail by Green and Sharpe, 2015; Kondo and Miura, 2010), has also provided interesting insights into the molecular interactions underlying the patterning and formation of digits (Fig. 3). In the reaction-diffusion model, an activator and an inhibitor interact, resulting in the establishment of a periodic pattern from an initially homogenous state. This system allows simulation of repetitive structures with the properties of a self-organising system (Fig. 3). Initially, it was proposed that the pattern of the cartilage models of the future limb skeletal elements could be determined by a Turing-type patterning system, in which TGFβ signals would act as 'activator' molecules (Leonard et al., 1991; Miura and Shiota,

**Box 2. *Cis*-regulatory landscapes and chromatin topology**

Gene expression is regulated by non-coding sequences called *cis*-regulatory modules (CRMs) that may be located rather distantly from proximal promoters. CRMs that drive expression of a reporter gene in transgenic animals (e.g. mice or zebrafish) in a specific pattern are referred to as transcriptional enhancers. A distant enhancer interacts with the promoter region by chromatin looping. During limb bud development, the limb bud-specific expression of certain genes, such as *Shh*, is controlled by a single distant enhancer (Lettice et al., 2003), whereas others, such as Hox genes and *Fgf8*, are controlled by multiple enhancers (Marinic et al., 2013; Montavon et al., 2011). Several enhancers, located several hundred kilobases up- or downstream of the gene, can loop simultaneously to the promoter region to regulate expression. Studies using Hi-C, a technique that allows the identification of all active chromatin interactions on a genomic scale (Lieberman-Aiden et al., 2009), have shown that these multiple interactions occur coordinately within what is called a topological chromatin domain (see text and Fig. 4; Dixon et al., 2012).

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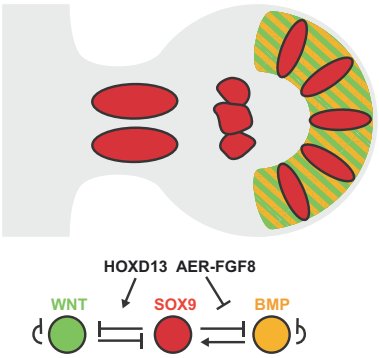


**Fig. 3. Data-driven simulations of digit patterning.** During hand plate development, the formation of digit ray primordia involves condensation of SOX9-positive digit progenitors (red), WNT signal transduction in the interdigit mesenchyme (green stripes) and BMP signalling from the interdigit (orange stripes) to the digit progenitors. WNT signal transduction inhibits *Sox9* expression in the prospective interdigit mesenchyme, and BMPs are expressed by the interdigit mesenchyme and signal to upregulate the expression of *Sox9* specifically in the digit-forming mesenchyme. Simulations of the resulting three-node Turing system can explain the periodic nature of the SOX9-positive digit ray primordia. Simulations and genetic analysis also reveal the important modulatory role of distal HOX transcription factors and AER-FGF signalling.

2000). However, these studies were done in micromass cultures, which are simplistic systems for studying chondrogenic differentiation *in vitro* but not spatial patterning *in vivo*. More recently, Turing network simulations were taken much further and have been used to model the results of genetic and experimental manipulation of digit patterning (Raspopovic et al., 2014; Sheth et al., 2012). These studies addressed whether the repeated pattern of digit/non-digit mesenchymal progenitors could be generated by a Turing-type mechanism. Previous studies had established that the limbs of mice lacking the transcriptional regulator *Gli3* are polydactylous (Hui and Joyner, 1993; Schimmang et al., 1992). Interestingly, increasing genetic reduction of distal Hox genes in the context of *Gli3*-deficient limb buds results in progressively more severe polydactyly with digits becoming thinner and more densely packed (Sheth et al., 2012). One possible explanation for this striking progression of digit phenotypes was that an (unknown) Turing-based digit patterning system control point was disrupted by changing the Hox gene dosage in the context of the *Gli3*-deficiency. This in turn would alter the 'tuning' of the wavelength and thereby digit periodicity and width. This proposal was indeed substantiated by data-driven model simulations, which provided evidence in favour of an intrinsically self-organising Turing-type mechanism operating during digit patterning. These simulations proposed that the wavelength (digit width) is modulated by distal Hox genes and AER-FGFs (Fig. 3; Sheth et al., 2012). Although this study provided evidence in favour of a Turing-type system, the nature of the two postulated Turing signals was not addressed. By combining fluorescence-activated cell sorting (FACS) and transcriptome analysis of mouse limb bud mesenchymal cells with experimental manipulation and model simulations, Raspopovic and colleagues (2014) provided convincing evidence for both the BMP and WNT signalling pathways being integral parts of a Turing-type digit patterning system (Fig. 3). In addition, they showed that the transcriptional regulator SOX9 is an integral part of what appears to be a three-node Turing system (Fig. 3). The dynamic and periodic expression of *Sox9* in the autopod territory is positively regulated by BMPs and marks the positions of the future digits. BMP ligands, which are expressed in the interdigit mesenchyme, signal to the SOX9-positive digit progenitors. By contrast, WNT signal transduction in the interdigit mesenchyme inhibits *Sox9* expression and thereby the formation of digit primordia (i.e. is out of phase with SOX9; Raspopovic et al., 2014).

Other data-driven simulations have been used to evaluate different aspects of limb patterning. A study by Uzkudun et al. (2015) simulated the progress zone and two-signal models for PD patterning and performed virtual perturbations that mimic real experimental manipulations. These simulations led the authors to conclude that the model best explaining the experimentally observed expression patterns of proximal and distal markers is a so-called 'cross-over model' whereby expression of a distal marker is controlled by proximal signals while proximal markers are controlled by distal signals. This cross-over model incorporates the timing component of the progress zone model and the morphogen gradient component of the two-signal model (Uzkudun et al., 2015). Given the significant predictive power of these data-driven models, it will be fascinating to simulate the spatiotemporal kinetics of complex gene networks during progression of limb bud development and explore their potential for *in silico* genetics (Green and Sharpe, 2015; Iber and Zeller, 2012).

## Molecular insights into the fin-to-limb transition

The origin of tetrapod limbs is of considerable fascination, as it enabled originally aquatic vertebrates to colonise land. The fossil record shows that fins evolved progressively more distal structures from ~400 million years ago. The first ancestral tetrapods had up to eight digit-like structures (Coates and Clack, 1990). That tetrapod limbs evolved from fish paired fins is evident as both develop from morphologically similar embryonic buds and exhibit striking molecular similarities (Davis et al., 2007; Sordino et al., 1995). However, one major difference between the fin and limb buds concerns the fate of the AER. During fin bud development in paired-fin fishes, an AER develops and promotes cell proliferation and the outgrowth of mesenchyme, which differentiates into endoskeletal elements as seen in limb development (Grandel and Schulte-Merker, 1998). In contrast to the AER in tetrapods, which is maintained during limb bud outgrowth, the AER in fish fin buds differentiates into an extended structure, the apical ectoderm fold (AEF), which lifts off the mesenchyme at an early stage and elongates to give rise to the dermoskeleton (Thorogood, 1991). Studies have shown that the repeated experimental removal of the AEF from zebrafish fin buds results in increased mesenchymal cell proliferation and excessive elongation of the mesenchyme, suggesting that prolonged exposure to AER signals induces distal elongation of mesenchyme and the endoskeleton (Yano et al., 2012). By contrast, overexpression of the distal *hoxd13a* gene in fin buds reduces the size of the AEF and distal expansion of chondrogenic tissue (Freitas et al., 2012). Two rows of rigid fibrils called actinotrichia keep the AEF straight, and the knockdown of two genes essential for their formation, *actinodin1* and *-2* (*and1/2*), results in a defective AEF (Zhang et al., 2010a). The loss of *and1/2* also causes expansion of the *hoxd13a* and *shh* expression domains and a significant reduction in *gli3* expression. Although actinodin genes are present in teleosts and in elephant sharks they are absent from extant tetrapods, suggesting that AEF impairment and/or the inactivation of actinodin genes may have been important during the fin-to-limb transition. This analysis suggests that the polydactylous fossils of early aquatic tetrapods could have formed by extending the expression of genes such as *Shh* and Hox genes as a consequence of losing of a functional AEF

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(Zhang et al., 2010a). A comparison of the expression patterns of genes in the regulatory networks that pattern fin and limb buds should provide further insight into the changes underlying the fin-to-limb transition.

A recent study by Onimaru et al. (2015) shows that during AP pre-patterning, *Gli3* is not repressed in the posterior mesenchyme in fin buds of catsharks, in contrast to mouse limb buds, despite the fact that *Hand2* is expressed posteriorly in fin buds. In this study, it was shown that the catshark *Gli3* enhancer active in fin buds lacks the elements normally responding to repressor activity, which underlies the observed posterior mesenchymal *Gli3* expression. Comparative analysis of the genome-wide spectrum of fin/limb bud enhancers will be necessary to understand how these enhancers evolved to control the spatiotemporal gene expression that is observed in the fin buds and limb buds of extant species.

It should be noted, however, that the fin skeletons of extant fish and the limbs of modern tetrapods are very different, making the identification of true homologies difficult. For example, the endoskeleton of teleost pectoral fins is composed of long proximal radials, followed by smaller distal radials. Lobe-finned fish, which share a common ancestor with tetrapods, have a similar but more elaborate endoskeleton (Fig. 4A,B; Thorogood, 1991). Although palaeontological data suggest progressive evolution of the bony elements of fins towards limb skeletal elements, it has proven difficult to determine by molecular analysis whether the tetrapod distal limb skeletal structures (carpals and digits) are homologous to or derived from teleost radials, or whether they arose *de novo*. Several of the seven distal limb enhancers controlling expression of the *Hoxd* gene cluster in tetrapod limb buds (Montavon et al., 2011) have been identified in teleosts (Amemiya et al., 2013; Gehrke et al., 2015; Schneider et al., 2011). This points to their evolutionary ancient nature, whereas the others could have arisen *de novo* in tetrapods. However, the genomes of living teleosts vary considerably, and sequence comparisons alone are not sufficient to evaluate the extent to which enhancers are conserved between fish and tetrapods. To overcome this, the chromatin topologies of the zebrafish *HoxA* and *HoxD* gene clusters have recently been analysed (Woltering et al., 2014). This study revealed that the zebrafish Hox clusters exhibit a bimodal topology strikingly similar to that in mice (Fig. 4C), which points to an ancient origin of the Hox chromatin topology that controls distal limb and digit development in tetrapods. To assess the extent to which a teleost Hox gene cluster is functional in mouse limb buds, the puffer fish *Hoxd* and *Hoxa* clusters were expressed in transgenic mice. The teleost distal Hox genes are indeed expressed in mouse limb buds, but their expression failed to extend into the forming hand plate. These data led Woltering et al. (2014) to conclude that the distal radials of teleost fish are not the homologues of tetrapod digits (Fig. 4).

Gehrke et al. (2015) reached a different conclusion by investigating Hox gene regulation in the spotted gar, which belongs to a lineage that diverged from teleosts such as zebrafish and puffer fish before the teleost genome was duplicated (Braasch et al., 2014). This study identifies the potentially orthologous distal enhancers for both Hox clusters, and these spotted gar distal enhancers are able to drive the expression of a reporter well into the hand plates of transgenic mouse embryos. In addition, an orthologous distal enhancer from the coelacanth, which is more closely related to tetrapods than to teleosts, is also partially active in hand plates of transgenic mouse embryos (Amemiya et al., 2013). The study by Gehrke and colleagues points to a potentially ancient origin of what might have become the digit enhancer in tetrapods

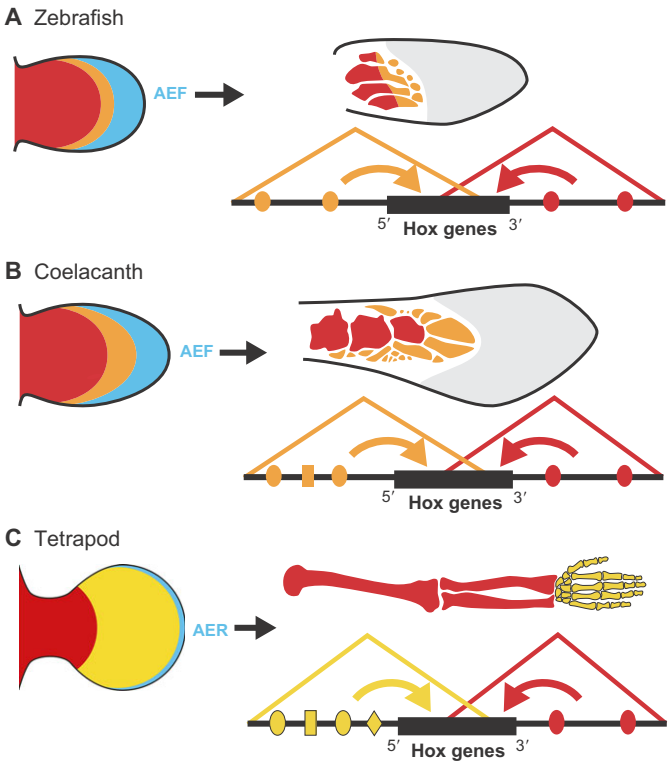


**Fig. 4. Enhancers and topological chromatin domains controlling *HoxD* locus expression are conserved between teleost fishes and tetrapods.** (A) Representation of a teleost (zebrafish) pectoral fin bud, highlighting its mesenchyme (red for proximal, orange for distal) and apical ectodermal fold (AEF, blue) and the formation of fin radials (red for proximal, orange for distal) and the dermoskeleton (grey). The *HoxD* gene cluster regulating fin development is schematically shown. The 5′ and 3′ *cis*-regulatory domains, which harbour enhancers that are conserved between teleosts and tetrapods, are shown. The distal (5′ orange disks) and proximal (3′ red disks) enhancers are located within the C-DOM (orange pyramid) and T-DOM (red pyramid) topological domains that correspond to early and late phase transcriptional enhancing activities, respectively. (B) Hypothetical representation of a coelacanth pectoral fin bud, showing its proximal mesenchyme (red), enlarged distal mesenchyme (orange) and AEF (blue), and its fin with more elaborate fin radials. The bi-modal chromatin topology of the *HoxD* cluster is conserved, although additional enhancers (orange squares) that may control the distal expansion of Hox expression (as compared with teleosts) are also detected in the coelacanth's genome. (C) Scheme of a tetrapod limb bud at the hand plate stage, showing the proximal mesenchyme (red, giving rise to stylopod and zeugopod), digit territory (yellow) and AER (blue). A tetrapod forelimb skeleton with its long bones (red) and carpals and digits (yellow) is also shown. The tetrapod *Hoxd* cluster has a chromatin architecture similar to that found in teleosts (A). The characteristic bi-model chromatin topology contains both conserved and novel distal enhancers (yellow rhomboids). It is debatable whether the distal radials of teleosts (orange) and digits of tetrapods (yellow) are homologous structures. Either novel distal enhancers transformed distal radials into digits, or digits were generated *de novo* from more distal mesenchyme (modified from Schneider and Shubin, 2013; Woltering et al., 2014).

and suggests that the so-called late phase, i.e. distal Hox expression domains, in mice and fish could be homologous (Amemiya et al., 2013; Gehrke et al., 2015). Obviously, more molecular analysis using enhancers from different fish lineages and the orthologous enhancers from genes other than Hox genes must be identified in fish and tested in transgenic mouse limb buds in order to understand the extent to which distal fin and limb structures might be orthologous or not and what might be the true evolutionary novelties in the making of tetrapod digits.

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### Digit reductions during tetrapod evolution

The colonisation of land also required adaptation to different habitats, which resulted in diversification of the basic pentadactylous bauplan of tetrapod limbs. Over evolutionary time, tetrapod limbs were adapted to a multitude of specialised uses, such as running, digging, swimming, flying and grasping. In extant tetrapods, the number of digits varies from predominantly five (e.g. humans) to one (e.g. horses). Recently, progress has been made in understanding the molecular alterations underlying digit reductions in different tetrapod species and clades. For example, digit reductions and loss have been studied in evolutionarily closely related lizards by comparing *Shh* expression in the embryonic limb buds of skinks with two to five digits (Shapiro et al., 2003). This analysis showed that the temporal window of *Shh* expression is progressively shorter when comparing limb bud development in five-toed skinks with that in skinks with fewer toes (Fig. 5A). By contrast, no changes in the kinetics of *Shh* expression (or the expression of other select posterior genes) are observed in the limb buds of birds and crocodiles that also lost digits during evolution (de Bakker et al., 2013).

Recently, the molecular mechanisms underlying the digit reductions and loss observed in ungulates, such as pigs (which possess four toes), cows and camels (two toes), horses (one toe) and the three-toed jerboa, have been analysed (Cooper et al., 2014). Analysis of key digit patterning genes such as *Hoxd13*, and of *Shh* and its transcriptional targets *Ptch1* and *Gli1*, showed that their expression was not significantly altered in age-matched limb buds of camel, horse and jerboa embryos in comparison to that in their mouse counterparts (Cooper et al., 2014). Only in pig limb buds, the transcriptional up-regulation of the SHH targets *Ptch1* and *Gli1* was altered, similar to bovine limb buds (see below; Lopez-Rios et al., 2014) This suggests that, at least in camel, horse and jerboa limb buds, digit loss cannot be attributed to a simple patterning defect. Another mechanism that controls digit numbers is inter-digital apoptosis, which occurs during post-patterning and is controlled by BMP signalling. Indeed, inter-digital apoptosis is increased and the regions with active BMP signalling expanded in the autopods of camel, horse and jerboa limb buds in comparison to the mouse (Cooper et al., 2014).

What, then, might explain the digit loss observed in pigs? Pigs are artiodactyls, i.e. they belong to even-toed ungulates, which also include bovine species. A recent in-depth molecular analysis of bovine limb bud development showed that the failure to upregulate *Ptch1* expression in the limb bud mesenchyme disrupts the sensing of SHH signalling, which is paralleled by a striking molecular loss of AP asymmetry (Lopez-Rios et al., 2014). In fact, the specific inactivation of *Ptch1* in the mouse limb bud mesenchyme phenocopies the molecular loss of asymmetry and digit reductions. Lopez-Rios and colleagues used 4C-seq analyses to identify a limb regulatory module (LRM) that controls the upregulation of *Ptch1* during mouse limb bud outgrowth and hand plate formation. The activity of the mouse LRM and the orthologous bovine LRM was assessed in transgenic mice, revealing that the activity of the bovine LRM had degenerated to low levels. This striking change provides a straightforward molecular explanation for the failure to upregulate *Ptch1* expression in the mesenchyme of

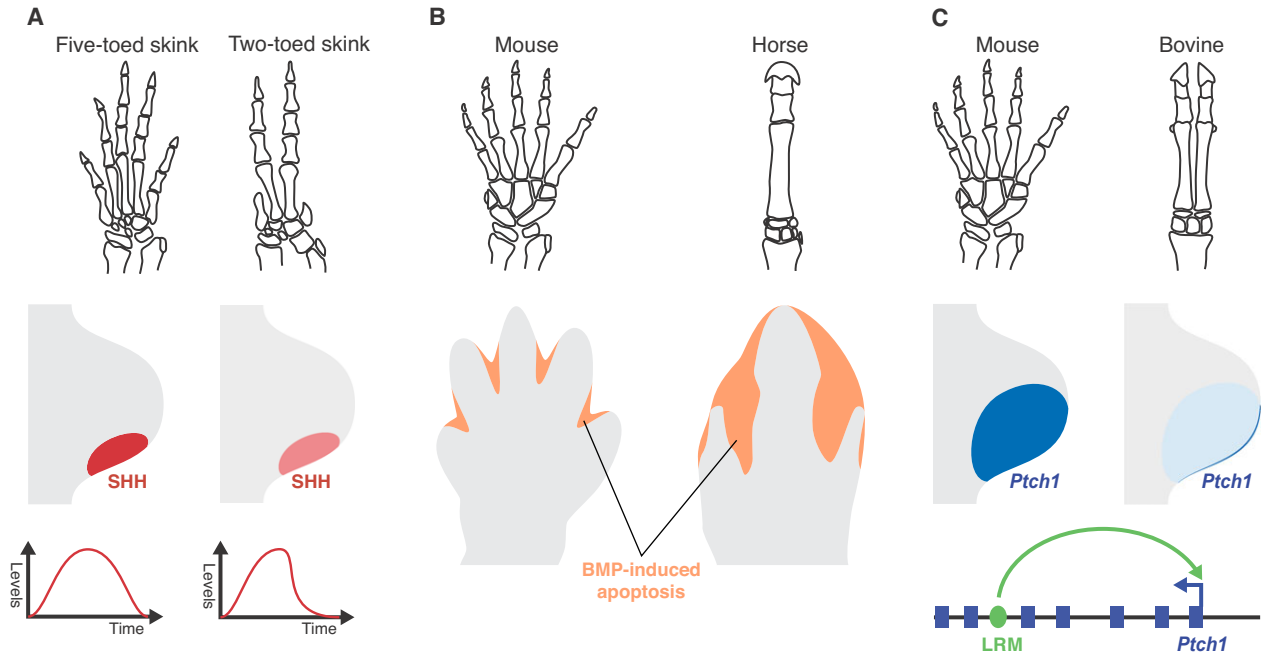


**Fig. 5. Different modes of digit reduction in tetrapods.** Digit reduction has been suggested to occur using the following different modes: (A) modulation of *Shh* expression; (B) modulation of inter-digital apoptosis; or (C) modulation of *Ptch1* expression. (A) The skink family *Hemiergis* encompasses closely related species that have maintained between two and five toes. Upper panel: Scheme of the forefoot skeletons of *Hemiergis initialis* (a five-toed skink; left) and *Hemiergis quadrilineata* (a two-toed skink; right). Lower panel: Molecular analysis shows that *Shh* expression is initially similar, but terminates prematurely in the limb buds of the skinks with reduced digit numbers. (B) Upper panel: Scheme of a *Mus musculus* (mouse; left) and *Equus ferus* (horse; right) forelimb skeleton. Lower panel: the area of BMP-induced apoptosis is significantly increased in the interdigit mesenchyme of horse limb buds in to the mouse. (C) Upper panel: Scheme of a *Mus musculus* (left) and *Bos taurus* (bovine; right) forelimb skeleton. Lower panel: *Ptch1* expression in the mesenchyme of bovine limb buds (right) is much reduced in comparison to its mouse counterpart (left). A limb bud-specific *cis*-regulatory module (LRM) controls the transcriptional upregulation of *Ptch1* in the distal mesenchyme of mouse limb buds. By contrast, the bovine LRM has functionally degenerated and is unable to upregulate expression in the distal limb bud mesenchyme. The failure to upregulate mesenchymal *Ptch1* expression also underlies the early loss of AP asymmetry in the limb buds of bovine and pig embryos, which are both artiodactyls.

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bovine and pig limb buds and the resulting inability to properly sense the morphogenetic SHH signal (Lopez-Rios et al., 2014). The fossil record suggests that the loss of asymmetry probably preceded the loss of digits in artiodactyls, as some of the early artiodactyls retained pentadactyly while having lost asymmetry of the middle digits, which is a defining feature of the paraxonic limb skeleton of extant artiodactyls (for more details, see Lopez-Rios et al., 2014).

In summary, at least three distinct molecular mechanisms underlie alterations in digit patterning and reductions in extant tetrapods. Two of these directly alter SHH signalling, either by shortening morphogenetic signalling (Fig. 5A) or by interfering with the sensing of SHH signalling (Fig. 5C), with both resulting in digit patterning defects. By contrast, the third mechanism involves a post-patterning defect that eliminates digit progenitors by BMP-induced apoptosis (Fig. 5B).

## Conclusions

Since the properties of the AER were discovered by John Saunders in the middle of the last century, studies of limb development have constantly been at the forefront of providing insight into the principles that govern vertebrate organogenesis. Indeed, the ongoing characterisation of the gene regulatory networks that control limb development continues to further our deep understanding of the complex processes that govern organ development and the causal alterations resulting in congenital malformations and disease. The knowledge gained from these studies is also relevant to regenerative medicine and tissue engineering, in particular to the generation of cartilage and bone from stem and progenitor cells.

The research described here represents only part of the very diverse research and comprehensive analysis of fish fin and tetrapod limb development; I have chosen to highlight only the new and exciting directions. Nevertheless, it is clear that more challenges and hurdles lie ahead if we are to arrive at a comprehensive understanding of limb bud organogenesis. For example, real-time live imaging of chicken limb buds represents a great advance to gain better insight into the cellular changes occurring during the progression of limb bud morphogenesis. However, as the chicken is not a genetically amenable model, these live-imaging techniques must be adapted to mouse limb buds in culture. Furthermore, whereas many of the network regulatory interactions had been previously based on gene expression patterns and genetic interactions, we now have the biochemical tools at hand to identify the range of direct transcriptional targets and interacting proteins regulated by these network interactions. One major challenge is to assign the often very distant enhancers to their target genes, but recent advances in techniques that allow the study of the kinetics of epigenetic modifications and chromatin architecture make this possible. As the expression of genes is often controlled by multiple enhancers scattered in large landscapes with changing activities over time, analysis of the epigenetic mechanisms that regulate promoter-enhancer interactions is key for understanding gene regulation during limb bud development. The next generation genomic tools available will also allow the identification of enhancers in non-model organisms, and new genome-editing tools may make some of these amenable to genetic manipulation. This will facilitate the analysis of how such enhancers might have evolved during tetrapod limb evolution and diversification. It should be noted, however, that these 'omic'-based studies are creating ever-expanding datasets and gene networks that render the understanding of increasingly complex processes difficult. We thus need more elaborate mathematical modelling, simulations and bioinformatics tools if we want to grasp the cellular and molecular complexity that underpins the formation of the versatile structure that has enabled me to write this text.

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The author declares no competing or financial interests.

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