Limb development: a paradigm of gene regulation

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Abstract | The limb is a commonly used model system for developmental biology. Given the need for precise control of complex signalling pathways to achieve proper patterning, the limb is also becoming a model system for gene regulation studies. Recent developments in genomic technologies have enabled the genome-wide identification of regulatory elements that control limb development, yielding insights into the determination of limb morphology and forelimb versus hindlimb identity. The modulation of regulatory interactions — for example, through the modification of regulatory sequences or chromatin architecture — can lead to morphological evolution, acquired regeneration capacity or limb malformations in diverse species, including humans.

Promoters
Cis-regulatory DNA sequences immediately upstream of transcription start sites at which RNA polymerases and transcription factors bind to initiate gene transcription.

The limb is a complexly patterned, easily observable and experimentally modifiable organ, leading it to be widely used as a model in developmental biology. Although the signalling pathways involved in limb development are largely conserved, wide morphological differences are observed among species, revealing the crucial role of gene regulation in limb patterning.

The limb originates from the lateral plate mesoderm and develops along three major axes, each of which is controlled by a different signalling centre[1,2]. The proximal–distal axis (running from the shoulder to fingers) is under the control of the apical ectodermal ridge (AER). The AER keeps the underlying mesenchyme in a proliferative state, allowing the limb to grow. The anterior–posterior axis (running from digit I to V) is specified by the zone of polarizing activity (ZPA) signalling centre, which is governed by the morphogen sonic hedgehog (SHH). Finally, the dorsal–ventral axis (running from the back of the hand to the palm) is thought to be regulated by WNT family member 7A (WNT7A) signalling in the overlying ectoderm (FIG. 1). The coordination of these developmental axes is crucial for proper limb development. This coordination is established by numerous genes that are under the control of regulatory elements such as promoters, enhancers, silencers and insulators. Enhancers, the focus of our Review, instruct gene promoters when, where and at what levels to activate gene transcription (BOX 1).

With recent advancements in genomics, limb development genes and their regulatory elements can now be identified in a genome-wide manner. Chromatin immunoprecipitation followed by massively parallel sequencing (ChIP–seq) enables the identification of specific transcription factors, cofactors and histone marks on a genome-wide scale. Using this technology, thousands of enhancers that are associated with limb development have been mapped in humans[3], monkeys[3], bats[4] and mice[3,5–8], including putative enhancers in the aforementioned AER and ZPA signalling centres[9]. ChIP can also be used to capture regulatory elements and their target promoters through chromatin interaction analysis with paired-end tagging (ChIA–PET). For example, one study using an antibody for a cohesin subunit that is involved in establishing tissue-specific DNA loops identified more than 2,000 interactions in the developing limb, many of which were enhancer–promoter interactions[10]. To match ChIP-identified regulatory elements to actively transcribed RNAs in a genome-wide fashion, RNA sequencing (RNA-seq) has been used to identify numerous genes and genetic pathways that drive limb development at various time points in humans[3], mice[8,11,12], bats[4,12,13] and other mammals[12].

Together, studies using sequencing techniques have 'sketched out' in a genome-wide manner the major building blocks (that is, genes and their regulatory components) that control limb development. In this Review, we show how playing with these blocks can lead to morphological differences between forelimb and hindlimb, morphological evolution or acquired regeneration capacity in diverse species. We also discuss how the disruption of these building blocks and their interactions can lead to limb malformations.

The forelimb–hindlimb enigma

How limb-type identity and morphology are determined remains an important biological enigma that has yet to be fully resolved. In this section, we introduce the main

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Enhancers
Cis-regulatory DNA sequences that, when bound by specific transcription factors, enhance the transcription of an associated gene. Enhancers can be located upstream or downstream of the gene, and at variable distances.

Silencers
Cis-regulatory DNA sequences that, when bound by specific transcription factors, repress the transcription of an associated gene. Silencers can be located upstream or downstream of the gene, and at variable distances.

Insulators
DNA sequence elements that protect genes from inappropriate regulatory signals emanating from their surrounding environment. Insulators act like barriers, blocking the effect of a regulatory element on a promoter, or preventing the advance of chromatin condensation.

ChIA–PET
A method that combines chromatin conformation capture and DNA paired-end high-throughput sequencing to analyse chromatin interaction (ChIA) across the genome.

RNA-seq
A technique that combines high-throughput sequencing of cDNA molecules obtained by reverse transcription within a biological sample to determine the sequence and relative abundance of each RNA molecule.

Topologically associating domains
(TADs). Conserved megabase-sized sub-orders of chromosome organization, that are delineated by boundaries enriched in architectural proteins (CCCTC-binding factor and cohesin). Within a TAD, chromatin interactions occur at a high frequency and allow enhancer–promoter contacts.

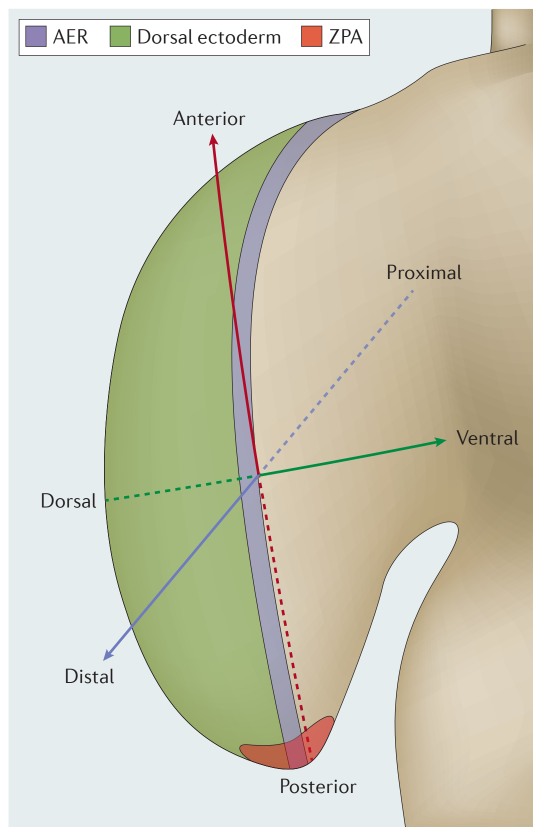

Figure 1 | **Overview of axes involved in limb development.** In vertebrates, limb development is orchestrated along three different axes. The proximal–distal axis (from the shoulder to fingers), controlled by the apical ectodermal ridge (AER) signalling centre located at the dorsoventral frontier of the limb bud, is responsible for the proximodistal outgrowth and differentiation of the limb. Fibroblast growth factor signalling is the major pathway involved in the AER. The anterior–posterior axis (from digits I to V) marked by the zone of polarizing activity (ZPA), located in the posterior mesenchyme, is responsible for the anteroposterior polarization and is controlled by sonic hedgehog signalling. The dorsal–ventral (from the back of the hand to the palm) axis is thought to be controlled primarily by WNT signalling. The signalling centres within these three axes have complex interconnections and fine-tune gene expression to achieve proper limb development.

factors known to play a part in these developmental processes, focusing on what is currently known about their regulation.

Early limb specification. Forelimbs and hindlimbs emerge from the lateral plate mesoderm that runs along the flank of the embryo. Both limbs initially form as buds composed of undifferentiated mesenchymal cells covered by a layer of ectoderm. Although forelimbs and hindlimbs appear morphologically indistinct during their initial formation, their identity has already been determined. Molecular evidence for the early determination of limb identity comes from the differential expression of two paralogous transcription factors: T-box 5 (TBX5) and TBX4 (REF. 14). *Tbx5* and *Tbx4* are expressed in the early prospective mesenchyme of the forelimb and the hindlimb, respectively. Changes in *Tbx4* and *Tbx5* expression are hypothesized to have contributed to the acquisition of paired appendages during vertebrate evolution[15]. During limb bud initiation, both genes trigger fibroblast growth factor 10 (*Fgf10*) expression in the limb mesenchyme[16–19]. FGF10 signalling then induces *Fgf8* expression in the ectoderm in a positive regulatory loop[20–22]. The establishment of this epithelial–mesenchymal feedback loop is necessary to maintain the proliferation of mesenchymal cells and thereby limb outgrowth. In *Fgf10*-deficient mice, limb bud formation is initiated but no further limb growth is observed, resulting in severe limb truncations with only rudimentary scapulae and pelves remaining[21]. *Tbx5* inactivation in mice abolishes *Fgf10* expression and forelimb skeletal formation[17,23]. Similarly, *tbx5* knockdown in zebrafish results in a failure to initiate pectoral fin bud formation[24], while *tbx4* mutation leads to pelvic fin loss[25]. However, although *Tbx4* knockout in mouse also disrupts hindlimb development, a small hindlimb is formed owing to the retention of low FGF10 levels[26]. Mid-gestation lethality has rendered detailed studies of *Tbx4*-knockout mice difficult; however, the loss of *Tbx4* in mice seems to be partially compensated for by other factors, including paired-like homeodomain transcription factor 1 (PITX1). Similarly to *Tbx4*, *Pitx1* is expressed preferentially in the hindlimb mesenchyme[27].

The determination of limb identity in tetrapods depends on the rostrocaudal positions of *Tbx4*, *Tbx5* and *Pitx1* expression, which in turn are influenced by the expression patterns of the architectural homeobox (Hox) family of genes (FIG. 2). Hox genes are characterized by their clustered organization in the genome and the spatiotemporal collinearity of their expression, which require complex transcriptional regulation. Most tetrapods harbour four Hox clusters. The *HoxA* and *HoxD* clusters have major roles during limb development and display similar expression patterns. These clusters harbour a bimodal regulatory landscape, whereby their regulatory elements are contained within two flanking topologically associating domains (TADs) that encompass the adjacent gene deserts (reviewed in REF. 28). A TAD is a genomic region in which chromatin interactions occur at a higher frequency compared with interactions across its boundary[29] (BOX 1). The *HoxA* and *HoxD* regulatory domains appeared during vertebrate evolution and underlie the collinear regulation of Hox genes in land vertebrate appendages[30,31]. At the limb initiation stage, an early wave of *HoxA* and *HoxD* expression takes place in the lateral plate mesoderm, controlled by the telomeric TAD. Hox genes are sequentially activated in a rostrocaudal pattern, which is crucial for the induction of limb growth at a specific position. This process has been primarily demonstrated for forelimb initiation, during which *Tbx5* expression is induced under a rostral Hox expression pattern, leading to the development of a forelimb[32,33]. Similarly, it is hypothesized that under a more caudal Hox expression pattern, *Pitx1* and *Tbx4* are induced, leading to the development of a hindlimb.

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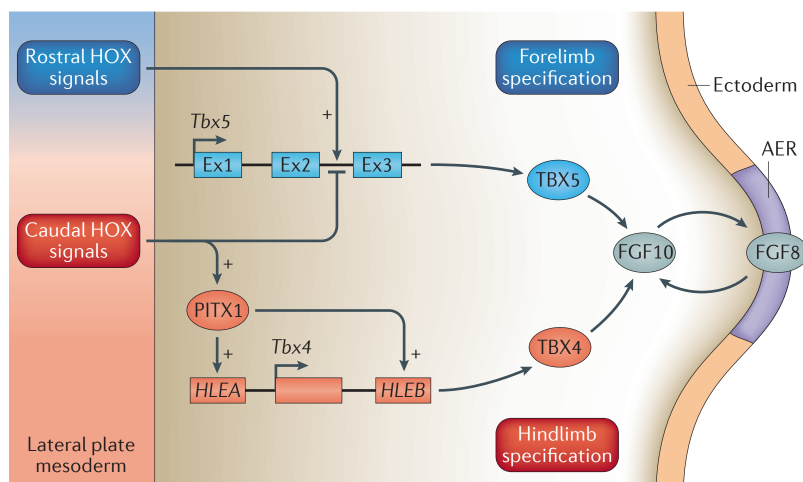

Figure 2 | **Establishment of limb bud identity in mammals.** In the prospective forelimb bud mesenchyme, T-box 5 (*Tbx5*) expression is induced by a combinatorial code of rostral homeobox (HOX) proteins that bind directly to its limb-specific enhancer that is located in intron 2. In the caudal lateral plate mesoderm, paired-like homeodomain transcription factor 1 (*Pitx1*) expression is induced by a combinatorial code of caudal Hox genes while *Tbx5* is repressed. PITX1 directly binds to hindlimb-specific enhancer A (*HLEA*) and *HLEB* to drive the expression of *Tbx4* in the prospective hindlimb bud mesenchyme. Both *Tbx4* and *Tbx5* trigger the expression of fibroblast growth factor 10 (*Fgf10*) in the mesenchyme. After this stage, FGF10 interacts in a positive regulatory loop with FGF8 in the ectoderm, and this reciprocal regulation is crucial for limb bud outgrowth. AER, apical ectodermal ridge; Ex, exon.

To date, two enhancers of *Tbx5* that are specific to the limb or fin have been identified. The first of these enhancers, which is conserved among mammals but is not found in other vertebrates, was identified within *Tbx5* intron 2 and can restrict early *Tbx5* expression to the prospective forelimb bud mesenchyme of mice[32]. The enhancer sequence contains binding sites for HOX proteins, which are direct upstream regulators of *Tbx5*. Through this enhancer, activating Hox signals (rostral *Hox4* and *Hox5* paralogous group genes) and repressing Hox signals (caudal *Hoxc8, Hoxc9 and Hoxc10*) function to restrict *Tbx5* expression to the appropriate rostrocaudal level[32,33] (FIG. 2). The intron 2 enhancer also contains retinoic acid response elements and a T cell factor/lymphoid enhancer factor (TCF/LEF)-binding site that are required for its activity[16]. A second *tbx5* enhancer for the pectoral fin was more recently identified in jawed fishes. This enhancer is located downstream of *tbx5* and is functionally conserved in zebrafish and mice[34]. As the pectoral fin-specific *tbx5* enhancer is non-functional in extant jawless fishes without paired appendages, its activity in jawed vertebrates has been hypothesized to be associated with appendage acquisition[34].

A comparative genomic study identified two limb-specific enhancers of *Tbx4*: hindlimb enhancer A (Hlea) and Hleb[35]. These enhancers seem to act synergistically to generate robust *Tbx4* expression in the hindlimbs. HLEA is present only in mammals, and both homozygous and heterozygous deletions of this enhancer in mice result in altered hindlimbs with smaller bones[35]. HLEB is highly conserved from fish to mammals and is important for hindlimb and phallus development in mice[36]. ChIP–seq experiments have shown that PITX1 binds directly to these two regulatory elements[5] (FIG. 2). A pelvic fin-specific enhancer of *Pitx1* (named *Pel*) has been described in three-spined stickleback fishes[37]. *Pel* is located in a fragile region of the genome that is susceptible to double-stranded DNA breaks, and several natural populations of stickleback fish lack pelvic structures owing to deletions encompassing this enhancer (FIG. 3). *Pitx1* regulatory elements have yet to be identified in mammals, but there is evidence to suggest that mammalian *Pitx1* is regulated by the caudal Hox family of genes (FIG. 2). In the hindlimb, HOXC9 acts as a repressor of *Tbx5* but as a transcriptional activator of *Pitx1*. Experimental misexpression of *Hoxc9* in the lateral plate mesoderm at the forelimb level induces the ectopic expression of *Pitx1* (REF. 33). Conversely, it is tempting to hypothesize that the rostral Hox gene products (*Hox4* and *Hox5* paralogous group genes) act as repressors of *Pitx1* in the forelimb; however, this hypothesis has yet to be tested.

Building a leg-like arm or vice versa. Animal and disease model studies have shown that playing with the developmental building blocks involved in limb-type identity and morphology can lead to the transformation of an arm into a leg or vice versa. Misexpression experiments involving the viral injection of Tbx transcription factors into chicks showed that ectopic expression of *Tbx5* in the hindlimb results in the conversion of a leg to a wing-like structure[38,39]. Conversely, *Tbx4* injection in the forelimb leads to the formation of a leg-like forelimb[38,39]. However, it was subsequently demonstrated that the Tbx transcription factors are not responsible for limb type-specific morphology in mice, as *Tbx4* can rescue its counterpart during forelimb development in *Tbx5*-knockout mice[19]. Therefore, Tbx genes are considered 'markers' of limb-type identity in mammals rather than primary identity generators.

Pitx1 is the only gene that has been clearly implicated in limb-type morphology determination across multiple species[39–42]. The hindlimbs of *Pitx1*-knockout mice are reduced in size and exhibit a loss of hindlimb structures such as the patella and a gain of forelimb structures such as a pisiform-like tarsal bone[43,44] (FIG. 3). Although the limb outgrowth defects of *Pitx1*-knockout mice are rescued by *Tbx4* or *Tbx5*, the morphological changes in the hindlimb are not. This result is consistent with the hypothesis that PITX1 determines hindlimb morphology independently from TBX4 (REF. 45). Conversely, ectopic expression of *Pitx1* in the developing forelimb of mouse or chick embryos (FIG. 3) induces *Tbx4* expression and results in a shift towards hindlimb morphology[40,41]. In humans, variable chromosomal rearrangements near *PITX1* cause Liebenberg syndrome (Online Mendelian Inheritance in Man (OMIM) 186550), in which patients exhibit arm malformations resulting in a leg-like morphology[46–48] (FIG. 3). In some patients, the chromosomal abnormalities lead to the mislocalization of an enhancer with upper-limb activity, which is thought to result in ectopic *PITX1* expression[47]. In pigeon breeds with large foot feathers (muffed breeds), *Pitx1* expression is reduced in the leg whereas *Tbx5* is ectopically expressed,

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Box 1 | **Enhancing transcription**

Enhancers are defined as gene regulatory DNA sequences that, when bound by specific transcription factors, enhance the transcription of an associated gene by activating its promoter. Enhancers are typically a few hundred base-pairs long and can be located upstream or downstream of their target gene or even on another chromosome. Most enhancers lie in non-coding DNA, but protein-coding DNA can also have enhancer activity (exonic enhancers (eExons))[115]. Enhancers are thought to influence promoter activity through direct or indirect physical interactions in 3D space. The spatial organization of chromatin has a crucial role in this process (reviewed in REF. 110). With the exploration of chromatin structure through diverse high-resolution microscopy and chromatin conformation capture technologies, several organizational levels at the chromosomal and sub-chromosomal scale have been discovered.

During interphase, each chromosome occupies a specific nuclear space known as a chromosome territory. Within these territories, the inactive chromatin is associated with the nuclear lamina and harbours repressive histone marks, whereas the active chromatin tends to localize towards the interior of the nucleus and interacts with RNA polymerase II foci in transcription factories. At the megabase level, the chromatin is functionally divided into regions of high regulatory interaction frequency called topologically associating domains (TADs), flanked by low-interaction regions called TAD boundaries that are enriched for CCCTC-binding factor (CTCF) binding. TADs define units where most of the enhancer–promoter contacts occur, facilitated by a limited search space within the nucleus. These functional chromatin regions are conserved among species, tissues and cell lines[29]. At the sub-TAD level, insulator elements, bound by architectural factors such as CTCF and cohesin, act as barriers and facilitate the organization of minor chromatin loops that isolate active genes from neighbouring inactive regions. The local chromatin composition may also affect enhancer–promoter interactions, both physically and functionally.

Enhancers can be recognized by specific histone marks. For example, poised enhancers tend to have a monomethylated lysine 4 on histone 3 (H3K4me1) mark, whereas active enhancers can be recognized by an acetylated lysine 27 on histone 3 (H3K27ac) mark. By contrast, inactive enhancers harbour repressive histone marks[116], such as H3K9 methylation. In addition to the spatial organization and modification of the chromatin, the specificity of enhancer–promoter contacts involves the binding of specific transcription factors, RNA polymerase II and other cofactors[117]. Transcription factors that bind to gene regulatory elements determine enhancer–promoter specificity by providing biochemical compatibility to the interacting loci[118]. EA1-binding protein p300 (EP300) and CREB-binding protein (CBP) are transcriptional co-activators that represent another signature of active enhancers[6]. EP300 and CBP mediate transcriptional activation by recruiting RNA polymerase II[119]. Some active enhancers undergo bidirectional transcription by RNA polymerase II, producing short enhancer RNAs (eRNAs). The level of eRNA transcription tends to correlate with mRNA synthesis at nearby genes, suggesting that eRNAs might be involved in enhancer activity, possibly by stabilizing enhancer–promoter contacts[120].

leading to a wing-like phenotype with musculoskeletal re-patterning[49] (FIG. 3). These changes in expression are related to *cis*-regulatory divergence among breeds. Although there is still much to learn about the downstream factors and the regulatory elements involved in limb specification, it is becoming evident that these elements play a major part in morphological changes among species and can also be disease-causing.

Evolution of vertebrate appendages

Limb evolution can be described as a variation on the theme of limb development. All limbed tetrapods possess three limb segments: the stylopod (upper arm), the zeugopod (forearm) and the autopod (hand). Although these segments are present across species, the number of skeletal elements within these segments and the proportions of these elements vary. The evolution of the tetrapod limb from the fish fin is a classic example of evolutionary transformation. However, although the homology of the stylopod and zeugopod of modern tetrapods and ancestral sarcopterygian fishes is generally accepted, the homology of the tetrapod autopod and the more distal structures of the fish fin remains more controversial.

Evidence from studies of Hox gene regulation and expression has played a central part in this debate, and has been used to support hypotheses of both the novelty and ancestral homology of the tetrapod autopod (BOX 2). Through evolutionary variations in skeletal element number and proportion, tetrapods have achieved remarkable diversity in limb forms that is closely associated with the diverse locomotion, feeding and other behaviours of this group. As examples, the bat arm has been modified into a wing that enables powered flight, and the aye-aye lemur has a thin and elongated III finger that it uses to find and remove larvae from cavities in wood. The developmental basis of limb evolution has been studied, to varying degrees, in a limited but growing number of non-model tetrapods, including bats[4,13,50–55], pigs[56,57], horses[56], jerboas[56,58], cattle[59], camels[56], whales[60], opossums[61,62], wallabies[63], anoles[64], snakes[36,65] and skinks[66]. The lengths of limb skeletal elements vary considerably among these species. For example, bats have greatly elongated III, IV and V metacarpals and manual digits, whereas jerboas have elongated metatarsals and reduced lateral digits. Cattle and camels have elongated metapodials (metacarpals and metatarsals) and reduced lateral digits. Anoles have also repeatedly experienced adaptive radiations in limb length. Species that forage primarily on the ground and on the trunks of trees (trunk-ground anoles) have relatively longer limbs than do those that forage on the trunks and crowns of trees (trunk-crown anoles)[64]. The specific sequence changes that underlie most of these evolutionary modifications remain largely unknown. However, in a few cases, researchers have linked evolutionary changes in limb form to the divergence of regulatory elements. In this section, we discuss how modifications in regulatory elements and gene interactions have contributed to limb loss in snakes and morphological diversification of the limb skeleton in cattle and bats.

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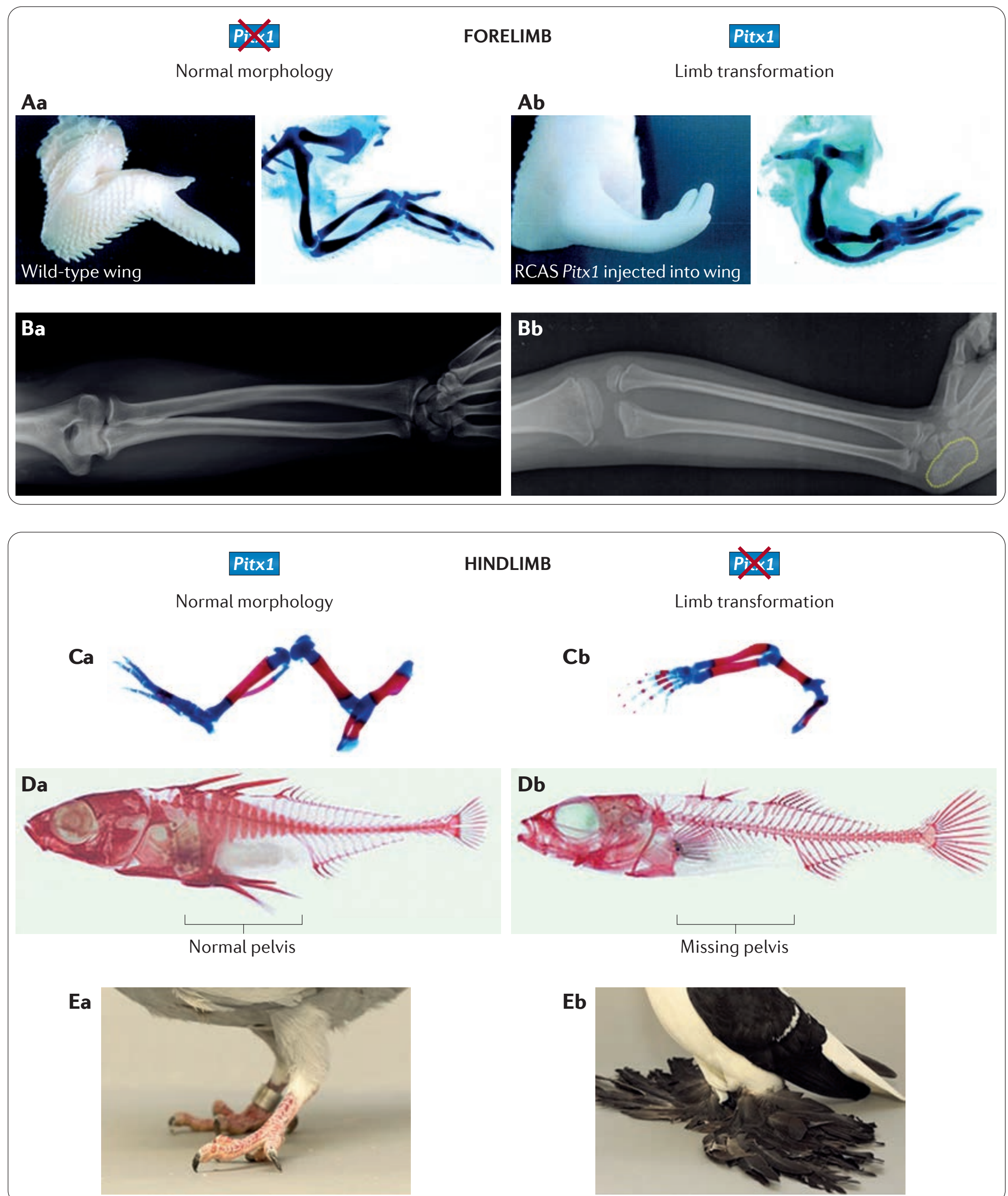

Figure 3 | ***Pitx1* disruption phenotypes.** Paired-like homeodomain transcription factor 1 (*Pitx1*) is responsible for hindlimb-type morphology and is usually only expressed in the hindlimb. The left panels show the normal morphology of forelimbs (panels **Aa, Ba**) and hindlimbs (panels **Ca, Da, Ea**) in diverse species. Ectopic expression in the forelimb (panels **Ab, Bb**) or loss of expression in the hindlimb (panels **Cb, Db, Eb**) is responsible for homeotic limb transformation phenotypes. **A** | Wing-to-leg transformation induced by ectopic *Pitx1* expression in the chick forelimb using replication-competent avian retroviruses (RCAS)[44]. **B** | In Liebenberg syndrome, chromosomal aberrations disrupt the *PITX1* topological domain. Patients have limb malformations in which the arm acquires the morphological characteristics of a leg: missing olecranon, and fusion of carpal bones, which forms a calcaneus-like element (shown in panel **Bb**)[47]. **C** | *Pitx1*$^{-/-}$ mouse embryos (panel **Cb**) harbour hindlimb malformations in which the diameter of the tibia is reduced, the fibula is enlarged and an abnormal knee occurs. By contrast, *Pitx1*$^{+/-}$ embryos do not exhibit skeletal malformations (panel **Ca**)[43]. **D** | Several populations of stickleback fish lack pelvic structures (panel **Db**) owing to deletions encompassing the *Pitx1* pelvic enhancer[37]. **E** | Feathered feet in pigeons result from a partial hindlimb-to-forelimb identity transformation that is mediated by *cis*-regulatory changes in *Pitx1* and T-box 5 (*Tbx5*). Compared with the scale-footed breeds (panel **Ea**), the muffed breeds (panel **Eb**) show reduced *Pitx1* expression in the leg while *Tbx5* is ectopically expressed[49]. Panel **A** is adapted with permission from REF. 44, Cold Spring Harbour Laboratory Press. Panel **Bb** is reproduced with permission from REF. 47, Elsevier. Panel **C** is reproduced with permission from REF. 43, The Company of Biologists. Panel **D** is courtesy of S. Ipakchin, Stanford University, California, USA. Panel **E** is adapted from REF. 49.

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Limb loss in snakes. The absence of limbs in snakes represents an extreme case of morphological evolution. Although adult snakes have lost both their forelimbs and hindlimbs (although some retain their pelves), developing snakes such as pythons initially form a hindlimb bud that stalls and regresses within a few days[65]. The regression of the hindlimb bud in pythons has been linked to a failure in AER and ZPA activation, and a resultant failure in limb tissue maintenance. One of the major events responsible for the termination of these developmental pathways appears to be the early arrest of *Shh* expression in the presumptive limbs of snakes[65,67]. Deletions of crucial binding sites for transactivators (for example, HoxD13 and Ets1) have been identified in the snake orthologue of the *Shh* limb-specific enhancer[67,68]. The activity of this enhancer, named the Zrs (ZPA regulatory sequence), is highly conserved across vertebrates, including fishes, but is progressively lost during snake body plan evolution through divergence in its genomic sequence[68]. Interestingly, the Zrs is completely absent in a subfamily of advanced snakes, such as the corn snake, which has lost all limb structures.

Beyond *Shh*, additional regulatory changes that contribute to snake limb loss have been identified. For example, the snake *Pitx1* HLEB enhancer was shown to have lost hindlimb enhancer activity but maintain its activity in the genitalia[36]. The disruption of limb formation in pythons has also been associated with the expansion of Hox gene expression boundaries along the body axis[65] (FIG. 4A). However, Hox expression in the python hindlimb bud surprisingly resembles the pattern in limbed tetrapods. Moreover, skeletal condensations corresponding to the stylopod, the zeugopod and the autopod are temporarily observed in developing snake limbs[67]. Despite an overall conservation of a bimodal, limb-associated *HoxD* chromatin structure in snakes and other vertebrates, researchers have identified differences in *HoxD* regulation in the corn snake relative to other vertebrates[69]. The regulatory elements that drive *HoxD* expression in the limb mesoderm are primarily located within the *HoxD* cluster in snakes, rather than outside the cluster as in other vertebrates. Furthermore, the snake orthologues of several *HoxD* enhancers (for example, *Prox* and *Island1*) do not drive limb expression as they do in other vertebrates (FIG. 4B). These findings suggest that changes in the activity of limb enhancers for crucial developmental genes have contributed to the loss of limbs during snake evolution. However, the changes that have been observed may also be related to the release of the negative selection pressure caused by earlier molecular events[68].

Digit reduction in mammals. Cattle present an extreme case of changes in skeletal element length, having lost digits I, II and V. The bovine autopod deviates from that of pentadactyl mammals (for example, mice) at the very earliest stages of digit formation, when only two elongated digit primordia form[59]. During this stage, cow and mouse limbs display similar *Shh* expression domains, but the expression of *Gli1*, a SHH-responsive transcription factor, expands into the posterior mesenchyme. In addition, an early distalization and loss of asymmetry in the expression of many additional SHH response genes, including *Grem1*, *Hoxd13* and *Fgf8*, is observed. Given the important role of *Shh* in the establishment of the anterior–posterior axis, these findings suggest that a change in SHH signalling might underlie digit reduction in cattle. This change is thought to arise through alterations in the expression of *Ptch1*, a SHH receptor. In mice, *Ptch1* expression is upregulated in the posterior mesenchyme and the ectoderm of the developing limb, whereas expression of this gene in cattle and pigs is restricted to the ectoderm (FIG. 4C). Intriguingly, when *Ptch1* is conditionally inactivated in the mesoderm of the developing mouse limb, the resulting mouse autopod displays an oligodactylous phenotype that is strikingly similar to that of cattle[70] (FIG. 4D).

Through bioinformatic, genetic and chromatin-based assays, a potential candidate enhancer for the differential regulation of *PTCH1* in cattle, the bovine *PTCH1* limb *cis*-regulatory module (LRM), was identified. Although both bovine and murine LRMs are capable of driving limb-specific expression, the patterns of expression differ in a manner that is consistent with an inability of the bovine LRM to respond to graded SHH signalling. These findings suggest that a change in *Ptch1* regulation disrupts SHH signalling and, because of the important role of SHH signalling in digit formation, contribute to the evolution of reduced digits in cattle. Given the similarity of *PTCH1* and *GLI1* domains in the developing limbs of cattle and pigs, it is likely that a similar mechanism underlies digit reduction in pigs (FIG. 4D). Additional digit reduction mechanisms have been identified in other artiodactyls, such as camels, and in

Box 2 | **Evolution of the tetrapod limb from the fish fin**

The evolution of the tetrapod limb from the fish fin is a classic example of evolutionary transformation driven by gene regulation. Early fossil evidence suggested that the autopod (hand) was a novel feature of tetrapods that lacked a precursor in fish[121]. This hypothesis was initially supported by developmental studies that seemingly identified a second wave of homeobox A (*HoxA*) and *HoxD* expression in the tetrapod autopod that was not present in zebrafish[122–124]. This 'late phase' of Hox expression was shown to partially control autopod development in tetrapods such as mice[125]. In addition, although researchers were able to identify an enhancer that drives late-phase *HoxD* expression in mice, the global control region (GCR) in the pufferfish *Tetraodon nigroviridis* could not drive reporter expression in the limbs of transgenic mice[123]. Another notable difference is the absence of *Hoxa11* expression in the distal limbs of tetrapods, which is in contrast to the expression of other distal *HoxA* genes (for example, *Hoxa10* and *Hox13*), for which limb expression largely overlaps with that in fins. The exclusion of *Hoxa11* from the distal *Hoxa* expression domain relies on an intronic enhancer driving antisense transcription at the *Hoxa11* locus when bound by Hoxa13 and Hoxd13. This enhancer is absent in fish, suggesting its possible emergence during the fin-to-limb transition[126]. Other findings, however, support the homology of skeletal elements of the fish fin and tetrapod autopod[121,127–132]. For example, computed tomography scans of early sarcopterygian fish fossils have identified skeletal elements (for example, distal radials) that resemble those of the tetrapod autopod[121]. A series of additional gene expression studies identified late-phase *HoxD* expression in the fins of several different fish species, including catshark, lungfish, paddlefish and zebrafish[127,130,133,134]. In addition, recent genetic and cellular assays suggest that fish fin rays and tetrapod digits arise from similar cell populations[135]. With regard to gene regulation, transgenic assays have indicated that some *HoxA* and *HoxD* enhancers (including GCR elements) from skate, gar and zebrafish can drive autopod expression in transgenic mice, and that enhancers from zebrafish, gar and mouse can drive reporter expression in the distal fin of transgenic zebrafish[132,136]. Taken together, these findings highlight the insights into evolutionary processes that can be gained from the study of gene regulation.

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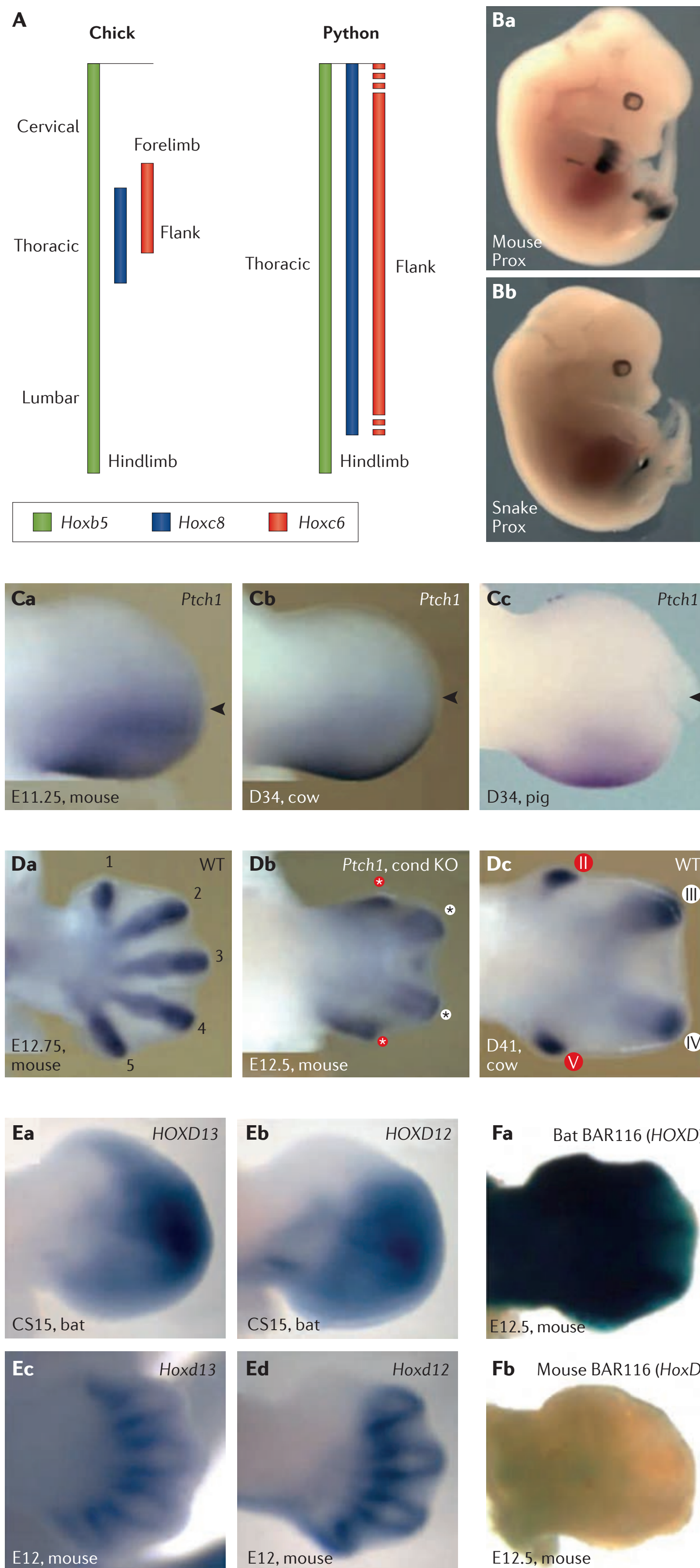

other mammals, such as jerboas and horses[56]. These mechanisms include *MSX2*-driven or other gene-driven changes that are involved in cell death during subsequent digit development. As the genes that regulate limb patterning and growth are essential to many other developmental events, it is likely that we will ultimately find that regulatory evolution is a primary driver of many, if not most, instances of digit reduction.

Wing acquisition in bats. Variation in limb segment length can arise early in development through differences in patterning, later through differences in long-bone growth or through a combination of both processes, as is the case in bats. Changes in the size of the AER (and the associated *FGF8* expression domain) and ZPA (and the associated *SHH* expression domain) during early bat limb development are thought to contribute to the larger overall size of the bat wing relative to the forelimbs of non-flying mammals[52,55]. A subsequent increase in the size of the zone of hypertrophic cells during long-bone growth has also been functionally linked to the elongated metacarpals of bats[50]. Significant changes in the expression of the HOX family of genes (FIG. 4E) as well as in *SHH*, *TBX3*, *FAM5C* (also known as *BRNP3*), *MEIS2*, *MLLT3* and *MAB21L2* have also

Figure 4 | **Morphological evolution of vertebrate appendages. A** | Homeobox (Hox) expression domains are expanded along the body axis in python (a snake) relative to chick. **B** | In transgenic mice at embryonic day 12.5 (E12.5), the mouse sequence for Prox, an established *HoxD* enhancer, drives limb-specific expression, as indicated by dark staining (panel **Ba**), whereas the orthologous snake sequence does not show LacZ staining in the limbs (panel **Bb**). **C** | *Ptch1* expression, indicated by dark staining, in mouse (panel **Ca**), cow (panel **Cb**) and pig (panel **Cc**) limb buds of equivalent developmental stages. The *Ptch1* expression domain is reduced in cattle and pig relative to mouse, consistent with differences in sonic hedgehog (SHH) signalling in these species. Black arrowheads indicate the limb bud apex. **D** | The pattern of digit condensations in autopods of mouse and cow of equivalent developmental stages, revealed by *Sox9* expression, indicated by dark staining. Wild-type (WT) mice have five digits with an axis of symmetry through digit III (panel **Da**), whereas mice in which *Ptch1* has been conditionally knocked out (cond KO; panel **Db**) display only four digits with an axis of symmetry between digits III and IV, similar to the phenotype in cow (panel **Dc**). Vestigial digits are indicated in red. **E** | *Hoxd12* and *Hoxd13* expression domains, indicated by dark staining, differ in bat (panel **Ea,b**) and mouse (panel **Ec,d**) autopods of equivalent developmental stages. **F** | The bat sequence for bat accelerated region 116 (BAR116), an enhancer located in the *HOXD* locus, drives limb-specific expression in transgenic mice at E12.5 (panel **Fa**), whereas the orthologous mouse sequence does not (panel **Fb**). CS, *Carollia* embryonic stages; D, days post fertilization. Panel **A** is adapted with permission from REF. 65, Macmillan Publishers Limited. Panel **B** is adapted from REF. 69. Panels **Ca**, **Cb** and **D** are adapted with permission from REF. 59, Macmillan Publishers Limited. Panel **Cc** is adapted with permission from REF. 56, Macmillan Publishers Limited. Panels **E** and **F** are adapted from REF. 51.

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been documented in developing bat wings[4,12,13,51,52,55,71]. Long non-coding RNAs (for example *HOTTIP* and *TBX5-AS1*) also exhibit changes in expression in bat wing development[4]. These bat-specific gene expression changes are thought to be driven by changes in the regulatory elements of these genes.

RNA-seq[4,12,13] and ChIP–seq[4] studies of developing bat forelimbs and hindlimbs at various developmental stages have identified numerous candidate genes and regulatory elements involved in wing acquisition. Using comparative genomics to annotate sequences that are specifically accelerated in the bat lineage and then layering these sequences on limb ChIP–seq peaks enabled the identification of numerous bat accelerated regions (BARs)[4,51]. Five of these BARs, located near genes associated with limb development (for example, *TWIST2*, *SPRY1*, *SHH*, *SPG20* and *HOXD*), were tested for enhancer activity, and all five were capable of driving limb-specific expression in mice[51]. Three of these BARs also showed differential enhancer activity compared with the orthologous mouse sequence. For example, BAR116, located near the *HOXD* cluster, generated robust forelimb expression for the bat sequence but was negative for the mouse sequence (FIG. 4F). Further functional assays are needed to associate these sequences with specific wing phenotypes. The potential of such assays to dissect the function of regulatory elements was demonstrated by the replacement of the mouse copy of a limb-specific paired-related homeobox protein 1 (*Prx1*) enhancer with the bat orthologous sequence, which led to mice with significantly longer forelimbs than controls[53].

Pathways to limb evolution. The growing body of research on the evolution of limb development, some of which is discussed above, suggests that diverse cellular processes and patterning changes underlie the evolution of limb length. Examples of cellular process changes include cell death-mediated digit reduction in camels, horses and jerboas, and alterations in chondrocyte maturation during bat wing development. Examples of patterning changes include digit reduction in cattle related to SHH signalling modulation, and limb loss in snakes related to *Pitx1* and *Shh* regulation changes. However, these diverse processes share at least two broad similarities. First, earliest limb development (that is, the ridge stage of development when the limb is first emerging (for example, embryonic day 10 (E10) in mouse)) seems to be highly conserved among most species. This statement applies even to species such as the dolphin and python because although these animals ultimately lose the entire limb, they do initially form a limb bud[60,65]. Second, certain pathways (for example, SHH, FGF and HOX) seem to be repeated targets for the evolution of limb development. This latter observation could be due to higher sampling — that is, genes known to be important for limb development are often studied as candidates for limb evolution — or it could be a true biological phenomenon.

Recent comparisons of forelimb transcriptomes during earlier and later stages of limb development across multiple mammals (that is, pig, opossum, bat and mouse) provide some support for the latter of these hypotheses and for the hypothesis that early limb development is conserved across species[12]. These comparisons suggest that the limb transcriptomes of mammals are more similar at an earlier stage (that is, ridge stage) than at a later stage (that is, paddle stage) in development, and that the expression levels of some pathways, including SHH signalling, vary more than others. Furthermore, computational perturbation assays suggest that these expression differences are likely to be due to the structure of the gene network that regulates limb development. That is, the structure of the network that regulates early limb development is more robust to perturbations than that regulating later limb development[12]. Taken together, these results suggest that the nature of development, in which development at later stages is dependent on proper development at earlier stages, can bias the distribution of variation over developmental and, as a result, evolutionary time. Furthermore, these results highlight the importance of evolutionary changes in the interactions among genes (that is, through changes in gene regulation) to the evolution of limb form.

Accelerated regions
Highly conserved sequences that have experienced a marked increase of substitution rates in a particular lineage. These regions are good candidates for the identification of regulatory DNA sequences that could have contributed to specific morphological differences in that lineage.

Limb regeneration

Urodele (tailed) amphibians, such as salamanders and newts, have a remarkable capacity to regenerate injured tissues, including limbs. After amputation, the injured extremity undergoes blastema formation (a blastema consists of a heterogeneous collection of restricted progenitor cells), outgrowth and differentiation. In the salamander *Ambystoma mexicanum* (the axolotl), the initial cell cycle response is induced by a MARCKS-like protein (MLP), an extracellularly released factor, the expression of which rises strongly after amputation[72]. Blastema outgrowth is then dependent on nerve-derived signals and on the presence of both anterior and posterior limb cells[73]. Anterior and posterior limb cells provide complementary cross-inductive signals of Fgf8 and Shh, which are expressed in the anterior and posterior compartments, respectively. Similar to the Shh–Gremlin–Fgf signalling loop that is involved in limb development, both the Fgf8 and Shh signals are necessary for blastema outgrowth and integration of limb position, and thereby the growth of a correctly oriented limb[74]. These signals are probably orchestrated through gene regulatory elements.

Although much remains to be learned about the regulatory elements that are involved in regeneration, a handful of studies of species such as frogs and zebrafish have linked developmental enhancers to regenerative processes. Adult frogs have limited regenerative capacity after limb amputation, whereas frog tadpoles can completely regenerate their limbs[75]. These age-related differences in frog regenerative capacity are thought to be related to epigenetic regulation. *Shh* is expressed in the regenerative blastema and has an important role in limb positioning[74]. The aforementioned Zrs, the limb-specific enhancer of *Shh*, is hypomethylated in the tadpole and in other amphibians with regenerative capacity, whereas it is highly methylated in the adult, thus precluding *Shh* expression[76]. This phenomenon is in contrast to the overall methylation

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profile of the transcription start sites of developmental genes, which is similar in intact and regenerating limbs, despite dynamic changes in developmental gene expression during limb regeneration. For example, the histone methylation profiles at the *Shh*, *Tbx4* and *Tbx5* loci remain unchanged in the regenerating hindlimb. Furthermore, limb bud regeneration is dramatically reduced when an inhibitor of histone methylation is applied to the blastema, and this effect is reversible after termination of the treatment. These findings suggest that histone modifications that activate gene regulatory elements are required for proper limb regeneration and may act as an epigenetic memory to maintain limb cell properties[77]. Recent work has demonstrated the existence of 'tissue regeneration enhancer elements' (TREEs) that are activated upon tissue damage and regulate the regenerative genetic programmes[78]. For example, research combining transcriptomic analyses and epigenetic profiling in zebrafish identified a bipartite enhancer that upregulates leptin B expression after injury in regenerative fin and heart tissue. Given progress to date, it is likely that future research will identify additional regulatory elements with roles in limb regeneration. Understanding their regulatory function could help to develop therapeutic targets in mammals, whose regenerative capacity is limited.

Limb malformations

Modifications of the developmental building blocks can also lead to limb malformations, an extremely common condition in humans that occurs in approximately 1 in every 500 births[79]. There is accumulating evidence to indicate that disruption of gene regulatory elements has a major role in the pathogenesis of isolated (non-syndromic) limb malformations (FIG. 5). This finding makes intuitive sense, as genes involved in limb development are usually also involved in the development of other organs. Consequently, mutations in their coding sequences would be expected to lead to multiple organ malformations, rather than just limb malformations. In support of this idea, coding mutations only explain a small portion of isolated limb malformations. In addition, several alterations in gene regulatory elements that lead to various isolated limb malformations have already been detected (see REFS 79,80 for reviews), some of which are described below.

Zrs mutations: a model for enhancer modulation. The most extensively characterized limb enhancer that has been shown to cause limb malformations is the Zrs[81]. The Zrs resides ~900 kb downstream to *Shh* in an intron of another gene (limb development membrane protein 1 (*Lmbr1*)) and interacts with the *Shh* promoter via chromatin looping[82,83] (FIG. 5a). The Zrs is a cooperative unit comprising two regulatory components: the 5′ part of the Zrs directs spatiotemporal activity and the 3′ part is thought to be required for long-range activity[84]. *Shh* and Zrs lie within a constitutively compact chromatin domain and are in close spatial proximity in all tissue types that have been investigated[82]. However, super-resolution 3D-fluorescence *in situ* hybridization (3D-FISH) imaging has shown that their colocalization occurs only in the limb within cells that express *Shh*[82]. So far, the molecular mechanisms that lead to the chromatin looping at the *Shh* locus are unclear and may depend on the embryonic stage. Complete deletion of the Zrs reduces *Shh* expression in mice but does not affect the interaction of the remaining sequence with *Shh* at E10.5 (REF. 83). By contrast, the enhancer–promoter interaction is disrupted at E11.5 in mouse embryos carrying a 3′ deletion of the Zrs[82,84]. The early expression of *Shh* in the ZPA at the posterior region of the limb bud is induced by the binding of HAND2 and 5′ HOXD (which are encoded by genes at the 5′ end of the *HoxD* cluster) proteins to this enhancer[85,86]. Later on in development, the spatial regulation of *Shh* expression is modulated by the direct binding of transcription activators (for example, ETS1 and GAPBα) and repressors (for example, ETV4, ETV5 and GATA6)[87,88]. A concentration gradient of the diffusible morphogen SHH is thereby established from the ZPA and is required for the differentiation of digits V to II[89,90]. Development of the first digit is SHH-independent, whereas specification of the identity of digits V to II is determined by the time of exposure for the most posterior digits and by the concentration of SHH for the most anterior ones[89].

In support of its importance in limb development, removal of the Zrs causes limb truncation in mice[91]. Gain-of-function mutations or gain-of-copies of this regulatory element cause anterior ectopic expression of *Shh* in the limb (FIG. 5a), and are associated with preaxial polydactyly in humans (OMIM 174500), cats, chickens and mice[81,92–94]. However, the pathogenic mechanisms of most of these mutations are currently unknown. For some mutations, the abolishment of a binding site for repressive ETV factors or the creation of a binding site for activatory ETS factors are thought to be responsible for the ectopic activity[87]. Interestingly, mutations at specific positions of the human ZRS have been linked to more severe limb phenotypes that are associated with long-bone deficiency (for example, Werner mesomelic syndrome (OMIM 188740) and Laurin–Sandrow syndrome (OMIM 135750)), the causes of which currently remain unknown.

Split hand/foot malformation: a model for gene regulatory rearrangements. Split hand/foot malformation (SHFM; OMIM 183600) is a clinically and genetically heterogeneous condition with variable severity that is typically characterized by a reduction of the central rays. SHFM is commonly associated with diverse genomic disorders that deregulate genes involved in AER function or maintenance (BOX 3). In addition to humans, SHFM has been observed in chickens, amphibians and mice, consistent with the conservation of the affected signalling pathways.

The *SHFM1* locus, which is located on 7q21, has been associated with isolated and syndromic SHFM. In addition to SHFM, patients may display craniofacial abnormalities, deafness and/or intellectual disability. Mutations in distal-less homeobox 5 (*DLX5*), which lies within the *SHFM1* locus, have been observed in

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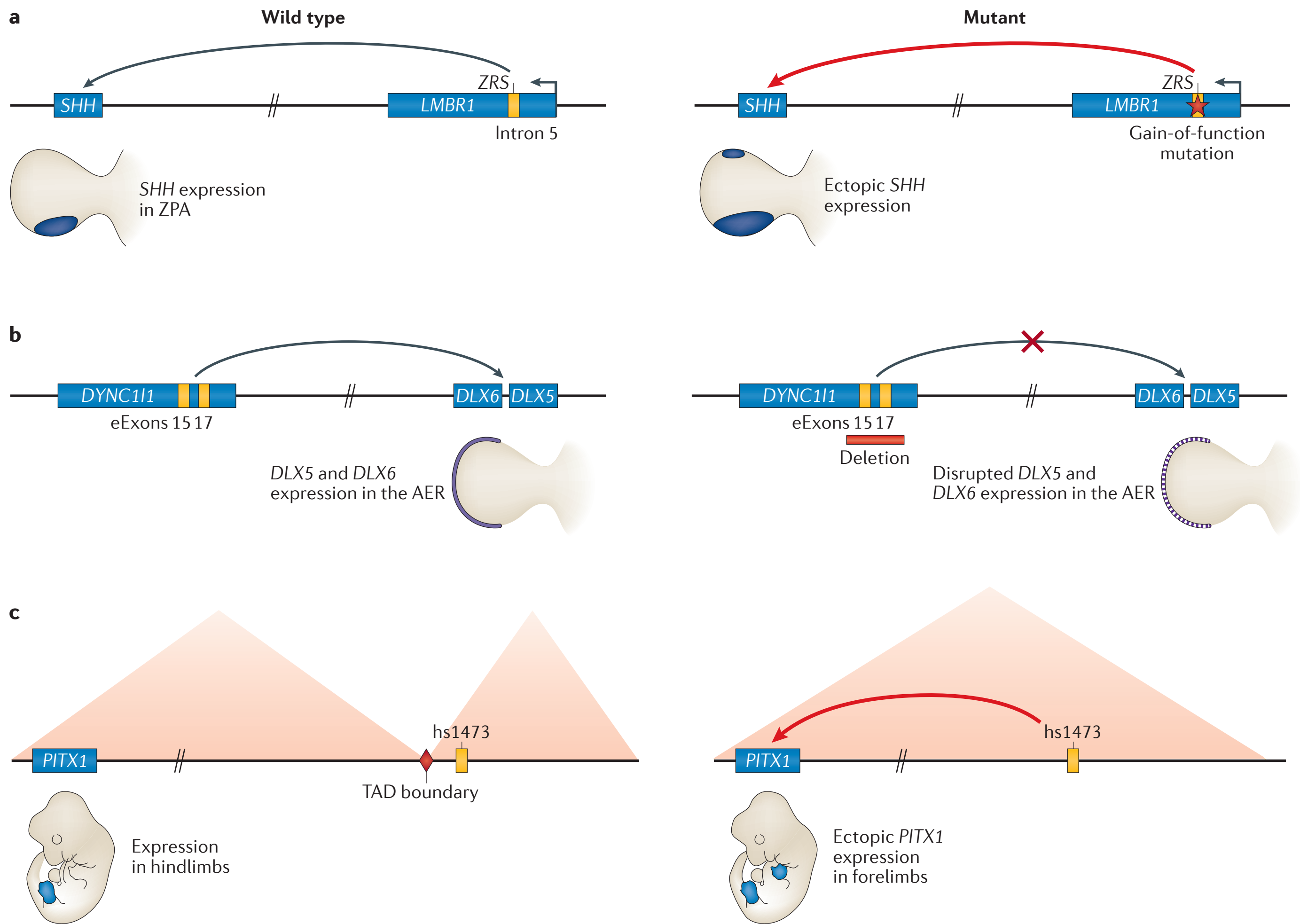

Figure 5 | **Regulatory mechanisms of limb malformations. a** | The zone of polarizing activity (ZPA) regulatory sequence (*ZRS*) is a limb-specific enhancer that drives sonic hedgehog (*SHH*) expression selectively in the posterior margin of the limb bud (that is, the ZPA). Gain-of-function mutations in the *ZRS* lead to the ectopic anterior expression of *SHH* in the limb bud, causing preaxial polydactyly in multiple species. **b** | Split hand/foot malformations (SHFMs) at the *SHFM1* locus are due to the altered expression of distal-less homeobox 5 (*DLX5*) and *DLX6* in the apical ectodermal ridge (AER), which is the signalling centre for the proximal–distal limb outgrowth. In this condition, diverse chromosomal rearrangements (deletions, inversions and translocations) disrupt the interaction of *DLX5* and *DLX6* with their limb enhancers, which are located in exons 15 and 17 of dynein cytoplasmic 1 intermediate chain 1 (*DYNC1I1*), a neighbouring gene that encodes a protein that is not involved in limb development. The right panel shows a 106 kb characterized deletion[146] (red line) that removes the *DYNC1I1* exon 15 and 17 enhancers, and leads to SHFM type 1. **c** | Liebenberg syndrome is a topologically associating domain (TAD)opathy caused by variable chromosomal rearrangements near paired-like homeodomain transcription factor 1 (*PITX1*) that are responsible for its ectopic expression in forelimbs. In the rearrangement shown as an example, the deletion of the boundary between the *PITX1* TAD and the adjacent TAD leads to the fusion of both TADs, enabling the abnormal interaction between *PITX1* and an enhancer with forelimb and hindlimb activity (hs1473). This enhancer adoption is responsible for driving ectopic *PITX1* expression in the forelimbs. The affected patients present with forelimb malformations consistent with an arm-to-leg homeotic transformation. TADs are represented by the triangles and the inter-TAD boundary by a red diamond. eExons, exonic enhancers; *LMBR1*, limb development membrane protein 1.

several families with autosomal dominant or recessive inheritance of SHFM[95–98]. However, most patients with SHFM type 1 carry chromosomal aberrations that disrupt the interaction between the *DLX5–DLX6* region and its tissue-specific enhancers (limb, branchial arch, inner ear and/or forebrain)[99] (FIG. 5b). A correlation between patient phenotype (SHFM, hearing loss and/or craniofacial anomalies) and the tissue specificity of the enhancers involved in the chromosomal aberration has been delineated[100]. Interestingly, two of the limb enhancers that are thought to regulate *DLX5* and *DLX6* in this region also function as coding exons for another gene, dynein cytoplasmic 1 intermediate chain 1 (*DYNC1I1*) (FIG. 5b), which has no known function in the limb[101]. These results highlight the multifaceted fluidity of enhancers; they can regulate genes at long distances and have multiple functions (that is, protein coding and enhancer activity).

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SHFM type 3 (OMIM 246560) and SHFM type 5 (OMIM 606708) are also caused by copy number variations that do not encompass clear candidate genes explaining the SHFM phenotype. Duplications at 10q24 (*SHFM3*), which are the most frequent cause of SHFM, are thought to disrupt the regulation of *FGF8* expression. Multiple and functionally interdependent *FGF8* enhancers are interspersed with unrelated flanking genes along a 220 kb region. The structural organization of this region is necessary for the correct spatiotemporal expression of *FGF8* during embryogenesis. Given its central role in AER function, deregulation of *FGF8* is probably responsible for the SHFM phenotype[102]. Deletions centromeric to the *HOXD* cluster (2q31 deletions) are associated with SHFM type 5. The *HOXD* cluster has a dual role in controlling the expression of FGF proteins in the AER during limb outgrowth and patterning[103], and the minimal critical interval for 2q31 deletions encompasses a conserved region containing *HOXD* regulatory elements[104,105]. Similar to SHFM type 3, disrupting the complex organization of *HOXD* regulatory modules is likely to have consequences for the expression of their target genes, leading to limb malformations.

SHFM with long-bone deficiency 3 (SHFLD3; OMIM 612576) serves as a good example of the importance of the fine-tuning of gene expression to achieve proper limb patterning. This condition arises from copy number gains involving *BHLHA9* (REF. 106), which encodes Fingerin, a transcription factor involved in the regulation of apoptosis during autopod development[107]. In SHFLD3, SHFM is generated by increased apoptosis within the AER. Conversely, *BHLHA9* loss-of-function mutations lead to defective interdigital apoptosis and mesoaxial syndactyly in mice and humans (OMIM 609432)[107,108]. These results demonstrate how changes in gene dosage can lead to opposing phenotypes, with a higher *BHLHA9* dose leading to SHFM and a lower dose to syndactyly. Patients with SHFLD3 also present with striking intra-individual phenotypic variability (that is, within left and right side), suggesting that highly subtle differences in gene regulation or dosage can lead to abnormal limb patterning.

A model for chromatin architecture disruption. Limb development has also become a model for chromatin architecture disruption, an important pathogenic mechanism that was recently shown to alter gene regulation and lead to limb malformations (FIG. 5c). It has long been known that genomic disorders lead to position effects. That is, a chromosomal rearrangement may disrupt the interaction between a gene and its regulatory elements and/or lead to novel gene–enhancer interactions (called enhancer adoption)[109]. Recent advances in genome editing and chromatin conformation capture techniques have enabled the study of the consequences of genomic disorders on chromatin architecture and gene regulation. As noted in BOX 1, chromatin is functionally divided into regions called TADs, within which DNA interactions occur at high frequency relative to interactions between TADs[29]. Chromosomal rearrangements that alter TAD boundaries can lead to various phenotypes, including isolated malformations, which are referred to as TADopathies[110].

This phenomenon is nicely illustrated by the effects of 2q35q36 chromosomal rearrangements, which lead to various human limb malformations[111]. This locus comprises three independent TADs, each of which

Box 3 | The apical ectodermal ridge regulatory network is disrupted in split hand/foot malformations

The apical ectodermal ridge (AER) is the signalling centre for proximodistal growth and differentiation of the limb bud. The AER corresponds to a thickening of the ectoderm at the dorsoventral frontier of the limb bud. This morphologically dynamic and transient structure enables the maintenance of undifferentiated and proliferating cells, which are necessary for limb outgrowth, in the underlying mesenchyme. The AER is induced through complex interactions between the ectoderm and the mesenchyme; fibroblast growth factor 10 (*Fgf10*) is expressed in the mesenchyme and *Fgf8* is expressed in the ectodermal cells of the AER. FGF8 is the first marker of AER cells, and its expression is maintained until the regression of this structure. The establishment of the epithelial–mesenchymal feedback loop involving FGF8 and FGF10 is regulated by the WNT–β-catenin pathway and by bone morphogenetic proteins (BMPs)[137,138]. Another major signalling pathway involved in AER morphology and function is the p63 network. p63 is a transcription factor that regulates the proliferation, differentiation and stratification of ectodermal cells[139], and is expressed in the AER during limb development. In *p63*-knockout mice, *Fgf8* expression is dramatically reduced and AER formation is impaired. These animals do not develop ectodermal structures and have variable reductive malformations of the limbs, from split hand/foot malformation (SHFM) to the absence of limbs[140]. Regulation of FGF8 by p63 is indirect and is thought to be carried out by members of the distal-less homeobox (DLX) and specificity protein (SP) families of transcription factors. Several DLX members are expressed in the AER, notably *Dlx5* and *Dlx6*. Double-knockout mice that lack both *Dlx5* and *Dlx6* exhibit SHFM[141], and p63 was shown via chromatin immunoprecipitation followed by massively parallel sequencing to bind to the promoters and enhancers of these genes[142,143]. Computational predictions have identified binding sites for DLX family members around *Fgf8*, suggesting that the DLX family may be the intermediate factors by which p63 regulates *Fgf8* expression[144]. *Sp6* and *Sp8* are expressed in the AER and more widely in the ectoderm of the limb bud. These factors act downstream of the WNT–β-catenin pathway and induce *Fgf8* expression[145]. In particular, SP8 binds directly to the promoter of *Fgf8* to trigger its expression. Binding sites for DLX factors were also computationally predicted to reside near *Sp8*, suggesting that this gene could be targeted by Dlx genes to modulate *Fgf8* expression[144]. In mouse models, progressive reduction of *Sp6* and *Sp8* expression leads to an absence of *Fgf8* induction and a malformation spectrum ranging from syndactyly to SHFM and the absence of limbs[145].

Chromatin conformation capture
A technique that is used to quantify DNA–DNA contacts in the nucleus, within a single locus or on a genome-wide scale, to study the 3D organization of the genome.

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contain the *WNT6*–indian hedgehog (*IHH*), *EPHA4* and paired box 3 (*PAX3*) genes. *EPHA4* encodes ephrin receptor A4, which is involved in axon guidance and is expressed in the limb bud (particularly in the preaxial limb). A deletion involving the telomeric TAD boundary places *PAX3* under the control of the *EPHA4* enhancer cluster and causes brachydactyly. Similarly, an inversion of the centromeric TAD boundary puts *IHH* under the control of the *EPHA4* enhancers and causes syndactyly. The ectopic interactions between enhancers and the misexpressed genes were demonstrated by chromatin conformation experiments in patient fibroblasts and in mouse limb buds. In both cases, the autopod malformations appeared more severe on the preaxial side, consistent with the level of activity of the adopted enhancers.

Another TADopathy mechanism is the formation of new chromatin domains (neo-TADs) as a result of genomic microduplications that contain a TAD boundary (inter-TAD duplications). These rearrangements may bring together, in the same neo-TAD, elements that normally reside in separate domains. Therefore, genes may be misplaced under the control of regulatory elements that normally belong to the adjacent TAD[112]. In patients with Cooks syndrome (OMIM 106995), which is characterized by short digits and aplasia of nails, a duplication involving *SOX9* regulatory elements, the TAD boundary and *KCNJ2* (which encodes potassium voltage-gated channel subfamily J member 2) from the adjacent TAD has been identified[112]. *SOX9* encodes a transcription factor that has an important role in chondrocyte differentiation and male sex determination. This inter-TAD duplication creates a neo-TAD containing duplicates of *SOX9* regulatory elements and *KCNJ2*. The misplacement of these elements within the same TAD leads to their ectopic interaction and ultimately to the *KCNJ2* misexpression responsible for a limb phenotype[112].

These examples demonstrate that the misguidance of a regulatory element towards an inappropriate promoter can lead to variable malformations depending on the nature of the gene that is inappropriately targeted. Furthermore, they highlight that the disruption of TAD boundaries can lead to altered gene regulation, and present the intriguing possibility that broken TADs or neo-TADs could be a cause of many other malformations and morphological differences among species.

Conclusions

The limb has proven to be an effective tool for understanding and modelling organ development. As described in this Review, the limb is also becoming an important model for studying gene regulation. However, there are several hurdles that need to be overcome. Genomic techniques such as ChIP–seq, ChIA–PET, RNA-seq and others described in this Review provide us with information about the building blocks that make up the molecular blueprint of the developing limb. However, the number of these sequences is in the thousands and, similar to a map, they are merely descriptive. Functional assays are therefore needed to substantiate the role of these sequences in the generation of normal and altered limb morphologies. In addition, as shown here with the multitude of different phenotypes that result from Zrs mutations, our understanding of the role of single gene regulatory mutations in the translation of phenotype from genotype remains fairly limited. Moreover, morphological changes could also be caused by additive gene regulatory changes (that is, caused by more than one regulatory element), as described here for bats. However, current functional assays are generally carried out on a one-by-one basis and are time consuming and cost prohibitive, limiting our ability to systematically unravel the regulatory underpinnings of limb development. The development of high-throughput functional assays, such as massively parallel reporter assays[113] and genome-editing screens[114], could circumvent these challenges and lead to a more comprehensive understanding of the function of these regulatory elements and how their alteration leads to specific phenotypes. The limb, with its outstanding morphological diversity and viable malformations, can continue to serve as an *in vivo* model for these assays, increasing our understanding of the regulatory code and grammar.

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