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Review Article

Evolutionary and Developmental Integration of the Fin-to-Limb Transition through Conserved Gene Regulatory Networks

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Abstract : The vertebrate limb is the archetypal example of evolutionary novelty. The evolution of limbs from ancestral sarcopterygian fins enabled vertebrates to conquer the land some 375 million years ago, enabling tetrapods to occupy virtually all terrestrial environments. To fully understand how this evolutionary transition occurred, it is necessary to integrate paleontological, molecular, and genomic discoveries that have been accumulating since the modern fields of paleontology and molecular biology were founded decades ago. Here we review the origin and evolution of limbs within the unifying framework of deep homology and regulatory divergence. We show that limb diversity is generated by the dynamic interplay between an ancient genetic toolkit and changes to cis-regulatory modules that control its spatial and temporal deployment. We first cover fossils that have captured the gradual acquisition of skeletal innovations leading to vertebrate limbs, such as *Elpistostege*, *Tiktaalik*, and *Acanthostega*. Then we discuss the central molecular components of the vertebrate limb developmental program: the biphasic *Hox* code, the *Shh*/*Fgf* signaling feedback loop, and retinoic acid and bone morphogenetic protein (BMP) signaling during limb axis patterning. We also address the role of Wnt signaling in dorsal-ventral axis specification, which integrates with the above pathways to coordinate three-dimensional limb patterning. Lastly, we showcase how modifications to this conserved developmental program through changes in cis-regulatory DNA has sculpted limbs into dramatically diverse forms—wings and flippers, horse hooves, snakes—including the elimination of limbs entirely. By weaving together these lines of evidence, this review presents a cohesive narrative of how a single ancestral blueprint, through the tinkering of its regulatory logic, can generate nearly infinite morphological variation.

Keywords : Limb evolution, Fin-to-limb transition, Deep homology, *Hox* genes, Signaling molecules, Wnt signaling, Dorsal-ventral patterning

INTRODUCTION: A FRAMEWORK OF DEEP HOMOLOGY AND REGULATORY DIVERGENCE

The evolution of four limbs with digits from the fins of fish-like ancestors is arguably one of the most significant evolutionary innovations in the history of life on Earth [1,2]. The origin of the tetrapod limb is unparalleled among major evolutionary transitions as it is both the cause and the result of the adaptive radiation of vertebrates onto land. Accord-

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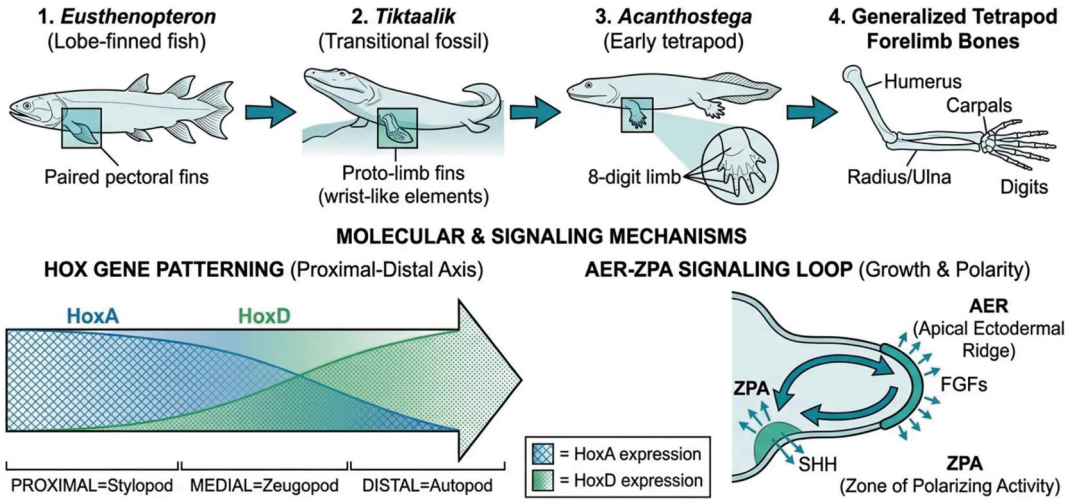

Fig. 1. Graphical abstract. This figure provides a visual summary of the major stages and molecular mechanisms underlying vertebrate limb evolution. The top panel illustrates a skeletal transition timeline, tracing the path from the Devonian sarcopterygian fin to the modern tetrapod forelimb. The bottom left panel depicts a generalized and simplified model of the overlapping *HoxA* and *HoxD* expression domains along the proximal-distal gradient of the developing limb; the reader is referred to Fig. 4 for a more detailed representation of the phase-specific expression of different Hox paralogs. The bottom right panel shows the critical AER-ZPA signaling feedback circuit that orchestrates limb outgrowth and patterning.

ingly, the origin and diversification of tetrapod limbs have required both the evolution of novel features and functions and the co-option and modification of existing ones. Insight into this transition, which occurred during the Late Devonian period, has historically come from two uniquely powerful fields that study its facets in isolation: paleontology explains patterns in evolution, and developmental biology explains processes. These fields have recently begun to overlap through the realm of evolutionary developmental biology, allowing us to study major evolutionary transitions with a truly integrative approach [3]. We propose that the origin and diversification of the vertebrate limb is best framed through an understanding of deep homology and regulatory divergence. Deep homology is the concept that even the most disparate, sometimes non-homologous traits across the tree of life are controlled by a conserved set of ancestral genes, commonly referred to as the genetic toolkit [4]. Gene regulatory networks (GRNs) are the complex webs of interactions through which transcription factors regulate the expression of other genes, orchestrating the spatial and temporal deployment of this toolkit. The specificity of these networks is often encoded in cis-regulatory modules (CRMs), which are non-coding DNA sequences that bind transcription factors to control the transcription of nearby genes [5]. Regulatory divergence acknowledges that changes to cis-regulatory elements (CREs) controlling expression of these toolkit genes can have large morphological effects and lead to the evolution of novelty [5]. We argue that limb evolution follows this same pattern; over evolutionary time, a conserved set of patterning genes (*Hox*, *Shh*, *Fgf*, et al.) establishes the fundamental blueprint for the limb, and co-option and modification of the regulatory elements controlling these genes drives evolutionary innovation. Additionally, loss or silencing of these regulatory modules has been shown to lead to the secondary loss of limbs. We will discuss this idea by highlighting three themes (Fig. 1). First, we discuss how paleontologists have uncovered the fin-to-limb transition by studying an increasingly rich fossil record that captures the stepwise acquisition of tetrapod-defining characteristics [6,7]. Then, we will discuss how genetic and signaling components necessary for limb development and patterning have been identified, highlighting both new discoveries that help fill in the gaps of previously established mechanisms—such as retinoic acid signaling—as well as conceptual changes to our understanding of key processes—such as proximal-distal limb specification [8,9]. Finally, we will review the remarkable diversity of limbs that have evolved since the origin of tetrapods. We will show that this diversity can be explained by differential modifications to the CREs controlling the expression of this ancestral developmental program, which have been used to build wings, flippers, hooves, and even abolish limbs altogether [10,11]. Under-

standing the ancestral limb blueprint and the regulatory logic that governs it is also fundamental to interpreting the evolutionary modifications that gave rise to the human hand—with its precision grip and dexterous manipulation—and the bipedal hindlimb, both of which are central topics in biological anthropology. Taken together, these lines of evidence help us weave a narrative of how evolution can create novel structures by tinkering with regulatory logic of ancient genes.

FIN-TO-LIMB TRANSITION: FOSSIL EVIDENCE AND SKELETAL HOMOLOGY

1. Evolutionary context: devonian paleoenvironment and key taxa

Numerous fossils preserve the origin of tetrapods in rock layers dating to the late Devonian period (419~359 million years ago [Mya]), through the Frasnian-Famennian stages (385~360 Mya). This was a time when shallow aquatic environments were extensive on Earth and plants were starting to colonize land. In these ancient ecosystems, several lineages of sarcopterygians (lobe-finned fishes) started developing more efficient limb-based locomotion strategies [2]. Giant genomes recently sequenced from lungfishes, the closest living relatives of tetrapods, have provided crucial insights into genetic 'pre-adaptations' that facilitated the eventual invasion of land [12]. The closest known fossil relatives of tetrapods are members of the Elpistostegalia, such as *Panderichthys*, *Elpistostege* and *Tiktaalik*.

2. Anatomical features of key fossil taxa (Fig. 2; Table 1)

1) *Elpistostege watsoni*: a hand within a fin

In a landmark 2020 study, Cloutier et al. re-examined a complete 1.57-meter-long specimen of *Elpistostege watsoni* from the Upper Devonian of Miguasha, Canada, using high-energy computed tomography (CT) scanning. Within the pectoral fin endoskeleton, they identified a humerus homolog, radius and ulna homologs, and four distinct rows of radial elements corresponding to carpal homologs [7]. Most

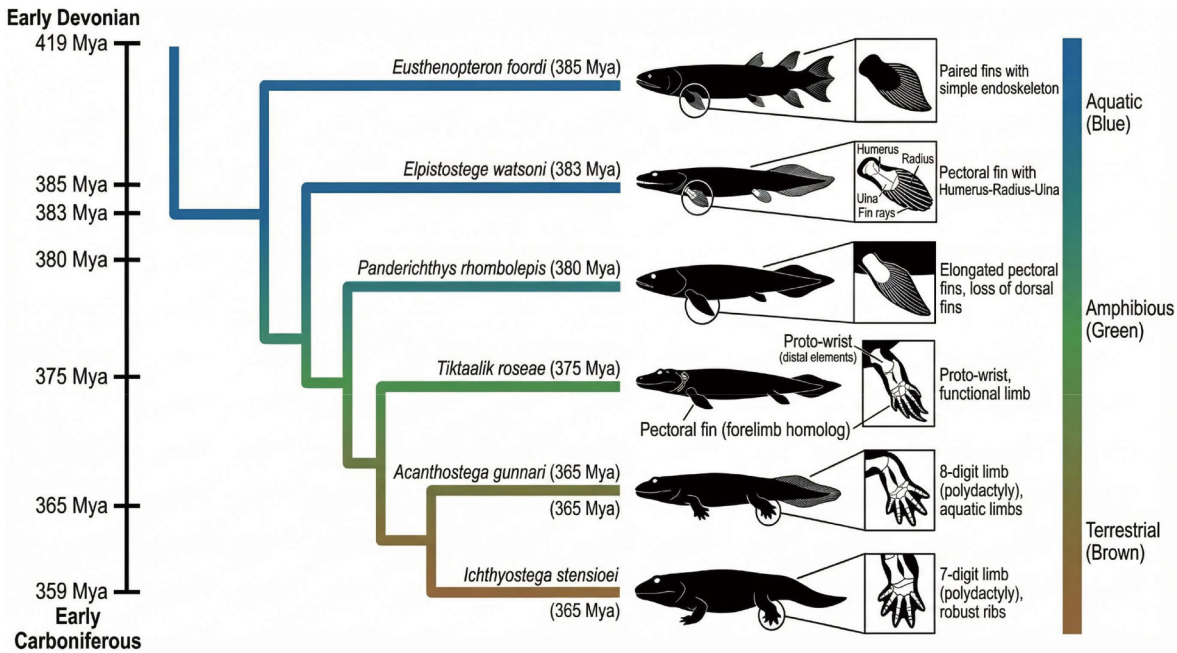

Fig. 2. Fossil timeline of the Devonian fin-to-limb transition. Six key taxa are plotted along a phylogenetic timeline from the Early Devonian (419 Mya) to the Early Carboniferous (359 Mya). Each taxon is illustrated with a body silhouette, a pectoral fin/forelimb skeletal diagram, and annotations of major anatomical innovations. From earliest to latest: *Eusthenopteron foordi* (385 Mya; paired fins with simple endoskeleton), *Elpistostege watsoni* (383 Mya; humerus-radius-ulna configuration), *Panderichthys rhombolepis* (380 Mya; elongated pectoral fins, loss of dorsal fins), *Tiktaalik roseae* (375 Mya; proto-wrist, pectoral fin as forelimb homolog), *Acanthostega gunnari* (365 Mya; eight-digit polydactylous aquatic limb), and *Ichthyostega stensioei* (365 Mya; seven-digit limb, robust ribs). A color gradient from blue (aquatic) through green (amphibious) to brown (terrestrial) represents the ecological transition.

Table 1. Summary of key morphological transitions in the fin-to-limb transition

Taxon	Age (Mya)	Habitat	Pectoral appendage	Key skeletal features	Molecular/ developmental significance
Eusthenopteron foordi	385	Aquatic	Paired fins with simple endoskeleton	Humerus, radius, ulna present; no wrist elements; lepidotrichia (fin rays) dominant	Basal sarcopterygian body plan; Hox expression presumably similar to extant lungfish
Elpistostege watsoni	383	Aquatic	Pectoral fin with humerus-radius-ulna	Distinct humerus, radius, ulna; rows of distal endoskeletal elements (digit homologs)	Earliest evidence of digit-like radials within a fin; supports fin-fold origin of digits
Panderichthys rhombolepis	380	Amphibious (shallow water)	Elongated pectoral fins	Loss of dorsal and anal fins; flattened skull; eyes dorsally positioned	Transitional morphology; loss of median fins suggests shift toward substrate locomotion
Tiktaalik roseae	375	Amphibious	Proto-limb with wrist-like elements (forelimb homolog)	Functional wrist joint; mobile pectoral girdle; flat skull with spiracle	Demonstrates proto-wrist and weight-bearing capability; SHH and HoxD expression domains inferred from morphology
Acanthostega gunnari	365	Primarily aquatic	8-digit limb (polydactyl)	Eight digits; internal gills retained; paddle-like limb not suited for terrestrial locomotion	Polydactyly indicates ancestral digit patterning before pentadactyl constraint; HoxD13 expansion
Ichthyostega stensioei	365	Semi-terrestrial	7-digit limb (polydactyl)	Seven digits; robust ribs; overlapping rib cage; specialized hindlimb for paddling	More terrestrially adapted than Acanthostega; robust rib cage suggests air-breathing capability

remarkably, the two most distal rows contained skeletal elements that can be interpreted as digits and putative digits, providing the most direct fossil evidence to date that the vertebrate hand evolved from within the endoskeleton of an aquatic fin [7]. This discovery solidified the paradigm that the genetic toolkit required to build digits was already present in our aquatic ancestors.

2) *Tiktaalik roseae*: the emergence of a functional wrist

Described in 2006, *Tiktaalik roseae* is a 375-million-year-old sarcopterygian possessing a striking mosaic of fish-like and tetrapod-like characteristics, earning it the designation of "key transitional fossil" [6]. Among its most notable features are a functional wrist joint and a pectoral fin that represents a key forelimb homolog in the fin-to-limb transition. In a companion paper, Shubin et al. demonstrated that the endoskeleton of *Tiktaalik*'s pectoral fin possessed a tetrapod-like arrangement of humerus-radius + ulna-distal radial elements, consistent with the capacity for substrate-supported locomotion in shallow-water environments [13]. In the same issue of *Nature*, Ahlberg and Clack assessed this discovery as a "firm step" in the transition from water to land [1].

3) *Acanthostega gunnari* and *Ichthyostega stensioei*: the paradox of an aquatic limb

Among the earliest near-complete tetrapod skeletons are *Acanthostega gunnari* and *Ichthyostega stensioei*, recovered from 365-million-year-old strata in East Greenland. These organisms possessed eight and seven digits, respectively [2,14]. Despite the presence of true digits, the limbs of *Acanthostega* were not functionally adapted for terrestrial weight-bearing, as evidenced by its weakly ossified radio-ulna and the retention of a complete gill skeleton. Using a functional adaptive landscape modeling approach, Dickson et al. confirmed that both *Acanthostega* and *Ichthyostega* were primarily aquatic and that their limbs likely first evolved to generate thrust during aquatic locomotion rather than for terrestrial walking [15].

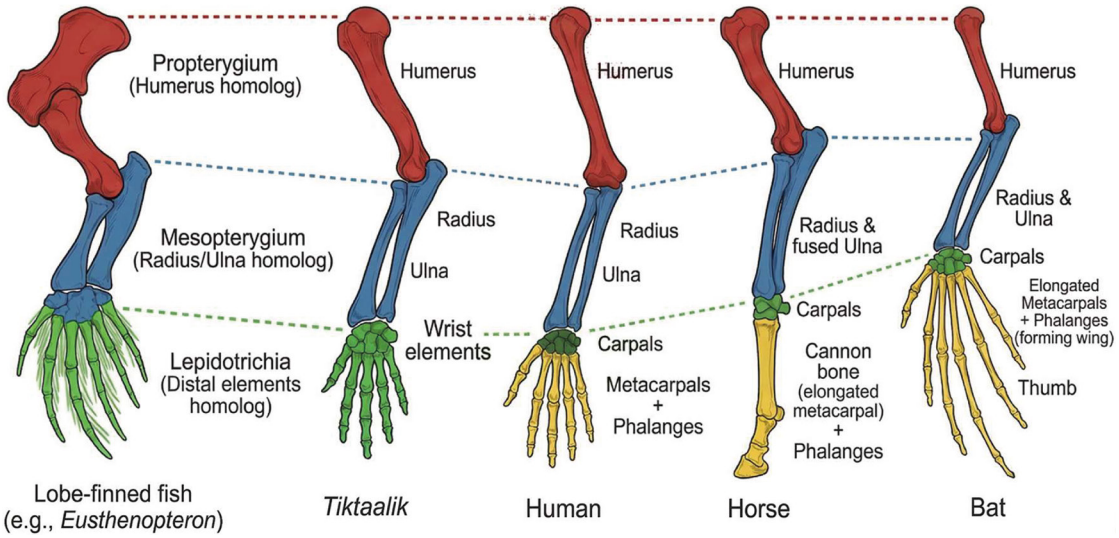

Fig. 3. Skeletal homology across vertebrate forelimbs. The forelimbs of five representative vertebrates—a lobe-finned fish (*Eusthenopteron*), *Tiktaalik*, human, horse, and bat—are compared, with homologous endochondral elements indicated by color coding: humerus/propterygium (red), radius and ulna/mesopterygium (blue), carpals and wrist elements (green), and metacarpals/phalanges (yellow). Dashed lines connect corresponding homologous elements across taxa. The dermal fin rays (lepidotrichia) of the lobe-finned fish, shown in green as distal element homologs, are not direct structural homologs of tetrapod digits, as they are derived from dermal rather than endochondral tissue. However, the shared gene regulatory networks (e.g., late-phase *HoxD* expression) controlling development of both distal fin radials and digits represent a case of deep homology at the regulatory level [16,47].

3. Skeletal homology: from fin radials to digits (Fig. 3)

Converging evidence from comparative developmental biology and fossil morphology has firmly established the skeletal homology between fish fins and tetrapod limbs (Fig. 3). Moving from proximal to distal, the pectoral fin of sarcopterygians contains a humerus homolog, radius and ulna homologs, and numerous distal endoskeletal elements. In tetrapods, these skeletal elements correspond to the stylopod (humerus/femur), zeugopod (radius-ulna/tibia-fibula), and autopod (carpals/tarsals + digits), respectively [13]. Although the dermal fin rays (lepidotrichia) of fish are not structurally homologous to the endochondral digits of tetrapods, recent studies have demonstrated that the enhancers controlling the development of distal fin radials in fish can drive reporter gene expression in the digits of transgenic mice, providing compelling evidence for a deep homology at the level of the underlying gene regulatory network [16]. It is important to note that this represents a regulatory-level homology rather than a structural homology; the shared gene regulatory networks (e.g., late-phase *HoxD* expression) do not imply that fin rays and digits are the same structure, but rather that they are built using a conserved developmental toolkit that has been co-opted and modified during evolution [16,47].

MOLECULAR MECHANISMS: GENETIC REGULATORY CIRCUITS IN LIMB DEVELOPMENT

1. Limb bud initiation: the role of retinoic acid and FGF10

Limb formation occurs when limb buds initially develop from the lateral plate mesoderm (LPM) of the vertebrate embryo. Prior to limb bud development, whether the field will become forelimbs or hindlimbs is determined by a combination of *Hox* genes expressed along the anterior-posterior axis [17]. The formation of the bud itself is initiated by a multi-step process involving Retinoic Acid (RA) and Fibroblast Growth Factor (FGF) signaling. RA is produced in the flank mesoderm, where it acts as a permissive signal that defines the limb-forming field by actively repressing *Fgf8* [8]. Within this permissive field, the transcription factor *Tbx5* (forelimb) or *Tbx4* (hindlimb) activates expression of *Fgf10* in the LPM [18,19]. FGF10 then signals to the overlying ectoderm, where it induces the formation of the Apical Ec-

todermal Ridge (AER), a specialized signaling center that will subsequently direct limb outgrowth [20].

2. Patterning the proximal-distal axis: a shifting paradigm

1) The progress zone mode

How the proximal-to-distal sequence of skeletal elements—stylopod, zeugopod, and autopod—is specified has been one of developmental biology's central questions for the past half century, and our understanding continues to evolve. The first model put forth to address this question was the "progress zone" model, proposed by Summerbell et al. [21]. In this model, mesenchymal cells residing in a region of rapid proliferation at the distal tip of the limb bud are assigned progressively more distal positional values the longer they remain within this zone. Cells that depart the progress zone early would form proximal limb elements (the humerus, for example), while cells that remain longer would adopt distal fates (digits, for example).

2) The early specification and two-signal models

This temporal model was subsequently challenged by experimental evidence suggesting that limb domains may be specified much earlier than originally envisioned. Dudley et al. provided evidence for an "early specification" model, in which proximal and distal domains are largely determined at the bud stage and subsequently expanded through growth [22]. More recent work has integrated these ideas into a "two-signal model." In this model, proximal-distal identity is established by the opposing actions of two signals: a proximal signal of RA emanating from the flank and a distal signal of FGFs from the AER [9,23]. RA activates genes that promote proximal identity (such as *Meis1/2*), while FGF signaling from the AER represses RA activity and promotes distal fates [9]. As the cells of the growing limb bud physically distance themselves from the RA-producing flank, they are progressively specified into zeugopod and then autopod identity through decreasing RA levels and increasing exposure to FGF signaling [24].

3) The permissive versus instructive role of retinoic acid

It should be noted that the role of RA in proximal-distal patterning remains a subject of active debate. While the two-signal model posits RA as an instructive morphogen that directly specifies proximal identity, an alternative view holds that RA plays a more permissive role, being required to establish a competent limb field but not directly encoding positional information along the proximal-distal axis. Cunningham et al. demonstrated that RA signaling is essential for forelimb bud initiation by restricting the domain of *Fgf8* expression, but questioned whether RA acts as a true proximal morphogen [8]. Similarly, Zhao et al. showed that conditional inactivation of RA synthesis in the limb bud did not prevent proximal-distal patterning once the bud had formed, suggesting that RA's primary role may be in the initiation rather than the ongoing patterning of the limb [59]. This ongoing debate highlights the complexity of limb patterning and underscores the need for further investigation into the precise mechanisms by which RA contributes to proximal-distal specification.

3. Hox genes: the blueprint for the limb skeleton (Fig. 4)

Hox genes from both the A and D clusters provide the fundamental molecular code for patterning the limb skeleton. These genes display a property known as spatial and temporal collinearity, whereby 3′ genes (e.g., *Hoxd9*) are expressed early and in the proximal limb bud, while 5′ genes (e.g., *Hoxd13*) are expressed later and more distally [25,26]. This process occurs in two distinct waves. In the early wave, 3′ *Hox* genes specify stylopod and zeugopod identity. In the late wave, the most 5′ genes—particularly *Hoxa13* and *Hoxd13*—are activated to pattern the autopod. These 5′ *Hox* genes are essential for digit formation, as mice lacking functional copies of both *Hoxa13* and *Hoxd13* fail to form digits entirely [27]. It should be noted that Fig. 1 presents a generalized and simplified model of the overlapping *HoxA* and *HoxD* expression domains; the reader is referred to Fig. 4 for a more detailed representation of the phase-specific expression of different *Hox* paralogs (9~13). Notably, fish fins also employ a two-wave system of *Hoxd* expression to pattern their endoskeletons. However, when fish regulatory elements are tested in transgenic mice, they can only drive the first (proximal) phase of expression, not the second. This observation provides key evidence that the autopod evolved through the co-option of a pre-existing developmental program, with novel regulatory inputs driving the late distal phase [28].

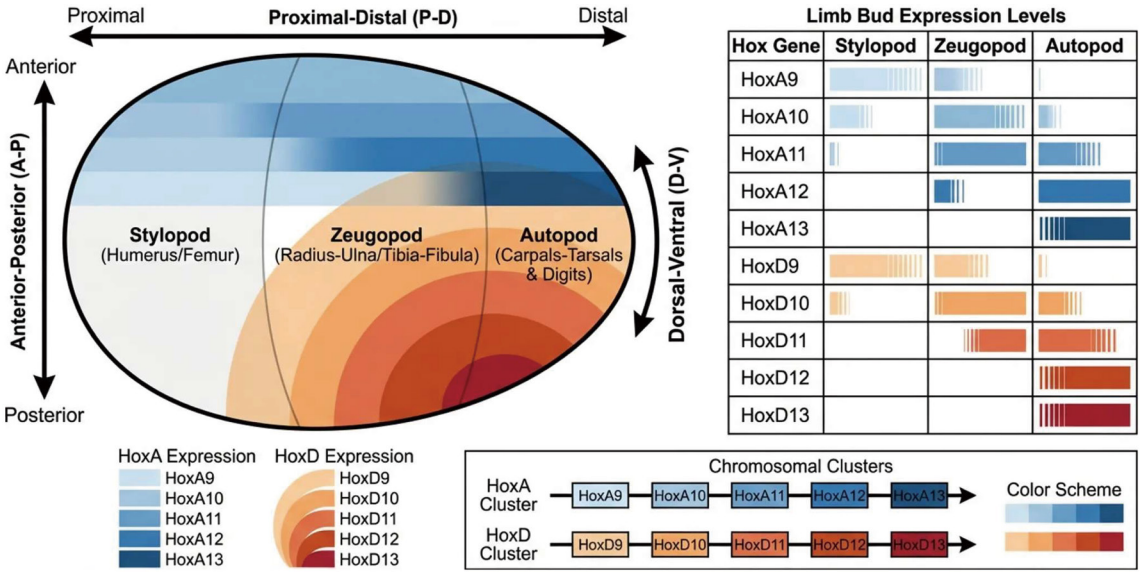

Fig. 4. *Hox* gene expression patterns in the vertebrate limb bud. Left: A schematic of the developing limb bud illustrates the nested, collinear expression domains of *HoxA* (blue shades) and *HoxD* (orange-red shades) genes along the proximal-distal (P-D), anterior-posterior (A-P), and dorsal-ventral (D-V) axes. The three major skeletal regions—stylopod (humerus/femur), zeugopod (radius-ulna/tibia-fibula), and autopod (carpals-tarsals and digits)—are labeled. Upper right: A table summarizes the relative expression levels of each *Hox* gene (*HoxA9-13*, *HoxD9-13)* across the three skeletal zones. Lower right: The genomic organization of the *HoxA* and *HoxD* clusters, illustrating the principle of collinearity [25,26].

4. The SHH/FGF signaling feedback loop: integrating growth and patterning (Fig. 5)

1) The zone of polarizing activity (ZPA) and sonic hedgehog

The outgrowth and anterior-posterior patterning of limbs are linked through a reciprocal feedback loop between two signaling centers: The Apical Ectodermal Ridge (AER) and the Zone of Polarizing Activity (ZPA). Anterior-posterior patterning is regulated by the morphogen Sonic hedgehog (*Shh*), which is secreted from the ZPA in the posterior mesenchyme of the limb bud [29]. The level and duration of SHH received by each cell determines digit identity. Along the posterior-to-anterior axis, the little finger receives the most SHH signal while the thumb receives no SHH signal at all. Transcription factor *Gli3* interprets this morphogen gradient. Anteriorly, where SHH is absent, *Gli3* is proteolytically processed into a repressor form of the protein (*Gli3R*). Posteriorly, where SHH is present, this processing is inhibited and instead a cleavage-resistant activator form (Gli3A) accumulates. The ratio of Gli3A to Gli3R then converts the SHH morphogen gradient into positional information, conferring digit identities along the AP axis of the limb bud [30]. The limb-specific expression of *Shh* is regulated by a highly conserved long-range enhancer called the ZPA Regulatory Sequence (ZRS) over 1 megabase away within an intron of the gene *Lmbr1* [31].

2) The AER-ZPA feedback loop

The ZPA and AER signal to one another in a positive feedback loop required for the maintenance of both structures. SHH from the ZPA causes expression of *Fgf4* and *Fgf8* in the AER. These FGFs then signal back to the ZPA and maintain *Shh* expression [32,33]. In this way, the limb bud is both maintained in its outward growth (FGFs) and its anterior-posterior patterning (SHH). This central circuitry is modified by several other signaling pathways including BMP and Wnt signaling, creating a self-regulatory circuit that controls limb development [34,35].

5. Dorsal-ventral axis specification: the role of Wnt signaling

In addition to the proximal-distal and anterior-posterior axes, the dorsal-ventral (D-V) axis of the limb bud is established by a distinct set of signaling molecules (Fig. 5). *Wnt7a*, a member of the Wnt family of secreted glycoproteins, is expressed specifically in the dorsal ectoderm of the limb

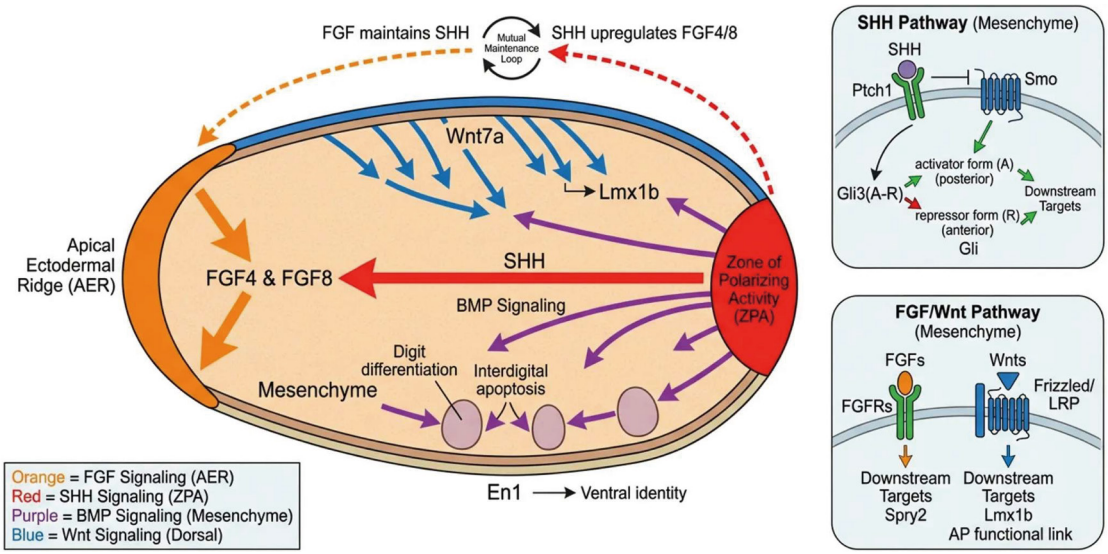

Fig. 5. The AER-ZPA signaling network in limb development. The main panel shows a schematic cross-section of the limb bud, illustrating the key signaling centers and their interactions. Signaling pathways are color-coded: orange, FGF signaling from the AER; red, SHH signaling from the ZPA; purple, BMP signaling in the mesenchyme; blue, Wnt signaling from the dorsal ectoderm. *Wnt7a* (blue arrows) from the dorsal ectoderm activates *Lmx1b* to specify dorsal identity, while *En1* specifies ventral identity. A mutual maintenance loop between AER-derived FGFs and ZPA-derived SHH ensures coordinated growth and patterning [32,33]. BMP signaling regulates both digit differentiation and interdigital apoptosis. The right insets provide molecular details of the SHH pathway and the FGF/Wnt pathway [30,34,35,58].

bud. *Wnt7a* signals to the underlying dorsal mesenchyme, where it induces the expression of the LIM-homeodomain transcription factor *Lmx1b*. *Lmx1b* is both necessary and sufficient to specify dorsal cell fates in the limb; loss of *Lmx1b* function results in a ventralized limb, while ectopic expression can dorsalize ventral structures [58]. Conversely, ventral identity is specified by the transcription factor *En1* (Engrailed-1), which is expressed in the ventral ectoderm. *En1* represses *Wnt7a* expression in the ventral ectoderm, thereby restricting dorsal specification to the dorsal side of the limb [58]. Importantly, the D-V patterning pathway is not independent of the other axes. *Wnt7a* also plays a role in maintaining *Shh* expression in the ZPA, thus linking D-V patterning to the A-P axis. This interconnection between the three axes ensures the coordinated three-dimensional patterning of the developing limb.

ADAPTIVE RADIATION: LIMB DIVERSITY THROUGH REGULATORY DIVERGENCE (FIG. 6)

1. Limb specializations evolved from evolutionarily conserved limb plan

Limbs have evolved into dramatically diverse forms since their emergence in the first tetrapods. Wings (bat), flippers (whale), a single hoof (horse), and loss of limbs entirely (snake) are all morphologies achieved from one evolutionarily conserved limb plan [4]. Novelty is not created through development of new genes, but through modification of how developmental toolkit genes are expressed by altering cis-regulatory elements (CREs) that regulate them [5].

2. Convergent flight adaptations: bat wings and bird wings

The forelimbs of bats (order Chiroptera) and birds (class Aves) are prime examples of evolutionary and functional convergence of limbs. Both structures are used for powered flight and have evolved this function independently of each other. The bat wing is formed by the extreme elongation of digits II~V with the intervening membranes still intact (creating the wing membrane or patagium). Bat wing digits show prolonged BMP signaling during development, resulting in extended chondrogenesis. Additionally, apoptosis does not occur in the regions between digits allowing membranes to remain intact [36,37]. The research of Cretekos et al. showed that divergence of a single limb enhancer for *Prx1* is enough to alter limb proportions between species, demonstrating direct experimental evidence that changes to CREs can have evolutionary effects [38].

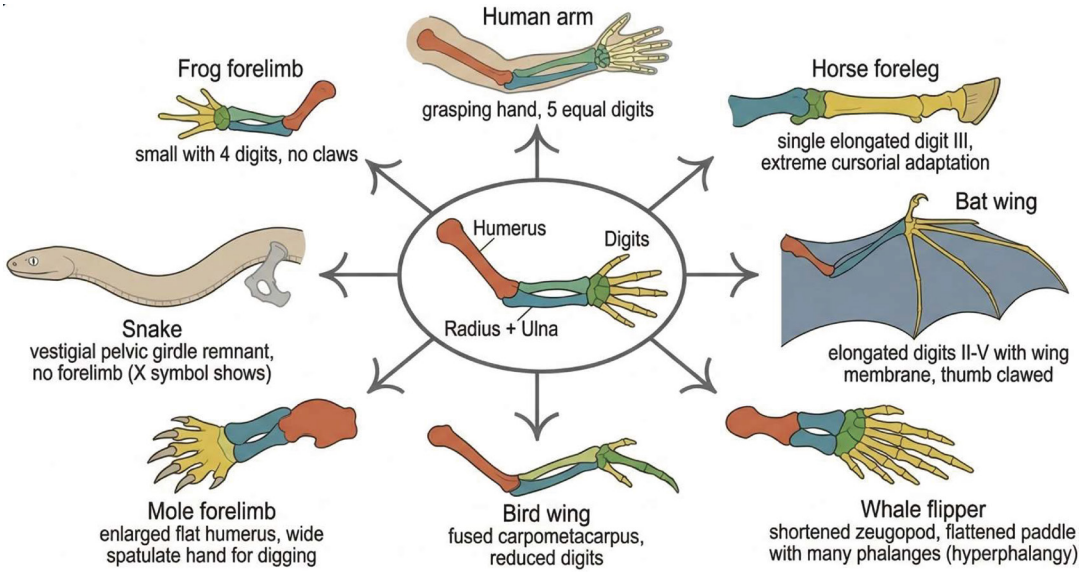

Fig. 6. Adaptive radiation of tetrapod forelimbs. A central diagram of the generalized pentadactyl forelimb skeleton—with labeled humerus, radius and ulna, and digits—is surrounded by eight examples of specialized forms, illustrating the concept of descent with modification. Homologous skeletal elements are color-coded across all forms: humerus (red), radius and ulna (blue), carpals (green), and metacarpals/phalanges (yellow). Radiating outward from the ancestral plan are: the human arm, the frog forelimb, the horse foreleg, the bat wing, the bird wing, the whale flipper, the mole forelimb, and the snake (vestigial pelvic girdle remnant only; complete forelimb loss indicated by X symbol). This figure demonstrates how evolutionary modification of a single conserved skeletal blueprint, driven largely through cis-regulatory changes in developmental genes, can generate a remarkable diversity of functional forms.

Bird wings show elongation of only three digits, however, there has been debate as to which digits these are. Fossil studies of theropod dinosaurs support that bird wing digits are composed of digits I, II, and III. Embryological studies seemed to contradict these conclusions, concluding digits II, III and IV were retained in the bird wing. The large amount of evidence supporting the theropod hypothesis caused a shift in thinking towards a homeotic shift in digit identity during bird development, coined the "frame shift" hypothesis [39-41]. As such, most evidence now supports bird wing digits are I-II-III. Regardless of digit identity, both bat and bird wings demonstrate how changes in degree and timing of developmental programs such as chondrogenesis can create vastly different morphologies that evolve to perform the same function.

3. Digit reduction and specialized adaptations: horses, moles, and frogs

The horse limb is an example of how mammals can radically reduce the number of digits. Originally starting with all five digits, during horse evolution digits I and V were completely lost, while digits II and IV were progressively reduced to the vestigial splint bones that flank the single weight-bearing central digit III [42]. Lopez-Rios et al. demonstrated that this reduction of digits in bovine and equine limbs correlates with attenuated SHH signaling, as detected by reduced expression of the SHH receptor *Ptch1* in the anterior autopod. This attenuation of SHH signaling leads to decreased progenitor cell proliferation, ultimately resulting in the loss of lateral digits [42].

Instead of losing digits, moles have evolved a massively expanded, spatulate hand for subterranean digging. Much of this adaptation is due to the development of an extra digit-like bone called the os falciforme or "sickle bone." This bone is accessory and shaped like a sickle making it part of the sesamoid class. Although it is not true polydactyly, development of this bone allows moles to escape the pentadactyl constraint [43]. Changes in transcriptional heterochrony of the mole autopod (advanced relative to the hindlimb) also play a role in mole limb evolution. This leads to an enlarged and flattened shape of the humerus and expansion of the hand [44].

The frog forelimb also possesses reduced digits (frog limbs usually have only 4 digits). They also lack claws on their digits. In anurans such as *Xenopus*, limb development follows a broadly conserved tetrapod program, yet with notable

modifications. The autopod initially forms as a continuous cartilaginous plate from which individual digits are sculpted through programmed cell death (interdigital apoptosis). The reduction to four digits in the forelimb is associated with altered expression domains of posterior *Hox* genes (particularly *HoxA13* and *HoxD13*), which specify a narrower digit-forming field compared to pentadactyl species. Furthermore, the unique metamorphic transition in frogs, regulated by thyroid hormone signaling, adds an additional layer of complexity to limb morphogenesis that is absent in amniotes [60,61].

4. Limb reduction and loss: whales and snakes

Reduction and complete loss of limbs have also evolved convergently multiple times. Though the limb is completely lost in snakes, we see an intermediate stage of reduction in the whale flipper. While the limb as a whole is much shortened and flattened into the paddle shape we associate with whales, the digits show hyperphalangy or increased number of phalanges per digit. This enhances flipper surface area for aquatic propulsion (Fig. 6). Whale hindlimbs do not develop because the ZPA does not form and *Shh* is not expressed [45].

Limb loss reaches its extreme in snakes. Mutations have accumulated through evolutionary decay of the ZRS enhancer that eliminate key transcription factor binding sites necessary for *Shh* expression in the limb field [11]. Interestingly, Kvon et al. showed that by reverting only a small number of snake-specific mutations in the ZRS enhancer they were able to restore enhancer activity in a mouse reporter assay [11]. This experiment highlights how even small CRE changes can have major effects on macroevolutionary scales. Leal and Cohn later discovered that modular evolution of *Shh* as well as *Hoxd* enhancers played a role in both losing and partially regaining snake hindlimbs in different snake lineages [46].

5. Deep homology: a shared regulatory logic

The diversity described above is underpinned by the fact that the gene regulatory network underlying limb development is highly conserved. Deep homology refers to the idea that the same toolkit genes that pattern the fins of fish also pattern the limbs of tetrapods [4,16]. Gehrke et al. provided compelling evidence for this by demonstrating that enhancers from both zebrafish and gar's *Hoxd* locus that drive expression in the distal portion of the fin could also drive expression in digits of transgenic mice [16]. Nakamura et al. took this a step further by showing that digits and fin rays share common developmental regulatory programs, though this finding should be interpreted as evidence for the re-deployment of a distal *HoxD* enhancer rather than as evidence that fin rays and digits are structurally homologous [47]. Indeed, the concept of deep homology has its limits: while it powerfully explains the conservation of regulatory logic across vast evolutionary distances, it does not imply that all structures controlled by the same toolkit genes are homologous in the traditional, structural sense. Rather, deep homology reflects the evolutionary tendency to co-opt and redeploy existing genetic circuits for new morphological purposes. Thus, deep homology and evolutionarily malleable CREs provide the mechanistic basis for the extraordinary adaptive radiation of the vertebrate limb.

CONCLUSION AND FUTURE PERSPECTIVES

1. Synthesis: a story of tinkering

The evolution of the vertebrate limb is a paradigmatic example of how evolution tinkers with a conserved toolkit to generate novelty. The fossil record provides clear evidence of a stepwise acquisition of limb-like features in our fish ancestors (Fig. 2). Critically, the underlying genetic toolkit required for limb formation—including *Hox* genes, *Shh*, and *Fgf*—was already present in these aquatic pioneers (Figs. 4, 5) [12,16]. The emergence of the autopod, and the subsequent explosion of limb diversity, was not driven by the invention of new genes, but by the repurposing and modification of ancient gene regulatory networks [48].

This review has framed this grand narrative through the lens of deep homology and regulatory divergence. Deep homology explains the conserved blueprint, while regulatory divergence explains the nearly infinite variations on that theme (Fig. 6). From subtle shifts in *Hox* expression that alter digit number [49] to the complete loss of a limb through enhancer decay [11], the story of the limb is written in the language of cis-regulatory DNA.

2. Unresolved questions and future directions

Despite enormous progress, key questions remain. The precise sequence of regulatory changes that drove the fin-to-

limb transition is still being reconstructed. The debate over proximal-distal patterning models continues, with recent evidence suggesting that neither the progress zone model nor the early specification model alone is sufficient to explain all experimental observations [24,50,51]. The interplay between RA and FGF signaling in establishing proximal-distal identity remains an active area of investigation, with some studies questioning the direct role of RA as a proximal morphogen [52,53]. As discussed in Section 3.2, the permissive versus instructive role of RA remains a central unresolved question, and resolving this debate will require more sophisticated conditional genetic approaches in diverse model organisms.

The next frontier lies in leveraging emerging technologies. Single-cell transcriptomics is already providing unprecedented resolution of the cell types and lineage trajectories within the developing limb bud, revealing previously unappreciated cellular heterogeneity and complex regulatory dynamics [54,55]. Comparative genomics in non-model organisms, particularly the lungfish whose giant genome has recently been sequenced [12], will continue to illuminate the genetic starting material for the conquest of land. Chromatin conformation capture techniques (Hi-C) have revealed that the two-phase *Hox* expression system is controlled by a switch between two topologically associating domains (TADs), adding a three-dimensional chromatin architecture layer to our understanding of limb patterning [56,57]. Finally, functional analysis of CREs using CRISPR-based tools across diverse taxa will allow us to directly test the causal links between specific regulatory mutations and evolutionary changes in morphology [11,46]. By integrating these approaches, we can move closer to a complete understanding of one of the most spectacular innovations in the history of life.

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