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The origins, scaling and loss of tetrapod digits

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Many of the great morphologists of the nineteenth century marvelled at similarities between the limbs of diverse species, and Charles Darwin noted these homologies as significant supporting evidence for descent with modification from a common ancestor. Sir Richard Owen also took great care to highlight each of the elements of the forelimb and hindlimb in a multitude of species with focused attention on the homology between the hoof of the horse and the middle digit of man. The ensuing decades brought about a convergence of palaeontology, experimental embryology and molecular biology to lend further support to the homologies of tetrapod limbs and their developmental origins. However, for all that we now understand about the conserved mechanisms of limb development and the development of gross morphological disturbances, little of what is presented in the experimental or medical literature reflects the remarkable diversity resulting from the 450 million year experiment of natural selection. An understanding of conserved and divergent limb morphologies in this new age of genomics and genome engineering promises to reveal more of the developmental potential residing in all limbs and to unravel the mechanisms of evolutionary variation in limb size and shape. In this review, we present the current state of our rapidly advancing understanding of the evolutionary origin of hands and feet and highlight what is known about the mechanisms that shape diverse limbs.

This article is part of the themed issue 'Evo-devo in the genomics era, and the origins of morphological diversity'.

1. Origin of the tetrapod autopod

> 'The Vertebrated animals enjoy as extensive and diversified a sphere of active existence as the Invertebrated. They people the seas and can move swiftly both beneath and upon the surface of water: they can course over the dry land, and traverse the substance of the earth: they can rise above that surface and soar in the lofty regions of aerial space. The instruments for effecting these different kinds of locomotion – diving and swimming, burrowing and running, climbing and flying – are accordingly very different in their configuration and proportions.'
>
> Sir Richard Owen [1, p. 5].

The origin of tetrapod limbs can be traced back to the appearance of paired appendages in jawless fishes (*Agnatha*) approximately 560 million years ago (Ma) [2,3]. Subsequent serial duplication in the earliest jawed fishes (*Gnathostomata*) resulted in two sets of paired appendages, the pectoral and pelvic fins. Within *Gnathostomata*, the bony vertebrates are further subdivided into ray-finned fishes (*Actinopterygii*: the vast majority of modern fish) and the lineage descended from lobed-finned fishes (*Sarcopterygii*: lungfish, coelocanths and tetrapods). The homology between the fins and tetrapod limbs is apparent morphologically in the integration of the fin/limb into the axial skeleton via a single proximal element, the stylopod [4–7]. Further evidence comes from shared mechanisms of induction, growth and patterning during embryonic development. Both originate as mesenchymal buds surrounded by a sheath of ectoderm. The pectoral fin and forelimb buds each require the transcription

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factor *Tbx5* for bud initiation, while the pelvic fin and hindlimb buds similarly require its paralogue *Tbx4* [8–15]. The distal margin at the border of dorsal and ventral ectoderm forms an epithelial thickening called the apical ectodermal ridge (AER) which secretes *Fgf8*, a signalling molecule both necessary and sufficient for subsequent fin and limb bud outgrowth [16–19]. Lastly, posteriorly restricted expression of *Shh* is observed in both fin and limb buds [20,21] where it is necessary for proper anterior–posterior appendage patterning [21–24].

Here, the clear homologies end, and the shared histories of distal appendages become murky. While the limb skeleton of tetrapods and the proximal part of the fin skeleton of fish form by endochondral ossification, whereby mineralized bone is laid down on a cartilage scaffold, the distal fin is composed of rays called *Lepidotrichia* that form by direct ossification in the dermal apical fold. The patchy fossil record of stem tetrapods indicates these lepidotrichia diminished in length and number as the distal endochondral skeleton expanded and branched to form true digits (figure 1). Indeed, the presence of a distal-most digit bearing limb segment, the autopod, is a hallmark feature of tetrapods. While the evolutionary mechanism remains controversial, it is thought that the origin of the autopod lies early within *Sarcopterygii* where homologies to a modern digit bearing autopod can be seen in Devonian stem tetrapods, *Acanthostega* and *Ichthyostega*, that appeared around 360 Ma [27–30]. Like their predecessors, these species were probably entirely aquatic as their limbs lacked flexion at the joints that would be later required for supporting body weight on land [31].

Recent advances in chromatin interrogation and expansion into 'non-canonical' animal models are shedding light on the temporal and spatial control of *Hox* genes and the evolutionary origins of our fingers and toes. Complete loss of both *HoxA* and *D* clusters results in severe limb agenesis [32], and a combined loss of *Hoxa13* and *Hoxd13* results in limbs that completely lack autopods [33–36]. Discrete early and late phases of *Hox* expression pattern the proximal and distal limb structures, respectively. While the early phase of *Hox* expression represents classical collinearity, the late phase of 5′ *Hox* gene expression in the distal limb (paralogous groups 10–13) is characterized by 'reverse collinearity' with *Hoxd13* expression extending to the anterior of the autopod and *Hoxd10–12* more posteriorly restricted [37]. While the genomic regions downstream (telomeric) from the mouse *HoxD* cluster control early *Hoxd* expression, the late autopod expression is regulated by enhancers in the upstream (centromeric) regions [38,39].

Chromosome conformation capture established that the *HoxD* cluster physically interacts with these flanking enhancer regions, and a switch from early telomeric to later centromeric binding brings about the biphasic *Hox* expression observed during limb development [40]. Further, the late *HoxD*–centromeric interactions can control *Hoxd13* expression and digit patterning in a quantitative manner [41]. Although telomeric *Hox* control regions are more ancient and present in the basal chordate *Amphioxous*, the centromeric control regions and the bipartite mechanism for biphasic *Hox* expression are a more recent tetrapod novelty [42]. Super-resolution imaging of the tetrapod *HoxD*–enhancer interactions has confirmed the existence of distinct, physically interacting telomeric and centromeric chromatin compartments called topologically associating domains (TADs) and has opened an avenue to explore the link between epigenetic signatures, chromatin organization, and temporal and spatial control of gene expression during development [43].

Two centromeric *cis*-regulatory modules called *CsB* and *CsC*, together with other centromeric enhancers, are necessary for late, autopod-specific, *Hoxd* expression [41,44,45]. A biphasic *Hox* expression pattern has also been observed in chondrichthyian [6] and basal actinopterygian fishes [46,47] suggesting that it is a common feature of gnathostomes. Consistent with this, homologues of *CsB* and other conserved upstream *HoxD* enhancers have been found in chondrichthyian (skate) and actinopterygian fishes (zebrafish and gar). Interspecies transgenic experiments revealed that *CsB* from skate, zebrafish and gar can promote lacZ reporter expression in the wrist and at the base of developing digits, but not throughout the autopod [48,49].

Interestingly, transgene expression in the zebrafish from mouse-derived *CsB*, tetrapod-specific *CsC* and other centromeric *HoxD* enhancers shows that these elements can be utilized in distal parts of the developing fin, suggesting *trans*-activating factors were present ancestrally and were co-opted during limb evolution [48–50]. Further, ectopic expression of *Hoxd13a* in the distal fin enhances proliferation, distal expansion of chondrogenesis and reduction in fin-folding [50]. These findings support the idea that additional *cis*-regulatory elements in the tetrapod lineage, perhaps including the tetrapod-specific *CsC*, served to modify a pre-existing and conserved gene regulatory network in the distal fin/limb bud. The result may have been to shape the tetrapod limb with its long bones of the upper and lower arm/leg by early *Hox* expression, the long bones of the autopod proper by a late phase of *Hox* expression, and a true wrist/ankle that allowed for flexion, extension and mobility on land by the formation of round mesopodial elements in a region with minimal *Hox* expression [51–53] that is not present in basal Gnathostomata.

Interspecies transgenesis and chromatin interrogation have begun to uncover a conserved *Hox* regulatory system and bimodal TADs in fishes [49,54]. Does a telomeric to centromeric TAD switch, similar to limbs [40], pattern distal fin elements? Do distal fin structures develop in *HoxA* and *D* mutant fish? What is the fate of fin cells experiencing a fish version of 'late' *Hox* expression? Answers to these questions will address some of the challenges in considering homology between fins and autopod [51]. Nevertheless, studies to date highlight the presence of an indelible genetic stamp for the origin of digit bearing autopods from the fins of our fish ancestors, but also forming the basis for the tetrapod limb homologies noted by Darwin [55].

2. Development and evolution of digit number

During the evolution of the tetrapod limb, the number and pattern of elements along the proximal–distal axis (running from the shoulder to the fingers) has remained invariant, while the number and pattern of elements along the autopod anterior–posterior axis (thumb to little finger, also designated digit I to V) has been subject to repeated modifications. In contrast to modern tetrapods with seldom more than five digits, the ancestral autopods were 'polydactylous'. Two striking early examples are Devonian stem tetrapods that existed 360 Ma. *Acanthostega* had eight digits

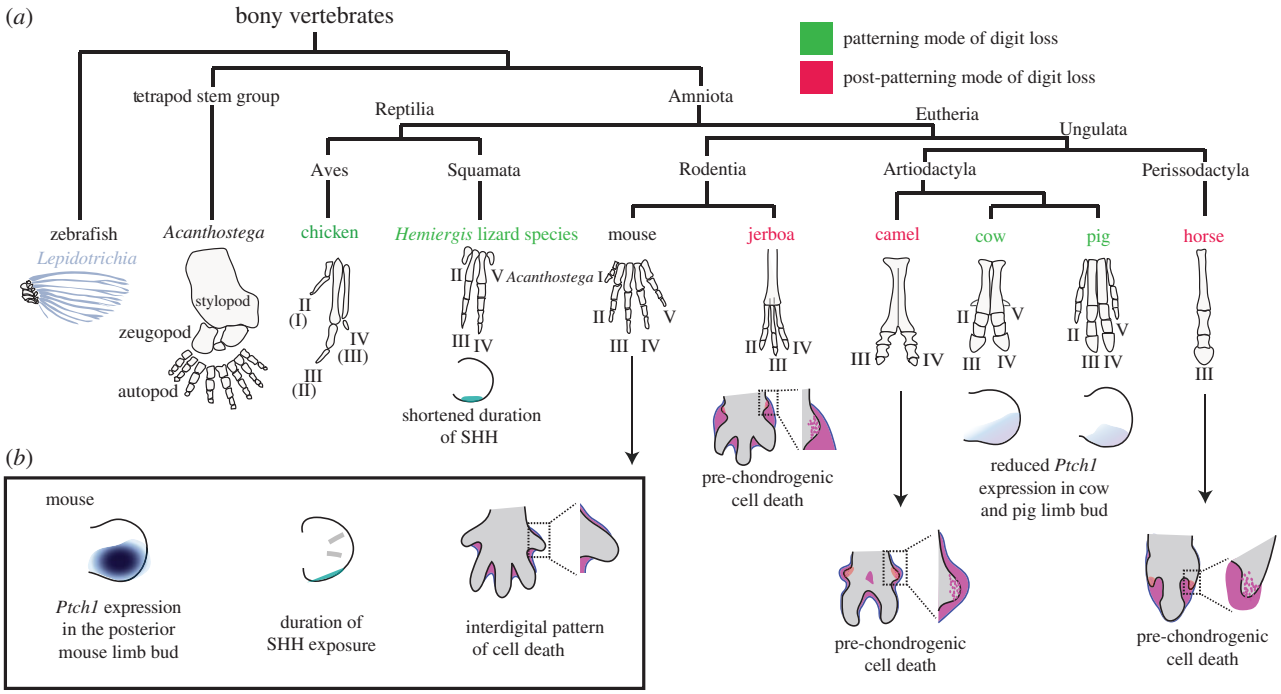

Figure 1. (*a*) Phylogeny of vertebrates illustrating the origin of the tetrapod autopod (*Acanthostega*) since a shared ancestor with modern fish (represented by the zebrafish). Digit loss has evolved repeatedly in tetrapods (six cases illustrated here) via mechanisms that affect the pre-pattern of digits or post-pattern chondrogenesis. For detail of alternate models of digit loss in Aves, see [25,26]. (*b*) Extent of *Ptch1* expression, duration of *Shh* expression, and pattern of interdigital cell death in the mouse as a model of pentadactyl limb development.

in the fossilized forelimb (figure 1) [27,28], and *Ichthyostega* had seven digits in the hindlimb [29,30]. Transition to a pentadactyl ground state is first observed in the limb of *Pederpes* fossils of the Carboniferous period, about 350 Ma [56]. It is still not known exactly when or even how many times digit number was reduced to five. Modern molecular methods in model systems and human syndromes reveal how polydactyly *can* arise and therefore give clues as to how our ancestors may have developed more than five digits. Similarly, mechanisms that give rise to oligodactyly, or fewer than five digits, in model systems provide insight into the mechanisms of convergent digit loss in multiple species since the stabilization of the pentadactyl ground state. It is interesting to note that while evolution of oligodactyly has converged again and again, polydactyly occurs aberrantly in a plethora of species, yet has only re-evolved in a single recognized species of amphibian [57]. This implies a unidirectional constraint on pentadactyly from which advantage is gained by a further reduction in digit count.

Much is known about how the number and identities of the digits are specified, and the secreted morphogen sonic hedgehog (SHH) fulfils a pivotal role. Functional inactivation of *Shh* eliminates digits in the chick wing and mouse hand while a single digit I forms in the feet of both species [58–60]. SHH is produced by the mesenchymal cells of the zone of polarizing activity (ZPA) found at the posterior margin of the limbs of all vertebrates with paired appendages, including the most primitive chondrichthyian fishes [61]. Its expression is driven by a well-conserved limb-specific enhancer called the ZRS (zone of polarizing region activity regulatory sequence) that is located approximately 1 Mb upstream of the coding sequence of *Shh* [62]. Many factors converge on the ZRS to drive the stereotyped expression of *Shh*, including HAND2 and the posteriorly restricted 5′ HOX transcription factors [63]. Inappropriate activation of the ZRS at the anterior margin of the developing limb bud is responsible for polydatcyly in several naturally occurring mutants and knockout mice [64–67]. The ability for *Shh* expression to be modulated specifically in the limb, and therefore not to affect other structures, has perhaps provided a basis for subsequent evolution of digit number. However, most known mutations in the ZRS, with the notable exception of chick *ozd* mutation that abolishes *Shh* expression [68], are associated with additional rather than fewer digits.

The SHH protein is distributed as a gradient across the posterior half of the limb bud. A wealth of evidence, arising from experimental embryology on the chick wing bud, suggests that low concentrations of SHH specify the anterior digit I, while increasing concentrations specify the more posterior digits II and III [69]. An important component of this positional information model involves promotion, by which cells are transiently specified with anterior digit fates before being promoted to more posterior fates [25,69]. Additionally, in the early chick wing bud, SHH promotes expansion of adjacent mesenchyme to generate sufficient progenitor cells for three digits [70,71]. It has also become evident from genetic analyses in the mouse limb that the duration of autocrine SHH signalling may be necessary for specifying the identities of the two posterior digits that arise from the cells of the ZPA (digits IV and V) [72–74]. Further, genetic analyses have indicated an additional role for SHH signalling in the proliferation and survival of specified pre-chondrogenic digit progenitor cells [75].

Members of the GLI family of transcription factors (GLI1–3) are the downstream effectors of SHH signalling, with GLI3 playing the critical role in limb development. SHH signalling prevents proteolytic conversion of GLI3 to its transcriptional repressor form, GLI3R, which is predominantly present in the anterior region of the limb bud where it suppresses *Shh* target genes [76]. Hence, a posterior high to anterior low

distribution of SHH results in an inverse gradient of GLI3R in developing limb buds [24,73,76,77]. Disruption of either *Gli3* protein coding region or deletion of its 3′ regulatory region results in the human Greig cephalopolysyndactyly syndrome (GCPS) [78]. A spontaneous mutation in mice called *Extra-toes* (*Xt*) recapitulates the 3′ *Gli3* deletion in human GCPS and results in polydactyly with as many as eight or nine digits [24,79,80]. *Xt* mutant mice have shown that *Gli3* controls digit number in developing autopods by regulating proliferation and bone morphogenetic protein (BMP)-mediated differentiation of digit-forming cells [81].

Interestingly, *Shh* expression is dispensable for the manifestation of polydactyly in *Gli3*/*Xt* mutants [24,76,82]. GLI3 normally suppresses *5′-Hox* genes in the anterior region of the limb bud [24,76], and loss of this suppression in *Gli3* mutants results in polydactyly, independently of *Shh* [82]. The extent of polydactyly that arises in *Gli3*;*Hoxa13* double mutant mice are impacted by the dosage of *5′-Hoxd* genes such that an additional reduction in *Hoxd11–13* can result in as many as 13 'generic' digits [83]. This finding formed the initial basis for a molecular explanation of the Turing-type reaction–diffusion mechanism long-proposed to regulate the number and periodic pattern of digits [83–85]. In this system, dynamic feedback between an activator and an inhibitor (recently proposed to be BMPs and WNTs, respectively) generates a pattern of digits with periodicity that is 'tuned' by levels of *5′-Hoxa/d* [86]. Interestingly, a WNT–BMP feedback-controlled Turing network has also been proposed in the pattering of chondrichthyian distal pectoral fin elements. This highlights the prevalence of Turing-type mechanisms in patterning of the non-homologous distal fin and limb structures [87]. While many open questions regarding the conserved mechanisms underlying the specification and pattern of digits remain, these data collectively provide a framework on which to build investigations into the mechanisms of autopod evolution.

A perpetually controversial area of limb evolution concerns the loss of digits in the bird wing that enabled flight. This controversy arose out of the long-standing debate over whether modern birds are derived from theropod dinosaurs [88] or a now obsolete group of archosaurian reptiles called the Thecodontia [89]. Nowadays, following the discovery of theropod dinosaurs with bird traits, including feathers, this debate is generally considered settled [90,91]. However, how the bird wing evolved from the dinosaur hand remains a contentious issue. The three digits of the bird wing are morphologically homologous to digits I, II and III of their theropod ancestors and a progressive phylogenetic loss of digits IV and V is documented in the fossil record. The early Triassic theropod *Herrerasaurus* (231 Ma) had four digits (I-II-III-IV) and a rudimentary fifth digit, while later Jurassic theropods, including *Allosaurus* (150 Ma), had three digits (I-II-III), a pattern still present in modern birds, although digit III in birds has fewer phalanges [92]. Other derived theropods exhibited further loss of digits in their forelimbs: *Tyrannosaurus* had an I-II pattern, and the enigmatic *Mononykus* had a single digit I [93]. Recent molecular evidence also supports an I-II-III identification of the digits in the bird wing. RNA sequencing of digit primordia revealed strikingly similar gene expression patterns between digit I of the chick wing and digit I of the chick leg, but with little concordance between the other digits [94]. This adds to previous work that demonstrated *Hoxd13* is expressed throughout the chick wing autopod and *Hoxd12* in all but the digit I forming area, similar to chick leg and mouse limbs [95,96].

Despite fossil and molecular evidence presenting a seemingly convincing argument that identifies the digits of the bird wing as I-II-III, this hypothesis has been difficult to reconcile with embryological evidence. It is suggested that the pre-cartilage condensations of five digit precursors can be detected in the wing buds of several modern birds, including chicken and ostrich, but that only the digits at positions II, III and IV continue to develop, segment and ossify [97–99]. This would imply a more conventional pattern of medial and lateral digit loss, as observed in many mammals and reptiles (discussed in §3), but that clearly contradicts the fossil evidence. Theropod dinosaurs that support a II-III-IV digit pattern have seldom appeared in the fossil record, an example being the Jurassic *Limusaurus* (160 Ma), but as a ceratosaur, it is distinct from the lineage that eventually gave rise to birds [100]. Opponents of the II-III-IV hypothesis have questioned whether the five pre-cartilage condensations could give rise to actual digits in the bird wing, and the possibility remains that the digit I condensation is the vestige of another element called the prehallux [101]. Despite this, weight has been given to the bird digit II, III and IV hypothesis because this would encompass the idea that digit IV is the first digit to form in most tetrapod limbs as part of the 'primary axis of condensation' that is aligned with the ulna [102]. However, digit II condenses first in the limbs of salamanders [103], suggesting that this developmental 'rule' is not universal, at least outside the amniotes.

An elegant solution to the paradoxical palaeontological and embryological evidence is the frameshift theory, also thought to have occurred in Italian skinks, which postulates that digits having the identities I, II and III instead arise from pre-cartilaginous cells found in positions II, III and IV in the limb [104,105]. Such transformations in digit identity have been observed experimentally in the chick wing following the inhibition of SHH signalling [26]. Loss of digits IV and V in the limbs of the earliest theropods implies the frameshift occurred later, yet precisely when is unclear. If indeed this were the case, then the primary axis running through the digit IV position would have been transiently lost, thus weakening the argument for its conservation in amniotes. An alternative theory is the axis-shift hypothesis, which suggests that the primary axis has been displaced into the digit III position in the bird wing [101,103]. This proposal is supported by long-term fate mapping studies of the ZPA in the chick wing using grafts of green fluorescent protein-expressing cells. These experiments revealed that the developmental origin of the digits of the bird wing is the same as digits I-II-III of the mouse limb [25]. However, other interpretations of short-term fate maps of dye-labelled cells in the chick wing support the frameshift theory [106]. Therefore, the debate regarding the pattern of digit loss in the theropod arm/bird wing is likely to continue.

Given the importance of *Shh* in determination of digit number, it is perhaps no surprise that the loss of digits in reptiles [107,108] and mammals [109,110] corresponds to changes in the expression of *Shh* pathway members. Comparison of five different *Hemiergis* lizard species, with digits ranging from two to five in number, showed that species with the fewest digits were those with the shortest duration of *Shh* expression in developing limb buds (figure 1). Premature termination of *Shh* expression correlates with reduced

cell proliferation in the posterior limb bud, and experimentally reduced cell proliferation leads to loss of skeletal elements [70,111]. These observations point to a possible mechanism for evolutionary loss of digits in the *Hemiergis* clade that may have been 'tuned' over time in different species [107]. Premature termination of *Shh* expression has also been suggested for hindlimb digit loss in *Calyptommatus* lizards [108].

The importance of *Shh* signalling in the evolution of digit loss is further illustrated in a subset of the artiodactyls, hoofed mammals including the cow and pig that have a central axis of limb symmetry extending through the interdigital space between digits III and IV. Cattle have two toes (digits III and IV) while pigs have two prominent toes (III and IV) and two that are reduced in size (II and V). Expression of the SHH receptor, *Ptch1*, is highly reduced and limited to the posterior autopod in developing cow [109] and pig [110] forelimb buds compared with mouse (figure 1). In addition to its role in derepressing the SMOOTHENED receptor in response to SHH binding, PTCH1 is thought to sequester and restrict SHH protein distribution [112,113]. Consistent with this hypothesis, the distribution of SHH protein extends farther into the anterior of cow forelimb buds that fail to upregulate *Ptch1* expression, and GLI1 is expanded across the limb field. Further, identification of a limb *cis*-regulatory module (LRM) showed the bovine LRM sequence contains a repeat expansion of variable length in many artiodactyl species, and it fails to promote mesenchymal expansion of a LacZ reporter in transgenic mice [109]. Consistent with these results, limb-specific *Ptch1* loss of function causes oligodactyly in mouse forelimbs in a pattern similar to the cow limb [109,114,115]. Together with *Shh* expression termination in the squamate reptiles, these studies show how modulation of the *Shh* signal or reception frequently contributes to evolution of digit loss.

However, not all mechanisms of digit loss manifest from alterations to the regional specification of the early limb field as evidenced by species that lose digit chondroprogenitors later in autopod development (figure 1). The three-toed jerboa, a desert adapted bipedal rodent, has three hind- and five forelimb digits. Expression of 5′ *Hox* genes and components of the *Shh* pathway do not differ from mouse, but *Bmp4* and *Msx2* are upregulated in the anterior and posterior margins specifically in the hind- but not forelimb [110]. *Bmp4* and *Msx2* together regulate the pattern of interdigital apoptotic cell death, and indeed their expansion corresponds with domains of apoptosis that encompass the tissue distal to the nascent first and fifth digits. As a result, cells that might have been incorporated into the growing metatarsal condensation are instead sculpted away.

Jerboa hindlimb morphology is strikingly similar to the three-toed ancestors of the horse lineage, leading to complete loss of all but the middle digit in modern horses with flanking remnants of the metatarsals of digits II and IV. Similar to the jerboa, cells that may have contributed to the continued development of these medial and lateral digits instead die by apoptotic cell death [110]. Surprisingly, camels, which are highly derived two-toed artiodactyls, do not have the posterior restriction of *Ptch1* that is shared among other species of the clade but instead have a more jerboa and horse-like pattern of apoptosis that carves away progenitors of digits II and V to leave only III and IV. In addition to these two *Shh*-dependent and apoptosis-mediated mechanisms of digit loss, *Fgf8* expression is lost from the AER overlying truncated and missing digits of cow, pig, camel, horse and three-toed jerboa limb buds at digit-forming stages. This suggests that loss of a mitogenic fibroblast growth factor (FGF) signal might play an additional role reinforcing each of the early limb pattern and late apoptotic mechanisms of digit loss [109,110].

Of all tetrapod orders, Amphibia represent extraordinary variation in digit number, with salamanders possessing four digits on the forelimb of most species, yet the least is known of the genetic mechanisms of digit specification in these taxa. The frog species with the most reduced morphology, *Psyllophryne didactyla*, has entirely lost digit I and severely reduced the phalangeal elements of digits II and V while maintaining normal morphology of the central digits. By contrast, salamanders appear to primarily lose digit V and subsequently reduce digits from the posterior to the anterior. In the most extreme example of the cave-dwelling salamander *Proteus anguinus*, only digits I and II remain on its hindfeet. These cases of reduction and loss in salamanders are thought to have evolved by three possible evolutionary mechanisms: reduction in size of the limb mesenchyme through reduced proliferation or developmental arrest [116], failure of digit primordium to separate, and fusion of initially separate condensations [117,118].

Morphometric and phylogenetic analysis has found that salamander digit loss is associated with global developmental arrest (such as in paedomorphosis) and a global slowdown in cellular proliferation (such as in dwarfism) [116,119]. Body size varies dramatically across species, and structures scale while maintaining pattern by altering levels or sensitivity to morphogens [120]. However, as morphogenetic mechanisms of pattern formation are size-dependent within species, sudden scaling of taxa without concomitant scaling of morphogens can have significant morphological and developmental consequences. Therefore, convergent miniaturization of embryos is often accompanied by digit loss. Although the molecular mechanisms that drive amphibian digit reduction and loss remain less clear, blocking SHH signalling with cyclopamine in salamanders and frogs [121,122] and inhibiting BMP signalling during late frog development [123] can both cause digit loss, indicating mechanisms associated with miniaturization may function by limiting the range of signalling pathways common to the development of all tetrapod limbs.

3. Evolution of limb segment proportion in mammals

As the autopod first appeared, its changing proportions and numbers of elements have made it a veritable evolutionary 'Swiss army knife' of diverse functions. The cetaceans shortened each of the phalangeal elements but added more per digit to produce long and flexible flippers while the bat retained the ancestral number of skeletal elements but elongated each to provide the support structure for a membranous wing. Yet for all the evidence of malleability in skeletal proportions, the mechanisms that make one bone longer or shorter than another remain a mystery.

Limb bone elongation proceeds by endochondral ossification at the cartilage growth plate [124]. Proliferating chondroprogenitors give rise to terminally differentiated

hypertrophic chondrocytes that increase in volume up to 20-fold and secrete the mineralized matrix that forms the scaffold upon which mature bone is structured. The increase in hypertrophic chondrocyte volume principally contributes to the daily rate of long bone growth and to the differences in growth rate throughout the body (i.e. proximal versus distal tibia) [125]. Furthermore, differences in cell size largely explain the differences in growth rate between mammal species as evidenced by large hypertrophic chondrocytes of the bat forelimb elements [126] and metatarsals of the three-toed jerboa [127] compared with homologous anatomical positions in the mouse. By contrast, birds vary growth plate cell number to control skeletal proportions [128]. Advanced methods of quantitative phase microscopy identified three distinct phases of chondrocyte volume increase in rodent growth plates [127]. Cells first enlarge by classic hypertrophy followed by a swelling phase of disproportionate cytoplasmic fluid increase and finally a continuation of cellular mass production at constant low density. It is the duration of this third phase that varies between growth plates elongating at different rates. A paracrine IGF1 signal, provided locally at each growth plate, is required in the mouse to establish the differences in cell size by promoting cellular mass production [127,129]. These results suggest a putative molecular mechanism for the control of differential bone growth and species-specific skeletal scaling [127]. Indeed, pathway analysis of RNASeq data from bat fore- and hindlimbs [130] raises the possibility that *Igf-1* may also play a role in morphological divergence of the wing in late fetal development.

The best insights into the molecular mechanisms that control the evolution of skeletal proportions come from research in various species of bats. Transcriptome and chromatin modification assays highlighted thousands of loci that are differentially expressed in the fore- versus hindlimb over developmental time including a number of known limb patterning genes (*5′-Hoxd*, *Tbx3-5*, *Pitx1*, *Msx1* and *2* and *Meis2*) and genes not previously identified in the limb, including *Fam5c*, *Mllt3* and *Lhx8* [130,131]. Speculation on the functions of these genes in bat limb development awaits a genome editing approach in bat or methods to replace homologous stretches of sequence in the mouse. To date, the latter approach of replacing homologous sequence has been attempted only for a single enhancer of the *Prx1* locus resulting in a small but statistically significant and forelimb-specific increase in the length of skeletal elements in part by increasing the mitotic index of proliferating chondrocytes [132].

4. Conclusion

Recent advances in genome sequencing, genome editing, transcriptomics, chromatin interrogation and advanced microscopy can be performed in a wider range of species and have changed the course of developmental biology [133]. Expansion of these approaches to a variety of non-canonical research species stands to further broaden our understanding of limb development and morphological evolution. How does evolution tinker with highly pleiotropic pathways to modify the limb without disrupting other parts of the body? Do long-range limb-specific enhancers reflect a mechanism to segregate some *cis*-regulatory elements responsible for highly evolvable limb-specific traits from other critical pleiotropic functions? Quantitative trait analyses of a variety of adaptive traits in a number of species often identify a single locus of major effect with a collection of modifiers. Are there similarly single major effect loci underlying evolutionary change at greater phylogenetic distances? Are the same gene networks, or even the same loci, utilized repeatedly in cases of convergent evolution? Does evolution of gene regulatory control proceed through modification of previously existing enhancers and/or de novo acquisition of new regulatory modules? We are in an exciting era where we can begin to understand the cause and effect of evolutionary changes that ultimately produced a seemingly endless diversity in limb form.

Competing interests. We have no competing interests.

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References

1. Owen R. 1849 *On the nature of limbs: a discourse*. London, UK: John van Voorst. (Reprinted by Chicago University Press, 2007.) See https://archive.org/stream/Owen1849br46D#page/9/mode/2up.
2. Forey P, Janvier P. 1993 Agnathans and the origin of jawed vertebrates. *Nature* **361**, 129–134. (doi:10.1038/361129a0)
3. Kumar S, Hedges SB. 1998 A molecular timescale for vertebrate evolution. *Nature* **392**, 917–920. (doi:10.1038/31927)
4. Holmgren N. 1933 On the origin of the tetrapod limb. *Acta Zool*. **14**, 185–295. (doi:10.1111/j.1463-6395.1933.tb00009.x)
5. Coates MI. 1994 The origin of vertebrate limbs. *Dev. Suppl*., 169–180.
6. Freitas R, Zhang G, Cohn MJ. 2007 Biphasic *Hoxd* gene expression in shark paired fins reveals an ancient origin of the distal limb domain. *PLoS ONE* **2**, e754. (doi:10.1371/journal.pone.0000754)
7. Johanson Z, Joss J, Boisvert CA, Ericsson R, Sutija M, Ahlberg PE. 2007 Fish fingers: digit homologues in sarcopterygian fish fins. *J. Exp. Zool*. **308B**, 757–768. (doi:10.1002/jez.b.21197)
8. Gibson-Brown JJ, Agulnik SI, Chapman DL, Alexiou M, Garvey N, Silver LM, Papaioannou VE. 1996 Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev*. **56**, 93–101. (doi:10.1016/0925-4773(96)00514-X)
9. Yonei-Tamura S, Tamura K, Tsukui T, Izpisúa Belmonte JC. 1999 Spatially and temporally-restricted expression of two T-box genes during zebrafish embryogenesis. *Mech. Dev*. **80**, 219–221. (doi:10.1016/S0925-4773(98)00219-6)
10. Ruvinsky I, Oates AC, Silver LM, Ho RK. 2000 The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev. Gene Evol*. **210**, 82–91. (doi:10.1007/s004270050014)
11. Szeto DP, Rodriguez-Esteban C, Ryan AK, O'Connell SM, Liu F, Kioussi C, Gleiberman AS, Izpisua-Belmonte JC. Rosenfeld M. G 1999 Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary

development. *Genes Dev*. **13**, 484–494. (doi:10.1101/gad.13.4.484)

12. Rodriguez-Esteban C, Tsukui T, Yonei S, Magallon J, Tamura K, Belmonte JCI. 1999 The T-box genes *Tbx4* and *Tbx5* regulate limb outgrowth and identity. *Nature* **398**, 814–818. (doi:10.1038/19769)
13. Ahn D, Kourakis MJ, Rohde LA, Silver LM, Ho RK. 2002 T-box gene *tbx5* is essential for formation of the pectoral limb bud. *Nature* **417**, 754–758. (doi:10.1038/nature00814)
14. Garrity DM, Childs S, Fishman MC. 2002 The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129**, 4635–4645.
15. Don EK *et al.* 2016 Genetic basis of hindlimb loss in a naturally occurring vertebrate model. *Biology Open* **5**, 359–366. (doi:10.1242/bio.016295)
16. Vogel A, Rodriguez C, Izpisua-Belmonte JC. 1996 Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737–1750.
17. Martin GR. 1998 The roles of FGFs in the early development of vertebrate limbs. *Genes Dev*. **12**, 1571–1586. (doi:10.1101/gad.12.11.1571)
18. Grandel H, Draper BW, Schulte-Merker S. 2000 Dackel acts in the ectoderm of the zebrafish pectoral fin bud to maintain AER signaling. *Development* **127**, 4169–4178.
19. Abe G, Ide H, Tamura K. 2007 Function of FGF signaling in the developmental process of the median fin fold in zebrafish. *Dev. Biol*. **304**, 355–366. (doi:10.1016/j.ydbio.2006.12.040)
20. Krauss S, Concordet J-P, Ingham PW. 1993 A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431–1444. (doi:10.1016/0092-8674(93) 90628-4)
21. Riddle RD, Johnson RL, Laufer E, Tabin C. 1993 Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401–1416. (doi:10.1016/0092-8674(93)90626-2)
22. Eeden FJ *et al.* 1996 Genetic analysis of fin formation in the zebrafish, *Danio rerio*. *Development* **123**, 255–262.
23. Neumann CJ, Grandel H, Gaffield W, Schulte-Merker S, Nusslein-Volhard C. 1999 Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of sonic hedgehog activity. *Development* **126**, 4817–4826.
24. Litingtung Y, Dahn RD, Li Y, Fallon JF, Chiang C. 2002 *Shh* and *Gli3* are dispensable for limb skeleton formation but regulate digit number and identity. *Nature* **418**, 979–983. (doi:10.1038/nature01033)
25. Towers M, Signolet J, Sherman A, Sang H, Tickle C. 2011 Insights into bird wing evolution and digit specification from polarizing region fate maps. *Nat. Commun*. **2**, 426. (doi:10.1038/ncomms1437)
26. Salinas-Saavedra M, Gonzalez-Cabrera C, Ossa-Fuentes L, Botelho JF, Ruiz-Flores M, Vargas AO. 2014 New developmental evidence supports a homeotic frameshift of digit identity in the evolution of the bird wing. *Front. Zool*. **11**, 33. (doi:10.1186/1742-9994-11-33)
27. Coates MI, Clack JA. 1990 Polydactyly in the earliest known tetrapod limbs. *Nature* **347**, 66–69. (doi:10.1038/347066a0)
28. Coates MI. 1996 The Devonian tetrapod *Acanthostega gunnari* Jarvik: postcranial anatomy, basal tetrapod interrelationships and patterns of skeletal evolution. *Earth Environ. Sci. Trans. R. Soc. Edinb*. **87**, 363–421. (doi:10.1017/S0263593300006787)
29. Säve-Söderbergh G. 1932 Preliminary note on Devonian stegocephalians from East Greenland. *Meddelelser om Grønland* **98**, 1–211.
30. Jarvik E. 1996 The Devonian tetrapod *Ichthyostega*. *Fossils Strata* **40**, 1–206. (doi:10.1111/j.1502-3931.1996.tb01839.x)
31. Pierce SE, Clack JA, Hutchinson JR. 2012 Three-dimensional limb joint mobility in the early tetrapod *Ichthyostega*. *Nature* **486**, 523–526.
32. Kmita M, Tarchini B, Zàkàny J, Logan M, Tabin CJ, Duboule D. 2005 Early developmental arrest of mammalian limbs lacking *HoxA/HoxD* gene function. *Nature* **435**, 1113–1116. (doi:10.1038/nature03648)
33. Fromental-Ramain C, Warot X, Messadecq N, LeMeur M, Dolle P, Chambon P. 1996 *Hoxa-13* and *Hoxd-13* play a crucial role in the patterning of the limb autopod. *Development* **122**, 2997–3011.
34. Zákány J, Fromental-Ramain C, Warot X, Duboule D. 1997 Regulation of number and size of digits by posterior *Hox* genes: a dose-dependent mechanism with potential evolutionary implications. *Proc. Natl Acad. Sci. USA* **94**, 13 695–13 700. (doi:10.1073/pnas.94.25.13695)
35. Tarchini B, Duboule D, Kmita M. 2006 Regulatory constraints in the evolution of the tetrapod limb anterior–posterior polarity. *Nature* **443**, 985–988. (doi:10.1038/nature05247)
36. Tarchini B, Duboule D. 2006 Control of *Hoxd* genes' collinearity during early limb development. *Dev. Cell* **10**, 93–103. (doi:10.1016/j.devcel.2005.11.014)
37. Zákány J, Duboule D. 2007 The role of *Hox* genes during vertebrate limb development. *Curr. Opin Genet. Dev*. **17**, 359–366. (doi:10.1016/j.gde.2007.05.011)
38. Spitz F, Gonzalez F, Duboule D. 2003 A global control region defines a chromosomal regulatory landscape containing the *HoxD* cluster. *Cell* **113**, 405–417. (doi:10.1016/S0092-8674(03)00310-6)
39. Tschopp P, Duboule D. 2011 A regulatory 'landscape effect' over the *HoxD* cluster. *Dev. Biol*. **351**, 288–296. (doi:10.1016/j.ydbio.2010.12.034)
40. Andrey G, Montavon T, Mascrez B, Gonzalez F, Noordermeer D, Leleu M, Trono D, Spitz F, Duboule D. 2013 A switch between topological domains underlies *HoxD* genes collinearity in mouse limbs. *Science* **340**, 1234167. (doi:10.1126/science.1234167)
41. Montavon T, Soshnikova N, Mascrez B, Joye E, Thevenet L, Splinter E, Laat W, Spitz F, Duboule D. 2011 A regulatory archipelago controls *Hox* genes transcription in digits. *Cell* **147**, 1132–1145. (doi:10.1016/j.cell.2011.10.023)
42. Acemel RD *et al.* 2016 A single three-dimensional chromatin compartment in amphioxus indicates a stepwise evolution of vertebrate Hox bimodal regulation. *Nat. Genet*. **48**, 336–341. (doi:10.1038/ng.3497)
43. Fabre PJ, Benke A, Joye E, Huynh THN, Manley S, Duboule D. 2015 Nanoscale spatial organization of the *HoxD* gene cluster in distinct transcriptional states. *Proc. Natl Acad. Sci. USA* **112**, 13 964–13 969. (doi:10.1073/pnas.1517972112)
44. Gonzalez F, Duboule D, Spitz F. 2007 Transgenic analysis of *Hoxd* gene regulation during digit development. *Dev. Biol*. **306**, 847–859. (doi:10.1016/j.ydbio.2007.03.020)
45. Lonfat N, Montavon T, Darbellay F, Gitto S, Duboule D. 2014 Convergent evolution of complex regulatory landscapes and pleiotropy at *Hox* loci. *Science* **346**, 1004–1006. (doi:10.1126/science.1257493)
46. Davis MC, Dahn RD, Shubin NH. 2007 An autopodial-like pattern of *Hox* expression in the fins of a basal actinopterygian fish. *Nature* **447**, 473–476. (doi:10.1038/nature05838)
47. Ahn D, Ho RK. 2008 Tri-phasic expression of posterior *Hox* genes during development of pectoral fins in zebrafish: implications for the evolution of vertebrate paired appendages. *Dev. Biol*. **322**, 220–233. (doi:10.1016/j.ydbio.2008.06.032)
48. Schneider I, Aneas I, Gehrke AR, Dahn RD, Nobrega MA, Shubin NH. 2011 Appendage expression driven by the *Hoxd* global control region is an ancient gnathostome feature. *Proc. Natl Acad. Sci. USA*. **108**, 12 782–12 786. (doi:10.1073/pnas.1109993108)
49. Gehrke AR *et al.* 2015 Deep conservation of wrist and digit enhancers in fish. *Proc. Natl Acad. Sci. USA*. **112**, 803–808. (doi:10.1073/pnas.1420208112)
50. Freitas R, Gómez-Marín C, Wilson JM, Casares F, Gómez-Skarmeta JL. 2012 *Hoxd13* contribution to the evolution of vertebrate appendages. *Dev. Cell*. **23**, 1219–1229. (doi:10.1016/j.devcel.2012.10.015)
51. Woltering JM, Duboule D. 2010 The origin of digits: expression patterns versus regulatory mechanisms. *Dev. Cell*. **18**, 526–532. (doi:10.1016/j.devcel.2010.04.002)
52. Villavicencio-Lorini P, Kuss P, Friedrich J, Haupt J, Farooq M, Türkmen S, Duboule D, Hecht J, Mundlos S. 2010 Homeobox genes d11–d13 and a13 control mouse autopod cortical bone and joint formation. *J. Clin. Invest*. **120**, 1994–2004. (doi:10.1172/JCI41554)
53. Beccari L *et al.* 2016 A role for HOX13 proteins in the regulatory switch between TADs at the *HoxD* locus. *Genes Dev*. **30**, 1172–1186.
54. Woltering JM, Noordermeer D, Leleu M, Duboule D. 2014 Conservation and divergence of regulatory strategies at *Hox* loci and the origin of tetrapod digits. *PLoS Biol*. **12**, e1001773. (doi:10.1371/journal.pbio.1001773)
55. Darwin C. 1859 *On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life*. London, UK: John Murray.
56. Clack JA. 2002 An early tetrapod from 'Romer's Gap'. *Nature* **418**, 72–76. (doi:10.1038/nature00824)

57. Hayashi S *et al.* 2015 Evidence for an amphibian sixth digit. *Zool. Lett.* **1**, 17. (doi:10.1186/s40851-015-0019-y)
58. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. 1996 Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407–413. (doi:10.1038/383407a0)
59. Chiang C, Litingtung Y, Harris MP, Simandl BK, Li Y, Beachy PA, Fallon JF. 2001 Manifestation of the limb prepattern: limb development in the absence of sonic hedgehog function. *Dev. Biol.* **236**, 421–435. (doi:10.1006/dbio.2001.0346)
60. Ros MA *et al.* 2003 The chick oligozeugodactyly (*ozd*) mutant lacks sonic hedgehog function in the limb. *Development* **130**, 527–537. (doi:10.1242/dev.00245)
61. Dahn RD, Davis MC, Pappano WN, Shubin NH. 2007 Sonic hedgehog function in chondrichthyan fins and the evolution of appendage patterning. *Nature* **445**, 311–314. (doi:10.1038/nature05436)
62. Lettice LA *et al.* 2003 A long-range *Shh* enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum. Mol. Genet.* **12**, 1725–1735. (doi:10.1093/hmg/ddg180)
63. Galli A, Robay D, Osterwalder M, Bao X, Bénazet J-D, Tariq M, Paro R, Mackem S, Zeller R. 2010 Distinct roles of Hand2 in initiating polarity and posterior *Shh* expression during the onset of mouse limb bud development. *PLoS Genet.* **6**, e1000901. (doi:10.1371/journal.pgen.1000901)
64. Chiang C. 2006 From oligodactyly to polydactyly. In *Hedgehog-Gli signaling in human disease*. New York, NY: Springer.
65. Lettice LA, Hill AE, Devenney PS, Hill RE. 2008 Point mutations in a distant sonic hedgehog *cis*-regulator generate a variable regulatory output responsible for preaxial polydactyly. *Hum. Mol. Genet.* **17**, 978–985. (doi:10.1093/hmg/ddm370)
66. Anderson E, Peluso S, Lettice LA, Hill RE. 2012 Human limb abnormalities caused by disruption of hedgehog signaling. *Trends Genet.* **28**, 364–373. (doi:10.1016/j.tig.2012.03.012)
67. Lettice LA *et al.* 2012 Opposing functions of the ETS factor family define *Shh* spatial expression in limb buds and underlie polydactyly. *Dev. Cell* **22**, 459–467. (doi:10.1016/j.devcel.2011.12.010)
68. Maas SA, Suzuki T, Fallon JF. 2011 Identification of spontaneous mutations within the long-range limb-specific *Sonic hedgehog* enhancer (ZRS) that alter *Sonic hedgehog* expression in the chicken limb mutants *oligozeugodactyly* and silkie breed. *Dev. Dyn.* **240**, 1212–1222. (doi:10.1002/dvdy.22634)
69. Yang Y *et al.* 1997 Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* **124**, 4393–4404.
70. Towers M, Mahood R, Yin Y, Tickle C. 2008 Integration of growth and specification in chick wing digit-patterning. *Nature* **452**, 882–886. (doi:10.1038/nature06718)
71. Bastida MF, Ros MA. 2008 How do we get a perfect complement of digits? *Curr. Opin Genet. Dev.* **18**, 374–380. (doi:10.1016/j.gde.2008.06.009)
72. Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. 2004 Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**, 517–528. (doi:10.1016/j.cell.2004.07.024)
73. Ahn S, Joyner AL. 2004 Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. *Cell* **118**, 505–516. (doi:10.1016/j.cell.2004.07.023)
74. Scherz PJ, McGlinn E, Nissim S, Tabin CJ. 2007 Extended exposure to Sonic hedgehog is required for patterning the posterior digits of the vertebrate limb. *Dev. Biol.* **308**, 343–354. (doi:10.1016/j.ydbio.2007.05.030)
75. Zhu J, Nakamura E, Nguyen M-T, Bao X, Akiyama H, Mackem S. 2008 Uncoupling sonic hedgehog control of pattern and expansion of the developing limb bud. *Dev. Cell* **14**, 624–632. (doi:10.1016/j.devcel.2008.01.008)
76. Welscher P *et al.* 2002 Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* **298**, 827–830. (doi:10.1126/science.1075620)
77. Wang B, Fallon JF, Beachy PA. 2000 Hedgehog-regulated processing of *Gli3* produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* **100**, 423–434. (doi:10.1016/S0092-8674(00)80678-9)
78. Vortkamp A, Gessler M, Grzeschik K-H. 1991 GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. *Nature* **352**, 539–540. (doi:10.1038/352539a0)
79. Johnson DR. 1967 Extra-toes: a new mutant gene causing multiple abnormalities in the mouse. *Development* **17**, 543–581.
80. Hui C, Joyner AL. 1993 A mouse model of Greig cephalo–polysyndactyly syndrome: the *extra–toes* mutation contains an intragenic deletion of the *Gli3* gene. *Nat. Genet.* **3**, 241–246. (doi:10.1038/ng0393-241)
81. Lopez-Rios J *et al.* 2012 GLI3 constrains digit number by controlling both progenitor proliferation and BMP-dependent exit to chondrogenesis. *Dev. Cell* **22**, 837–848. (doi:10.1016/j.devcel.2012.01.006)
82. Sheth R, Bastida MF, Ros M. 2007 *Hoxd* and *Gli3* interactions modulate digit number in the amniote limb. *Dev. Biol.* **310**, 430–441. (doi:10.1016/j.ydbio.2007.07.023)
83. Sheth R *et al.* 2012 *Hox* genes regulate digit patterning by controlling the wavelength of a turing-type mechanism. *Science* **338**, 1476–1480. (doi:10.1126/science.1226804)
84. Wilby OK, Ede DA. 1975 A model generating the pattern of cartilage skeletal elements in the embryonic chick limb. *J. Theor. Biol.* **52**, 199–217. (doi:10.1016/0022-5193(75)90051-X)
85. Newman SA, Frisch HL. 1979 Dynamics of skeletal pattern formation in developing chick limb. *Science* **205**, 662–668. (doi:10.1126/science.462174)
86. Raspopovic J, Marcon L, Russo L, Sharpe J. 2014 Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science* **345**, 566–570. (doi:10.1126/science.1252960)
87. Onimaru K, Marcon L, Musy M, Tanaka M, Sharpe J. 2016 The fin-to-limb transition as the re-organization of a Turing pattern. *Nat. Commun.* **7**, 11582. (doi:10.1038/ncomms11582)
88. Huxley TH. 1868 On the animals which are most nearly intermediate between birds and reptiles. *Ann. Mag. Nat. Hist.* **2**, 66–75.
89. Heilmann G. 1926 *The origin of birds*. New York, NY: D. Appleton and Co.
90. Xu X, Zhou Z, Wang X, Kuang X, Zhang F, Du X. 2003 Four-winged dinosaurs from China. *Nature* **421**, 335–340. (doi:10.1038/nature01342)
91. Zhou Z, Barrett PM, Hilton J. 2003 An exceptionally preserved Lower Cretaceous ecosystem. *Nature* **421**, 807–814. (doi:10.1038/nature01420)
92. Weishampel DB, Dodson P, Osmólska H (eds) 2007 *The dinosauria*, 2nd edn. Berkeley, CA: University of California Press.
93. Currie PJ, Padian K (eds) 1997 *Encyclopedia of dinosaurs*. San Diego, CA: Academic Press.
94. Wang Z, Young RL, Xue H, Wagner GP. 2011 Transcriptomic analysis of avian digits reveals conserved and derived digit identities in birds. *Nature* **477**, 583–586. (doi:10.1038/nature10391)
95. Vargas A, Fallon J. 2005 Birds have dinosaur wings: the molecular evidence. *J. Exp. Zool.* **304B**, 86–90. (doi:10.1002/jez.b.21023)
96. Nelson CE *et al.* 1996 Analysis of *Hox* gene expression in the chick limb bud. *Development* **122**, 1449–1466.
97. Burke AC, Feduccia A. 1997 Developmental patterns and the identification of homologies in the avian hand. *Science* **278**, 666–668. (doi:10.1126/science.278.5338.666)
98. Feduccia A, Nowicki J. 2002 The hand of birds revealed by early ostrich embryos. *Naturwissenschaften* **89**, 391–393. (doi:10.1007/s00114-002-0350-y)
99. Larsson HCE, Wagner GP. 2002 Pentadactyl ground state of the avian wing. *J. Exp. Zool.* **294**, 146–151. (doi:10.1002/jez.10153)
100. Xu X *et al.* 2009 A Jurassic ceratosaur from China helps clarify avian digital homologies. *Nature* **459**, 940–944. (doi:10.1038/nature08124)
101. Chatterjee S. 1998 Counting the fingers of birds and dinosaurs. *Science* **280**, 355. (doi:10.1126/science.280.5362.355a)
102. Cohn M, Lovejoy C, Wolpert L, Coates M. 2002 Branching, segmentation and the metapterygial axis: pattern versus process in the vertebrate limb. *Bioessays* **24**, 460–465. (doi:10.1002/bies.10088)
103. Shubin NH, Alberch P. 1986 A morphogenetic approach to the origin and basic organization of the tetrapod limb. In *Evolutionary biology* (eds MK Hecht, B Wallace, GT Prance). New York, NY: Springer.
104. Wagner GP, Gauthier JA. 1999 1,2,3=2,3,4: a solution to the problem of the homology of the

digits in the avian hand. *Proc. Natl Acad. Sci. USA* **96**, 5111–5116. (doi:10.1073/pnas.96.9.5111)

105. Young RL, Caputo V, Giovannotti M, Kohlsdorf T, Vargas AO, May GE, Wagner GP. 2009 Evolution of digit identity in the three-toed Italian skink *Chalcides chalcides*: a new case of digit identity frame shift. *Evol. Dev.* **11**, 647–658. (doi:10.1111/j.1525-142X.2009.00372.x)
106. Tamura K, Nomura N, Seki R, Yonei-Tamura S, Yokoyama H. 2011 Embryological evidence identifies wing digits in birds as digits 1, 2, and 3. *Science* **331**, 753–757. (doi:10.1126/science.1198229)
107. Shapiro MD, Hanken J, Rosenthal N. 2003 Developmental basis of evolutionary digit loss in the Australian lizard *Hemiergis*. *J. Exp. Zool.* **297B**, 48–56. (doi:10.1002/jez.b.19)
108. Roscito JG, Nunes PMS, Rodrigues MT. 2014 Digit evolution in gymnophthalmid lizards. *Int. J. Dev. Biol.* **58**, 895–908. (doi:10.1387/ijdb.140255jg)
109. Lopez-Rios J *et al.* 2014 Attenuated sensing of SHH by *Ptch1* underlies evolution of bovine limbs. *Nature* **511**, 46–51. (doi:10.1038/nature13289)
110. Cooper KL *et al.* 2014 Patterning and post-patterning modes of evolutionary digit loss in mammals. *Nature* **511**, 41–45. (doi:10.1038/nature13496)
111. Alberch P, Gale EA. 1983 Size dependence during the development of the amphibian foot. Colchicine-induced digital loss and reduction. *J. Embryol. Exp. Morphol.* **76**, 177–197.
112. Chen Y, Struhl G. 1996 Dual roles for Patched in sequestering and transducing Hedgehog. *Cell* **87**, 553–563. (doi:10.1016/S0092-8674(00)81374-4)
113. Jeong J, McMahon AP. 2005 Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1. *Development* **132**, 143–154. (doi:10.1242/dev.01566)
114. Butterfield NC, Metzis V, McGlinn E, Bruce SJ, Wainwright BJ, Wicking C. 2009 Patched 1 is a crucial determinant of asymmetry and digit number in the vertebrate limb. *Development* **136**, 3515–3524. (doi:10.1242/dev.037507)
115. Zhulyn O *et al.* 2014 A switch from low to high Shh activity regulates establishment of limb progenitors and signaling centers. *Dev. Cell* **29**, 241–249. (doi:10.1016/j.devcel.2014.03.002)
116. Alberch P, Gale EA. 1985 A developmental analysis of an evolutionary trend: digital reduction in amphibians. *Evolution* **39**, 8–23. (doi:10.2307/2408513)
117. Shubin N, Wake D. 1996 Phytogeny, variation, and morphological integration. *Am. Zool.* **36**, 51–60. (doi:10.1093/icb/36.1.51)
118. Shubin NH. 2002 Origin of evolutionary novelty: examples from limbs. *J. Morphol.* **252**, 15–28. (doi:10.1002/jmor.10017)
119. Wiens JJ, Hoverman JT. 2008 Digit reduction, body size, and paedomorphosis in salamanders. *Evol. Dev.* **10**, 449–463. (doi:10.1111/j.1525-142X.2008.00256.x)
120. Uygur A, Young J, Huycke TR, Koska M, Briscoe J, Tabin CJ. 2016 Scaling pattern to variations in size during development of the vertebrate neural tube. *Dev. Cell* **37**, 127–135. (doi:10.1016/j.devcel.2016.03.024)
121. Stopper GF, Wagner GP. 2007 Inhibition of Sonic hedgehog signaling leads to posterior digit loss in *Ambystoma mexicanum*: parallels to natural digit reduction in urodeles. *Dev. Dyn.* **236**, 321–331. (doi:10.1002/dvdy.21025)
122. Stopper GF, Richards-Hrdlicka KL, Wagner GP. 2016 Hedgehog inhibition causes complete loss of limb outgrowth and transformation of digit identity in *Xenopus tropicalis*. *J. Exp. Zool.* (*Mol. Dev. Evol.*) **326**, 110–124.
123. Jones TEM, Day RC, Beck CW. 2013 Attenuation of bone morphogenetic protein signaling during amphibian limb development results in the generation of stage-specific defects. *J. Anat.* **223**, 474–488. (doi:10.1111/joa.12098)
124. Long F, Ornitz DM. 2013 Development of the endochondral skeleton. *Cold Spring Harb. Perspect. Biol.* **5**, a008334. (doi:10.1101/cshperspect.a008334)
125. Breur GJ, Vanenkevort BA, Farnum CE, Wilsman NJ. 1991 Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. *J. Orthop. Res.* **9**, 348–359. (doi:10.1002/jor.1100090306)
126. Farnum CE, Tinsley M, Hermanson JW. 2008 Forelimb versus hindlimb skeletal development in the big brown bat, *Eptesicus fuscus*: functional divergence is reflected in chondrocytic performance in autopodial growth plates. *Cells Tissues Organs* **187**, 35–47. (doi:10.1159/000109962)
127. Cooper KL, Oh S, Sung Y, Dasari RR, Kirschner MW, Tabin CJ. 2013 Multiple phases of chondrocyte enlargement underlie differences in skeletal proportions. *Nature* **495**, 375–378. (doi:10.1038/nature11940)
128. Kember NF, Kirkwood JK, Duignan PJ, Godfrey D, Spratt DJ. 1990 Comparative cell kinetics of avian growth plates. *Res. Vet. Sci.* **49**, 283–288.
129. Wang J, Zhou J, Bondy CA. 1999 Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. *FASEB J.* **13**, 1985–1990.
130. Eckalbar WL *et al.* 2016 Transcriptomic and epigenomic characterization of the developing bat wing. *Nat. Genet.* **48**, 528–536. (doi:10.1038/ng.3537)
131. Wang Z, Dai M, Wang Y, Cooper KL, Zhu T, Dong D, Zhang J, Zhang S. 2014 Unique expression patterns of multiple key genes associated with the evolution of mammalian flight. *Proc. R. Soc. B* **281**, 20133133. (doi:10.1098/rspb.2013.3133)
132. Cretekos CJ, Wang Y, Green ED, Martin JF, Rasweiler JJ, Behringer RR. 2008 Regulatory divergence modifies limb length between mammals. *Genes Dev.* **22**, 141–151. (doi:10.1101/gad.1620408)
133. Delgado I, Torres M. 2016 Gradients, waves and timers, an overview of limb patterning models. *Semin. Cell Dev. Biol.* **49**, 109–115. (doi:10.1016/j.semcdb.2015.12.016)