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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

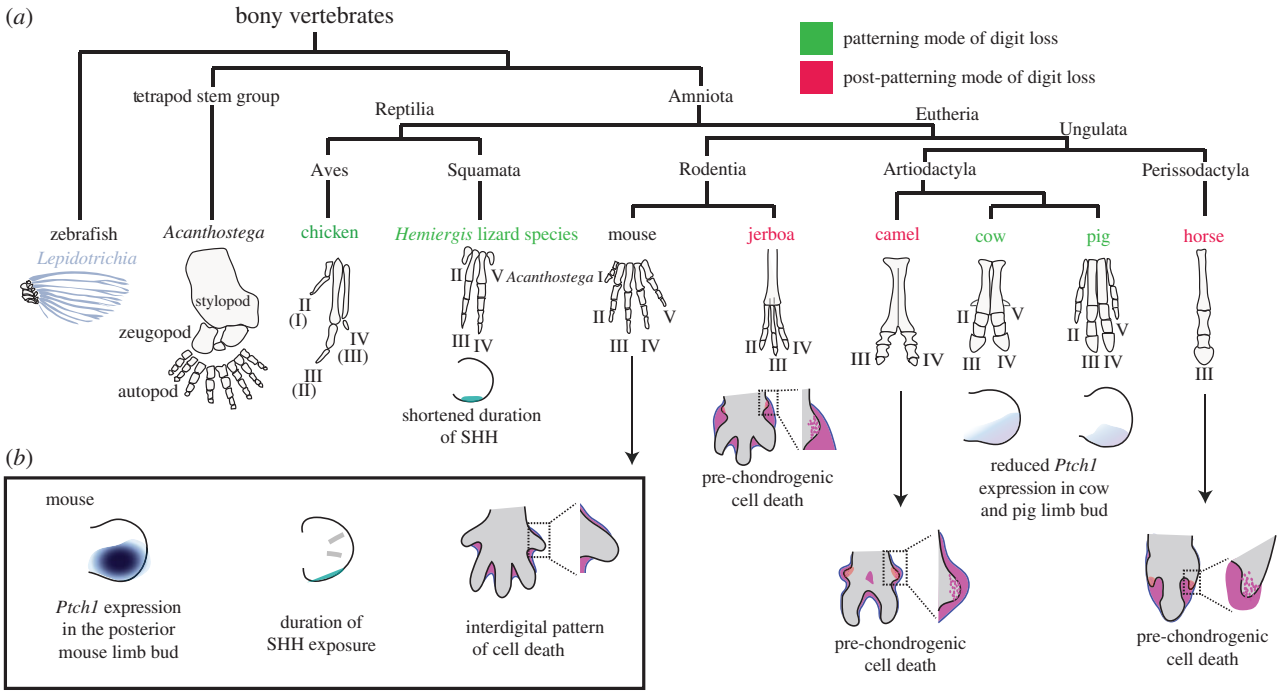

Figure 1. (*a*) Phylogeny of vertebrates illustrating the origin of the tetrapod autopod (*Acanthostega*) since a shared ancestor with modern fish (represented by the zebrafish). Digit loss has evolved repeatedly in tetrapods (six cases illustrated here) via mechanisms that affect the pre-pattern of 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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