Organogenesis in deep time: A problem in genomics, development, and paleontology

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The fossil record is a unique repository of information on major morphological transitions. Increasingly, developmental, embryological, and functional genomic approaches have also conspired to reveal evolutionary trajectory of phenotypic shifts. Here, we use the vertebrate appendage to demonstrate how these disciplines can mutually reinforce each other to facilitate the generation and testing of hypotheses of morphological evolution. We discuss classical theories on the origins of paired fins, recent data on regulatory modulations of fish fins and tetrapod limbs, and case studies exploring the mechanisms of digit loss in tetrapods. We envision an era of research in which the deep history of morphological evolution can be revealed by integrating fossils of transitional forms with direct experimentation in the laboratory via genome manipulation, thereby shedding light on the relationship between genes, developmental processes, and the evolving phenotype.

fossil record | development | genomics | evolution | limb

Paleontologists in recent decades have discovered a host of new taxa that reveal transitional stages in the evolution of birds, whales, mammals, tetrapods, frogs, salamanders, and arthropods (1–9). This pulse of discovery is not an accident, but the result of an elaboration of our ability to identify likely sites for fossil recovery by using increasingly refined phylogenies, stratigraphic maps, and geological records. Likewise, imaging techniques, such as high-energy CT, have opened up old and understudied fossil collections as new vehicles for discovery. With advances in both fieldwork and imaging, the discovery of the phenotypic basis for morphological innovation is at a critical moment in its long history: Novel perspectives on classical questions of anatomical evolution are within our reach.

Fossils, when placed in a phylogenetic context, can reveal taxa with novel combinations of characters that could not be predicted by studying extant creatures alone. If we lacked fossil evidence of mammal-like reptiles, for example, then the physiological and morphological similarities of birds and mammals would likely be interpreted as homologies rather than examples of parallel evolution (e.g., the discredited "Haemothermia" clade) (10, 11). In addition to identifying solid taxonomic groupings, these same fossils reveal transitional series in the origin of the mammalian dentition, ear, and cranium (3). Our understanding of numerous other transformations, from the origin of birds to the origin of tetrapods, is seriously limited without the knowledge of extinct stem taxa.

A rich fossil record permits us to document robustly supported transformation series in the evolution of an anatomical feature, organ system, or body plan. However, to understand the pattern and process of evolutionary transitions, paleontologists have increasingly turned their attention to development. In recent years, the combination of technologies from developmental biology and abundant genomic resources for a multitude of model and nonmodel organisms has greatly enriched our understanding of the genetic and developmental processes underlying organogenesis. This broad set of tools provides a new framework for testing hypotheses derived from paleontological findings, thereby forming an interdisciplinary research program with comparative genomics as well as genetic manipulation of embryonic development (12–15).

Here, we use the evolution and diversification of the vertebrate limb as an exemplar to reveal how discoveries in paleontology can leverage experimental and comparative work in molecular biology, genomics, and embryology. First, we review how fossil analyses of early gnathostomes, coupled with embryological studies, offer the foundation for hypotheses on the origin of paired appendages. Then, we discuss current research on model and nonmodel species that shed light on the origin of digits by comparing gene expression and regulatory mechanisms underlying fin and limb development. Next, we examine recent studies that identify the genetic and developmental basis for digit reduction in tetrapods. Finally, we highlight novel technologies that are enabling biologists to solve century-old evolutionary puzzles with state-of-the-art molecular approaches. The synthesis of modern technology with paleontological findings has been an ongoing topic of interest (16–18). Continued advances in technology now give morphologists an ever-expanding toolkit to test genome function and, ultimately, manipulate genomes in a phylogenetic framework. When these new technologies are coupled with paleontological discovery, new insights into classical questions in evolutionary morphology lie in the offing.

Origin of Paired Appendages

The origin of paired fins is one of the critical events in the history of vertebrates. Two hypotheses, dating back to the 19th century, have been generated to explain this transition: (*i*) the gill-arch hypothesis, in which the posterior-most gill arch is considered to be a precursor to the pectoral girdle and paired fins (Fig. 1*A*) (19), and (*ii*) the fin-fold hypothesis, which holds that paired fins are derived from lateral longitudinal folds that appear early in development and evolution (Fig. 1*B*) (21–23). Both hypotheses were originally proposed from observations of comparative embryology and anatomy of extant sharks. Here, we review how evidence

Significance

Phylogenetic data inclusive of rich paleontological records can be used to inform hypotheses on evolutionary transformations. These data, when combined with developmental studies and functional genomic assays in model and nonmodel organisms, expand our understanding of the evolutionary processes that build and pattern the vertebrate body plan. Here, we highlight a direction of the fossil record, one of "experimental paleontology," where morphological transformations inferred from the fossil record can be experimentally assayed in the lab. With the addition of genomic techniques to test hypotheses, researchers can now begin to explore genomic states that have influenced both past and present morphological diversity.

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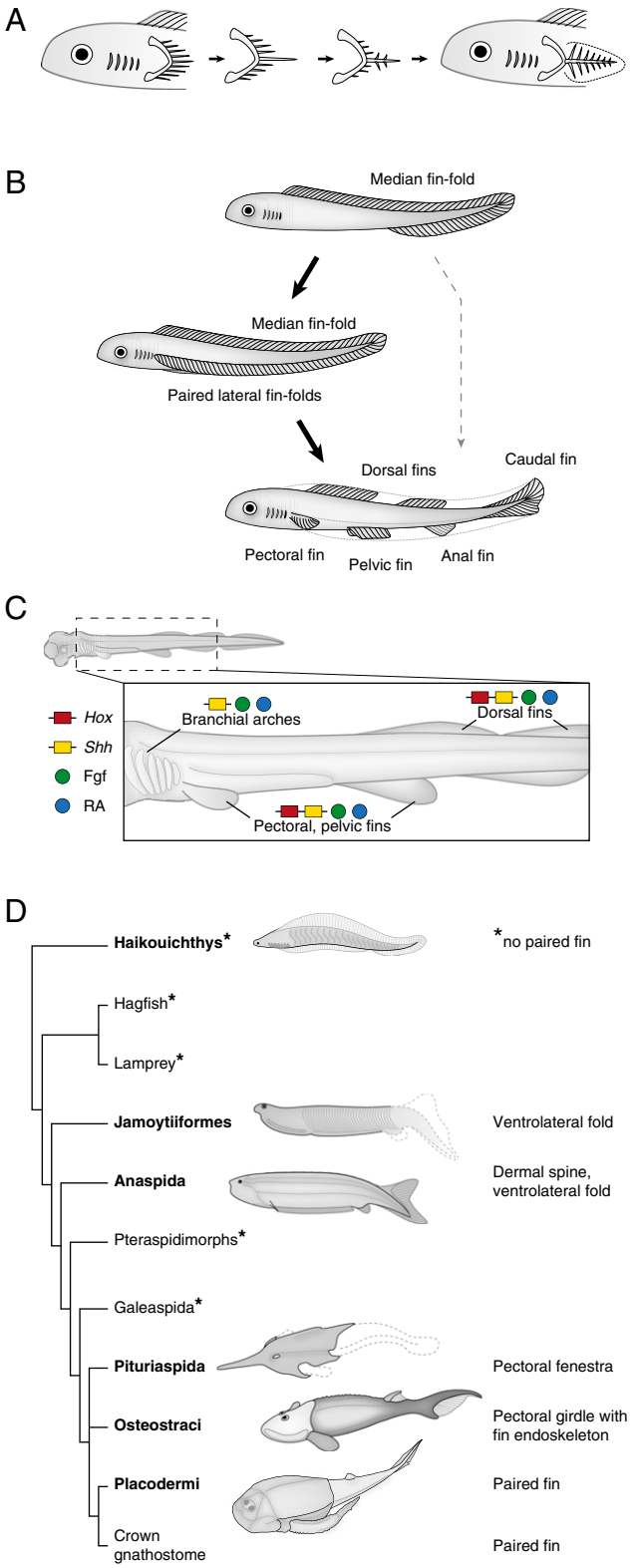

Fig. 1. Origin of vertebrate paired fins. Scheme of the gill-arch hypothesis (*A*) and the fin-fold hypothesis (*B*), which supposes redeployment of the median fin developmental program through paired lateral fin folds or an unknown process leading to pectoral and pelvic fins. (*C*) Shared genetic features of gill arches, paired, and median fins in a generalized gnathostome embryo. (*D*) A phylogenetic tree of early vertebrates, modified from ref. 20. Taxa with associated illustrations are shown in bold. All vertebrates listed are agnathans except Placodermi and crown gnathostomes.

accumulated over the past 120 years has helped evaluate these two hypotheses and assess new fossil, developmental, and molecular data from a variety of species that combine to reveal a third scenario for the origins of paired fins.

The general structure of paired fins of extant vertebrates consists of a shoulder girdle connected to a series of radials that, themselves, articulate with a distal dermoskeleton. According to the gill-arch hypothesis, the shoulder girdle and fin skeleton gradually evolved from the gill endoskeleton and constituent gill rays (Fig. 1*A*). Currently, two pieces of evidence from chondrichthyan biology are used as support for this hypothesis. Morphological evidence shows that gill structures and pectoral girdles of sharks exhibit similar shape and position within the body (19). Another line of evidence comes from chondrichthyan embryology—namely, skates. A recent study showed that the fibroblast growth factor-sonic hedgehog-retinoic acid (FGF-SHH-RA) signaling axis, key to fin development and patterning, is also deployed in skate gills (Fig. 1*C*) (24). However, *Shh*, *Fgf*, and RA pattern other tissues during development, including median fins (25, 26), implying that whatever similarities exist between gill arches and fins may not reflect transformations of arches into appendages, but the co-option of gill signaling networks by fins. Morphological observations of Paleozoic fossils further weaken the gill-arch hypothesis, showing that primitive sharks have an osteichthyan-like gill structure. This finding suggests that modern chondrichthyan gills represent a derived condition (27). Another shortcoming of this hypothesis is the absence of fossils that show transitional gill-like fin structures. Thus, more evidence from both fossil and developmental work is needed to provide robust support of the gill-arch hypothesis.

Founders of the fin-fold hypothesis proposed that lateral fin folds are an iteration of median fin folds of ancestral agnathans, which were then specialized into two separate paired appendages, the pectoral and pelvic fins (Fig. 1*B*) (21–23). Central to this hypothesis was the similar anatomical configuration of median and paired fins (21, 22). The presence of a transient ectodermal thickening along each side of the body in chondrichthyan embryos seems to support an ancestral lateral fin fold (23). In addition, it has been shown that the flank region has the competency to produce extra limbs, which could be a developmental remnant of a lateral fold (28, 29). Finally, as mentioned before, similar signaling cues pattern median and paired appendages (Fig. 1*C*) (25, 26, 30). Together, these findings support the recruitment of median fin developmental programs to the paired fins, yet the precise mechanism of this process remains elusive (Fig. 1*B*).

Initially, fossil data appeared to support the fin-fold hypothesis, because extinct agnathans, such as jamoytiids and anaspids, possess ventrolateral fin folds (Fig. 1 *B* and *D*). However, lateral folds found in these stem gnathostomes are unevenly distributed in the phylogenetic tree and are interpreted as having convergently evolved (Fig. 1*D*) (20, 31, 32). Furthermore, these lateral folds differ substantially from paired appendages in lacking bony pectoral or pelvic girdles (31–33).

A reevaluation of gnathostome fossils provides an alternative scenario for the origin of paired fins. Placoderms, the sister group to the crown gnathostomes, possess pectoral and pelvic fins supported by girdles comparable to those of modern fishes. Their closest relatives, osteostracans and pituriaspids, have pectoral fins morphologically similar to those of primitive placoderms and lack lateral fin folds or pelvic fins (Fig. 1*D*) (32, 33). Furthermore, both basal placoderms and osteostracans have a shoulder girdle with a single endoskeletal element in articulation (31–35). These observations suggest that the primitive gnathostome condition is a paired pectoral fin with a single skeletal element connecting the fin to the girdle. This finding contrasts with the fin-fold hypothesis, which predicts a lateral fold that gives rise simultaneously to both anterior and posterior fins, each composed of multiple radials.

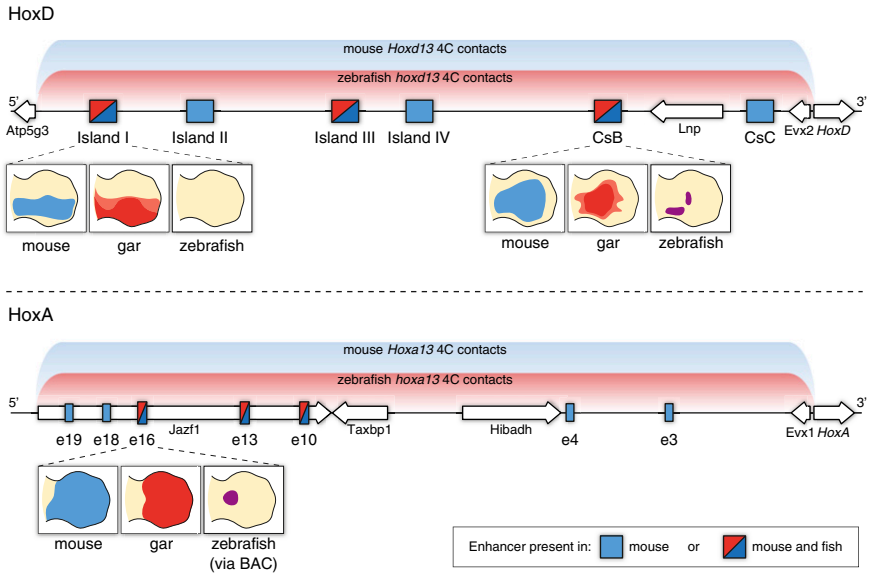

Fig. 2. Epigenetic profiles and enhancer conservation of the vertebrate "autopod" *Hox* regulatory region. *HoxD13* and *HoxA13* show extensive contacts (as defined by 4C-seq and shown generally as red and blue regions above genomic areas) with the region 5′ to the cluster, defining an "autopod building" regulatory topology that is shared in both mouse and zebrafish (blue and red regions above the clusters, respectively) (39–42). A number of individual enhancers that drive expression in the wrists and digits of mouse are also present in fish genomes (Island I, CsB, e16, e13, and e10) (42–44). Both zebrafish and pufferfish sequences were unable to drive reporter activity in the digits of transgenic mice, whereas those of gar (a nonteleost fish with an unduplicated genome) were able to drive robust expression throughout the autopod of transgenic mice (41, 42).

Although the full details of the origin of paired fins, whether directly from median fin folds or via lateral fin folds (Fig. 1*B*), remain to be determined, it seems likely that a redeployment of the median fin developmental program occurred in the origin of paired appendages. Although we know a great deal about paired appendage development, little is known of median fin initiation and patterning. Comparative analyses of gene expression and regulation in median fins may provide us with new clues as to the origins of paired fins.

Fins to Limbs

As we move crownward along the gnathostome tree of life, both fossil and molecular data conspire to reveal transformations in the structure of fins and limbs. In particular, considerable attention has been focused on the evolution of the defining feature of limbs: the presence of the wrist and digits (autopod). The autopod has held a particular fascination with evolutionary biologists for two main reasons. First, this structure provides a level of flexibility and precise tactile motion that was likely crucial to the radiation of tetrapods in diverse environments. Second, the autopod appears to be an anatomical novelty, in that there is no obvious counterpart to the wrist and digits in living fishes based on morphology (36, 37). The fossil record has greatly improved our understanding of the evolution of the autopod, with extinct intermediate forms that reveal an aquatic origin of tetrapod apomorphies in sarcoptyergian fish that was progressively built through elaboration of the distal endochondral skeleton and reduction of the dermal one (36). These insights from fossils lead to a number of questions: Notably, do the fins of living fishes have the equivalent of an autopod? Is the autopod a true anatomical novelty that first appeared in extinct sarcopterygian fish such as *Tiktaalik* (4), or is it a part of even more ancient fins? Do the genetic mechanisms that build the wrists and digits of extant tetrapods have antecedents in fish? These questions are difficult to answer through morphology alone, but they can be addressed in concert with data from developmental biology and functional genomics.

The molecular mechanisms of tetrapod appendage development have been studied in detail in mouse limbs and provide a framework for comparison with fish fins. Limb development in mouse relies on expression of the *Hox* family of transcription factors, where specific deletions of *Hox* activity manifest as losses of discrete portions of the limb (38). The autopod is built via a distinct "late" phase of *HoxD* and *HoxA* gene expression that is controlled by a series of enhancers that lie 5′ to the clusters (Fig. 2) (39, 40). Studies in a variety of fish species (i.e., paddlefish, catshark, zebrafish) have found a late-phase-like pattern of *Hox* gene expression in the distal portion of developing pectoral fins (30, 45, 46). Although these patterns are intriguing, comparisons of gene expression patterns alone can be misleading (47). Thus, dissecting the regulatory architecture underlying the expression of these genes is necessary to define homologous domains of activity.

Recent work has sought to elucidate this regulatory landscape using functional genomics and developmental biology in a variety of fish species (41). Woltering and colleagues reasoned that if fish do contain a late-phase *cis*-regulatory apparatus, their chromatin state at the 5′ end of the *Hox* clusters should be "open" later in fin development, an epigenetic state that has been well documented during mouse digit formation (Fig. 2) (39, 41). This hypothesis is reasonable: Only open chromatin can be transcribed during development. The authors performed chromatin conformation capture experiments on whole-body zebrafish embryos and found that the 5′ genomic region was indeed in an open and accessible conformation (implying regulatory action) in comparison with the region 3′ to the cluster (Fig. 2) (41). However, when tested in transgenic mice, these *cis*-regulatory domains were not able to drive reporter expression in developing digits (Fig. 2) (41). These results led the authors to conclude that the late-phase regulatory region present in fishes is insufficient to build an elaborate distal endoskeleton comparable to an autopod, making it an innovation of tetrapods (41).

Further insight has come from nonmodel species. The majority of genomic work in this area has been performed by using model species (*Fugu*, *Tetraodon*, and zebrafish), all of which are teleosts. This group may not be the ideal genomic model because the

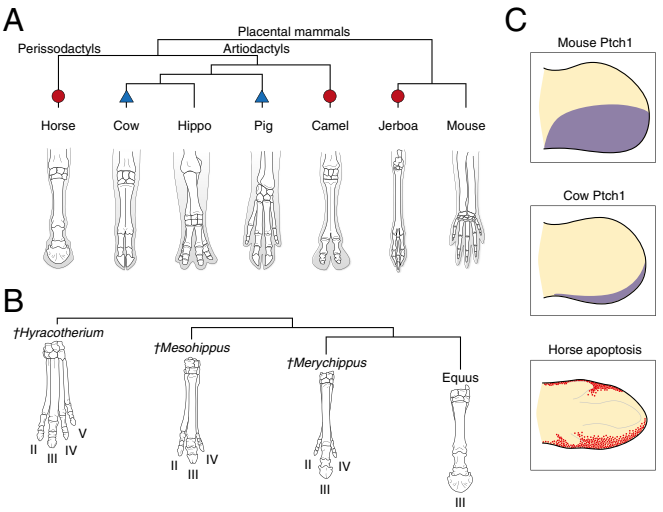

Fig. 3. Evolutionary diversification of limbs. (*A*) Phylogeny of mammalian taxa as per ref. 48, showing groups that have independently lost digits. Symbols denote the developmental mechanism sculpting limbs: cell apoptosis (red circle) and reduced *Ptch1* expression (blue triangle) (49, 50). Skeletal limb morphology of adult organisms highlights the extent of digit reduction among taxa. (*B*) Equine fossil specimens document deviations from a pentadactyl state. (*C*) Pre- and postdigit patterning changes associated with alterations to limb morphology. (*Top*) Mouse forelimb showing broad *Ptch1* expression in ectoderm and mesenchyme at embryonic day 11.25. (*Middle*) Cow forelimb expressing *Ptch1* restricted to ectoderm at gestational day 34 (50). (*Bottom*) Schematic of labeled apoptotic cells in horse forelimb at 34 d after conception (49).

teleost lineage-specific genome duplication could potentially allow reshuffling of genomic sequence around the duplicated *Hox* clusters. Gehrke and colleagues used the genome of a nonmodel bony fish that diverged before the teleost genome duplication—the spotted gar—to identify specific enhancers that are common between mouse and fish, which was not possible using the genomic sequence of teleosts (Fig. 2) (42). The authors found that these gar enhancers were able to drive robust expression of the wrist and digits of transgenic mice, in a pattern nearly identical to their orthologs in mouse (Fig. 2) (42). These findings suggest that the inability of teleost fish enhancers to drive expression in the digits of transgenic mice is due to the derived nature of teleost genomes, and the unduplicated genome of the gar better represents the ancestral condition. These regulatory data define the late-phase *Hox* expression in fish fins and tetrapod limbs as homologous, in turn suggesting that at least a portion of the autopod is an ancestral feature that is represented by the distal bones of fish pectoral fins, particularly Devonian sarcopterygians (42). This example reveals the reciprocal illumination of paleontological, phylogenetic, and molecular approaches: Fossils revealed that an autopod is definitively present in at least one lineage of ancient fish, thereby suggesting new molecular studies of phylogenetically relevant nonmodel organisms.

With such conserved developmental networks, we can now ask the question: How does morphological disparity in limbs arise? Recent work in digit evolution suggests that subtle modifications to ancient networks may be the answer.

Limb Diversification: Convergence in Mechanism and Phenotype

Tetrapod limbs are often described as pentadactyl. The reality is that, when one considers both the fossil record and variation of extant taxa in a phylogenetic context, a five-digited appendage is neither primitive nor fixed (Fig. 3 *A* and *B*). Basal Devonian tetrapods have appendages with six or more digits, as do plesiosaurs (51, 52). Indeed, between fossil and extant appendages, there is a rich diversity of digital number, with patterns of limb reduction evolving numerous times independently within lissamphibians, squamates, mammals, and archosaurs. As a rule, however, extant tetrapod taxa do not have more than five digits per limb, despite the fact that polydactylous mutant phenotypes are common in a number of taxa, including humans.

The quest from both a paleontological and developmental perspective has been to look for underlying regularities and mechanisms behind the process of reduction. Among vertebrates, the loss of peripheral digits before central ones is a common sequence best described by Morse's law (53), in which digits I and V are more readily lost (Fig. 3*B*). Urodele amphibians are the only taxon that has a major violation of this rule—salamander species lose digits V and IV sequentially (54, 55).

Two general classes of developmental shifts could account for the loss or reduction of digits. One way is through a change in the specification of digit primordia during early limb development—i.e., fewer, or smaller, digital condensations are generated. Digital reduction could also arise from the specification of digital primordia with a later deviation from this "ground state" through cell death or changes in development of the digit primordia themselves, resulting in changes to cell proliferation, diminished growth, or fusion.

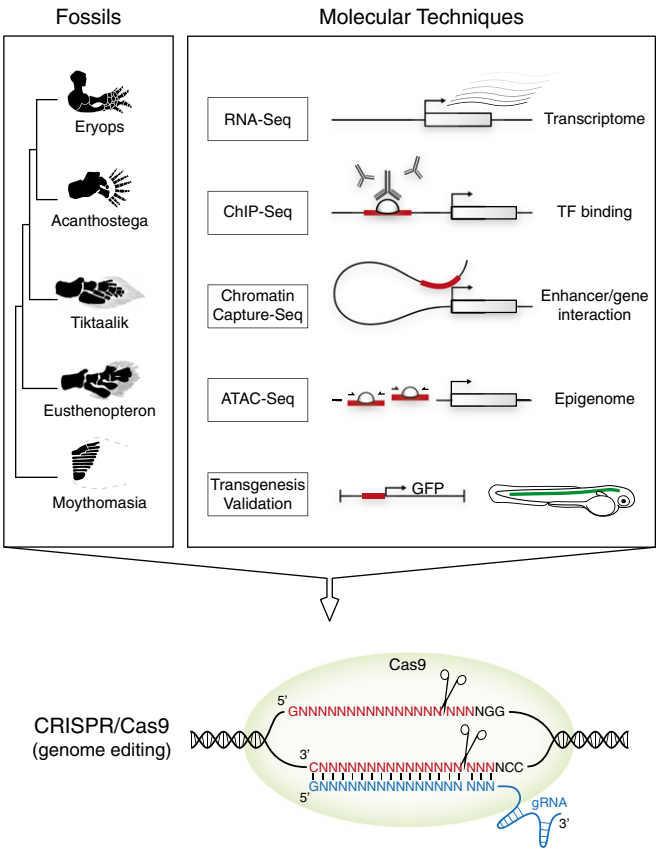

Fig. 4. Experimental paleontology. (*Left*) Fossil evidence demonstrates an anatomical transition of body plan. (*Right*) Modern epigenomic techniques can identify candidate loci that contributed to this transition in the genome of extant organisms (41, 50, 66–68). This synthesis of paleontological data and functional genomics form a model of phenotypic change that can be tested by direct manipulations of the genome (*Lower*) (69), bringing us closer to understanding the evolutionary course of morphological transformations.

Classic experiments by Alberch and Gale sought to explore taxon-specific developmental mechanisms of digit loss by using anurans and urodeles as exemplars (55, 56). Digit loss in these taxa appears unrelated to cell death, but dependent on cell number in the developing limb bud; mitotic inhibitors brought about taxon-specific patterns of reduction. More recent analyses in urodeles extended these results to show that the duration of *Shh* expression, and likely extent of cell proliferation, is correlated to the number of digits that ultimately form (54). A similar relation between *Shh* expression and digit loss was uncovered in squamates (e.g., *Hemiergis*) (57). Together, these data reveal one likely pattern of parallel evolution in lissamphibians and amniotes: a relation of digital loss to changes in the duration of *Shh* activity and cell number during the specification of digit primordia (58, 59).

However, how general are these mechanisms phylogenetically and developmentally? Digital reduction has happened multiple times in amniotes; mammals with reduced appendages, for example, reveal a range of cursorial and saltatory adaptations. Comparative studies of perissodactyls, artiodactlys, and rodents offer clues to likely genetic factors involved. In mouse, the SHH receptor *Patched1* (*Ptch1*) is expressed in both the posterior mesenchyme and ectoderm of developing limb buds and restricts the movement of SHH across the limb (Fig. 3*C*, *Top*) (60, 61). In the highly reduced digital pattern of a bovid (*Bos taurus*), *Ptch1* expression is restricted to the posterior ectoderm, resulting in attenuation of SHH signaling (Fig. 3*C*, *Middle*) (50). Experimentally disrupting *Ptch1* during mouse limb development resulted in both a change in the central axis (a kind of paraxonic pattern seen in artiodactyls) and oligodactyly, indicating that loss of *Ptch1* is sufficient to phenocopy digital features of the cow limb (50). As such, the reduction of *Ptch1* expression in cow provides a molecular clue for the loss of digit asymmetry in bovids. Pigs, another artiodactyl taxon, also reveal posteriorly restricted *Ptch1* expression in forelimbs, suggesting a role for alterations of the SHH pathway in mammalian limb reduction.

As more taxa are added to the analysis, other mechanisms for reduction emerge. In a basal artiodactyl, such as a camel, Cooper et al. discovered that *Ptch1* expression is unaltered in camel limb mesenchyme. Rather, programmed cell death sculpts the autopod after digit patterning has taken place (49). Investigation of perissodactyl (horse; *Equus*) forelimbs (Fig. 3*C*, *Bottom*) and rodent (three-toed jerboa; *Dipis saggita*) hind limbs found that autopodial remodeling results from cell apoptosis and expansion of *Msx2* expression, a transcription factor associated with apoptotic pathways (62). This finding suggests the possibility that the mechanisms for digit reduction were coopted from pathways controlling interdigital cell death in the limb (49).

These studies show that digit reduction through cell apoptosis appears to be a convergently evolved trait among rodents (jerboa), perissodactyls (horse), and some artiodactyls (camel) (Fig. 3*A*). However, among other amniotes—squamates (e.g., *Hemiergis*), derived artiodactyls such as cow, and pig—alterations to SHH signaling causes early patterning changes during limb development. The message from both paleontology and development is one of extraordinary flexibility: The independent evolution of common patterns of digital reduction can result from the parallel evolution of different kinds of genetic and developmental perturbations in diverse taxa.

A Future of the Fossil Record

For decades, paleontologists have, in fits and starts, discussed ways to synthesize molecular and geological data to understand the rates and patterns of evolution (16, 17, 63). This interdisciplinary integration can conspire to explore a range of issues including the analysis of rates of evolution, topologies of phylogenetic trees, the mechanics of evolutionary diversification, and the evolution of novelties, whether genetic, developmental, or morphological. Fossils, when placed in a phylogenetic context, can reveal extinct conditions of stem taxa, unique combinations of characters, and the temporal sequence in the development of novelties (17, 64, 65). These features give paleontology the power to shape experiments on the genome, epigenome, and development and explore the patterns and processes of morphological transformations (65).

The arsenal of genomic tools available to understand morphological diversity is ever growing, putting evolutionary-developmental biologists in a position to rapidly identify—and ultimately characterize—the developmental and morphological roles of candidate genes and their regulatory elements in both model and nonmodel organisms (Fig. 4). However, these epigenomic and transgenic techniques offer a "passive" snapshot of a particular time point and locus in the organism of interest, begging the question of functional assays. Recent revolutionary techniques in genome editing may finally allow biologists to modify genomes and access an unprecedented new level of functional data.

Until recently, rigorous genetic approaches to modify endogenous loci were limited for use only in model organisms, and in a time-consuming and expensive manner. Jinek et al. have used the breakthrough CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) system to cause double-stranded breaks at targeted genomic sites by taking advantage of the adaptive immune response discovered in bacteria (69). CRISPR/Cas9 has been rapidly applied to produce targeted knockouts in a range of organisms (e.g., mouse, zebrafish, *Xenopus*, *Drosophila*, and human cell lines) (70), allowing researchers to directly manipulate genomes and test hypotheses of morphological evolution.

We are at an age in which expeditionary paleontological investigation for transitional forms, and high-resolution fossil imaging, yielding new insights into previously hidden parts of the fossil record, can be part of a research program that encompasses genomic and developmental biology (Fig. 4). Paleontological discovery of critical stem taxa with intermediate conditions or character combinations and the elucidation of the sequence of the assembly of complex morphological novelties can shape molecular research. Genomic and developmental biology, with an ever-expanding array of experimental tools, can be used to test paleontological hypotheses, amplify them, or reveal where critical fossils may be lacking in the tree of life. A kind of "experimental paleontology" is on the horizon, in which morphological transformations revealed by the phylogenetic analysis of the fossil record may be physically assayed in the laboratory (71–73). What do we need for this future to happen? It will take new or newly interpreted fossils from critical nodes of the tree of life, genomes from diverse species, and the further expansion of our molecular toolkit for the identification, characterization, and modification of genomes from nonmodel organisms. This synthesis is one of many new and promising futures of the fossil record.

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