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The Deep Homology of the Autopod: Insights from Hox Gene Regulation

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Synopsis The evolution of tetrapod limbs from fish fins was a significant functional and morphological shift, but how significant was it in terms of the underlying genetic mechanisms? The fossil record provides insight into the morphological changes. However, to understand the underlying mechanisms, we must peer into the gene regulatory networks of living vertebrates. Analysis of HoxA/D expression in a basal actinopterygian, the North American paddlefish, Polyodon spathula, reveals patterns of expression long considered to be a unique developmental signature of the autopod (hands/feet, digits) and shown in tetrapods to be controlled by a “digit enhancer” regulatory landscape. These data, along with recent interspecific transgenic experiments, expression results from chondrichthyans, and data from fossils support the notion that the autopod shares a deep homology with the distal endoskeleton of the fin (distal radials) of other gnathostomes.

Introduction Fossils record the evolutionary transition from paired fins to paired limbs in sarcopterygian tetrapodomorphs during the latter half of the Devonian period (Long and Gordon 2004; Coates and Ruta 2007). In recent years, our interpretation of the invasion of land by vertebrates has evolved somewhat—careful analysis of taxa close to the transition, such as Tiktaalik (Shubin et al. 2006), Acanthostega (Coates 1996), and Ichthyostega (Pierce et al. 2012), support the notion of a unique and rather long-standing transitional ecology. Nonetheless, a major remodeling of many ancient vertebrate systems—for example, lateral lines, hearing, vision, jaw mechanics, structural support of the body and internal viscera, and unpaired and paired appendages—define this period as a major morphological event in vertebrate evolution. Within the paired appendages themselves, the origin of the autopod (hands, feet, and digits) is seen as one of the defining adaptations that make tetrapods unique. Here, we will address the origin and homology of the autopod by placing recent studies of gene expression and function in the context of datasets on fossils and comparative anatomy.

To evaluate possible changes in gene regulation that may associate with the fin-to-limb transition and the origin of the autopod, it is first necessary to phylogenetically bracket tetrapodomorph fish and stem tetrapods between living taxa that are amiable to developmental and genetic analysis (Fig. 1). Until recently, insights have primarily come from the use of established tetrapod (the mouse, Mus, and to a lesser extent the chicken, Gallus) and teleost (the zebrafish, Danio, and to a lesser extent the pufferfish, Fugu) genetic systems. These taxa provided the most powerful tools for functional assays and were already part of large and established communities in which techniques and results could be exchanged. It is also worth considering that these systems were not specifically chosen for their phylogenetic position, or to address evolutionary questions per se. However, a certain danger comes in bracketing a fossil transition with two highly derived clades,
as when variation is present one cannot ascertain which character state is primitive. Outgroups are needed to polarize character states—for both morphology and developmental mechanisms.

Recently, more basal taxa of gnathostomes have come under scrutiny. Chief among these are a non-teleost actinopterygian, the North American paddlefish, Polyodon spathula (Davis et al. 2004a, 2007; Modrell et al. 2011), and chondrichthyan taxa for which eggs are readily available—the skate Raja erinacea (Dahn et al. 2007) and the catshark Scyliorhinus canicula (Tanaka et al. 2002; Freitas et al. 2007). With these additional taxa as outgroups, we can more effectively address the following questions: (1) What are the fundamental differences in gene expression and regulation between fins and limbs and (2) Do these differences offer insights into the origin of the autopod? In addition, we may ask whether the comparison of regulatory differences between fins and limbs can actually pinpoint the genetic changes behind this evolutionary transition.

**Homologies and novelties: the skeletons of appendages**

Phylogenetic analyses and evidence from fossils support the hypothesis that a fin endoskeleton with one or more anterior radials (homologues of the propterygium and mesopterygium) and a posterior metapterygium (with associated radials) is the primitive condition for gnathostomes (Janvier 1996; Zhu and Yu 2009). The paired fin endoskeletons of early fossil gnathostomes were often unossified or weakly ossified, limiting the number of specimens for which radials are easily identified. However, this conserved arrangement is present in the extinct placoderms (e.g., Ctenurella, Ørvig 1962) and acanthodians (e.g., Acanthodes, Coates 1994), and in phylogenetically basal members of all three extant clades,
Teleosts appear to have lost the metapterygium (Fig. 1A). An alternative, and underexplored, hypothesis is that the teleost metapterygium may have lost its morphological identity, having been transformed into simply the most posterior radial (See Danio in Fig. 1A). Because the number of mesopterygial radials can vary among taxa, it would be difficult to distinguish between these hypotheses using anatomical criteria alone. However, evidence from the descriptive embryology of chondrichthyans (Balfour 1881) and from non-teleost actinopterygians (Davis et al. 2004a) demonstrates that the metapterygium has a distinct mode of condensation and chondrogenesis when compared with the anterior radials. Such differences have not been described for the posterior radial of well-studied teleosts (e.g., Danio, Grandel and Schulte-Merker 1998). However, it is possible that loss of mesopterygial identity and mode of formation may be causally linked. As no anterior radial or metapterygial-specific markers are yet known, these hypotheses have yet to be rigorously tested.

Sarcopterygians possess fin skeletons composed exclusively of the metapterygium, apparently having lost the anterior radials. Alternatively, the anterior radials may have lost their morphological identity or become highly modified, a notion supported by similar expression patterns for key anterior–posterior patterning genes in tetrapods and the fins of fish that possess anterior radials (discussed below). However, recently described shoulder girdles for two fossil stem sarcopterygians demonstrate that anterior radials were primitive to the clade (Zhu and Yu 2009). With the exception of these most basal forms, sarcopterygian fins consist of a single proximal stylopod (humerus/femur) articulating with the pectoral or pelvic girdles. Tetrapodomorphs show a branching arrangement of two elements, the zeugopod (radius/ulna; fibula/tibia), articulating with the distal margin of the stylopod.

In general, all gnathostomes’ paired fins possess an arc of distal radials, articulating with the distal margins of the more proximal anterior radials and/or metapterygium. The only exception to this rule are numerous fossils for which distal radials are not described, most often due to lack of preservation from weak or absent ossification of these elements, and the heterochronic retention of larval fin characters in some teleosts. These distal radials form as discrete and separate condensations in chondrichthyans (Balfour 1881; Davis, personal observation), actinopterygians (Grandel and Schulte-Merker 1998; Davis 2004a), and lungfish (Johanson et al. 2007). Tetrapodomorphs also possess a distal zone of skeletal elements, the autopodium, although greatly more elaborate than the arc of simple nodular elements or short rods seen in the other clades. In tetrapodomorphs, there are generally two or more proximodistal segments. A more proximal mesopodium is generally recognizable, with “wrist” elements, such as the ulnare and intermedium, recognizable in even basal tetrapodomorph clades (e.g., the rhizodontid Sauripterus, Davis et al. 2004b; Fig. 1A). Distal to the mesopodial elements are a series of radials considered homologous to the digits of tetrapods. It is significant to note that no tetrapodomorph has been described that possesses both an autopodium and distal radials, supporting the notion that these two distal regions are homologs.

### Homologies and novelties: Hox genes

Gene expression and functional studies demonstrate a remarkably conserved regulatory program for building paired appendages that may help elucidate homologous skeletal regions shared by fins and limbs. Among the genes that are best studied and best characterized are the Hox genes, which encode transcription factors that provide positional identity along animal axes, including the limbs (McGinnis and Krumlauf 1992). In general, Hox genes are arranged in genomic clusters, with multiple clusters present in vertebrates (Duboule 2007). All vertebrates possess two or more clusters of Hox genes, the result of a duplication event sometime after the evolutionary split of the vertebrate lineage from the rest of “invertebrate” bilaterian diversity, which possesses only a single Hox cluster (e.g., Drosophila). Two additional major duplication events of Hox clusters occurred within vertebrates: the first of these, resulting in four clusters (A–D), typify phylogenetically basal jawed fishes and tetrapods; and the second (resulting in eight ancestrally or seven due to subsequent loss of one cluster) characterizes the teleost lineage of actinopterygian fish (Prohanska and Stadler 2004). With these duplications came opportunity for the additional clusters to assume novel regulatory roles. For example, Hox clusters A and D became the key positional regulators of the development of appendages (unpaired and paired fins in fish, and limbs in tetrapods).
Hox genes exhibit a correspondence between their genomic position within a cluster and their domain of expression along the A-P axis of the embryo. The more 3’ (more telomeric) genes in a Hox cluster are expressed more anteriorly, and generally earlier, than more 5’ (more centromeric) genes of the cluster, a phenomenon known as spatial colinearity. This colinearity expresses itself not only during A-P patterning of the body but also in the patterning of the proximal to distal (P-D) and A-P axes of the appendages.

Experiments demonstrate that the conserved phenotypic pattern of tetrapod limbs (stylopod, zeugopod, and autopod regions) is clearly tied to Hox regulation during embryonic outgrowth of limb buds. In transgenic knockout mice, targeted deletion of individual Hox genes affects skeletal patterning in the specific region of the limb where that Hox gene would normally have been expressed (Davis et al. 1995). For example, double knockouts of HoxA11/D11 in combination result in severe truncation of the zeugopod. In wild-type mice, highest levels of HoxA11 and D11 would normally occur in the presumptive zeugopod (Davis and Capecchi 1996; Zákány et al. 1997). Similarly, an almost complete loss of the autopod (digits are lost, only the mesopodium remains) is observed in HoxA13/D13 double knockouts, the genes with the most distal expression within the limbs of wild-type mice (Fromental-Ramain et al. 1996).

A look at Hox A/D expression in fins and limbs reveals a biphasic pattern consisting of a phylogenetically conserved early phase during initial bud outgrowth, with some key differences specific to fins and limbs appearing as development proceeds. In the early buds of teleosts and tetrapods, 5’ Hox A/D members are expressed in a conserved, spatiotemporally collinear fashion. The more 5’ genes are progressively activated and then expressed in progressively restricted domains along the P-D and A-P axes, respectively. However, at later stages when the autopod is being specified, a distinct late-phase of 5’ HoxA/D expression with inverted spatial colinearity is activated along the A-P axis. This late phase, although once considered a unique developmental hallmark of autopod formation, does exhibit distinct expression patterns in tetrapods; these are not seen in other gnathostomes. For example, Hoxa11 and Hoxa13 resolve into mutually exclusive proximal and distal domains, with Hoxa13 expressed throughout the autopod. The late-phase has not been definitively confirmed in teleosts, and prior to the addition of more basal taxa (see sections below), it was proposed that the regulatory changes in Hox expression seen in teleosts and tetrapods may underlie the origin of the autopod (Sordino et al. 1995; Wagner and Chiu 2001). However, Ahn and Ho (2008) demonstrated a possible degraded or remnant late-phase expression for both HoxA and HoxD in zebrafish pectoral fins, a result further supported by the presence of late-phase enhancers in zebrafish capable of driving expression in the mouse autopod (Schneider et al. 2011, and the following section). These recent results, along with the simplified fin skeleton of teleosts (Davis et al. 2004a), suggest that teleosts may have lost, modified, or truncated portions of an ancestral Hox regulatory program that is retained in tetrapods. If so, teleosts may be poor proxies for the ancestral state of fin development.

**Hox regulation of the development of appendages**

*In vivo* chromosomal engineering studies in mice have offered much insight into the transcriptional regulation of biphasic HoxA/D expression in paired appendages. The HoxD cluster has been best characterized and will be the primary focus of our discussion here. Regulatory sequences sit outside of a tightly packed Hox cluster, flanking its 5’and 3’ends (Spitz et al. 2001; Fig. 2). Furthermore, the enhancers for early and late phases of HoxD expression consist of distinct and physically separated regulatory domains, strongly supporting the notion of separate evolutionary origins for the two phases (Tarchini and Duboule 2006; Gonzalez et al. 2007).

Early-phase HoxD appears to be regulated by an activation–inhibition interaction between sequences located on either side of the cluster. A 3’ (telomeric) early limb control region (ELCR; Fig. 2) is hypothesized to drive temporal colinearity of HoxD expression in the early bud (Zákány et al. 2004). Hox genes more proximal to the ELCR would be transcribed earlier in development than genes more distal to this control region. However, this mechanism alone does not explain the posterior restriction of more 5’ HoxD genes during the early phase. In a series of deletion experiments in mice, Tarchini and Duboule (2006) demonstrated that specific HoxD genes could be posteriorized in their pattern of expression by the removal of their 5’Hox neighbors. From these results, they proposed that a distinct regulatory sequence (POST; Fig. 2), centromeric to the cluster, spatially restricts Hox genes to more exclusive posterior domains, again based on their proximity to the regulatory sequence. Although both the ELCR and POST regions remain poorly
characterized, the mechanism does explain the temporal and spatial colinearity of early-phase expression.

Late-phase HoxD expression, associated with formation of the autopod, possesses a separate regulatory mechanism. Located 5' (centromeric) of the cluster is the global control region (GCR; Fig. 2), which maps to the intergenic region between the ATP5G3 and Lunapark (Lnp) genes (Spitz et al. 2003). Within the GCR are two conserved regulatory domains, CsA and CsB. Of these, only the CsB region appears to contain limb-specific regulatory function. A further regulatory region (Prox) containing a conserved regulatory domain (CsC; Fig. 2) has also been identified, mapping to the intergenic region between Lnp and the gene Evx2. Gonzalez et al. (2007) demonstrated that neither CsB nor CsC alone could fully recapitulate normal autopod expression, suggesting a synergetic role for these two separate elements in regulating colinearity of late-phase HoxD expression from the 5' side of the cluster. It has also been observed that the two non-Hox genes that lie within the 5' regulatory landscape, Lnp and Evx2, are also expressed in the mouse autopod, despite not having any clear functional role in autopod development. This demonstrates the lack of specificity in these 5' enhancers—They promote a suite of genes (Lnp, Evx2, and HoxD) that are not structurally, functionally, or phylogenetically related to each other.

Early-phase and late-phase HoxD expression interact through the Sonic hedgehog pathway (Shh). ELCR-POST induced restriction of more 5' HoxD genes into more exclusively restricted posterior domains within the bud sets up a unique posterior regulatory “compartment” that breaks Gli3-mediated symmetry of the early limb (Zákány et al. 2004). These unique populations of mesenchymal cells in the posterior bud are the only ones expressing the full compliment of HoxD transcription factors, inducing the localized expression of Shh transcripts and, thus, defining the zone of polarizing activity (ZPA). Shh expression in the ZPA then mediates, but is not mandatory for, late-phase expression in the autopod (Litingtung et al. 2002; te Welscher et al. 2002).

Recent experimental work by Sheth et al. (2012) provides additional insights into the role that Shh/Gli3 play in late-phase HoxD patterning of the autopod. Sheth and colleagues demonstrated that titration of 5' HoxA/D genes from a Gli3 null mice results in increasing polydactyly, with increasingly thinner and more closely packed digits at the lowest Hox dosages. Intriguingly, the extreme phenotypes bear striking similarities to the radials of basal gnathostomes such as Polypterus (a basal actinopterygian) and chondrichthyans, suggesting that digit patterning may involve modification of an ancestral Turing-type reaction diffusion mechanism across the A-P extent of the appendage.

**Insights from basal taxa**

As we addressed in the first section, the fossil record demonstrates that the distal skeleton of the appendage, the autopod, was already present in
tetrapodomorph fins prior to the origin of tetrapods (Davis et al. 2004b; Shubin and Davis 2004; Shubin et al. 2006). However, these data were somewhat at odds with early comparative analyses of gene expression and regulation in teleosts and tetrapods. In these model systems, the results supported the notion that the autopod was likely an evolutionary novelty with its own unique pattern of expression and regulation (Sordino and Duboule 1996; Shubin et al. 1997; Spitz et al. 2003). Further analysis has revealed evidence of a highly conserved late-phase of Hox regulation in teleosts (Schneider et al. 2011). However, data from phylogenetically more basal extant taxa, such as non-teleost actinopterygian fishes and chondrichthyans, were lacking until recently.

Our analysis of 5' HoxA/D genes in Polyodon spathula reveals conserved expression patterns for both early and late phases, while demonstrating some key differences that point to those aspects of autopod regulation that may be truly developmental novelties (Davis et al. 2007). Early expression of Polyodon 5' HoxD genes exhibits the same posteriorly nested pattern observed in other vertebrates, with HoxD13 being the most restricted posteriorly. However, Polyodon also possesses a separate late-phase expression of 5' HoxD that is initially restricted to the distal-most mesenchyme (presumptive distal radial region) but later expands proximally into the region surrounding, but not including, the developing skeletal radials. In a pattern remarkably similar to that of the mouse autopod, HoxD13 expression extends more anteriorly than does either HoxD11 or HoxD12 (Fig. 1B). This last observation is all the more intriguing considering the presence of anterior radials in Polyodon and their absence (at least based on morphological criteria) in tetrapod limbs—a pattern that may support the hypothesis of transformation of anterior radials, rather than their loss, within the sarcopterygian lineage.

Late-phase HoxD11 expression in Polyodon embryos exposed to retinoic acid is expanded both anteriorly and proximally, in agreement with the observed Shh responsiveness of late-phase HoxD in tetrapods (Tarchini and Duboule 2006). Expression of the ZPA genes Shh and dHand mimic the expression of the posterior pattern of early-phase HoxD13 and appear developmentally earlier than does the onset of late-phase HoxD expression. In addition, application of retinoic acid results in an ectopic ZPA, as assayed by anterior expression of Shh.

Gene expression in the catshark, Scyliorhinus canicula, provides further evidence for the deep conservation of 5' HoxD function and regulation (Frietas et al. 2007). Scyliorhinus fins show a typical nested and collinear 5' HoxD early phase, a distally restricted late-phase, and the same anterior expansion of late-phase HoxD13 expression observed in Polyodon and tetrapods (Fig. 1B). Together, the data from Polyodon and chondrichthyans suggest that the presence of two HoxD phases (the early-phase Shh independent and posteriorly nested, the late-phase mediated by Shh and exhibiting distal expression) is the primitive gnathostome condition.

Early 5' HoxA expression is remarkably similar in tetrapods, zebrafish, and Polyodon. In all, HoxA11 and HoxA13 exhibit a proximodistally nested collinear expression with HoxA13 being the most distally restricted (Sordino et al. 1995; Sordino and Duboule 1996; Wagner and Chiu 2001; Davis et al. 2007). Later expression is markedly different. During the specification of the autopod, tetrapod HoxA11 becomes restricted to the zeugopod region and HoxA13 becomes restricted to the developing autopod (Sordino et al. 1995; Wagner and Chiu 2001). In zebrafish and Polyodon, HoxA11 and HoxA13 become progressively more distally restricted, yet expression remains nested and overlapping, with no observed tetrapod-like P-D segregation (Sordino and Duboule 1996; Davis et al. 2007; Fig. 1C).

One issue does merit further investigation: Polyodon possesses a lineage-specific whole-genome duplication (WGD), estimated to have occurred approximately 42 million years ago, and independent of the more-ancestral WGD characteristic of teleosts (Crow et al. 2012; our own transcriptome analysis). However, both our data and that of Crow et al. suggest that the “beta” HoxA/D paralogs are transcriptionally inactive. This functional diploidization, at least as far as the 5' HoxA/D genes are concerned, is consistent with our original results on gene expression, suggesting that only a single paralog of each 5' HoxA/D is expressed in the developing fin (Davis et al. 2007). Likewise, the presence of a HoxD late-phase in chondrichthyans (Frietas et al. 2007) strongly supports our interpretation that the expression patterns observed in Polyodon are representative of the primitive gnathostome condition, rather than an independently derived condition.

Perhaps the most impressive support for the homology of distal radials and the autopodium comes from a recent interspecific transgenic analysis of the late-phase enhancer CsB (Schneider et al. 2011). Researchers first demonstrated that the CsB region in zebrafish promotes expression in the presumptive endoskeleton of the distal fin. Then they swapped CsB regions from zebrafish and skate into mice.
and looked at expression. Remarkably, both zebrafish and skate CsB enhancers promote expression in the mouse autopod. Specifically, each showed expression at the base of the digits, in the mesopodial (wrist) region. Further support comes from Frietas et al. (2012) who demonstrated that the mouse CsC enhancer, an element that has not yet been identified in a non-tetrapod, promotes expression in the presumptive distal radials of the zebrafish fin.

Conclusions

The study of fossils, comparative gene expression, and gene regulation strongly support the hypothesis that the autopod is a regulatory “variant” of the ancient gnathostomes’ endoskeletal cell population of the distal fin, sharing a deep homology (sensu Shubin et al. 1997) with distal radials. First, all gnathostomes possess two skeletal regions in the appendage: an early developing proximal region and a later developing distal region. Paired appendages either possess distal radials or an autopodium—no appendage has been described that possesses both. Second, our results from the analysis of 5′ HoxD gene expression in Polyodon reveal that initial late-phase expression is restricted to the distal radials in a manner similar to late-phase expression in the mouse autopod (Davis et al. 2007). Finally, interspecific transgenic experiments demonstrate that the late-phase HoxD enhancer CsB from zebrafish and skate can promote expression in the proximal autopod (the mesopodium) in mice (Schneider et al. 2011) and that the CsC enhancer from mouse can promote expression in the distal radials in zebrafish (Frietas et al. 2012).

Our results support a model in which early and late phases of HoxA/D expression are ancestral to tetrapods, and when placed in the phylogenetic context of similar findings in chondrichthyans (Freitas et al. 2007), and recent transgenic results (Schneider et al. 2011; Frietas et al. 2012) suggest that this biphasic expression is the primitive condition for gnathostomes. Different regulatory mechanisms act on these two phases, suggesting that the proximal and distal appendages have different phylogenetic histories. Thus, we conclude that the origins of the autopod (and distal radials) as an evolutionary novelty lie much deeper in the evolutionary history of vertebrates—perhaps before the origin of gnathostomes.

The autopod is, however, structurally and functionally more complex than are distal radials. So, despite all the evidence to support homology, there is still much to explore about what makes the autopod distinct. Our results, along with previous studies, demonstrate that expression patterns are most divergent between fins and limbs in the P-D segregation of gene expression. In zebrafish and Polyodon, Hoxa11 and Hoxa13 transcripts are expressed in an overlapping P-D pattern within the fin bud, quite unlike the P-D segregation of these genes in the tetrapod limb. Likewise, in late stages of Polyodon, 5′ HoxD expression extends proximally beyond its initial restriction to the distal radials, whereas in tetrapods, early and late phases of 5′ HoxD remain segregated. Key insights into the novelty of the autopod are likely to be gained by further exploring the regulatory landscape surrounding the HoxA cluster, which is not nearly as well understood as is the HoxD cluster. Likewise, the expression patterns for the HoxA genes have not been described for chondrichthyans. Finally, the CsC enhancer has, so far, not been identified in a non-tetrapod, raising the possibility of an autopod-specific modifier. Although we have come to discover that fins and limbs share the same conserved regulatory mechanisms, we have also narrowed down the list of candidate mechanisms that confer the autopod with its uniqueness.

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