Fins, limbs, and tails: outgrowths and axial patterning in vertebrate evolution

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Summary

Current phylogenies show that paired fins and limbs are unique to jawed vertebrates and their immediate ancestry. Such fins evolved first as a single pair extending from an anterior location, and later stabilized as two pairs at pectoral and pelvic levels. Fin number, identity, and position are therefore key issues in vertebrate developmental evolution. Localization of the AP levels at which developmental signals initiate outgrowth from the body wall may be determined by Hox gene expression patterns along the lateral plate mesoderm. This regionalization appears to be regulated independently of that in the paraxial mesoderm and axial skeleton. When combined with current hypotheses of Hox gene phylogenetic and functional diversity, these data suggest a new model of fin/limb developmental evolution. This coordinates body wall regions of outgrowth with primitive boundaries established in the gut, as well as the fundamental nonequivalence of pectoral and pelvic structures. *BioEssays* **20**:371–381, 1998.

Introduction

Vertebrate appendages include an amazing diversity of form, from the huge wing-like fins of manta rays or the stumpy limbs of frogfishes, to ichthyosaur paddles, the extraordinary fingers of aye-ayes, and the fin-like wings of penguins. The functional diversity of these appendages is similarly vast and, in addition to various modes of locomotion, fins and limbs are also used for feeding, defense, attack, communication, and even internal fertilization (e.g., sharks). Vertebrate fins and limbs have diversified repeatedly around conserved anatomical themes throughout their evolutionary history, and recognition of these has been used over and again to exemplify fundamental concepts in biological theory. The striking uniformity of teleost pectoral fin skeletons illustrated Geoffroy Saint-Hilair's discussion of "special analogies,"[1] while tetrapod limbs exemplified Owen's[2] related concept of "homology"; Darwin[3] then employed precisely the same example as evidence of evolutionary descent from common ancestry. Most recently, remarkable similarities have been found at the developmental-genetic level underpinning these shared anatomical patterns.[4–13] The aim of this article is therefore to review these new developmental data to see how they inform questions about evolutionary patterns and processes underlying fin-limb origin and diversification. However, most studies have focused on the distal zones of limbs and fins: the sets of digits and fin supports outside the body wall and the morphogenetic activity at the tip of a developing fin or limb bud. Rather, this article directs attention to the earliest phase of fin outgrowth, addressing developmentally and phylogenetically earlier questions about fin position and number along the head–tail axis.

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Patterns of fin and limb evolution

Anaspid fishes are the most primitive vertebrates with paired fins,[14,15] placing their origin at a minimum of 450 million years (Myr) ago (Fig.1, node 3). Paradoxically, anaspids, although

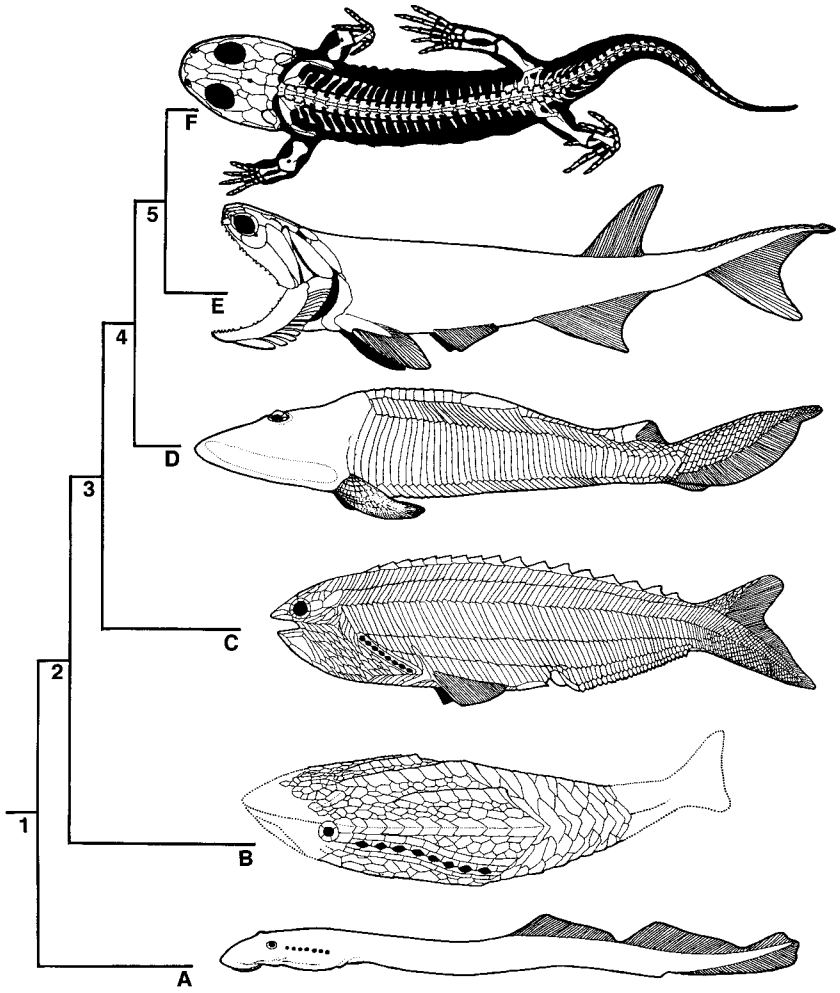

Figure 1. Primitive vertebrates illustrating gross patterns of paired fin evolution. While these are arranged according to their degree of relatedness, they do not represent an ancestor-descendent sequence. Evolutionary tree topology from Janvier.[15] Node numbers relate to those identified in Figures 2 and 5: node 1, vertebrates; node 2, base of jawed vertebrate lineage; node 5, last point of common ancestry of living jawed vertebrates. Terminal taxa: A, *Petromyzon*, a living lamprey; B, *Astraspis*, a 450-Myr-old finless pre-jawed fish[82]; C, *Rhyncholepis*, an anaspid displaying an early (420-Myr BP) example of lateral fins[17]; D, an osteostracan *Hemicyclaspis,*[17] showing more substantial muscular lateral fins; E, *Cheirolepis,*[83] an extremely primitive ray-finned osteichthyan, representative of primitive jawed fishes; F, *Balanerpeton,*[84] an early tetrapod.

jawless, branch from the ancestral lineage unique to jawed vertebrates.[15] This finding suggests that paired appendages are specializations of jawed vertebrates and their ancestry and that they evolved after the evolutionary split from the origins of living jawless fishes: hagfishes and lampreys (Fig.1, node 1). The most primitive paired fins may have been little more than bony lateral keels with or without flimsy scaled webs and needle-like spines[14] (Fig. 1C). These always extend from a pectoral-like position in ''pre-jawed'' fishes, and no primitive fish is known with only pelvic paired fins. The internal skeleton of such primitive fins, if any was present, is unknown. Although certain anaspids have paired fins running the length of the flank, there is no strong support for the theory that continuous lateral fin-folds represent the ancestral condition.[14] Similarly, while primitive paired fins always border with, or extend into, the branchial region, there is insufficient evidence to justify resurrecting the theory of fin and limb skeletons as transformed gill-bar extensions.[16] Osteostracans (Fig. 1D) present the earliest (indirect) evidence of paired fins with an internal skeleton, displaying facets and canals for such tissues on a cranially integrated shoulder girdle.[17] These lobate fins, extending from the rear of a bony headshield, are a relatively late innovation (Fig. 1, node 4), because such pre-jawed fishes are the closest known relatives of primitive jawed vertebrates.

All living jawed vertebrates belong to two major divisions: cartilaginous fishes (chimaeroids, sharks, and rays) and bony fishes (ray-fins and lobe-fins). Two great extinct radiations of jawed fishes—placoderms and acanthodians—also emerge at about the same time in the fossil record. Of these, acanthodians may include the ancestry of bony fishes,[14,17] but placoderm affinities are uncertain.[17,18] A likely minimum date for this key stage in vertebrate evolution (Fig. 1, node 5) is around 435 Myr ago (the Ordovician–Silurian boundary), although isolated scales suggest an earlier date of around 450 Myr ago.[19] Irrespective of their precise evolutionary

relationships, these fossil and recent fishes demonstrate that the presence of two fin pairs (pectoral and pelvic) is unique to jawed vertebrates. Nevertheless, its noteworthy that secondary loss of one or both pairs has happened repeatedly during evolution (e.g., cetaceans, snakes, and eels). All jawed vertebrate *pectoral* fin skeletons primitively share the following features: a series of internal skeletal supports (radials) articulating with the girdle, the posteriormost of which articulates with secondary radials mostly along the anterior edge. This complex posterior radial is the metapterygium. Soft fin-rays, constructed of collagen-like protein, fringe the outermost rim of pectoral and pelvic fins; in the bony fishes, these soft rays are enclosed by, or incorporated within, dermal bony rays, the lepidotrichia. Pelvic fins, in contrast to pectorals, are primitively smaller and anatomically simpler. Furthermore, there seems to be no example in which the pelvic appendage is a direct copy or identical serial homologue of the pectoral (irrespective of size, and contra Shubin et al.[13]). Primitive absence of a pelvic metapterygium highlights this difference in all groups, with the exception of the lobe-fins, and perhaps male cartilaginous and placoderm fishes, all of which have a metapterygium-like ''clasper'' (specialized intromittent organ). The metapterygium has been lost in at least one major vertebrate group, the teleosts. These include most living ray-finned fishes, such as the zebrafish (*Danio rerio*) and puffer fish (*Fugu rubripes*). However, metapterygial absence does not equate with loss of anteroposterior fin skeletal asymmetry, which is a primitive and persistent characteristic of all vertebrate paired appendages.

Lobe-fin fishes display an exceptional and evolutionarily specialized degree of close pectoral–pelvic similarity. Yet, even in these, the pelvic fin is primitively shorter and of a consistently different pattern relative to the pectoral.[20] Tetrapods (Fig. 1F) are a subdivision of the lobe-finned group and, as such, the primitive pre-limb condition is possession of fins with a short, bony internal skeleton extending exclusively from the metapterygium. This has the characteristic 1:2 proximodistal branching pattern, long recognized as an important feature shared with the proximal and midregions of tetrapod limbs. Details of the fin-limb evolutionary transition are incomplete but, since 1990, a considerable new body of evidence has emerged. Previous assumptions about the primitive primacy of the pentadactyl limb have been overturned by discoveries of 6-, 7-, and 8-digit limbs in the most primitive tetrapods.[14,21] Pentadactyly may have stabilized independently on two or more occasions during subsequent tetrapod evolution, and digits probably evolved before the elaboration of wrist and ankle joints.[21] The fin-limb transition probably occurred more or less simultaneously in pectoral and pelvic appendages, although in the most primitive known limbed tetrapod, *Acanthostega*, the forelimb is arguably more primitive (i.e., fin-like) than the hindlimb.

Limb development

Patterning

Fin and limb development involves a hierarchy of decisions during embryogenesis. Early decisions include bud position (where the outgrowth will be specified relative to the primary body axis) and, in tetrapods, at least, appendage type (forelimb vs hindlimb). In limb buds, after initiation at an appropriate position, outgrowth must be maintained, and the bud must be patterned along three primary axes to generate correct structures at appropriate positions, and to specify subsequent differentiation and growth. Considerable progress has been made in understanding how pattern is established in developing limbs,[4] although comparative investigations of paired fin development have only recently become available.

Tetrapod limb buds emerge from the lateral plate mesoderm by localized maintenance of cell proliferation, and consist of mesenchyme encased in an ectodermal jacket. After initiation, limb outgrowth is maintained by a thickened epithelium at the bud apex known as the apical ectodermal ridge (AER).[22] Signals from this ridge maintain a high rate of cellular proliferation and an undifferentiated state in the underlying, distalmost mesenchyme. Within this region, known as the progress zone, cells acquire positional identities, specifying whether they will form proximal (i.e., humerus) or distal (i.e., digits) structures.[23] The ridge also maintains the zone of polarizing activity (ZPA) in the posterior mesenchyme. The ZPA is involved in digit patterning along the anterior (thumb) to posterior (little finger) axis[24] and is the source of a secreted molecule that specifies positional identity in a dose-dependent manner.[25]

Recently identified genetic pathways operating in tetrapod limbs provide a molecular basis for the cellular interactions described above. Limb bud initiation from the lateral plate mesoderm is controlled by members of the fibroblast growth factor (FGF) family.[26–29] Radical fringe (R-fng) is expressed in the dorsal ectoderm of the emerging bud, and the AER appears to be positioned at the boundary of R-fng-expressing and nonexpressing cells.[30,31] Moreover, it is now clear that dorsal and ventral ectoderm are lineage-resticted compartments,[32,33] meeting at the apical boundary. FGFs produced subsequently by the AER mediate ridge signaling activities,[34,35] while polarizing activity is mediated by the Sonic hedgehog gene (Shh), which is normally expressed in the ZPA.[36] Shh can be induced by retinoic acid, and application of either signal to limb buds is sufficient to induce digit duplications.[36,37] A positive feedback loop, coordinating limb outgrowth and patterning, involves reciprocal maintenance between Shh in the ZPA, and FGF-4 in the AER.[38,39] Shh expression is also maintained by the Wnt7a signal secreted by the dorsal ectoderm.[40] Wnt7a contributes to limb patterning along the dorsoventral (palm to back of hand) axis[41] by inducing expression of Lmx-1, a transcription factor in the

dorsal mesenchyme.[42,43] Engrailed-1 expression in the ventral ectoderm antagonizes Wnt-7a expression and thereby inhibits formation of dorsal structures on the ventral surface.[44] Engrailed-1 also represses R-fng ventrally, creating the border of fringe expression that positions the AER at the limb bud apex. However, in the absence of engrailed-1 in the mouse, an apical ridge is induced that expands into the ventral ectoderm. This is surprising, because one might predict the complete absence of an AER, given that such conditions remove apical expression boundaries. It is noteworthy that, in contrast to chick embryos, mouse Fringe gene expression has not been detected in mouse limb buds.[45]

Limb outgrowth is therefore linked to interconnected genetic networks that pattern all three primary axes; these same networks are deployed during the development of many different organ systems within embryos.[4] Moreover, these networks may be evolutionarily conserved because they are used by crustacea, insects, and vertebrates.[13] Several parallels have been identified between gene expression patterns in paired appendages of tetrapods and teleosts (the major subgroup of ray-finned fishes; cf. Figs. 1F and 3A), and functional comparisons of gene regulation are now possible. Sonic hedgehog is expressed at the posterior margin of zebrafish fin bud mesoderm[5] and, like tetrapods, retinoic acid treatment induces ectopic Shh expression anteriorly within the fin bud.[6] Of several known zebrafish FGF receptors, at least one is expressed in the mesoderm underlying the apical ridge,[7] and an Engrailed protein is expressed in the ventral half of the pectoral bud.[8] Msx genes are expressed in the mesenchyme, and Msx and Distal-less (dlx) genes are expressed in the apical ridge,[9] as in tetrapod limb buds. Significantly, in fins it is this ridge that extends distally as an ectodermal fold, enclosing the developing dermal fin rays. Sordino and Duboule[10] speculate that these rays may physically interrupt ectodermal signaling to the mesoderm, thereby truncating fin endoskeletal outgrowth. If this is correct, a key question in the fin-limb evolutionary transition concerns the nature of the mechanism which evolved to prevent early fin-ray development, *before* such primitive bud-truncation occurs. Further analyses of zebrafish mutants and experimentally manipulated fin buds are likely to demonstrate additional differences and similarities between fin and limb development as exemplified by emerging patterns of Hox gene expression (discussed below).

Pectoral versus pelvic appendages and positioning

Specification of fin or limb buds at appropriate positions along the body is a fundamental problem of axial patterning. Developmental control of appendage type is linked closely to this issue: forelimb versus hindlimb, or pectoral fin versus pelvic fin. Transplantation studies show that limb type is specified long before bud initiation and patterning.[46] One possibility is that as the node or organizer establishes the primary body axis, the same positional information that determines head-to-tail coordinates also determines the type of appendage that will form. It appears significant that most of the genes involved in patterning limb axes are expressed in both fore- and hindlimbs. Therefore, early specification of positional identity within lateral plate mesoderm would allow these same patterning genes to operate in a different context in each bud-pair, generating different fore- and hind-fin/limb structures. Several candidate genes may be involved in lateral plate mesoderm regionalization into limb-forming and non-limb-forming regions, and, more specifically, in generating positional differences that may influence limb type. These are discussed in detail later (setting the level).

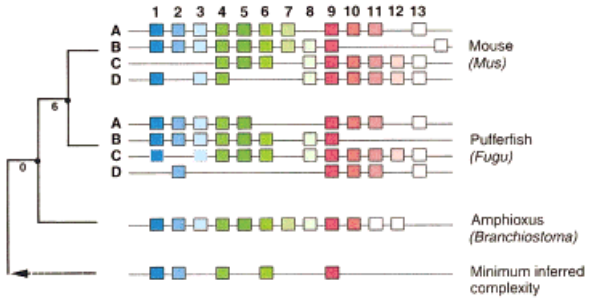

Figure 2. Hox gene cluster diversity and evolution. Simple tree shows evolutionary relationships of representative chordates in which Hox cluster organization is known. Node 0 predates node 1, and node 6 postdates node 5, as shown in Figure 1. Numbers identify paralogous gene groups between clusters; letters identify homologous clusters between taxa. Mouse Hox gene network from Sharman and Holland[50]; *Hoxb13* from Zeltser et al.[85] *Fugu* Hox gene network from Aparicio et al.[52] Amphioxus Hox gene cluster from Sharman and Holland.[50] Minimum inferred complexity of arthropod–chordate ancestral Hox gene cluster, and interrelationships of anterior *(blue)*, medial *(green)*, and posterior *(red/*orang*e)* Hox gene groups from Kappen.[47] No attempt is made to indicate intergene distances, with the exception of mouse *Hoxb13*, which is out of register with other group 13 genes.[85] *Open boxes in amphioxus cluster*, incompletely characterized genes[50]; *dotted boxes*, pseudogene remnants of former Hox gene cluster members.[52]

Hoxology: a quick introduction

Hox genes (Fig. 2) are involved in specification of positional identity during metazoan embryogenesis. Gain or loss of function mutations involving Hox genes can produce dramatic transformations in which one body part or segment is substituted for another (homeotic mutations). Hox genes were first identified in *Drosophila* and are characterized by the following criteria[47]: (1) sequence similarity to the *Drosophila* Hom–C complex, (2) organization in gene clusters, and (3) their expression pattern along the embryonic axis corresponds to their arrangement on the chromosome. In all animals examined so far, genes located at the 3′ end

of Hox clusters are expressed anteriorly, and genes located at the 5′ end are expressed posteriorly (termed "spatial colinearity").[48] Position within a cluster also correlates with expression timing, so that 3′ genes are generally expressed before their 5′ neighbors (termed "temporal colinearity"). This results in an embryonic expression pattern in which 3′ Hox genes are activated anteriorly, before expression of more 5′ genes in more posterior body regions.

The evolutionary diversity of chordate, and therefore vertebrate, Hox gene clusters is becoming increasingly clear (Fig. 2). Amphioxus, a cephalochordate, has only a single cluster, but this has members of at least 10 of the 13 paralogue gene groups identified in the three lamprey clusters and four jawed vertebrate clusters.[49–51] This finding suggests that, during early vertebrate evolution, a Hox gene cluster resembling that of amphioxus underwent fourfold duplication, beginning at a locus preceding the divergence of lamprey and jawed vertebrate lineages[11,51] (Fig. 1, node 1). Moreover, the amphioxus Hox gene complement must itself have arisen from lateral duplications of the genes in successively simpler, more primitive Hox clusters.[47] Clues about the pattern of these events derive from Hox gene sequence analyses that identify three interrelated sets[47]: anterior (1–3), medial- (4–8), and posterior groups (9–13) (Fig. 2).

During the period since these gene duplication episodes, jawed vertebrate Hox cluster evolution seems to have been characterized by gene deletions. This much is apparent from direct comparison of amphioxus with mouse clusters[49] (Fig. 2). Cluster structures in other nonmammalian vertebrates are less well documented. Information about the Hox cluster organization of a pair of teleosts, the zebrafish (*Danio rerio*)[11] and puffer fish (*Fugu rubripes*, an advanced form lacking ribs and pelvic fins),[52] shows major dissimilarities relative to mammalian clusters, indicating quite separate patterns of gene loss in tetrapod and teleost lineages. Furthermore, the observation that *Fugu* lacks several Hox genes that are present in *Danio* (which may have an additional Hox cluster)[11,52] already points to variation within the teleosts, which are known to have diversified for more than 100 Myr.[53]

Hox genes and axial patterning

The vertebrate head–tail body axis is established during gastrulation, when cells are assigned anteroposterior positional identities. The paraxial mesoderm, which forms the vertebrae, musculature, and skin, is a useful model for studying axial patterning because segmented somites have particular identities; changes in these can be detected in the vertebral pattern. Retinoid treatment alters Hox gene expression boundaries along the paraxial mesoderm A–P axis, resulting in apparent homeotic transformations of vertebral identity.[54] Further evidence for the Hox gene role in specifying vertebral identity emerges from knockout or overexpression experiments. Initial comparative studies have already begun to relate 'transposed'[55] vertebral anatomical landmarks and regions to shifts in conserved expression boundaries.[56,57] In amniote tetrapods, the anteroposteriorly dispersed expression domains[54] of Hox genes from the same paralogue group (Fig. 2) are probably evolutionarily derived, and differentiated with the evolution of axial regional boundaries such as the lumbar–thoracic division and sacrum.[56]

This interpretation is based on the premise that members of each paralogue group primitively shared coincident expression boundaries. A clear prediction of this hypothesis is that simpler colinear expression patterns should be found in experimental subjects with less highly regionalized equivalent domains of axial organization (e.g., the vertebral column of the zebrafish, *Danio rerio*)(Fig. 3A). Preliminary results from such a study appear to support this hypothesis. Prince et al.[58] report that, in comparison with tetrapods, zebrafish Hox genes of paralogue groups 6–8 (Fig. 2) share significantly closer anterior expression boundaries in the paraxial mesoderm.[58] Because these paralogues probably derive from a single ancestral gene (Fig. 2), they suggest that this is a primitive expression pattern.

These data are complemented by patterns of morphological change emerging from analyses of vertebrate phylogeny. Major structural transformations affect the axial skeleton during the fish–tetrapod evolutionary transition. These include the spread of intervertebral articulation, reorientation of neural (and haemal) arches and spines (implying modification of the chevron-shaped muscle pattern characteristic of jawed fishes), loss of axial fin supports and dermal rays, and enlargement of the ribs. When plotted as an evolutionary series, the tetrapod-like features replace fish-like features in a consistently anterior to posterior direction[21] (Fig. 3B). However, it is noteworthy that, while such axial landmarks move tailward, there is no corresponding shift in girdle and limb position.

Hox genes in limbs and fins

Hox gene expression in tetrapod limbs has been the focus of considerable attention. Most concerns AbdB-related members of the A and D clusters (Fig. 2), but it is now clear that members of all four clusters are activated during bud outgrowth.[59] The same HOXA and HOXD genes are expressed in the posterior part of fore and hindlimb buds. These expression patterns are probably mediated via the signaling systems centred on the ZPA. Early expression patterns correlate with proximal and mesial limb domains, and a later, distal expression phase maps onto the domain of digit development in which gene expression extends anteroposteriorly across the bud apex.[59] In this region, digit size and number are apparently dependent on cumulative expression of group 11, 12 and 13 genes from HOXA and HOXD clusters.[60] Unlike these HOXA and HOXD genes, the HOXC gene contribution is not restricted so closely to the 5′ end of the cluster, and the expression domain lies within the anterior

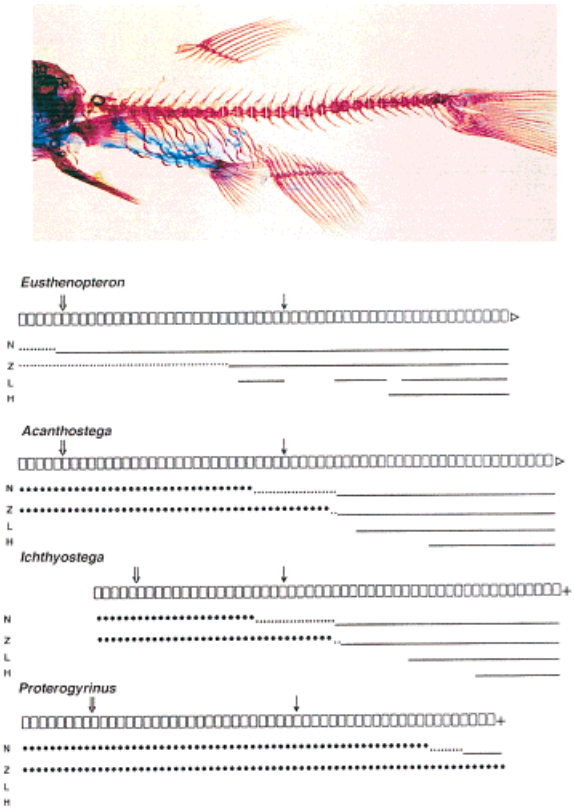

Figure 3. A: Cleared and double-stained postcranial skeleton of the teleost zebrafish (*Danio rerio*) axial skeleton showing lack of strong regionalization in the vertebral column relative to tetrapod conditions. B: Schematic representations of axial skeletons spanning the fish–tetrapod transition illustrating the caudal displacement of fish-like morphologies[21] (*Eusthenopteron* is a fully finned sarcopterygian fish; *Acanthostega* and *Ichthyostega* are primitive tetrapods with limbs and fish-like finned tails; *Proterogyrinus* has limbs and a finless tail.) *Rectangles*, vertebral segments; *bold outlines* in presacral region: ⇓, level of pectoral girdle; ↓ level of pelvic girdle; +, continuation of vertebral series and/or incomplete data; >, terminus of vertebrae; *horizontal line*, fish-like condition; *dotted line*, incomplete or transitional condition; *bold dotted line*, tetrapod condition; N, posteriorly inclined neural (and haemal) arches; Z, presence of zygapophyses; L, lepidotrichial outgrowth; H, haemal radials.

proximal region of each bud. Fore- and hindlimb buds express different HOXC genes, and these respect the colinear expression sequence along the body axis. Of the HOXB cluster, *Hoxb5*, *Hoxb8*, and *Hoxb9* are expressed during limb development, and have been linked to the relationship of limbs to patterning along the body axis (described below).

As in the axial skeleton, Hox gene expression in zebrafish paired fins provides an informative comparison with tetrapod limbs. 5′ HOXA and HOXD genes are expressed in pectoral and pelvic fins, although these are morphologically dissimilar.[5,12] *Hoxc6* is expressed in a wedge-shaped domain at the anterior-proximal region of pectoral limb and fin buds[61] and is involved in tetrapod shoulder girdle formation. However, Hox gene expression domains in zebrafish fin-pairs are abbreviated distally, and appear to be monophasic or biphasic instead of triphasic, as in limb buds.[59] These simpler expression patterns correlate impressively with shorter and simpler appendicular skeletons of zebrafish fins, relative to their tetrapod homologues. HOXB gene expression patterns in fin pairs are currently undescribed. Therefore, while zebrafish fin data are less detailed than those of tetrapod limbs, it appears that similar 5′ Hox genes sharing similar patterns of proximal (but not distal) expression, are found in pectoral and pelvic paired appendages whose last point of common ancestry dates from a minimum of 410 Myr ago. Moreover, experimental evidence now suggests that the HOXA cluster was predominantly active in the distal domain of primitive polydactylous limbs,[21] with subsequent recruitment of the HOXD cluster contributing to the evolution of more recent digit patterns.[60]

Induction and initiation of limbs

During normal limb initation, Fgf10 is expressed in the mesenchyme and Fgf8 is expressed in ectoderm of the prospective limbs and in the mesonephros.[27–29] Additional limbs can be induced in chick embryos at inappropriate axial levels by applying FGF-soaked beads to flank region lateral plate mesoderm between the prospective limbs.[26–29] Interestingly, FGF can induce either ectopic wings or legs according to the position at which it is applied: application to the anterior flank induces an additional wing, whereas application to the posterior flank induces an additional leg Unexpectedly, cell labeling studies show that the same population of prospective flank cells can be respecified by FGF to form either wing or leg.[62] This uncovers a surprising lability of lateral plate mesoderm cells: their positional value can be anteriorized to make a forelimb or posteriorized to make a hindlimb; significantly, this occurs without vertebral respecification.

FGF application to the flank induces complex changes to the pattern of Hox gene expression in the lateral plate mesoderm. Hox patterns are anteriorized or posteriorized according to the position at which FGF is applied, indicating that expression in the lateral plate mesoderm may mediate limb position and identity along the primary body axis. After Hox gene flank expression is reprogrammed, signaling molecules are induced which control ectopic limb outgrowth and patterning. Overlying ectoderm expresses Fgf-8 and flank mesenchyme cells with high polarizing potential activate Shh to make a ZPA anteriorly in the ectopic limb. As a consequence, ectopic buds produce morphologically normal limbs, but with polarity reversed in the AP axis. This suggests that endogenous FGF could be the signal that initiates normal

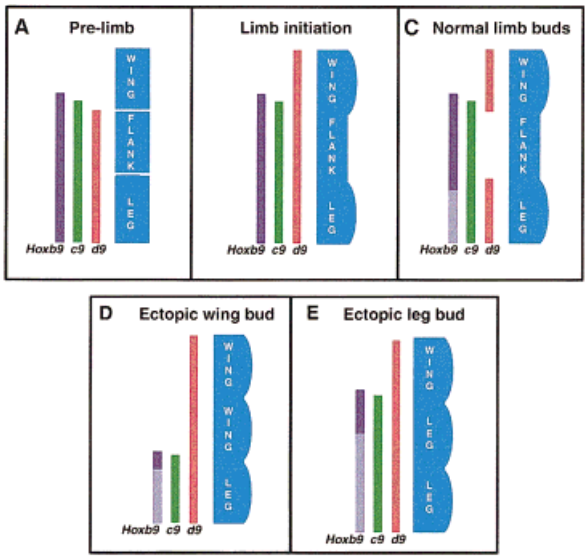

Figure 4. Expression of *Hox9* genes in lateral plate mesoderm during normal and ectopic limb development. (Adapted from ref. 62.) A: Before limb initiation, *Hoxb9, Hoxc9,* and *Hoxd9* are expressed throughout lateral plate mesoderm with anterior boundaries staggered in the region of the wing bud. B: As limb budding is initiated, *Hoxd9* expression shifts anteriorly to mark the anterior limit of the emerging wing bud. C: Later in limb development, *Hoxd9* switches off in the flank, and *Hoxb9* expression is reduced in the leg. Thus, the wing expresses *Hoxb9* and *Hoxc9* posteriorly, and *Hoxd9* throughout, the flank expresses *Hoxb9* and *Hoxc9*, and the leg expresses low levels of *Hoxb9* and high levels of *Hoxc9* and *Hoxd9*. D: Application of FGF to anterior flank reprograms Hox gene expression in the lateral plate such that the wing pattern is recreated in the flank and an ectopic wing develops from flank cells. E. Application of FGF to posterior flank results in the leg pattern of Hox gene expression being recreated in the flank and an ectopic leg develops from the flank.

limb outgrowth, and therefore control of FGF localization, or localized competence to respond to FGF, is the likely key to bud positioning.

Setting the level

Several lines of evidence suggest that Hox genes are involved in establishing positional differences in the lateral plate mesoderm. Loss of function of the *Hoxb5* gene causes the forelimb to shift anteriorly along the body axis.[63] *Hoxb9*, *Hoxc9,* and *Hoxd9* genes are expressed in the lateral plate mesoderm of chick embryos, with staggered anterior boundaries within the prospective forelimb territory (Fig. 4A). These patterns of Hox gene expression undergo specific changes as limb budding is initiated such that different combinations and levels of expression characterize wing, flank, and leg (Fig. 4B,C). When FGF is applied at positions that result in ectopic wing development, lateral plate mesoderm Hox gene expression patterns are reprogrammed such that flank cells acquire a pattern characteristic of the wing level (Fig. 4D). The new expression pattern is consistent with the positional identity of flank cells being anteriorized from "flank" to "wing." Application of FGF to posterior flank induces a leg-like pattern of Hox gene expression, and subsequent formation of an ectopic leg (Fig. 4E). Interestingly, Hox expression boundaries in the somites are unaffected. This highlights the apparent mutual independence of paraxial and lateral plate mesodermal patterning, and may explain why vertebrae are not transformed when ectopic limbs develop. Moreover, anterior extension of the *Hoxb8* expression domain in transgenic mice leads to formation of an ectopic polarizing region and extra digits anteriorly in the forelimb, suggesting that *Hoxb8* is involved in positioning the forelimb polarizing region.[64] Thus, Hox genes are involved in positioning limbs (and fins) along the body axis, and positioning signaling centers within these appendages. Members of the T-box family of transcription factors are differentially expressed in pectoral and pelvic appendages, and may also play role in generating morphological differences between the two.[86]

Mechanisms controlling normal FGF expression at discrete positions are not yet understood, but given the tight link between limb initiation signal position, and lateral plate regional identity (i.e., outgrowth signals must be coordinated with forelimb and hindlimb positional values), Hox genes must be considered strong candidates for encoding both. It is becoming apparent that, although they are not interdependent, systems regulating Hox gene expression and cellular regional identity in different tissues are generally the same. It is therefore possible that fin/limb morphological diversity may have arisen by similar, but independent, mechanisms to those that generated morphological diversity of the axial skeleton.

In light of these results, new pathways for paired fin and limb developmental evolution must be considered. Hox gene expression dynamics, the independence of Hox gene regulation in paraxial and lateral plate mesoderm, and the fact that morphological transformations can occur in one tissue without altering the other, raise the following possibilities. The first possibility is that paired appendage evolution included a mechanism that staggered Hox gene expression along the lateral plate mesoderm. This would resemble patterns in the paraxial domain and is likely to have been achieved by modifying genes that regulate Hox gene expression, rather than the Hox genes themselves. The second possibility is that paired appendages originated without an obligatory reorganization of the axial skeleton. Modular control of Hox genes, perhaps by tissue-specific regulatory genes, would enable decoupling of anatomical systems and freedom for variation

to occur without inducing wholesale body plan transformations.

A developmental model of the evolution of vertebrate paired appendages

In conclusion, we consider independent regulation of Hox gene expression in lateral plate mesoderm to be a key factor in paired fin evolution. Increased complexity of the vertebrate genome[50] (Fig. 2), could have permitted this innovation by allowing new developmental role acquisition without disrupting preexisting patterning mechanisms. Staggered Hox gene expression boundaries within primitive vertebrate lateral plate mesoderm (pre-node 3, Figs. 1, 5) would therefore have supplied differential positional values along the body wall. These would then be available for co-option to determine outgrowth boundaries at the origin of paired appendages.

This scenario demands an explanation of how such boundaries came to be positioned during pre-finned stages of vertebrate evolution. The answer seems to lie within gut regionalization. Before the origin of vertebrates, Hox gene expression was involved in gut regional specialization.[65,66] Splanchnic mesoderm (producing the smooth muscle of the gut), and somatic mesoderm (producing body wall and paired appendages), are both lateral plate derivatives, and Hox genes are expressed in both layers in early embryos.[67,68] Furthermore, mutations in Hox genes can have coordinated effects on limb and gut development,[66,69] and FGF application to the lateral plate mesoderm can modulate Hox gene expression and morphological patterning in the gut as well as the body wall (M.J. Cohn and K. Patel, unpublished data). Together, these data suggest that Hox genes are co-regulated in somatic and splanchnic mesoderm, independent of paraxial Hox gene expression. We suggest that, before the origin of paired fins, gut regionalization provided morphological phenotypes subject to selection pressures that stabilized Hox boundaries within the lateral plate mesoderm.

An attempted synthesis of these phylogenetic and embryological data is presented as a conjectural model of axial and appendicular evolution in early vertebrates in Figure 5. This model is mostly described in the figure caption, but certain points deserve emphasis. First, it is assumed that evolutionarily related Hox genes (Fig. 2) primitively shared the same expression domains, with divergence and dispersal being derived features that permitted the evolution of morphological novelties. In this respect the model resembles Averof and Akam's[70] hypothesis of Hox gene functional evolution in the protostome–arthropod lineage. Second, we suggest that in chordate evolution, staggered boundaries of paralogous Hox genes appeared in the lateral plate mesoderm concomitant with gut regionalization (Fig. 5, stages A and B). Third, that *Hoxb9*, *Hoxc9,* and *Hoxd9* are expressed in lateral plate mesoderm at a pectoral level within the early phase of Hox gene expression along the body axis (Fig. 4A). The anterior limit of *Hox9* expression is probably important for limb specification and subsequent 5′ Hox gene activation, because *Hoxd9* expression is a likely prerequisite for expression of more 5′ Hox genes such as *Hoxd13* within outgrowing fin/limb buds[10,60] (Fig. 5, stages B– D). Fourth, that boundaries within the lateral plate allowed spatially restricted activation of outgrowth signals such as FGF, and the resultant fin buds would have inherent differences in their positional values at different loci along the body axis (Fig. 5, stage C). The same signaling molecules (e.g., Shh, Fgf) would therefore operate in different contexts in anterior and posterior appendages, and the resultant differences in signal interpretation during development may explain morphological differences between anterior and posterior appendages. Finally, this transformational scenario is consistent with evidence from the fossil record that indicates coordinated evolution of the stomach and pectoral fins[17,71] and that pelvic and pectoral appendages evolved as morphologically distinct structures.

This model stresses the relationship between Hox gene expression in somatic and splanchnic mesoderm, and this is independent of paraxial mesoderm regulation. However, there is a strikingly conserved relation between the *Hoxc6* anterior expression boundary in paraxial mesoderm at the vertebral cervical–thoracic transition, and the anteroposterior level of the pectoral girdle.[56,57] This begs the question of whether such a correlation reflects an underlying causal connection. Limb development and spinal cord patterning clearly need coordination, so that specialized motor neurons are specified at the same axial level as limbs. The developmental mechanisms involved in this process are unclear. Tissue- and region-specific promotors of Hox gene expression identified in zebrafish and tetrapods[72,73] indicate highly modular expression control in adjacent tissues. Interactions between the paraxial mesoderm and spinal cord can influence Hox gene expression in the cord,[72,74] perhaps by activation of neuroectoderm-specific Hox promoters. Similarly, interactions between lateral plate mesoderm and paraxial mesoderm have been shown to influence somite patterning,[75] although this occurs at a later stage than limb and spinal cord specification. We favor the hypothesis that tissue patterning in the limb and spinal cord may be coordinated by secondary or tertiary signaling, perhaps under the control of Hox gene expression in one tissue, which may in turn influence Hox gene expression in adjacent tissues. This modularity probably involves differences in timing and specificity of the response to signaling molecules. For example, Fgf can alter Hox gene expression in different tissues at different development stages.[62,76–79] Furthermore, the apparent conservation of Hox gene promoters between zebrafish and tetrapods indicates that such modular control of Hox genes evolved at a deeper node in vertebrate phylogeny.

There are numerous evolutionary and functional reasons for stabilizing the spatial relation between the pectoral girdle

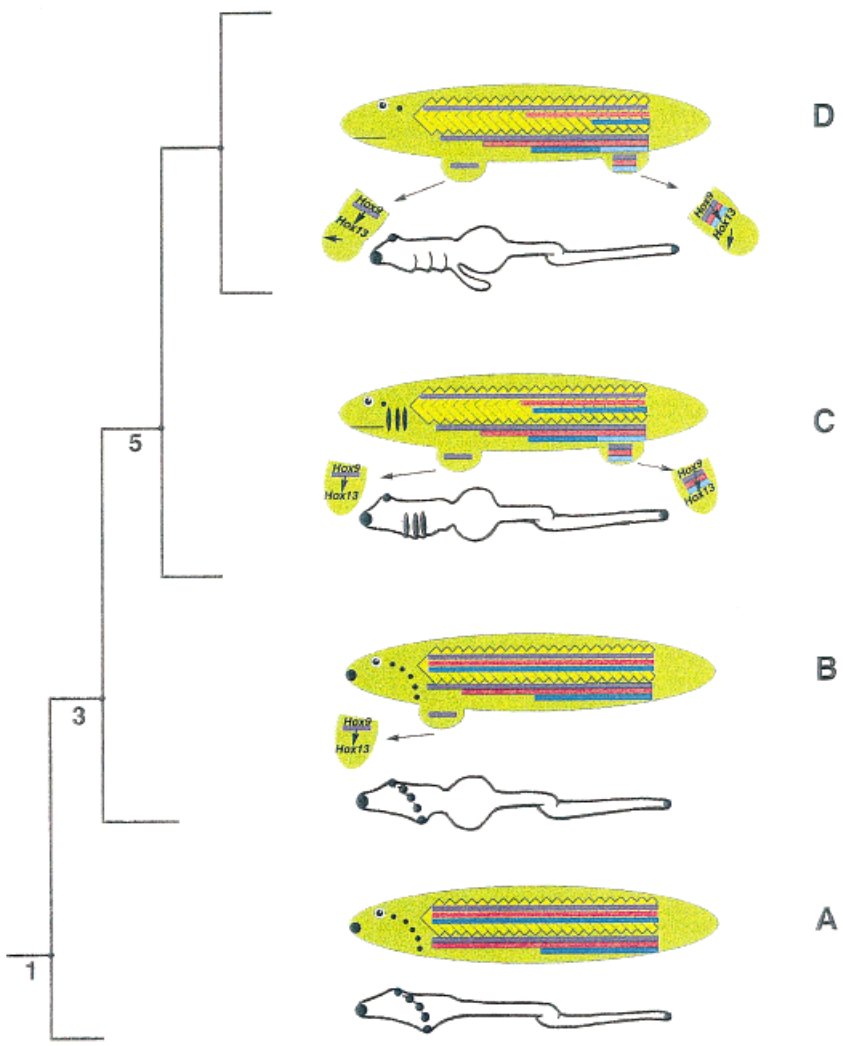

Figure 5. Model of evolving Hox gene expression and function in vertebrate axial and appendicular domains. Changing morphological and developmental conditions are illustrated by cartoons of the whole body, fin/limb buds and gut, and these are related to specific nodes on the evolutionary tree in Figure 1. Horizontal bars represent general features of Hox gene expression, rather than specific clusters or paralogous groups. Chevrons represent somites and paraxial mesoderm (PM); lower region represents body wall and lateral plate mesoderm (LPM). Isolated fin and limb buds show developmentally later patterns of Hox gene distal expression, after initial conditions shown in whole body cartoon. Stage A (node 1): Primitively, paralogous Hox gene domains in paraxial and lateral plate mesoderm mostly shared coincident anterior expression boundaries; single posteriorly restricted Hox gene expression domain *(blue)* is associated with simple division of anterior and posterior gut regions. Stage B (node 3): Staggering of Hox boundaries anteriorly in lateral plate mesoderm coordinates specification of anterior fins and a specialized foregut/stomach. The pattern of Hox gene expression in lateral plate mesoderm from which the fin bud emerges determines the initial profile of Hox gene expression in the bud. Stage C (node 5): Emergence of new Hox boundaries in posterior lateral plate mesoderm is associated with posterior fin specification. This posterior fin is initiated in a different, "posterior" context of Hox gene expression. Subsequent expression in the bud proceeds in conventional 5′ sequence. The sum of subsequent expression of more 5′ Hox genes resembles, but is not identical to, the total expression pattern in the anterior bud. Limited axial skeletal regionalization reflects emerging Hox gene boundaries in PM. Stage D. Fin–limb transition includes new, distal expression phase in bud outgrowth; increased regionalization of axial skeleton associated with greater AP spread of PM Hox gene expression boundaries.

and the skull. The girdle originates as an integral skullpart, forming the rear wall of an internal gill chamber, a shield for the pericardial cavity, and a secure insertion for the pectoral fins. It is also possible that this spatial correlation reflects a developmental link present in the earliest vertebrates, but subsequently lost with the evolution of increased morphogenetic complexity, while the topographic positions of structures are maintained by fuctional demands and the integrity of tissue systems. A possible clue about such primitively shared regulation should come from analysis of Hox regulatory elements in primitive chordates and jawless vertebrates.

Finally, the absence of vetebrates with more than two sets of paired appendages has often been used as an illustration of evolutionary constraint. Developmental mechanisms responsible for this anatomical limitation remain unclear. Arguably, the nearest approach to a third pair of lateral appendages may be the lateral caudal keels of certain fishes, such as tuna and various sharks. Moreover, the tail flukes of whales and dolphins also fall within this category of extra, lateral, caudal outgrowths. However, none of these keels or flukes contains internal skeletal supports (or even dermal rays). And, within the context of this discussion, it appears unlikely

that any fin pair with endoskeletal content will emerge posterior to the Øanalia,Ø[11] (although regenerative tails can be induced to form limbs[80,81]). Even the most elongate lateral fins of primitive fishes terminate in front of the anal level. Clearly, lateral caudal keels can and do emerge, but articulated endoskeletal paired appendages require the lateral plate mesoderm, and this is linked intimately to the extent and pattern of the gut.

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