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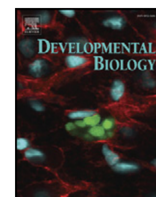

Evolution of Developmental Control Mechanisms

Tri-phasic expression of posterior *Hox* genes during development of pectoral fins in zebrafish: Implications for the evolution of vertebrate paired appendages

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ABSTRACT

During development of the limbs, *Hox* genes belonging to the paralogous groups 9–13 are expressed in three distinct phases, which play key roles in the segmental patterning of limb skeletons. In teleost fishes, which have a very different organization in their fin skeletons, it is not clear whether a similar patterning mechanism is at work. To determine whether *Hox* genes are also expressed in several distinct phases during teleost paired fin development, we re-analyzed the expression patterns of *hox9–13* genes during development of pectoral fins in zebrafish. We found that, similar to tetrapod *Hox* genes, expression of *hoxa/d* genes in zebrafish pectoral fins occurs in three distinct phases, in which the most distal/third phase is correlated with the development of the most distal structure of the fin, the fin blade. Like in tetrapods, *hox* gene expression in zebrafish pectoral fins during the distal/third phase is dependent upon *sonic hedgehog* signaling (*hoxa* and *hoxd* genes) and the presence of a long-range enhancer (*hoxa* genes), which indicates that the regulatory mechanisms underlying tri-phasic expression of *Hox* genes have remained relatively unchanged during evolution. Our results suggest that, although simpler in organization, teleost fins do have a distal structure that might be considered comparable to the autopod region of limbs.
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Introduction

Among modern-day vertebrates, paired appendages (fins and limbs) vary widely in their form and function (Fig. 1; Goodrich, 1930; Coates and Cohn, 1998). According to the current interpretation of the phylogeny, much of the differences among various types of vertebrate paired appendages largely result from differential losses of skeletons that were present in the common ancestor of jawed vertebrates (Fig. 1; Coates, 1994, 1995). In archetypal paired appendages, skeletal supports consisted of several large endoskeletal bones (pro-, meso-, and metapterygium of Gegenbaur, 1878) lying at the base, which articulate proximally with pectoral or pelvic girdles and distally with numerous smaller bones supporting dermal fin rays. In the fins of most sharks and primitive ray-finned fishes of today (Fig. 1A), this basic pattern has been retained with little modification, while in paired appendages of the teleosts and tetrapods, which together comprise the vast majority of modern-day vertebrate species, only portions of the ancestral skeletons remain. In the paired fins of teleosts, skeletal supports now consist mostly of the pro- and mesopterygial components of endoskeletons plus the dermal fin rays (Fig. 1B), while in limbs of tetrapods only the metapterygium and its distal branches can be seen (Fig. 1C) (Coates, 1994).

In spite of the major differences in structure and function between fins and limbs (Fig. 1), however, remarkably few differences have been found in the genetic regulation of their development (Sordino and Duboule, 1996; Ruvinsky and Gibson-Brown, 2000). Genes such as *Tbx4/5*, *Fgf8/10*, or *Wnt7a*, which are known to play critical roles in the initiation, growth, and patterning of tetrapod limbs (Capdevila and Izpisúa-Belmonte, 2001; Logan, 2003), are expressed in comparable spatiotemporal domains in teleost fins, and the analysis of relevant zebrafish mutants has revealed that many aspects of their functions are also conserved during fin development (Grandel et al., 2000; Ruvinsky et al., 2000; Garrity et al., 2002; Fischer et al., 2003; Norton et al., 2005). Most notably, genes that are known to be involved in antero-posterior patterning of fin/limbs such as *Shh* (Krauss et al., 1993; Riddle et al., 1993), *Han2* (Charité et al., 2000; Yelon et al., 2000), or *Gli3* (Masuya et al., 1997; Tyurina et al., 2005) also seem to have little difference in expression or function, which indicates that mechanisms responsible for the differences between fin and limb structures (Figs. 1B, C) may reside in changes in patterning events lying more downstream. One such possible change in developmental mechanisms has been proposed to occur at the level of the regulation of *Hox* gene expression during fin/limb development (Sordino et al., 1995).

Hox genes are a family of transcriptional regulator genes that are involved in axial patterning of many structures in vertebrates (Kessel and Gruss, 1991; Krumlauf, 1994; Burke et al., 1995; Deschamps et al., 1999), including fins and limbs (Yokouichi et al., 1991; Sordino et al., 1995; Nelson et al., 1996). In both fish and tetrapods, *Hox* genes belonging to the paralogous groups 9–13 of *Hox* A and D clusters have been shown to be expressed in nested domains along the antero-posterior as well as proximo-distal axes of developing fins and limbs

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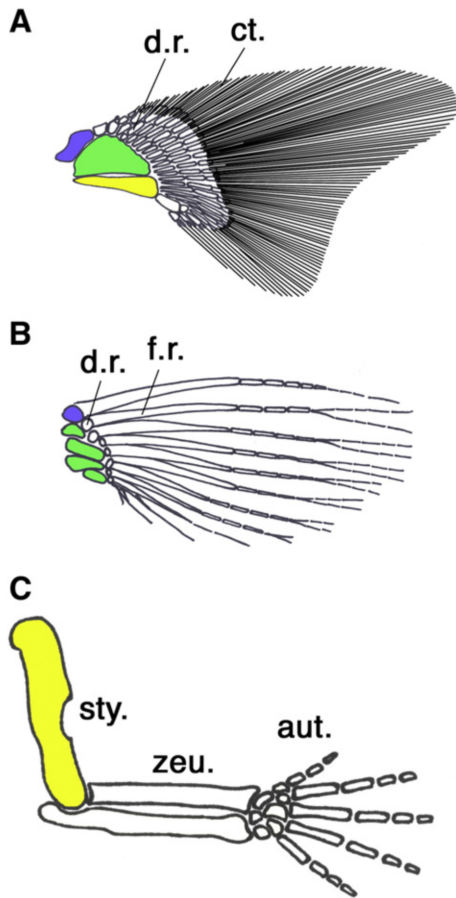

Fig. 1. Diversity in vertebrate appendicular skeletons. (A) Dogfish shark (*Squalus acanthias*), a chondrichthyan. In most cartilaginous fishes, the bauplan of pectoral fins consist of three large cartilages lying at the base of the fin which articulate proximally with the pectoral girdle and distally with numerous smaller cartilages ("distal radials") supporting the fibrous fin rays known as ceratotrichia. (B) Zebrafish (*Danio rerio*), a teleost. In most teleosts, the skeletons of paired appendages ("fins") consist of five basal bones and variable number of smaller, more distally-lying nodular bones (distal radials) articulating with the segmented fin rays. (C) Mouse (*Mus musculus*), a tetrapod. In tetrapods, paired appendages ("limbs") are characterized by the presence of several large, proximo-distally arranged series of endochondral bones which are organized into the three major segments known as the stylopod (upper arm/leg), zeugopod (lower arm/leg), and autopod (wrist/ankle and digits). In all panels anterior is to the top and proximal is to the left. Only pectoral appendages are shown. Homology of pro- (blue), meso- (green), and metapterygium (yellow) is based on Mabee (2000). A: after Shubin and Alberch (1986). B: after Grandel and Schulte-Merker (1998). C: after Williams et al. (2006). d.r.: distal radials. cer.: ceratotrichia. f.r.: fin rays. sty.: stylopod. zeu.: zeugopod. aut.: autopod.

(Sordino et al., 1995, 1996; Nelson et al., 1996), which, at least in tetrapods, appears to be regulated in three spatio-temporally distinct phases called the phases I, II, and III (Nelson et al., 1996; Shubin et al., 1997). In teleosts such as zebrafish and medaka, however, a simpler pattern of expression has been reported for *Hox* genes during development of pectoral fins (Sordino et al., 1995, 1996; Takamatsu et al., 2007). Notably, changes in expression similar to the ones associated with the development of the most distal regions in tetrapod limbs (i.e., phase III), have not been seen in the distal fin bud region (Sordino et al., 1995, 1996; Takamatsu et al., 2007). These differences have led to the idea that the proximal regions of tetrapod limbs (corresponding to the stylopod and zeugopod: Fig. 1C) are patterned by conserved mechanisms shared with teleost fins while distal parts of the limbs (autopod: Fig. 1C) are patterned by a novel mechanism unique to tetrapods and therefore digits and associated distal wrist/ankle bones could be considered evolutionary novelties (Shubin et al., 1997; Wagner and Chiu, 2001).

These interpretations, however, have recently been criticized for the lack of a proper phylogenetic perspective, ignoring the fact that bones in teleost fins and tetrapod limbs have different evolutionary origins (Fig. 1), and therefore may not be compared directly (Mabee, 2000). A more fundamental problem in these arguments, though, is that much of these interpretations are based upon incomplete data that may not represent the full dynamics of *Hox* gene expression in teleost paired fins. There is now increasing evidence that the development of pectoral fins begins much earlier and involves more complex morphogenetic processes than were thought at the time of initial study of teleost *hox* gene expression (van Eeden et al., 1996; Grandel and Schulte-Merker, 1998; Fischer et al., 2003). Furthermore, recent revelations that teleosts as a group had undergone a whole genome duplication event close to their evolutionary origin (Taylor et al., 2001; Amores et al., 2004; Hurley et al., 2007) generated a list of "missing" *Hox* genes that were not included in the original data (Amores et al., 1998). It is therefore possible that large portions of the dynamics of *Hox* gene expression during development of teleost paired fins might still remain undocumented.

In this paper we present a more complete picture of the expression dynamics of teleost *Hox* genes during development of paired appendages using zebrafish pectoral fins as an illustrative model. We show that detailed examination of *hox* gene expression during zebrafish pectoral fin development reveals the presence of an additional, distal phase of expression, which is similar but not identical to the late phase of *Hox* gene expression seen in the autopod region of tetrapod limbs. We also show that, similar to tetrapods, this late/distal phase of *hox* gene expression in zebrafish pectoral fins is also dependent upon *sonic hedgehog* (*shh*) signaling, which in tetrapods has been shown to be essential for proper patterning of the autopod region (Chiang et al., 2001; Litingtung et al., 2002). Lastly, we show that, in zebrafish, expression within the distal portion of pectoral fins is not a feature unique to the posterior *hox* genes but is a trait shared by genes neighboring the 5′ end of the *hox* cluster, suggesting that, as in tetrapods, distal/late expression of *hox* genes in zebrafish pectoral fins is likely to be due to the presence of a long-range enhancer located 5′ to the *hox* cluster (Spitz et al., 2003; Lehoczky et al., 2004). Our results demonstrate that contrary to the prevailing notion in current literature (e.g., Coates, 1995; Sordino and Duboule, 1996; Shubin et al., 1997; Wagner and Chiu, 2001; Freitas et al., 2007) expression of *Hox* genes during teleost paired fin development is quite similar to the expression in tetrapod limbs, indicating a widespread conservation in genetic mechanisms controlling the development of paired appendages among different vertebrates.

Materials and methods

Care and maintenance of fish

Embryos of wild-type zebrafish and zebrafish *sonic you* (*syu*^{t4}; Schauerte et al., 1998) mutant were collected from pairwise matings and maintained in embryo medium (Westerfield, 1993) at a low density (about 60 embryos per 100 to 150 ml of medium). In order to ensure a consistent staging of old (>24 hpf (hours post-fertilization)) embryos, temperature settings of the incubator were calibrated so that development up to 24 hpf consistently occurred close to the schedules described in Kimmel et al. (1995). We found that under this condition development of pectoral fins occurred slightly slower than the descriptions given in Kimmel et al. (1995), which might reflect differences in growth conditions or differences in genetic background of the fish.

Cloning of zebrafish hox genes and genes adjacent to the hox cluster

Posterior *hoxa/d* genes belonging to the paralogous groups 9–13 were cloned by PCR using nested primer sets designed to amplify most

or all of the coding sequences of the respective cDNA. The cDNA sequences of the *hox* genes used in primer designs are currently available in GenBank (see Amores et al., 1998 for details). Zebrafish homologs of mouse genes lying 5′ to the *Hox* A and D clusters—*Evx1*, *Hibadh*, and *Tax1bp1* for *Hox* A (Lehoczky et al., 2004), and *Evx2* and *Lnp* for *Hox* D (Spitz et al., 2003)—were also cloned by PCR using sequence information obtained from GenBank or through BLAST searches on zebrafish genomic sequence. These had led to the identification of two zebrafish homologs for each mouse gene except for *Evx1* and *Evx2*, which were represented by single homologs in zebrafish genome. Linkage of these genes to a *hox* cluster was confirmed by available mapping data from zebrafish radiation hybrid panels (e.g., LN54) or by a manual annotation of coding sequences from large genomic clones encompassing the *hox* cluster and neighboring regions. GenBank accession numbers for these genomic clones are: CR382300 (*hoxa13a*, *evx1*), BX601646 (*hibadh-a*, *tax1bp1-a*), AC107364 (*hoxa13b*, *hibadh-b*, *tax1bp1-b*), BX322661 (*hoxd13a*, *evx2*), and BX546447 (*lnp-b*). Each member of the paralogous pair was then labeled as either "*-a*" or "*-b*" depending on the designations given to the neighboring *hox* cluster. The GenBank accession numbers for these genes are: *evx1* (AF305889), *hibadh-a* (BC078207), *tax1bp1-a* (BX601646), *hibadh-b* (NM201160), *tax1bp1-b* (NM212664), *evx2* (NM131232), *lnp-a* (AY423028), *lnp-b* (BC057494).

Whole mount in situ hybridization, immunohistochemistry, and photography

Embryos were fixed in 4% paraformaldehyde at 4 °C for 3–4 days before storage in methanol at −20 °C. Whole mount *in situ* hybridization was performed using protocols given in Harland (1991) and Thisse et al. (1995). After *in situ* hybridization, some embryos were processed further for the labeling of myotomes using a monoclonal antibody against myosin (A4.1025, Developmental Studies Hybridoma Bank). Staining of muscles was achieved by peroxidase reaction using hydrogen peroxide and diamino benzidine as substrates following instructions given in Vectastain ABC kit (Vector Laboratories). For photography, embryos were mounted in 60% glycerol on a depression slide with their left sides facing up at an appropriate angle. Photographs were taken on Nikon D1 digital camera (Nikon corp.) attached to a Zeiss Axioplan compound microscope equipped with Normarski optics.

Cyclopamine treatment

Cyclopamine (Toronto Research Chemicals Inc.) was dissolved in 100% ethanol at 10 mM concentration and kept in dark at 4 °C. Dilution to the final concentration (50 µM) was made in embryo medium containing 0.5% DMSO just before use. Treatments were done by replacing normal embryo medium (containing 20 dechorionated embryos each) with 1 ml of embryo medium containing cyclopamine. Embryos were allowed to develop in dark in the presence of cyclopamine until fixation at 48 hpf. Control embryos were treated in the same way without cyclopamine.

Results

Expression of hoxa9a, a11a, and a13a during zebrafish pectoral fin development

In zebrafish there are 12 posterior *hox* genes that are known to be homologous to the posterior *Hox* A and D genes of tetrapods, with 3 genes located on *hoxaa* cluster (on chromosome 19), 4 on *hoxab* cluster (on chromosome 16), and 5 on *hoxda* cluster (on chromosome 9) (Amores et al., 1998). Previously, expression patterns of some of the posterior *hox* genes belonging to the *hoxab* and *hoxda* clusters had been described in zebrafish fin bud (Sordino et al., 1995, 1996; Neumann et al., 1999; Grandel et al., 2000), but expression patterns of *hoxaa* cluster genes have not been reported. Here we show that in zebrafish, posterior *hox* genes of the *hoxaa* cluster are expressed within the muscles and distal mesenchyme cells during pectoral fin development.

hoxa9a/a11a

Because expression of *hoxa9a* and *hoxa11a* are almost completely identical to each other during most of the stages examined (Fig. 2), expression of these genes will be described together. Expression of

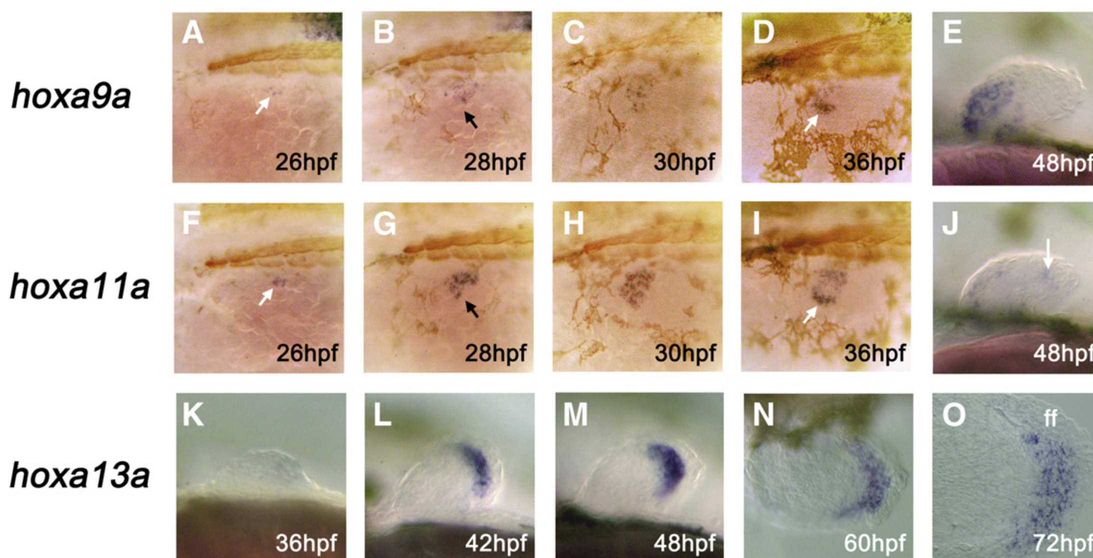

Fig. 2. Expression of *hoxa9a*, *hoxa11a*, and *hoxa13a* during zebrafish pectoral fin development. Expression of *hoxa9a* (A–E) and *hoxa11a* (F–J) is largely confined to the prospective myogenic cells of the fin bud, whereas expression of *hoxa13a* (K–O) is seen exclusively within the distal mesenchyme cells that later give rise to the connective tissues of the fin blade. Oblique dorsal (A–D, F–I), lateral (E, J, K–M), or dorsal (N, O) views with anterior to the left in all panels. Only left side is shown for each embryo. Some embryos are also stained for muscle myosin (brown staining in panels A–D, F–I) to show the position of myotomes. Arrows in panels A/F, B/G, and D/I mark the expression within the nascent myogenic cells (A, F), myogenic cells invading the fin bud proper (B, G), and lateral cluster of prospective pectoral fin muscle cells (D, I), respectively. ff: fin fold (later, fin blade). hpf: hours post fertilization.

hoxa9a/a11a is first detected at 26 h post-fertilization (hpf) in a small group of cells lying postero-medial to the fin bud, in an area slightly anterior to the border between the third and fourth myotomes (Figs. 2A, F, arrows). These cells are subsequently joined by additional groups of *hoxa9a/a11a*-positive cells appearing more anteriorly and, to a lesser extent, more posteriorly, eventually forming a continuous stream of *hoxa9a/a11a* expression occupying a narrow zone centered in an area lateral to the third myotome (not shown).

As the fin bud begins to form visibly, expression of *hoxa9a/a11a* undergoes a further, dynamic change in distribution, first forming several finger-like extensions coming out of the main body of expression (Figs. 2B, G, black arrows) which subsequently spread out at an angle toward the lateral/outer edge of the fin bud (Figs. 2C, H). These patterns are reminiscent of the behavior of myogenic precursor cells of the pectoral fin bud, which in zebrafish are known to originate from the neighboring paraxial mesoderm but migrate away antero-laterally to populate the proximal portion of the growing fin bud (Neyt et al., 2000).

By 30 hpf, expansion of *hoxa9a/a11a* expression within the prospective fin bud area is nearly complete (Figs. 2C, H), and *hoxa9a/a11a* expression begins to organize itself into a pair of clusters that lie parallel to the long axis of the fin bud. This is more clearly seen in embryos at 36 hpf (Figs. 2D, I) at which time it is clear that *hoxa9a/a11a* expression co-localizes with the progenitor cells of pectoral fin muscles (compare with *myoD* expression in Fig. 1b of Neyt et al., 2000; also see Supplementary Figure 1). Expression of *hoxa9a/a11a* tends to be stronger in the lateral cluster (Figs. 2D, I, arrows) compared to the medial cluster, which often does not express *hoxa9a*.

After 36 hpf, the relative intensity of expression between *hoxa9a* and *hoxa11a* begins to change, with *hoxa11a* expression becoming much weaker than *hoxa9a*, which continues to increase its level of expression within the pectoral fin muscle cells. By 48 hpf, differences in level of expression become noticeable between the two genes (Figs. 2E, J). Interestingly, by this time expression of *hoxa11a* diverges from that of *hoxa9a* since a small group of scattered cells in the distal fin bud weakly express *hoxa11a* but not *hoxa9a* (Fig. 2J, arrow). Expression of both genes within the fin muscle cells remains weakly visible during later stages of development and eventually disappears after 72 hpf (not shown).

hoxa13a

In contrast to *hoxa9a* and *hoxa11a*, *hoxa13a* is not expressed in the progenitor cells of pectoral fin muscles. Instead, expression of *hoxa13a* is noted within the distal fin bud mesenchyme cells in a pattern

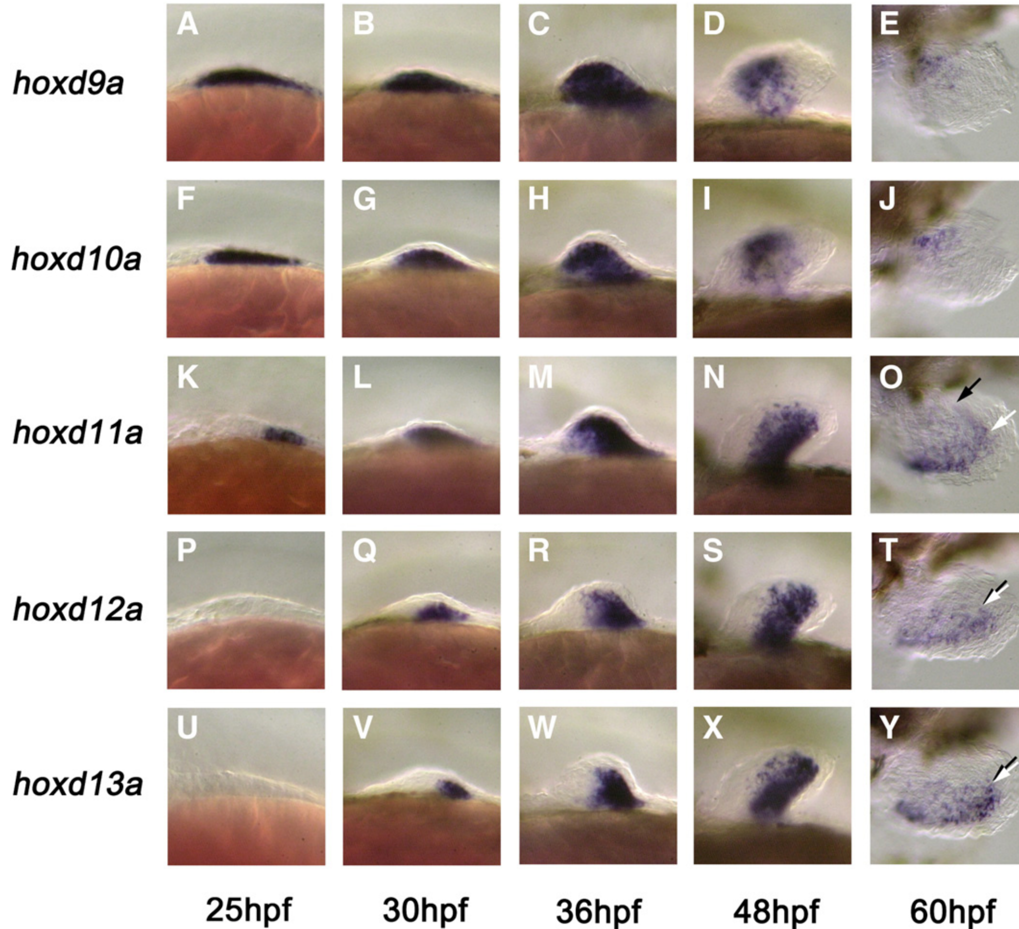

Fig. 3. Expression of *hoxda* cluster genes during pectoral fin development in zebrafish. Expression of *hoxda* genes occurs in three phases during pectoral fin bud development. Phase I, which occurs at the beginning of fin bud morphogenesis, is characterized by a uniform expression of *hoxd9a* within the early fin bud mesenchyme (A, F, K, P, U). Phase II, which immediately follows phase I (and for *hoxd10a* (F), occurs simultaneously with phase I), is characterized by a sequential activation of *hoxd10–13a* gene expression, each of which occupies successively smaller regions within the fin bud centered at the posterior margin ("early" phase II: B, G, L, Q, V). During later stages ("late" phase II) distal expression domains of these genes extend variably into more anterior regions, thereby causing an appearance of the distal bending of the expression (C, H, M, R, W). Phase III, which is limited to the distal fin bud mesenchyme cells, occurs last and is characterized by the posteriorly limited expression of *hoxd11–13a* genes within the most distal group of cells (O, T, Y). Note that, unlike *Hoxd13* in tetrapod limbs, expression of *hoxd13a* in zebrafish pectoral fins during this phase does not cover the entire distal fin bud region. During phase III, no expression is seen for *hoxd9a* and *hoxd10a* in distal cells (E, J). Lateral (A–D, F–I, K–N, P–S, U–X) or dorsal (E, J, O, T, Y) views with anterior to the left in all panels. Only left pectoral fin buds are shown. Black and white arrows in panels O, T, Y show the anterior limits of expression within the proximal and distal mesenchyme cells, respectively. hpf: hours post fertilization.

similar to the one reported for the tetrapod *Hoxa13* gene (Nelson et al., 1996). Expression of *hoxa13a* begins weakly at 36 hpf in the distal posterior portion of the fin bud mesenchyme under the surface ectoderm in a weak posterior-to-anterior gradient (Fig. 2K). Subsequently, expression within this region expands and becomes upregulated, so that by 42 hpf a much stronger expression of *hoxa13a* can be seen postero-distally in a crescent-shaped domain (Fig. 2L). Expression of *hoxa13a* remains distal and within the fin bud proper (Fig. 2M) up until 60 hpf when it co-localizes with the distal mesenchyme cells (as defined in Grandel and Schulte-Merker, 1998) migrating into the fin fold (Fig. 2N). During later stages, expression gradually recedes towards the distal edge of the fin and eventually is seen to be contained in a thin layer of cells within the proximal portion of the fin blade (Fig. 2O). Expression of *hoxa13a* continues weakly within the distal mesenchyme cells up until 6 days of development, although after 4 days *hoxa13a* expression within the fin blade becomes practically undetectable.

Examination and re-interpretation of the expression of posterior hox genes during pectoral fin development in zebrafish: early expression (phase I and phase II)

Studies of *Hox* genes in tetrapods have indicated that expression of *Hox* genes during limb development is regulated in three distinct phases (Nelson et al., 1996). Previously, it was reported that *hox* gene expression during development of zebrafish pectoral fins lacks a pattern that resembles the most distally occurring third phase of expression in tetrapod limbs (Sordino et al., 1995). This interpretation, however, was based upon data that did not include expression of posterior *hoxaa* genes as well as *hoxd9a*. To determine whether an additional phase of regulation of *hox* gene expressions could be found, we re-investigated the expression patterns of posterior *hox* genes during zebrafish pectoral fin development using the full complement of posterior *hoxa/d* genes except for *hoxa9a/a11a*, which are expressed almost exclusively in the muscle cells (Figs. 2A–J).

Similar to tetrapod limbs, we found that, in zebrafish, the onset of pectoral fin bud morphogenesis (24 hpf) is marked by a uniform expression of the 3′-most posterior *hoxd* gene—*hoxd9a*—within the fin bud mesenchyme (Fig. 3A). Expression of *hoxa* genes also begins at this stage, although, unlike *hoxd* genes, the initial expression of the 3′-most posterior *hoxa* gene—*hoxa9b*—is limited to a small group of mesenchyme cells within the anterior fin field (Fig. 4A). During subsequent hours, however, *hoxa9b* expression gradually expands to more posterior regions (Fig. 4B), which eventually encompasses almost all of the fin bud mesenchyme cells by 30 hpf (Figs. 4C, 5A). In tetrapods, uniform expression of several 3′-most posterior *Hox* A/D genes during early stages of limb development (*Hoxa9, d9* and *d10* in chickens (Nelson et al., 1996) and *Hoxd9* in mouse (Tarchini and Duboule, 2006) constitutes phase I expression (Nelson et al., 1996). This indicates that, at least with respect of the *Hox* D cluster genes, the earliest part of the regulatory mechanism controlling *Hox* gene expression is likely to be similar between fins and limbs.

As development progresses, genes from more 5′ parts of the *hoxa/d* clusters begin to show expression. *hoxd10a* begins its expression simultaneously with *hoxd9a* at 24 hpf (Fig. 3F), which is followed by *hoxd11a*, *hoxa10b*, and *hoxa11b* at 25 hpf (Figs. 3K, 4E, 4I), and then by *hoxd12a*, *hoxd13a*, and *hoxa13b* about one hour later (data not shown). Interestingly, the onset and subsequent unfolding of expression domains seem to be regulated differently for genes of the *hoxa* and *hoxd* clusters, and therefore they will be described separately.

For *hoxd* cluster genes, the initial expression of more 5′ genes—*hoxd11a*, *d12a*, and *d13a*—is limited to the posterior part of the incipient fin bud (Fig. 3K and data not shown). During subsequent hours, expression of these genes variably expands into more anterior regions (Figs. 3K, L and data not shown). By 30 hpf, combined expression domains of *hoxd* genes roughly form a nested concentric series centered at the posterior margin of the fin bud, with *hoxd9a* occupying the largest area, followed by *hoxd10a*, *d11a*, *d12a*, and *d13a* occupying successively smaller areas (Figs. 3B, G, L, Q, V). This pattern is reminiscent of the early phase II expression of posterior *Hox* D genes of tetrapods (as seen in stage 19 chicken (Nelson et al., 1996) or E10.0 mouse (Tarchini and Duboule, 2006) embryos), indicating that like the phase I regulation, the early phase II regulation of *Hox* D genes is also likely to be conserved between fins and limbs.

In zebrafish pectoral fins, the phase II regulation of *hoxd* genes seems to be in effect up until 36 hpf, by which time expression

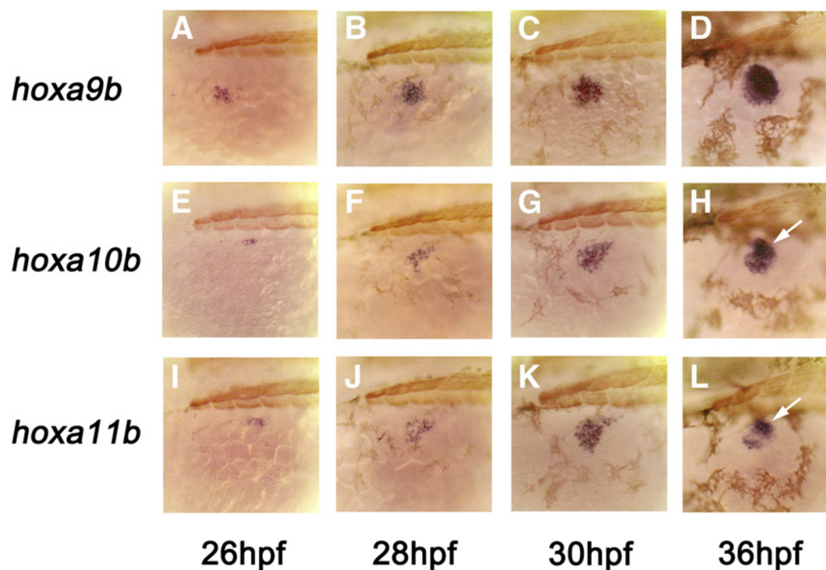

Fig. 4. Early expression of *hoxab* cluster genes in zebrafish pectoral fins. Expression of *hoxa9b* starts within the mesenchyme cells of anterior fin field (lying lateral to the second myotome) and subsequently spreads toward the posterior, eventually encompassing the entire fin bud mesenchyme by 30 hpf (A–C: phase I expression). For *hoxa10b* and *hoxa11b*, the initial expression is confined to the myogenic mesenchyme cells (E–G, I–K). For these genes, expression within the chondrogenic mesenchyme cells begins around 30 hpf in distal posterior cells (phase II expression) roughly overlying the medial group of myogenic cells (H, L: arrows). In dorsal views, this creates a misleading impression of enhanced expression in the medial cluster of prospective pectoral fin muscle cells (compare Figs. 4H, L with Figs. 2D, I). Oblique dorsal views with anterior to the left in all panels. Each embryo is also counter-stained for muscle myosin (brown) to show the position of myotomes. hpf: hours post fertilization.

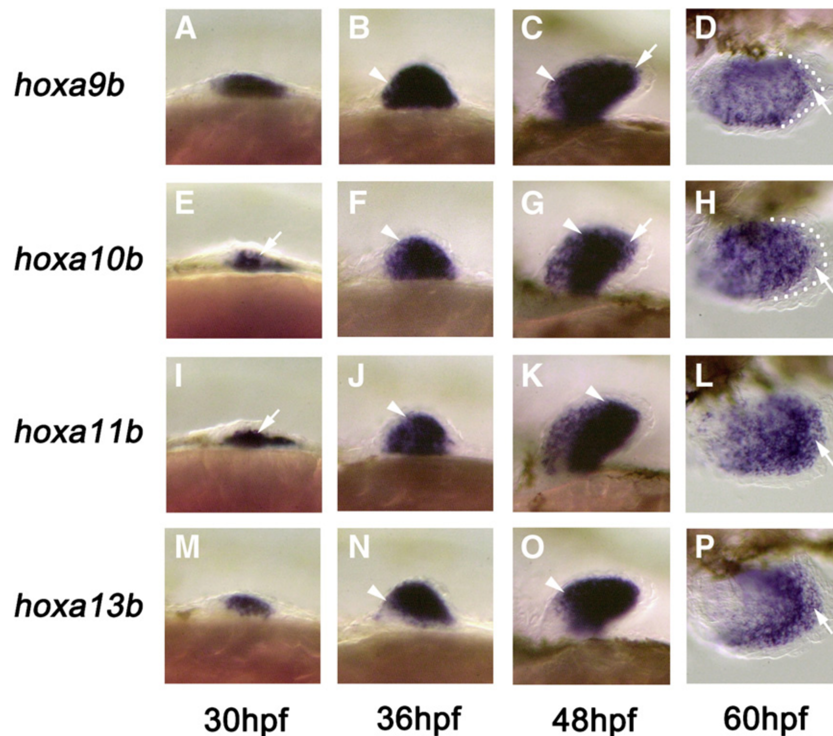

Fig. 5. Late expression of *hoxab* cluster genes during zebrafish pectoral fin development. Similar to *hoxda* genes, expression of *hoxab* genes occurs in three distinct phases during pectoral fin bud development. Phase I, which gradually occurs during early stages of fin bud morphogenesis, is characterized by a uniform expression of *hoxa9b* within the early fin bud mesenchyme (A). Phase II expression begins with the onset of *hoxa10/11/13b* expression within the distal posterior fin bud mesenchyme just underneath the surface ectoderm (E, I, M), which establishes nested domains of expression for *hoxa9–11b* genes during later stages (B, F, J, C, G, K). Note that, for *hoxa13b* (N, O), colinearity is not observed, even though its expression is otherwise similar to the phase II expressions of *hoxa10b/a11b* during the same period. Phase III, which is limited to the distal fin bud mesenchyme cells, occurs last and is characterized by the expression of *hoxa11b* and *hoxa13b* genes within distal cells (L, P: arrows). During phase III, little expression is seen for *hoxa9b* and *hoxa10b* genes in the same region (D, H: arrows). Lateral (A–C, E–G, I–K, M–O) or dorsal (D, H, L, P) views with anterior to the left. Only left pectoral fin buds are shown. Arrowheads in panels B, C, F, G, J, K, N, and O show the anterior limits of expression within the chondrogenic mesenchyme cells which at these stages are flanked on both sides by myogenic cells which tend to show weaker expression. Small dots in panels D and H represent the outermost extent of the distal mesenchyme cells. hpf: hours post fertilization.

domains of several *hoxd* genes have diverged between proximal and distal regions of the fin bud (Figs. 3C, H, M), although the colinear nature of the expression of these genes can still be seen in their anterior-most domain of expression (late phase II: Figs. 3C, H, M, R, W). Interestingly, similar to the corresponding tetrapod genes during late phase II expression (as seen in stage 23 chicken (Nelson et al., 1996) or E10.5 mouse (Tarchini and Duboule, 2006) embryos), for *hoxd9a*, *d10a*, and *d11a*, this distal expression also variably covers anterior regions of the fin bud (Figs. 3C, H, M), which, in prior studies, was reported to be devoid of the expression of any of these genes (Sordino et al., 1995).

Compared to the *hoxd* genes, however, the interpretation is not as straightforward for *hoxa* genes. Unlike *hoxd* genes, which initiate their expression within the fin bud proper and therefore (at least initially) only in the prospective chondrogenic mesenchyme cells (Figs. 3A, F, K, P, U), *hoxa* genes are often seen to begin their expression within the prospective myogenic cells and thus outside of the fin bud (Figs. 4E, I). Specifically, expression of *hoxa10b* and *hoxa11b* begins within the myogenic mesenchyme cells at their birth (Figs. 4E, I), and remains restricted to the prospective muscle cells until 30 hpf (Figs. 4F, G, J, K). From 30 hpf on, expression of *hoxa10b* and *hoxa11b* genes is also seen in prospective chondrogenic mesenchyme cells, which is initially limited to small groups of cells immediately underneath the posterior fin bud ectoderm (Fig. 5E, I, arrows) but later expands to include more proximal cells as well.

Curiously, the other two posterior *hox* genes within the *hoxab* cluster—*hoxa9b* and *hoxa13b*—show the opposite pattern, initiating their expression within the prospective chondrogenic cells (Fig. 4A and data not shown), and do not incorporate muscle cells into their expression domains until much later. As a consequence, unlike *hoxd* genes, expression domains of *hoxa* genes do not stabilize until fin bud development is well under way. Nevertheless, by 36 hpf (and more clearly at 48 hpf; compare the positions of arrowheads in Figs. 5C, G, K), nested domains of expression similar to the ones seen for *hoxd* genes during late phase II could be recognized for *hoxa9b*, *hoxa10b*, and *hoxa11b* genes in the prospective chondrogenic mesenchyme cells (Figs. 5B, F, J, arrowheads). This indicates that like *Hox* A genes in tetrapods (Nelson et al., 1996), *hoxab* genes in teleosts are also subject to a phase II-like regulation during fin development.

Examination and re-interpretation of the expression of posterior hox genes during pectoral fin development in zebrafish: late expression (phase III)

By 36 hpf, developmental processes producing embryonic fin buds come to an end and the transformation of fin buds into larval pectoral fins is about to begin. This event is marked by the onset of expression of *hoxa13a*, which is the last *hox* gene to be expressed within the fin bud (Fig. 2K). Expression of other *hox* genes also begins to change, most notably for *hoxd9a* and *hoxd10a*. Up to 36 hpf, expression of these two genes was present throughout the fin bud except for a small group of cells at the anterior proximal corner (Figs. 3A–C, F–H). By 48 hpf, however, expression of *hoxd9a* and *hoxd10a* disappears in the most distal group of cells and expression in the center of the fin bud becomes weak, thereby leaving large patches of *hoxd9a/d10a* expression only in the middle segment of the fin (Figs. 3D, I). Intriguingly, such lack of *hoxd9a/d10a* expression in this distal fin bud region is closely mirrored by a strong expression of *hoxa13a* in the same area (Fig. 2M). In tetrapods, distal limb mesenchyme cells that express *Hoxa13* but not *Hoxd9* are known to develop into the autopod (Nelson

et al., 1996), suggesting that this distal zone of *hoxa13a*-expressing cells within the fin bud has the genetic profile partly similar to the autopod region of tetrapod limb bud.

Since the phase III expression of *Hox* genes within the tetrapod limbs, particularly that of *Hox* D genes, is known to characterize the development of the autopod (Nelson et al., 1996), we investigated whether a similar phase of expression could also be found for zebrafish *hoxd* genes in this distal autopod-like region. In the forelimbs of mouse and chicken, phase III expression begins at stage 22/23 (chicken) or E10.5 (mouse) with the onset of *Hoxd10–13* expression within the posterior distal corner of the limb bud (Nelson et al., 1996; Tarchini and Duboule, 2006). Initially, these expressions are fully masked by the overlapping late phase II expressions of the same genes which by this stage also have expanded into more anterior regions of the limb bud (Nelson et al., 1996; Tarchini and Duboule, 2006). However, as the limbs grow out distally, these two domains begin to separate from each other such that by stage 26/27 (chicken) or E11.5–12.0 (mouse), a spatially distinct set of *Hoxd10–13* expression develops within the distal limb bud, in which *Hoxd13* has the widest domain of expression covering the entire distal autopod while *Hoxd10–12* share a common expression domain that is more posteriorly restricted (Nelson et al., 1996; Tarchini and Duboule, 2006).

We found that similar to tetrapod *Hoxd10–13* during late phase II–early phase III (stage 23–25 chicken wings (Nelson et al., 1996) or E10.5 mouse forelimbs (Tarchini and Duboule, 2006)), the zebrafish *hoxd11–13a* genes show variable degrees of expansion toward the anterior in their distal domains of expression by 36 hpf (Figs. 3M, R, W). Like tetrapods, colinearity of expression is observed within these distal domains, although by 48 hpf most of the differences in expression have disappeared (Figs. 3N, S, X). By 60 hpf, expression of *hoxd11–13a* genes in the distal fin bud region occupies approximately the posterior half of the distal mesenchyme cells (compare with *hoxa13a* expression in Fig. 2N) in which they also share a common anterior limit of expression (Figs. 3O, T, Y, white arrows). Unlike tetrapod genes, however, in zebrafish *hoxd11–13a*, these distal domains of expression remain continuous with more proximal expression and *hoxd13a* expression remains posteriorly restricted in the distal fin bud region (Figs. 3O, T, Y). This indicates that, as shown previously (Sordino et al., 1995), *hoxd11–13a* genes in zebrafish do not show expression patterns fully compatible with those seen in tetrapod *Hoxd10–13* genes during development of the limb autopod regions.

Nevertheless, despite apparent continuity of expression for zebrafish *hoxd11–13a* genes between proximal and distal regions of the fin bud, the two regions still seem to be subject to separate regulations. This is most clearly seen in *hoxd11a* (Fig. 3O) which, unlike *hoxd12a* and *hoxd13a* (Figs. 3T, Y), transiently shows a divergence in expression domains between proximal (Figs. 3O, T, Y, black arrows) and distal (Fig. 3O, T, Y, white arrows) groups of cells. Furthermore, although *hoxd13a* expression does not cover the entire distal fin bud region (Fig. 3Y), *hoxd11a* and *hoxd12a* do share common anterior limits of expression in distal regions (Figs. 3O, T). This indicates that these genes might be regulated similarly to their tetrapod counterparts. Therefore, although not exactly alike, *hoxd11–13a* genes of zebrafish still show some similarities to tetrapod *Hoxd10–13* genes with respect to their distal/late expression and thus may also utilize a distinct control in distal region that is separate from phases I and II regulations of more proximal regions.

In zebrafish, such differential regulation between proximal and distal domains of expression does not appear to be limited to *hoxd* cluster genes, since genes from the *hoxa* cluster also show a similar pattern of divergence in expression. Similar to *hoxd9a*/*d10a*, expression of *hoxa10b*, and to a lesser extent *hoxa9b*, begins to diminish in distal fin bud regions at 48 hpf (Figs. 5C, G, arrows), which by 60 hpf show little or no expression of these genes (Figs. 5D, H, arrows). This is in contrast to the behavior of *hoxa11b* and *hoxa13b*, which maintain relatively high levels of expression in all distal cells (Figs. 5L, P, arrows) and thus are quite similar to *hoxa13a* in this regard (compare with Fig. 2N). Interestingly, the combined outcome of such differential expression of posterior *hoxab* genes is a generation of a distinct distal domain of expression characterized by the presence of *hoxa13b* and *hoxa11b* and the absence of *hoxa10b* and *hoxa9b* transcripts (Fig. 5D, H, L, P, arrows). This is highly reminiscent of the *Hox* A expression within the autopod region of tetrapod limbs which is also characterized by differential expression such as the presence of *Hoxa13* but not *Hoxa11* or *Hoxa10* transcripts (see the expression of these genes in stage 28 chicken wings (Nelson et al., 1996)). Therefore, at least for *hoxa* genes the distal domains of expression are regulated in much the same way as the phase III expression of tetrapod *Hox* A genes, indicating that, unlike the case of *hoxd* genes, the phase III expression of *hoxa* genes in zebrafish is likely to be governed by a mechanism fully conserved between fins and limbs.

Regulation of the distal expression of hox genes during development of pectoral fins: role of sonic hedgehog (shh) signaling

One important characteristic of the phase III *Hox* expression during tetrapod limb development is its reliance on *Shh* signaling (Nelson et al., 1996; Chiang et al., 2001; Zákány et al., 2004). In tetrapods, while *Hox* gene expression during earlier phases can initiate independently of *Shh* signaling (but is maintained at higher levels in the presence of *Shh*), the expression of *Hox* A and D genes during phase III is known to be absolutely dependent upon *Shh* signaling (Ros et al., 1996; Chiang et al., 2001). In zebrafish, the effect of the loss of *shh* signaling on the expression of *hox* genes during pectoral fin development was previously examined by Neumann et al. (1999) but to date their results have not been analyzed in terms of the influence on the individual phases of *hox* gene expression. We found that, in zebrafish embryos lacking *shh* function—zebrafish *sonic you* mutants (syu^{t4}: Schauerte et al., 1998), development of pectoral fins does not progress further than the 30 hpf-like stage (data not shown), thereby making it unlikely that $shh^{-/-}$ fin buds will ever show a phase III *hox* expression which normally begins at 36 hpf with the onset of *hoxa13a* expression (see above).

Consistent with this, in $shh^{-/-}$ (or syu^{t4}/syu^{t4}) embryos, *hoxd* genes failed to show any expression characteristic of the late phase II (anterior deflection of distal expression from which distinct late expression domains arise postero-distally: Figs. 3C, H, M, R, W) and later, although, like the *Hox* D genes in tetrapods, the phase I as well as most of the early phase II expressions occurred normally (not shown; also see Fig. 1 of Neumann et al., 1999). For *hoxa* genes, the consequences were even more severe. As expected, no *hoxa13a* expression was ever seen in $shh^{-/-}$ fin buds (not shown) but surprisingly, except for *hoxa9b*, *hoxa* genes also failed to show an expression within the prospective chondrogenic mesenchyme cells (not shown). This indicates that in addition to phase III, phase II expression also failed to be initiated for posterior *hoxa* genes in the absence of *shh* function. Expression within the prospective myogenic cells, on the other hand, was initiated normally and progressed to a 30 hpf-like stage (data not shown) but during later stages this often failed to undergo proper organization into lateral and medial clusters, thereby leaving a single malformed cluster or, in some cases, several small disorganized clusters of *hoxa*-positive cells in the proximal part of the mutant fin bud (not shown; also see Fig. 2 of Neumann et al., 1999).

In order to circumvent the problem of early developmental arrest in $shh^{-/-}$ pectoral fin buds, we took advantage of the availability of cyclopamine, a plant alkaloid known to inhibit *hedgehog* signaling (Incardona et al., 1998). Treatment of wild-type embryos with 50 µM cyclopamine beginning at 36 hpf resulted in no gross abnormality in development of these fish, although they tend to show abnormal pooling of blood in their brains (data not shown). However, when examined for the expression of *hox* genes at 48 hpf these fish were found to completely lack *hoxa13a* expression as well as most of the distal expression of *hoxd11–13a* genes in their pectoral fins (Figs.

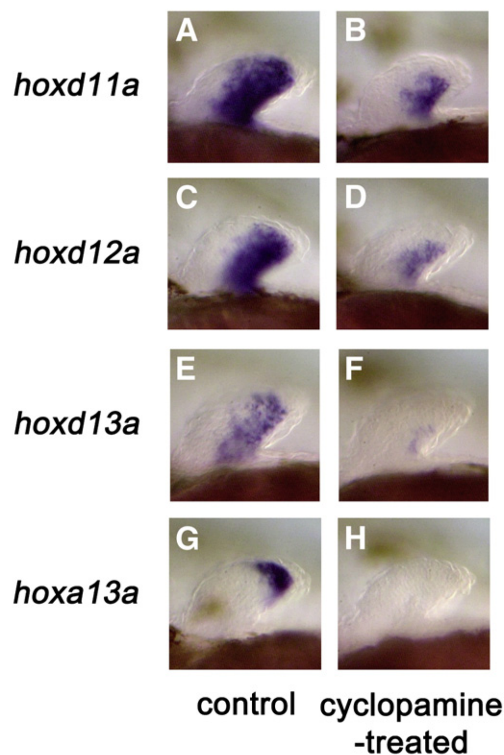

Fig. 6. Effect of cyclopamine treatment on *hox* gene expression in the pectoral fin bud. (A, C, E, G) Control embryos showing normal expression of *hox* genes. (B, D, F, H) Embryos treated with 50 μM cyclopamine for 12 h from 36 hpf. Note the complete absence of *hox* gene expression within the distal mesenchyme cells in cyclopamine-treated embryos. Lateral views of the left pectoral fins with anterior to the left in all panels. All embryos are approximately at 48 hpf. hpf: hours post fertilization.

6A–H). This indicates that, as shown in tetrapods, initiation and/or maintenance of the distal (*hoxd11–13a*) or phase III (*hoxa13a*) expression of *hox* genes are also dependent upon the presence of *shh* signaling in teleosts.

Regulation of the distal expression of hox genes during development of pectoral fins: coordination by a prospective long-range enhancer element

Another and perhaps more important feature of the phase III expression of *Hox* genes in tetrapod limbs is its regulation via a distinct enhancer element. In mouse, distal limb expression of both *Hoxa13* (Lehoczky et al., 2004) and *Hoxd10–13* (Hérault et al., 1999; Kmita et al., 2002; Spitz et al., 2003; Tarchini and Duboule, 2006) is known to be controlled by a nearby regulatory element located outside of the *Hox* cluster. This element, which seems to be able to influence the expression of several *Hox* genes at a great distance (Spitz et al., 2003), also directs the expression of genes neighboring the *Hox* A and D clusters to the autopod region (Spitz et al., 2003; Lehoczky et al., 2004). Similar, highly conserved blocks of non-coding sequences have also been found in corresponding regions of the pufferfish (*Fugu rubripes*) genome (Spitz et al., 2003; Lehoczky et al., 2004), although in transgenic mice, these fish sequences failed to show any phase III enhancer-like activities (Spitz et al., 2003). To determine whether the *hox* genes in zebrafish are also subject to a similar regulatory control, we examined the expression of genes whose murine homologs are known to be located outside of the *Hox* cluster but are expressed in the autopod region due to the regulatory influence of nearby phase III (digit/autopod) enhancers.

We found that the two genes adjacent to the *hoxaa* cluster—*evx1* and *hibadh-a*—are also expressed in distal fin mesenchyme cells at 48 hpf in a pattern similar to *hoxa13a* (Fig. 7A). High level expression in the distal mesenchyme cells is also occasionally seen for *tax1bp1-a* (not shown), the next 5′ gene on the chromosome, although in the majority of specimens (95%) expression is not detected above the background level within the fin bud mesenchyme (Fig. 7A). These results suggest that, similar to the mouse *Hoxa13* (Lehoczky et al., 2004), expression of *hoxa13a* in zebrafish is also likely to be controlled by a long-range enhancer element.

A similar conclusion could also be reached for the regulation of the distal expression of *hoxa13b*, although the evidence here is not as strong. Like *hoxa13b*, the two genes 5′ to the *hoxab* cluster—*hibadh-b* and *tax1bp1-b*—are expressed at relatively high levels throughout fin bud mesenchyme, including the distal-most group of cells (Fig. 7B). This suggests that similar to the genes from the corresponding genomic region containing the *hoxaa* cluster, genes surrounding the 5′ end of the *hoxab* cluster might also be subject to a co-regulation by a long-range enhancer. However, unlike *hoxa13a*, *evx1*, *hibadh-a*, and also possibly *tax1bp1-a*, there is little distinction between the proximal and distal expression of these genes within fins, and therefore at present it is difficult to determine whether there is indeed a separate regulation homologous to the phase III regulation of tetrapod *Hox* A genes governing their distal expression. Further studies involving the identification of distinct regulatory elements within the promoter regions of these genes might help to resolve this issue.

In contrast to the genes of *hoxaa* and also possibly *hoxab* clusters, posterior *hox* genes of *hoxda* cluster do not seem to be subject to a long-range control similar to the phase III regulation of tetrapod *Hox* D genes in limbs (Figs. 3O, T, Y; also see Sordino et al., 1995). The two genes adjacent to the *hoxda* cluster, *evx2* and *lnp-a*, which in mouse are known to be expressed within the limb autopod region in a very *Hox*-like manner due to the influence of a nearby long-range enhancer (Spitz et al., 2003), either fails to be expressed across the entire distal mesenchyme cells (*evx2*) or is expressed at low levels throughout undifferentiated mesenchyme including the distal regions of the fins (*lnp-a*) (Fig. 7C). This pattern suggests that a functional phase III enhancer associated with the *hoxda* cluster could be present in teleost genome 5′ to *lnp-a* (Spitz et al., 2003), but if present its regulatory influence does not extend beyond *lnp-a*. Therefore, none of the genes in *hoxda* cluster is likely subject to the type of long-range regulation governing the phase III/autopod expression of *Hox* D genes in tetrapod limbs. In spite of this, however, similarity of late distal expression between *hoxd11-13a* and *evx2* genes (Figs. 3N, S, X; Fig. 7C) is still highly suggestive of the coordination of their distal expression within the fin bud.

Therefore, in conclusion, among the zebrafish *hox* genes showing expression in distal mesenchyme cells during pectoral fin development, only genes from *hoxaa* and possibly also *hoxab* clusters seem to be regulated by a long-range enhancer that is functionally similar (and likely homologous) to the phase III/autopod enhancer of tetrapods. This indicates that, in zebrafish pectoral fins, a distal phase of *hox* gene expression that could be considered a true phase III expression is represented by at most four *hox* genes lying close to the 5′ ends of the *hoxaa* and *hoxab* clusters, namely *hoxa11a* and *hoxa13a*, and possibly also *hoxa11b* and *hoxa13b*.

Discussion

Hox genes and evolution of paired appendages in vertebrates

In this study, we found that taken as a whole, patterning of zebrafish pectoral fins by *Hox* genes is much more similar to the patterning of forelimbs of the mouse and chicken than was previously thought. Like in tetrapods, expression of *hox* genes during development of pectoral fins occurs in three distinct phases (see Fig. 8), and other aspects of *hox* gene expression such as the split expression between prospective myogenic and chondrogenic mesenchyme cells

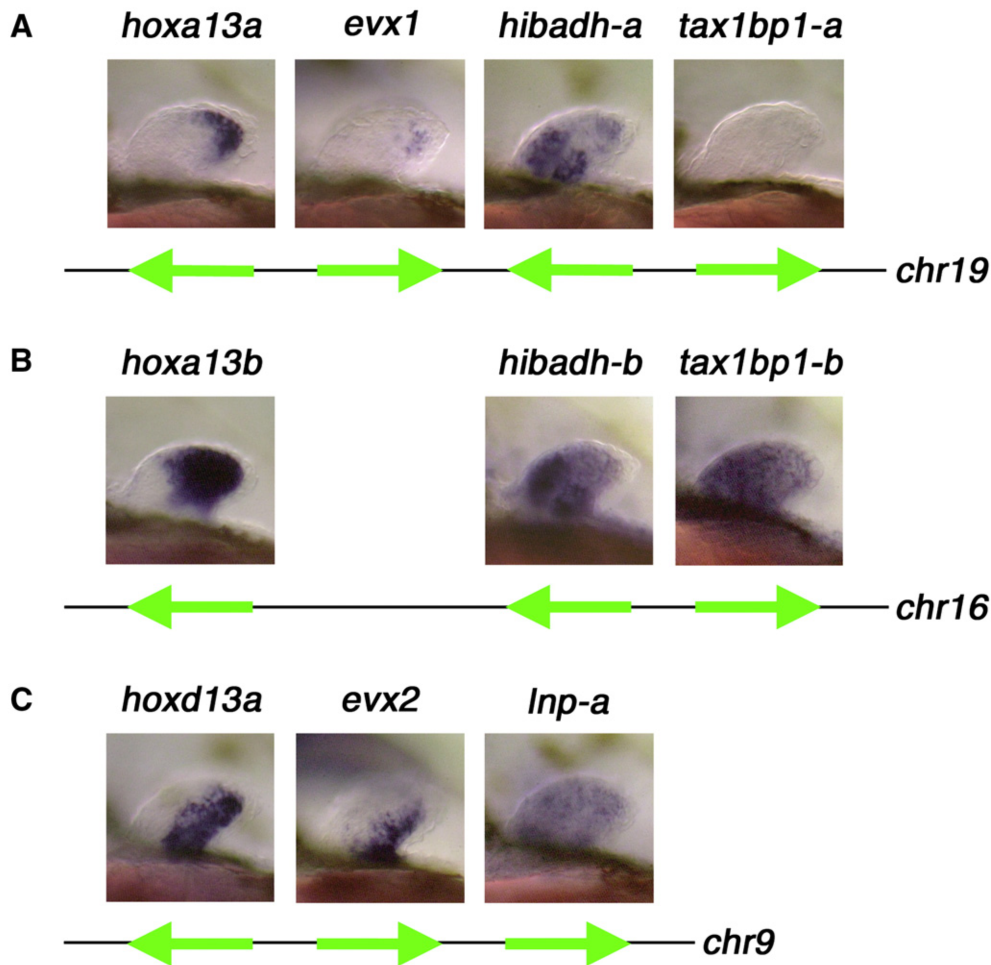

Fig. 7. Expression of the genes neighboring the *hoxa* and *hoxd* clusters during zebrafish pectoral fin development. (A) Expression of the genes near the 5′ end of the *hoxaa* cluster. For *evx1* and *hibadh-a*, strong expression is clearly seen within the distal region of the fin bud in a pattern similar to *hoxa13a*. For *tax1bp1-a*, little expression is seen except for a small subset of specimens (about 5%; not shown), which show an elevated level of expression in the distal region of the fin in a pattern similar to *hoxa13a*. (B) Expression of the genes near the 5′ end of the *hoxab* cluster. Similar to *hoxa13b*, both *hibadh-b* and *tax1bp1-b* are expressed in both proximal and distal regions of the fin bud. (C) Expression of the genes near the 5′ end of the *hoxda* cluster. For *evx2*, expression in distal regions is confined to the posterior, which is similar to its 3′ neighbor, *hoxd13a*. For *lnp-a*, low level expression is seen throughout the fin bud mesenchyme. Lateral views of left pectoral fin buds with anterior to the left in all panels. All embryos are at 48 hpf. Strong proximal staining of *hibadh-a* (A) and *hibadh-b* (B) within the fin bud represents expression within the myogenic cells. The diagram below each set of photographs represents arrangement of genes on the zebrafish genome and the direction of transcription for each gene. Distances between genes are not drawn in scale. For all genes except *lnp-a*, linkages and precise syntenic relationships are independently confirmed by examining large genomic clones covering the corresponding region. For *lnp-a*, synteny is presumed based upon its map position in radiation hybrid panels (ZFIN database) and the relative direction of transcription is tentative. chr: chromosome.

in *hoxa* genes during early stages (*hoxa9a*, *a10b*, *a11a*, and *a11b* in zebrafish (this work) and *Hoxa10* and *Hoxa11* in mouse (Haack and Gruss, 1993)) as well as a lack of distal expression in 3′-lying posterior *hoxa/d* genes during late stages (*hoxa9b*, *a10b*, *d9a*, and *d10a* in zebrafish (this work) and *Hoxa10*, *a11*, and *d9* in chicken (Nelson et al., 1996)) also appears to be the same.

Most significant still is our finding that contrary to the prevailing notion in current literature (e.g., Coates, 1995; Sordino and Duboule ,1996; Shubin et al., 1997; Wagner and Chiu, 2001; Davis et al., 2007; Freitas et al., 2007; Johanson et al., 2007) zebrafish does seem to have a distinct distal phase of *Hox* gene expression similar to the phase III expression of *Hox* genes in tetrapod limbs. This distal phase, which shows the greatest similarity to its tetrapod counterparts with respect to the genes of *hoxa* clusters, appears to be regulated by the same molecular processes governing the phase III expression of *Hox* genes in tetrapod limbs such as *shh* signaling (*hoxa* and *hoxd* genes) and long-range enhancer elements (*hoxa* genes) (Figs. 6, 7). Similarly, like its counterparts in tetrapods, this distal expression seems to be involved in the formation and patterning of the most distal part of the fin, the fin blade. Viewed in a phylogenetic context, this indicates that the tri-phasic expression of *Hox* genes must already have been in place in the common ancestor of teleosts and tetrapods, which must also have been using the regulated expression of *Hox* genes to pattern its paired appendages.

Consistent with this, recent studies on the expression of *Hox* genes during the development of paddlefish (Davis et al., 2007) and catshark (Freitas et al., 2007) pectoral fins have demonstrated that the tri-phasic expression of *Hox* genes—at least for *Hox* D cluster genes—is already operational in the paired appendages of both the primitive ray-finned fishes and the cartilaginous fishes. In addition, fossil ray-finned fishes and lobe-finned fishes often had structurally complex paired appendages (Shubin, 1995; Coates, 2003; Friedman et al., 2007), which indicates that the patterning mechanisms regulating development of paired appendages must have been already fairly advanced by the time the first group of bony fishes appeared in late Silurian sea. Indeed, it has been suggested that the regulatory mechanisms governing the tri-phasic expression of *Hox* genes is likely to be highly ancient in origin, possibly predating even the origin of paired appendages themselves (Lehoczky et al., 2004; Freitas et al., 2006). If such is the case, then the use of the tri-phasic expression of *Hox* genes in patterning of paired appendages must be nearly universal among jawed vertebrates.

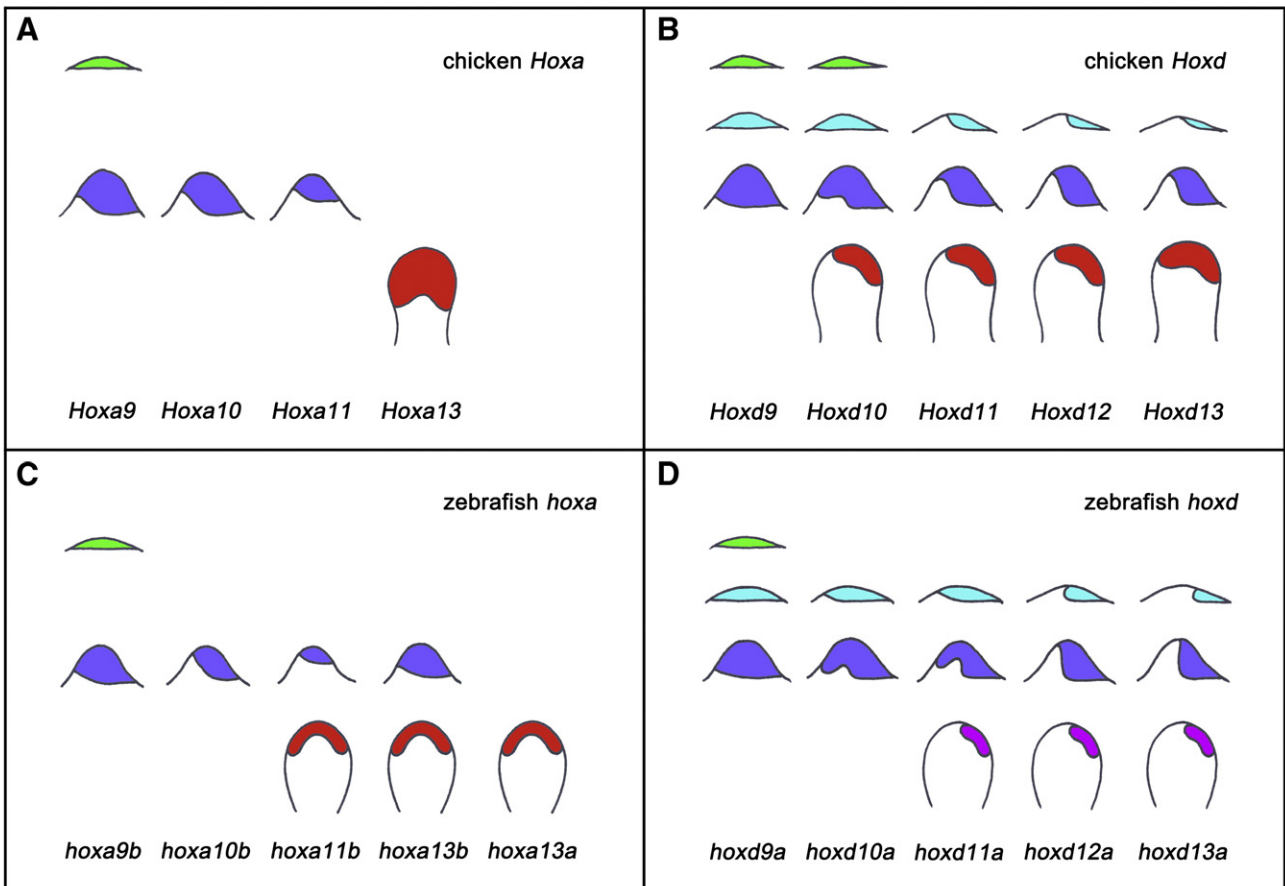

Fig. 8. Summary of the *Hox* gene expression in fins and limbs. (A) Expression of chicken *Hoxa* genes during forelimb (wing) development. (B) Expression of chicken *Hoxd* genes during forelimb (wing) development. (C) Expression of zebrafish *hoxa* genes during pectoral fin development. (D) Expression of zebrafish *hoxd* genes during pectoral fin development. Each panel shows schematic diagrams representing expression domains of various *Hox* genes during each phase of regulation. Green: phase I expression. Note that, unlike *Hoxa9* in forelimb of chickens, the phase I expression of *hoxa9b* in zebrafish pectoral fins becomes established gradually rather than all at once. Turquoise: early phase II expression of *Hox* D genes. Blue: late phase II expression of *Hox* D genes and phase II expression of *Hox* A genes. Note the similarity in pattern between phase II expression of *Hox* A genes and the late phase II expression of *Hox* D genes. Red: phase III expression. Purple: phase III-like distal expression of zebrafish *hoxda* genes which shows only partial similarities to the phase III expression of *Hoxd* genes of tetrapods but is likely to be an independent phase. Data for chicken *Hox* genes are taken from Nelson et al. (1996). For panels A and B, dorsal views of right wing buds in all drawings with anterior to the left. For panels C and D, lateral (phases I and II) or dorsal (phase III and phase III-like) views of left pectoral fin buds with anterior to the left.

In spite of the high-level conservation of the tri-phasic expression of *Hox* genes during development of paired appendages in vertebrates, however, it is also clear that the tri-phasic expression had occasionally undergone regressive changes during evolution. This is most clearly seen in the simplicity of late distal expression of *hoxd* genes in zebrafish pectoral fins (Fig. 3) compared to the complex distal expression of homologous genes in the pectoral fins of more primitive fishes (Davis et al., 2007; Freitas et al., 2007) as well as in the limbs of tetrapods (Nelson et al., 1996; Tarchini and Duboule, 2006) (Fig. 8). It has been suggested that the relative simplicity in the structure of teleost paired fins compared to the complexity of structures in paired appendages of many other vertebrates (Fig. 1) might be causally linked to the complete absence of the phase III expression of *Hox* D genes in teleosts (Sordino and Duboule, 1996; Shubin et al., 1997; Davis et al., 2007; but also see Mabee, 2000).

Our results indicate that the actual evolutionary change in phase III regulation of teleost *hoxd* genes was not as extreme as previously thought. Nevertheless, teleost *hoxd* genes still lack important regulatory features such as reverse colinearity in their distal domains of expression (Fig. 8), which suggests that during evolution teleosts must have lost at least some of the regulatory mechanisms necessary for the complete phase III expression of *hoxd* genes in their paired fins (Fig. 7). Perhaps a better approach to the issue of possible causal connection between structural complexity of paired appendages and complexity of underlying regulatory mechanisms might be to examine the expression of *Hox* genes in several distantly related vertebrate species, each of which had undergone an independent but significant alteration in the structure of its paired appendages during evolution (e.g., some lizards (Wiens and Slingluff, 2001), dolphins (Richardson and Oelschläger, 2002), and lungfishes (Friedman et al., 2007)). Such tests might even help us to determine what the precise patterning roles are for the individual phases of *Hox* gene expressions during development of vertebrate paired appendages (see below).

Hox genes and development of paired appendages in vertebrates

Our results also suggest that, given the potentially universal use of the tri-phasic expression of *Hox* genes in the patterning of paired appendages (Lehoczky et al., 2004), many of the current hypotheses on the potential roles of *Hox* genes in the development of paired appendages may need to be re-evaluated. Presently, due to the predominance of tetrapod models in the study of *Hox* gene functions, most hypotheses on the roles of *Hox* genes in appendage development (or evolution) are based on the assumption that they are involved in the construction of a fundamentally limb-like structure. For example, one of the most commonly held assumptions on the role of tri-phasic expression of *Hox* genes in development is that successive deployment of individual phases during development of limbs represents the sequential specification of each of the three segments of the limbs (Yokouichi et al., 1991; Nelson et al., 1996; Shubin et al., 1997).

Likewise, the nested expression domains formed by five posterior *Hox* D genes during late phase II was once viewed as morphogenetic codes for the specification of the five digit types present in most limbs (Tabin, 1992; but also see Graham, 1994). More recently, the anteriorward deflection of the distal domains of expression of *Hox* D genes during late phase II was interpreted as a molecular representation of the hypothetical bending of the metapterygial axis (Coates, 1995; but also see Cohn et al., 2002).

Our observation that in zebrafish pectoral fins, the structure of which does not readily conform to the morphological norm of tetrapod limbs (Figs. 1B, C), *Hox* genes are still expressed in a very limb-like fashion (Fig. 8) indicates that the apparently close correspondence between features of *Hox* gene expressions and the structure of tetrapod limbs is likely to be a coincidence and the actual roles of the *Hox* genes in fin/limb development might be more general, possibly defining only the most fundamental aspects of the fin/limb structures that are shared by all paired appendages such as the asymmetry in internal structures (Friedman et al., 2007). Consistent with this, recent studies on the function of *Hox* A and D cluster genes in mouse have shown that, in limbs, the early expression (phases I and II) of *Hox* genes has a previously unrecognized role of limiting *Shh* expressions to the posterior of the early limb bud (Zákány et al., 2004), which is essential for the proper establishment of the asymmetry in limb structures as well as the subsequent initiation of distal outgrowth of the limbs (Kmita et al., 2005; Tarchini et al., 2006). We predict that, given the generic and versatile nature of *Hox* gene functions in development (Greer et al., 2000), more of such basic patterning roles will likely emerge for *Hox* genes in the development of fins and limbs.

Expression of Hox genes in fins vs limbs: change in long-range regulations?

In spite of the high-level conservation in the tri-phasic expression patterns of *Hox* genes between fins and limbs, subtle differences were also evident between zebrafish and tetrapod *Hox* clusters in the operation of the underlying regulatory mechanisms. This was most clearly seen in the aberrant behavior of the *Evx2* gene, which is located just 5′ to the *Hox* D/*hoxda* clusters and is known to be expressed very similarly to its closest neighbor, *Hoxd13*/*hoxd13a* (Fig. 7C; Dollé et al., 1994; Sordino et al., 1996). However, in mouse, *Evx2* is expressed relatively late in limb development and only in the autopod (i.e., subject only to the phase III regulation) (Tarchini and Duboule, 2006), while in zebrafish *evx2* begins its expression shortly after *hoxd13a* and in a domain similar to *hoxd13a* (Fig. 7C and data not shown). This indicates that in zebrafish, *evx2* is subject to a phase II regulation which is not seen in mouse.

Such changes in regulation, however, may not have been limited to the *Hox* D cluster. *hoxa11a*, a gene lying just 3′ to *hoxa13a*, also shows an *evx2*-style change in developmental regulation by showing a phase III expression (Fig. 2J) while its tetrapod homolog, *Hoxa11*, shows only phase II expression (Nelson et al., 1996; Post and Innis, 1999). Likewise, earlier onset of expression and more proximally expanded expression domains of *hoxa13b* compared to its paralog *hoxa13a* (Figs. 7A, B) seem to indicate the presence of a phase II-like regulation in addition to the phase III regulation, although, compared to *hoxa9–11b*, the anterior limit of expression is not colinear for *hoxa13b* (Figs. 5B, F, J, N). Lastly, the complete lack of even the phase I and phase II expressions of zebrafish *hoxa9a* and *hoxa11a* in fins is in stark contrast to the behavior of their paralogs *hoxa9b* and *hoxa11b* as well as their mouse homologs *Hoxa9* and *Hoxa11*, both of which show phase I (*a9* and *a9b*) and phase II (*a9*, *a9b*, *a11*, and *a11b*) expressions during fin/limb development (Nelson et al., 1996; this study).

Considering these, it appears likely that during evolution, the two long-range enhancers located near the *Hox* A and D clusters, each of which is responsible for the onset of early/proximal (phases I and II) and late/distal (phase III) expression of *Hox* and nearby genes (Spitz et al., 2003; Lehoczky et al., 2004; Zákány et al., 2004; Tarchini and Duboule, 2006), had undergone changes in regulatory activities such that homologous genes in teleosts and tetrapods have fallen into different regulatory sub-domains (or “regulatory landscapes” *sensu* Spitz et al., 2003) in each group of animals. Perhaps for a mechanism as indispensable in development of paired appendages as the triphasic expression of *Hox* genes (Pöpperl et al., 2000; Kmita et al., 2005), a small-scale change in regulatory parameters might have been one of the few (and possibly the only) viable way of introducing evolutionary variations in patterning processes while maintaining the overall integrity of the system. Such subtle modifications in regulation might also have been instrumental in maintaining the overall expression patterns of *Hox* genes while at the same time allowing generation of morphological differences during divergence between fins and limbs.

Hox genes and origin of digits: an alternative view

Our results also suggest that the transition from fins to limbs during the evolution of tetrapods (reviewed in Shubin, 1995; Vorobyeva and Hinchliffe, 1996; Wagner and Chiu, 2001; Coates et al., 2002) might have taken a somewhat different course than is presently thought. Evolution of tetrapod limbs from sarcopterygian fins involved a series of modifications in internal structures, the most important of which being the acquisition of digits (Wagner and Chiu, 2001; Coates et al., 2002). Digits are the most distal set of limb skeletal elements consisting of several spool-shaped bones articulated in a proximo-distal direction (Coates et al., 2002), which have organizational as well as developmental features that are distinct from the bones of more proximal regions (Shubin and Alberch, 1986; Shubin et al., 1997; Wagner and Chiu, 2001; Coates et al., 2002; Johanson et al., 2007).

Previously, digits have been variously interpreted as either modified radials of sarcopterygian fins or an entirely new entity that does not have a corresponding structure in the fins of tetrapods' sarcopterygian relatives (reviewed in Shubin and Alberch, 1986; Coates, 1994; Shubin, 1995; Laurin, 2006). More recently, evolutionary origin of digits has also been explored in the context of comparative gene expression studies (Sordino et al., 1995; Sordino and Duboule, 1996; Wagner and Chiu, 2001; Metscher et al., 2005; Davis et al., 2007; Freitas et al., 2007; Johanson et al., 2007), which demonstrated that at least with respect to the *Hox* D genes a distinct late phase of expression similar to the phase III expression associated with the development of digits in tetrapod limbs (Nelson et al., 1996; Tarchini and Duboule, 2006) can be delineated in the fins of a diverse array of fish species such as paddlefish (Davis et al., 2007), catshark (Freitas et al., 2007), and lungfish (Johanson et al., 2007). Interestingly, in cases of paddlefish and lungfish, such phase III-like expression of *Hox* D genes appears late in development and in close association with the chondrogenesis of fin radials (Davis et al., 2007; Johanson et al., 2007), suggesting that digits and fin radials might share a common patterning mechanism. These observations are also consistent with the notion of homology between digits and radials, which has become an increasingly preferred view among paleontologists studying the evolution of early tetrapods (e.g., Shubin et al., 2006; Friedman et al., 2007; but also see Ahlberg and Clack, 2006 and Laurin, 2006).

In spite of these recent advances, however, it might still be premature to conclude digits as homologues of fin radials, since current models fail to take into consideration the possible roles of *Hox* A cluster genes, even though these genes have been shown to be at least as important as *Hox* D genes in the formation and patterning of digits (Zákány et al., 1997, Kmita et al., 2005). Here we show that, based on our data, an entirely different scenario can be constructed for the evolution of digits, if we consider the expression patterns of *Hox* A genes. We notice that one of the key characters separating tetrapods from sarcopterygian fishes is the complete absence of fin rays (Fig. 9), and by inference, the entire distal fin blades in their paired

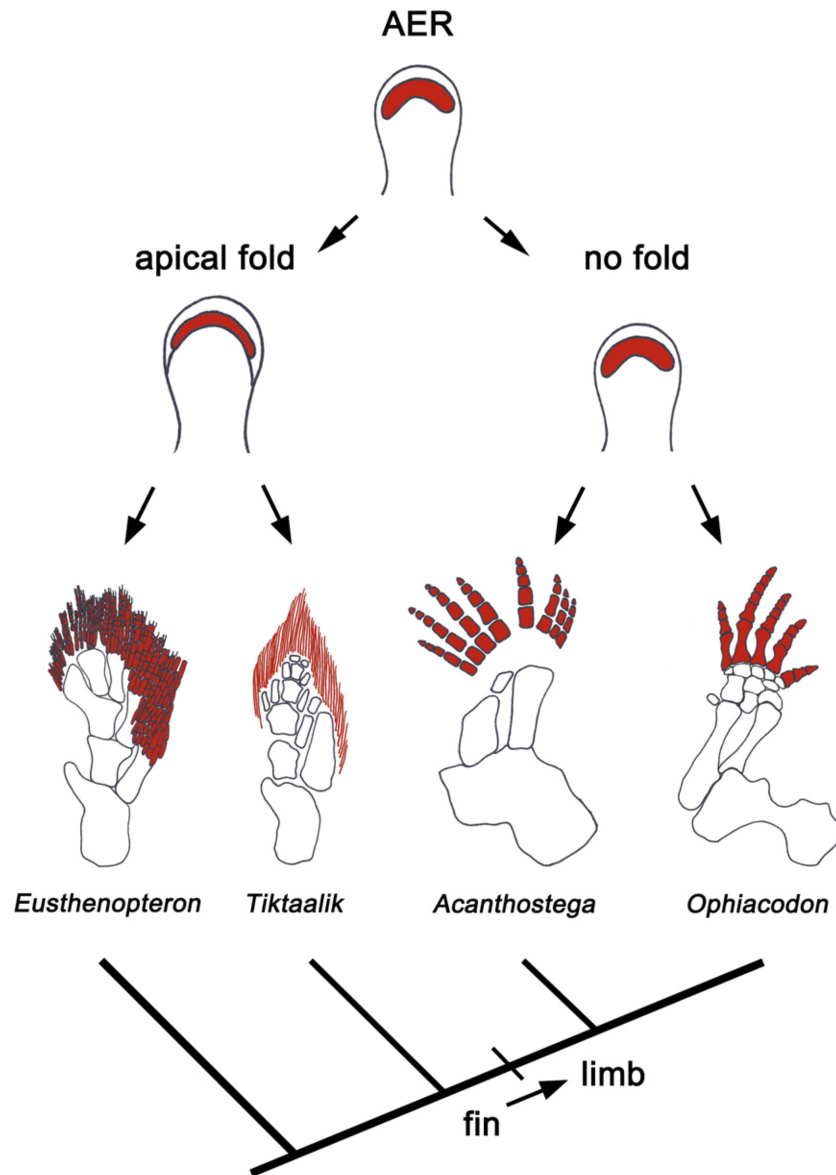

Fig. 9. A developmental scenario for the evolutionary origin of digits. The fin–limb transition in the fossil record involved loss of fin rays and acquisition of digits, which in phylogeny falls roughly between *Tiktaalik*, the most tetrapod-like fossil sarcopterygian fish known to date (Shubin et al., 2006), and *Acanthostega*, the most primitive "limbed" tetrapod (Coates and Clack, 1990). Both animals probably showed phase III expression of *Hox* A genes (red) in their paired appendages during early stages of development, which they used to earmark a distinct group of distal cells under the apical ectodermal ridge (AER). In *Tiktaalik* as well as other sarcopterygian fishes such as *Eusthenopteron*, these cells probably migrated into the fin fold, which derives from the distal outgrowth of the AER, thus giving rise to the connective tissues of the fin blade, including its skeletal support, the fin rays (red). In tetrapods such as primitive *Acanthostega* and more modern *Ophiacodon* (a mammal-like reptile), which did not have fin rays and thus probably did not develop fin folds in its limbs, these cells remained within the limb proper, thereby contributing to the generation of the most distal set of skeletal elements of the limbs, the digits (red). Only pectoral appendages are shown. In each diagram of fin/limb buds, red represents only those cells that later give rise to the fin rays and associated structures (*Eusthenopteron* and *Tiktaalik*) or digits (*Acanthostega* and *Ophiacodon*). *Eusthenopteron*: after Shubin (2002); *Tiktaalik*: after Shubin et al. (2006); *Acanthostega*: after Coates (1991); *Ophiacodon*: after Williston (1925).

appendages (Coates et al., 2002). In zebrafish and other teleosts, cells populating the distal fin blades derive from the distal-most group of cells within the fin bud ("distal mesenchyme cells"; Grandel and Schulte-Merker, 1998) which in zebrafish express *hoxa13a* during development (Fig. 2N). These cells then become motile and migrate into the fin fold during late embryogenesis (Wood and Thorogood, 1984; Grandel and Schulte-Merker, 1998) and eventually differentiate into the connective tissues of the fin blade, including its skeletal support, the fin rays (Grandel and Schulte-Merker, 1998).

If similar processes were responsible for the development of fin blades in fossil sarcopterygian fishes, then the evolutionary loss of fin blades in the appendages of tetrapods' ancestors would have generated a group of "spare" cells that could have been used for building a new structure such as digits. These new cells would certainly have occupied the most distal portion of the fin bud from the beginning and would already have exhibited a phase III expression of at least *Hox* A cluster genes, thereby starting their new roles in development in an already autopod-like configuration (Fig. 9, top half). Furthermore, due to their origin, these cells would have been predisposed to develop into a series of parallel structures emanating from the distal ends of the ancestral endoskeleton (as most structures within the fin blade would be), thereby precipitating the development of new terminal structures in a very digit-like configuration (Fig. 9, bottom half; compare the spatial arrangement of fin rays in *Eusthenopteron* with digits of *Acanthostega*).

Viewed in this way, evolution of digits probably did not require a significant modification of the expression or function of *Hox* genes (contra. Sordino and Duboule, 1996 and Wagner and Chiu, 2001: also see Davis et al., 2007 and Freitas et al., 2007). More likely, the phase III expression of *Hox* A genes as well as the distinct cell–cell interactions

within the distal fin regions would have been sufficient to provide enough developmental background for the evolution of digits by producing a group of cells that have distinct developmental potentials, which in the fins of tetrapods' ancestors might have included the formation of fin rays and associated tissues (blood vessels, nerves, etc) within the fin blade.

By inference, our model predicts that the digits and fin rays would share the embryonic origin of skeletogenic mesenchyme cells. At present, however, it is usually hypothesized that unlike digits, which are usually considered to be derivatives of the mesoderm, fin rays of paired fins are derivatives of the neural crest, an ectodermal structure (Smith and Hall, 1990). These views, however, still remain hypothetical, having yet to be subject to a rigorous experimental testing (see discussions in Smith et al., 1994). If, as our model indicates, digits and fin rays share not only the phase III expression of *Hox* A genes but also the embryonic origins of constituent cells, then it is possible that either there is a neural crest contribution to the developing limb buds or the fin rays of paired fins actually derive from the mesoderm rather than the neural crest (see review by Witten and Huysseune, 2007 on this subject).

Our model, of course, also assumes that the development of paired fins in teleost fishes accurately represents the development of paired fins in sarcopterygian fishes. At present, however, little information is available for either the behavior of mesenchyme cells or the expression patterns of *Hox* A genes during development of paired fins in most sarcopterygian fishes. Nevertheless, limited studies of *Polyodon spathula*, a primitive ray-finned fish that still has metapterygium in its paired fins (Mabee, 2000), indicate that the expression of *Hoxa11* and *Hoxa13* genes in such fins is essentially identical to that of zebrafish *hoxa11b* and *hoxa13b* genes at least during early stages (Metscher et al., 2005; Davis et al., 2007). Furthermore, a brief histological study of the development of pectoral fins in at least one sarcopterygian fish, the Australian lungfish *Neoceratodus*, seems to indicate that the differentiation between proximal and distal mesenchyme cells may also occur in the fins of sarcopterygian fishes (Vorobyeva and Hinchliffe, 1996).

Such similarities in gene expression and behavior of cells may be indicative of an extensive conservation of molecular and cellular mechanisms regulating the development of paired appendages not just between teleosts and tetrapods but also between ray-finned and lobe-finned fishes in general. It is even possible that such mechanisms might have evolved much earlier, possibly even before the origin of paired appendages (Freitas et al., 2006). At present, however, even less is known about the developmental mechanisms underlying the origin of paired appendages (Coates and Cohn, 1998; Ruvinsky and Gibson-Brown, 2000). In this regard, further studies comparing expression patterns of *Hox* genes in more basal groups of vertebrates such as jawless fishes (e.g., Freitas et al., 2006) would be instructive in determining the precise roles played by *Hox* genes in the origin and diversification of paired appendages in vertebrates, which might have been instrumental in their becoming one of the most dominant groups of modern-day animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.032.

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