**HoxD expression in the fin-fold compartment of basal gnathostomes and implications for paired appendage evolution**

Frank J. Tulenko\(^1\), Gaius J. Augustus\(^1,2\), James L. Massey\(^3\), Seth E. Sims\(^1\), Sylvie Mazan\(^4\) & Marcus C. Davis\(^1\)

The role of Homeobox transcription factors during fin and limb development have been the focus of recent work investigating the evolutionary origin of limb-specific morphologies. Here we characterize the expression of *HoxD* genes, as well as the cluster-associated genes *Evx2* and *LNP*, in the paddlefish *Polyodon spathula*, a basal ray-finned fish. Our results demonstrate a collinear pattern of nesting in early fin buds that includes *HoxD14*, a gene previously thought to be isolated from global *Hox* regulation. We also show that in both *Polyodon* and the catshark *Scyliorhinus canicula* (a representative chondrichthyan) late phase *HoxD* transcripts are present in cells of the fin-fold and co-localize with *And1*, a component of the dermal skeleton. These new data support an ancestral role for *HoxD* genes in patterning the fin-folds of jawed vertebrates, and fuel new hypotheses about the evolution of cluster regulation and the potential downstream differentiation outcomes of distinct *HoxD*-regulated compartments.

One of the challenges of evolutionary morphology is to gain insight into how changes in developmental programs contribute to anatomical diversification in lineages descended from a common ancestor. *Hox* subfamily transcription factors are critical organizers of regional patterning during embryonic development, and have received much attention for their role in morphological evolution\(^1-7\). Invertebrates typically possess a single *Hox* cluster, whereas vertebrates possess at least four *Hox* clusters—designated as *HoxA, B, C,* and *D*—resulting from two rounds of whole genome duplication\(^8\). Additional duplication events further expanded the repertoire of *Hox* genes in several vertebrate lineages including lamprey (6 clusters: ref. 9), teleosts (8 ancestrally, with patterns of both loss and further duplication within derived clades; refs 10–12), and paddlefish, a non-teleost actinopterygian\(^13\). The genes within each *Hox* cluster fall into a series of paralogy groups based on sequence homology. Comparative genomic studies support the hypothesis that the ancestral vertebrate *Hox* cluster included 14 paralogues\(^8,14,15\).

While there is significant experimental and clinical data describing paralogy groups 1–13, little is known about the role of *Hox14* paralogues during development due to their loss from the genomes of teleosts and tetrapods thus far sampled\(^8,12,15,16\).

How paired fins gave rise to limbs during the invasion of land is one of the compelling questions in vertebrate evolution\(^17,18\). This morphological transition involved several key changes in appendage anatomy, including the loss of the dermal skeleton of the fin, and an expansion/remodeling of the distal endoskeleton to form an autopod with digits\(^19,20\) (Fig. 1). *HoxA/D* cluster genes are active during both fin and limb development, and over the last two decades, have been the focus of many studies aimed at gaining insight into the evolutionary origin of limb-specific morphologies\(^21-29\).

In tetrapods, the expression of *HoxD* genes are temporally and spatially dynamic during limb ontogeny, occurring in two phases, an early phase and a late phase, each driven by distinct regulatory elements positioned outside the cluster\(^30-32\). This has been particularly well-studied in mice, where during the early phase, transcripts of *HoxD1–9* are present throughout the initial limb bud, followed by the sequential, posteriorly-nested activation...
of HoxD10–13 during bud outgrowth\(^3\). This first phase of collinear expression (i.e., HoxD13 is most posteriorly restricted) patterned the proximal limb (stylopod and zeugopod), and is predominantly regulated by enhancer elements positioned telomeric (3′) to the cluster\(^3,34\). In contrast, during the late phase of expression, HoxD9–13 are activated in the autopod, forming an inverse collinear arrangement (i.e., HoxD13 is most broadly expressed) that patterned the digits\(^3\). The enhancer elements that drive this late phase are interspersed across a region centromeric (5′) to the cluster and have been shown to regulate the expression of Evx2 and LNP, two non-Hox genes within the 5′ genomic neighborhood\(^35–37\). Together, these observations from mice underscore a topological switch between domains of chromatin interaction to pattern the zeugopod and autopod, respectively. Interestingly, one current model for limb development derived from these data posits that this regulatory transition creates a zone of low Hox dosage that manifests anatomically as the small, nodular bones of the wrist and ankle\(^34,38\).

Among non-tetrapod gnathostomes, the distribution of HoxD transcripts during paired fin development has been partially characterized in teleosts [zebrafish: refs 21, 24 and 39], a basal actinopterygian [paddlefish: ref. 22], a basal sarcopterygian [lungfish: ref. 40], and two chondrichthyans [catshark and skate: refs 23, 41 and 42]. These in situ studies reveal a conserved, collinear pattern of expression in the proximal region of early fin buds, similar to the first phase of HoxD expression in tetrapods\(^3\). In later stages of paired fin development, HoxD boundaries shift, suggesting a change in regulatory control. The dynamics of these shifts, however, vary with lineage. In zebrafish, a late phase of HoxD11a–13a expression appears to extend into the mesenchyme of the dermal fin-fold, creating a patterning compartment in which the anterior and posterior boundaries of each paralogue are in register\(^24\). In contrast, current data sets for paddlefish and catshark indicate that 5′ HoxD transcripts form an inverse collinear profile\(^22,23,41\). In paddlefish, this late phase overlaps early expression at the site of distal radial formation in the mid-fin, and has been interpreted as evidence of a deep developmental homology (sensu}\(^\prime\)HoxD\(^\prime\) expression relative to the proximo-distal fin compartment. Overall, these data from non-tetrapods suggest an evolutionary scenario in which distinct proximal and distal HoxD patterning compartments were already in place in the paired fins of ancestral gnathostomes\(^18,23,45\).

Recent studies have focused on testing whether the regulatory landscapes that control Hox expression in fins and limbs are functionally conserved. Significantly, this work has shown that several of the enhancer elements that drive digit expression in mice are represented in non-tetrapods\(^17,25,29,35,37,46\), and that a subset of these homology regions are not only active distally during paired fin development [zebrafish CsB: ref. 25; zebrafish Island 1: ref. 29; Gar CsB and Island 1: ref. 29], but when inserted into mice drive reporter expression in the proximal autopod [skate CsB: ref. 25; zebrafish CsB: ref. 25; pufferfish BAC clones: ref. 28] and digits [Gar CsB and Island 1: ref. 29]. Additionally, there is evidence that trans-acting factors are at least partially conserved between fins and limbs, as a tetrapod specific element (mouse CsC) has been shown to drive distal fin expression when inserted into zebrafish\(^26\). Together, these results provide evidence that the origin of limb-specific morphologies involved modification of an ancient, conserved regulatory architecture for HoxD activation already in place in fins.

Current models for the fin to limb transition that integrate these data focus on the role of HoxD genes in patterning the endoskeleton, though with alternative perspectives on the relationship between cluster regulation and anatomical homology\(^17,18,28,29,36,45\). In these models, the fin-fold is considered a Hox-free zone, and its formation is thought to interrupt the ectodermal/mesenchymal signaling interactions that determine the relative size of a Hox-patterned endoskeletal mesenchyme\(^17,23,24\). Key to further informing models of appendage evolution is a better understanding of the phylogenetic distribution of HoxD expression relative to the proximo-distal fin compartments in basal gnathostomes. Herein, we characterize the expression of HoxD genes, as well as the cluster-related genes Evx2 and LNP, in a basal actinopterygian, the American paddlefish Polyodon spathula. Our results demonstrate a posteriorly restricted, collinear pattern of nesting in the early fin buds that includes HoxD14Beta, a gene
previously thought to be insulated from canonical Hox regulation in the mesoderm\textsuperscript{14,47}. Additionally, we show that in later stages of fin development, the collinear expression profile established in early fin buds is maintained along the site of distal radial formation, with no “autopodial-like” anterior expansion of \textit{HoxD13} (contra a previous report for \textit{Polyodon}\textsuperscript{22}), \textit{Evx2}, or \textit{LNP}. Most significantly, we observed a proximo-distal dynamic to \textit{HoxD} expression in which late phase transcripts of both 3′ and 5′ paralogues extended into the fin-fold mesenchyme, co-localizing with \textit{And1}, the elastoidin component of the actinotrichia\textsuperscript{48}. In order to determine the phylogenetic distribution of this condition, we compared the expression domains of \textit{And1} and \textit{HoxD12} in a representative chondrichthyan, the lesser-spotted catshark \textit{Scyliorhinus canicula}. These \textit{in situ} in situ revealing an overlap between distal \textit{HoxD} and \textit{And1} expression, much like that of paddlefish, suggesting \textit{HoxD}-positive cells contribute to fin-fold specific tissues in both taxa (the ceratotrichia and actinotrichia of chondrichthyans and actinopterygians, respectively). Our results support an ancestral role for \textit{HoxD} genes in patterning the fin-fold, bringing new information to current models for fin/limb evolution and fueling novel hypotheses about the relationship between cluster regulation and appendage morphology.

### Results

#### Expression of \textit{HoxD} cluster genes in paddlefish paired fins

A whole genome duplication event occurred in the paddlefish lineage approximately 42 million years ago, resulting in the formation of unique \textit{Alpha} and \textit{Beta} \textit{HoxD} clusters\textsuperscript{13} (Fig. 2a). In order to determine if both clusters are transcriptionally active during development, we surveyed our \textit{Polyodon} transcriptome assembly using published genomic BAC sequences for specific \textit{HoxD} \textit{Alpha} and \textit{Beta} paralogues\textsuperscript{13}. These blast searches identified both \textit{Alpha} and \textit{Beta} sequences of \textit{HoxD11}, the elastoidin component of the actinotrichia\textsuperscript{48}. In order to determine the phylogenetic distribution of this condition, we compared the expression domains of \textit{And1} and \textit{HoxD12} in a representative chondrichthyan, the lesser-spotted catshark \textit{Scyliorhinus canicula}. These \textit{in situ} in situ revealing an overlap between distal \textit{HoxD} and \textit{And1} expression, much like that of paddlefish, suggesting \textit{HoxD}-positive cells contribute to fin-fold specific tissues in both taxa (the ceratotrichia and actinotrichia of chondrichthyans and actinopterygians, respectively). Our results support an ancestral role for \textit{HoxD} genes in patterning the fin-fold, bringing new information to current models for fin/limb evolution and fueling novel hypotheses about the relationship between cluster regulation and appendage morphology.
to the HoxD4 clone used in our analyses in the absence of available genomic sequence from the 3′ end of each duplicated cluster.

In Stage 42 embryos, transcripts of HoxD4 and HoxD8Beta were detected along much of the length of the pectoral fin buds (Fig. 2b). In contrast, transcripts of HoxD11Beta–14Beta were more restricted in distribution, forming a collinear, posteriorly nested pattern of expression that mirrored the 3′–5′ arrangement of paralogues within the cluster (Fig. 2a,b). In more advanced embryos (Stages 44 and 46), the expression domains of HoxD4, HoxD8Beta, and HoxD11Beta–HoxD13Beta persisted in the middle of the fin following outgrowth of the distal fin and fin-fold (see arrowheads in Fig. 2b). Although an anterior expansion of HoxD13 expression in stage 46 fins has been reported in Polyodon (ref. 22 and see additional discussion in Supplemental Fig. 1), we observed that the collinear pattern of HoxD nesting established in early fin buds was maintained through later stages (Fig. 2b, Supplemental Fig. 1, and Supplemental Fig. 2). We also examined the distribution of LNP and Evx2 to infer if these non-Hox genes are coordinately regulated with 5′ HoxD paralogues, as in mice where Evx2 and LNP co-localize with HoxD135. In paddlefish, Evx2 expression was restricted to the posterior most region of the fin and appeared similar in distribution to HoxD14Beta (Fig. 2b). LNP, in contrast, was detected throughout the pectoral fins, with no distinct regionalization (Fig. 2b). Pelvic fins mirrored the 5′ HoxD and Evx2 expression patterns observed in pectoral fins (Fig. 2c). Transcripts of the more 3′-ward paralogues HoxD4 and HoxD8Beta, however, were difficult to detect in whole mount.

**HoxD expression in the fin-fold compartment of paddlefish.** During limb development, HoxD genes are expressed in well-characterized proximal and distal zones that correspond to the stylopod/zeugopod and autopod, respectively. Given that fins are highly regionalized along the proximo-distal axis into endoskeletal radials (proximally) and a fin-fold/dermal skeleton (distally) (Fig. 1), we sought to characterize the distribution of HoxD transcripts relative to the early patterning zones that give rise to these morphologically distinct compartments. Actinodin genes encode non-collagenous (elastoidin) components of the actinotrichia and provide an early molecular marker for cells contributing to the fin-fold48. We identified a homologue of Actinodin1 (And1) in paddlefish, which we used for both single and double colorimetric in situ hybridizations in whole-mount, representative cross sections, and magnifications. Stages are shown in rows (a) Actinodin1 (And1), an early molecular marker for cells contributing to the fin-fold. And1 transcripts (red) appear in the presumptive distal fin and fin-fold of early fin buds (stage 41) and persist as the fin-fold elongates (stages 42 and 45). (b) Double in situ for the pre-chondrogenic marker Sox9 (purple) and And1 (red) reveal that endochondral and dermal compartments remain separate throughout fin development. Cross sections (and magnification) reveal And1 transcripts in both the fin-fold mesenchyme (m) and the ectoderm (e) adjacent to the basement membrane (bm). The slight proximo-distal overlap between And1 and Sox9 expression presages the arrangement of the dermal fin skeleton later in development. Scale bars = 200 nm.

**Figure 3. Actinodin1 expression in the fin-fold compartment of paddlefish.** (a,b) Pectoral fin in situ hybridizations in whole-mount, representative cross sections, and magnifications. Stages are shown in rows (a) Actinodin1 (And1), an early molecular marker for cells contributing to the fin-fold. And1 transcripts (red) appear in the presumptive distal fin and fin-fold of early fin buds (stage 41) and persist as the fin-fold elongates (stages 42 and 45). (b) Double in situ for the pre-chondrogenic marker Sox9 (purple) and And1 (red) reveal that endochondral and dermal compartments remain separate throughout fin development. Cross sections (and magnification) reveal And1 transcripts in both the fin-fold mesenchyme (m) and the ectoderm (e) adjacent to the basement membrane (bm). The slight proximo-distal overlap between And1 and Sox9 expression presages the arrangement of the dermal fin skeleton later in development. Scale bars = 200 nm.
Figure 4. *HoxD* expression in the fin-fold compartment of paddlefish. (a, b) Pectoral fin double in situ for *HoxD* genes (purple) and *And1* (red) in whole-mount (top row), representative cross sections (middle row) and magnifications of select sections (bottom row). Numbers mark planes of section. Genes are shown in columns. (a) Co-expression of 5′ *Hox* genes *HoxD11Beta*, *HoxD12Beta*, and *HoxD11Beta* (purple) with *And1* (red) reveal the posteriorly-restricted pattern observed in 5′ *HoxD* single in situ (compare with Fig. 2b). Significantly, cross sections confirm that 5′ *HoxD* expression extends distally to overlap the *And1* expression domain. 5′ *HoxD* transcripts were not detected in the anterior fin (sections labeled 1). More posterior sections reveal expression throughout the fin-fold mesenchyme, with a proximal boundary that roughly aligned with ectodermal *And1* (sections labeled 2). In sections near the caudal margin of the fin, 5′ *HoxD* expression is continuous between the fin-fold and proximal fin mesenchyme (sections labeled 3). (b) Co-expression of the more 3′-ward *HoxD* genes *HoxD8Beta*, and *HoxD4* (purple) with *And1* (red) reveals transcripts of both *Hox* paralogues within the fin-fold mesenchyme and aligned proximally with the *And1* labeling boundary in the ectoderm (sections labeled 1, 2). Anterior is left, distal is up for all whole mounts. Ventral is left, distal is up for all sections. Scale bars = 200 nm.

Our *And1/Sox9* data make visible the proximal boundary of the fin-fold patterning compartment at various stages of pectoral fin development, and show that prechondrogenic cells are excluded from the population of mesenchyme distal to this boundary. We next performed a series of double colorimetric in situ to determine if *HoxD* paralogues co-localize with *And1* in the fin-fold mesenchyme. In whole mount pectoral fins at Stage 45, transcripts of *HoxD11Beta*–*HoxD13Beta* formed the same posteriorly-restricted pattern of nesting along the mid-fin (Fig. 4a) as in single in situ (Fig. 2b). Significantly, the expression of these 5′ *HoxD* paralogues extended distally to overlap with the *And1* expression domain. Cross sections revealed differences in the profile of *HoxD* and *And1* boundaries, depending on their position along the anterior-posterior axis (Fig. 4c). Whereas no 5′ *HoxD* transcripts were detected in the anterior portion of the fin, in more posterior sections *HoxD11Beta*–*HoxD13Beta* were expressed throughout the fin-fold mesenchyme, with a proximal boundary for strongest labeling that roughly aligned with that of *And1* in the ectoderm. In sections near the caudal margin of the fin, no such alignment was observed, as *HoxD11Beta–HoxD13Beta* labeling was continuous between the fin-fold and proximal fin mesenchyme.

In mice, early phase *HoxD* patterning of the proximal limb is primarily driven by the 3′ telomeric landscape and involves the upregulation of paralogues along the length of the cluster (i.e., *HoxD1–13*)5,33,34. Regulatory control over *HoxD* expression then transitions to the 5′ centromeric landscape to pattern the autopod, where only *HoxD9–13* (i.e., those paralogues neighboring the 5′ end of the cluster) are active5,37. In paddlefish, our in situ data demonstrate that 5′ *HoxD* paralogues are expressed in the fin-fold (Fig. 4a), which like the autopod in mice represents the distal-most compartment of the appendage. In order to determine if *HoxD* expression in the fin-fold is restricted to genes at the 5′ end of the cluster (similar to late phase autopod patterning), we compared the expression of *And1* and the more 3′-ward *HoxD* paralogues *HoxD4* and *HoxD8Beta*. In whole mount fins, strong *And1* labeling in the ectoderm made it difficult to discern 3′ *HoxD* labeling within the fin-fold. In section, however, transcripts of both *HoxD4* and *HoxD8Beta* were detected in the fin-fold mesenchyme (Fig. 4b), and like the 5′ paralogues examined (Fig. 4a), aligned proximally with the *And1* labeling boundary in the ectoderm.

**HoxD** expression in the fin-fold compartment of chondrichthyans. To test whether *Hox* patterning of the fin-fold compartment is a derived condition of paddlefish or a more general feature of gnathostomes, we extended our analysis to the lesser-spotted catshark *Scyliorhinus canicula* as a representative chondrichthyan. Chondrichthyan dermal fin skeletons are composed of ceratotrichia, which are histologically and chemically similar to actinotrichia31. Blast searches of the catshark transcriptome assembly returned a putative *Actinodin*
homologue, which we identified as \( \text{ScAnd1} \). Whole mount in situ expressions revealed \( \text{ScAnd1} \) expression in Stage 30 pectoral and pelvic fins is restricted to the distal fin. (a') Cross section of \( \text{ScAnd1} \) Stage 30 pectoral fins (and magnification) reveal \( \text{ScAnd1} \) positive cells mark the boundaries of the fin-fold with both mesenchymal and ectodermal expression domains. (b) In situ gene expression for \( \text{S. canicula HoxD12} \). Whole-mount \( \text{HoxD12} \) expression in Stage 30 pectoral and pelvic fins form distinct proximal and distal domains. (b') Cross section of \( \text{HoxD12} \) Stage 30 pectoral fins (and magnification) reveal overlap with the distal domain of \( \text{ScAnd1} \) (compare to a'), indicating \( \text{HoxD} \) positive cells in the fin-fold. For all whole mounts: anterior is left, distal is up in pectoral fins, distal is down in pelvic fins. Dashed lines correlate to plane of section in (a,b'). Scale bars = 200 nm.

**Figure 5. HoxD expression in the fin-fold compartment of catshark.** (a,b) In situ hybridizations in whole-mount and representative cross sections. (a) In situ expression for the catshark \( \text{S. canicula Actinodin1} \) homologue (\( \text{ScAnd1} \)), shown to be an early molecular marker for cells contributing to the fin-fold in teleosts. Whole-mount \( \text{ScAnd1} \) expression in Stage 30 pectoral and pelvic fins is restricted to the distal fin. (a') Cross section of \( \text{ScAnd1} \) Stage 30 pectoral fins (and magnification) reveal \( \text{ScAnd1} \) positive cells mark the boundaries of the fin-fold with both mesenchymal and ectodermal expression domains. (b) In situ gene expression for \( \text{S. canicula HoxD12} \). Whole-mount \( \text{HoxD12} \) expression in Stage 30 pectoral and pelvic fins form distinct proximal and distal domains. (b') Cross section of \( \text{HoxD12} \) Stage 30 pectoral fins (and magnification) reveal overlap with the distal domain of \( \text{ScAnd1} \) (compare to a'), indicating \( \text{HoxD} \) positive cells in the fin-fold. For all whole mounts: anterior is left, distal is up in pectoral fins, distal is down in pelvic fins. Dashed lines correlate to plane of section in (a,b'). Scale bars = 200 nm.

Discussion
In this study, we have characterized the expression of \( \text{HoxD} \) and \( \text{HoxD} \) cluster-related genes during paired fin development in the American Paddlefish \( \text{Polyodon spathula} \). These results demonstrate an early collinear arrangement of 5' \( \text{HoxD} \) nesting, similar to that of other vertebrates\(^{21-24,33} \) and consistent with a model in which the first phase of \( \text{HoxD} \) activation is part of a conserved pathway for establishing \( \text{Shh} \) in the ZPA and appendage asymmetry.\(^{25-35} \) Our results also demonstrate that in more advanced fins, collinear \( \text{HoxD} \) nesting is maintained along the presumptive site of distal radial formation following outgrowth of the fin-fold. This persistent mid-fin expression resembles the later dynamics of early phase patterning in limbs, where \( \text{HoxD} \) expression in the nascent limb bud continues in the fore-limb forming territory following outgrowth of the autopod.\(^{33,38} \) Notably, we did not observe an extended anterior sweep of expression of \( \text{HoxD13} \) (contra a previous report\(^{22} \)), \( \text{HoxD14} \), or the cluster-related genes \( \text{Evx2} \) or \( \text{LNP} \), in any of the stages examined. These observations provide evidence that the inverse collinear
profile considered a hallmark of digit patterning in tetrapods may not be present in paddlefish (or a more inclusive clade, see ref. 24).

In addition to early patterning of the proximal fin bud, our paddlefish in situ results reveal a late phase of HoxD expression that co-localizes with And1 in the mesenchyme of the fin-fold, a region absent of pre-chondrocytes. These new data, together with a report of late HoxD expression in the fin-fold of zebrafish, suggest this character may be shared among actinopterygians. Chondrichthians provide a critical outgroup for testing this hypothesis. Our data for catshark confirm the spatially distinct early and late phases of HoxD activation described by Freitas and colleagues, and demonstrate overlap between the late distal phase of HoxD12 labeling and that of And1. In tetrapods, most studies of Hox function during limb development have focused on endoskeletal patterning and the effects of gene abrogation on the formation of the stylopod, zeugopod, and autopod. Recent work, however, has shown that Hox genes have a broader role in limbs, and are required for normal muscle and tendon formation independent of skeletal phenotype. While the anatomical outcome of HoxA/D loss in non-tetrapods remains elusive, the co-localization of HoxD and And1 labeling in a representative actinopterygian and chondrichthyan provide evidence of Hox-positive cells contributing to fin-fold specific tissues (i.e., the elastoidin component of the actinotrichia in paddlefish and ceratotrichia in catshark), and suggest an ancient role for Hox genes in integrating aspects of appendage formation beyond endoskeletal patterning.

Our unexpected find, the first report of appendicular expression of HoxD14Beta, is contra recent hypotheses that Hox14 paralogues are isolated from canonical regulation of the cluster. Instead, our results demonstrate that transcripts of HoxD14Beta are posteriorly-restricted within the fins, and together with other cluster paralogues form a collinear arrangement of nesting, as would be expected with canonical early phase regulation. These results suggest an ancestral role for HoxD14 in appendage patterning in gnathostomes, one that has been lost in most lineages yet retained, at least in terms of expression, in paddlefish.

Current evidence from comparative gene expression, genomics, and transgenic assays support a model in which bimodal regulation of the HoxD cluster is partially conserved between fins and limbs. The apparent antiquity of this regulatory strategy, together with the data presented here, raise the intriguing and underexplored possibility that HoxD patterning of the proximal fin and fin-fold may reflect separate controls from the 3′ and 5′ landscapes, respectively. In tetrapods, late phase HoxD-related expression in the autopod is limited to HoxD9–13 (those paralogues nearest the 5′ end of the cluster) and the 5′ flanking genes Evx2 and LNP, both of which are co-regulated with HoxD13. We predicted a similar restriction in cluster activity in the fin-folds of paddlefish, but found transcripts of the 3′-ward members HoxD4 and HoxD8Beta throughout the fin-fold mesenchyme. This difference, combined with a lack of anteriorized HoxD13, Evx2 and LNP may reflect lineage specific variation in landscape/cluster interactions between tetrapods and paddlefish, or more generally actinopterygians. Additional comparative data characterizing the expression of 3′ HoxD paralogues during fin development in a chondrichthyan will inform inferences about ancestral patterns of cluster activation, and fuel new hypotheses as to the regulatory dynamics of the 3′ and 5′ landscapes during appendage formation in non-tetrapods. Interestingly, current models for limb development posit that chromatin re-organization between early (proximal) and late (distal) HoxD expression creates an intermediate zone of low Hox dosage that manifests anatomically as the small bones of the wrist. In fins, a series of small, endochondral bones (distal radials) often separate the proximal radials and fin-fold (e.g., Fig. 1). An ancestral role for HoxD genes in patterning the distal fin compartment makes it tempting to speculate that a similar mechanism may explain appendage morphologies in non-tetrapods. However, not all patterns meet the predictions of this model, (e.g., strong HoxD labeling in the presumptive site of distal radial formation in paddlefish), suggesting that modulation of Hox dosage and its effects on cell behavior in the context of local signaling environments differ across lineages. Our results demonstrate that further comparative work, with an increasing focus on basal gnathostomes, will be necessary to elucidate the ancestral roles of Hox genes in appendage patterning, roles that may involve a great flexibility in skeletogenic differentiation outcomes.

Methods
Animal husbandry and staging. Embryos of the American Paddlefish Polyodon spathula were obtained from Osage Catfisheries Inc. (Osage Beach, MO, USA), and were raised at 18 °C in recirculating large-volume freshwater tanks (pH 7.5 ± 0.2, salinity of 1.0 ± 0.2ppt), fixed overnight in Carnoy’s solution, and dehydrated to 100% ethanol for storage at −20 °C. Animals were staged according to refs 49, 50, and 51.

Transcriptome. Paddlefish larvae from early pectoral fin development stages (stages 41–44) were preserved in RNA later and then dissected into pectoral fin and reference tissue (operculum) samples. After RNA library construction, Illumina™ sequencing of the transcriptome was performed by the Centre for Applied Genomics – Toronto (Toronto, Canada) producing a total of 65,682,405 pectoral fin raw reads (72 bp nonpaired). We used the Trinity program (default settings) to assemble 92,446 likely transcripts. Transcripts were annotated and validated against the National Centre for Biotechnology Information (NCBI) non-redundant (nr) database using BLAST (cut off E value of 10−5).

cDNA synthesis for cloning. RNA was isolated from Stage 26–46 paddlefish embryos with Trizol reagent (Invitrogen) plus the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen), and used
as template to generate single-strand cDNA with the Superscript III First Strand Synthesis kit (Invitrogen), all per manufacturer's instructions. Paddlefish primers were designed from transcriptome sequences as follows (F: forward; R: reverse): Evx2: CGCGAGTCCTCATACCTTTF (F), CCGTTCACAACTCTTGACG (R); Sox9: CGATCGTACATGAGTGCCAC (F), GATGTCAGTTTGGCTCAGATCCG (R); LNP: GCCCTGAGACTGACAGAA (F), GAGTCTTCCGAGCAGGAATTTAGAGA (R); HoxD4: TGGGACCTTCAATTTCCACCTTGCG (F), GCACAGTTTGAAATGCTGGCTG (R); HoxD8Beta: ACTACGACGTCTCAGTTTCCAGGG (F), CGTCCTTTGATGTAAGATGAATGGAAT (R); HoxD12Alpha: TTTTAATTTCCCTACCCGGGACCTC (F), CTCACAAAGCGCTTGTAAC (R). Primers for catshark were as follows: HoxD12: TATTGCCAACACCTGGCTCC (F), CTGTGTCGCTCCTAGCTGAC (R); HoxD4: TATGGATGACATGATGTCTCTTC (F), CGTCCTACCTGCTTTTCTGCC (R); And1: CTCGTAACCTGCTAGTACTGCT (F), CTGACGATCTGGACACACTGGTC (R). PCR products were cloned into pGEM®-T Easy Vector (Promega), which was purified and sequenced using Genscript DNA Sequencing Services. All sequences were analyzed using Uniprot UGENE and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Orthology for the Actinodin family members was confirmed using maximum likelihood in Mega6.06. GenBank accession numbers for this work are KU744647-KU744655.

For in situ hybridization experiments, the entire PCR product obtained from each primer set was used as template for in vitro transcription (see below). Plasmids containing paddlefish fragments of HoxD11Alpha, HoxD11Beta, HoxD13Alpha, and HoxD13Beta were obtained through Life Technologies Gene Art Services and were based on published genomic BAC clones9. All new probes generated during this study ranged from approximately 480 to 900 base pairs in length. Paddlefish probes for HoxD4, HoxD8Beta, and HoxD14Beta were complementary to regions of coding exons one and two, including the homeodomain. Probes for HoxD11Alpha and HoxD11Beta were complementary to a region of coding exon one, coding exon two, including the homeodomain, and 3' UTR. Probes for HoxD12Alpha, HoxD12Beta, HoxD13Alpha, and HoxD13Beta were complementary to the first coding exon and excluded the homeodomain. Probes for Sox9, And1, and LNP were targeted against coding region. Probe for Evx2 was targeted against coding region and 3' UTR. Probes HoxD13-EMS28721 and ScHoxD12 were from Refs 22, and 23 respectively.

**In situ hybridization.** Plasmids were linearized for probe template using restriction enzymes (New England Biolabs, NEB) and any resultant 3' overhangs were blunted using DNA Polymerase I, Large Klenow (NEB). Probe synthesis was carried out using SP6, T7, or T3 RNA polymerases (Promega) and either Digoxigenin or Fluorescein RNA labeling mixes (Roche) per manufacturer's instructions. Whole-mount in situ hybridizations were as described in ref. 67 at a hybridization temperature of 69–70 °C, with the following modifications for double colorimetric in situ: Both Digoxigenin (Sox9, HoxD4, HoxD8Beta, HoxD11Beta–HoxD13Beta) and Fluorescein (And1) labeled probes were included during the hybridization step. Digoxigenin-labeled probes were detected with Anti-Digoxigenin-AP, Fab fragments (1:2000 dilution; Roche) and developed with BM-Parl (Roche). Following the first color reaction, embryos were incubated in 0.1M Glycine (pH 2.2) for 30 minutes to inactivate AP, and then washed thoroughly with MABT. Fluorescein-labeled probes were detected with Anti-Fluorescein-AP, Fab fragments (1:1000 dilution; Roche) and developed using Fast Red Tablets (Roche) per manufacturer's instructions.

For paddlefish, the total number of antisense in situ specimens examined were as follows: HoxD4: St41–43 (n = 3); St44–45 (n = 8); St46–1dps (n = 4); HoxD8: St41–43 (n = 3); St44–45 (n = 4); St46–1dps (n = 3); HoxD11Alpha: St41–43 (n = 4); St44–45 (n = 3); St46–1dps (n = 4); HoxD11Beta: St41–43 (n = 11); St44–45 (n = 11); St46–1dps (n = 8); 2dp5–3dps (n = 4); HoxD12Alpha: St41–43 (n = 7); St44–45 (n = 5); St46–1dps (n = 5); HoxD12Beta: St41–43 (n = 4); St44–45 (n = 3); St46–1dps (n = 4); 2dp5–3dps (n = 3); HoxD13Alpha: St41–43 (n = 6); St44–45 (n = 12); St46–1dps (n = 13); 2dp5–3dps (n = 5); 5–15dps (n = 5); HoxD13Beta: St41–43 (n = 7); St44–45 (n = 9); St46–1dps (n = 12); 2dp5–3dps (n = 7); 5–15dps (n = 4); HoxD13EF52721: St41–43 (n = 4); St44–45 (n = 7); St46–1dps (n = 3); HoxD14Beta: St41–43 (n = 11); St44–45 (n = 13); St46–1dps (n = 10); 2dp5–3dps (n = 3); 5–15dps (n = 3); Evx2: St41–43 (n = 10); St44–45 (n = 7); St46–1dps (n = 7); 2dp5–3dps (n = 5); 5–15dps (n = 3); LNP: St41–43 (n = 6); St44–45 (n = 4); St46–1dps (n = 3); And1: St41–43 (n = 4), St44–45 (n = 5); And1 plus Sox9: St41–43 (n = 7); St44–45 (n = 10); And1 plus HoxD4: St44–45 (n = 7); And1 plus HoxD8Beta: St44–45 (n = 7); And1 plus HoxD11Beta: St44–45 (n = 6); And1 plus HoxD12Beta: St44–45 (n = 9); And1 plus HoxD13Beta: St44–45 (n = 8). For catshark the total number of in situ specimens examined were as follows: HoxD12: St28–31 (n = 3); And1: St28–31 (n = 3). Sense probe was used with stage-matched specimens as a negative control for antisense hybridization experiments (see Supplemental Fig. 4). Whole mount imaging was done using a Zeiss Discovery.V12 Stereo microscope equipped with an AxioCam MRc5 camera and Zen 2012 (blue edition) software.

**Histology.** Embryos used for sectioning were incubated in successive 10%, 20%, and 30% sucrose in PBS solutions for a minimum of 3 hours each at room temperature, and then transferred to a 1:1 solution of TBS Tissue Freezing Medium (Fisher): 30% Sucrose in PBS for 8 hours. Embryos were then embedded in TBS Tissue Freezing Medium in disposable plastic moulds and frozen for 10 minutes using isopentane chilled with liquid nitrogen. Embryos were cryosectioned at 16 μm on a Leica CM1850 cryostat, dried for two hours at 35 °C on a slide warmer, and cover-slipped with 25% glycerol in PBS. Sections were imaged using a Zeiss Axioimager.M2 compound microscope equipped with an Axiocam 503 color camera and Zen 2012 (blue edition) software.
References

1. Duboule, D. & Dollé, P. The structural and functional organization of the murine HOX gene family resembles that of Drosophila homeotic genes. EMBO J. 8, 1497–1505 (1989).

2. Graham, A., Papalopulu, N. & Krumlauf, R. The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell 57, 367–378 (1989).

3. Burke, A. C., Nelson, C. E., Morgan, B. A. & Tabin, C. Hox genes and the evolution of vertebrate axial morphology. Development 121, 333–46 (1995).

4. Cohn, M. J. & Tickle, C. Developmental basis of limblessness and axial patterning in snakes. Nature 399, 474–479 (1999).

5. Duboule, D. The rise and fall of Hox gene clusters. Development 134, 2549–2560 (2007).

6. Freitas, R., Guangjun, Z. & Cohn, M. J. Evidence that mechanisms of fin development evolved in the midline of early vertebrates. Nature 442, 1033–1037 (2006).

7. Head, J. H. & Polly, P. D. Evolution of the snake body form reveals homoplasies in amniote gene function. Nature 520, 86–89 (2015).

8. Pascual-Anaya, J., D'Aniello, S., Kuratani, S. & Garcia-Fernandez, J. Evolution of Hox gene clusters in deuterostomes. BMC Dev. Biol. 13, 26 (2013).

9. Mehta, T. K. et al. Evidence for at least six Hox clusters in the Japanese lamprey (Lethenteron japonicum). Proc. Natl. Acad. Sci. USA 110, 16044–16049 (2013).

10. Chambers, K. E. et al. Hox cluster duplication in the basal teleost Hiodon alosoides (Osteoglossomorpha). Theory Biosci. 128, 109–120 (2009).

11. Kuraku, S. & Meyer, A. The evolution and maintenance of Hox gene clusters in vertebrates and the teleost-specific genome duplication. Int. J. Dev. Biol. 53, 765–773 (2009).

12. Munggat瞌. et al. Differential evolution of the 13 Atlantic salmon Hox clusters. Mol. Biol. Evol. 25, 1333–1343 (2008).

13. Crow, K. D., Smith, C. D., Cheng, J. F., Wagner, G. P. & Amemiya, C. T. An independent genome duplication from Hox paralogs in ray-finned fishes. Proc. Natl. Acad. Sci. USA 105, 6679–6683 (2008).

14. Powers, T. P. & Amemiya, C. T. Evidence for a Hox14 paralog group in vertebrates. Curr. Biol. 14, R183–R184 (2004).

15. Lee, A. P., Koh, E. G., Tay, A., Brenner, S. & Venkatesh, B. Highly conserved syntenic blocks at the vertebrate Hox loci and conserved regulatory elements within and outside Hox gene clusters. Proc. Natl. Acad. Sci. USA 103, 6994–6999 (2006).

16. Schneider, I. & Shubin, N. The origin of the tetrapod limb: from expeditions to enhancers. Proc. Natl. Acad. Sci. USA 108, 12782–12786 (2011).

17. Schneider, I. & Shubin, N. The pectoral fin of Tiktaalik roseae and the origin of the tetrapod limb. Nature 440, 764–771 (2006).

18. Sordino, P., van der Hoeven, F. & Duboule, D. Hox gene expression in teleost fins and the origin of vertebrate digits. Nature 375, 678–681 (1995).

19. Davis, M. C., Dahn, R. D. & Shubin, N. H. An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. Nature 447, 473–476 (2007).

20. Freitas, R., Zhang, G. & Cohn, M. J. Biphasic Hox gene expression in shark paired fins reveals an ancient origin of the distal limb domain. PLoS ONE 15, e7574 (2014).

21. Ahn, D. & Ho, R. K. Tri-phenotypic expression of posterior Hox genes during development of pectoral fins in zebrafish: implications for the evolution of vertebrate paired appendages. Dev. Biol. 322, 220–233 (2008).

22. Shubin, N. H. & Davis, M. C. in Modularity in development and evolution (eds Schlussler, G. & Wagner, G.) Ch. 19, 429–440 (University of Chicago Press, 2004).

23. Shubin, N. H., Daeschler, E. B. & Jenkins, F. A. J. The pectoral fin of Tiktaalik roseae and the origin of the tetrapod limb. Nature 440, 764–771 (2006).

24. Tarchini, B. & Duboule, D. Conservation and divergence of regulatory strategies at Hox Loci and the evolution of tetrapod digits. Dev. Cell 23, 1219–1229 (2012).

25. Stothert, R. et al. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. Science 338, 1476–1480 (2012).

26. Woltering, J. M., Noordermeer, D., Leleu, M. & Duboule, D. Conservation and divergence of regulatory strategies at Hox Loci and the origin of tetrapod digits. PLoS Biol. 12, e1001773 (2014).

27. Gehlke, A. R. et al. Deep conservation of wrist and digit enhancers in fish. Proc. Natl. Acad. Sci. USA 112, 803–808 (2015).

28. Nelson, C. E. et al. Analysis of Hox gene expression in the chick limb bud. Development 122, 1449–1466 (1996).

29. Spitze, F. & Duboule, D. Global control regions and regulatory landscapes in vertebrate development and evolution. Adv. Genet. 61, 175–205 (2008).

30. Andrey, G. & Duboule, D. SnapShot: Hox gene regulation. Cell 156, 856.e1 (2014).

31. Tarchini, B. & Duboule, D. Control of Hox genes’ collinearity during early limb development. Dev. Cell 10, 93–103 (2006).

32. Andrey, G. et al. A switch between topological domains underlies HoxD genes collinearity in mouse limbs. Science 340, 1234167 (2013).

33. Spitze, F., Gonzalez, F. & Duboule, D. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. Cell 113, 405–417 (2003).

34. Gonzalez, F., Duboule, D. & Spitze, F. Transgenic analysis of HoxD gene regulation during digit development. Dev. Biol. 306, 847–859 (2007).

35. Montavon, T. et al. A regulatory archipelago controls Hox genes transcription in digits. Cell 147, 1132–1145 (2011).

36. Woltering, J. M. & Duboule, D. The origin of digits: Expression patterns versus regulatory mechanism. Dev. Cell 18, 526–532 (2010).

37. Takamatsu, N. et al. Duplicated Abd-B class genes in medaka hoxAa and hoxAb clusters exhibit differential expression patterns in pectoral fin buds. Dev. Genes Evol. 217, 263–273 (2007).

38. Johanson, Z. et al. Fish fingers: digit homologues in sarcopterygian fish fins. J. Exp. Zool. B, Mol. Dev. Evol. 308, 757–68 (2007).

39. Sakamoto, K. et al. Heterochronic shift in Hox-mediated activation of sonic hedgehog leads to morphological changes during fin development. PLoS One 4, e5121 (2009).

40. O’Shaughnessy, K. L., Dahn, R. D. & Cohn, M. J. Molecular development of chondrichthyan claspers and the evolution of copulatory organs. Nat Commun. Apr 14;6:6698. doi: 10.1038/ncomms6698 (2015).

41. Head, J. H. & Polly, P. D. Evolution of the snake body form reveals homoplasies in amniote gene function. Nature 520, 86–89 (2015).

42. Shubin, N., Tabin, C. & Carroll, S. Fossils, genes and the evolution of animal limbs. Nature 388, 639–648 (1997).

43. Shubin, N., Tabin, C. & Carroll, S. Deep homology and the origins of evolutionary novelty. Nature 457, 818–823 (2009).

44. Freitas, R., Gómez-Skarmeta, J. L. & Rodrigues, P. N. New frontiers in the evolution of fin development. J Exp Zool B Mol Dev Evol. 322B, 540 – 552 (2014).

45. Amemiya, C. T. et al. The African coelacanth genome provides insight into tetrapod evolution. Nature 496, 311–316 (2013).

46. Chambers, K. E. et al. Revisiting the origin of the vertebrate Hox14 by including its relict sarcopterygian members. J. Exp. Zool. B, Mol. Dev. Evol. 316, 515–25 (2011).

47. Zhang, J. et al. Loss of fish actinotrichia proteins and the fin-to-limb transition. Nature 466, 234–237 (2010).
49. Davis, M. C., Shubin, N. H. & Force, A. Pectoral fin and girdle development in the basal actinopterygians Polyodon spathula and Acipenser transmontanus. J. Morph. 262, 608–628 (2004).
50. Mahee, P. M. & Noordsy, M. Development of the paired fins in the paddlefish. Polyodon spathula. J. Morphol. 261, 334–344 (2004).
51. SantaMaria, J. A., Santos, R. L. & Becerra, J. An antiserum against ceratotrichia (selachian) recognizes actinotrichia in teleost regenerating fins. Int. J. Dev. Biol. Suppl 1, 175S–176S (1996).
52. Dahn, R. D., Davis, M. C., Pappano, W. N. & Shubin, N. H. Sonic hedgehog function in chondrichthyan fins and the evolution of appendage patterning. Nature 445, 311–314 (2007).
53. Zakany, J., Kmita, M. & Duboule, D. A dual role for Hox genes in limb anterior-posterior asymmetry. Science 304, 1669–1672 (2004).
54. Kmita, M. et al. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. Nature 435, 1113–1116 (2005).
55. Tarchini, B., Duboule, D. & Kmita, M. Regulatory constraints in the evolution of the tetrapod limb anterior-posterior polarity. Nature 443, 985–988 (2006).
56. Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. & Capecchi, M. R. Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. Nature 375, 791–795 (1995).
57. Fromental-Ramain, C. et al. Hox-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. Development 122, 2997–3011 (1996).
58. Fromental-Ramain, C. et al. Specific and redundant functions of the paralogous Hox-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. Development. 122, 461–472 (1996).
59. Wellik, D. M. & Capecchi, M. R. Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 301, 363–367 (2003).
60. Boulet, A. M. & Capecchi, M. R. Multiple roles of Hoxa11 and Hoxd11 in the formation of the mammalian forlimp zeugopod. Development 131, 299–309 (2004).
61. Zákany, J. & Duboule, D. The role of Hox genes during vertebrate limb development. Curr. Opin. Genet. Dev. 17, 359–366 (2007).
62. Swinehart, I. T., Schlientz, A. J., Quintanilla, C. S., Mortlock, D. P. & Wellik, D. M. Hox11 genes are required for regional patterning and integration of muscle tendon and bone. Development 140, 4574–4582 (2013).
63. Bemis, W. E. & Grande, L. Early development of the actinopterygian head. I. External development and staging of the paddlefish Polyodon spathula. J. Morphol. 213, 47–83 (1992).
64. Ballard, W. W. & Needham, R. G. Normal embryonic stages of Polyodon spathula (Walbaum). J. Morphol. 114, 465–477 (1964).
65. Ballard, W. W., Mellinger, J. & Lechenault, H. A series of normal stages for development of scyliorhinus-canicula, the lesser spotted dogfish (Chondrichthyes, Scyliorhinidae). J. Exp. Zool. 267, 318–336 (1993).
66. Grabher, M. G. et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Commun. 2, 496 (2011).

Acknowledgements
We thank Osage Catfisheries, Inc. and the Kahrs family for their continued support of paddlefish research. We thank A. Burke, D. Menke, and S. Nowak for critical reading of the manuscript. This research was supported by a grant from the NSF (IOS 1144965 to M.C.D.) and by instrumentation supplied by the Georgia Research Alliance.

Author Contributions
M.C.D. and F.J.T. conceived the project. M.C.D., F.J.T. and G.J.A. designed the experiments; S.E.S. designed and constructed the custom iBLAST server used to analyze transcriptome data. M.C.D. facilitated this project through aquaculture support and expertise for the paddlefish data. S.M. facilitated this project through aquaculture support and expertise for the chondrichthyan data. F.J.T., G.J.A. and J.L.M. performed all cloning, in situ aquaculture support and expertise for the paddlefish data. S.M. facilitated this project through aquaculture support and expertise for the paddlefish data. Constructed the custom iBLAST server used to analyze transcriptome data. M.C.D. facilitated this project through hybridization assays, and imaging. F.J.T. and M.C.D. generated all figures and artwork and wrote the manuscript with significant contributions from G.J.A., J.L.M. and S.E.S.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Tulenko, F. J. et al. HoxD expression in the fin-fold compartment of basal gnathostomes and implications for paired appendage evolution. Sci. Rep. 6, 22720; doi: 10.1038/srep22720 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/