

The Origin of Digits: Expression Patterns versus Regulatory Mechanisms

Joost M. Woltering¹ and Denis Duboule^{1,2,*}

¹National Research Centre “Frontiers in Genetics,” Department of Zoology and Animal Biology, University of Geneva, Sciences III, Quai Ernest-Ansermet 30, 1211 Geneva 4, Switzerland

²School of Life Sciences, Federal Institute of Technology (EPFL), CH-1015, Lausanne, Switzerland

*Correspondence: denis.duboule@unige.ch

DOI 10.1016/j.devcel.2010.04.002

In the emerging discipline of Evo-Devo, the analysis of gene expression patterns can be deceptive without a clear understanding of the underlying regulatory strategies. Here, we use the paradigm of hand and foot evolution to argue that the consideration of the regulatory mechanisms controlling developmental gene expression is essential to resolve comparative conundrums. In this context, we discuss the adaptive relevance of evolving stepwise, distinct developmental regulatory mechanisms to build an arm, i.e., a composite structure with functional coherence.

Along their proximal to distal axes, limbs can be broadly divided into three parts: the most proximal part or stylopod contains the humerus (the femur in hindlimbs), an intermediate part or zeugopod contains the ulna and radius (tibia and fibula in hindlimbs), and a distal part or autopod contains the bones of the hand (foot in hindlimbs) including both the carpals (or tarsals) and digits (Figure 1; see Tabin and Wolpert, 2007). When and how did digits appear? And how well do we understand the evolutionary relationships between the different kinds and numbers of digits found in various animals?

Over the past 25 years, the discovery of molecular markers has greatly helped to address these questions. Among these markers, *Hox* genes belonging to the “posterior” halves of the *HoxA* and *HoxD* clusters are critical for the development of the proximo-distal organization, as illustrated by multiple series of gene disruption experiments (Davis et al., 1995; Davis and Capecchi, 1996; Dollé et al., 1993; Zákány et al., 1997; Zákány and Duboule, 1996; Kmita et al., 2005). These analyses have uncovered functions for these genes during both the patterning and the subsequent growth of limb elements; while mutant specimens can indeed be polydactylous, oligodactylous, or even adactylous, the relative sizes and shapes of particular skeletal elements are generally affected too.

Both expression and functional analyses have established distinct spatial and temporal signatures for *Hoxa* and *Hoxd* genes, which clearly distinguish proximal from distal limb regions. In the *HoxA* cluster, *Hoxa11* functions in the zeugopod whereas *Hoxa13* labels the autopod (Yokouchi et al., 1991; Nelson et al., 1996; Tamura et al., 2008). Likewise, posterior *Hoxd9* to *Hoxd13* are coordinately expressed in two subsequent phases (Figure 1). In the early phase, *Hoxd9* to *Hoxd12* are transcribed in proximal regions, up to the boundary between the zeugopod and the autopod, i.e., in those cells that will ultimately build the humerus, radius, and ulna. Subsequently, a second phase of expression develops into a clearly distinct, more distal domain covering most of the autopod (Figure 1; Dollé et al., 1989; Nelson et al., 1996). During this second phase, *Hoxd10* to *Hoxd13* are expressed concomitantly, in the same domain yet with decreasing transcriptional efficiencies, such that *Hoxd13*

is transcribed at the highest level (Montavon et al., 2008). As a consequence of this robust transcription, this latter gene is expressed throughout the five digit primordia found in amniotes, whereas *Hoxd12*, *Hoxd11*, and *Hoxd10* transcripts are detected in all digits but the future thumb, a quantitative effect sometimes referred to as “reverse collinearity” (Nelson et al., 1996). This uneven anterior to posterior (AP) distribution reflects both the graded transcriptional efficiencies of *Hoxd* genes and the activity of *sonic hedgehog* (*Shh*), which is expressed at the posterior margin of the limb bud (Riddle et al., 1993; Drossopoulou et al., 2000; Harfe et al., 2004). These specific distributions of posterior *Hoxa* and *Hoxd* transcripts are globally conserved in tetrapod limbs and similar patterns were described for *Axolotl* and *Xenopus* (Torok et al., 1998; Christen et al., 2003; Satoh et al., 2006; Ohgo et al., 2010).

Hoxd Gene Expression and the Origin of Digits

Comparisons between modern limbs, fossils, and recent sarcopterygian fins reveal likely homologies between proximal and intermediate limb bones and parts of fin skeletons (Coates, 1994; Cohn et al., 2002). However, when the most distal parts of the appendages are considered, in particular the digits, structural relationships become problematic and hence homologies between the autopod and ancestral fin elements have been controversial (Coates, 1994, 1995; Coates et al., 2002; Wagner and Chiu, 2001). Coates and colleagues consider digits as generally related to fin radials (e.g., Coates, 1994; Friedman et al., 2007), based on shared ontological and anatomical characters (Friedman et al., 2007). Anatomical criteria, however, do not exclude convergence, and the ontological argument (both structures develop as buds and use similar genetic programs; see Friedman et al., 2007) does not consider the proximal versus distal parts separately, which makes it only moderately informative regarding the origin of digits.

Alternatively, structural and molecular differences have led others to postulate a more recent origin for the autopodial field including the digits, as opposed to that of more proximal limb elements. This hypothesis implies different evolutionary trajectories for distal versus more proximal parts of the tetrapod limb and

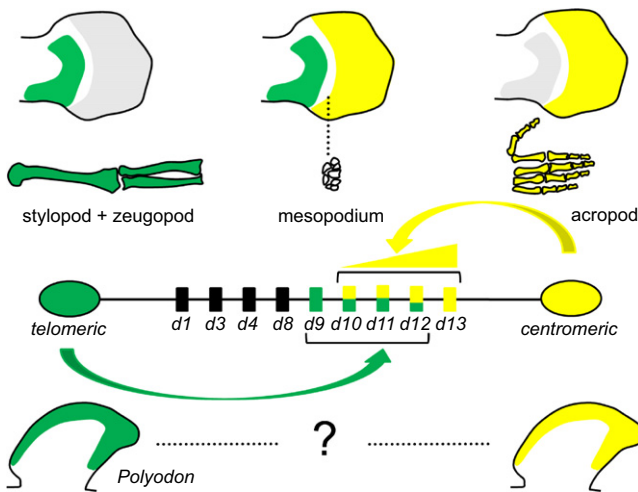


Figure 1. Expression Patterns Reflect Regulatory Strategies
 Posterior *Hoxd* genes are regulated by long-range transcriptional enhancers (green and yellow), located on opposite sides of the gene cluster. Enhancers regulating the early and proximal phase (green) locate on the telomeric side, whereas enhancers regulating the late and distal expression (yellow) locate on the centromeric side. The centromeric (distal) enhancers interact asymmetrically with *Hoxd10–13* in developing digits, resulting in higher levels of transcription for *Hoxd13* than for the other genes. This leads to the absence of *Hoxd10*, *Hoxd11*, and *Hoxd12* transcripts from the most anterior digit (Montavon et al., 2008). The mesopodium (carpals) will develop from the “no-*Hoxd* land” located between the proximal and distal expression domains. In fishes such as *Polyodon*, which show distal expression of *Hoxd* genes, it is as yet unknown whether different regulatory modules exist or if the detected distal expression derives from an extension of the early domain. This raises the problem of relying upon expression patterns, rather than mechanisms, when inferring homologies, and indicates that homologies can be ascertained only when the phylogeny of the underlying *cis*-regulation is considered.

suggests that the autopod is a neomorphic structure. In this view, the tetrapod limb is made out of two independent pieces bearing distinct ontogenetic and phylogenetic histories (Holmgren, 1952; Sordino et al., 1995; Wagner and Chiu, 2001; reviewed in Wagner and Larsson, 2007). The conceptual distinction between homology and neomorphy must nevertheless be handled carefully considering the facts that novel structures (1) seldom arise entirely *de novo* and (2) obligatorily implement preexisting genetic pathways and hence bear genetic signatures related to preexisting morphologies.

Support for the latter view came from a comparison of *Hox* gene expression between tetrapods and fishes, which revealed the absence, in fins, of the clear bimodal signature of proximal versus distal domains. In the zebrafish fin buds, a single phase of *Hoxd* expression was distinguished, extending to the most distal part of the presumptive endoskeleton, suggesting that the fin to limb transition involved the acquisition of a new phase of *Hoxd* transcription, functionally associated with the emergence of the autopod and digits (Sordino and Duboule, 1996). This interpretation was recently challenged after the examination of fish species such as *Polyodon*, shark (*Scyliorhinus*), and lungfish (*Neoceratodus*), whose fins more closely resemble those of tetrapod ancestors, for instance via the presence of a metapterygium (Mabee, 2000). In these species, two phases of expression were observed during pectoral fin development (*Scyliorhinus*, *Polyodon*) and strong distal *Hoxd13* signal was reported in fin

radials (*Neoceratodus*), suggesting the existence of the same proximal and distal expression domains as in tetrapod limbs (Davis et al., 2007; Freitas et al., 2007; Johanson et al., 2007).

In support of this view, *Polyodon* and shark *Hoxd13* transcripts extend more anteriorly than those of *Hoxd12*, as expected from reverse collinearity (Davis et al., 2007; Freitas et al., 2007). *Hoxd* expression was also reconsidered in zebrafish pectoral fin buds and both the proximal and distal expression phase were reported to exist in this fish too (Ahn and Ho, 2008). Altogether, it was proposed that the two-phased *Hoxd* expression, including that in the autopod, is an ancestral gnathostome character, rather than a neomorphic trait of tetrapods (see Shubin et al., 2009). By extension, distal fin cells labeled by the second phase of *Hoxd* expression could possess an “autopodial identity” (Freitas et al., 2007), implying homology between fin radials and digits (Johanson et al., 2007).

Do Fishes Have Digits?

These new expression data for *Hoxd* genes in fishes imply an ancient origin for the distal *Hoxd* domain and the presence of structures homologous to digits in fishes. However, a careful examination of these data sets calls for some caution in several respects. The description of multiple expression phases (see Ahn and Ho, 2008) does not rely upon clear temporal and/or topological observations, and *Hoxd11* “proximal” and “distal” expression in fins refers to a unique and continuous domain, unlike in tetrapods, wherein two distinct positive areas of the limb are visible *concomitantly*, separated by a zone of no (or low) *Hox* activity (see below). Also, while it is clear that *Hoxd* expression in *Polyodon* fins becomes distal (Davis et al., 2007), a similar trend occurs in the developing mouse limb *within* the proximal domain itself (i.e., during the early phase) before the autopod is formed (e.g., Nelson et al., 1996; Tarchini and Duboule, 2006). In other words, should the autopod be absent, expression of tetrapod *Hox* genes during the early proximal phase would develop into a “distal” domain as observed in *Polyodon*. In this latter case, a single extended expression domain seems to include all of the future endoskeletal components, and it is thus unclear how the *Hox* expression patterns in the *Polyodon* fin would relate to the bimodal pattern of the tetrapod limb.

It should also be noted that *Hoxa11* and *Hoxa13* transcripts are expressed in the same domain in fin buds (Sordino et al., 1995; van der Hoeven et al., 1996; Metscher et al., 2005; Davis et al., 2007), a situation drastically different from tetrapods wherein the two domains segregate early on to label proximal versus distal parts. Therefore, qualifications such as “distal,” “proximal,” “early,” or “late,” while helpful in an ontogenetic context, are of little use whenever phylogenetic issues are being considered, in particular when trying to homologize expression patterns between structures as distinct as fins and limbs. This problem can be partially addressed by considering a deeper level of comparison, that of the underlying regulatory circuits, rather than looking at the resulting expression patterns.

Patterns versus Mechanisms

Extensive genetic analysis of the *HoxD* locus *in vivo* has shown that the proximal and distal expression phases are mechanistically disconnected from one another (Figure 1). Therefore, the

morphological boundary, in tetrapod limbs, between those elements that can be clearly homologized with an ancestral fin and those that cannot matches a region of transition between two completely distinct regulatory modules controlling different, yet partially overlapping, subsets of *Hoxd* genes. Different sets of regulatory sequences are indeed located on opposite sides of the gene cluster (Spitz et al., 2003, 2005; see Deschamps, 2007), such that the proximal domain (the forearm) is controlled by sequences mapping telomeric to the cluster, whereas the distal domain (the digits) is driven by enhancers lying on the centromeric side (Gonzalez et al., 2007). Because *Hoxd13* is “the closest” to the centromeric enhancers, it shows preferential interactions and is thus expressed twice as strongly as *Hoxd12*. This difference impacts the AP distribution of *Hoxd13* in presumptive digits and causes it to be expressed in future digit I (the thumb), where other *Hoxd* genes are not transcribed. This biased interaction of centromeric enhancers with posterior *Hoxd* genes thus leads to the observed reverse collinearity in digits (Montavon et al., 2008).

The distal regulation triggered by these global enhancers also affects *Lunapark* (*Lnp*) and *Evx2*, two genes located on the centromeric side of the cluster (Spitz et al., 2003) and expressed in the same presumptive digit domain. In tetrapods, some of these enhancers have been identified, in particular two sequences referred to as CsB (part of the GCR sequence; Spitz et al., 2003) and CsC (part of the Prox sequence; Gonzalez et al., 2007), which can, on their own, elicit expression in distal limbs in a transgenic context. While CsB is highly conserved in all vertebrate genomes, the CsC sequence is found neither in fugu, nor in tetraodon, whereas it is present in birds, *Xenopus*, and *Anolis* (Gonzalez et al., 2007; data not shown). What do we know about *Hoxd* gene regulation during pectoral fin development, which could help ascertain homologies?

Unfortunately, a quantitative assessment of *Hox* transcripts in budding fins is lacking, which makes the comparison with reverse collinearity, as mechanistically defined in tetrapods, illegitimate. In addition, the pufferfish CsB sequence, when introduced into a mouse transgenic context, is unable to elicit expression in distal limbs, whereas it triggers expression in neuronal populations corresponding to another expected regulatory potential of this sequence (Spitz et al., 2003). Finally, the zebrafish *Lnp* gene, even though located at the same relative distance from *Hoxd13*, does not show expression in developing fins (Ahn and Ho, 2008) equivalent to that seen in the mouse limb bud. Given the absence of regulatory and quantitative analyses of posterior *Hoxd* gene regulation, the lack of apparent reverse collinearity in zebrafish (Ahn and Ho, 2008), and the conflicting data regarding genes known to be coregulated in the distal expression phase in tetrapods (*Lnp* and *Evx*), the conclusion that this distinctive tetrapod digit regulation may have a counterpart in fishes (see Shubin et al., 2009) should perhaps await more evidence.

A No-*Hoxd* Land: When Two Domains Make Three

A defining feature of tetrapods (Gaffney, 1979) is the presence of an interface between the digits and the radius/ulna, a collection of nodular-shaped mesopodial elements (the carpals/tarsals) that form the wrist and ankles and allow the hands and feet to properly articulate with the rest of the limbs (see Coates et al., 2002; Wagner and Chiu, 2001; Johanson et al., 2007). In terms

of adaptive value, the emergence of fully functional limbs thus required the coevolution of a mesopodium (carpus/tarsus) so as to optimize the use of hands and feet in land-based locomotion (Carroll, 1997). In this context, the mesopodium was an essential novelty to be added to a sarcopterygian fin, either as a new structure or via the transformation of preexisting elements.

We would like to propose that the evolution of a mesopodium was made possible by the existence of two independent *Hox* regulatory modules as opposed to a single ancestral one. *Hoxd* expression levels influence both the pattern of mesenchymal condensations and their subsequent growth capacities during limb development, in a dose-dependent manner. They have also been associated with the induction of growth plates (Boulet and Capecchi, 2004). The segregation of the two *Hoxd* domains in tetrapods creates a no-*Hoxd* land, situated between two series of elongated bones (the ulna-radius and the metacarpals), precisely at the future mesopodial position. Consequently, this region includes those cells producing the lowest amount of posterior *Hox* transcripts during limb development (Nelson et al., 1996; Reno et al., 2008). A causal relationship between high amounts of *Hox* products and long-shaped bones is further supported by combined inactivations of *Hoxd* genes, which produce ill-formed, less elongated bones (e.g., Zákány and Duboule, 1996; Bruneau et al., 2001).

Interestingly, the only *Hox* gene permanently expressed in this region is *Hoxa13*, whose ectopic gain of function can induce zeugopodial to mesopodial homeotic transformations (Yokouchi et al., 1995). In digits, this function is likely overruled by the expression of *Hoxd* genes, leading to long bones with joints (Figure 2). Mesopodial (carpus and tarsus) identity is thus determined by *Hoxa13* expression together with low (if any) *Hoxd* gene transcription. Mechanistically, the *Hoxd* bimodal regulation thus provided an elegant and parsimonious solution in evolving via the same genetic circuitry as both the digits and the accompanying carpal articulation by merely segregating apart a proximal and a distal domain, leaving a *Hoxd*-free zone in between. Understandably, this zone could not have been generated in a system where *Hox* expression in limbs would be controlled by a single regulatory module (Figure 2).

Should We Number Digits?

This zone of low *Hox* expression also includes the most anterior digit, commonly referred to as “digit I” or the thumb (or big toe). Unlike other presumptive digits, which express a broader range of *Hoxd* genes from *Hoxd10* to *Hoxd13*, the Most Anterior Digit (MAD) indeed expresses *Hoxd13* only, at low dose, in addition to *Hoxa13*. This difference in *Hoxd* gene expression between the MAD and other digits has been used as a signature of digit I (Vargas and Fallon, 2005a, 2005b; Vargas et al., 2008; Vargas and Wagner, 2009), an instrumental concept when discussing autopod evolution. Tetrapods have evolved all kinds of digital formulae, well adapted to various locomotive behaviors, illustrating the high flexibility of our distal limbs when compared with the more constrained proximal parts. In particular, the number of digits can vary substantially, either between distinct classes of animals (compare a bird wing with a human hand), or within highly related species such as among lizards.

Much like the case of homology between limbs and fins, the homology relationships between digits of various animals have

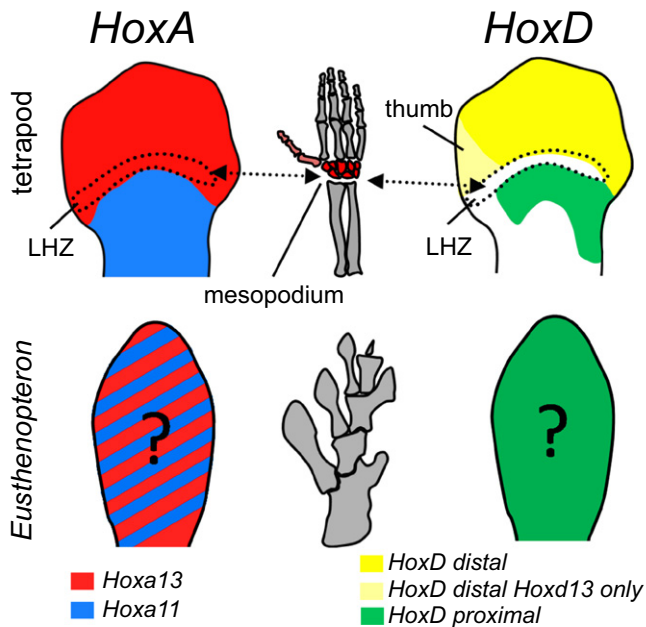


Figure 2. The Origin of the Wrist

The mesopodium (red) will develop from the low *Hox* zone (LHZ), located between the early (green) and late (yellow) phases of *Hoxd* gene expression. In this region, the only *Hox* gene permanently expressed is *Hoxa13* (red). Both the absence of *Hoxd* function and the expression of *Hoxa13* are responsible for the nodular character of mesopodial bones. In addition to *Hoxa13*, the most anterior digit (dark red) expresses low amounts of *Hoxd13* alone, in contrast to more posterior digits, which express the full set of posterior *Hoxd* genes. In this view, the thumb can be genetically considered to be an intermediate between the carpus and the digits. While tetrapod limbs show this clear separation of two series of long bones by the nodular mesopodium, the sarcopterygian ancestral fin skeleton is entirely made of long bones. Accordingly, the presumed primitive pattern of *Hox* gene expression in sarcopterygian fins would consist of both a single *Hoxd* phase (green) and the overlapping expression of *Hoxa11* and *Hoxa13* (shown here as stripes). Tetrapod limbs may have evolved an autopod after the segregation of the *Hoxa11* (blue) and *Hoxa13* (red) domains and the emergence of a second global regulation for *Hoxd* genes. The nonoverlap between both phases of *Hoxd* gene expression resulted in the formation of a mesopodium, and hence the necessary articulation.

been controversial (see Galis et al., 2005), in particular concerning the dinosaurs to bird transition. Birds have three digits in their wings, and developmental patterns indicate that the MAD corresponds in fact to digit II of the pentadactylous forelimbs (Burke and Feduccia, 1997), a correspondence further supported by the transient presence of a digit I rudiment in developing wings (e.g., Welten et al., 2005). However, this bird “digit II” does not transcribe any *Hoxd* genes besides *Hoxd13*, an expression pattern which qualifies it as digit I, as defined above. Wagner and Gauthier (1999) proposed a solution to this conflict, arguing that a frameshift occurred such that the most anterior digit of the wing develops at the position of digit II, yet with the program of digit I, and hence should be considered as a wrongly placed digit I. Here again, mechanistic considerations can help clarify the issue.

Unlike *Hoxa* genes, *Hoxd* genes have expression domains skewed toward the posterior limb margin, reflecting the effect of *Shh* signaling. *Shh* itself is transcribed posteriorly, in the limb, partially in response to HOX proteins produced during the

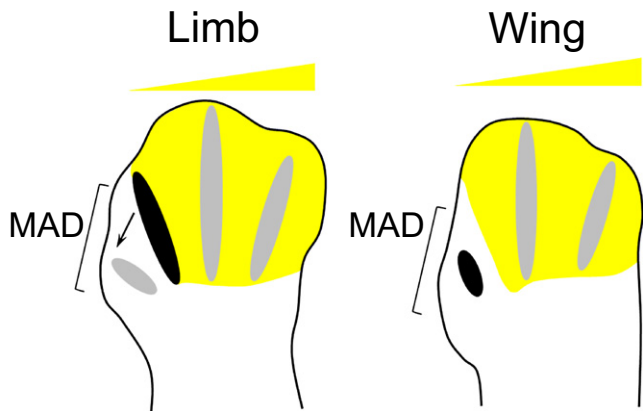


Figure 3. Hox Genes and the Genetics of MADness

In the chicken limb, digit I is the shortest likely due to absence of *Hoxd10*, *Hoxd11*, and *Hoxd12* (yellow) expression. In the chicken wing, three digits develop with a recognizable morphology with the shortest positioned at the anterior side. The morphology thus suggests that the Most Anterior Digit (MAD) is digit I. Embryological evidence however indicates that it develops from the second digital condensation and should thus be considered digit II (Burke and Feduccia, 1997). However, upon loss of the most anterior digit DI, DII (black in limb) shifts into the most anterior position (black in wing), thereby creating a new MAD. Because the most anterior digital condensation will always receive a lower *Hox* dose, due to the underlying regulatory mechanism, it will always develop a short “DI morphology.” Here again, gene expression cannot be used to trace homologous relationships between digits of different species, and the MAD will be defined by the exposure to the lowest HOX concentration, regardless of its relative “identity” (digit I or II, etc).

early phase (Mackem and Knezevic, 1999; Zákány et al., 2004; Tarchini et al., 2006). In turn SHH directs the AP expansion of the autopod and quantitatively regulates the late phase of *Hoxd* expression, resulting in the absence of *Hoxd12* to *Hoxd10* in the most anterior cells, those located opposite the source of SHH. Therefore, the AP polarity of the limb cannot be disconnected from its growth, and the asymmetric expression of *Hoxd12* to *Hoxd10* is an intrinsic property of *Shh* signaling, a constraint imposed by the logic of the regulatory system (Tarchini et al., 2006). As a consequence, every tetrapod hand or foot, regardless of its digit number (provided it is more than one), will display this unbalanced *Hoxd* gene expression, and hence will have digits of distinct morphologies, unless the *Shh* pathway is modified in one way or another. Because one effect of the late phase of *Hoxd* gene expression is to elongate digital bones, the most anterior digit will always be (among) the shortest, independently of its developmental origin (Figure 3). The “identification” of digits for phylogenetic purposes can thus hardly rely upon expression patterns only (Coates, 1993), which are not directly associated with particular digits, but instead with the general regulatory strategy that gives rise to the pattern in the autopodial field.

Is the Thumb Part of the Carpus?

Hoxd13 for instance is the only *Hoxd* gene transcribed in presumptive digit I. Yet the MAD is not functionally characterized by the expression of *Hoxd13*, whose absence can be largely compensated for by the gain of function of *Hoxd12* in anterior cells, giving rise to an almost genuine thumb (Kmita et al., 2002). Instead, the MAD is defined by a reduced dose of

HOXD proteins altogether, because gain of function of either *Hoxd12* or *Hoxd11* anteriorly elongates the thumb toward the shape of other digits (Morgan et al., 1992; Knezevic et al., 1997). Also, the inactivation of *Hoxd13* alone does not preclude the formation of a thumb (albeit one that is short, misshapen, and lacking joints; Dollé et al., 1993) because, in addition to a low dose of HOXD products, MADness is determined by the expression of *Hoxa13*, whose inactivation leads to thumb agenesis (Fromental-Ramain et al., 1996).

In this view, the extension of transcriptional activation of *Hoxd13* anteriorly (reverse collinearity) is critical, for it slightly elongates the MAD and allows joints to appear. In the case of the big toe, the inactivation of *Hoxd13* transforms the metatarsal into a nonelongated, round-shaped bone, as if the absence of *Hoxd* genes would transform “long” bones into mesopodial elements, which normally express *Hoxa13* only. In genetic terms, the MAD may be as related to the carpus as it is to its neighboring digits, in particular in those species where expression of *Hoxd13* is expectedly low anteriorly, associated with a short and poorly elongated thumb. In these cases, *Hoxa13* would be the major determinant (Figure 2). In contrast, a re-enforced expression of *Hoxd13* in the presumptive MAD, on the top of *Hoxa13*, may have allowed a more elaborated thumb to develop in species wherein such a structure would have an adaptive value.

Limb elements associated with the low *Hox* zone have undergone interesting evolutionary modifications. One is the enlargement of a carpal bone to act as an additional digit either in pandas or in moles, an adaptation to a fossorial existence (Gould, 1980; Galis et al., 2001; Sánchez-Villagra and Menke, 2005). This change in morphology may have been caused by a shift in expression of the second phase of *Hoxd* genes into the presumptive territories for these bones. Conversely, an expansion of carpal identity is observed in mesozoic marine reptiles, the most extreme case being ichthyosauri, where the complete distal fin consists of arrays of nodular bones lacking a perichondrium (Caldwell, 1997). A loss of the second phase of expression could have been associated with this shift from digital to carpal identity. Accordingly, the mouse *spd* mutant, caused by a polyalanine expansion in *Hoxd13* leading to the functional inactivation of several *Hox* genes (Bruneau et al., 2001), exhibits a transformation of digits toward nodular-like bones (Muragaki et al., 1996; Villavicencio-Lorini et al., 2010).

Conclusions

Both the fin to limb transition and the question of digital identity illustrate a recurrent problem in the young field of Evo-Devo that is apparent whenever expression patterns are interpreted without a full consideration of the underlying mechanisms (discussed in Arthur, 2002; De Robertis, 2008). In order to infer deeply homologous relationships (senso Shubin et al., 2009), it is essential that a phylogeny of regulations be established, much in the same way precise modifications in defined regulatory sequences can be associated with variations in morphological patterns (e.g., Prud'homme et al., 2006). Whether the tetrapod autopod is a neomorphic structure (senso Sordino et al., 1995) or preexisted in sarcopterygian fishes in one form or another (Johanson et al., 2007) cannot be solved by the mere contemplation of expression patterns and thus remains to be determined. Future studies on the underlying *cis*-regulation

in fishes will indicate whether our digits were built as modifications (adaptations) of both preexisting radials and their regulatory circuitry or, alternatively, if they appeared along with the emergence of a novel regulatory mechanism, which added both these structures and the associated articulation to the distal tip of preexisting endoskeletal elements present in ancestral fins.

ACKNOWLEDGMENTS

We thank C. Tabin and M. Coates for insightful discussions and comments. J.M.W. is supported by a long-term EMBO fellowship. The laboratories are funded by the University of Geneva, the Federal Institute of Technology (EPFL, Lausanne), the Swiss National Research Fund, the National Research Center “Frontiers in Genetics,” the EU program *Crescendo* and the European Research Council (ERC).

REFERENCES

- Ahn, D., and Ho, R.K. (2008). Tri-phasic 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.
- Arthur, W. (2002). The emerging conceptual framework of evolutionary developmental biology. *Nature* 415, 757–764.
- Boulet, A.M., and Capecchi, M.R. (2004). Multiple roles of *Hoxa11* and *Hoxd11* in the formation of the mammalian forelimb zeugopod. *Development* 131, 299–309.
- Bruneau, S., Johnson, K.R., Yamamoto, M., Kuroiwa, A., and Duboule, D. (2001). The mouse *Hoxd13*(*spd*) mutation, a polyalanine expansion similar to human type II synpolydactyly (SPD), disrupts the function but not the expression of other *Hoxd* genes. *Dev. Biol.* 237, 345–353.
- Burke, A.C., and Feduccia, A. (1997). Developmental Patterns and the Identification of Homologies in the Avian Hand. *Science* 278, 666–668.
- Caldwell, M.W. (1997). Modified perichondral ossification and the evolution of paddle-like limbs in Ichthyosaurs and Plesiosaurs. *J. Vertebr. Paleontol.* 17, 534–547.
- Carroll, R.L. (1997). *Patterns and Processes of Vertebrate Evolution* (Cambridge: Cambridge University Press).
- Christen, B., Beck, C.W., Lombardo, A., and Slack, J.M. (2003). Regeneration-specific expression pattern of three posterior Hox genes. *Dev. Dyn.* 226, 349–355.
- Coates, M.I. (1993). Ancestors and homology (the origin of the tetrapod limb). *Acta Biotheor.* 41, 411–424.
- Coates, M.I. (1994). The origin of vertebrate limbs. *Dev. Suppl.*, 169–180.
- Coates, M.I. (1995). Limb evolution. Fish fins or tetrapod limbs—a simple twist of fate? *Curr. Biol.* 5, 844–848.
- Coates, M.I., Jeffery, J.E., and Rut, M. (2002). Fins to limbs: what the fossils say. *Evol. Dev.* 4, 390–401.
- Cohn, M.J., Lovejoy, C.O., Wolpert, L., and Coates, M.I. (2002). Branching, segmentation and the metapterygial axis: pattern versus process in the vertebrate limb. *Bioessays* 24, 460–465.
- Davis, A.P., and Capecchi, M.R. (1996). A mutational analysis of the 5' *HoxD* genes: dissection of genetic interactions during limb development in the mouse. *Development* 122, 1175–1185.
- Davis, A.P., Witte, D.P., Hsieh-Li, H.M., Potter, S.S., and Capecchi, M.R. (1995). Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*. *Nature* 375, 791–795.
- Davis, M.C., Dahn, R.D., and Shubin, N.H. (2007). An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. *Nature* 447, 473–476.
- De Robertis, E.M. (2008). Evo-devo: variations on ancestral themes. *Cell* 132, 185–195.

- Deschamps, J. (2007). Ancestral and recently recruited global control of the *Hox* genes in development. *Curr. Opin. Genet. Dev.* 17, 422–427.
- Dollé, P., Izpisua-Belmonte, J.C., Falkenstein, H., Renucci, A., and Duboule, D. (1989). Coordinate expression of the murine *Hox-5* complex homoeobox-containing genes during limb pattern formation. *Nature* 342, 767–772.
- Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbauer, B., Chambon, P., and Duboule, D. (1993). Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. *Cell* 75, 431–441.
- Drossopoulou, G., Lewis, K.E., Sanz-Ezquerro, J.J., Nikbakht, N., McMahon, A.P., Hofmann, C., and Tickle, C. (2000). A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range *Shh* signalling and *Bmp* signalling. *Development* 127, 1337–1348.
- Freitas, R., Zhang, G., and Cohn, M.J. (2007). Biphasic *Hoxd* gene expression in shark paired fins reveals an ancient origin of the distal limb domain. *PLoS ONE* 15, e754.
- Friedman, M., Coates, M.I., and Anderson, P. (2007). First discovery of a primitive coelacanth fin fills a major gap in the evolution of lobed fins and limbs. *Evol. Dev.* 9, 329–337.
- Fromental-Ramain, C., Warot, X., Messadecq, N., LeMeur, M., Dollé, P., and Chambon, P. (1996). *Hoxa-13* and *Hoxd-13* play a crucial role in the patterning of the limb autopod. *Development* 122, 2997–3011.
- Gaffney, E.S. (1979). Tetrapod monophyly: a phylogenetic analysis. *Bull. Carnegie Mus. Nat. Hist.* 13, 92–105.
- Galis, F., van Alphen, J.J.M., and Metz, J.A.J. (2001). Why five fingers? Evolutionary constraints on digit numbers. *Trends Ecol. Evol.* 16, 637–645.
- Galis, F., Kundrát, M., and Metz, J.A.J. (2005). *Hox* genes, digit identities and the theropod/bird transition. *J. Exp. Zool. B Mol. Dev. Evol.* 304, 198–205.
- Gonzalez, F., Duboule, D., and Spitz, F. (2007). Transgenic analysis of *Hoxd* gene regulation during digit development. *Dev. Biol.* 306, 847–859.
- Gould, S.J. (1980). *The Panda's Thumb* (New York: Norton).
- Harfe, B.D., Scherz, P.J., Nissim, S., Tian, H., McMahon, A.P., and Tabin, C.J. (2004). Evidence for an expansion-based temporal *Shh* gradient in specifying vertebrate digit identities. *Cell* 118, 517–528.
- Holmgreen, N. (1952). An embryological analysis of the mammalian carpus and its bearing on the question of the origin of the tetrapod limb. *Acta Zool.* 33, 1–115.
- Johanson, Z., Joss, J., Boisvert, C.A., Ericsson, R., Sutija, M., and Ahlberg, P.E. (2007). Fish fingers: digit homologues in sarcopterygian fish fins. *J. Exp. Zool. B Mol. Dev. Evol.* 308, 757–768.
- Kmita, M., Fraudeau, N., Hérault, Y., and Duboule, D. (2002). Serial deletions and duplications suggest a mechanism for the collinearity of *Hoxd* genes in limbs. *Nature* 420, 145–150.
- Kmita, M., Tarchini, B., Zákány, J., Logan, M., Tabin, C.J., and Duboule, D. (2005). Early developmental arrest of mammalian limbs lacking *HoxA/HoxD* gene function. *Nature* 435, 1113–1116.
- Knezevic, V., De Santo, R., Schughart, K., Huffstadt, U., Chiang, C., Mahon, K.A., and Mackem, S. (1997). *Hoxd-12* differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* 124, 4523–4536.
- Mabee, P.M. (2000). Developmental Data and Phylogenetic Systematics: Evolution of the Vertebrate Limb. *Am. Zool.* 40, 789–800.
- Mackem, S., and Knezevic, V. (1999). Do 5' *Hoxd* genes play a role in initiating A-P polarizing signals in the limb? *Cell Tissue Res.* 296, 27–31.
- Metscher, B.D., Takahashi, K., Crow, K., Amemiya, C., Nonaka, D.F., and Wagner, G.P. (2005). Expression of *Hoxa-11* and *Hoxa-13* in the pectoral fin of a basal ray-finned fish, *Polyodon spathula*: implications for the origin of tetrapod limbs. *Evol. Dev.* 7, 186–195.
- Montavon, T., Le Garrec, J.F., Kerszberg, M., and Duboule, D. (2008). Modeling *Hox* gene regulation in digits: reverse collinearity and the molecular origin of thumbness. *Genes Dev.* 22, 346–359.
- Morgan, B.A., Izpisua-Belmonte, J.C., Duboule, D., and Tabin, C.J. (1992). Targeted misexpression of *Hox-4.6* in the avian limb bud causes apparent homeotic transformations. *Nature* 358, 236–239.
- Muragaki, Y., Mundlos, S., Upton, J., and Olsen, B.R. (1996). Altered growth and branching patterns in synpolydactyly caused by mutations in *HOXD13*. *Science* 272, 548–551.
- Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., and Tabin, C. (1996). Analysis of *Hox* gene expression in the chick limb bud. *Development* 122, 1449–1466.
- Ohgo, S., Itoh, A., Suzuki, M., Satoh, A., Yokoyama, H., and Tamura, K. (2010). Analysis of *hoxa11* and *hoxa13* expression during patternless limb regeneration in *Xenopus*. *Dev. Biol.* 338, 148–157.
- Prud'homme, B., Gompel, N., Rokas, A., Kassner, V.A., Williams, T.M., Yeh, S.D., True, J.R., and Carroll, S.B. (2006). Repeated morphological evolution through cis-regulatory changes in a pleiotropic gene. *Nature* 440, 1050–1053.
- Reno, P.L., McCollum, M.A., Cohn, M.J., Meindl, R.S., Hamrick, M., and Lovejoy, C.O. (2008). Patterns of correlation and covariation of anthropoid distal forelimb segments correspond to *Hoxd* expression territories. *J. Exp. Zool. B Mol. Dev. Evol.* 310, 240–258.
- Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416.
- Sánchez-Villagra, M.R., and Menke, P.R. (2005). The mole's thumb — evolution of the hand skeleton in tapids (Mammalia). *Zoology (Jena)* 108, 3–12.
- Satoh, A., Endo, T., Abe, M., Yakushiji, N., Ohgo, S., Tamura, K., and Ide, H. (2006). Characterization of *Xenopus* digits and regenerated limbs of the froglet. *Dev. Dyn.* 235, 3316–3326.
- Shubin, N., Tabin, C., and Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. *Nature* 457, 818–823.
- Sordino, P., and Duboule, D. (1996). A molecular approach to the evolution of vertebrate paired appendages. *Trends Ecol. Evol.* 11, 114–119.
- Sordino, P., van der Hoeven, F., and Duboule, D. (1995). *Hox* gene expression in teleost fins and the origin of vertebrate digits. *Nature* 375, 678–681.
- Spitz, F., Gonzalez, F., and Duboule, D. (2003). A global control region defines a chromosomal regulatory landscape containing the *HoxD* cluster. *Cell* 113, 405–417.
- Spitz, F., Herkenne, C., Morris, M.A., and Duboule, D. (2005). Inversion-induced disruption of the *Hoxd* cluster leads to the partition of regulatory landscapes. *Nat. Genet.* 37, 889–893.
- Tabin, C., and Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* 21, 1433–1442.
- Tamura, K., Yonei-Tamura, S., Yano, T., Yokoyama, H., and Ide, H. (2008). The autopod: its formation during limb development. *Dev. Growth Differ.* 50 (Suppl 1), S177–S187.
- Tarchini, B., and Duboule, D. (2006). Control of *Hoxd* genes' collinearity during early limb development. *Dev. Cell* 10, 93–103.
- Tarchini, B., Duboule, D., and Kmita, M. (2006). Regulatory constraints in the evolution of the tetrapod limb anterior-posterior polarity. *Nature* 443, 985–988.
- Torok, M.A., Gardiner, D.M., Shubin, N.H., and Bryant, S.V. (1998). Expression of *HoxD* genes in developing and regenerating axolotl limbs. *Dev. Biol.* 200, 225–233.
- van der Hoeven, F., Sordino, P., Fraudeau, N., Izpisua-Belmonte, J.-C., and Duboule, D. (1996). Teleost *HoxD* and *HoxA* genes: Comparison with tetrapods and functional evolution of the *HoxD* complex. *Mech. Dev.* 54, 9–21.
- Vargas, A.O., and Fallon, J.F. (2005a). Birds have dinosaur wings: The molecular evidence. *J. Exp. Zool. B Mol. Dev. Evol.* 304, 86–90.
- Vargas, A.O., and Fallon, J.F. (2005b). The digits of the wing of birds are 1, 2, and 3. A review. *J. Exp. Zool. B Mol. Dev. Evol.* 304, 206–219.
- Vargas, A.O., and Wagner, G.P. (2009). Frame-shifts of digit identity in bird evolution and Cyclopamine-treated wings. *Evol. Dev.* 11, 163–169.

- Vargas, A.O., Kohlsdorf, T., Fallon, J.F., Vandenbrooks, J., and Wagner, G.P. (2008). The evolution of HoxD-11 expression in the bird wing: insights from *Alligator mississippiensis*. *PLoS ONE* 3, e3325.
- Villavicencio-Lorini, P., Kuss, P., Friedrich, J., Haupt, J., Farooq, M., Türkmen, S., Duboule, D., Hecht, J., and Mundlos, S. (2010). Hox genes control bone formation in the mouse limb. *J. Clin. Invest.*, in press.
- Wagner, G.P., and Chiu, C.H. (2001). The tetrapod limb: a hypothesis on its origin. *J. Exp. Zool.* 297, 226–240.
- Wagner, G.P., and Gauthier, J.A. (1999). 1,2,3 = 2,3,4: a solution to the problem of the homology of the digits in the avian hand. *Proc. Natl. Acad. Sci. USA* 96, 5111–5116.
- Wagner, G.P., and Larsson, H.C.E. (2007). *Fins and Limbs in the Study of Evolutionary Novelty: Fins into Limbs* (Chicago, USA: The University of Chicago Press), pp. 49–61.
- Welten, M.C., Verbeek, F.J., Meijer, A.H., and Richardson, M.K. (2005). Gene expression and digit homology in the chicken embryo wing. *Evol. Dev.* 7, 18–28.
- Yokouchi, Y., Sasaki, H., and Kuroiwa, A. (1991). Homeobox gene expression correlated with the bifurcation process of limb cartilage development. *Nature* 353, 443–445.
- Yokouchi, Y., Nakazato, S., Yamamoto, M., Goto, Y., Kameda, T., Iba, H., and Kuroiwa, A. (1995). Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev.* 9, 2509–2522.
- Zákány, J., and Duboule, D. (1996). Synpolydactyly in mice with a targeted deficiency in the HoxD complex. *Nature* 384, 69–71.
- Zákány, J., Fromental-Ramain, C., Warot, X., and Duboule, D. (1997). Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. *Proc. Natl. Acad. Sci. USA* 94, 13695–13700.
- Zákány, J., Kmita, M., and Duboule, D. (2004). A dual role for Hox genes in limb anterior-posterior asymmetry. *Science* 304, 1669–1672.