Trends in Genetics

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Review

Reassessing the Role of *Hox* Genes during Vertebrate Development and Evolution

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Since their discovery *Hox* genes have been at the core of the established models explaining the development and evolution of the vertebrate body plan as well as its paired appendages. Recent work brought new light to their role in the patterning processes along the main body axis. These studies show that *Hox* genes do not control the basic layout of the vertebrate body plan but carry out region-specific patterning instructions loaded on the derivatives of axial progenitors by *Hox*-independent processes. Furthermore, the finding that *Hox* clusters are embedded in functional chromatin domains, which critically impacts their expression, has significantly altered our understanding of the mechanisms of *Hox* gene regulation. This new conceptual framework has broadened our understanding of both limb development and the evolution of vertebrate paired appendages.

The Vertebrate Basic Body Plan Is Laid Out Independently of *Hox* Genes

Vertebrates display a remarkable variety of body sizes and shapes, typically involving features along their main body axis and their **paired appendages** (see Glossary). However, the basic developmental principles generating the various structures are largely shared among vertebrates. The various body attributes along the main body axis are assembled sequentially in a head-to-tail sequence as the embryo extends at its posterior end. This results from the activity of dedicated axial progenitors producing the raw material that eventually forms the various embryonic tissues [1,2]. Although continuous, axial extension can be divided into three major steps each regulated by a distinct gene network [3–5]. During the first step, typically known as head development, the embryo generates the brain and heart primordia together with musculoskeletal structures of the head and neck. This is followed by formation of the trunk, which holds most of the internal organs involved in vital and reproductive functions. The final step of axial extension is devoted to tail formation, essentially comprising vertebrae, muscles, and a variable amount of neural tissue. Differences in the overall extent of body elongation during development, as well as the portions of this axial growth devoted to making head, trunk, or tail structures, are among the most relevant parameters generating anatomical diversity in vertebrates. For this reason both the mechanisms controlling these processes and their role in vertebrate evolution have attracted plenty of attention over the past few decades.

Since their discovery *Hox* genes (Box 1) have been considered the major drivers of morphological evolution in the animal kingdom [6]. Comparative expression analyses in embryos of vertebrates with clearly distinct body distributions revealed a close correlation between the expression boundaries of particular *Hox* genes and specific landmarks in the axial skeleton [7,8], suggesting key roles for these genes in setting up the basic vertebrate body plan. A large variety of genetic studies, mostly in the mouse, confirmed the relevant contributions of *Hox* genes to patterning processes along the main body axis [9,10]. For instance, *Hox4* genes are involved in proper patterning of the neck vertebrae [11], *Hox5*, *Hox6*, and *Hox9* genes control

Highlights

The basic layout of the vertebrate body is outlined in the axial progenitors mostly through *Hox*-independent mechanisms.

Hox genes are carriers of patterning information loaded onto the derivatives of axial progenitors that guides the production of body structures congruent with their axial level.

Selective target inactivation allows the shutting down of a subset of *Hox*-dependent functions while keeping others active in the same domain. This increases the flexibility of evolutionary processes.

The processes regulating *Hox* gene expression in the proximal and distal regions of the limb buds occur in two alternative functional chromatin domains.

Modification of *Hox* regulatory processes within chromatin domains might have played a role in the evolution of vertebrate paired appendages.

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various aspects of ribcage development [12,13], and *Hox10* and *Hox11* genes are essential for the formation of the lumbar and sacral areas of the axial skeleton, respectively [14,15]. However, these studies consistently failed to show significant changes in the basic head/trunk/tail distribution of the body, even in cases where alterations in *Hox* gene activity or expression produced extreme phenotypes in the axial skeleton. Only *Hox13* genes have been found to contribute to this process, by playing a role in determining the final length of the tail region [16,17]. Overall these data indicated that the basic head/trunk/tail structure of the embryo is most likely not under direct *Hox* regulation, which seemed to contradict the prevailing model for the evolution of the vertebrate body plan along the main body axis.

Gdf11 and *Oct4* Are Upstream of *Hox* Genes in the Body-Patterning Hierarchy

Recent findings brought new light to this issue, identifying *Oct4* and Gdf11 signaling as major players in the establishment of the basic body plan, acting upstream of *Hox* genes. *Gdf11*, a member of the Tgfβ family expressed in the posterior embryonic area starting at mid-gestation [18,19], was shown to be a key activator of the trunk-to-tail transition [20], a process in which it shows partial redundancy with *Gdf8* [21]. Mice with impaired Gdf11 signaling have longer trunks resulting from delayed activation of this transition from the early stages of development, as reflected in the significantly more posterior position of the hindlimbs and cloaca, which mark the posterior end of trunk-associated structures such as the lateral mesoderm and the endodermal tissues contributing to the internal organs [19,20]. Conversely, premature activation of Gdf11 signaling resulted in more anterior induction of the trunk-to-tail transition, which placed the hindlimb next to the forelimb bud and thus led to embryos without a trunk [20]. *Gdf11* expression in different vertebrates seems to give further support for the role of this signaling in the evolution of vertebrate trunk length [22,23].

Other studies revealed that the pluripotency factor *Oct4* plays a somewhat complementary role in the layout of the basic body plan, as it promotes extension through the trunk region. Such new role for *Oct4*, consistent with its expression dynamics during mouse development [24,25], was suggested by genetic studies. In particular, conditional *Oct4* inactivation in mouse embryos at early stages of trunk formation resulted in embryos without a trunk but that still contained recognizable tails [26]. A different set of studies showed that *Oct4* is also sufficient to extend the vertebrate trunk [23]. Prolonging the period of *Oct4* activity in mouse embryos resulted in longer trunks at the expense of the tails. In addition, molecular analyses in snake embryos indicate that their remarkably long trunks might be the result of an increased period of *Oct4* activity during embryonic development [23]. Altogether, various lines of evidence place the balance between *Oct4* and *Gdf11* activities at the top of the hierarchy of regulatory processes controlling the basic features of the vertebrate body plan by playing fundamental roles in determining the relative contributions of the different body sections to the animal's anatomy.

Where Do *Hox* Genes Fit in This Scheme?

Expression analyses indicated that in mouse embryos with modified *Gdf11* or *Oct4* activity, **5′ *Hox* genes** became activated at axial levels congruent with the new position of the hindlimb bud and, thus, the trunk-to-tail transition [19,20,23,27,28]. Interestingly, some **3′ *Hox* genes of 3′ groups** showed a complementary behavior, best seen in embryos with longer trunks (i.e., *Gdf11* mutants or transgenics with sustained *Oct4* expression), where their expression spread into more posterior embryonic areas [19,20,23,28] (Figure 1). The same global patterns of *Hox* gene expression were observed in the natural setting presented by the snake embryo [8,29]. These studies thus place *Hox* gene expression downstream of *Oct4* and Gdf11 signaling. A

Glossary

3′ and 5′ *Hox* genes: a convenient way of grouping *Hox* genes in the clusters (sometimes also referred to as anterior and posterior *Hox* genes, respectively). Although this can vary slightly depending on the specific sources, 3′ *Hox* genes normally include those of groups 1–9 and 5′ *Hox* genes those of groups 10–13.
Amniotes: a clade of limbed vertebrates, including reptiles, birds, and mammals. Embryos of amniote species develop in a fluid-filled cavity formed by a series of extraembryonic membranes including the amnion, which gives name to the clade.
Paired appendages: locomotor structures (normally two pairs per animal) typical of jawed vertebrates; include the pectoral and pelvic fins of fishes and the limbs of tetrapods.
Teleosts: a group of bony fishes containing a movable premaxilla. The largest part of living species of fish belongs to this group.
Topologically associating domains (TADs): 3D chromatin structures found in interphase nuclei; identified, by methods allowing high-throughput analysis of mutual genomic DNA contacts, as regions that interact with each other more frequently than with other parts of the genome. Regulatory interactions are typically contained in TADs.
Zeugopod, stylopod, and autopod: the three basic sections of the tetrapod limb, from proximal to distal. The zeugopod typically contains one bone (the humerus in the forelimb and the femur in the hindlimb), the stylopod contains two bones (the ulna and radius in the forelimb and the tibia and fibula in the hindlimb), and the autopod contains a variable number of digits.

Box 1. Basic Concepts of Vertebrate *Hox* Gene Organization and Expression

In mammals, which provide a paradigm to outline the basic concepts of *Hox* gene organization (Figure I), *Hox* genes are distributed in four clusters, named *HoxA*, *HoxB*, *HoxC*, and *HoxD*, thought to result from two successive duplications of an ancestral cluster. *Hox* genes are subdivided in 13 groups (from *Hox1* to *Hox13*) based on sequence homology and their position in the cluster. *Hox* genes with lower numbers are located at the 3′ side of the cluster and those with higher numbers toward the 5′ end. In general *Hox* gene activation is sequential, following their order in the cluster in a 3′to-5′ direction, a property known as collinearity. Because *Hox* gene activation is concurrent with axial extension and limb growth, the different areas of the body and limbs express distinct combinations of *Hox* genes. Other vertebrates have different numbers of *Hox* clusters. For instance, the zebrafish has seven clusters, known as *HoxAa*, *HoxAb*, *HoxBa*, *HoxBb*, *HoxCa*, *HoxCb*, and *HoxDa*, resulting from an additional duplication followed by the loss of one whole cluster.

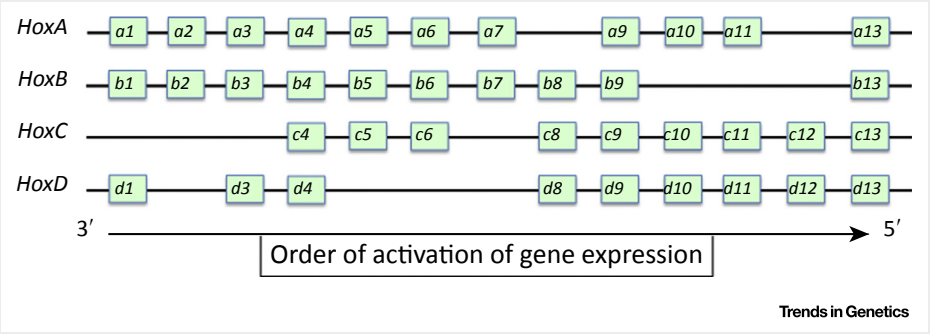

Figure I. Basic Structure of the Mammalian *Hox* Clusters.

similar hierarchy was observed at the cell/tissue level, as *Oct4* and *Gdf11* regulate patterning directly on the axial progenitors (and thus at the top of the cellular hierarchy controlling body formation) whereas *Hox* gene activity is most important in tissues derived from those progenitors [20,23]. Hence, the global picture emerging from these studies (Figure 2) is that, in a first step, the basic layout of the vertebrate body is outlined in the axial progenitors mostly through *Hox*-independent mechanisms. As an integral part of these basic processes, the different cell types produced by these progenitors become loaded with patterning information corresponding to the axial level where they will differentiate. *Hox* genes thus belong to this second patterning layer providing axial identity to mesodermal, neural, and maybe also endodermal derivatives of the axial progenitors [9,30,31]. Thus, their role in the evolution of the vertebrate anatomy is exerted in these tissues where specific combinations of *Hox* gene expression determine regional variations in the main body domains. Again, snakes provide a natural example to illustrate this hypothesis. In particular, the morphology of the trunk vertebrae of different snake species revealed that this region is not uniform as was classically considered but regionalized along the anterior–posterior axis following species-specific patterns, most likely resulting from specific variations in *Hox* gene expression, set independently of their trunk length [32].

So far little is known about how *Oct4* and Gdf11 signaling controls *Hox* gene expression. The two-way complementarity of *Hox* regulation by *Oct4* and *Gdf11* (i.e., that 5′ *Hox* genes are repressed by *Oct4* and activated by *Gdf11* whereas 3′ *Hox* gene expression expands posteriorly following extended *Oct4* activity or on *Gdf11* inactivation) might provide clues to understand this process. The regions of the *Hox* cluster under differential regulation by *Oct4* and *Gdf11* roughly correspond to their distribution within the two **topologically associating domains (TADs)** covering the *HoxA* and *HoxD* clusters [33,34]. TADs demarcate chromatin territories facilitating regulatory interactions [35,36] and the TAD structure associated with the

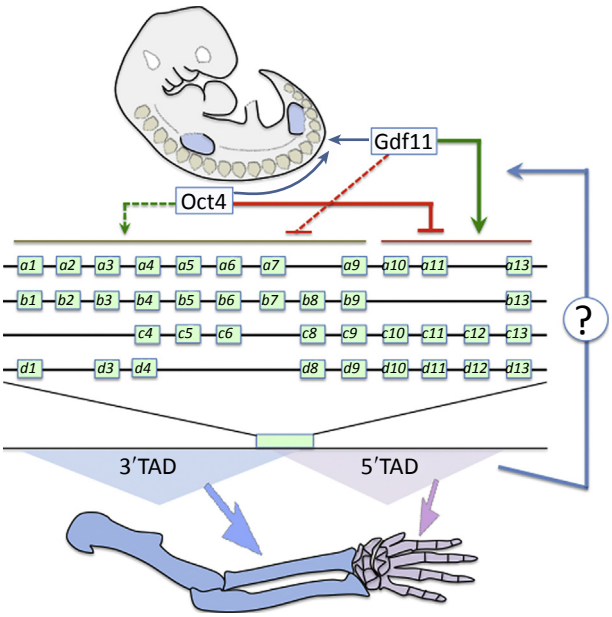

Figure 1. *Oct4* Promotes Trunk Elongation and *Gdf11* Activates the Trunk-to-Tail Transition (Marked by the Hindlimb Position). As part of these activities, they regulate *Hox* gene expression. 5′ *Hox* genes are kept inactive at trunk levels by *Oct4* and become activated when Gdf11 signaling takes over. Regulation of several 3′ *Hox* genes follows a complementary pattern. *Hox* gene expression in the areas of the limb bud generating the arm and forearm is under the control of regulatory interactions in the 3′ topologically associating domain (TAD) (in blue). The regulatory landscape switches to the 5′ TAD in the distal limb (purple) to produce hand-specific *Hox* gene expression. Does TAD organization also impact *Oct4*/*Gdf11*-mediated *Hox* regulation in the main body axis?

Hox clusters has been shown to be relevant for the regulation of *Hox* gene expression during limb bud development [33,37]. In particular, as mentioned below, as the limb bud grows distally the *Hox* regulatory landscape switches from the 3′ to the 5′ TAD coincident with the activation of *Hox* genes at the 5′ end of the cluster (Figure 2). *Hox* gene regulation in the major body axis might also fit in a similar general scheme involving a regulatory switch between TADs orchestrated by the balance between *Oct4* and Gdf11 signaling activities. Involvement of the TAD structure in *Hox* gene regulation in the main body axis is supported by recent findings showing

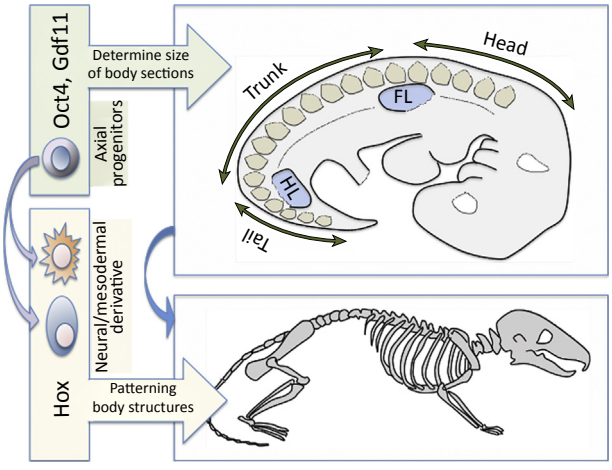

Figure 2. Patterning in the Main Body Axis Occurs in Two Consecutive Stages. In the first stage, the final size of the head/trunk/tail region is determined in the axial progenitors mostly through *Hox*-independent mechanisms, including *Oct4* and *Gdf11* activities. The transition between these regions can be identified in the developing embryo by the positions of the forelimb (FL) and hindlimb (HL). The second stage occurs in the derivatives of the axial progenitors by loading them with axial level-specific patterning information (including *Hox* genes) that guides their differentiation into the appropriate body structures.

that Wnt3/Wnt3a signaling and Cdx proteins sequentially activate 3′ *HoxA* genes in the epiblast through specific regulatory interactions occurring in the 3′ TAD [38,39]. Additional findings suggest that *Oct4* activity might also fit within this regulatory scheme. In particular, it has been shown that in embryonic stem cells Oct4 primes *Hoxa1* and *Hoxb1* for activation on exposure to retinoic acid [40], a physiological activator of *Hox* gene expression. These observations indicate that *Oct4* might indeed be involved in activation of 3′ *Hox* genes. In addition, Oct4 has been reported to bind the *HoxA* cluster at the intersection of the TADs, and this binding might be important for proper chromatin structure and *Hox* gene expression in cell lines [41]. Even less is known about *Hox* regulation by Gdf11 signaling. Currently, the only connection between Gdf11 signaling and *Hox* gene expression was suggested by the study of an enhancer in the *Hoxd11* 3′ UTR required for proper activation of *Hoxd11* [42]. This enhancer contains a phylogenetically conserved Smad binding site essential for its activity in transgenic assays [43]. Whether this enhancer plays a more general role in the regulation of the *HoxD* cluster remains to be determined.

Selective Inactivation of Specific Hox Targets Adds Evolutionary Flexibility

Although a large part of *Hox*-dependent morphological variations in the axial skeleton might result from changes in *Hox* gene expression, modulation of specific downstream aspects of Hox activity can also have a relevant impact on this process. An interesting example is the origin of the large rib numbers of Paenungulata (including elephants and manatees), extending further posteriorly than in other mammals to cover most of their presacral skeleton [44]. This feature looks surprisingly similar to the skeletal phenotype of mutant mice totally lacking *Hox10* activity [14]. Since *Hox10* genes play other essential roles in mammalian development (e.g. [45]) it is unlikely that Paenungulata just happened to lose these genes. However, the genome of animals belonging to this clade contain an SNP in an enhancer mediating *Hox10* activity during rib development [13] that precludes binding of Hox10 proteins [46], thus simulating a functional *Hox10* inactivation restricted to the developing axial skeleton. Interestingly, the same polymorphism was found in snakes, where it is also very likely to interfere with *Hox10* rib-repressing activity. In particular, expression of the snake *Hoxa10* gene, which blocks rib formation when tested in mouse embryos [46], extends well into rib-forming somites of the snake embryo without apparent negative effects on rib development [8]. This example illustrates well how regulation of *Hox* gene activity by selective interference with specific downstream targets generates substantial evolutionary flexibility, as it can affect a subset of the protein's functions while keeping other relevant activities in the same domain or even allowing the acquisition of additional functions in that area.

The Chromatin Structure of *Hox* Clusters Impacts Limb Morphogenesis . . .

Hox genes also play essential roles in the morphogenesis and evolution of vertebrate paired appendages. Their expression in the tetrapod limb bud occurs in two sequential phases, the first associated with proximal limb segments (**stylopod** and **zeugopod**) and the second with the distal domain, the **autopod** [47]. Recent data indicate that the regulation of these two phases of *Hox* gene expression is closely connected to the 3D chromatin topology covering the *Hox* clusters [33,34,37]. Interaction analyses performed on the *HoxA* and *HoxD* clusters, the major *Hox* players in limb development, revealed that during the first expression phase *Hox* genes in the 3′ TAD (up to *Hox11*) are under the control of regulatory regions in this TAD [33,48]. This regulation is maintained in the proximal limb domain at later developmental stages. However, in the developing autopod the *Hox* regulatory landscape switches drastically. In this region *Hox* genes in the 5′ TAD (those of the *Hox9* to *Hox13* groups), change their functional interactions to become regulated by enhancers in this topology domain [33] (Figure 2). These latter interactions are required for the autopod-type *Hox* gene expression, including the

'reverse collinearity' [49] (i.e., the strength and extension of *Hox* gene expression decreases in a 5′-to-3′ direction) and, importantly, the inactivation of *Hoxa11* in the autopod [50], which, as discussed below, is directly linked to *Hox13* gene activity. This regulatory switch creates a '*Hox*-free' area between the proximal and distal limb domains thought to be required for wrist/ankle joint development [33,47].

Although the mechanisms involved in this regulatory switch remain incompletely understood, it is clear that *Hox13* genes are a key component of the process. Consistent with this, *Hox13* mutants are unable to elicit the switch and as a consequence characteristic features of the proximal limb bud extend into the prospective autopod region, hindering proper morphogenesis in this area [51,52]. *Hox13* genes play several roles in this process. Besides disconnecting regulatory activities that involve the 3′ TAD [51], Hox13 proteins also interact with specific enhancers in the 5′ TAD to promote the late phase of *Hox* gene expression in the limbs [51,52]. Finally, Hox13 proteins control *Hoxa11* expression as well, by activating an enhancer that promotes the transcription of an antisense *Hoxa11* transcript (*Hoxa11as*) that silences in-*cis* *Hoxa11* gene expression [50].

. . . and the Evolution of Paired Appendages

Progress in understanding the control of Hox activity during limb development also provided new insights into how the tetrapod limb might have evolved from paired fins of fishes. While the early stages of fin and limb development are similar, they clearly differ during the formation of their distal domains. The distal-most limb domain, the autopod, is dominated by mesenchymal tissue that eventually provides the substrate for digit formation, whereas the distal fin domain comprises an epithelial structure, the fin fold, that eventually holds the fin radials. The origins of the differences between distal fin and limb development remain not totally understood, but recent data suggest that they might involve the acquisition by **amniotes** of novel regulatory regions involved in the second phase of *Hox* gene expression in the limbs. 3D structure and interaction analyses indicate that *Hox* clusters of **teleosts** and amniotes largely share both their TAD structure and the preferred contacts between *Hox13* genes and genomic regions in the 5′ TAD [37,53]. Despite this, functional analyses revealed different regulatory potentials for the 5′ TAD sequences of teleosts and mammals. In particular, when pufferfish BAC clones containing the *HoxAa*, *HoxAb*, or *HoxDa* cluster together with the adjacent 5′ region were introduced into mice, they activated fish *Hox* gene expression in proximal limb domains, failing to extend into the autopod [37]. Consistent with this, the zebrafish 5′ TADs lack several key regulatory elements required for autopod-type *Hox* gene expression, including regions controlling *Hox13* activity [54,55] and the Hox13-responsive enhancer promoting *Hoxa11as* transcript expression [50]. Typical features of fin *Hox* gene expression, like overlapping *Hoxa11* and *Hoxa13* expression domains [56], non-detectable *Hoxa11as* transcript [50], and expression of 5′ *Hox* genes with no sign of reverse collinearity [57], are consistent with the lack of those enhancer elements.

Interestingly, at least some of the 5′ regulatory elements absent from teleosts can activate reporter expression in the zebrafish fin bud with appropriate spatial distribution [50,54], suggesting that teleost paired appendages contain the molecular machinery required to control those enhancers. This observation, together with the similar chromosomal architecture and interaction profiles of fish and amniote *Hox* clusters, indicate that the teleost fin could easily acquire an autopod-type regulatory landscape on incorporation of the relevant enhancers into their 5′ TADs. This scenario has been suggested to have contributed to the fin-to-limb evolutionary transition [37]. Interestingly, the gar *HoxA* region seems to have a regulatory structure somewhere between teleosts and amniotes, as it contains at least one of the

enhancers absent from zebrafish that is able to reproduce autopodal *Hoxa13* gene expression when tested in mouse embryos [54].

A Central Role for *Hox13* Genes in Distal Development of Paired Appendages

A variety of functional experiments suggest that the specific expression features of *Hox13* genes, including their expression levels, are relevant for the differential developmental characteristics of the distal limb and fin. Gradual reduction of the *Hox13* dosage in the mouse correlated with the progressive acquisition of several fin-like features, including the distal extension of *Hoxa11* expression that invades the *Hox13* domain, digit shortening, and the absence of the small skeletal elements characteristic of the zeugopod/autopod joint [58,59]. Conversely, increasing *Hoxa13* levels in zebrafish fins expanded the mesodermal core at the expense of the ectodermal fold and promoted the expression of some typical distal limb bud markers [55].

Hox13 genes are also required for distal fin development. Cell-tracing studies indicate that distal radials derive from *Hox13*-positive mesenchyme entering the fin fold and genetic experiments revealed that those structures fail to form in the absence of *Hox13* activity [60]. These observations reopen the old discussion about the homology between digits and distal radials. Such homology could actually help to explain the larger number of radials versus digits on the basis of quantitative and qualitative aspects of *Hox* gene expression. The characteristic *Hoxa11* and *Hox13* expression overlap in fin buds [56] could be part of the mechanism, as experimental *Hoxa11* activation in the *Hox13* expression domain resulted in polydactyl limbs [50]. In addition, reduced *Hox13* activity in a *Gli3*-null context (thus with reduced hedgehog activity) leads to a significant increase in digit number [59]. It was suggested that under these conditions *Hox13* activity would determine the wavelength of the Turing-type mechanism controlling digit number [59,61], with this number increasing with the reduction of *Hox13* activity. It is thus possible that a Turing system acting in the relatively low *Hox13* context of the fin could contribute to the large radial fin numbers, although the precise components of this system might not be the same as in mammals.

Can Snakes Blame *Hox* Genes for Having Lost Their Limbs?

In addition to the fin-to-limb evolutionary transition, *Hox* genes have been suggested to play a role in the loss of paired appendages by snakes [62], although the precise mechanisms or even whether this is indeed true awaits direct experimental proof. It has been reported that *Tbx5*, a key regulator of forelimb bud induction [63], is activated in the lateral mesoderm by *Hox4* and *Hox5* genes [64], suggesting a possible mechanism by which changes in *Hox* gene expression might have contributed to the loss of forelimbs characteristic of snakes. However, regardless of whether these *Hox* genes are essential for forelimb induction [11,12], expression studies showed that members of those *Hox* groups as well as *Tbx5* itself are expressed in the lateral mesoderm of snake embryos [8,29], indicating that the lack of forelimbs in snakes is most likely to require alternative explanations.

Still, modification of *Hox* gene activity might account for the inability of the hindlimb buds of ancient snakes to develop into full-grown limbs. It has long been shown that expression of *Shh*, a key player in limb bud growth and patterning [65], is defective in the snake limb buds [62]. *Shh* limb expression relies on a distal enhancer that is functionally compromised in snakes [66,67]. Interestingly, the modifications found in the python *Shh* enhancer render it unable to respond to Hox proteins [67]. Considering that *Hox* gene activity is required to initiate and/or maintain *Shh* expression in the mouse limb buds [68], it is likely that the lack of response of the *Shh* enhancer

to Hox proteins has played a fundamental role in the developmental arrest of the snake hindlimb bud.

Concluding Remarks and Future Perspectives

Over the past years a considerable amount of work has changed the place held by *Hox* genes in the overall hierarchy of the patterning cascade regulating the vertebrate body structure and their variations among taxa. The resulting new model not only describes how patterning information can traverse the various hierarchical levels controlling axial patterning and the production of functional body structures, but also sheds light on some unexplained aspects of *Hox* mutant phenotypes. So far it is unclear how *Oct4* and *Gdf11* control *Hox* genes (see Outstanding Questions). The effect of these two factors on *Hox* gene expression suggests a mechanism relying on the modulation of regulatory activities organized in TADs, akin to the model recently described in the limb buds. However, direct experimental evaluation is needed to elucidate whether this is indeed the case or whether these factors operate according to entirely different principles. It might also be interesting to determine whether Gdf11/Gdf8 signaling participates in *Hox* gene regulation in the limb buds, as both *Gdf11* and *Gdf8* are expressed in these appendages [18,69] and their simultaneous inactivation led to strong limb malformations [21]. Another interesting question is the extent to which other aspects of *Hox* regulation identified in the limb buds, like those involving *Hox13* genes, also operate in the main body axis. The subsequent findings will show the extent to which basic mechanisms of *Hox* gene regulation are conserved throughout developmental territories. Finally, it will be important to understand how the mechanisms of *Hox* gene regulation coordinate with other relevant features associated with *Hox* gene activation, like the progressive opening of the *Hox* clusters [70], as well as their relationship with other factors known to regulate patterning processes in *Hox*-expressing embryonic areas, like the main body axis or paired appendages.

Outstanding Questions

How do *Oct4* and Gdf11 signaling control *Hox* gene expression?

Does *Hox* gene regulation in the main body axis also involve dynamic regulatory processes associated with the TAD structure of the *Hox* clusters?

Does *Hox* gene regulation follow the same basic principles in the different embryonic areas or does it obey region-specific rules? If those principles are to some extent conserved, how extensive is this conservation?

What are the mechanistic links between *Hox* regulatory processes and the progressive opening of the *Hox* cluster chromatin?

It is known that growth and patterning processes along the main body axis and in the paired appendages are also under the control of a variety of factors other than *Hox* genes, including many signaling pathways. What is the functional relationship between these activities and *Hox* gene regulation?

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