



Published in final edited form as:

Dev Dyn. 2014 January ; 243(1): 182–191. doi:10.1002/dvdy.24019.

“Self-Regulation,” A New Facet of *Hox* Genes’ Function

Rushikesh Sheth^{1,2,*}, Maria Félix Bastida^{1,3}, Marie Kmita^{2,4,*}, and Marian Ros^{1,3,*}

¹Instituto de Biomedicina y Biotecnología de Cantabria,. CSIC-SODERCAN-Universidad de Cantabria, Santander, Spain

²Institut de Recherches Cliniques de Montréal, Montréal, QC, Canada

³Department de Anatomía y Bécología Celular, Universidad de Cantabria, Santander, Spain

⁴Department De Médecine, Université de Montréal, Montréal, QC, Canada

Abstract

Background—Precise temporal and spatial expression of the clustered *Hox* genes is essential for patterning the developing embryo. Temporal activation of *Hox* genes was shown to be cluster-autonomous. However, gene clustering appears dispensable for spatial colinear expression. Generally, a set of *Hox* genes expressed in a group of cells instructs these cells about their fate such that the differential expression of *Hox* genes results in morphological diversity. The spatial colinearity is considered to rely both on local and long-range *cis* regulation.

Results—Here, we report on the global deregulation of *HoxA* and *HoxD* expression patterns upon inactivation of a subset of HOXA and HOXD proteins.

Conclusions—Our data suggest the existence of a “self-regulation” mechanism, a process by which HOX proteins establish and/or maintain the spatial domains of the *Hox* gene family and we propose that the functionally dominant HOX proteins could contribute to generating the spatial parameters of *Hox* expression in a given tissue, i.e., HOX controlling the establishment of the ultimate HOX code.

Keywords

Hox genes; transcriptional regulation; limb development

INTRODUCTION

During embryogenesis, developmental events are orchestrated by changes in gene expression. Appropriate spatial and temporal gene expressions execute pattern formation, a process in which cells become sequentially specified and differentiate to form a morphological structure. A gene or subset of genes’ transcriptional outcome, in a cell or group of cells, is regulated at multiple levels, including interactions between promoter, enhancer, transcription factors, epigenetic modifiers and noncoding RNAs. How

*Correspondence to: Rushikesh Sheth, Institut de Recherches Cliniques de Montréal, Montréal, QC, H2W 1R7, Canada. rushikesh.sheth@ircm.qc.ca; Marie Kmita, Institut de Recherches Cliniques de Montréal, Montréal, QC, H2W 1R7, Canada Marie.Kmita@ircm.qc.ca; Marian Ros, Instituto de Biomedicina y Biotecnología de Cantabria. CSIC-SODERCAN-Universidad de Cantabria, 39011 Santander, Spain Rosm@unican.es.

transcriptional networks are regulated, resulting in complex patterns of gene expression, remain an open question in biology.

The discovery of the *Hox* cluster in *Drosophila* revealed an intriguing correspondence between the physical order of the genes within the *Hox* cluster and their expression domain along the embryonic anterior to posterior (A–P) axis, a phenomenon known as the spatial colinearity (Lewis, 1978). Subsequent studies of orthologous *Hox* complexes uncovered the conservation of the spatial colinearity in vertebrates and in addition showed that *Hox* genes are sequentially activated in time from the 3' to the 5' end of the cluster (referred to as temporal colinearity) (Gaunt et al., 1986, 1989; Dolle et al., 1989; Duboule and Dolle, 1989; Graham et al., 1989; Izpisua-Belmonte et al., 1991). Spatial and temporal control of *Hox* gene expression is essential for patterning the vertebrate body plan; yet, the mechanisms underlying spatial and temporal “colinearity” remain largely elusive. After initial activation, spatial expression domains of each *Hox* gene are progressively refined and further maintained during embryonic development through mechanisms that most likely vary between tissues. The resulting spatial expression domains or the differential qualitative and quantitative combination of different *Hox* genes products along the axes (referred to as the “HOX Code”) is essential for patterning the developing embryo (Kessel and Gruss, 1991). Therefore, elucidating the mechanisms by which *Hox* spatial domains are established and maintained is crucial for our understanding of embryonic development and disease.

Genetic analyses in mice suggest that *cis*-regulatory elements could be implicated in the sequential activation of *Hox* genes from 3' to 5' (Kmita et al., 2002; Spitz et al., 2003; Deschamps, 2007). Sequential posttranslational modifications of histones, i.e., from transcriptionally silent-specific to active-specific forms, also correlate with sequential gene activation (Soshnikova and Duboule, 2009). Furthermore, it was proposed that changes in higher order chromatin organization, e.g., chromatin de-condensation (Noordermeer and Duboule, 2013) and looping out of chromosome territories, contribute to the sequential activation of *Hox* genes (Chambeyron and Bickmore, 2004; Chambeyron et al., 2005; Morey et al., 2007). Although it remains unclear whether these latter changes are a cause or a consequence of the sequential activation of *Hox* genes. While sequential activation determines the spatial coordinates for *Hox* gene expression in some animal phyla, clustering per se appears dispensable for spatial colinearity (Deschamps, 2007; Duboule, 2007). It has thus been proposed that spatial colinearity is an ancestral property whereas clustering and temporal colinearity were imposed during evolution (Duboule, 2007).

During limb development, genes from *HoxA* and *HoxD* clusters are activated in a sequential manner following their order within the cluster, leading to expression domains that are colinear both in space and time (Kmita and Duboule, 2003; Zakany and Duboule, 2007; Montavon and Duboule, 2013). Expression of *HoxD* genes occurs in two independent phases (referred to as phase one and phase two) that rely on distinct *cis*-regulatory elements located on both sides of the cluster (Nelson et al., 1996; Tarchini and Duboule, 2006; Montavon and Duboule, 2013). The 3' regulatory region (early limb control region; ELCR) controls the sequential timing of gene activation (phase one expression) (Zakany et al., 2004). Concomitantly, a 5' regulatory region (POST) exerts a repressive effect to spatially restrict 5'*Hoxd* expression to the posterior mesenchyme of early limb buds (Tarchini and

Duboule, 2006). Subsequently, these early expression domains evolve to later be fixed as the presumptive zeugopod domain (Tarchini and Duboule, 2006). The phase two expressions of *HoxD* genes occurs exclusively in the presumptive digit-forming region and is mechanistically unlinked to the first expression phase (Spitz et al., 2003; Tarchini and Duboule, 2006; Gonzalez et al., 2007; Montavon et al., 2011). Distinct remote regulatory elements, located in the gene desert on the 5' side of the cluster, drive the phase two transcription (Montavon et al., 2011; Tschopp and Duboule, 2011). This occurs in a reverse colinear manner as the most 5' transcription unit, i.e., *Hoxd13*, is expressed strongly in the entire presumptive digit territory while *Hoxd12* to *Hoxd9* are transcribed with progressively lower efficiency and are excluded from the digit-1 region (Kmita et al., 2002; Montavon et al., 2008). In both phases, the position of transcription unit relative to the regulatory regions determines the spatial and temporal parameters of *HoxD* expression. Changes in the genomic position of a transcription unit relative to the 3' regulatory region (e.g., by targeted deletion in the cluster) alter its time of activation, while spatial expression is affected depending on its relative position to the 5' regulatory regions. It is believed that similar mechanisms apply for the *HoxA* cluster.

In this study, through the analysis of different *HoxA* and *HoxD* mutants, we provide evidence that HOX proteins themselves are important to set up the spatial parameters of *HoxA* and *HoxD* expression in distal limb buds. As previously reported, we observed that deletion of 5'*Hoxd* genes affects only *HoxD* expression. In contrast, loss of HOXA13 protein has a more general impact upon the expression of both *HoxA* and *HoxD* genes. Furthermore, we show that presence of the HOX paralogous group 13 proteins is a prerequisite for the separation of “zeugopod” and “autopod” expression domains of *HoxA* and *HoxD* genes.

RESULTS

Expression of the Remaining *Hox* Genes in Limb Buds of *HoxD^{del(11-13)/del(11-13)}* Embryos

The *HoxD^{del(11-13)}* allele used in this study carries the deletion in *cis* of the *Hoxd13* and *Hoxd12* loci, as well as the insertion of a *lacZ* reporter transgene within the first exon of the *Hoxd11* gene (i.e., combined loss of function of *Hoxd13*, *12*, and *11*) (Zakany and Duboule, 1996). This deletion modifies the position of the remaining transcription units relative to the 5' enhancers, such that *Hoxd11-lacZ* is relocated at the former position of *Hoxd13*. Based on our current understanding of *HoxD* genes regulation, deletion of the 5'*Hoxd* genes should not alter the time of activation of the remaining *Hoxd* genes, as their position relative to the 3' regulatory region (ELCR) is unchanged (Zakany et al., 2004; Tarchini and Duboule, 2006). In contrast, this deletion brings the remaining *HoxD* genes closer to the 5' regulatory landscape, which is expected to posteriorly restrict their phase one expression in the early limb bud and subsequently modify their expression in the presumptive zeugopod domain (e.g., *Hoxd10* should be expressed like *Hoxd12*). In addition, it has been shown that 5' deletion within the *HoxD* cluster affects the phase two expression of only the first transcription unit nearest to the deletion site (Kmita et al., 2002), i.e., *Hoxd11-LacZ* should be expressed like *Hoxd13* without affecting the remaining *Hoxd* genes.

Therefore, we first checked the effect of the deletion on *Hoxd4*, *Hoxd9* and *Hoxd10* expression at embryonic day (E) 11.5, when the presumptive zeugopod domain (phase one) and the presumptive autopod domain (phase two) can be simultaneously observed. At E11.5, *Hoxd4* was expressed in a small central domain in the wild-type limb bud (Fig. 1A), whereas its domain slightly extended into the posterior limb mesenchyme in *HoxD^{del(11-13)}* homozygote embryos (yellow arrow in Fig. 1B). At this stage, while the presumptive zeugopod domain of *Hoxd9* shows an anterior bias in wild-type limb buds (Fig. 1C), a posterior bias is observed in *HoxD^{del(11-13)}* homozygote limb buds (yellow arrow in Fig. 1D). No difference is observed for *Hoxd10* expression in the presumptive zeugopod, which is already posteriorly biased in wild-type limb buds (Fig. 1E,F). The pattern of *Hoxd9* and *Hoxd10* observed in mutant limb buds at E11.5 is maintained at E12.5 (Fig. 1J,L). Phase two expression of *Hoxd9* and *Hoxd10* in the presumptive digit-forming region is mostly unaffected by the deletion both at E11.5 and E12.5 (Fig. 1C–F,I–L). Nonetheless, there is an unexpected ectopic anterior expansion of *Hoxd10* expression into the presumptive digit-1 territory (red arrow in Fig. 1L), even though *Hoxd10* is not adjacent to the deletion breakpoint. No alteration of *HoxA* genes expression has been reported in the absence of *HoxD* genes. Accordingly, no modification of *Hoxa13* expression is observed in *HoxD^{del(11-13)}* homozygous limb buds (Sheth et al., 2007). Similarly, *Hoxa11* expression is unchanged in *HoxD^{del(11-13)}* homozygous limb buds and remains restricted to the zeugopod both at E11.5 (Fig. 1G,H) and E12.5 (Fig. 1M,N).

Loss of HOXA13 Affects *HoxA* and *HoxD* Expression in the Presumptive Zeugopod Domain

A few examples of cross-regulatory interactions among *Hox* genes have been reported but this is not considered as a general mechanism of *Hox* gene regulation (Arcioni et al., 1992; Popperl et al., 1995; Studer et al., 1998; Manzanares et al., 2001). It has been suggested that HOXA13 may participate in transcriptional repression of *Hoxa11*, and shown that *Hoxa11* expression marginally extends into the presumptive digit-1 region of *Hoxa13* mutant limb buds (Yokouchi et al., 1995; Post and Innis, 1999). Surprisingly, in *Hoxa13* mutant limb buds, in which the Homeobox containing region of *Hoxa13* is disrupted (Fromental-Ramain et al., 1996), we found that *Hoxd4* expression is upregulated and distally expanded in the anterior and posterior mesenchyme (Fig. 2A,B, red arrow in Fig. 2B). In addition, *Hoxd9* (Fig. 2D,E and 2M,N), *Hoxd10* (Fig. 2G,H and 2P,Q), and *Hoxd11* (not shown) transcripts are found in the presumptive digit-1 region (yellow arrow in Fig. 2N and 2Q), from which they are excluded in wild-type limb buds (Fig. 2M,P). Similar ectopic expression of these *Hoxd* genes is observed in *Hoxa13^{-/-};HoxD^{del(11-13)}/+* limb buds (Fig. 2C,F,I,O,R and not shown). The ectopic expression of *Hoxd4*, *Hoxd9*, *Hoxd10*, and *Hoxd11* in mutant limb buds is accompanied by an expansion of *Hoxa11* expression in that region (Fig. 2J–L and 2S–U, red arrow in 2K and 2T).

The presumptive zeugopod domain of *HoxD* genes at E11.5 and E12.5 has been suggested to be the continuation of phase one regulation (Tarchini and Duboule, 2006). In this view, the ectopic expressions described above, which look like an extension of the presumptive zeugopod domain, could reflect a modification of the phase one expression in early buds resulting from *Hoxa13* inactivation. However, *Hoxa13* expression starts at E10.5 in a small

distal-posterior domain, mostly nonoverlapping with the phase one expression. Moreover, *Hoxd10*, *Hoxd11*, and *Hoxd13* expression does not show any alteration of phase one expression in early limb buds of *Hoxa13*^{-/-} embryos (Fig. 3). Therefore, the effect of *Hoxa13* loss of function on *HoxD* expression is most likely unrelated to the phase one regulation in early limb bud but rather reflects the role of HOXA13 protein in preventing the phase one expression in the presumptive autopod (mesopod and acropod) domain. Accordingly, while the phase one and phase two expression domains of *Hoxd9*, *Hoxd10*, and *Hoxd11* are normally separated by a stripe of cells devoid of either transcripts, this gap is reduced in *Hoxa13*^{-/-} and *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/+} limb buds (Fig. 2D–I, 2M–R and not shown). This reduction in the gap between phase one and phase two expression domains may be secondary to growth defect in the distal region. However, the broadening of *Hoxa11* expression pattern in *Hoxa13*^{-/-} and *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/+} limb buds (Fig. 2S–U) suggest a deregulation of *HoxD* expression in the autopod territory rather than reduced growth in the mesopod region. This is further supported by the virtually normal expression of *Hoxa13-exon1* in mutant limb buds (see below). Altogether, these results suggest a mechanism whereby HOXA13 protein suppresses the expression of *Hoxa11* and phase one regulation of *HoxD* genes in the presumptive mesopod-acropod territory.

Paralogous HOX proteins are characterized by some redundancy in their function (Wellik and Capecchi, 2003). In addition to *Hoxa13*, *Hoxd13* is the only *HoxD* gene expressed in the presumptive digit-1 region. Surprisingly, in *Hoxa13*^{-/-} limb buds, *Hoxd13* expression is excluded from digit-1 region (white arrow in Fig. 2X, compared with 2V,W) and its down-regulation correlates with the up-regulation of the other *HoxD* genes and *Hoxa11* (compare Fig. 2N,Q,T with 2X). This raises the possibility that the absence of digit-1 in the *Hoxa13* mutant may actually be due to the loss of HOX paralogous group 13 function in this region. In addition, the down-regulation of *Hoxd13* in the presumptive digit-1 raises the question of whether *Hoxd13* together with *Hoxa13* share a similar function in controlling the expression of *Hoxa11* and the other *HoxD* genes.

Cells Destined to Form Autopod are Present in the Absence of 5' Hox Genes

To further assess the functional relevance of 5'HOX products in regulating expression of *HoxA* and *HoxD* genes, we decided to analyze the double *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/del(11-13)} mutant. These mutant embryos show complete digit agenesis similar to the *Hoxa13*^{-/-}; *Hoxd13*^{-/-} mutant (Fromental-Ramain et al., 1996; Sheth et al., 2012), with no reported defects in the zeugopod and stylopod segments (Zakany et al., 1997). It is frequently assumed that digit loss upon 5'*Hox* inactivation could be due to the lack of autopod progenitors. Therefore we first tested whether *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/del(11-13)} limb bud cells are properly specified along the proximal–distal (P–D) axis. A proximal signal (presumably retinoic acid) induces *Meis1* expression (Cooper et al., 2011; Rosello-Diez et al., 2011), while a distal signal, fibroblast growth factors from the apical ectodermal ridge, regulates expression of the retinoic acid-degrading enzyme *Cyp26b1* in the distal mesenchyme (Probst et al., 2011). We thus used *Meis1* and *Cyp26b1* as markers of proximal and distal cell identities, respectively. In both wild-type and mutant early buds, *Meis1* expression gets restricted to the proximal region by E11.5 (Fig. 4A–D), confirming normal specification of the proximal cells. Similarly, *Cyp26b1* is expressed with

a wild-type pattern in mutant buds (Fig. 4E–H), indicating the normal specification of the distal cells.

Hoxa13 is so far the best available marker for specified autopod cells (Tabin and Wolpert, 2007). Its expression starts in the posterior-distal mesenchyme of wild-type forelimb buds around E10.5 and progressively covers the entire digital plate as it forms. The homeobox region of *Hoxa13* is disrupted in the *Hoxa13*^{-/-} mutant but *Hoxa13-exon1* remains transcribed (Fromental-Ramain et al., 1996). Therefore, we used *Hoxa13-exon1* specific riboprobe to check for the presence of autopod progenitors. In the wild-type limb bud, *Hoxa13-exon1* expression is observed in the presumptive autopod and recapitulates the reported *Hoxa13* expression pattern. No obvious changes in *Hoxa13-exon1* expression is observed in mutant limb buds (Fig. 4I–L), thus indicating that cells destined to form the autopod are present in these mutants. Consequently, *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/Del(11-13)} limb buds provide an appropriate context to study the HOX-dependent regulation of *Hox* genes in developing limbs.

Function of HOX Paralogous Group 13 is Required for Proper Separation of the Zeugopod and Autopod Expression Domains of *HoxA* and *HoxD* Genes

A striking spatial redistribution of *HoxA* and *HoxD* transcripts is observed in *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/Del(11-13)} limb buds suggesting a synergistic effect of the combined mutations (Fig. 5). Notably, *Hoxd4* is ectopically expressed in the distal mesenchyme (Fig. 5B) and the separation between the phase one and two expression of *Hoxd9* and *Hoxd10* is completely lost (Fig. 5D,F compared with 5C,E). In addition, *Hoxa11* expression, which marks the boundary between the prospective zeugopod and autopod in wild-type limbs, expands distally to cover the entire presumptive autopod region of the mutant limb bud (Fig. 5H compared with 5G). Altogether, these modifications indicate that the presence of 5'HOX products is required first to suppress *Hoxa11* and the phase one expression of *HoxD* genes in the presumptive autopod domain and second for the proper separation of the presumptive zeugopod and autopod expression domains.

The most 5'*Hox* genes are functionally dominant when co-expressed with other *Hox* genes, a phenomenon described as posterior prevalence (Duboule and Morata, 1994). Therefore, to test whether the observed effects are primarily due to loss of HOX paralogous group 13 proteins, we analyzed mutant limb buds in which HOXA13 and HOXD13 are inactivated. This also permits the evaluation of the possible contribution of HOXD11 and HOXD12. Our results show that *Hoxd4* and *Hoxa11* expression is extended distally and covers the entire distal limb bud in *Hoxa13*^{-/-}; *Hoxd13*^{-/-} mutants (Fig. 5I,J), recapitulating the situation in *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/del(11-13)} buds (Fig. 5B,H). This confirms that the modified *HoxD* and *Hoxa11* expression in *Hoxa13*^{-/-}; *HoxD*^{del(11-13)/del(11-13)} buds is actually due to the lack of HOXA13 and HOXD13 proteins. These results, for the first time, show that the function of HOX paralogous group 13 is required for the correct spatial expression of the genes from both the *HoxA* and *HoxD* clusters.

DISCUSSION

During limb development, proximal cells, marked by *Meis1* expression, are fated to form the stylopod (arm), while more distal mesenchyme eventually gives rise to two distinct anatomical segments: the zeugopod (forearm) and the autopod that includes the mesopod (wrist) and the acropod (digits). The distal mesenchyme is molecularly subdivided by the expression of *HoxA* and *HoxD* genes, with *Hoxa11* expressed in the presumptive zeugopod region while *Hoxa13* is expressed in the presumptive mesopod and acropod region. The biphasic regulation of *HoxD* genes triggers expression in the presumptive zeugopod region (phase one) and acropod region (phase two). A fundamental question is how this molecular subdivision, which is specific to tetrapod limbs, is generated and maintained.

Alterations in *HoxA* and *HoxD* expression have been observed in limb buds of different mutants. Curiously, most of these alterations are along the A–P axis, while alterations along the P–D axis have been reported only for *HoxD* genes upon *cis*-deletions (Kmita et al., 2002; Tarchini and Duboule, 2006). The changes in *HoxA* and *HoxD* expression reported in this work is, to our knowledge, the first evidence for the most 5' HOX products controlling the expression of both *HoxA* and *HoxD* genes along the P–D axis.

Function of HOX Paralogous Group 13 Proteins is Required for the Segregation of Hox Domains Along the P–D Axis

Our results reveal that the deletion of the three most 5'*Hoxd* genes, in *cis*, besides affecting the expression of the *HoxD* gene adjacent to the deletion breakpoint (Zakany and Duboule, 1996), also has some previously unappreciated effects. In the *HoxD^{del(11–13)}* allele, *Hoxd4* and *Hoxd9* are separated from the deletion breakpoint by four and two transcription units, respectively, and their expression is thus expected to remain as in wild-type buds. Yet, we found that their expression in the presumptive zeugopod domain is posteriorly biased in the mutant context. This may be the consequence of their modified position with respect to the 5' repressive region (POST), which in turn would suggest that POST exert its effect over longer distance than previously reported (Tarchini and Duboule, 2006). However, such change in expression is not observed for *Hoxd10* even though it is located closer to the deletion breakpoint than *Hoxd9* and *Hoxd4*. Of interest, a recent report shows that deletion of the 5' regulatory landscape only affects the phase two expression of 5'*Hoxd* genes (*Hoxd10–12*), while the presumptive zeugopod domains remain unaffected (Montavon et al., 2011). Together these results suggest that the establishment of presumptive zeugopod expression domains may be differently regulated for *Hoxd1–9* than for *Hoxd10–12*. Nonetheless, in this mutant background, the biphasic expression of *HoxD* genes and the segregation of phase one and phase two of *HoxD* are maintained and the expression of *HoxA* genes is unaffected.

In contrast to *HoxD^{del(11–13)/del(11–13)}*, inactivation of *Hoxa13* results in two main changes: (i) *Hoxa11* and *HoxD* genes are ectopically expressed distally, mainly in the presumptive digit-1 territory and (ii) separation between phase one and phase two expression domains is significantly reduced. These results suggest that the mechanism that suppresses phase one regulation of *HoxD* genes and *Hoxa11* expression in the presumptive mesopod and digit-1 region is impaired upon *Hoxa13* inactivation. This indicates that HOXA13 may play a

critical role in segregating *Hox* expression in the presumptive zeugopod and auto-pod domains. Furthermore, our results show that HOXA13 and HOXD13 act in a synergistic manner as when both proteins are inactivated, *Hoxa11* and *Hoxd4* transcription occurs in the entire distal limb bud and segregation of the zeugopod and autopod domains of *HoxD* genes is lost. Together our data provide compelling evidence that HOX paralogous group 13 proteins are crucial for the proper segregation of *Hox* domains along the P–D axis.

Even though HOX13 proteins act in a synergistic manner (Fromental-Ramain et al., 1996), our results indicate a prominent role for HOXA13. Most of the global change in regulation is observed only when *Hoxa13* is inactivated while the effect of the 5'*HoxD* deletion is only detectable for the remaining *HoxD* genes. The predominant effect of *Hoxa13* could be due to its expression pattern, which covers the entire autopod and mesopod domain more rapidly than *Hoxd13*, rather than differences in HOXA13 and HOXD13 functional properties per se.

Possible Mechanisms by Which HOX Proteins Contribute to Cluster-wide Regulation

The absence of HOX paralogous group 13 proteins results in ectopic expression of *Hoxa11* and *Hoxd4* distally and loss of segregation of the zeugopod and autopod expression domains of *HoxD* genes. As far as *HoxD* genes are concerned, several possibilities could account for their deregulation: (i) the phase one regulation is ectopically active in the presumptive autopod cells; (ii) phase two regulation loses its restriction to the most 5' genes, thereby triggering ectopic expression of more 3' genes in the presumptive autopod; and (iii) a combination of the two latter scenarios. Based on the progressive distalization of *Hoxd4* expression in the mutant contexts, we favor the ectopic activity of phase one in autopod cells as being responsible for the *HoxD* deregulation in absence of HOX paralogous group 13 proteins. However, a detailed study of chromatin organization of the *HoxD* locus in the mutant contexts is needed to unambiguously resolve which phase of regulation is affected. Nonetheless, our results reveal that the function of HOX paralogous group 13 proteins, in particular HOXA13, is crucial to establish the “dichotomy” of *HoxD* expression (zeugopod and autopod domains), thus generating an intermediate *HoxD*-less domain considered as the presumptive wrist domain (Woltering and Duboule, 2010)

A very recent study, identified a regulatory domain located 3' to the *HoxD* cluster (T-DOM) controlling the phase one expression of *HoxD* genes in the early limb bud and presumptive zeugopod (Andrey et al., 2013), while the second phase of expression is controlled by the regulatory domain located 5' to the *HoxD* cluster (C-DOM) (Montavon et al., 2011; Andrey et al., 2013). The switch from phase one to phase two regulation in distal cells is accompanied by posttranslational histone modifications, switching the chromatin state from active to inactive at the T-DOM and from inactive to active at the C-DOM. Of interest, the silencing of the phase one regulation in distal cells is independent of phase two activation and it was proposed that the gap between *HoxD* zeugopod and autopod expression domains corresponds to cells in which phase one is switch off but phase two is not activated (Andrey et al., 2013). Based on our results revealing the loss of this gap in absence of HOX paralogous group 13 proteins, we propose that these proteins, may act as a switch to turn-off the phase one regulation. The evidence that HOX proteins can interact with histone/ chromatin modifying complexes (Shen et al., 2001; Lu et al., 2003; Luke et al., 2006), raises

the possibility that HOX paralogous group 13 proteins may influence the chromatin state at the *HoxD* cluster and/or its regulatory landscapes. Alternatively, HOX paralogous group 13 proteins could induce minor changes in the chromatin conformation at the *HoxD* locus disrupting its interaction with phase one regulatory sequences while favoring the interaction underlying phase two regulation in the presumptive autopod domain.

Random Incident or a Coordinate Strategy?

Over the years, *Hox* genes have been studied either as individual genes or as a cluster. Only in few cases, such as during hindbrain patterning, “local” auto- or cross-regulatory interactions among *Hox* genes have been reported (Arcioni et al., 1992; Popperl et al., 1995; Studer et al., 1998; Manzanares et al., 2001). Here, we show that the HOX proteins themselves are likely involved in generating “global” spatial parameters of *Hox* expression in developing limbs. We propose that the establishment of *Hox* expression patterns involves a “self-regulatory” mechanism whereby functionally dominant HOX proteins determine the spatial parameters of the other *Hox* genes’ expression in a given tissue. In this view, in animals, in which *Hox* genes are either clustered or non-clustered, the functionally dominant HOX protein ultimately establishes and/or maintains the spatial expression specificities or HOX code, eventually defining cell fate.

Simple Step and Big Leap Forward

The appearance of paired appendages was a major step in tetrapod evolution and land colonization (Coates, 1994; Clack, 2005). Fossil data suggest that limbs evolved from fins, but how this morphological transformation occurred is not yet resolved (Cohn et al., 2002; Schneider and Shubin, 2013). Curiously, *HoxA* and *HoxD* cluster genes are expressed in overlapping domains in fins while the hallmark of tetrapod limbs is the segregation of *Hox* expression domains in the distal limb bud, at least in the species studied so far (Metscher et al., 2005; Woltering and Duboule, 2010). Segregation of *Hox* expression domains has been suggested as an important change in the evolution of well-articulated, functional tetrapod limbs (Metscher et al., 2005; Woltering and Duboule, 2010).

The acquisition of new *cis*-regulatory elements modulating *Hox* expression or increased distal cell proliferation by prolonged AER function have been suggested as possible mechanisms involved in this evolutionary transformation (Sordino et al., 1995; Freitas et al., 2012; Schneider and Shubin, 2013). Recent studies in mice show that *HoxA* and *HoxD* genes are important for limb bud growth in addition to patterning (Kmita et al., 2005; Sheth et al., 2013). HOXD13 overexpression in zebrafish fins results in distal overgrowth, segregation of *Hox* domains and expression of markers specific to autopod (Freitas et al., 2012). Here we show that HOX13 proteins are important for the segregation of the other *Hox* domains along the P–D axis. Upon inactivation of HOX13, the other *HoxA* and *HoxD* genes are expressed in overlapping domains resembling *Hox* expression patterns in fish-fins. Therefore, we speculate that, in the course of evolution, acquisition of new *cis*-regulatory elements and/or modulation of AER signaling contributed to the distal specific expression of *Hoxa13*. In turn, distal expression of *Hoxa13* ensured the expansion of autopod progenitors and generated the conditions required for segregation of the autopod and zeugopod domains of

HoxA and *HoxD* genes, allowing for the development of the wrist and digits and thus giving rise to well-articulated, functional tetrapod limbs.

EXPERIMENTAL PROCEDURES

Mice

The *Hoxa13* mutant allele is generated by insertion of neomycine cassette in *Hoxa13* coding region (Fromental-Ramain et al., 1996). The *HoxD^{del(11-13)}* allele is the deletion of *Hoxd13, Hoxd12* loci plus the insertion of *lacZ* reporter transgene in *Hoxd11* therefore represents *Hoxd11-13* loss of function (Zakany and Duboule, 1996). In *Hoxd13* mutant allele, a *lacZ* reporter transgene is inserted in the first exon of *Hoxd13* (Kmita et al., 2000). The *Hoxa13*, *HoxD^{del(11-13)}* and *Hoxd13* mutant lines were maintained in a mixed background.

Noon of the day the vaginal plug was observed was considered as E0.5. The embryos were obtained by caesarean and genotyping was performed by PCR as described.

In Situ Hybridization

Digoxigenin-labeled antisense riboprobe were prepared, and whole-mount in situ hybridization was performed according to standard procedure (Sheth et al., 2007). The probes used were *Hoxd4*, *Hoxd9*, *Hoxd10*, *Hoxd11*, *Hoxd13*, and *HoxA11* (kindly provided by D. Duboule) and *Hoxa13-exon1* (kindly provided by S. Stadler) and *Meis1* and *Cyp26b1* (kindly provided by M. Torres).

Acknowledgments

Grant sponsor: the Spanish Government; Grant number: BFU2011-24972; Grant sponsor: the Canadian Institutes for Health Research; Grant number: MOP-82880; Grant number: 126110.

We thank Denis Duboule and Pierre Chambon for providing the mutant mice. This work was supported by the Spanish Government to M.R. and by the Canadian Institutes for Health Research as well as the Canada Research Chair program to M.K. R.S was supported by a Formación Profesorado Universitario fellowship from the Spanish Ministry of Science and Innovation and currently supported by the Angelo Pizzagalli postdoctoral fellowship.

References

- Andrey G, Montavon T, Mascrez B, Gonzalez F, Noordermeer D, Leleu M, Trono D, Spitz F, Duboule D. A switch between topological domains underlies HoxD genes collinearity in mouse limbs. *Science*. 2013; 340:1234-1237. [PubMed: 23744951]
- Arcioni L, Simeone A, Guazzi S, Zappavigna V, Boncinelli E, Mavilio F. The upstream region of the human homeobox gene HOXD3 is a target for regulation by retinoic acid and HOX homeoproteins. *EMBO J*. 1992; 11:265-277. [PubMed: 1346761]
- Chambeyron S, Bickmore WA. Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev*. 2004; 18:1119-1130. [PubMed: 15155579]
- Chambeyron S, Da Silva NR, Lawson KA, Bickmore WA. Nuclear reorganisation of the Hoxb complex during mouse embryonic development. *Development*. 2005; 132:2215-2223. [PubMed: 15829525]
- Clack JA. Getting a leg up on land. *Sci Am*. 2005; 293:100-107. [PubMed: 16323697]
- Coates MI. The origin of vertebrate limbs. *Dev Suppl*. 1994:169-180. [PubMed: 7579518]

- Cohn MJ, Lovejoy CO, Wolpert L, Coates MI. Branching, segmentation and the metapterygial axis: pattern versus process in the vertebrate limb. *Bioessays*. 2002; 24:460–465. [PubMed: 12001269]
- Cooper KL, Hu JK, ten Berge D, Fernandez-Teran M, Ros MA, Tabin CJ. Initiation of proximal-distal patterning in the vertebrate limb by signals and growth. *Science*. 2011; 332:1083–1086. [PubMed: 21617075]
- Deschamps J. Ancestral and recently recruited global control of the Hox genes in development. *Curr Opin Genet Dev*. 2007; 17:422–427. [PubMed: 17870464]
- Dolle P, Izpisua-Belmonte JC, Falkenstein H, Renucci A, Duboule D. Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature*. 1989; 342:767–772. [PubMed: 2574828]
- Duboule D. The rise and fall of Hox gene clusters. *Development*. 2007; 134:2549–2560. [PubMed: 17553908]
- Duboule D, Dolle P. The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. *EMBO J*. 1989; 8:1497–1505. [PubMed: 2569969]
- Duboule D, Morata G. Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet*. 1994; 10:358–364. [PubMed: 7985240]
- Freitas R, Gomez-Marin C, Wilson JM, Casares F, Gomez-Skarmeta JL. Hoxd13 contribution to the evolution of vertebrate appendages. *Dev Cell*. 2012; 23:1219–1229. [PubMed: 23237954]
- Fromental-Ramain C, Warot X, Messadecq N, LeMeur M, Dolle P, Chambon P. Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development*. 1996; 122:2997–3011. [PubMed: 8898214]
- Gaunt SJ, Miller JR, Powell DJ, Duboule D. Homeobox gene expression in mouse embryos varies with position by the primitive streak stage. *Nature*. 1986; 324:662–664. [PubMed: 2879244]
- Gaunt SJ, Krumlauf R, Duboule D. Mouse homeo-genes within a subfamily, Hox-1.4, 2.6 and 5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. *Development*. 1989; 107:131–141. [PubMed: 2576400]
- Gonzalez F, Duboule D, Spitz F. Transgenic analysis of Hoxd gene regulation during digit development. *Dev Biol*. 2007; 306:847–859. [PubMed: 17448461]
- Graham A, Papalopulu N, Krumlauf R. The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. *Cell*. 1989; 57:367–378. [PubMed: 2566383]
- Izpisua-Belmonte JC, Tickle C, Dolle P, Wolpert L, Duboule D. Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature*. 1991; 350:585–589. [PubMed: 1673231]
- Kessel M, Gruss P. Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell*. 1991; 67:89–104. [PubMed: 1680565]
- Kmita M, Duboule D. Organizing axes in time and space; 25 years of colinear tinkering. *Science*. 2003; 301:331–333. [PubMed: 12869751]
- Kmita M, Kondo T, Duboule D. Targeted inversion of a polar silencer within the HoxD complex re-allocates domains of enhancer sharing. *Nat Genet*. 2000; 26:451–454. [PubMed: 11101844]
- Kmita M, Fraudeau N, Herault Y, Duboule D. Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. *Nature*. 2002; 420:145–150. [PubMed: 12432383]
- Kmita M, Tarchini B, Zakany J, Logan M, Tabin CJ, Duboule D. Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. *Nature*. 2005; 435:1113–1116. [PubMed: 15973411]
- Lewis EB. A gene complex controlling segmentation in *Drosophila*. *Nature*. 1978; 276:565–570. [PubMed: 103000]
- Lu Y, Goldenberg I, Bei L, Andrejic J, Eklund EA. HoxA10 represses gene transcription in undifferentiated myeloid cells by interaction with histone deacetylase 2. *J Biol Chem*. 2003; 278:47792–47802. [PubMed: 14512427]
- Luke MP, Sui G, Liu H, Shi Y. Yin Yang 1 physically interacts with Hoxa11 and represses Hoxa11-dependent transcription. *J Biol Chem*. 2006; 281:33226–33232. [PubMed: 16963455]
- Manzanares M, Bel-Vialar S, Ariza-McNaughton L, Ferretti E, Marshall H, Maconochie MM, Blasi F, Krumlauf R. Independent regulation of initiation and maintenance phases of Hoxa3 expression in

- the vertebrate hindbrain involve auto- and cross-regulatory mechanisms. *Development*. 2001; 128:3595–3607. [PubMed: 11566863]
- Metscher BD, Takahashi K, Crow K, Amemiya C, Nonaka DF, Wagner GP. 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*. 2005; 7:186–195. [PubMed: 15876191]
- Montavon T, Duboule D. Chromatin organization and global regulation of Hox gene clusters. *Philos Trans R Soc Lond B Biol Sci*. 2013; 368:20120367. [PubMed: 23650639]
- Montavon T, Le Garrec JF, Kerszberg M, Duboule D. Modeling Hox gene regulation in digits: reverse collinearity and the molecular origin of thumbness. *Genes Dev*. 2008; 22:346–359. [PubMed: 18245448]
- Montavon T, Soshnikova N, Mascrez B, Joye E, Thevenet L, Splinter E, de Laat W, Spitz F, Duboule D. A regulatory archipelago controls Hox genes transcription in digits. *Cell*. 2011; 147:1132–1145. [PubMed: 22118467]
- Morey C, Da Silva NR, Perry P, Bickmore WA. Nuclear reorganisation and chromatin decondensation are conserved, but distinct, mechanisms linked to Hox gene activation. *Development*. 2007; 134:909–919. [PubMed: 17251268]
- Nelson CE, Morgan BA, Burke AC, Laufer E, DiMambro E, Murtaugh LC, Gonzales E, Tessarollo L, Parada LF, Tabin C. Analysis of Hox gene expression in the chick limb bud. *Development*. 1996; 122:1449–1466. [PubMed: 8625833]
- Noordermeer D, Duboule D. Chromatin architectures and hox gene collinearity. *Curr Top Dev Biol*. 2013; 104:113–148. [PubMed: 23587240]
- Popperl H, Bienz M, Studer M, Chan SK, Aparicio S, Brenner S, Mann RS, Krumlauf R. Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell*. 1995; 81:1031–1042. [PubMed: 7600572]
- Post LC, Innis JW. Altered Hox expression and increased cell death distinguish Hypodactyly from Hoxa13 null mice. *Int J Dev Biol*. 1999; 43:287–294. [PubMed: 10470645]
- Probst S, Kraemer C, Demougin P, Sheth R, Martin GR, Shiratori H, Hamada H, Iber D, Zeller R, Zuniga A. SHH propagates distal limb bud development by enhancing CYP26B1-mediated retinoic acid clearance via AER-FGF signalling. *Development*. 2011; 138:1913–1923. [PubMed: 21471156]
- Rosello-Diez A, Ros MA, Torres M. Diffusible signals, not autonomous mechanisms, determine the main proximodistal limb subdivision. *Science*. 2011; 332:1086–1088. [PubMed: 21617076]
- Schneider I, Shubin NH. The origin of the tetrapod limb: from expeditions to enhancers. *Trends Genet*. 2013; 29:419–426. [PubMed: 23434323]
- Shen WF, Krishnan K, Lawrence HJ, Largman C. The HOX homeodomain proteins block CBP histone acetyl-transferase activity. *Mol Cell Biol*. 2001; 21:7509–7522. [PubMed: 11585930]
- Sheth R, Bastida MF, Ros M. Hoxd and Gli3 interactions modulate digit number in the amniote limb. *Dev Biol*. 2007; 310:430–441. [PubMed: 17714700]
- Sheth R, Marcon L, Bastida MF, Junco M, Quintana L, Dahn R, Kmita M, Sharpe J, Ros MA. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science*. 2012; 338:1476–1480. [PubMed: 23239739]
- Sheth R, Gregoire D, Dumouchel A, Scotti M, Pham JM, Nemeč S, Bastida MF, Ros MA, Kmita M. Decoupling the function of Hox and Shh in developing limb reveals multiple inputs of Hox genes on limb growth. *Development*. 2013; 140:2130–2138. [PubMed: 23633510]
- Sordino P, van der Hoeven F, Duboule D. Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature*. 1995; 375:678–681. [PubMed: 7791900]
- Soshnikova N, Duboule D. Epigenetic temporal control of mouse Hox genes in vivo. *Science*. 2009; 324:1320–1323. [PubMed: 19498168]
- Spitz F, Gonzalez F, Duboule D. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell*. 2003; 113:405–417. [PubMed: 12732147]
- Studer M, Gavalas A, Marshall H, Ariza-McNaughton L, Rijli FM, Chambon P, Krumlauf R. Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development*. 1998; 125:1025–1036. [PubMed: 9463349]

- Tabin C, Wolpert L. Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* 2007; 21:1433–1442. [PubMed: 17575045]
- Tarchini B, Duboule D. Control of Hoxd genes' collinearity during early limb development. *Dev Cell.* 2006; 10:93–103. [PubMed: 16399081]
- Tschopp P, Duboule D. A regulatory 'landscape effect' over the HoxD cluster. *Dev Biol.* 2011; 351:288–296. [PubMed: 21195707]
- Wellik DM, Capecchi MR. Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. *Science.* 2003; 301:363–367. [PubMed: 12869760]
- Woltering JM, Duboule D. The origin of digits: expression patterns versus regulatory mechanisms. *Dev Cell.* 2010; 18:526–532. [PubMed: 20412768]
- Yokouchi Y, Nakazato S, Yamamoto M, Goto Y, Kameda T, Iba H, Kuroiwa A. Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes cell adhesiveness in chick limb buds. *Genes Dev.* 1995; 9:2509–2522. [PubMed: 7590231]
- Zakany J, Duboule D. Synpolydactyly in mice with a targeted deficiency in the HoxD complex. *Nature.* 1996; 384:69–71. [PubMed: 8900279]
- Zakany J, Duboule D. The role of Hox genes during vertebrate limb development. *Curr Opin Genet Dev.* 2007; 17:359–366. [PubMed: 17644373]
- Zakany J, Fromental-Ramain C, Warot X, Duboule D. Regulation of number and size of digits by posterior Hox genes: a dose-dependent mechanism with potential evolutionary implications. *Proc Natl Acad Sci U S A.* 1997; 94:13695–13700. [PubMed: 9391088]
- Zakany J, Kmita M, Duboule D. A dual role for Hox genes in limb anterior-posterior asymmetry. *Science.* 2004; 304:1669–1672. [PubMed: 15192229]

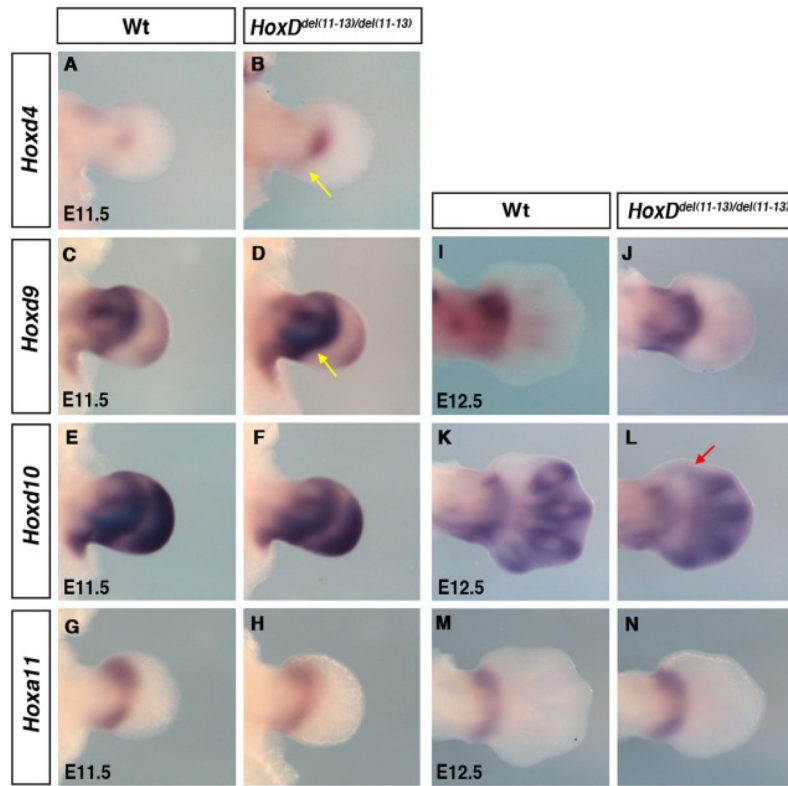


Fig. 1. Effect of *HoxD*^{del(11-13)} deletion on *Hox* genes expression. **A–M:** Limb buds hybridized with *Hoxd4* (A,B), *Hoxd9* (C,D), *Hoxd10* (E,F), and *Hoxa11* (G,H) at embryonic day (E) 11.5 and *Hoxd9* (I,J), *Hoxd10* (K,L), and *Hoxa11* (M,N) at E12.5. B,D,J: Yellow arrow points at posteriorly biased expression of *Hoxd4* (B) and *Hoxd9* (D,J). L: Red arrow point at ectopic second phase expression of *Hoxd10* in digit-1 region. Note that the slight difference in the morphology of *HoxD*^{del(11-13)/del(11-13)} presumptive autopod at E12.5 prefigures the eventual skeletal phenotype of *HoxD*^{del(11-13)} homozygous mice, i.e., shortening of digits and up to six digits per limb.

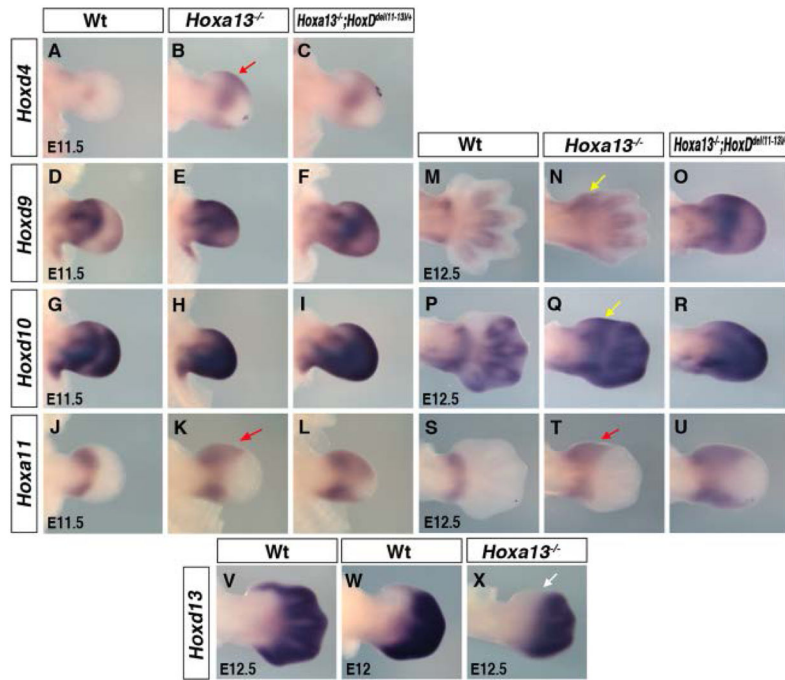


Fig. 2. Deregulation of *Hox* genes expression in *Hoxa13*^{-/-} and *Hoxa13*^{-/-};*HoxD*^{del(11-13)/+} mutant limb buds. **A–X:** Limb buds hybridized with *Hoxd4* (A–C), *Hoxd9* (D–F), *Hoxd10* (G–I), and *Hoxa11* (J–L) at embryonic day (E) 11.5, and *Hoxd9* (M–O), *Hoxd10* (P–R), *Hoxa11* (S–U), *Hoxd13* (V–X) at E12.5. B,C,E,F,H,I: Note deregulation of *Hoxd4* (B,C), *Hoxd9* (E,F) and *Hoxd10* (H,I) expression. N,Q: The yellow arrow points at ectopic first phase expression of *Hoxd9* (N) and *Hoxd10* (Q) in presumptive digit-1 region. B,K,T: The red arrow points at the anterior distal extension of *Hoxd4* (B) and *Hoxa11* (K,T) expression. V–X: Note that *Hoxd13*, which is normally expressed in presumptive digit-1 (V,W), is excluded from this region in *Hoxa13* mutants (white arrow in X). The difference in the shape of the presumptive digit domains of *Hoxa13*^{-/-} and *Hoxa13*^{-/-};*HoxD*^{del(11-13)/+} embryos at E12.5 prefigures the eventual oligodactyl phenotype.

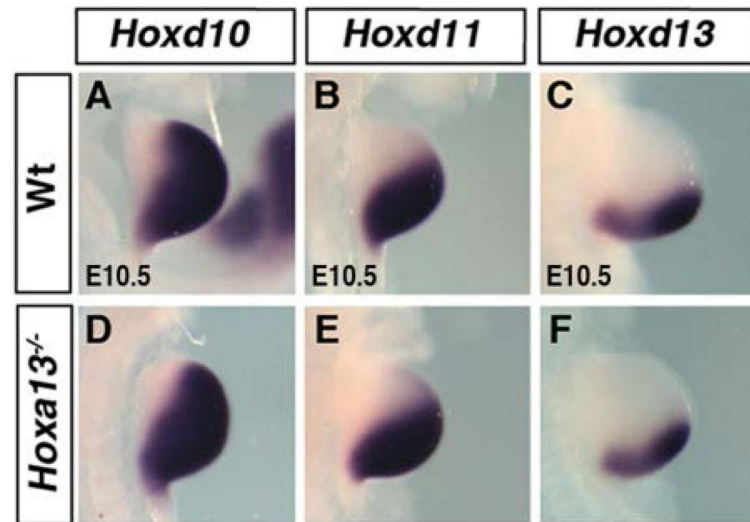


Fig. 3. Expression of 5'*Hoxd* genes in *Hoxa13*^{-/-} limb bud. Embryonic day (E) 10.5 forelimb buds hybridized with *Hoxd10* (A,D), *Hoxd11* (B,E), and *Hoxd13* (C,F). Note that the phase one expression of 5'*Hoxd* genes is unaltered in absence of *Hoxa13*. Genotypes are indicated on the left.

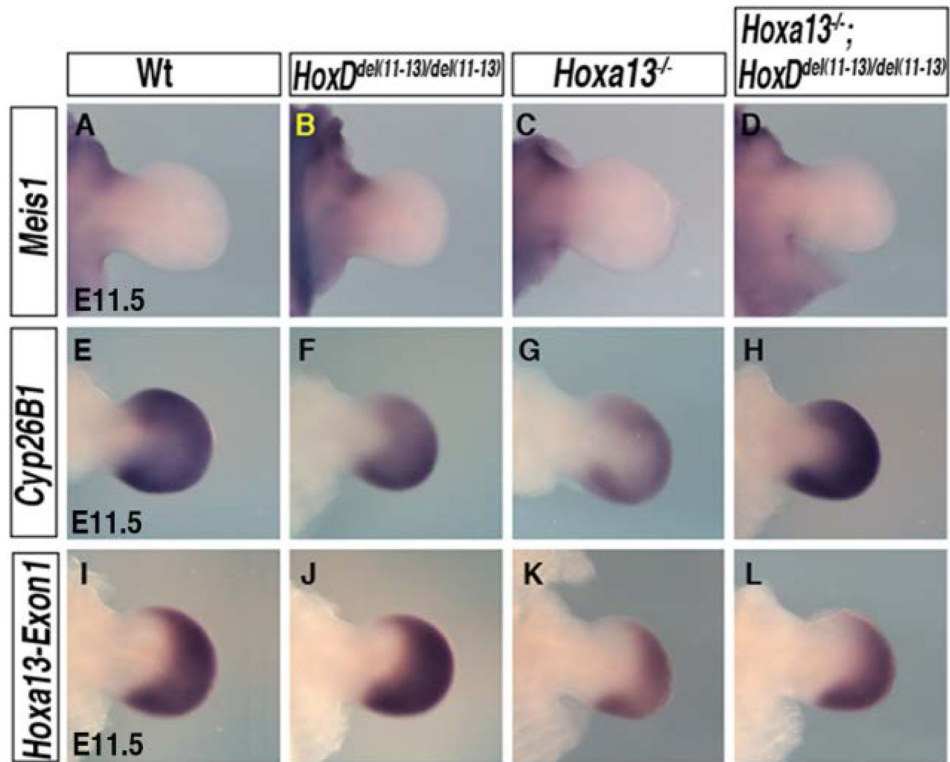


Fig. 4.

Proximal and distal limb cells are properly specified in mutant limb buds. **A–H:** Embryonic day (E) 11.5 forelimb buds show proper specification of proximal limb cells, marked by *Meis1* expression (A–D), and of distal cells, marked by *Cyp26b1* expression (E–H). **I–L:** *Hoxa13-Exon1* expression marks the cells destined to form autopod.

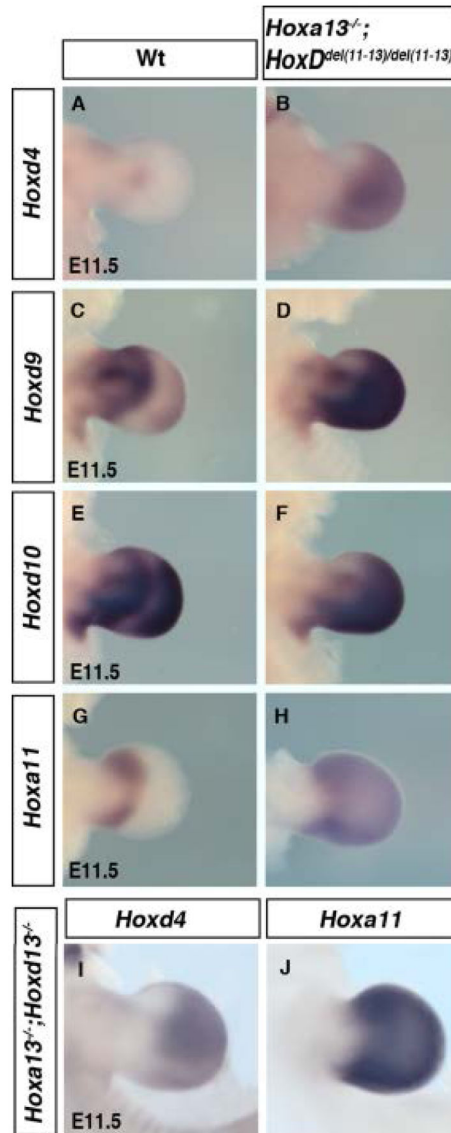


Fig. 5. Spatial redistribution of remaining *Hox* genes in *Hoxa13*^{-/-};*HoxD*^{del(11-13)/del(11-13)} and *Hoxa13*^{-/-};*Hoxd13*^{-/-}. **A–J:** Embryonic day (E) 11.5 forelimb buds hybridized with *Hoxd4* (A,B,I), *Hoxd9* (C,D), *Hoxd10* (E,F), and *Hoxa11* (G,H,I). B,H–J: Note similar distal expansion of *Hoxd4* (B,I) and *Hoxa11* (H,J) in both genotypes.