LETTERS

Early developmental arrest of mammalian limbs lacking *HoxA/HoxD* gene function

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Vertebrate *HoxA* and *HoxD* cluster genes are required for proper limb development[1–3]. However, early lethality, compensation and redundancy have made a full assessment of their function difficult[3–5]. Here we describe mice that are lacking all *Hoxa* and *Hoxd* functions in their forelimbs. We show that such limbs are arrested early in their developmental patterning and display severe truncations of distal elements, partly owing to the absence of *Sonic hedgehog* expression. These results indicate that the evolutionary recruitment of *Hox* gene function into growing appendages might have been crucial in implementing *hedgehog* signalling, subsequently leading to the distal extension of tetrapod appendages. Accordingly, these mutant limbs may be reminiscent of an ancestral trunk extension, related to that proposed for arthropods[6].

Vertebrate limbs bud out of flank mesoderm through interactions with the overlying ectoderm. The subsequent outgrowth and patterning of skeletal elements require signals from both the apical ectodermal ridge (AER) and the zone of polarizing activity, a cohort of cells at the posterior margin of the bud. These cells express *Sonic hedgehog* (*Shh*)[7], whose signalling promotes distal limb growth and patterning, notably through its effect on *Hox* genes belonging to both the *HoxA* and *HoxD* clusters. Before responding to *Shh* signalling, up to seven *Hox* genes are expressed in the early bud, with a restriction in the posterior part, where they may promote *Shh* transcription[1].

Functional analyses have highlighted the function of *Hox* genes in developing limbs. In particular, compound mutants revealed synergistic and redundant mechanisms, because phenotypic alterations were significantly more severe than merely additive. Although this raises problems in assigning gene-specific phenotypes, it indicates that *Hox* products might act quantitatively in both the production and the organization of the structure, a conclusion supported by the truncations observed in mice lacking one of group 13, 11 or 10 *Hox* genes[2,3,5]. In contrast to the *HoxA* and *HoxD* clusters, *HoxB* and *HoxC* are unlikely to have a major function in forelimb development[5], a conclusion based on expression analyses. Furthermore, normal limbs developed in the absence of these latter clusters[8,9]. Consequently, to evaluate the extent of forelimb development in the absence of any relevant *Hox* function, we engineered a combined deletion of the *HoxA* and *HoxD* clusters.

Because loss of *Hoxa13* is embryonic lethal[3], we floxed the *HoxA* cluster to generate tissue-specific deletions (Fig. 1a; $HoxA^{flox}$) and used *Prx1–Cre* mice, where recombination occurs from early limb bud stage onwards[10]. Mice homozygous for a conditional *HoxA* deletion ($HoxA^{c/c}$, referred to hereafter as $A^{c/c}$) were viable but infertile. We assessed the efficiency of recombination through the disappearance of *Hoxa* transcripts from developing $A^{c/c}$ limbs (Fig. 1b). Although some *Hoxa13* and *Hoxa11* transcripts were still detectable in early buds, by late in day 11 *Hoxa* RNAs were no longer seen in forelimbs (Fig. 1b). To decrease residual *Hoxa* transcripts further, we generated embryos *trans*-heterozygous for a full *HoxA* deletion ($HoxA^{-}$, or A^{-}) and the conditional allele. Such embryos ($A^{c/-}$) had no trace of *Hoxa* transcripts in forelimb buds (Fig. 1b). Because *Prx1–Cre* was less efficient in hindlimbs (Fig. 1), only mutant forelimbs are documented.

Forelimb skeletons of $HoxA^{flox/-}$; *Prx1–Cre* adult mice ($A^{c/-}$) showed abnormal zeugopods (forearms) and autopods (hands; Fig. 1c). The thumb was absent and all digits were reduced in size. The zeugopod defects seemed stronger than the mere inactivation of *Hoxa11*, indicating that *Hoxa10* also participates in patterning this structure[4]. We verified that the *Prx1–Cre* transgene completely deleted *HoxA* before the time at which it became functional by generating compound mutants, in which *Hoxd11* was concomitantly inactivated. $A^{c/-}$;$Hoxd11^{-/-}$ mice displayed markedly reduced zeugopods (Fig. 1c), comparable to those of *Hoxa11*;*Hoxd11* mutant

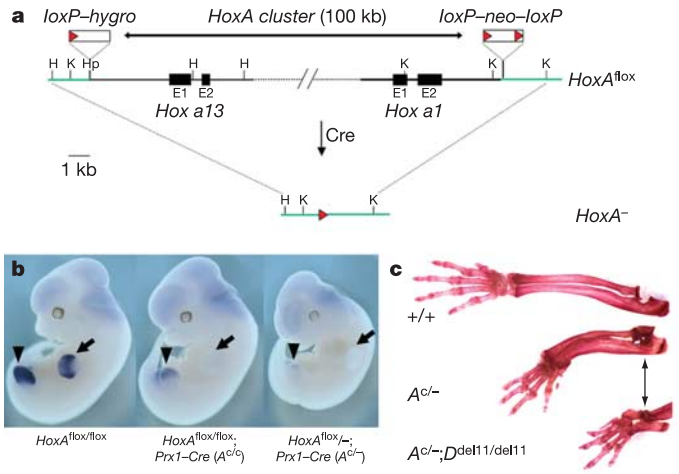

Figure 1 | Conditional deletion of the *HoxA* cluster. **a**, The cluster, flanked by *loxP* sites, was selectively deleted in limb buds. **b**, Control of deletion using a *Hoxa13* RNA probe. Floxed mice express *Hoxa13* in developing hindlimb (arrowhead) and forelimb (arrow). After deletion, *Hoxa13* expression in forelimbs is virtually undetectable, whereas transcripts are weakly detected in hindlimb buds. In E10 embryos carrying one floxed copy and one deleted allele ($A^{c/-}$), transcripts are undetectable in forelimbs (arrow) and barely scored in hindlimb buds (right). **c**, Adult forelimb of the *HoxA* conditional deletion (middle), showing a reduction in the length of bones and ill-formed radius and ulna. When combined with a *Hoxd11* inactivation (bottom), both radius and ulna were further reduced, resembling the double *Hoxd11*;*Hoxa11* mutant phenotype[2], indicating that *HoxA* function was absent from developing forelimbs.

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animals[2], showing that the conditional *HoxA* deletion occurred efficiently.

We combined a full deletion of *HoxD* ($D^{-/-}$)[11] together with one floxed and one deleted *HoxA* allele and *Prx1–Cre* (*HoxD*$^{-/-}$; *HoxA*$^{c/-}$). Double homozygous mutants died soon after birth; we therefore analysed fetal and newborn animals. Forelimbs deficient for *HoxA*/*HoxD* function were drastically truncated (Fig. 2b). At fetal stages, a single cartilage model was observed, articulating with the scapula. This cartilage element, bent in the middle, had a Y-like shape distally. We interpret this as a truncated humerus, bent distally and followed by a bifurcation prefiguring the formation of a zeugopod (Figs 2 and 3a–c). At birth, this cartilage was ossified proximally, whereas structure after bifurcation remained cartilaginous (Fig. 2b). Small and atypical distal rays were scored, which were identified not as proper digits but as distal derivatives of an abnormal cartilaginous plate (Fig. 3). Altogether, mutant forelimbs seemed delayed in their development, as though patterning had been arrested at an early stage. These skeletal elements are probably not produced through the persistence of undetectable *Hoxa* transcripts, because the phenotype of mice carrying a conditional *Tbx5* allele[12] indicates that the *Prx1–Cre* transgene might be active early on and throughout the proximal-to-distal extent of the limb bud. In addition, mice mutant for proximally expressed *Hox* genes[13,14] have defects in the humerus, but not in its most proximal part, further indicating that the skeletal elements present in the mutant forelimbs of our mice might develop independently of any *Hoxa*/*Hoxd* function.

We compared this phenotype with fetuses lacking all relevant *Hox* gene function except that of *Hoxd13* by bringing the *HoxA* deletion over a *HoxD* deletion, removing *Hoxd9* to *Hoxd12* (*HoxD*$^{Del9–12}$; B.T. and D.D., unpublished work). Proximal forelimb elements of such animals were comparable to those of $A^{c/-};D^{-/-}$ mutants, yet they displayed clear digits as early as embryonic day 15.5 (E15.5) (Fig. 3a). At birth, it was evident in these animals that the Y-shaped part was indeed a radius/ulna primordium, which generated two separate cartilages articulating with the humerus (Fig. 3d). Such individualized elements articulating with the humerus were not observed in forelimbs lacking all *Hoxa* and *Hoxd* genes. Comparison of both mutants confirmed that the atypical distal rays in $A^{c/-};D^{-/-}$ animals were not digits. They might reflect an intrinsic property of mutant distal mesenchyme in producing fragmented condensations. The fact that several digits normally derive from cells expressing *Shh*[15], added to the absence of *Shh* expression in the *Hox*-deficient forelimbs we report here (see below), supports this conclusion.

The early developmental arrest of $A^{c/-};D^{-/-}$ forelimb buds was confirmed by the expression of *Meis1* and *dHand*. *Meis1* transcripts normally mark the proximal half of the bud in E9.5–10.5 fetuses. Expression is subsequently maintained proximally as development proceeds (Fig. 3g). In mutant buds, *Meis1* expression at embryonic day 12.5 resembled the wild-type pattern at day 10 (Fig. 3f). Similarly, expression of *dHand* in E11.5 mutant limbs was identical to that of E10–10.5 wild-type buds (Fig. 3h, i) with a distal part devoid of transcript, whereas *dHand* expression in wild-type limbs showed a robust distal domain from E11 onwards (Fig. 3i). These results indicated that the early developmental programme was initiated but arrested soon afterwards.

Critical of this early-to-late transition is the activation of *Shh* transcription in the posterior limb bud. Forelimbs lacking *Shh* display severe distal and posterior agenesis, involving both the autopod and zeugopod, the humerus being less affected[16–18]. We looked at *Shh* expression and observed an almost complete downregulation in conditional double-mutant forelimbs ($A^{c/-};D^{-/-}$) (Fig. 4a–e), with only few cells weakly positive (Fig. 4e). However, a single copy of either *HoxD* or *HoxA* was enough to trigger *Shh* transcription at a level similar to that in the wild type (Fig. 4b). To investigate further the requirement of *Hox* function for *Shh* transcription, we analysed embryos deficient for both clusters obtained by means of *trans*-heterozygous crosses ($A^{-/-};D^{-/-}$), before embryonic lethality. Two such embryos were obtained (out of 577) and *Shh* transcription was undetectable in the bud (Fig. 4, compare k to i and j), whereas other sites showed normal expression levels (Fig. 4, compare h to f, g).

These results indicate that the early expression of *Hox* genes in developing limbs is mandatory for *Shh* transcription to proceed. Forelimbs that lack *Shh* but have functional *Hox* clusters are less severely affected than is shown here. In particular, the radius and a distal element are produced, whereas they are absent from *Hox* mutant limbs. This difference reflects the persistence of some *Hox* transcripts derived from the budding stage in *Shh* mutant limbs[16,19]. Downregulation of *Shh* signalling was further confirmed by the analysis of *Gli3* and *Fgf8* expression in double-mutant forelimbs. *Gli3* was expressed throughout the bud, unlike in control animals but as in *Shh* mutant mice. Similarly, *Fgf8* expression in the AER was abnormal and mostly absent from the distal parts (Fig. 4l, m).

Forelimbs of *HoxA*/*HoxD* mutants are therefore arrested in their development, at about the time at which SHH starts to accumulate posteriorly. It is difficult to infer from these results whether *Hox* genes act directly on *Shh* transcription and/or maintenance, or whether they sustain the growth of the bud by ensuring proper AER function. Nevertheless, the fact that early ectopic *Hox* expression can trigger *Shh* transcription[1,20] argues in favour of the former

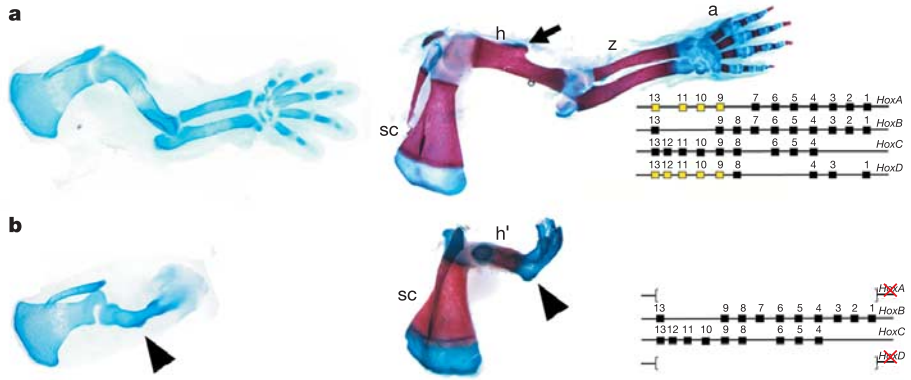

Figure 2 | *HoxA/HoxD* double-mutant forelimbs. Wild type (**a**) and mutant ($A^{c/-};D^{-/-}$) (**b**) cartilage and skeletal patterns in E15 (left) and newborn (right) specimens. Mutant animals (**b**) show a severe truncation of a single cartilage model (left), which is bent distally (arrowhead) and adopts a Y-shaped aspect. The scapula (sc) is apparently normal. After ossification occurred, a single bone rod is observed proximally, articulating with the scapula. This truncated humerus (h′) is extended, after the bend but without articulation, by a piece of cartilage fragmented at its distal tip. h, humerus; z, zeugopod; a, autopod.

alternative. This raises the possibility that the limb distal extension, in the course of tetrapod evolution, used the capacity of *Hox* gene function to trigger *Shh* transcription, which in turn antagonized the repressive effect of *Gli3* (refs 21,22). This required AER signalling to be fully functional, and defects in—or the absence of—either one of these components could prevent distal extension. In this view, the situation in teleost fishes, in which both the early *Hox* and the *Shh* patterns are present but no extension is observed, probably reflects a derived situation generated by modification in AER signalling after the ectodermal folding process in the fin bud[23].

Accordingly, the truncated proximal skeleton shown here might reflect an ancestral trunk extension, before the genetic machinery was co-opted to develop more distal limb pieces. Strikingly, most of these skeletal elements develop from cells expressing *Meis1*, a gene orthologous to the arthropod *homothorax* gene (*hth*). In *Drosophila*, *hth*, in combination with *exd*, specifies an ancient limb proximal piece, whereas more distal structures emerge along with *Hedgehog* signalling[24,25]. In vertebrates, limb bud expression of *Meis* represses *Shh* transcription and is antagonized by *HOX* products[26]. This indicates

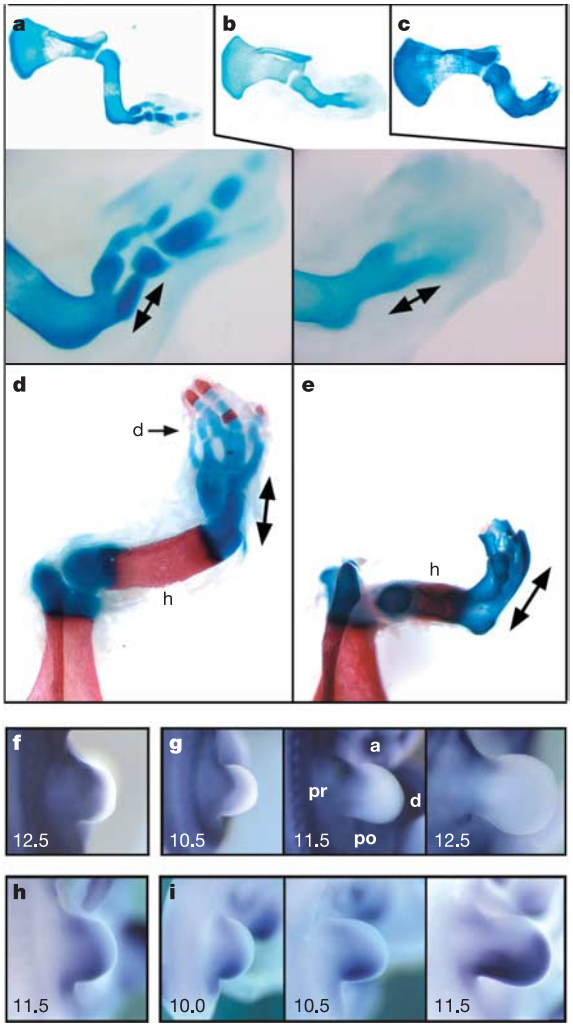

Figure 3 | Developmental arrest in forelimbs lacking *Hox* function. **a**–**c**, Cartilage patterns in E15.5 (**b**) and E17.5 (**c**) fetuses lacking *Hoxa*/*Hoxd* function in forelimbs ($A^{c/-};D^{-/-}$), or with *Hoxd13* function only ($A^{c/-};D^{del9-12/del9-12}$) in an E15.5 fetus (**a**). *Hoxd13* alone triggers the appearance of digits, distal to the Y-shaped structure (double arrow), absent from the double mutant (**b**). **d**, **e**, In postnatal skeletons (**d**), these condensations generated independent elements, remnants of radius and ulna, whereas the *HoxA*/*HoxD* mutant displayed a single cartilage plate (**e**, double arrow), with atypical rays. **f**, **g**, Expression of *Meis1* in mutant ($A^{c/-};D^{-/-}$) (**f**) and wild-type (**g**) buds. The mutant pattern at E12.5 resembles that of wild-type buds at E10.5, indicating a developmental arrest before E10. **h**, **i**, This is confirmed by *dHand* expression, which in E11.5 mutant ($A^{c/-};D^{-/-}$) buds (**h**) is expressed similarly to that in the wild type (**i**) at E10–10.5. Abbreviations: a, anterior; po, posterior; pr, proximal; d, distal.

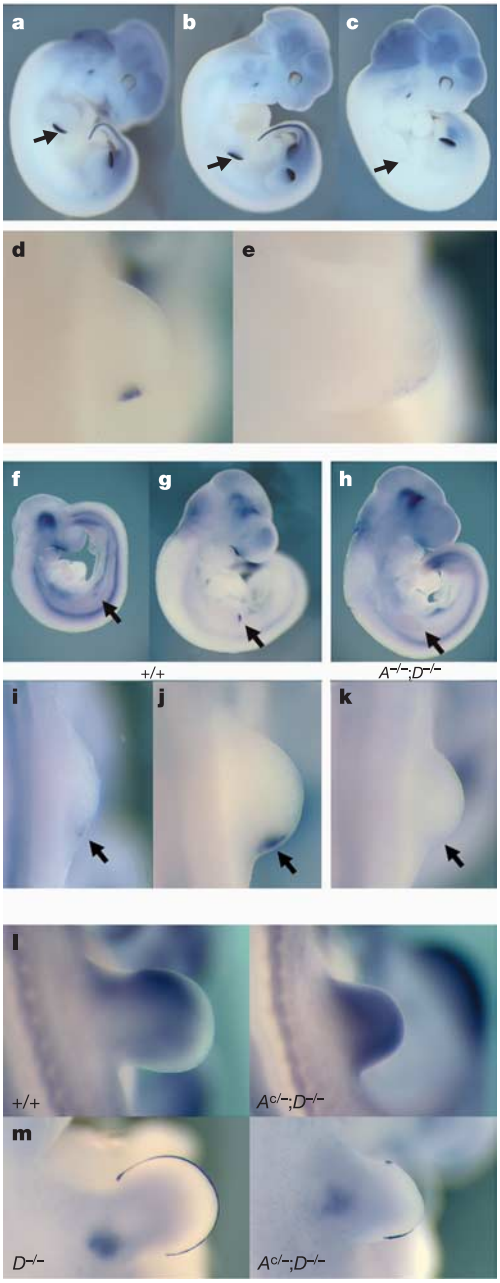

Figure 4 | Lack of *Shh* expression in double-mutant forelimbs. **a**–**c**, At day 11, control animals (**a**) express *Shh* at the posterior margin of the bud (arrow). Embryos lacking *HoxD* and one copy of *HoxA* ($A^{+/-};D^{-/-}$) (**b**) still have a normal *Shh*. However, the conditional double mutant ($A^{c/-};D^{-/-}$) no longer shows *Shh* transcripts in forelimbs (**c**), unlike in hindlimbs where the deletion is incomplete. **d**, **e**, *Hox* genes maintain *Shh* transcription. At E10, some *Shh* expression is observed in conditional double-mutant ($A^{c/-};D^{-/-}$) forelimbs (**d**), as a result of residual *Hoxa* transcripts produced before completion of the deletion. In contrast, E11 buds (**e**) contain only few cells, if any, expressing *Shh*. **f**–**k**, *Shh* is not expressed in forelimbs fully deficient for both the *HoxA* and *HoxD* clusters (**h**, **k**), despite the delayed growth, as in both aged-matched (**g**, **j**) and younger (**f**, **i**) wild-type limb buds *Shh* expression is unambiguously detected. **l**, **m**, Expression of *Gli3* (**l**) and *Fgf8* (**m**) in double conditional-mutant ($A^{c/-};D^{-/-}$) forelimb buds. *Gli3* is expressed throughout the bud, unlike in the control, and *Fgf8* expression in the AER is patchy and interrupted. In both cases, transcript patterns resemble those observed in mice lacking *Shh* function[16,17].

that a generic animal appendage might be divided into two pieces: an ancestral, 'trunk'-dependent extension characterized by both the expression of *Meis* (*hth*) and the absence of *Shh* (*Hh*), whereas more distal parts evolved independently in various taxa[27], following different strategies, but only through the repression of *Meis* gene activity[25]. In tetrapods, the same *Hox*/*Shh* module was co-opted in both the forelimbs and the hindlimbs, which provides an explanation for serial homology and subsequent concerted evolution of paired appendages[28]. We therefore propose that the basic proximal/distal distinction proposed for arthropods[6] be extended to tetrapods. The position of this morphological transition, in both arthropods and vertebrates, might nevertheless not correspond precisely to an articulation. Instead, it might occur at a developmental stage at which these morphological landmarks are not yet formed, leading to the above uncertainty in identifying the remaining structures present in the *HoxA/HoxD* deficient forelimbs.

METHODS

To generate mice with their *HoxA* complex flanked with *loxP* sites, we used ES cells (a gift from F. Rijli) containing a *loxP–TKneomycin–loxP* cassette downstream of *Hoxa1* (ref. 29) and further electroporated with a *loxP–pGKhygromycin* cassette at an *Hpa*I site 3.9 kilobases (kb) upstream of the *Hoxa13*. The resulting floxed allele ($HoxA^{flox}$) was introduced into the germ line of chimaeric males. The conditional deletion in limbs was obtained with mice expressing the Cre recombinase under the control of the *Prx1* promoter[10]. All animals and embryos were genotyped by Southern blot analysis. The $HoxD^{Del9-12}$ mice were produced with the TAMERE strategy (B.T. and D.D., unpublished work). Whole-mount *in situ* hybridizations, skeletal preparations and cartilage staining were performed with standard procedures and previously described riboprobes.

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