LETTERS

Regulatory constraints in the evolution of the tetrapod limb anterior–posterior polarity

Basile Tarchini[1]†, Denis Duboule[1] & Marie Kmita[1]†

The anterior to posterior (A–P) polarity of the tetrapod limb is determined by the confined expression of *Sonic hedgehog* (*Shh*) at the posterior margin of developing early limb buds[1,2], under the control of HOX proteins encoded by gene members of both the *HoxA* and *HoxD* clusters[3–6]. Here, we use a set of partial deletions to show that only the last four *Hox* paralogy groups can elicit this response: that is, precisely those genes whose expression is excluded from most anterior limb bud cells owing to their collinear transcriptional activation. We propose that the limb A–P polarity is produced as a collateral effect of *Hox* gene collinearity, a process highly constrained by its crucial importance during trunk development. In this view, the co-option of the trunk collinear mechanism, along with the emergence of limbs, imposed an A–P polarity to these structures as the most parsimonious solution. This in turn further contributed to stabilize the architecture and operational mode of this genetic system.

The exquisite functionalities of tetrapod limbs largely derive from their various polarities. Among them, the A–P asymmetry (from the thumb/radius to the minimus/ulna, respectively) results from the presence of an organizing centre, the zone of polarizing activity, at the posterior margin of the developing limb buds[1]. The polarizing activity is achieved by the product of the gene *Sonic hedgehog* (*Shh*) (ref. 2), whose transcripts are found in posterior mesenchymal cells only, near the apical ectodermal ridge, from which growth factors such as *Fgf4* are necessary for *Shh* transcriptional onset and maintenance[7–12]. *Shh* signalling is required to maintain the apical ectodermal ridge and hence to sustain the growth of the structure, as well as to organize limb patterns along the A–P axis[13–16]. Therefore, the mechanism that restricts *Shh* transcription in posterior limb bud cells is critical for the generation of this polarity.

Gain-of-function experiments have suggested that some *Hox* gene products can trigger *Shh* transcription at ectopic sites of developing limb buds[3–5], probably through a direct interaction between HOX proteins, their cofactors and the *Shh* limb enhancer[6], leading to modifications in A–P patterning. Recently, the deletion of both *HoxA* and *HoxD* clusters in developing forelimbs was reported to abrogate *Shh* expression, thus confirming that HOXA and/or HOXD proteins are required for *Shh* transcription[17]. However, several *Hoxa/d* genes are expressed throughout the emerging limb bud[18], including in anterior cells, making the posterior restriction of *Shh* transcripts difficult to explain on this basis alone. Therefore, we set out to determine, in physiological conditions, which *Hox* product(s) could elicit *Shh* transcription in limb buds.

We generated a series of *Hoxa/Hoxd* double-mutant mice and started with a deletion of all *Hoxa* genes combined with the absence of the *Hoxd8* to *Hoxd13* interval. This resulted in the absence of *Shh* expression (Fig. 1b), even though the remaining *Hoxd1*, *Hoxd3* and *Hoxd4* genes were expressed throughout the limb mesenchyme at the expected stage for *Shh* transcription[18], suggesting that the latter gene products were unable to trigger *Shh* transcription. We next tested whether the addition of both *Hoxd8* and *Hoxd9* function could elicit *Shh* expression. There again, *Shh* transcripts were not detected (Fig. 1c). Interestingly, deleting from *Hoxd10* to *Hoxd13* led to *Hoxd9* upregulation in posterior cells (Fig. 1d), showing that even a robust

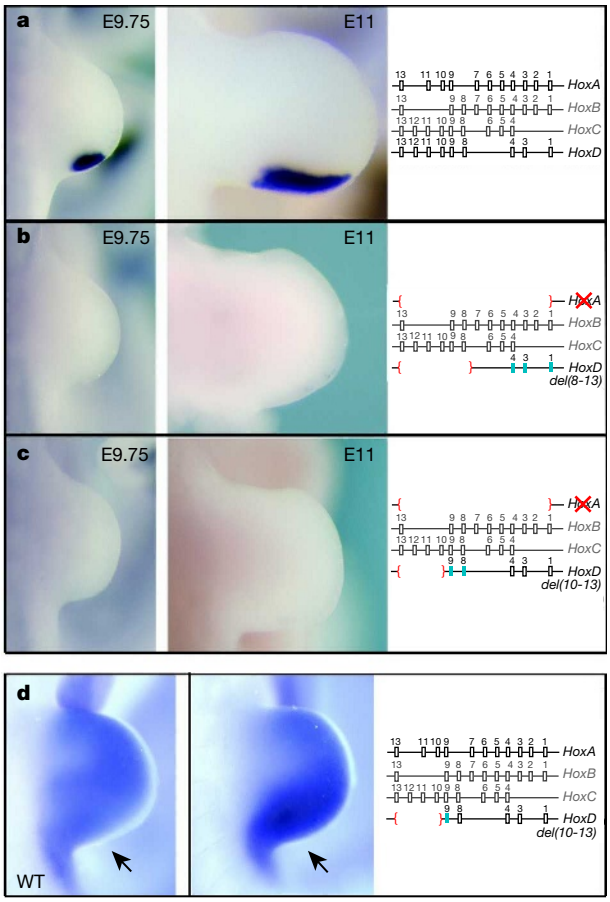

Figure 1 | Control of *Shh* expression by *Hoxa* and *Hoxd* genes. **a**–**c**, *Shh* expression in embryonic day (E) 9.75 and E11 limb buds, anterior to the top. The genotypes are depicted on the right-hand side. **a**, Wild type. **b**, *Shh* transcripts were absent from limb buds lacking all *Hoxa* genes and those from *Hoxd8* to *Hoxd13*. **c**, The addition of *Hoxd8* and *Hoxd9* functions did not elicit a *Shh* signal, even though *Hoxd9* was overexpressed posteriorly (**d**). *Hox* gene products from groups 1 to 9 are unable to activate and/or maintain *Shh* expression.

[1]Department of Zoology and Animal Biology and National Research Centre 'Frontiers in Genetics', University of Geneva, Sciences III, Quai Ernest Ansermet 30, 1211 Geneva 4, Switzerland. †Present addresses: Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal H3A 1B1, Quebec, Canada (B.T.); Laboratory of Genetics and Development, Institut de Recherches Cliniques de Montréal (IRCM), Université de Montréal, 110 avenue des Pins Ouest, H2W 1R7, Montréal, Quebec, Canada (M.K.)

dose of *Hoxd9* delivered in the right cells was unable to trigger *Shh* expression (Fig. 1c).

We then used two additional combinations to re-introduce either *Hoxd10* or *Hoxd13* function to the former deletion (Fig. 2a). In both cases, *Shh* transcripts were detected unambiguously, indicating that either *Hoxd10* or *Hoxd13* products can elicit *Shh* transcription. However, neither of these two HOX products, in isolation, appeared to fully recapitulate the normal *Shh* pattern. *Shh* messenger RNAs were detected rather distally, in the presence of *Hoxd13* only (Fig. 2a, bottom), but more proximally in the presence of *Hoxd10* alone (Fig. 2a, top). Even though these differences may be slightly biased by distortions in the growth of the mutated limb buds themselves, these results suggested that the wild-type *Shh* spatial pattern is established in response to multiple HOX proteins. This was supported by limb buds lacking all *Hoxd* genes, as well as *Hoxa13*, the most distally expressed gene of the *HoxA* cluster, where *Shh* was expressed in a proximal domain only (Fig. 2b), probably in response to *Hoxa11* and/or *Hoxa10*. A quantitative aspect was also revealed by comparing *Shh* expression in $HoxA^{+/+};HoxD^{del(8–13)/del(8–13)}$ versus $HoxA^{+/-};HoxD^{del(8–13)/del(8–13)}$ early limb buds (Fig. 2c). A reduced *Shh* expression was associated with the removal of one dose of *Hoxa* genes. All together, this analysis showed that *Shh* expression is controlled both by quantitative and qualitative combinations of *Hoxa* and *Hoxd* genes, belonging to paralogous groups 10 to 13.

The corresponding phenotypic analyses indicated that forearm and hand development requires the function of those *Hoxa* and *Hoxd* genes that can trigger *Shh* expression (Fig. 3a). Forelimbs carrying *Hox* deletions preventing *Shh* activation were virtually identical in morphology to the full *HoxA/HoxD* deficiency[17], with no genuine forearm and digits (Fig. 3b). Instead, addition of *Hoxd13* alone induced the formation of both digits and a remnant of forearm, whereas *Hoxd10* function induced a clear, yet truncated forearm followed by a series of rays (Fig. 3a). Therefore, the recruitment of the *Hox* system in growing limbs and its impact upon *Shh* activation led to both an increased number of bony elements along the A–P axis and their concomitant patterning, which illustrates a remarkable parsimony in producing appendages with high adaptative values. The resulting heteromeric nature of distal appendages was probably the ground for the architectural versatility and associated functional features that accompanied the emergence of tetrapods[19]. Also, while confirming the function of *Hoxd9* in proximal limb structures[20] (Fig. 3b), this analysis indicated that 3′-located genes (for example, *Hoxd4*, *Hoxd3*) have no function during limb development, despite their expression there. We discuss below (Fig. 4) why, paradoxically, these genes are nevertheless important in this context.

We believe the co-option of the collinear mechanism from the developing trunk to developing appendages is at the origin of the limb A–P polarity. In limb buds, *Hox* genes are activated in a spatial-temporal sequence following their genomic topography, leading to the progressive depletion of *Hox* gene transcripts from the anterior quadrant of the early bud, such that *Hoxd1* is transcribed throughout the bud, whereas *Hoxd13* is only expressed in a small posterior-distal domain[18] (Fig. 4a). Interestingly, the transition between *Hox* genes expressed throughout the bud and those excluded from anterior cells is precisely between *Hoxd9* and *Hoxd10*, that is, between the last gene unable to activate *Shh* and the first one to achieve this task, respectively.

The existence of this transition (Fig. 4, arrowhead) is critical for the morphology of the limb. Should it disappear, or be shifted towards more 'posterior' (5′) genes (for example, between *Hoxd12* and *Hoxd13*; Fig. 4b, arrowhead), expression of *Hox* genes restricted to the posterior mesenchyme would extend in anterior cells and trigger ectopic *Shh* transcription anteriorly. We would expect such limbs to be polydactylous with a bilateral symmetry[3–5]. Conversely, a premature occurrence of this transition would ultimately prevent *Shh* transcription owing to the lack of appropriate HOX products, and concurrent limb truncation (Fig. 4c). In this view, *Hox* gene collinearity in the early limb bud is a critical parameter in organizing the A–P polarity. Such a mechanism must be well controlled to avoid variations that would override the buffering capacity of the system, and which would thus severely affect the final morphology. Expression of 3′-located genes (for example, *Hoxd1*, *Hoxd3*) in the early buds may help in this respect, despite the absence of morphological

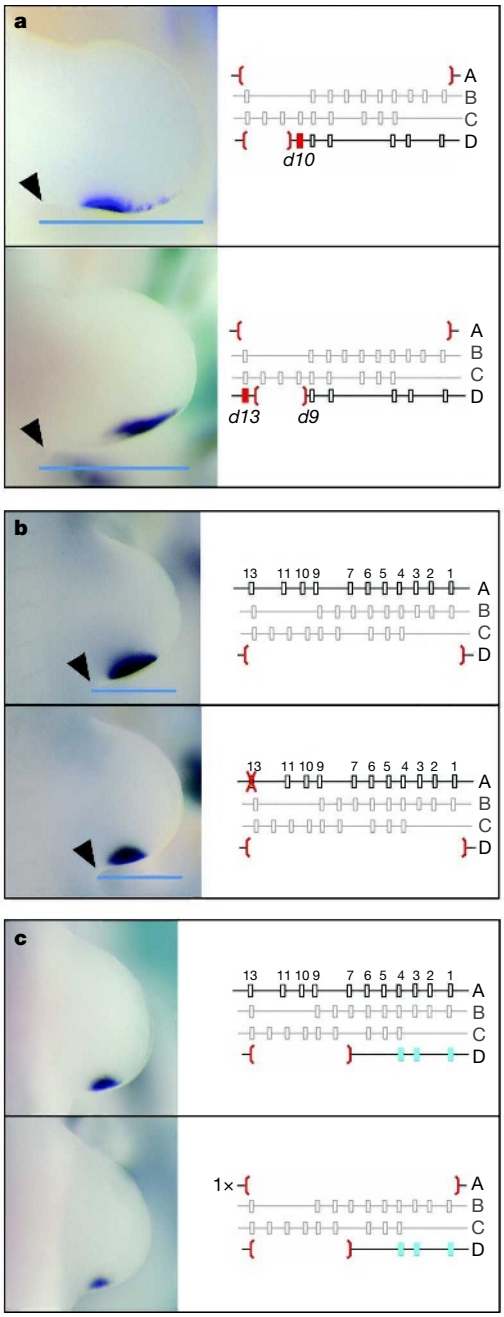

Figure 2 | 5′-located *Hox* genes induce *Shh* expression in a gene-specific and dose-dependent manner. **a**, The addition of either *Hoxd10* (top panel, red) or *Hoxd13* (bottom panel, red) function was enough to trigger *Shh* expression, yet with some qualitative differences; *Hoxd13* elicited *Shh* expression more distally than *Hoxd10* (arrowheads). **b**, Likewise, in the absence of *HoxD*, further removal of *Hoxa13* function seemed to restrict *Shh* expression proximally. **c**, A quantitative input was also observed, for example when comparing the potential of either two copies (top panel) or one (bottom panel) copy of *Hoxa* genes to activate *Shh* transcription, in the absence of all posterior *Hoxd* genes. Identical staining conditions showed a reduction in *Shh* transcripts (compare top and bottom panels). Blue lines in **a** and **b** indicate the proximal–distal extent of the limb bud at these stages.

impact, because even just their presence participates in delaying the activation of 5′-located genes (Fig. 4).

The early collinear mechanism acting in limb buds has recently been proposed to rely upon opposite regulations, located at both extremities of the *HoxD* cluster, one acting as a timer for gene activation, the other preventing expression in anterior cells[18]. Consequently, each gene of the cluster will show a unique response

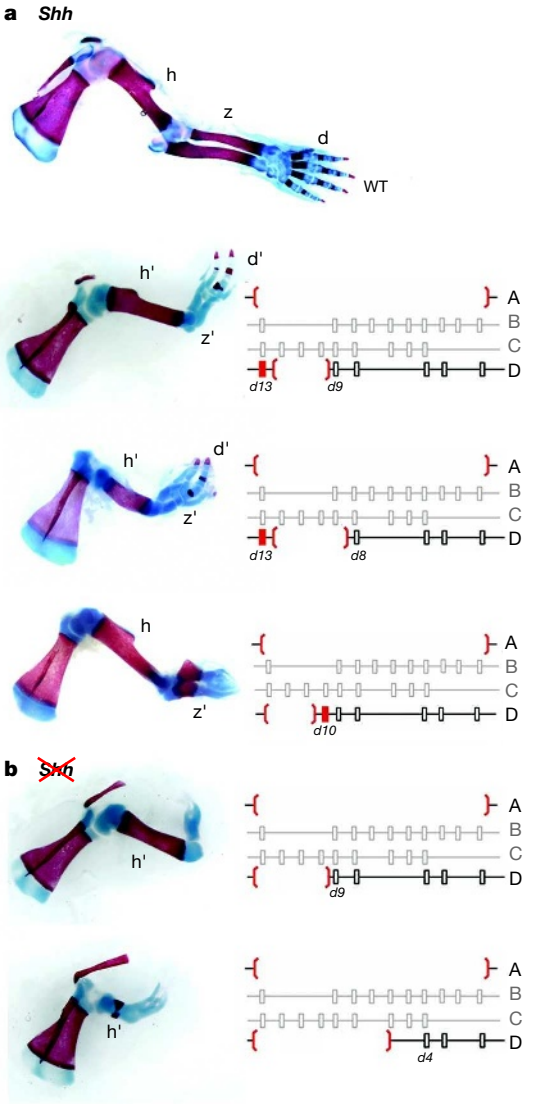

Figure 3 | Skeletons of the various mutant stocks in either the presence (top) or absence (bottom) of *Shh* signalling. **a**, On the top is a wild-type newborn forelimb. Below are mutant phenotypes. Genotypes are indicated, with all specimens carrying a conditionnal *HoxA* cluster deletion ($A^{c/-}$). As long as *Hoxd13* was present, both digits and a clear forearm (zeugopod; z, z′) were observed, though ill-formed, reduced and poorly ossified. In contrast, the introduction of *Hoxd10* (bottom panel) led to a better-developed and ossified zeugopod, whereas only rod-like structures were present distally. The presence of an A–P polarity, as scored for example at the articulation between the humerus (h, h′) and radius/ulna (z, z′) coincides with the presence of *Shh* signalling. **b**, In contrast, when all posterior *Hoxd* genes were missing, neither digit nor radius/ulna were detected, just an abnormal and segmented cartilaginous condensation. These phenotypes were virtually identical to the full *HoxA/HoxD* cluster deletion[17] and illustrated the absence of both *Shh* signalling and A–P polarity. The severity of the humerus truncation correlates with the number of remaining *Hox* genes, regardless of *Shh* activity, pointing to the function of groups 8 and 9 in the making of limb proximal structures (**b**, compare top and bottom panels). The presence, absence or orientation of the pectoral girdle results from the dissection of the specimen.

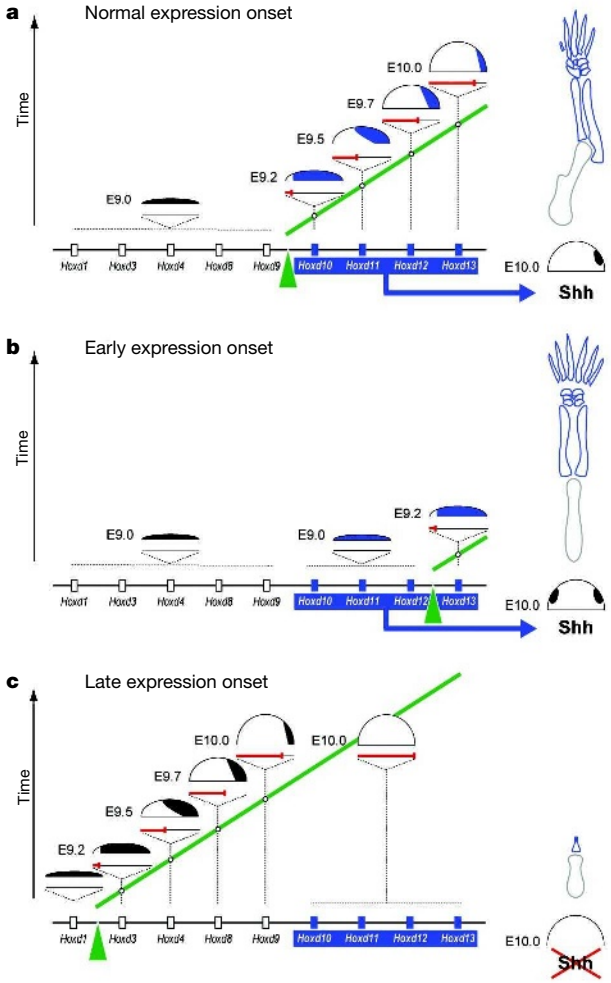

Figure 4 | Regulatory constraints in the emergence of the limb A–P polarity. This scheme shows how collinear *Hoxd* gene activation in early limb buds generates an A–P pattern and how temporal modifications (heterochronies) of this mechanism may affect limb morphology. **a**, Wild type. The expression of the different *Hoxd* genes is shown between E9.0 and E10.0, at transcriptional onset. The *HoxD* cluster is on the *x* axis and time on the *y* axis. Temporal collinearity thus appears as a positive slope (green line), posterior genes (for example, *Hoxd13*) being activated later than more anterior neighbours (for example, *Hoxd11*). Spatial collinearity, the progressive exclusion of *Hox* transcripts from the anterior-most region of the limb bud, is depicted with red bars. Genes and expression patterns in blue are those able to elicit *Shh* transcription, unlike those in black. Correspondingly, the skeletal elements are shown either in black or in blue, illustrating which *Hox* gene functions they require to properly develop. The beginning of *Hox* transcripts exclusion from anterior cells is indicated with a green arrowhead on the *X* axis. Anterior exclusion starts with *Hoxd10*, that is, precisely with the first HOX product able to trigger *Shh* transcription. Qualitative and quantitative inputs will lead to the activation of *Shh* at the posterior margin only, allowing for the formation of a fully grown, A–P polarized, limb skeleton. **b**, A substantial heterochronic shift of temporal collinearity towards premature *Hox* genes activation would lead to anterior exclusion only for the very last *Hox* gene transcripts (for example *Hoxd13*), with some 'blue' genes expressed throughout the early bud leading to *Shh* activation at both posterior and anterior margins and consequent bilateral symmetry of the limb skeleton. **c**, Conversely, a much delayed *Hox* gene activation would induce an anterior exclusion of 3′ genes, impeding the activation of the 'blue' genes at the appropriate stage, similar to the case of *Evx2* in the normal situation[18]. In such a case, *Shh* transcripts would be absent and the limb truncated. Therefore, the mechanism underlying *Hox* genes collinearity in early limb buds is critical for proper A–P limb polarity. Its co-option from trunk development makes the polarity of our appendages dependent on genetic constraints imposed by the general architecture of our body plan.

to these regulatory controls, determining its spatio-temporal expression. In many respects, this process resembles that implemented during the development of the major body axis, which also relies upon sequential activation of *Hox* genes in time and space[21], as illustrated by common regulatory re-allocations in both early limb buds and extending tail buds observed in some cases after genetic alterations of the *HoxD* cluster[22].

Consequently, we suggest that tetrapod limbs evolved along with the recruitment of the *Hox* collinear mechanism implemented in the developing body axis. This regulatory co-option imposed collinearity within limb buds, leading to the posterior-only expression of 5′-located *Hox* genes. Because these latter genes have the capacity to elicit *Shh* transcription, *Shh* signalling was confined to posterior limb bud cells, and hence the limb emerged with a built-in A–P polarity. Alternatively, all HOX proteins originally may have had the capacity to regulate *Shh*, this property being subsequently restricted to some genes only, to generate an A–P polarized structure. However, the absence of animals with appendages showing a truly bilateral symmetry, including ancestral tetrapod fossils[19,23], makes the latter scenario unlikely.

In the former view, the limb A–P polarity is a collateral effect of the genetic strategy co-opted for the distal extension of our limbs, a process highly constrained by the intrinsic logic of our body architecture. The necessity of restricting expression of 5′-located *Hox* genes caudally during gastrulation to prevent the deleterious effects of their products if expressed too rostrally in the main body axis (for example, see ref. 24), is thus probably the origin of their posterior expression in limb buds and of the consequent appendage A–P polarity. In this context, rather than the result of the selection of an independent regulatory strategy applied to an appendage because of the obvious adaptative advantages associated with such a polarity, we may consider that this polarity simply reflects the most parsimonious way of producing a limb.

METHODS

The production of the various mutant strains used in this study was previously described[17,18,25]. Genotyping of mice and embryos was performed using Southern blot analysis. Whole-mount *in situ* hybridizations were carried out on 9.5- to 11-day-old fetuses, using standard procedures and the previously described *Shh* riboprobe[26]. For skeletal preparations, adult or newborn animals were processed according to ref. 27. Embryos were carefully staged and the slight differences observed sometimes between the sizes of the limb buds reflect the phenotypic consequences of the genetic alterations rather than different ages.

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