Tinkering with constraints in the evolution of the vertebrate limb anterior–posterior polarity

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Abstract. Genes belonging to both *HoxA* and *HoxD* clusters are required for proper vertebrate limb development. Mice lacking all, or parts of, *Hoxa* and *Hoxd* functions in forelimbs, as well as mice with a gain of function of these genes in the early limb bud, have helped us to understand functional and regulatory issues associated with these genes, such that, for example, the tight mechanistic interdependency that exists between the production of the limb and its anterior to posterior (AP) polarity. Our studies suggest that the evolutionary recruitment of *Hox* gene function into growing appendages was crucial to implement *hedgehog* signalling, subsequently leading to the distal extension of tetrapod appendages, with an already built-in AP polarity. We propose that this process results from the evolutionary co-option, in the developing limbs, of a particular regulatory mechanism (collinearity), which is necessary to pattern the developing trunk. This major regulatory constraint imposed a polarity to our limbs as the most parsimonious solution to grow appendages.

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The development and evolution of tetrapod appendages provides a particularly enlightening example of what François Jacob quoted as 'tinkering' (Jacob 1977), or at least of one of the various interpretations one can give to this quotation. It is indeed understandable that, using this term, Jacob did not explicitly refer to a tinkering involving entire genetic pathways, but instead, the multiple usage of a core of building blocks and basic processes and their combinations, to generate biological diversity. This landmark paper was soon followed by a series of

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discoveries (e.g. the inter-species conservation of genes and networks), which not only revolutionized our views on developmental genetics, but also provided the conceptual tools to contemplate neo-Darwinism from a new perspective (see for example Duboule & Wilkins 1998, Kirschner & Gerhart 2006). In this novel context, the concept of 'tinkering' has survived well, to say the least, even though the associated notion of evolutionary 'genetic constraints' and the difficulty to reconcile this notion with an orthodox gradualist view of Darwinism still makes the impact of Jacob's prediction difficult to acknowledge for many of us.

In this respect, the historical and heuristic values of tetrapod limbs are of interest, as they represent the original example of structures that developed and evolved through the redeployment of a range of genetics pathways necessary for the ontogeny of the major body axis (Dollé et al 1989). Nowadays, the mere fact that limbs are rather recent innovations logically implies that they find their origin in the co-option of regulatory circuits that had previously evolved in a different context. While this is well accepted, the problems that it creates regarding the genetic constraints applied to the realm of available morphological possibilities, should not be overlooked. In this paper, we would like to discuss this issue and show that a large part of our basic limb morphology, hence its functionalities, is strongly constrained by those genetic mechanisms recruited to evolve these structures.

Hox genes, *Sonic hedgehog* (*Shh*) and limb development

Vertebrate limbs bud out of flank mesoderm through interactions with the overlaying ectoderm. The subsequent outgrowth and patterning of skeletal elements require signals from both the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), a cohort of cells at the posterior margin of the bud, near the AER. These cells express *Sonic hedgehog* (*Shh*; Riddle et al 1993) whose product promotes distal limb growth and patterning, notably via its effect upon *Hox* genes belonging to both *HoxA* and *HoxD* clusters. Before responding to *Shh* signalling, several *Hox* genes are expressed in the early bud, some with a restriction for the posterior part where they may promote *Shh* transcription and/or maintenance (Zakany et al 2004).

Functional analyses have highlighted the role of *Hox* genes in developing limbs. In particular, compound mutants revealed synergistic and redundant mechanisms, as phenotypic alterations were significantly more severe than merely additive. While this raises problems in assigning gene specific phenotypes, it suggests that *Hox* products act quantitatively in both the production and organization of the structure. This conclusion is supported by the truncations observed in mice lacking either group 13, 11 or 10 *Hox* genes (Davis et al 1995, Fromental-Ramain et al 1996, Wellik & Capecchi 2003). In contrast to the *HoxA* and *HoxD* clusters, *HoxB*

and *HoxC* are unlikely to play a major role in forelimb development (Wellik & Capecchi 2003, Nelson et al 1996), based on expression analyses. Furthermore, normal limbs developed when either of these gene clusters was deleted (Suemori & Noguchi 2000, Medina-Martinez et al 2000). Consequently, to evaluate the extent of forelimb development in the absence of any relevant *Hox* function, we engineered combined deficiencies of both the *HoxA* and *HoxD* clusters (Kmita et al 2005).

Because loss of *Hoxa13* is embryonic lethal, we floxed the *HoxA* cluster to generate tissue-specific deletions and used *Prx1-Cre* mice (Logan et al 2002), where *Cre*-mediated recombination occurs from early limb bud stage onwards. In this way, we produced mice lacking all *HoxA* and *HoxD* gene functions in forelimbs, which induced dramatic truncations of the appendages (Kmita et al 2005). At fetal stages, a single cartilage model was observed, articulating with the scapula. This cartilage element, bent in the middle, displayed a Y-like shape distally. We interpreted this as a truncated humerus, bent distally and followed by a bifurcation, which prefigured the formation of the zeugopod. Overall, mutant forelimbs appeared delayed in their development, as if patterning had been arrested at an early stage. Forelimbs lacking *Shh* also display severe distal and posterior agenesis, involving both the autopod and zeugopod, whereas the humerus is less affected (Chiang et al 2001, Kraus et al 2001). We looked at *Shh* expression and observed a virtually complete down-regulation in conditional *HoxD/HoxA* double mutant forelimbs, with only a few cells weakly positive. However, a single copy of either *HoxD* or *HoxA* was enough to trigger *Shh* transcription at a level similar to wild-type. To further investigate the requirement of *Hox* function for *Shh* transcription, we looked at early embryos deficient for both clusters obtained via *trans*-heterozygous crosses ($A^{-/-}; D^{-/-}$), before embryonic death had occurred. Two such embryos were obtained and *Shh* transcription was undetectable in the bud, whereas other sites showed normal expression levels in both cases (Kmita et al 2005). While these results indicated that the early expression of *Hox* genes in developing limbs is mandatory for *Shh* transcription to proceed, they paradoxically raised the question as to what restricts *Shh* transcription posteriorly, for several *Hox* genes are expressed throughout the early limb bud, including in most anterior cells where *Shh* is not normally activated.

Collinearity in limbs

In developing early limb buds, *Hox* genes are expressed following a collinear regulation whereby several 3′-located genes are expressed throughout the limb bud, whereas the transcription of more 5′-located genes (from *Hoxd10* onwards) is progressively restricted to more and more posterior cells. ZPA cells thus express distinct qualitative and quantitative combinations of *Hox* transcripts as compared

to more anterior cells, and this may lead to the observed difference in *Shh* regulation. In support of this explanation, we analysed a stock of mice carrying a partial deletion of the *HoxD* cluster, leaving in place *Hoxd11*, *Hoxd12* and *Hoxd13*. Due to some regulatory re-allocations, these three genes were found expressed throughout the early bud, i.e. not only in posterior cells, as expected, but also in anterior cells from which their transcription is normally excluded (Zakany et al 2004).

In such mice, expression of *Hoxd11*, *Hoxd12* and *Hoxd13* in anterior limb bud cells induced the ectopic transcription of *Shh* anteriorly, leading to double posterior, mirror-image distal limbs (Zakany et al 2004). This result demonstrated that the ectopic expression of 'posterior' *Hox* genes in anterior limb bud cells was able to induce another ZPA. Therefore, it strongly suggested that the normal ZPA, in particular *Shh* transcription, is under the control of these 'posterior' *Hox* genes, which are normally only transcribed in posterior limb bud cells. From these experiments, it appears that the anterior–posterior (AP) polarity of the limb buds is partly fixed by the restricted expression of *Hox* genes posteriorly, which in collaboration with factors released by the overlying ectoderm (AER) trigger *Shh* expression in the posterior mesenchyme. Therefore, a key step in our understanding of this polarity is to uncover the mechanism that restricts *Hox* gene expression in posterior cells.

Hoxd genes are activated in limb buds following multiple collinear strategies. Early on, in the incipient limb buds, genes are activated in a time sequence starting with the most 3′ located members such as *Hoxd1* and *Hoxd3*. These genes are expressed throughout the emerging bud, with a rather homogeneous expression observed up to *Hoxd9*. Starting from *Hoxd10*, however, the expression domains become progressively restricted to successively more posterior limb cells, until *Hoxd12* and *Hoxd13*, as a set of nested patterns (Dollé et al 1989, Nelson et al 1996). Therefore, two collinear processes can be observed in the early limb bud, in time and space, the former hypothetically controlling the latter.

After early limb budding has occurred, once *Shh* is transcribed, a second wave of *Hoxd* gene collinear activation takes place, in the presumptive autopod domain, i.e. in those cells fated to generate the hands and feet. This expression of the most 5′-located *Hoxd* genes is controlled by sequences located far upstream of the cluster (GCR; Spitz et al 2003), following regulatory modalities that have began to be uncovered (Kmita et al 2002).

The mechanism(s) of collinearity

The collinear mechanism(s) underlying the first wave of *Hoxd* gene activation in limbs, in both time and space, was recently studied (Tarchini & Duboule 2006). A previous deletion of this gene cluster indicated that the main corresponding

regulatory sequence(s) were localized outside the cluster itself (Spitz et al 2001). Subsequently, an engineered inversion of the same gene cluster revealed that the *Hoxd13* promoter, when placed at the position of *Hoxd1*, was expressed throughout the early limb bud, in a pattern related to this latter gene (Zakany et al 2004). These results indicated that the mechanism at work is promoter-independent and suggested that the progressive posterior restriction depends upon the mere position of a transcription unit within the cluster. They also led to the hypothesis that a critical element required for this collinear activation was located at the telomeric (3′) side of the cluster (ELCR; Zakany et al 2004).

To gain insights into this elusive early collinear mechanism, we produced and analysed of a set of mouse strains carrying a variety of deletions and duplications of parts of the *HoxD* cluster. These alleles were engineered using the targeted meiotic recombination strategy (TAMERE; Hérault et al 1998), starting with a selected set of parental lines such that breakpoints were readily comparable between various configurations. In these mice, gene topography was reorganized in many different ways, leading to important reallocations in their transcriptional controls during early limb budding. The analysis of such regulatory reallocations indicated that *Hoxd* gene collinearity in early limb buds is the result of two antagonistic regulations, implemented from either side of the cluster, which together establish the observed nested expression patterns in time and space (Tarchini & Duboule 2006).

The temporal aspect appears to be controlled by a sequence located telomeric to the *HoxD* cluster (ELCR), following a 'relative distance effect'. *Hoxd* genes located at the closest relative position (i.e. *Hoxd1*; *Hoxd3*) are activated first, whereas genes located at the other extremity of the cluster are activated last. This again seems to be promoter-independent and solely fixed by the genomic topography of a given gene. Therefore, the position of a given gene within the cluster will determine its timing of activation. However, the spatial collinearity is not solely determined by this timing process and also depends upon the existence of another, equally elusive, regulatory sequence located centromeric to the *HoxD* cluster, i.e. opposite to the ELCR. Here again, the relative distance between *Hoxd* genes and this sequence seems to be critical for the extent of posterior restriction (i.e. anterior suppression) in transcript distribution. Indeed *Hoxd* genes lying at the 5′ end of the series are strongly repressed in anterior limb bud cells whereas more 3′ located genes escape this repression and are transcribed throughout the limb bud (Tarchini & Duboule 2006).

Regulatory co-options

Regarding the evolutionary origins of these enhancer sequences, two alternative schemes—not exclusive from each other—can be considered. Firstly, a novel limb

enhancer sequence may have emerged and been selected outside the cluster. Alternatively, pre-existing regulatory modules, positioned outside *HoxD*, may have been co-opted for yet another functional output in parallel with limb evolution. As far as the early phase of collinearity is concerned, it is likely that the two opposite regulatory influences derive from the second kind of scenario. Several aspects of this phenomenon are indeed reminiscent of the regulatory strategy implemented during the formation of the major body axis (the trunk), raising the possibility that part of this ancient trunk collinear regulation was recruited into the context of the newly growing limbs. In particular, the existence of two types of collinearities, temporal and spatial, which can be somehow disconnected from each other (reviewed in Kmita & Duboule 2003), suggests that the collinear strategy used during trunk development relies upon opposite mechanisms, much like the process described above for the early wave of activation in limbs. This is supported by the preliminary survey of the effects of our set of deletions/duplications upon the timing and location of *Hoxd* and *Evx2* gene expression in the developing trunk. A detailed analysis of this particular aspect will be informative in this respect and may shed light on this fundamental mechanism.

Therefore, we speculate that the early collinear activation in limb was recruited from the trunk mechanism, allowing for the distal growth of an ancestral appendage up to the wrist area. Subsequently, a second global regulation evolved (GCR; Spitz et al 2003), also located outside the cluster, which was necessary to accompany the emergence of the autopods (hands and feet). The existence of distinct regulatory processes for the two waves of *Hoxd* activation in limbs is coherent with the proposal that the proximal and distal parts of our limbs have different phylogenetic histories. In this context, it is noteworthy that the mechanisms resembling those implemented during the development of the trunk may control the early and proximal *Hoxd* gene expression, i.e. at a time and in locations where *Hox* genes are necessary to build the 'ancient' proximal part, whereas an apparently newly evolved enhancer accompanied the emergence of digits, i.e. of a rather recent evolutionary novelty. In this view, the various types of regulatory innovations, and their distinct mechanisms of co-option, may tell us about the phylogenetic history of the structure (Duboule & Wilkins 1998).

The limb AP polarity: recruitment of a regulatory constraint

One important effect of the early phase of collinear activation is the restriction of *Shh* signalling to the most posterior margin of the limb bud (Zakany et al 2004, Kmita et al 2005). Since *Shh* signaling is a major factor in the establishment of the limb AP polarity (Riddle et al 1993), this polarity appears to be the morphological translation of the asymmetry in the expression of some *Hox* genes, as a result of their early collinear expression. Consequently, the limb AP polarity may reflect

nothing but a particular type of gene topography and its associated asymmetric regulations. Yet the major function of *Hox* genes in limb development is not to AP pattern the structure, but rather to trigger its growth, as the absence of *Hox* function leads to very severe truncations along the proximo-distal axis (Davis et al 1995, Kmita et al 2005). This apparent paradox suggests that the mechanism underlying the limb AP polarity did not evolve separately from, or in parallel with, the growth of the limbs. Instead, this mechanism was likely imposed as a collateral effect of the regulatory processes recruited to promote limb emergence and outgrowth.

In this view, an AP polarized limb is the expected consequence of using asymmetrically located enhancer sequences to control *Hox*-dependent outgrowth. Due to regulatory constraints imposed by the essential function of this gene family for trunk development, the co-option of this genetic system to promote limb development led to the impossibility to produce symmetrical limbs. During early trunk development, various combinations of *HOX* proteins are delivered at particular body levels, in specific cohorts of cells, which in turn will generate a given morphology. In tetrapods, axis formation and elongation are processes occurring along a time sequence; rostral structures are produced and determined before caudal structures. Therefore, it is crucial that the transcription of those *Hox* genes delivering 'caudal' information (e.g. *Hoxd13*) be postponed until the appropriate body level is produced, to prevent the premature formation of the caudal part of the body at a too rostral position. We believe this is the major evolutionary constraint maintaining temporal collinearity in vertebrates (Duboule 1994).

The co-option of this regulatory mechanism, along with the distal extension of appendages, transposed this repressive strategy into the context of growing limbs. As a result, 'caudal' *Hox* gene transcripts (e.g. *Hoxd11*, *Hoxd13*) are progressively restricted to the most posterior part of the emerging limb buds. Because these genes are able to activate and/or maintain the transcription of *Shh*, this later gene product became restricted to the posterior margin of the limb bud, hence generating an AP polarized structure. Therefore, our limb AP polarity reflects a major constraint that our body axis meets during its development and the regulatory solution that evolved to accommodate this constraint. In this scenario, the anterior to posterior polarity of our limbs merely results from the necessity to display caudal trunk structures at the extremity of our rostral to caudal major axis.

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DISCUSSION

Brakefield: I can quite accept the idea that the vertebrate limbs are fundamentally evolved through *bricolage* setting up the potential, but I see no paradox in thinking that what you are looking at is a beautiful adaptive trait, but one whose origin is in the type of effects that you are talking about. Setting up the potential and capacity is part of evolvability.

Duboule: Absolutely. The best example for me to mention is the expression in the developing digit, because this is our own work. I can admit that we attributed a high adaptative value to this pattern over the past 10 years. Ultimately, it turns out that it is a rather stochastic process that simply generates a pattern. We contemplate this pattern and try to give it a sense it may not have.

Brakefield: Evolution is so opportunistic that it just makes use of this *bricolage* capacity for building, and ends up with something that is extraordinarily functionally efficient.

Carroll: The main subject of your paper was mice, which is an amniote, a mammal. But how do you explain how salamanders, and an extinct group of amphibians from the Palaeozoic, the branchiosaurs, develop their fore and hind limbs from anterior to posterior rather than the other way round, but retain the adult structure, with the same phalangeal sequence, as the amniotes. This is a well known conundrum in living salamanders. In fact, we have superb fossils from the carboniferous, 300 million years ago, which preserve developmental sequences from hatchlings up to near metamorphosis, in which we can actually see the sequence across the metacarpals, metatarsals, and ulna, radius, tibia and fibula. So this is doing it the wrong way round all the way down the limb. This is a totally different mechanism of development, it is ancient, but it doesn't change the adult morphology. Can you explain this?

Duboule: When you say a reversal of structure, you mean the sequence of the cartilage condensation and ossification.

Coates: There is variability distally (in modern salamander limbs) but the chondrification and ossification is not always back to front, relative to the patterns in amniotes and frogs.

Duboule: I don't think this is necessary linked to the patterns of Hox gene expression I showed.

Carroll: It must be a different set of rules.

Duboule: It may be, but as far as I know, in all cases of amphibians that have been looked at, rather comparable patterns of Hox genes were observed. Even in fish pectoral fins, the same patterns are found at an early stage.

Wagner: Well that is not true for the distal Hox gene expression. In urodeles the Hox gene expression pattern is quite different from that in frogs and amniotes.

Also zebrafish does not seem to have a phase III expression as one finds in mouse and chicken.

Duboule: Of course. In the pectoral fin, the growth of the structure is interrupted at some point. We don't know why this is, but we proposed it is because of the fin fold structure, which prevents growth factors signalling from the epithelium to reach the mesoderm. But looking at the early fin bud, everything is there to produce a genuine limb. It is a mechanical problem: at some point the signalling doesn't go through. In salamanders, axolotls and some frogs, comparable distributions of both Hox genes and hedgehog were reported. The morphological result may be different, but at the end there is a polarity.

Hanken: You talked about the digit enhancer as being at a distance from the Hox genes. Has this been demonstrated yet outside the mouse?

Duboule: Yes, it is present in all tetrapods. It is extremely well conserved.

Hanken: I ask because Günter Wagner has argued, in light of the differences that Bob Carroll just mentioned, that the digits in salamanders may have evolved independently from those in other tetrapods. In other words, the tetrapod limb has evolved twice. If so, one might expect that we wouldn't see the exact same mechanisms underlying digit formation in all these groups.

Duboule: This enhancer sequence is large. It is a 40 kb piece of DNA that is incredibly conserved, and is present in all tetrapods. It is also present in *Danio* (zebrafish), coelocanths and tetraodon. The rate of conservation fits well with what we would expect. If the *Danio* enhancer is introduced into mouse though, we don't get expression in digits unlike if we use a tetrapod enhancer.

Hanken: The innovation you claim for tetrapods was not the evolution of the enhancer itself, because it is found in zebrafish.

Duboule: No, in zebrafish we find what we call the global control region (GCR). It is a 40 kb region that contains multiple enhancer sequences. Within this GCR we can see several boxes of conservation. One of them is digit specific. If you take the fish GCR you will find that this box is the least conserved, yet we can see some conservation. If you bring it into the mouse it doesn't work in digits. I would tend to say that this enhancer doesn't work in *Danio*.

Hanken: One of your opening slides showed that if you eliminate HoxA and D clusters you get a runt of a humerus. I noticed, however, that the scapula was fully formed. This is interesting.

Duboule: The piece of that is left, the scapula and mid humeral part, is roughly up to the deltoid crest. This corresponds to the part of the early limb bud that expresses a transcription factor called Meis1. This is a cofactor for Hox function and there is an antagonism between Meis1 and the posterior Hox genes. The interest here is that Meis1 is the gene in vertebrates that is orthologous to homothorax in insects. Homothorax is a gene that is expressed in the insect coxopodite. In insects, the limbs are composed of a sort of trunk extension, the coxa, and of a

most distal piece, the telopodite. Insect trunk extension is hedgehog-independent but homothorax-dependent. The early phase of Hox gene activation is hedgehog-independent and is Meis1-dependent. This is why in 2005 we proposed that there is a difference between the morphological and the genetic definitions of a limb (Kmita et al 2005), and the limb starts at the mid-humerus part. I had interesting discussions with morphologists about this! I would argue that this part of our limb is the remnant of the arthropod coxa. It is not a limb, but more a kind of a trunk extension.

Wagner: Is Meis1 working without an association with the 5′ Hox genes?

Duboule: Meis1 is associated with the 3′ Hox genes but its transcription is repressed by the 5′ Hox genes products.

Wagner: But Meis1 is physically interacting with A13, D13 and so on, isn't it?

Duboule: It has been shown by Miguel Torres and Juan-Carlos Izpisua-Belmonte that if Hox group 13 genes are overexpressed, Meis1 is down-regulated. There is a strong expression boundary between these two transcription factors.

Morriss-Kay: In terms of *bricolage*, what is the difference between a fish fin and a tetrapod limb? Is it a small change in the GCR? You said that the fish GCR won't make digits in the mouse, but it is present in the fish.

Duboule: My own view is that the fish GCR lost the capacity to work because there was a morphological transformation of the fin, with the folding of the ectoderm to allow for crest cells to come in and make the exoskeleton. This has a strong adaptative advantage in an aquatic environment. Reducing the endoskeleton and pushing the exoskeleton stops signalling from the ectoderm. This reduces the role of the endoskeleton to the first phase of Hox gene expression and thus reduces the function of hedgehog. It is still there, but it is no longer needed for the endoskeletal compartment

Wilkins: I have a comment on the philosophical considerations you raised rather than the details of the limb. I agree with your account of our idea of transitionism, and I still hold with it. I would add, however, that the best formulation of transitionism I have ever read was in a book that came out 14 years earlier, called *The Biology and Evolution of Language* by Philip Lieberman. He doesn't use the term transitionism, and his focus was morphological rather than molecular-genetic, but the same idea of cumulative small changes leading to qualitatively new properties when critical thresholds are passed is there. With regard to your last comments about *bricolage*, however, I have to say that I think they are not entirely correct because they leave out the population transformation aspect that is essential to evolution. There must be elements of *bricolage* created all the time in all populations through mutations that alter development. The ones that persist and are perpetuated, however, can do this either because they are neutral, and through drift persist, or because they enjoy a selective advantage. I don't think it is just Darwinian storytelling to attribute selective functions to these things. I would suggest that in the

particular case you have argued, there probably was some selective advantage in the AP symmetry that may have been created in just the way you claim. But, of course, that would be hard to test.

Wagner: You talked about how the global enhancer and the digit enhancer causes the polarity of the Hox expression in the digit region. How does this mechanism interact with the sonic hedgehog polarity? It gets its identity through hedgehog, so there must be some link between hedgehog, Gli3 and Hox gene activity.

Duboule: It has been shown that the Hox regulates hedgehog likely by direct binding to the hedgehog enhancer that is located a megabase away from the gene. On the other hand, you mentioned the Gli3 effector of the hedgehog signalling pathway. The only genetic mutants we have where this early collinear phase is clearly disrupted is indeed Gli3.

Wagner: How does the digit expression depend on sonic hedgehog?

Duboule: It doesn't depend on sonic hedgehog, but it is modified by it. If sonic hedgehog and Gli3 are removed, you get perfect Hox expression, although it is not polarized, as shown by John Fallon and Rolf Zeller's groups. Hox is repressed by Gli3 and sonic hedgehog de-represses this. But you don't need sonic hedgehog to activate Hox.

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