Tetrapod axial evolution and developmental constraints; Empirical underpinning by a mouse model

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A B S T R A C T
The tetrapod vertebral column has become increasingly complex during evolution as an adaptation to a terrestrial life. At the same time, the evolution of the vertebral formula became subject to developmental constraints acting on the size of the cervical and thoraco-lumbar regions. In the course of our studies concerning the evolution of Hox gene regulation, we produced a transgenic mouse model expressing fish Hox genes, which displayed a reduced number of thoraco-lumbar vertebrae and concurrent sacral homeotic transformations. Here, we analyze this mutant stock and conclude that the ancestral, pre-tetrapodial Hox code already possessed the capacity to induce vertebrae with sacral characteristics. This suggests that alterations in the interpretation of the Hox code may have participated to the evolution of this region in tetrapods, along with potential modifications of the HOX proteins themselves. With its reduced vertebral number, this mouse stock violates a previously described developmental constraint, which applies to the thoraco-lumbar region. The resulting offset between motor neuron morphology, vertebral patterning and the relative positioning of hind limbs illustrates that the precise orchestration of the Hox-clock in parallel with other ontogenetic pathways places constraints on the evolvability of the body plan.

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1. Introduction
The vertebrate spine is built as a sequence of serial homologous elements, the vertebrae, which develop with different morphologies at different positions along the anterior–posterior axis. Various vertebral formulae reflect both the requirements and the constraints associated with a skeleton that needs to accommodate protective, respiratory and locomotor functions (Romer, 1956; Woltering, 2012). The differentiation of initially similar somites into distinct type of vertebrae (i.e. cervical, thoracic, lumbar, sacral and caudal in mammals) is established early on during embryogenesis, mainly due to a collinear pattern of Hox gene expression (Knita and Duboule, 2003) along the antero-

Posterior axis (Casaca et al., 2014; Deschamps and van Nes, 2005; Mallo et al., 2010; Wellik, 2009). These coordinated expression patterns indeed generate various combinations of HOX proteins at distinct body levels (or a ‘Hox code’ (Kessel and Gruss, 1991)), which genetically instruct somites about their fates in terms of morphology. In addition, the correspondence between particular combinations of HOX proteins and critical morphological transitions are maintained throughout tetrapods, suggesting an instructive role for these proteins in setting up these boundaries (Burke et al., 1995; Gaunt, 1994). However, the fact that various HOX proteins display some functional hierarchies in these processes makes a pure combinatorial system unlikely (see Duboule and Morata, 1994).

The evolution of land vertebrates was paralleled by an increasing complexity of axial regionalization, as an adaptation to a terrestrial lifestyle: the sacrum evolved during the fish–tetrapod transition as a connection between the pelvic girdle and the axial skeleton (Carroll and Holmes, 2007), whereas the lumbar region first appeared in mammals as an adaptation to sagittal flexion during locomotion as well as to accommodate the diaphragm (Carroll, 1988). In mammals, the sacral and lumbar regions are genetically characterized by the transcription of Hox genes belonging to paralogy groups 10 and 11 (Hox10 and Hox11 genes), evolutionary related to the insect posterior gene Abd-B (Izpisua-Belmonte et al., 1991). Functional approaches have revealed that HOX10 proteins suppress rib formation whereas HOX11 proteins can induce sacral
processes (Carapuco et al., 2005; Wellik and Capecci, 2003). Interestingly, this collinear distribution of Hox transcripts predates the origin of vertebrates and fish for instance already express Hox10 and Hox11 genes in paraxial mesoderm, in spite of the absence of both sacral and lumbar regions (Oulion et al., 2011; Prince et al., 1998; van der Hoeven et al., 1996). Therefore, it is likely that these particular region-specific morphologies did not arise through mere changes in Hox gene expression domains. Instead, they may have involved concomitant alterations in the activation of downstream target genes, for example via modification in the interpretation of the ‘code’ either following changes in the cis-regulatory modules controlling these targets, or due to changes in the HOX proteins themselves. Altogether, it is currently unknown whether the emergence of these particular body regions involved a simple exaptation of a pre-existing Hox pattern, or if it was accompanied by essential structural changes in HOX proteins leading to novel functions.

Regardless of which evolutionary mechanism leads to such critical modifications of the tetrapod spine, its realm of action was likely reduced due to the strong developmental constraints applied to the axial formula. The existence of developmental constraints applied to the organization of segmental patterns in animals was recognized more than a century ago, by the mere observations of natural ‘rules’ governing the formation of metamereized body plans (see for example the work of Lankester, described in Jeffs and Keynes, 1980). Nowadays, such constraints or limitations in the evolution of otherwise potentially adaptive traits are thought to derive in part from the way the underlying regulatory processes are implemented and shared between various developmental contexts, leading to severe pleiotropic effects at least in vertebrates (Duboule and Wilkins, 1998; Kirschner et al., 2005).

The comparative analysis of vertebral columns provides many instances of such canalized processes (Asher et al., 2011), as for example the well-known constraint that fixes the number of cervical vertebrae to seven in all mammals but manatees and sloths (see e.g. Galis, 1999; Galis and Metz, 2007; Varela-Lasheras et al., 2011), even though natural selection favored in some instances either the increase or the decrease in neck length, as in giraffes and whales, respectively. In such cases however, variations occurred through changes in the sizes of vertebrae rather than in their number. It was suggested that this constraint was generated by a potential interference with the migration of the diaphragm muscles, thus leading to an impaired respiration (Buchholtz et al., 2012; Hirasaawa and Kuratani, 2013). Likewise, in the thoraco-lumbar region, a constraint seems to restrict the overall number of vertebrae to 19 or 20 in most mammals (Narita and Kuratani, 2005), perhaps associated with the proper implementation of locomotor mechanisms (Buchholtz, 2014; Galis et al., 2014).

Unfortunately, even though the mechanisms underlying both the time-sequence production of somites (Pourquie, 2003) and the concurrent progressive activation of Hox genes (Noordermeer et al., 2014) start to be understood, evolutionary scenarios accounting for the macroevolution of the axial skeleton remain complex to address experimentally and lack empirical support. In this study, we investigated the phenotypic abnormalities in a transgenic mouse containing a HoxAa BAC from the pufferfish (Tetraodon nigroviridis) genome. This transgenic line was produced in the aim of studying inter-gate the phenotypic abnormalities in a transgenic mouse containing HoxAa cluster and containing from HoxA9a through HoxA13a (Woltering et al., 2014). Three F0 males were obtained, which all showed locomotory incapitation of the hind limbs (paraplegia), as well as a trunk shortened along the anterior to posterior axis (Fig. 1A, Supplementary movies 1–2). One male died shortly after weaning and the cadaver was lost; a second male died without any apparent pathological cause, at approximately two years of age, but never reproduced; the third male proved capable of reproducing and was used to establish a line through natural mating. As further paternal transmission of this transgenic condition was never achieved in this line, it was maintained through hemizygous maternal crosses. In addition to the male reproductive defects, the problems in hind limb coordination with an abnormal gait caused by (partial) hind-limb paralysis persisted in this line. Adult animals improved in this respect after 5 months of age. To try and understand the etiology of these various phenotypes, we initially analyzed these mice for potential skeletal and/or neural abnormalities. Alizarin red-alcian blue staining in newborns and adults revealed major homeotic transformations in the posterior trunk, including a large anterior shift of the sacrum leading to a reduction of the lumbar region from usually six (sometimes five) lumbar vertebrae in wild-type mice, to only three lumbar vertebrae in the transgenic condition. In addition, the second and third lumbar vertebrae (L2 and L3) showed partial sacral transformation, as shown by clearly broadened lateral processes (Fig. 1B, C, Supplementary Fig. 1). This phenotype was also scored in the skeleton of the second F0 male, for which no line could be established (Fig. 1B [tni HoxAa#2]). The early innervation pattern of the hind limbs was investigated in 12.5 days old fetuses (E12.5), using immune-staining of neurofilaments (Fig. 1D). In transgenic mice, an abnormal truncation of the peroneal nerve, which innervates the dorsal aspect of the hind limbs, was observed consistent with the locomotory abnormalities detected in these mice. Both the neuronal and reproductive phenotypes proved very similar to those observed for the loss of function of Hox10 group genes. These latter mutants indeed display a misspecification of the sciatic part of the lumbosacral plexus, which normally innervates the hind limbs (Carpenter et al., 1997; Tarchini et al., 2005; Wu et al., 2008).

2.2. Gain of function of Tetraodon Hoxa11a

In order to associate the phenotypic abnormalities observed in the transgenic line with the potential expression of the transgenic Tetraodon Hox genes present in the BAC, we performed in situ hybridization for the Tetraodon Hoxa9a to Hoxa13a genes (Fig. 2). Interestingly, these genes were expressed during mouse development with the expected spatial collinear pattern along the main body axis, with Hoxa9a being expressed most anteriorly and Hoxa13a being confined to the posterior tail region. Comparison with the expression of the endogenous mouse Hoxa genes however, indicated the presence of transgenic Hoxa11a transcripts at a too anterior position, i.e. about three to four somites more anterior than the corresponding pattern for the mouse Hox11 gene. Such an anteriorized transcriptional pattern associated with a Hox11 gene was previously reported, associated with the replacement in vivo of an endogenous Hox11 enhancer by its telost counterpart. Mice carrying this fish enhancer at the correct endogenous position expressed their own Hox11 gene too anteriorly (Gerard et al., 1997). Therefore, an anterior gain of function of the Tetraodon Hoxa11a gene as reported here may reflect a specific cis-regulatory difference between fish and mammals, as somewhat supported by the high divergence in non-coding DNA sequences between the fish and tetrapods posterior...
HoxA clusters (Supplementary Fig. 2A). This anteriorized expression was equally observed at earlier stages (E10.5). In such embryos, the transgenic Hoxa11a gene was expressed more anteriorly than the three mouse paralogous genes Hoxa11, Hoxc11 and Hoxd11 (Fig. 3A).

This ‘anteriorized’ Hoxa11a transcript domain coincided in space with the observed sacral homeotic transformations of the lumbar region. In addition, the morphological transformations, which were scored precisely within this very region where Hoxa11a was gained, gave the expected phenotype for an ectopic expression of a tetrapod Hox11 group gene. Hox11 genes indeed determine sacral vertebral identity (Wellik and Capcchi, 2003) and their expression at ectopic anterior positions was shown to produce sacral transformations (Carapuco et al., 2005; Gerard et al., 1997). Likewise, the ectopic expression of Hoxd11 induced the mis-specification of the motor neurons innervating the dorsal aspects of hind limbs (Misra et al., 2009), a nerve pattern normally specified by Hox10 genes (Carpenter et al., 1997). Noteworthy, the spatial expression patterns of the endogenous Hox11 group genes were not noticeably modified in transgenic embryos (Fig. 3A), ruling out a potential auto-regulatory interaction between Hox11 proteins and their own promoters as a cause of the observed phenotypic alterations. Therefore, we concluded that the mis-expression of the Tetraodon Hoxa11a gene was directly responsible for the severe abnormalities detected in these transgenic animals. In this view, the anteriorization of the axial Hox11 domain, due to the expression of the transgenic Tetraodon Hoxa11a gene, induced both neuronal and vertebral homeosis.

2.3. Hind-limb bud formation and Hox-mediated axial patterning

The anterior shift of the sacrum observed in our Tetraodon Hoxa11a transgenic line was accompanied by a more anterior positioning of the hind-limbs along the body axis, as illustrated by the positioning of the hip joint (Supplementary Fig. 1). In newborn mice, an evaluation of the approximate hind-limb position indicated a two to three somites offset when compared to wild type specimen, consistent with the extent of the shift observed for the sacrum and thus suggesting that the entire posterior part of the animal had been shifted anteriorly. The position of forelimb buds seems to be determined by the combination of HOX proteins found in the lateral plate mesoderm (Nishimoto et al., 2014). Also, hind limb buds were shifted two somites backwards in mice mutant for multiple group 9 Hox genes (McIntyre et al., 2007). Despite these reports, however, our observation was unexpected since the determination of hind limb positioning along the trunk was recently suggested to be a Hox independent process (Jurberg et al., 2013), as assessed by both loss- and gain of function approaches. In this latter case, even the Hox11 gene was unable to shift hind limb buds anteriorly when overexpressed at an early stage (Jurberg et al., 2013). Potential...
reasons for this apparent discrepancy such as differences in the strength of transgene expression in the lateral plate mesoderm (Fig. 3A) are discussed below.

We further investigated if any alteration in hind limb positioning could already be observed at the stage of limb bud formation (E10.5) and hence whether additional evidence could be found to document a direct link between hind limb induction on the one hand, and the sacral 'Hox code', on the other hand. To better evaluate this parameter, somites were visualized by using DAPI staining in combination with fluorescent in situ hybridization for the MyoD transcripts (Hebrok et al., 1997). Analysis of E10.5 transgenic embryos showed that the hind limb buds developed at an axial position comparable to the wild type situation, i.e. at around somite level 25 (Fig. 3B). Therefore, the anteriorized positioning of hind limbs in adult mice was likely the mere manifestation of an anterior shift of the entire sacral region, rather than of a local change in the positioning of the limb field, the territory from where limbs bud out of the lateral plate mesoderm.

It has been noticed (e.g. Burke et al., 1995) that hind limbs initially emerge at a body level corresponding to lumbar somites. Subsequently, these buds adopt a more caudal position, at the level of the sacral somites, where they will become attached through the pelvic-sacral connection. This relative shift of the buds with respect to the somites may reflect a more general offset between paraxial and lateral plate mesoderm in the growing embryo. The possibility thus exists that the sacrum itself provides a cue for an arrest of this relative posterior shift of the hind limbs. In such a case, animals displaying a genetically 'sacralized' lumbar region may concomitantly show anteriorized hind limbs, due to a premature termination of this relative transition. In this view, the more anterior location of hind limbs in adult mice would be an indirect result of repositioning the sacrum, rather than be caused by the direct influence of ectopic Hoxa11 upon hind limb bud induction.

This anteriorization of the hind limbs becomes apparent at E11.5 already, when neuro-filament staining revealed an axial position corresponding to the innervation of spinal nerves 22 to 24 in transgenic

![Fig. 2. Expression of mouse HoxA and Tetraodon HoxA genes in either wild type or tni HoxAa mice. In situ hybridization was performed on E11.5 wild type and transgenic embryos (genotypes are indicated on the right hand side). Wild type embryos were analyzed with mouse specific probes to visualize the expression of the endogenous Hox genes and with Tetraodon specific probes to exclude potential cross reactivity with the endogenous mouse genes. Tni HoxAa transgenic embryos were processed for Tetraodon specific probes. Probe names are indicated above as well as whether the probe used was for a wild type or transgenic specimen (indicated on the left hand side). The expression pattern of the Tetraodon probes in the transgenic context shows the expected collinear pattern with Hoxa9a being expressed most anteriorly (although not as far as the endogenous mouse Hoxa9) and Hoxa13a restricted to the posterior most tail. There is however marginal differentiation, if any, between the anterior expression limits of Hoxa10a and Hoxa11a. A clear difference is observed in expression between mouse Hoxa11, which has an anterior expression limit close to the posterior limit of the hind limb buds, and the Tetraodon Hoxa11a, that has a limit around three to four somites more anterior, coinciding with the anterior limit of the hind limb buds (the anterior level of axial expression in both wild type and tni HoxAa panels is indicated with a dotted line). This particular area where the difference is observed is the part of the body where sacral transformations are scored (Fig. 1). The lower row shows that there is no cross reactivity in the in situ hybridization between the Tetraodon probes and the endogenous mouse genes.](image)
mice (Fig. 3C), whereas spinal nerves 24 to 26 are involved in wild type littersmates. This observation also illustrated a shift in the positioning of the sciatic plexus along the axis. This shift was accompanied by the absence of the peroneal branches of the newly specified sciatic nerve, similar to the effect of Hoxd10 loss of function (Carpenter et al., 1997; Tarchini et al., 2005). In this case, it is possible that, due to its new anterior position, the sacrum became located outside the territory of Hoxd10-dependent motoneurons thus leading to a position-dependent loss of function within those neurons entering the limb bud.

3. Discussion

3.1. Exaptation of the Hox code; ancestral versus novel regulation

Even though the concept of biological complexity is delicate to handle in many respects (Carroll, 2001; Hall, 1999), vertebrate evolution tends to show a trend towards an increasingly complex body plan. In this context, modifications in Hox gene regulation and function have been invoked as potential causes to the origin of morphological novelties (e.g. Head and Polly, 2015; Rijli et al., 1993; Sordino et al., 1995; Wagner et al., 2003).
Within such a conceptual framework, two distinct regulatory levels can be identified where important changes in the function of Hox genes may have occurred. The first case is well illustrated by neomorphic structures such as digits and external genitals, which evolved concomitantly partly via the recruitment of novel Hox expression domains (Dolle et al., 1991; Montavon et al., 2008). These regulatory specificities were acquired through both the emergence of new enhancer sequences and the co-optation of regulatory sequences already used for another purpose (Lonfat et al., 2014; Montavon et al., 2011). In such cases, morphological novelties coincide with regulatory innovations, even when the latter are based upon pre-existing modules (Lonfat et al., 2014; Woltering et al., 2014). Likewise, modifications in the organization of the spine may have relied upon the mere displacement of Hox expression boundaries, as illustrated by the different positions of the cervical–thoracic transitions in mammals and birds (Burke et al., 1995; Gaunt, 1994).

In an alternative – yet not exclusive – scenario, morphological novelties can evolve not only as the result of new regulatory modalities but, rather, through different responses from the systems downstream of Hox control, for example via the loss or gain of target genes. For instance a modification in the response of a target gene following changes in binding sequence can lead to Hox-derived phenotypic alterations (Guerreiro et al., 2013). Also, variations in the Hox protein structures could potentially lead to important quantitative and/or qualitative modifications in large sets of target loci, as shown for example in insects where a modification of the Ubx protein is thought to have accompanied the emergence of the hexapod body plan (Ronshaugen et al., 2002). Interestingly, changes in the Hoxa11 coding sequence were shown to have paralleled the evolution of pregnancy in mammals (Lynch et al., 2008). In addition, sequence analyses of this gene in various species showed potential signatures of adaptive sequence change across the fin-to-limb transition (Chiu et al., 2000). The comparison between the mouse HOXA11 and Tetraodon HOXA11A sequences indeed shows a very strong conservation of the homeodomain peptide sequence, whereas the N-terminal parts of the proteins are much more divergent (Supplementary Fig. 28). However, despite this divergence in protein sequences outside the homeodomain, the pufferfish Hoxa11a protein was clearly capable of inducing the sacrum, a well-defined tetrapod novelty and hence this protein already had the capacity to control sacral characteristics well before the evolution of a sacrum. Consequently, modifications in its coding sequence were likely not necessary for this functional expatation.

How other features associated with the vertebrates evolved or disappeared, and to which degree the above patterning concepts may provide explanatory frameworks for these key events remains to be investigated. Structures that would be of particular interest in this context would be for example the occipital–syringal complex found in cartilaginous fishes and extinct placoderms (Davis et al., 2012; Soshnikova et al., 2013), the ostarriphysan Weberian vertebrae (Bird and Mabee, 2003), the teleost specific homocercal caudal fin vertebrae (Moriyama and Takeda, 2013), the anuran urostyle (Rokkova and Rocek, 2005), the snake’s forked lymphapophyses (Woltering, 2012) or the enigmatic interlocking lumbar vertebrae of hero shrew (Stanley et al., 2013).

It is also noteworthy that the general principle of collinear distribution of Hox expression domains along the rostro-caudal body axis observed in vertebrates (Gaunt et al., 1988) largely predates their appearances (Duboule and Dolle, 1989; Graham et al., 1989; Lewis, 1978) and thus could not be initially associated with the evolution of a complex axial skeleton. It is possible that the Hox system was originally linked to the organization of either neural (Deutsch and Le Guyader, 1998) or endodermal (Kondo et al., 1996) structures along the AP axis, and was subsequently recruited by mesodermal derivatives due to its capacity to specialize particular body segments. From there onwards, modifications in either the regulation and structures of these genes, or of their specific targets (either at the regulatory or at the functional level) may have produced the variety of vertebral formulae known today in extant vertebrates and in the fossil record.

3.2. The Hox constraint on the body plan

This diversity in vertebral formulae is however not infinite and the observed anatomical bias towards certain prototypes at the detriment of others, is hard to explain on pure adaptive grounds. Therefore, it is likely that developmental constraints restrict the realm of possibilities for a vertebral column to combine and associate various vertebral types, both in their number and qualities (Asher et al., 2011). The identification of the developmental processes associated with these constrained morphologies has been problematic and without empirical support. The mouse line we describe in this study displays a lumbar region with only three vertebrae without a concomitant increase in the number of thoracic vertebrae. As such, it ‘violates’ the thoracolumbar constraint identified by Narita and Kuratani (2005) and may help thinking about both the nature of the underlying constraints and the mechanisms involved.

In our mutant mice, the reduction in the number of thoracolumbar vertebrae is caused by the anterior expression of the transgenic Hoxa11a gene, presumably as a result of a different interpretation of cis-regulatory information between the fish and the mouse contexts as previously noted (Gerard et al., 1997). The resulting transformation of the posterior lumbar region into a sacrum likely induced a morphological offset between structures and/or cell types, leading to the severe phenotypic condition observed. In the case of hind limb positioning, this offset seems to be compensated for, to some extent, since the final position of the hind limbs is in register with the anteriorized sacrum. This could be due to an instructive relationship between both structures, the sacrum helping to position the hind limbs. Alternatively, the strong gain of function of Hoxa11a observed in the lateral plate mesoderm may directly participate in the rostral positioning of the hind limbs, even though Hoxa11 was apparently not able to achieve a similar result in another experimental context (Jurbeg et al., 2013).

In contrast, the differentiation of the appropriate columns of motor neurons innervating the limbs, which is greatly influenced by Hox gene expression in the developing spinal cord (Jung et al., 2014), may not have been equally influenced by our gain of function. As a result, the motor neurons innervating the mutant hind limbs may originate from a territory of the spinal cord lacking the proper combination of Hox proteins, in particular HoxD10, the targeted or spontaneous mutation of which generates similar paraplegia phenotypes (Carpenter et al., 1997; Misra et al., 2009; Tarchini et al., 2005; Wahba et al., 2001). This case potentially illustrates the result of interfering with the collinear distribution of Hox proteins, having Hoxa11a transcripts abruptly produced more anteriorly (or at the same body level; see Fig. 2) than the native Hoxd10 mRNAs. The resulting compromised locomotion and accompanying reproductive incapacitation would of course be strongly selected against under natural conditions.

3.3. Mechanisms and constraints underlying the phylotypic progression

In vertebrates, the general body architecture (Bauplan) materializes during late gastrulation, where all vertebrate embryos tend to share important morphological features (the zootype). This ‘phylotypic stage’ was associated with the expression of particular transcription factors, including Hox genes (Slack et al., 1993). It was subsequently argued that early embryos, regardless of their various modes of gastrulation had to ‘converge’ towards this particular body plan to set the general ground from which different adult morphological traits can be subsequently derived. This ‘Hourglass model’ (Duboule, 1994) implies that embryos progress through a short period (the phylotypic progression) where the underlying developmental mechanisms must be maximally constrained, thus making alternative solutions impossible. While the existence of the developmental hourglass has been recently documented in a variety of contexts (see e.g. Irie and Kuratani, 2014) but also (Richardson, 1995) the nature of the constraints responsible for the phylotypic progression (into the bottleneck of the hourglass) is elusive.
Initially, two kinds of constraints were proposed: On the one hand, constraints based on meta-trans regulations (Duboule, 1994), i.e. due to particular interactions between networks of genes, necessary at this stage to properly set up the body plan (Raff, 1996). On the other hand, a single mechanism may underlie the passage through the hourglass bottleneck, provided that this mechanism is invariant and requires a particular context to be implemented. In this view, early meta-trans regulations are necessary to bring the developing system into a point where this invariant mechanism can now operate, during the phylotypic period. The fact that this period covers the extension and patterning of the rostro-caudal axis suggested that genetic mechanisms at work to orchestrate these critical steps might be particularly constrained, for some reasons. Hox genes are the major players in the patterning of the body axis (see Mallo et al., 2010) and possibly in its extension (Denans et al., 2015; Di-Poi et al., 2010; Young et al., 2009) and their sequential activation (temporal collinearity) relies upon a meta-cis mechanism (the Hox clock) that appears difficult to evolve as it relies upon a process that reads the linearity of DNA at the Hox loci (see Noordermeer and Duboule, 2013). On this ground, the Hox clock was proposed as the major constraint acting during the phylotypic progression (Duboule, 1994). Subsequently, the Hox clock was reported to closely interact with the segmentation clock, the mechanism whereby the vertebrate body becomes segmented (Palmeirim et al., 1997). Hox genes indeed can be regulated as a read out of the segmentation clock (Zakany et al., 2001) and, conversely, the amount of caudal non-segmented mesoderm available as substrate for the segmentation clock may be regulated by the Hox clock (Denans et al., 2015). Therefore, a perfect coordination between these two precise mechanisms must be secured and hence the vertebrate embryo may have to converge towards this point where both clocks will click in concert for about two days, before the system relaxes, giving more opportunities again for variable interactions between gene networks.

Offsets between these two clocks can naturally lead to the variety of vertebral formulae found in vertebrates. Here, we show an example of how the anteriorization of a single Hox11 group gene can lead to a complete reorganization of the spine. However, this gain of function did not respect the constraint applied to the system, as it was not accompanied by a gain of function of the entire set of Hox genes, bringing Hoxa11a expression at the rostral position of Hox10 genes. While the effect on the spine may not have been in itself a cause of counter selection, the interactions of body parts with the spine was affected in spite of some intrinsic re-organization, such as the anteriorization position of the hind limbs. This suggests the existence of developmental check-points where the relative connections between various structures can be adjusted. The hind legs were nevertheless not properly innervated, likely due to the absence of Hox10-positive/Hox11-negative motor neurons, which are required for the peroneal branch of the sciatic nerve (Carpenter et al., 1997; Tarchini et al., 2005).

It has also been argued that high developmental constraints may apply at the phylotypic stage due to the necessity to coordinate interactions between the various nascent tissue types (Galis and Metz, 2001; Raff, 1994; Sander, 1983). In other words, to produce a coherent organism, tissues that differentiate within spatially segregated embryonic domains during gastrulation (such as for instance neurons, vascular tissues that differentiate within spatially segregated embryonic domains) are required for the proper functioning of the body plan. These processes are not naturally interconnected with one another, which may re-enforce the constraints acting at this stage upon the evolvability of the body plan.

Claudes outside mammals can show high diversity in both the number of pre-caudal vertebra and the positioning of the posterior paired appendages along the body. In this regard, the teleost fish are particularly interesting as they can have their pelvic fins positioned as far anterior as their pectoral fins (Murakami and Tanaka, 2011; Murata et al., 2010; Tanaka, 2011). In fishes the initial position of the pelvic fins is specified by GDF11, as in vertebrates (see Jurberg et al., 2013), generally at the position of the trunk to tail transition (Murata et al., 2010). In some species, however, the pelvic fin buds subsequently migrate towards the anterior, along the trunk. The appropriate innervation of these anteriorly displaced pelvic fins occurs through locally exiting motor neurons, which are thus apparently rather independent from the combination of Hox genes they express (Murakami and Tanaka, 2011; Murata et al., 2010; Tanaka, 2011), perhaps due to a lesser complexity in the realm of movements implemented by these fins. This great flexibility in the fish body plan may be accounted for by a lower interdependence between specific motor neurons and hind limbs such that fishes may not need to tightly orchestrate the connection between a specific set of neurons and the pelvic fins to secure a proper functional outcome. The evolution of the sacrum and of a more generic connection between the posterior appendages and the spine, may have introduced yet another strong constraint, thus further decreasing the evolvability of the body plan.

4. Materials and methods

4.1. Mouse strains

The mouse tni HoxAa stock (Woltering et al., 2014) was generated by pronuclear injection following well-established procedures. All experiments were performed in agreement with the Swiss law on animal protection (LPA) with the appropriate legal authorization to D.D. Because of the severity of the phenotype, this transgenic line is no longer maintained as living animals.

4.2. In situ hybridizations

Whole mount in situ hybridizations using mouse and Tetraodon probes were performed as described previously, using 1.3 × SSC concentration in the hybridization buffer to prevent cross reactivity between mouse RNAs and Tetraodon probes (Woltering et al., 2009, 2014). Unpublished probes for Tetraodon Hoxa9a and Hoxa10a correspond to sequences within exon 1 and were cloned from BAC DNA using the following primers:

\[ a9a-FW: \text{ATGTGACATCCGGAACGCTG} \]
\[ a9a-RV: \text{TTGATCGAGGCCTGGCTGCAC} \]
\[ a10a-FW: \text{ATGCGATGTTCGGACACCC} \]
\[ a10a-RV: \text{CTTGGGGCCTGTTGCTGAC} \]

The mouse Hoxc11 probe was cloned from genomic DNA using the following primers:

\[ FW: \text{AACCAGACGAGCTGGATTTC} \]
\[ RV: \text{AGACTAAGCGATAACCGG} \]

Fluorescent in situ hybridization for the MyoD probe (Hebrok et al., 1997) was performed using staining with Fluorescein Thyramid Amplification System (Perkin-Elmer). Antibody staining for neurofilaments was carried out as described previously (Tarchini et al., 2005) using anti-NF160 (clone NN18, Sigma N-5264) and anti-mouse Ig Fab HRP conjugate (Sigma A-3882). For DAPI staining, embryos were incubated with 0.1 mM DAPI in TBS-T after in situ hybridization. Whole mount in situ images in Figs. 2 and 3 were constructed as overlays of gray-
scale DAPI fluorescence images and bright field images using Adobe Photoshop.

4.3 In silico sequence analysis

Analysis of non-coding regions in the Hoxa clusters was done using LAGAN-VISTA (http://genome.lbl.gov/vista/lagan/submit.shtml) (Frazer et al., 2004). Alignment of HOXA11 and HOXA11A proteins was done at EMBL (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Conserved domains were predicted using NCBI’s conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al., 2015). Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mod.2015.07.006.

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References


