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Delineating the anuran axial skeleton

SARA S. SÁNCHEZ*,[1] and ROMEL S. SÁNCHEZ[1,2,3]

[1]Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, [2]Cátedra de Biología General, Facultad de Ciencias Naturales e Instituto Miguel Lillo and [3]Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT. Cátedra de Fisiología, Departamento Biomédico-Fisiología, Facultad de Medicina, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina

ABSTRACT The axial skeleton of the *anurans* has undergone an evolutionary reduction of its bone elements. This structural plan is strongly preserved throughout the order and would have emerged as a highly specialized anatomical adaptation to its locomotor jumping pattern. The development programs that direct the vertebral morphogenesis of the anurans are poorly described and the molecular bases that have caused their pattern to differ from other tetrapods are completely unknown. In this work, we review the ontogeny of the spinal column of the anurans and explore the genetic mechanisms that could explain the morphological difference and the maintenance of the body plan during evolution. Here, we propose that the absence of caudal osseous elements, as a consequence of the inability of sclerotomes to form cartilaginous condensations in frogs, could be due to changes in both pattern and expression levels of *Hox, Pax1, Pax9* and *Uncx4.1* genes along the anteroposterior axis. The anteriorised expression of the *Hox* genes together with the reduction in the expression levels of *Pax1, Pax9* and *Uncx4* in the posterior somites could explain, at least partly, the loss of caudal vertebrae in the anurans during evolution.

KEY WORDS: *anurans, Xenopus, vertebral column, sclerotome, pax1, pax9, uncx, Hox*

The vertebral column of anurans

The vertebral column is a crucial structure provides both stability and mobility of vertebrate's body. This role is possible thanks to its segmental nature that gives it a certain degree of flexibility to adapt to locomotion.

The vertebral column of anurans diverges widely from that of other tetrapods. One of the most evident morphological specializations in this order is the evolutionary reduction in the skeletal elements of the vertebral column (Fig. 1A). In the closest nonanuran ancestor, the amphibian *†Triadobatrachus massinoti*, the axial skeleton consisted of at least 21 vertebrae, including 14 pre-sacral, 1 sacral and 6 exposed caudal vertebrae (Rage *and* Rocek, 1989). The earliest frog fossils currently known date back to the Early Jurassic period and it has been shown that they acquired their peculiar body plan at an early stage of their evolutionary history. In the fossil frog *†Vieraella herbsti*, the pre-sacral complement was reduced to ten vertebrae and the post-sacral skeleton to urostyle, while in *†Notobatrachus degiustoi* and the pre-sacral skeleton was formed by only nine vertebrae (Báez *and* Basso, 1996). This morphology is almost perfectly conserved among extant anurans and has undergone few modifications in the intervening 200 million years. In extant anurans, there are no more than nine pre-sacral vertebrae (with the exception of *Ascaphus truei* with nine) followed by a single sacral vertebra and, post-sacrally, the urostyle (Lynch, 1973) (Fig. 1B). However, during late ontogeny, the number of vertebrae can be reduced even more. In pipids and bufonids, the first and second pre-sacrals may be fused or the last pre-sacral may fuse with the sacrum, thus reducing the number of pre-sacral vertebrae from eight to seven or even six (Pugener, 2002).

The reduced number of vertebra has been attributed to two developmental processes: the fusion of discrete vertebrae or vertebral rudiments during late ontogeny and the loss of the vertebral-forming capacity by caudal somites during early ontogeny (Handrigan and Wassersug, 2007). During early morphogenesis of vertebral column of the pipid *Xenopus laevis* are formed XII vertebrae; however, the adult has the axial formula (1-7-1-1). That is, a cervical vertebra (atlas or pre-sacral I), seven post-cervical

Abbreviations used in this paper: LSF, lateral somitic frontier; MSC, multipotent somitic cell; Myo, myotome; N, notochord; NP, neural plate; NT, neural tube; PSM, presomitic mesoderm; Scl, sclerotome; Shh, sonic hedgehog.

***Address correspondence to:** Sara S. Sánchez. 1Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco 461, San Miguel de Tucumán (T4000ILI), Argentina. Tel /Fax: 54-381-4107214 ext 111. E-mail: ssanchez@fbqf.unt.edu.ar - https://orcid.org/0000-0001-6698-3095

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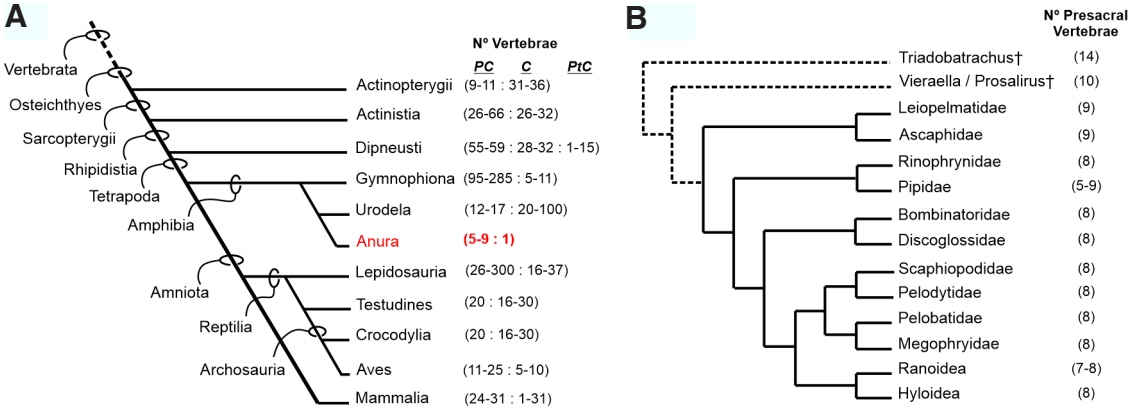

Fig. 1. A partial phylogeny of the Vertebrates and Anura *(adapted from Handrigan and Wassersug, 2007; Montero and Autino, 2009).* **(A)** *Number of precaudal and caudal vertebrae in the different groups of vertebrates, including coelacanths, bone fish, lungfish, caecilians, urodels, anurans, scaled lizards, turtles, birds and mammals. The axial skeleton of the anurans showed a big reduction of its bone elements.* **(B)** *Number of presacral vertebrae in the different groups of Anurans. The number of presacral vertebrae varies from 5-9 in the different groups. The caudal axial skeleton is represented by the urostylo. PC, pre-caudal; C, caudal; PtC, post-caudal.*

vertebrae (pre-sacrals II - VIII), a sacral vertebra (sacrum), and post-sacrally, the urostyle. This reduction of bone elements occurs due to the fusion of post-sacral vertebrae. Once the ossification has begun the X-XII post-sacral vertebrae fuse together to form a single structure. Finally, this block vertebrae fuses with the ossified hypochord that lie ventrally to form the urostyle (Trueb and Hanken, 1992).

The anuran truncated axial skeleton and the loss of tail (along with other features such as hinge-like ilio-sacral articulation, large hind limb muscles, reduced fore limbs and a fenestral skull) have been considered as key anatomical adaptations to the anuran locomotor pattern of saltation (Emerson, 1988; Pugener and Maglia, 2009). Handrigan and Wassersug (2007) have described the rationale for the fact that anuran saltation is more efficient with a short-compact torso and tailless. Briefly, the longest distance any large mass projectile will travel occurs with a take-off angle of approximately 45°, which can be achieved more easily when the body is short. A longer torso would need proportionally longer front limbs to raise the head, thus adding mass, increasing locomotion cost. On the other hand, the presence of a tail in anurans post-metamorphosis would decrease the efficiency of the jump since it would not allow them to raise their snouts up to 45°. The absence of caudal vertebrae in the anuran tail has the advantage of being able to be eliminated quickly at the time of metamorphosis. The absence of caudal vertebrae in the anuran allows the tail to be rapidly removed at the time of metamorphosis. Rapid regression of the tail is important in the transition because a tadpole that has simultaneously both forelimbs exposed and the tail has neither efficient aquatic nor terrestrial locomotion (Wassersug and Sperry, 1977).

Vertebral development in anurans

The morphogenesis of the vertebral column is a complex process that involves the coordination of a series of cellular events that include somite morphogenesis, specification, proliferation and migration of sclerotomal cells and formation of mesenchymal condensations that are finally replaced by cartilage and bone (Christ and Ordahl, 1995; Christ and Wilting, 1992; Keynes and Stern, 1988; Peters *et al.*, 1999).

Vertebral column development starts with somite morphogenesis. Somites are paired blocks of mesoderm tissue on either side of the neural tube formed from a regular wave of segmentation in a rostral to caudal direction throughout the trunk and tail (Delarue *et al.*, 1996; Keller, 1999; Pearson, M; Elsdale, 1979; Sparrow, 2008). Somitogenesis has been highly conserved during evolution and although the behavior of the cells that drive this process differs among different vertebrate groups, morphological formation of the somite principally involves a mesenchymal-to-epithelial transition (MET) of the paraxial mesoderm (Henry *et al.*, 2000; Kulesa and Fraser, 2002; Ostrovsky *et al.*, 1988) (Fig. 2A-B). In contrast, somitogenesis in anurans is different from that in most vertebrates because it does not form epithelial somites (Brustis *et al.*, 1976; Brustis, 1979; Brustis and Delbos, 1975; Brustis and Delbos, 1979; Gatherer and Del Pino, 1992; Hamilton, 1969; Keller, 1999; Kiełbówna, 1981; Youn and Malacinski, 1981a; Youn and Malacinski, 1981b) (Fig. 3). Anuran somite formation is characterized by an orchestrated rotation and/or elongation of blocks of cells, a process that depends of a series of cell rearrangements and in which adhesion molecules play a fundamental role in the coordination of the movements of the individual cells (Afonin *et al.*, 2006; Sánchez and Sánchez, 2015). Studies based on confocal imaging approach showed that somite morphogenesis in *Xenopus* begins with the mediolateral elongation of the blocks of presomitic cells which then bend anteriorly to effect a 90° turn that brings the alignment of myotome fibers parallel to the notochord (Afonin *et al.*,2006) (Fig. 2C). During somite rotation, the distal pole of each presomitic cell exhibits filopodial extensions while the flattened medial pole moves anteriorly along the notochord. Shortly after formation, in response to inductive signals from adjacent tissue, somites differentiate into three compartments with different cell fates (myotome, dermatome, and sclerotome) (Brand-Saberi *et al.*, 1996; Christ and Ordahl, 1995; Keynes and Stern, 1988). However, several studies have shown that compartmentalization begins before somite formation in the frog *Xenopus laevis* (Della Gaspera *et al.*, 2012; Sabillo *et al.*, 2016).

In anurans, most somites are myotomal, consisting of cells that will form muscle. Dermatome, which is made up only of a thin dorsolateral sheet covering the myotome, will give rise to the dermis of the back. Finally, the sclerotome (the precursor of ribs and vertebrae) has been described as a small population of pluripotent mesenchymal stem cells adjacent to the notochord and neural tube (Sparrow, 2008).

Due to its small size and less accessibility to observation and experimentation, the amphibian sclerotome compartment has

been practically ignored, so little is known about either the way in which sclerotomal cells behave during spinal morphogenesis or the genes involved in this process.

In anurans the sclerotome is described as a small polymorphic cell population located in the ventral-medial (*Xenopus laevis*) or medial (*Bufo bufo* and *Rana dalmatina*) somite compartment (Brustis *et al*., 1976; Ryke, 1953). Sclerotomal cells, like dermatome cells, differ from the myotome by not presenting the typical elongated cell shape. It is still unclear whether sclerotomal cells participate with myotomal cells in elongation and rotation/segmentation and later round up or if they could segment by some mechanism that does not require elongation.

Recent experiments have determined that sclerotome cells originate from the lateral somitic frontier (LSF) during early neurulation in *Xenopus* (Della Gaspera *et al*., 2019). The LSF region is made up of multipotent somitic cells that give rise not only to the sclerotome but also to the dermomyotome (Fig. 4A-B). Using *Twist1* gene as a cell marker, it was shown that sclerotome progenitor cells migrate ventrally from the lateral to the medial region, between the myotome and the endoderm until they reach the notochord (Fig. 4C-D).

A general description of how the vertebrae are formed from sclerotome in anurans emerges from a histological study of the development of the vertebral column using embryos of *Rana temporaria*, *Bufo melanostictus*, *Bombina*, *Xenopus laevis* and *Eleutherodactylus johnstonei* (Brustis *et al*., 1976; Meza-joya *et al*., 2013; Mookerjee, 1931).

In the tailbud stage, sclerotome progenitor cells are released from the ventro-medial corners of the mesenchymal somites and progressively scatter around and along both the neural tube and the notochord (Fig. 4E). Then, sclerotome cells surrounding the notochord change their shape, become fibrous connective tissue cells, and aggregate to form the perichordal rings that encircle the notochord and the dorsolateral corners of the notochord at the caudal end of the myotome.

Subsequently, the rings extend in a cranial as well as in a caudal

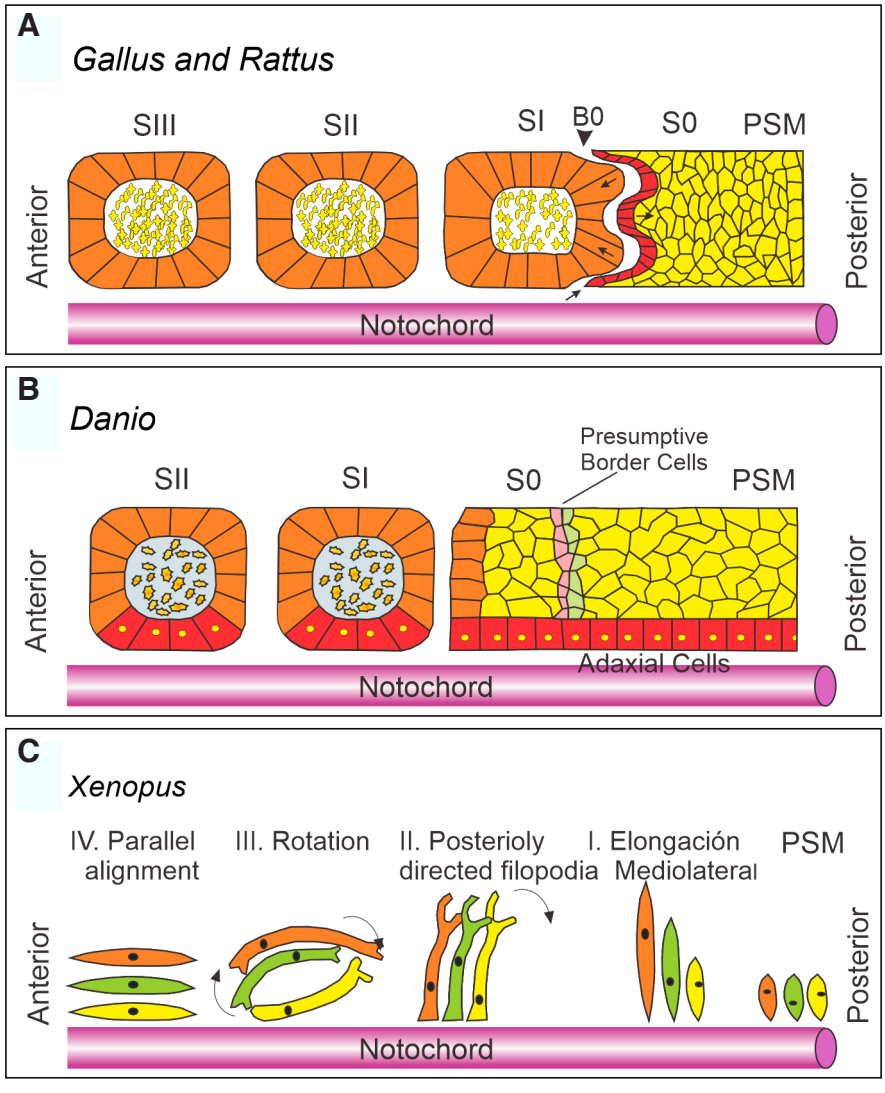

Fig. 2. Comparative vertebrate somitogenesis. (A) *Schematic representation of somite morphogenesis in chicken and mouse (Kulesa and Fraser, 2002). The epithelialization of the presomitic mesoderm (PSM) begins at its anterior border (S0 somitomere). Next, the mesenchymal-epithelial transition (MET) extends caudally towards the posterior end of somitomere where the intersomitic boundary B0 begins to form. Finally, the cells of the posterior border become epithelial and the somite eventually separates from the segmental plate.* **(B)** *Schematic representation of somite morphogenesis in zebrafish (Henry* et al., *2000). Similarly to amniotes, fish somite formation involves an MET; however, the cellular events that take place differ in some respects. In* Danio rerio *the structure of the somite presents the same pattern as in birds and mammals, with an external epithelioid surface (border cells) and an internal mass of mesenchymal cells (somitocoele). Initially, presumptive epitheloid border cells (EBC) are distributed in discontinuous mediolateral stripes within the morphologically homogeneous PSM. Morphogenesis of intersomitic boundaries is accomplished by the segregation of the presumptive border cells into two discrete rows of epitheloid border cells, which exhibit epithelial morphological characteristics. Next, the border cells undergo selective loss of adhesion along the intersomitic boundary, thereby forming the definitive intersomitic groove. Finally, these border cells surround the mesenchymal cells forming the new somite.* **(C)** *In* X. laevis *the formation of the somites involves a series of coordinated cell movements. Although somatic cells move as a single block of tissue, they do not form epithelial tissue. Afonin* et al., *(2006) proposed four-step model.* **I.** *Initially, the cells of somitomere S0 that are perpendicular to the notochord undergo mediolateral elongation.* **II.** *The number of posteriorly directed philopodial protrusions increases.* **III.** *Elongated cells bend around the dorsoventral axis such that the anterior cell moves to the lateral edge of the somite and a more posterior cell moves to the medial region of the somite.* **IV.** *Cell achieve a parallel alignment along the antero-posterior axis, thus separating itself from the PSM and forms a new somite. Arrows indicate the direction of movements. S0, somitomere 0; SI-SIII, somites I to III; B0, intersomitic boundary.*

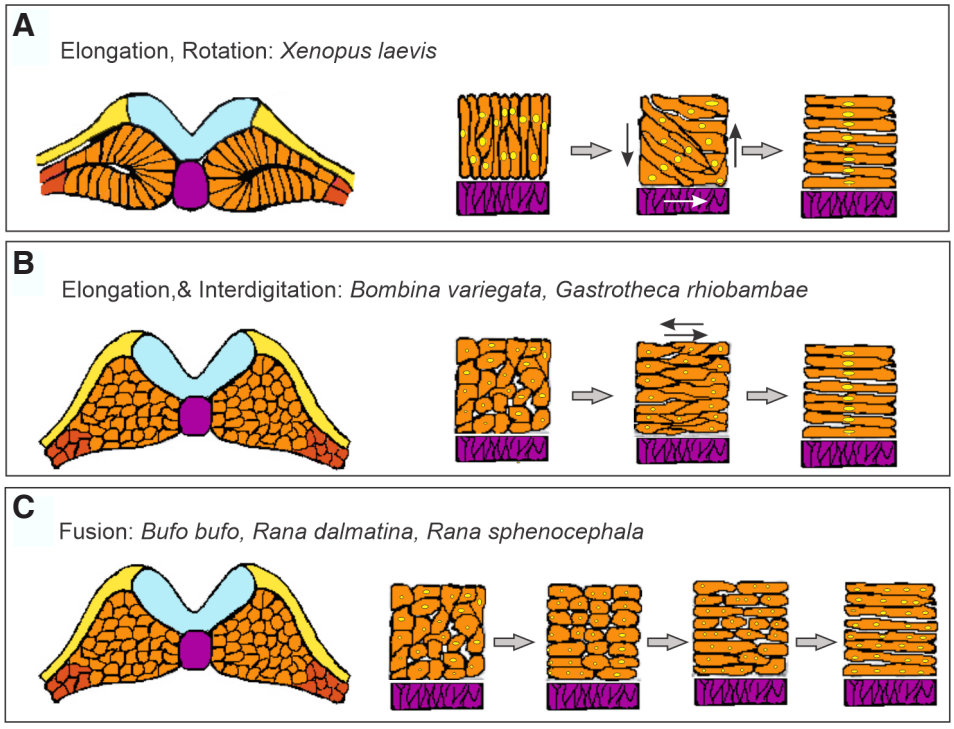

Fig. 3. Variations of cell behavior in the process of segmentation and myotome formation in anurans. *To the left, cross-sectional view of the PSM are shown. In the series at the right, the sequence of cell behaviors bringing about myotome formation are diagrammed with anterior to the right.* **(A)** *In* Xenopus, *the cells elongate mediolaterally prior to segmentation, and then, during segmentation they rotate, moving anteriorly next to the notochord, laterally anteriorly, and medially posteriorly to span the full length of the somite.* **(B)** *In* Bombina *and* Gastrotheca, *the cells are initially rotund and polymorphic. When they do elongate, they also interdigitate, and span the full length of the somite. As in* Xenopus, *no fusion occurs and the cells remain mononucleate until stage 45.* **(C)** Pelobates, Bufo, *and* Rana *initially have cuboidal cells, although some may elongate somewhat. These a line up and fuse to form multinucleate myotoma cells. Myocoel does not form in any of these anurans. Modified after Keller (1999).*

direction so as to form a continuous tube (perichordal tube). The sclerotomic cells at the dorsolateral corners of the notochord proliferate, aggregate and modify to become pro-cartilaginous cells. The pro-cartilaginous structures, extending up on both sides of the neural tube as well as ventrally at the caudal level of the myotome, will form the future neural arches (Fig. 4F).

On the other hand, once generated the rudiments of the neural arch, in the dorsal region appears a slight projection of cartilaginous tissue with a mass of sclerotomic cells at its free end. Later, these projections grow, aggregate and form the transverse processes (diapophyses).

According to Gegenbaur (quoted by Keller, 1999), the remainder of the vertebra is formed by one of two modes, depending on the species. In the "perichordal mode," additional sclerotomal cell aggregations form chondrogenic masses beneath the notochord in the intermyotomal region; these encircle the notochord and eventually unite with the neural arches to form the centrum of the vertebra. As the vertebra develops at the caudal end of the myotomal segment, the notochord becomes constricted in the future vertebral region (Fig. 4G-H). In the "epichordal mode," the notochord degenerates and the centrum of the vertebra is formed from ventral extensions of the neural arch elements (Fig. 4G´-H´).

An evolutionary innovation that distinguishes all anurans is the emergence of the urostyle, the only post-sacral element of the vertebral column. This structure, which articulates with the posterior end of the sacrum, is formed by coccyx fusion with the ossified hypochord at the onset of metamorphosis.

The hypochord it is a spindle-shaped rod located ventral to the notochord in the region comprised between the beginning of the aorta and the commencement of the tail. This structure arises independently of the vertebral column and is derived from mesoderm arising in the superficial epithelial layer of the gastrula (Shook *et al.*, 2004). On the midventral aspect of the perichordal tube, the hypochord begins to form as a mass of connective tissue cells. Then, in the center of this mass a rod of round cells becomes differentiated and later becomes cartilaginous and finally ossifies (Mookerjee, 1931). In a recent study, Senevirathne *et al.,*2020) suggested that the ossifying hypochord-induced loss of the tail during metamorphosis has enabled the evolution of the unique anuran bauplan.

On the other hand, coccyx development initiates before metamorphosis and is formed by the fusion of caudal vertebrae.

Chondrification and subsequent ossification of components of the vertebral column occur in a dorso-ventral pattern (Fig. 2F-H). A detailed histological analysis of vertebral column morphogenesis in a wide range of frog families including Ascaphidae, Hylidae, Dendrobatidae, Pipidae, Microhylidae, and Leptodactylidae showed that neural arches are first formed in cartilage before any trace of chondrification appears in the area of the vertebral body; later, ossification is completed within the arches while the centra are still not fully ossified (Carroll *et al.*, 1999; Enault *et al.*, 2015). Similarly, direct-developing frogs of the family Leptodactylidae (*Eleutherodactylus coqui* and *Eleutherodactylus johnstonei*) present the same sequence of chondrification and ossification of the vertebrae as frogs with indirect development (Hanken *et al.*, 1992; Meza-joya *et al.*, 2013). However, an analysis of the sequence of events using heterochrony plots showed a significant difference in the timing of vertebral column morphogenesis in the direct developing in comparison to metamorphic frogs such as a delay in the chondrification and ossification of the vertebral centers. These ontogenetic peculiarities may represent derived traits in direct-developing frogs and are possibly correlated with their unusual life history (Meza-joya *et al.*, 2013).

Molecular aspects of early vertebral development

Anuran body plan diverges widely from that of other vertebrates. However, no matter how divergent their forms, most animals share

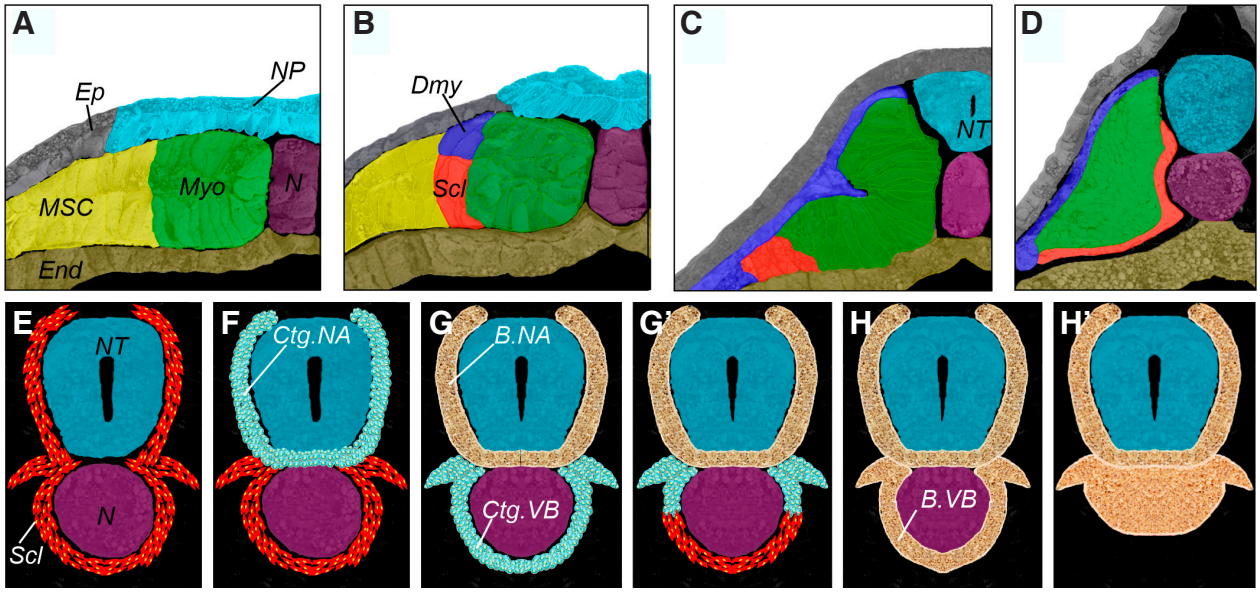

Fig. 4. Vertebral ontogeny in anurans. (A-D) *Schematic representation of somite compartmentalization in* Xenopus. **(E-H)** *Schematic representation of vertebral development in anurans.* **(G', H')** *Perichordal ossification.* **(G', H')** *Epichordal ossification. Abbreviations: B.NA, bone of the neural arch; B.VB, bone of the vertebral body; Ctg.BV, cartilage of the vertebral body; Ctg.NA, cartilage of the neural arc; Dmy, dermomyotome; End, endoderm; Ep, epidermis; MSC, multipotent somitic cell; Myo, myotome; N, notochord; NP, neural plate; NT, neural Tube; Scl, sclerotome. Modified after* Bruno Della Gaspera et al., *(2019), Sabillo* et al., *(2016) and Handrigan and Wassersug (2005).*

specific families of genes that regulate major aspects of the body pattern (Carroll *et al*., 2005). The developmental and genetic programs that establish and maintain the vertebrate body plan are tightly conserved. Nevertheless, although vertebrates have a fixed repertoire of such developmental programs, these programs have a certain degree of flexibility. This flexibility in the development machinery has allowed the generation of morphological diversity.

The anuran vertebral column has had an evolutionary reduction in its vertebral complement. The vertebral column of frogs and toads is made up of only a small number of skeletal elements and is characterized by the absence of discrete caudal vertebrae. Based on the fact that sclerotomal cells are segregated in the tail and that caudal sclerotomes undergo some degree of differentiation, Handrigan and Wassersug (2007) proposed that the formation of caudal vertebrae in anurans is precluded due to the failure of sclerotomes to form cartilaginous condensations, perhaps resulting from an altered expression of a suite of genes.

The molecular mechanisms involved in anurans sclerotome development is poorly described so the general conclusions are inferred primarily from studies conducted in amniotes. The anurans sclerotome is especially small and this small size could be associated with the reduction of skeletal elements of the vertebral column. It is well known that in amniotes, that size of the sclerotome depends on of balance of dorsal and ventral signals that create opposing antagonistic gradients on the somites.

The ventral signals promote the sclerotome development and it depends on both *Sonic Hedgehog* (*Shh*) and noggin emanating from notochord and floor plate of the neural tube (Christ *et al*., 2000). In *Shh* knock-out mice, sclerotomes are markedly reduced in size and the axial skeleton, including both dorsal and ventral elements, does not form. In addition, the mice lacking *Shh* initially display a normal expression of *Pax1* sclerotomal marker but later it is drastically reduced (Chiang *et al*., 1996). These observations indicate that *Shh* does not initiate but maintain the sclerotome program. On the other hand, McMahon *et al.,* (1998) showed that in homozygous *Noggin* mutants mice the *Pax1* expression is delayed and that Noggin alone can induce *Pax1*. Additional studies, showed that noggin regulate the sclerotomal development antagonizing with *BMP4* and *BMP2* signals, which act as inhibitors of sclerotome induction mediated by *Shh* (Liem *et al*., 1995).

Dorsal signals promote the development of the dermomyotome and inhibit the sclerotome. These signals belong to the *Wnt* family of genes emanating from ectoderm and the dorsal neural tube. Several studies have shown that overexpression of some members *Wnt* genes produce an expansion of the dermomyotome to the detriment of sclerotomal compartment. *Wnt*-secreting cells ectopically grafted adjacent to the notochord produce an expansion of the dorsal epithelial compartment and promote *Pax3* and *MyoD* expression (muscle marker). On the other hand, the sclerotome is reduced in size and *Pax1* expression is downregulated evidencing a competitive interaction between Wnts and *Shh* in the somites (Goulding *et al*., 1993; Goulding *et al*., 1994; Lee, 2000; Wagner *et al*., 2000).

Interestingly, in frog *Xenopus*, loss of function of *dishevelled 3* (*dvl3*), a transducer of *wnt* signaling path, leads to the elimination of perinotochordal pax1 expression (Gray *et al*., 2008). The *Xenopus dvl3* expression pattern diverges widely with respect mouse and chick. In mouse, early expression of *Dvl3* is ubiquitous, but *Dvl3* transcription is subsequently upregulated in neural tissue and somites (Tissir and Goffinet, 2006; Tsang *et al*., 1996). Chick *Dvl3* also was quite broadly expressed. At early stage, was observed in the brain, notochord, presomitic mesoderm and somites and, later, *Dvl3* expression remained within the neural tube, notochord, and the somites (Gray *et al*., 2008). In *Xenopus*, *dvl3* expression domain is more restricted, are located to the paraxial mesoderm and then latter to presomitic mesoderm. This pattern is maintained

until the late tailbud stages, at which time *dvl3* is also expressed in the developed somites.

This *Xenopus*-specific expression and function for *dvl3* may reflect the evolutionarily derived mechanisms of muscle and sclerotome development in amphibian (Gray *et al.*, 2008).

A few years ago, we started to study early vertebral column morphogenesis in anurans under the hypothesis that the reduction in vertebral complement in anurans could have emerged during evolution because of changes in the patterns and/or levels expression of genes involved in the regulation of proliferation, adhesion and differentiation of sclerotomal cells.

The first molecular markers appearing when the prospective sclerotome is induced are *Pax1*, *Nkx3*.1 and *Nkx3.2/Bapx1* (Monsoro-Burq, 2005). Later on, during sclerotome formation, genes such as *Pax9*, *Msx1/2* and *Uncx4.1* (among others) are activated in sub-domains of the sclerotome, prior to cartilage differentiation (Müller *et al.*, 1996; Neidhardt *et al.*, 1997; Takahashi *et al.*, 1992).

In amniotes, *Pax1* and *Pax9* genes play an important role in regulating cell proliferation and chondrogenic specification in the sclerotome (Peters, 1999; Rodrigo *et al.*, 2003; Wallin *et al.*, 1994). *Pax1* deficient mice lack vertebral bodies and intervertebral discs whereas neural arches are nearly normal (Wallin *et al.*, 1994). On

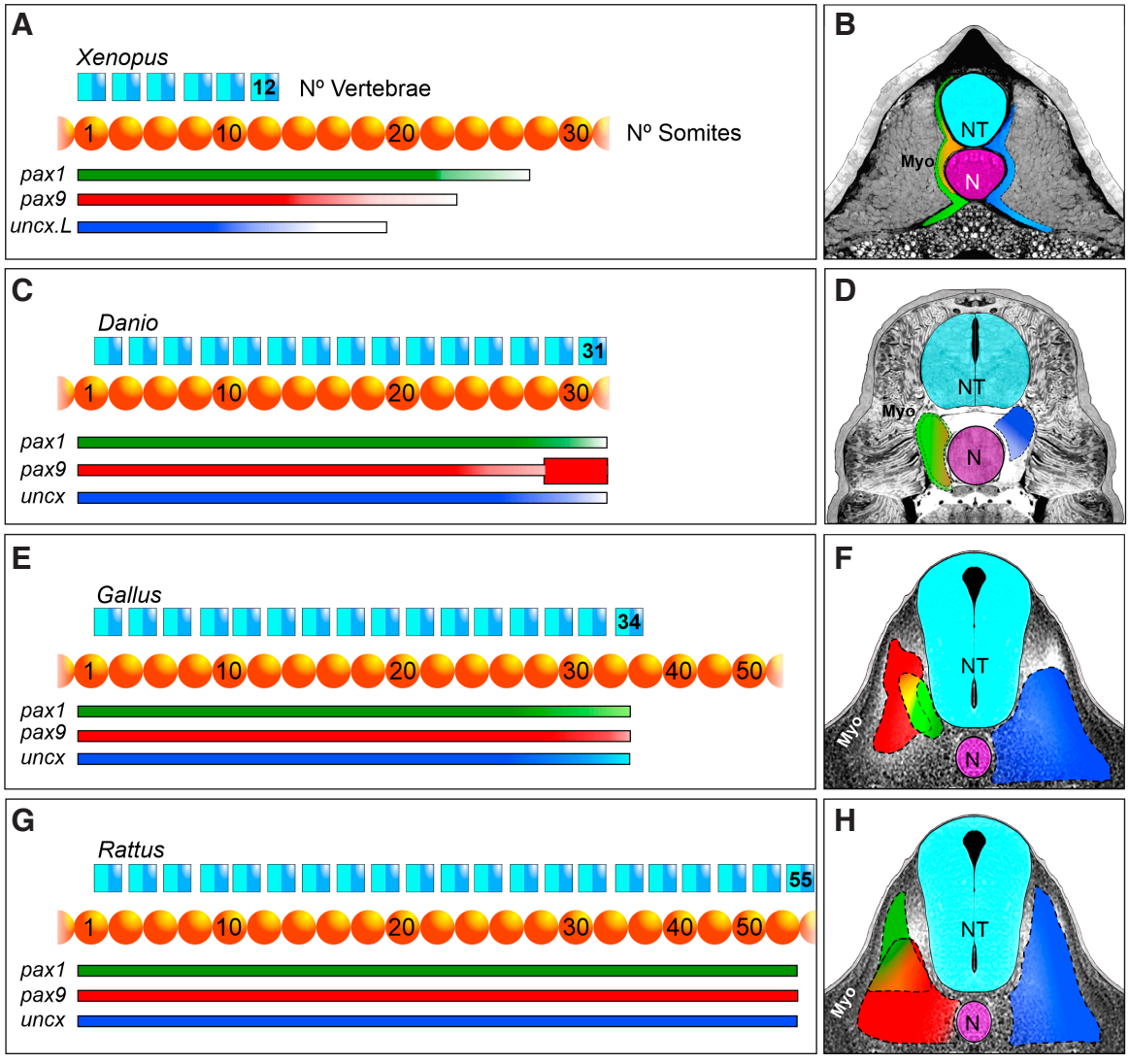

Fig. 5. Expression pattern of the *pax1, pax9* and *uncx* genes in the vertebrate sclerotome. (A,C,E,G) *Comparative analysis of expression patterns along the antero-posterior axis.* **(A)** *In the* Xenopus laevis *frog embryos (Nieuwkoop and Faber stage 35), the genes* pax1, pax9 *and* uncx *show a homogeneous level expression up to ~22*nd*, ~12*th*h and ~10*th*h somite, respectively. From here onwards, the genes undergo progressive reduction of their expression in the most caudal somites. In contrast, fish* (Danio rerio) **(C)**, *chicken (*Gallus gallus*)* **(E)** *and mouse* (Rattus norvegicus) **(G)**, *these genes are expressed at high levels along the entire anterior-posterior axis in all those somites that will lead to vertebrae formation. The reduction in the expression in caudal somites could explain, at least in part, the shortened of the anteroposterior axis and the absence of caudal vertebrae in anurans.* **(B,D,F,H)** *Comparative analysis of expression patterns in transversal section of frogs* **(B)**, *fish* **(D)**, *chicken* **(F)** *and mouse* **(H)**. *The expression domain of the* pax1, pax9 *and* uncx *genes within the sclerotome changes in the different groups of vertebrates. This divergence in the domains of expression would be related to the establishment of the pre-pattern of the vertebral elements, which is necessary for the vertebrae to acquire the morphology of each species. Expression colour-coded: green,* pax1*; red,* pax9*; blue,* uncx*; orange/yellow,* pax1/pax9 *co-localization. Abbreviations: N, notochord; NT, neural tube; Myo, myotome.*

the other hand, *Pax9* null allele does not exhibit morphological abnormalities (Peters, 1999). However, in *Pax1/Pax9* double mutant mice, the medial derivatives of the sclerotomes are completely missing. This phenotype is much more severe than that of *Pax1* single homozygous mutants and shows a functional redundancy between *Pax1* and *Pax9* during vertebral column development. In teleost fish (anamniotes), morpholino knockdown experiments revealed that both *Pax1* and *Pax9* are indispensable not only for the development but also of the vertebral body and neural arch (Mise *et al.*, 2008). When analyzing the spatial expression of *pax1* and *pax9* in the anuran *Xenopus*, we found certain differences in relation to the pattern and the level of expression observed in other vertebrates (Fig. 5 A-B). In medaka fish (*Oryzias latipes*), the expression of *pax1* and *pax9* in the sclerotome is restricted to the ventromedial region of the somite and no differences were detected in their spatial patterns (Mise *et al.*, 2008) (Fig. 5D). On the other hand, the level of expression is homogeneous along the antero-posterior axis except for a strong expression of Pax9 in mesodermal cells of the tail bud that was not observed for Pax1 (Fig. 5C).

In contrast, in amniotes, *Pax1* and *Pax9* have subfunctionalized their roles in the development of the sclerotome. In chicken, *Pax1* is expressed in almost all sclerotome cells, whereas *Pax9* expression is found mainly in dorsolaterally located sclerotomal cells (Müller *et al.*, 1996) (Fig. 5F). Similarly, in mice, while *Pax9* is mainly expressed in the lateral region of the sclerotome, *Pax1* is expressed medially and ventromedially (Deutsch *et al.*, 1988; Neubüser *et al.*, 1995) (Fig. 5H). Regarding to expression levels, *pax1* and *pax9* genes were homogeneously expressed along the anterior posterior axis of chicken and mouse embryos. Furthermore, *Pax9* was strongly expressed in the posterior mesoderm, as was also observed for fish *Pax9* (Fig. 5E and G).

In the anuran *Xenopus laevis*, we found that the expression of *pax1* is located in the sclerotomal cells of the center of the somites surrounding the notochord and neural tube, whereas *pax9* is expressed mainly in the anterior half of the sclerotome around the neural tube (Fig. 5B) (Sánchez and Sánchez, 2013). In addition, contrary to the other vertebrate, *pax9* gene is expressed at very low level in caudal somites. In fish, *pax9* loss of function leads to abnormal morphology in the tail hypural skeletal element. In this sense, we think that the low expression levels of *pax9* in the posterior mesoderm of *Xenopus* could be related to the inability to form cartilaginous condensations in the caudal region. Although *Xenopus pax9* is expressed beyond ~12th somite, from here onwards its level expression is lower which it would not be sufficient to produce the high rate cell proliferation necessary for the subsequent formation of the mesenchymal condensations.

Preliminary experiments indicate that *pax1* and *pax9* play an important role in the development of frog axial skeleton. We found using morpholino approaches, that loss of function of these gene leads to shortening of the axis and scoliosis, possibly caused by failures in the formation cartilaginous condensations (unpublished results).

Another gene involved in the development of the axial skeleton is *Uncx* (also known as *Uncx4.1*, *Phd1* and *Chx4*). This gene encodes a paired type homeobox transcription factor expressed in the developing somite and sclerotome compartment (Mansouri *et al.*, 1997; Mise *et al.*, 2008; Saito *et al.*, 1996; Sánchez and Sánchez, 2013). Functional studies have shown that *Uncx4.1* is required for the condensation of mesenchymal cells of the lateral sclerotome, which is necessary for the specification of pedicles, transverse processes, and proximal ribs (Leitges, 2000; Mansouri, 2000). Moreover, disruption of the establishment of antero-posterior somite polarity in *Uncx* mutant mice suggests that this gene is required for the maintenance of posterior somite characteristics. In mouse, *Uncx4.1* is expressed initially in the entire caudal half of each newly formed somite and later, during differentiation of the somites, is restricted to the caudal sclerotome (Mansouri *et al.*, 1997; Neidhardt *et al.*, 1997) (Fig. 5H). In zebrafish, *uncx* is expressed initially in low level in the Presomitic Mesoderm (PSM). As development proceeds, the signal of the *uncx* transcripts increases and is restricted to sclerotome (Nittoli *et al.*, 2019) (Fig. 5D).

Unlike mice, *uncx* factor were only observed in the sclerotomal surrounding the notochord in *Xenopus* embryo (Sanchez *and* Sanchez, 2013) (Fig. 5B). We have recently isolated other ortholog *uncx* gene in *Xenopus* (u*ncx.S*) This gene is first located in the migrating prospective sclerotomal cells and finally restricted to the sclerotomal cells surrounding the notochord and neural tube (unpublished data). Like *pax9* of *Xenopus*, both *uncx* genes are expressed at very low levels in the caudal somites while in vertebrates with caudal vertebrae it is strongly expressed along the entire antero-posterior axis. (Fig. 5A, C, E and F). Expression levels of *uncx.L* gene in *Xenopus* begin to progressively decrease from ~11th somite to finally disappear approximately in the ~22nd somite. In recent studies carried out in our laboratory, using a morpholino approach, we evidenced that *uncx* gene is required for normal development of vertebral column in *Xenopus* and that knockdown of function of *uncx* leads to severe alterations of the axial skeleton due to failures in cartilage formation (unpublished data).

This leads us to propose that the low expression level of *uncx* in frogs could be related to the absence of cartilage in the tadpole tail.

In addition, when comparing the expression patterns of the *pax1*, *pax9* and *uncx* genes between the different groups of vertebrates, it can be seen that these genes differ in their location. For example, whereas mouse Pax1 is strongly expressed medially and ventromedially; in chicken, fish and frogs is located mainly in almost all the sclerotome cells, in ventromedial region and in the sclerotomal cells that surrounding the neural tube, respectively (Fig. 5 B, D, F, H). The vertebral column has several components, including the neural arch, vertebral body, intervertebral disk, and rib or hemal arch. The divergence in the pattern of expression these genes in the sclerotome between the vertebrates could be involved in the regionalization of the sclerotome and differentiation of the components of the vertebral column. This hypothesis is supported by functional studies carried out on mouse and medaka fish. In mouse *pax1* and *pax9* have a central role in the formation of the vertebral body and intervertebral disks (Peters *et al.*, 1999), whereas in teleosts *pax1* and *pax9* are required for morphogenesis of the neural arches and vertebral body (Mise *et al.*, 2008). New functional studies should be carried out in other organisms to determine the role of these genes in the morphogenesis of the vertebral column.

Analyzing the vertebral column shows that it is divided into regions that exhibit different identities, such as cervical, thoracic, lumbar, sacral and caudal. Within a given species, the number of each type of vertebrae is usually constant. However, as we mentioned, the number of vertebrae within each region varies greatly among different species. The determination of these identities is

regulated by the Hox genes (Iimura *et al.*, 2009; Krumlauf, 1994; Wellik, 2007).

The Hox family genes encode transcription factors are the primary patterning genes of the segmental plate in vertebrates (Iimura and Pourquié, 2006). The vertebrate Hox genes generally are organized into four clusters in the genome. In each cluster, the genes are arranged on the chromosome in a sequence that reflects the timing of their expression during embryogenesis (temporal colinearity) and the position of their expression domain along the AP axis (spatial colinearity) (Dollé *et al.*, 1989; Gaunt *et al.*, 1988; Graham *et al.*, 1989). The spatial colinearity of the expression domains of Hox genes results in a specific combination of genes to be expressed in each somite (Kesseland and Gruss, 1991). This combination of Hox genes is involved in the control of the specification of vertebral identities (Carapuço *et al.*, 2005; Deschamps and van Nes, 2005; Wellik, 2007). However, whereas the expression domain of many Hox genes extends from the posterior end of the embryo to a defined anterior level in the somites, their action is essentially restricted to their anterior-most expression domain. Thus, the identity of a segment is controlled only by the posterior most Hox genes that are expressed in this segment (Burke *et al.*, 1995; Duboule and Morata, 1994). Hox expression boundaries in the paraxial mesoderm coincide with morphological boundaries between the different regions of the future axial skeleton (Burke, 2000; Gaunt, 2000). A comparative analysis of Hox genes expression patterns in tetrapods revealed that expression boundary in the frog orthologs has been anteriorised relative to the amniotes (Burke *et al.*, 1995; Christen *et al.*, 2003; Gaunt, 1994; Lombardo and Slack, 2001). Various evidences indicate that anteriorized expression of Hox genes could be responsible for the shortening of the trunk and the vertebral elements reduction in anurans.

The *Hoxc10* and *Hoxd10* genes, both markers of the presacral-sacral transitional zone in tetrapods, have their expression termini at *Xenopus* trunk in the somite 8 -around 20 somites anterior relative to the chick and mouse (somite 26) (Handrigan and Wassersug, 2007). Accordingly, the sacrum and cranium of frogs are separated by no more than eight vertebrae, and the anuran trunk is short compared to all other vertebrates. The “pushed forward” of the *Hox10* expression could be part of the reduction mechanism of presacral vertebrae.

The absence of caudal vertebrae in anurans also may be the product of anteriorised expression of *Hox13* genes. Mice knockout for *Hoxb13* show overgrowth in all major structures derived from the tail bud, including the neural tube and the caudal vertebrae. This points to a role for *Hoxb13* as a general repressor of caudal development in the mouse and perhaps other vertebrates by mediating cell proliferation and apoptosis rates in the tail (Economides *et al.*, 2003). Similarly, a recent study in zebrafish showed that *Hoxc13* knockout using CRISPR/Cas9 gene editing, led to the generation of additional caudal vertebrae (M Allende, personal communication, October 30, 2019) so that *Hox13* and other 5´ *Hox* genes would be the main candidates responsible, at least in part, for the absence of caudal vertebrae in the anurans.

Concluding remarks

The *pax1*, *pax9* and *uncx* genes play a central role in the formation of the axial skeleton of vertebrates. As in other vertebrates, the expression of the *pax1*, *pax9* and *uncx* genes in *Xenopus* was subregionalized within the sclerotome, so that they could possibly regulate the morphology of different components of the individual vertebra. The spatio-temporal expression pattern of these scleromal genes in *Xenopus* differs from those observed in other vertebrates. Their expression levels in the posterior somites have experienced a marked reduction that could be related to the inability of the sclerotome to form cartilaginous condensations.

On the other hand, the analysis of patterns expression of *Hox* genes revealed that have been anteriorly displaced in the paraxial mesoderm of *Xenopus* relative to other vertebrates, corresponding with the shortening of the trunk. Further, loss of function of the *Hox13* gene in mice and fish leads to overgrowth in all major structures derived from the tail bud, including the formation of additional caudal vertebrae in zebrafish.

This diferrences in both pattern and expression levels of Hox, Pax1, Pax9 and Uncx along the anteroposterior axis in frogs relative to other vertebrates could be related to the reduction of the vertebral elements in the spine, and be partly responsible for the divergent morphogenesis of the vertebral column in anurans. Additional studies are necessary to understand the genetic mechanisms that determine the divergent anuran body plan and its subsequent maintenance throughout evolution.

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