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# Cellular dynamics and molecular control of the development of organizer-derived cells in quail-chick chimeras

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**ABSTRACT Malformations affecting the nervous system in humans are numerous and various in etiology. Many are due to genetic deficiencies or mechanical accidents occurring at early stages of development. It is thus of interest to reproduce such human malformations in animal models. The avian embryo is particularly suitable for researching the role of morphogenetic movements and genetic signaling during early neurogenesis. The last ten years of research with Nicole Le Douarin in the Nogent Institut have brought answers to questions formulated by Etienne Wolff at the beginning of his career, by showing that Hensen's node, the avian organizer, is at the source of all the midline cells of the embryo and ensures cell survival, growth and differentiation of neural and mesodermal tissues.**

KEY WORDS: *neurulation, Hensen's node, floor plate, quail-chick chimera, caudal dysplasia*

## Foreword

Etienne Wolff, the founder of the Nogent Institute, was a pioneer in teratogenic studies. He was the first to use the chick embryo as an animal model to construct phenocopies of human developmental malformations (Wolff, 1936). During his PhD thesis under the supervision of Paul Ancel in Strasbourg University, he experimentally reproduced cyclopia and symmelia, two dramatic malformations occasionally observed in human embryos, affecting organ bilaterality. He obtained phenocopies of such malformations in bird embryos by performing axial organ destruction using X irradiation at early stages of development (Fig. 1). Sixty years after these pioneer experiments, with Nicole Le Douarin, a former student of Etienne Wolff and his successor at the head of the Nogent Institute, we used the quail-chick cell labeling technique (Le Douarin, 1969) and microsurgical excision to elucidate the role of the avian organizer, Hensen's node, during the development of the central nervous system in the avian embryo. The results that we obtained confirmed and extended Wolff's observations and, like them, could be related to known human malformations. In the present paper we will review several aspects of our recent work, the aim of which was to improve our general understanding of the early development of the vertebrate nervous system.

## The first stages of neurogenesis: «primary» *versus* «secondary» neurulation

In birds, as in other chordates, a region of the primitive ectoderm is precociously induced to form the neurectoderm (Stern, 2002 for a review). Neural induction leads to the formation of a prismatic cell layer organized into the so-called neural plate. This plate will undergo a series of subsequent morphogenetic movements called neurulation, giving rise to the neural tube, i.e., the primordium of the central nervous system (CNS). Vertebrate neurulation proceeds according to two different mechanisms regarding the type of cell movements implicated (Catala *et al.*, 1995; Colas and Schoenwolf, 2001 and references therein). In the first, which affects the anterior moiety of the bird embryo, the neural plate bends due to the formation of several hinge points (one median and two lateral points running on the rostro-caudal axis). These hinge points are produced by the apical shrinkage of epithelial neurectodermal cells due to the constriction of actin microfilaments. The formation of the median hinge point leads to the folding of the lateral edges of the neural plate resulting in the formation of the neural groove. The appearance of the lateral hinge points then allows the lateral borders of the neural plate to converge to the dorsal midline and eventually to fuse forming the neural tube. This mode of formation of the neural tube has been termed «primary neurulation».

Another mechanism of neurulation occurs in the posterior moiety of the bird embryo. In this region, the neural tube arises by cavitation of a solid row of cells, the medullary cord, which results

*Abbreviations used in this paper:* APH, axial-paraxial hinge; CNH, chordo-neural hinge; CNS, central nervous system; HH, Hamburger and Hamilton stage; LFP, lateral floor plate; MFP, medial floor plate; Ptc, Patched ; Shh, sonic hedgehog ; ss, somite stage

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from the juxtaposition of cells of the posterior primitive neural plate. This mode of formation of the neural tube is called «secondary neurulation».

For a long time, primary and secondary neurulation have been opposed (Catala *et al.*, 1995; Colas and Schoenwolf, 2001 and references therein); however, in terms of phylogenesis, it is far from clear why they are present. Indeed, the amphibian neural tube is entirely formed by folding of the neural plate while the fish neural tube results from cavitation of a cell cord; in contrast, in mammalian like in avian embryos, both types of neurulation take place according to the rostro-caudal level of the neuraxis considered. In the chick embryo, primary neurulation starts at the anterior level when the head fold first separates the anterior neural plate from the anterior extra-embryonic tissues, before the appearance of the first pair of somites. It is completed when the caudal neuropore closes at the 16- to 22-somite-stage (16-22-ss) (Schoenwolf, 1979). At this time, the cells of the late primitive streak and the caudal medullary cord issued from the posterior neural plate form a mass of morphologically undifferentiated cells called the tail bud. The tail bud is responsible for the caudal extension of the body and emergence of the lumbo-sacro-caudal part of the spinal cord by secondary neurulation.

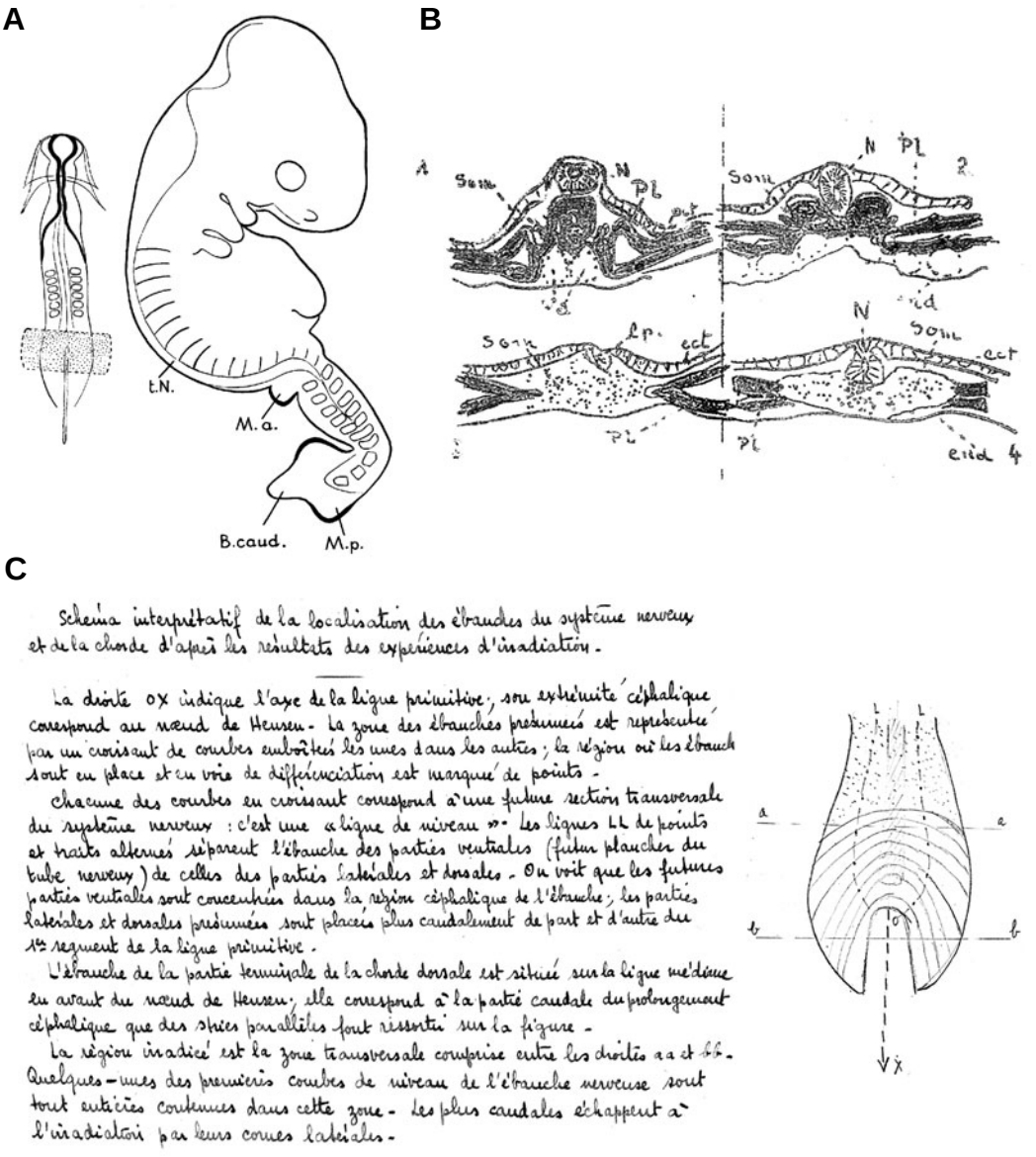


**Fig. 1. Illustrations from Hensen's node X irradiation experiments by Wolff. (A)** *Schematic representation of the location of the irradiation at 6-somite-stage and of the caudal malformations observed two days after Hensen's node destruction. The neural tube is smaller than normal or absent and the somites are fused by pair (figure 92, Wolff, 1936).* **(B)** *Sections in the caudal region of the irradiated embryo show the absence of notochord and fusion of the somites under a small round neural tube (figure 102, Wolff, 1936, original drawings).* **(C)** *Localization of the nervous system and notochord anlage inferred from Hensen's node irradiation experiments (figure 101, Wolff, 1936, original drawing and manuscript).*

## Neural tube ventral midline cells have a distinct origin from the rest of the neuroepithelium

It was of particular interest to analyze cell movements which occur inside the tail bud of the avian embryo during secondary neurulation. For this study, we used the quail-chick cell marking technique (Le Douarin, 1969) and performed a fate map of the avian tail bud at 25-ss (Catala *et al.*, 1995). At this stage, the most caudal region of the embryo is limited by an ectodermal fold (Fig. 2A). The anterior neural tube formed by primary neurulation is in perfect continuity with the tail bud-derived tissue in which secondary neurulation is on going. Ventrally, the notochord ends within the tail bud, merging with the caudal neural tissue, forming the so-called chordo-neural hinge (CNH) described by Pasteels (Pasteels, 1937 and see Fig. 2A).

The main results of our mapping are as follows (Catala *et al.*, 1995):

1- during elongation of the embryo, the CNH moves caudally and lays down the floor plate of the future neural tube and the notochord, from the lower sacral region down to the tip of the tail.

2- the caudal neural tube has a dual origin: the floor plate derives from the CNH, whereas the rest of the tube arises from the dorsal tissue situated in the rostral two thirds of the tail bud (Fig. 2 A,B).

3- the caudal mesoderm is

produced by progenitors distributed in the primitive streak remnant along the entire tail bud.

Analysis of the morphogenetic movements acting in this region (Catala *et al.*, 1995, Fig. 10) demonstrates that both the notochord and the floor plate progenitors elongate axially (from rostral to caudal). In contrast, lateral mesoderm progenitors issuing from the primitive streak diverge laterally to be added to the forming pre-segmental plate. These movements are the continuation of the gastrulean movements that take place earlier and more rostrally in both Hensen's node and the primitive streak. These results prompted us to propose that the tail bud represents a late gastrulation center in which the CNH is the remnant of Hensen's node and the other tissues of the tail bud consist in the association of the posterior neural plate and the late primitive streak and its derivatives (Catala *et al.*, 1995).

In order to further document this view, we constructed the fate map of the posterior region of the chick embryo at mid-gastrulation (i.e.5-6-ss) (Catala *et al.*, 1996). At that stage, the caudal part of the chick embryo presents a widely opened neural plate forming a lozenge-like structure, the *sinus rhomboidalis*. Hensen's node sits in a depression lying in the middle of the *sinus rhomboidalis*. Our results showed that the 5-6-ss node gives rise to the midline tissues belonging to the three germ layers (floor plate within the spinal cord, notochord and dorsal endoderm) from the brachial level to the tip of the tail (Fig. 2 C-E). It is thus noticeable that the 5-6-ss-Hensen's node contains all the progenitors that will eventually be found in the CNH at later stages. This shows that the formation of the notochord and floor plate is a unique and continuous process taking place through the whole gastrulation process up until the completion of neural tube formation. It is interesting to note that grafting the latero-caudal part of the neural plate at the same stage results in the formation of the dorsal medullary cord responsible for the formation of the caudal spinal cord by secondary neurulation (Catala *et al.,* 1996).

Thus, the neural plate as it stands at mid-gastrulation gives rise to the entire length of the neural tube including the secondary neural tube with the exception of the floor plate which is generated first by Hensen's node and then by the CNH which derives from the latter (Catala *et al.*, 1996).

The main conclusions of our two fate map studies are as follows: 1- midline cells of the avian embryo derive from Hensen's node, relayed by the CNH.

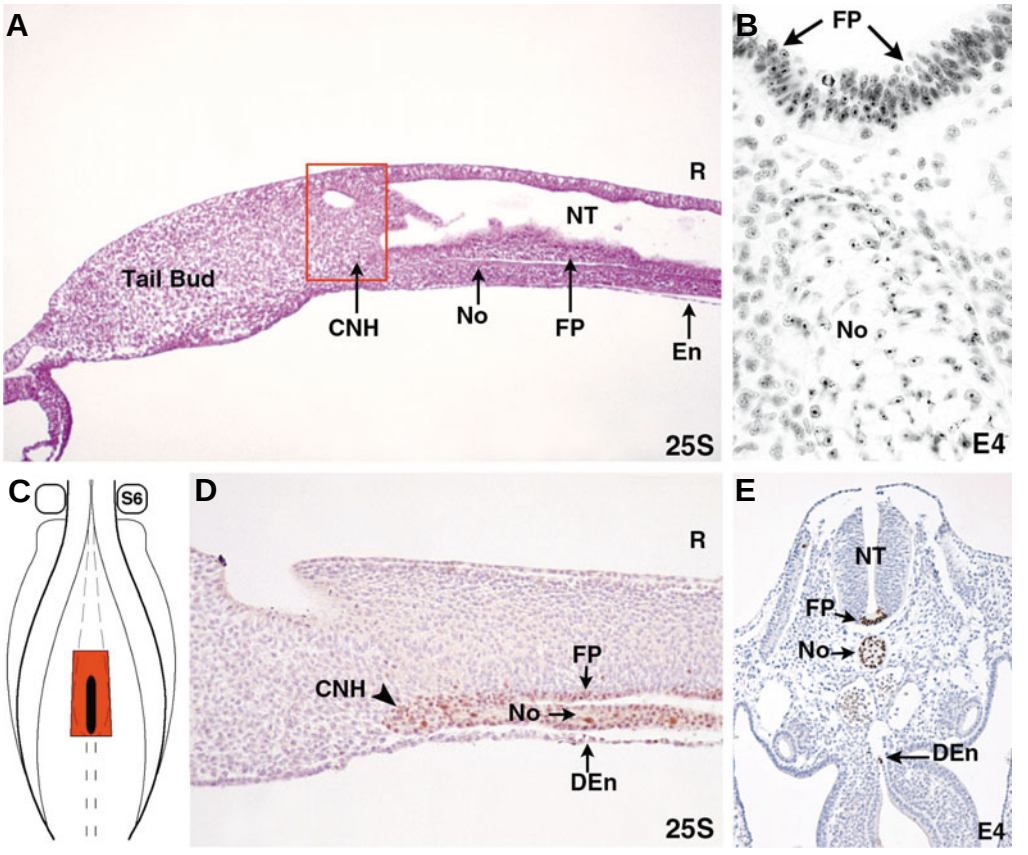


**Fig. 2. CNH and Hensen's node quail-chick chimeras. (A)** *Sagittal section of a 25-somite-stage (25S) chick embryo showing the replacement of the chordo-neural hinge (CNH) and overlaying medullary cord (red rectangle) by its quail counterpart.* **(B)** *This experiment results in the quail labeling of the notochord (No) and floor plate (FP) of the neural tube (NT) in the whole caudal region of the chick embryo as seen on a cross section of tip of the tail, two days after the graft (E4). Lateral and dorsal neural tube is labeled only in the grafted region.* **(C)** *Schematic representation of the replacement of Hensen's node in a 6S chick embryo by its quail counterpart. This experiment gives rise to quail labeled FP, No and dorsal endoderm (DEn) as seen on a sagittal section* **(D)** *the day after the graft (25S) and on a cross section* **(E)** *at E4. R, rostral. (B) Feulgen-Rossenbeck staining; (D,E) QCPN immunostaining.*

2- morphogenetic movements taking place during both primary and secondary neurulations are very similar, indicating that these two phenomena are closely related.

## «Early» *versus* «late» Hensen's node

At the onset of avian gastrulation, formation of the primitive streak from the posterior marginal zone settles the future rostro-caudal axis of the embryo. It is interesting to note that, at early stages, a fragment of posterior marginal zone grafted ectopically is able to induce a supplementary axis without contributing to the primitive streak (Bachvarova *et al.*, 1998). This specific property led to the proposal that the posterior marginal zone is homologous to the amphibian Nieuwkoop's center (see Skromne and Stern, 2002 for a review). It expresses both *Wnt8C* and *cVg1,* the products of which co-operate to induce an extra-axis (Shah *et al.*, 1997). This induction leads to the expression of *Nodal* by epiblastic cells located immediately in contact with the posterior marginal zone (Skromne and Stern, 2002). *Nodal* -expressing cells, which also express *Goosecoid*, eventually migrate to form the extreme

rostral tip of the primitive streak, namely the «early node» (Izpisua-Belmonte *et al.*, 1993). It is known that early Hensen's node, corresponding to the maximal extension of the primitive streak, is also able to induce an extra-axis when grafted in the *area opaca* (Dias and Schoenwolf, 1990 and references therein). Early node gives rise to the prechordal plate, the entire notochord and the floor plate posterior to the hindbrain (Patten *et al.*, 2003). In contrast, the late Hensen's node (node at somitic stages) is not able to induce an extra-axis (Dias and Schoenwolf, 1990; Inagaki and Schoenwolf, 1993). It gives rise to both the notochord and the floor plate of the truncal and caudal parts of the body (Catala *et al.*, 1996). At the molecular level, some genes such as *Otx2* (Bally-Cuif *et al.*, 1995), *Goosecoid* (Izpisua-Belmonte *et al.*, 1993) and *Nodal* (Patten *et al.*, 2003) are expressed by the early node and not by the late one.

## Hensen's node molecular pattern

Early Hensen's node, at Hamburger and Hamilton's stage 4 (HH4) consists of a group of cells expressing *HNF3β*, *Shh* and *Chordin*, located at the rostral end of the primitive streak (Lawson *et al.*, 2001). *Chordin* transcripts appear very early in the medio-rostral part of the growing primitive streak, whereas *HNF3β* transcripts are detected in this region just before complete extension of the streak. *Shh* expression begins just after that of *HNF3β* in the same region and becomes rapidly asymmetric (Levin *et al.*, 1995) under the influence of BMP4 (Monsoro-Burq and Le Douarin, 2001). These gene expression patterns (Lawson *et al.*, 2001) are maintained with some regional modifications during gastrulation.

We recently defined three rostro-caudal sub-regions (Fig. 3 A,C,E) within 5-6-ss Hensen's node (Charrier *et al.*, 1999): *zone a* corresponds to the region immediately ahead of the median depression located in the middle of the *sinus rhomboidalis*. This anterior part of the node is composed of a superficial epithelial cell layer and a deeper mesenchymal cell layer clearly separated by a basement membrane and corresponding respectively to the future floor plate and notochord. At this stage, cells of both layers express *Chordin*, *HNF3β* and *Shh*. In a more posterior position, *zone b* forms the bulk of the node and the source of most of the midline cell precursors. It lies within the median pit. In this region, the superficial pseudo-epithelial layer is not separated from the deep mesenchymal layer by a basement membrane. Cells of both layers express *Chordin*, *HNF3β* and *Shh*, although there are slightly less transcripts for *Chordin* and *Shh* in the superficial layer than the deep layer, principally in its caudal region. Between *zone b* and the rostralmost part of the primitive streak which strongly expresses the *Tbox* gene *Tbx6L*, *zone c* is represented by a discrete area of randomly distributed cells expressing *HNF3β* but neither *Shh* nor *Chordin* and *Tbx6L*. *Zone c* is immediately caudal to the median pit and is covered by a superficial columnar epithelium expressing neural markers *Sox1* and *Sox2*. This superficial epithelium is neural in nature and in continuity, with the lateral and caudal neural plate areas of the *sinus rhomboidalis* (Charrier *et al.*, 1999). It will form the basal plates of the future neural tube after medial insertion of the node-derived floor plate (Catala *et al.*, 1996 and see Fig. 2 B,E).

At later stages, the morphology of Hensen's node progressively changes as it becomes the so-called chordo-neural hinge (CNH), but gene expression pattern allows to distinguish the three sub-regions described at midgastrulation stage (Fig. 3 B,D,F). As stated previously, in the avian tail bud, the CNH is equivalent to Hensen's node (Catala *et al.*, 1995). It lies under the medullary cord which will give

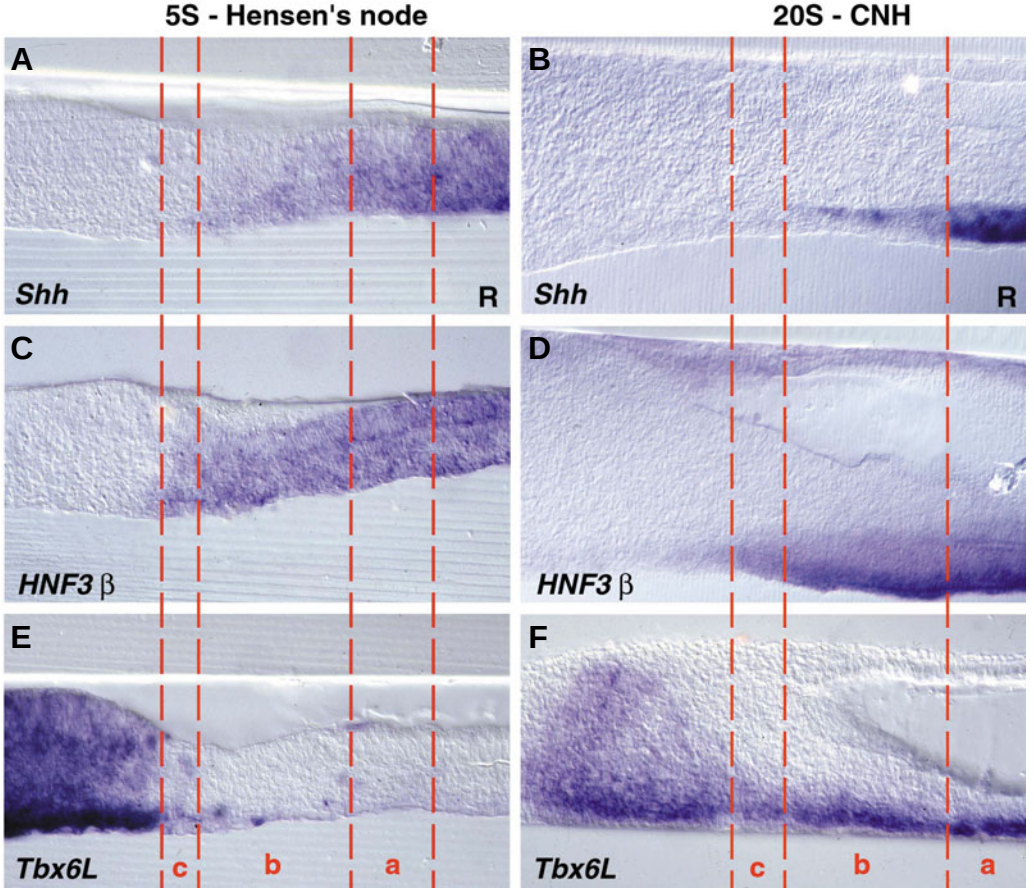


**Fig. 3. Conserved gene expression pattern in Hensen's node and CNH subregions. (A,B)** *In 5S-Hensen's node zone a,* Shh *is expressed in both the dorsal and the ventral cell layers separated by a basement membrane while in 20S-CNH zone a,* Shh *is expressed only in the ventral cell layer (future notochord). In Hensen's node and CNH zone b* Shh *is expressed exclusively in the ventral cell layer.* Shh *is not expressed in zone c.* **(C,D)** HNF3β *is expressed in dorsal and ventral cell layers of a and b zones and in zone c.* **(E,F)** Tbx6L *is expressed in primitive streak remnant at 5S and 20S.* **(F)** *Parasagittal section showing the paraxial mesoderm* Tbx6L *+ at the level of zones a, b and c.*

rise to the posterior neural tube by the process designated as «secondary neurulation». The caudalmost part of the medullary cord partially overlaps with the remnant of the primitive streak *(Tbx6L+)*, which extends caudally the midline structures and the CNH (Catala *et al.*, 1995 and see Fig. 3F). In the tail bud, *HNF3β* is expressed throughout the CNH and its derivatives, the floor plate and the caudal notochord (Ruiz i Altaba *et al.*, 1995). However, *Shh* is confined to the latero-ventral regions of the CNH and to the notochord and is not expressed in either the dorsal medial CNH nor in the floor plate which is situated immediately ahead of it (Marti *et al.*, 1995; Teillet *et al.*, 1998a). Asynchrony in *Shh* expression between the notochord and the floor plate first appears around 10-ss and increases with age. The delay of apparition of the *Shh* transcripts in the floor plate compared to the underlying notochord provided one of the principal arguments in favor of the hypothesis of the floor plate induction by the notochord (Dodd *et al.*, 1998; Tanabe and Jessell, 1996). However, although it is possible to induce experimentally a supplementary floor plate by the action of a notochord (see another section of this paper), we have demonstrated that the prolonged presence of the notochord under the floor plate territory is not necessary for its differentiation (Teillet *et al.*, 1998a).

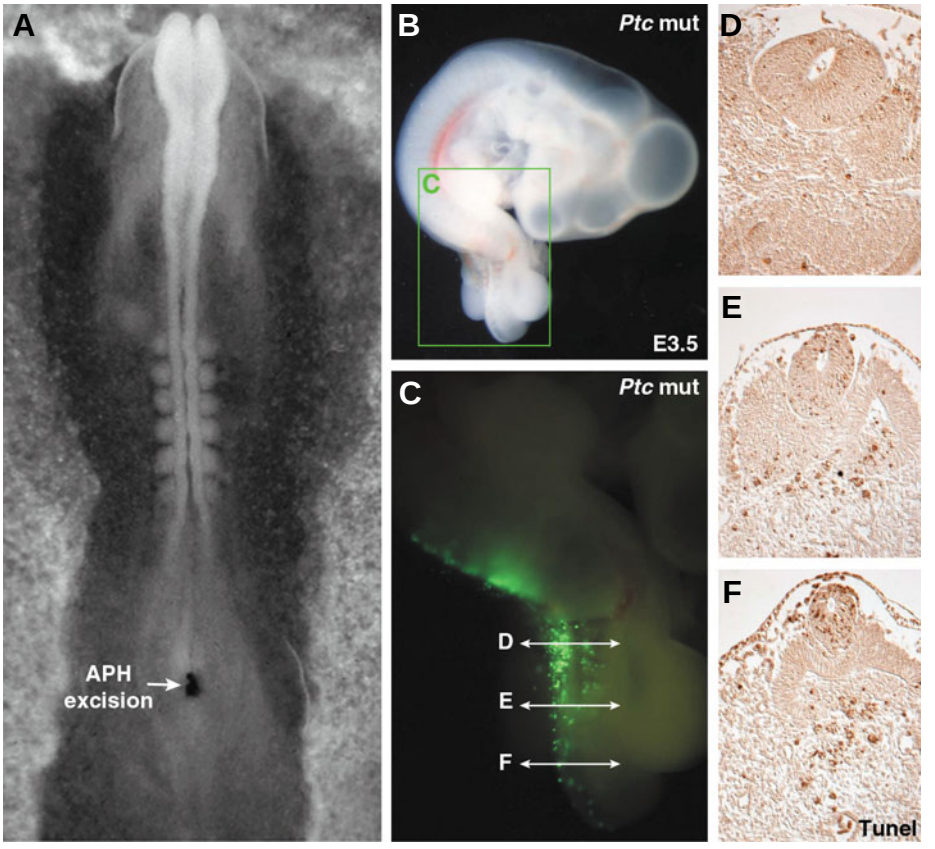


**Fig. 4. SHH Patched receptor is responsible for neural tube cell death. (A)** *Dorsal view of a 6-somite-stage chick embryo after microsurgical excision of Hensen's node zone c + rostral part of the primitive streak (axial paraxial hinge, APH). This experiment results in Hensen's node arrest, absence of notochord and floor plate in the caudal region, massive neural tube and somite cell death comparable to the result of Wolff Hensen's node irradiation (see figure 1). The same result is obtained after introduction of a solid barrier in the APH region.* **(B)** *Chick embryo 2 days after stopping Hensen's node caudal movement.* **(C)** *The neural tube has been electroporated with the dominant negative Ptc, here evidenced with GFP fluorescence.* **(D)** *Section in a well electroporated region shows few TUNEL positive cells, while other sections* **(E,F)** *in more caudal region where electroporation was not so efficient, TUNEL positive cells are more numerous.*

## Preventing Hensen's node rostro-caudal movement causes absence of midline cells

It has been known for many years, that excising Hensen's node before head-process development is followed by complete regeneration due to active cell proliferation and cell migration and gives rise to normal embryonic development (Waddington, 1932; Abercrombie and Bellairs, 1954; Yuan *et al.*, 1995; Psychoyos and Stern, 1996). A recent reinvestigation of the node regeneration process in the chick embryo suggested that early Hensen's node is a site where precursor cells are recruited and acquire specific molecular characteristics conferring them an organizer status (Joubin and Stern, 1999). In contrast, node excision after head-process formation gives rise to midline malformations (Psychoyos and Stern, 1996). We also found that selective excision of the previously described Hensen's node sub-regions at 5-6-ss results in reproducible specific consequences (Charrier *et al.*, 1999). Firstly, it has to be noticed that, although quail into chick grafts of *zone a+b* give rise to quail type midline cells from the thoracic region to the tip of the tail (Catala *et al.*, 1996), a simple excision of these structures without quail replacement does not result in the complete absence of the corresponding midline cells as expected (Charrier *et al.*, 1999). Instead, the floor plate and notochord are interrupted at the thoracic level, the site of excision. These structures appear separated from the neural tissue in their posterior end which is hypertrophied. Most of the lumbo-sacral region of the excised embryos is devoid of notochord and floor plate and pairs of somites are fused in the midline under a small unpolarized neural tube. Interestingly, this phenotype is reminiscent of Wolff's observations following X irradiation of the node region (see Fig. 1 A,B) (Wolff, 1936). In *zone a+b* excised embryos, the floor plate reappears first alone in the sacral region, then accompanied by the notochord in a more caudal region. These neo-formed midline structures continue to the tip of the tail, unless *zone c* has been removed along with *zone b*. In the which case, the midline cells are missing all along the thoraco-lumbo-sacro-caudal neural tube. Surprisingly, the complete absence of midline cells from the thoracic region to the tip of the tail can also be obtained by selectively excising *zone c* along with the rostral tip of the primitive streak (Charrier *et al.*, 1999) or by inserting a solid barrier between *zone c* and the tip of the streak (Thibert *et al.*, 2003). When these experiments are performed at 5-6-ss, Hensen's node does not yield midline structures. The neural tube and the somites

which develop caudal to Hensen's node arrest in the absence of midline cells express only dorsal molecular markers. Thus, *Pax3* is expressed all around the neural tube and throughout the somites, long after segmentation. Moreover, somites often fuse along the midline under the unpolarized neural tube. Therefore the caudalward movement of the node depends upon the integrity of *zone c*, the most posterior region, which is normally in close contact with the rostral part of the primitive streak, identified respectively by *HNF3β* and *Tbx6L* expression (see Fig. 3). We called this region the axial-paraxial hinge (APH), since it corresponds to the junction between the presumptive midline axial structures (notochord and floor plate) and the paraxial mesoderm (Charrier *et al.,* 1999).

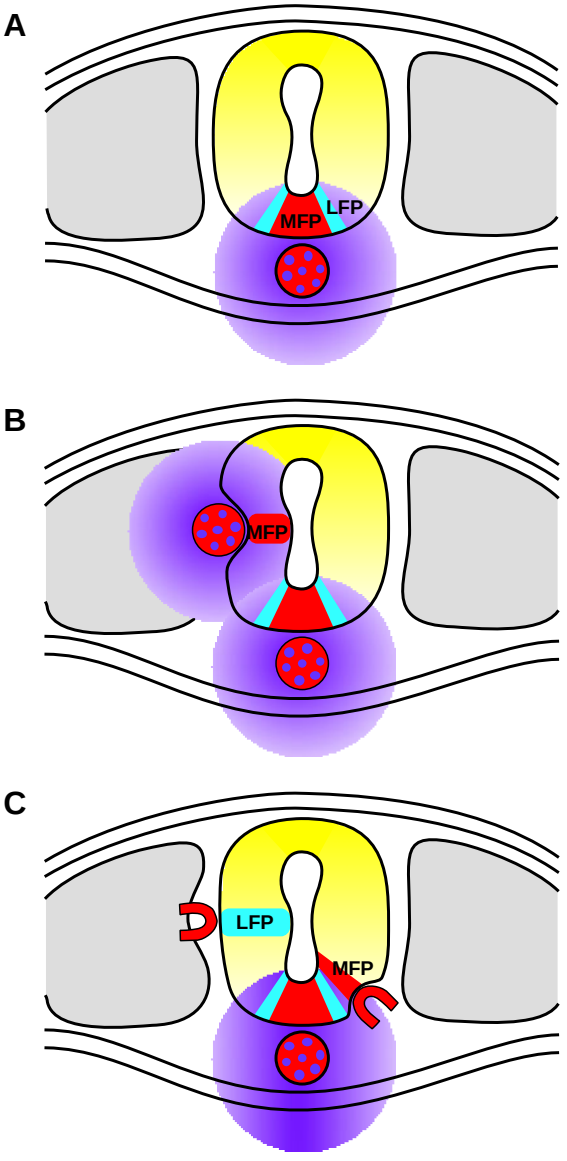


**Fig. 5. SHH is necessary but not sufficient to obtain floor plate differentiation in the lateral neuroepithelium. (A)** Medial floor plate (MFP) and notochord have a common origin in Hensen's node. The lateral f*loor plate (LFP) is induced in the neuroepithelium by action of SHH.* **(B)** *A typical MFP can be induced by grafting a notochord close to the lateral neuroepithelium.* **(C)** *A MFP or SHH producing cells grafted laterally to the neural tube induce a LFP. To obtain MFP development, MFP graft has to be made in the vicinity of the host notochord. These results show that another signal than SHH, present in the notochord, is necessary, with SHH, to obtain a MFP. This signal may be a BMP antagonist.*

## Absence of midline cells causes apoptosis in neural tube and somite cells

As stated previously, interruption of the caudalward movement of Hensen's node can be obtained by selective excision of the APH (Charrier *et al.*, 1999) or by inserting a barrier between *zone c* and the tip of the primitive streak (Thibert *et al.*, 2003). These microsurgical manipulations do not prevent the formation of the caudal neural tube posterior to the operation. However, the neural tube which develops in these conditions is devoid of midline cells (i.e. notochord and floor plate). As a consequence, the thoraco-lumbo-sacro-caudal neuraxis develops without any contact, at any time of development, with Hensen's node-derived midline cells showing that the presence of the notochord or floor plate is required for neither primary nor secondary neurulation. However, we did observe that the neural tube and somites formed in the absence of midline structures undergo massive cell death by apoptosis within a few hours following the operation, resulting in the disappearance of nearly all of the tissues within this region and often the truncation of the embryo posterior to the forelimbs at E4. Backgrafting notochord or floor plate fragments close to the neural tube in the caudal region devoid of midline cells, one day after APH excision, results in the rescue from cell death of the caudal tissues that are in contact with the graft. Their growth and differentiation take place demonstrating that midline cells exert a positive effect on survival and growth of the neural tube (Charrier *et al.*, 2001) as they do on axial myogenic and skeletogenic somitic cells (Teillet *et al.*, 1998b).

## SHH is necessary for the survival and patterning of neuroepithelial and somitic cells

Interestingly, either one of the two midline structures (notochord or floor plate) is sufficient in the avian embryo to restore both survival and early patterning of the neural tube and somites suggesting that the factor(s) involved in this process is(are) produced by both of them and able to exert a long-range action. One candidate factor is the protein SHH whose role in cell proliferation and cell survival has been amply documented in various systems, particularly in the nervous system (Jensen and Wallace, 1997; Teillet *et al.*, 1998a,b; Ahlgren and Bronner-Fraser, 1999; Borycki *et al.*, 1999; Dahmane and Ruiz-i-Altaba, 1999; Rowitch *et al.*, 1999; Wallace and Raff, 1999; Wechsler-Reya and Scott, 1999; Litingtung and Chiang, 2000; Dahmane *et al.*, 2001; Britto *et al.*, 2002). Fibroblastic cells engineered to produce the SHH glycoprotein (Duprez *et al.*, 1998) were grafted in the vicinity of the caudal neural tube previously deprived of midline cells by APH excision. Grafted SHH-producing cells insured the survival, growth and patterning of the neural tube and paraxial mesoderm (Charrier *et al.*, 2001). In these conditions, *Patched* (*Ptc* ) transcripts were strongly and homogeneously up-regulated in the wall of the neural tube and in the somites at the level of the graft. This up-regulation of the SHH receptor in the vicinity of the grafted SHH-producing cells demonstrates that the SHH protein exerts a powerful signaling activity on neuroepithelial and somitic cells. This result, concomitantly with the observation of cell death decrease in the

presence of SHH, supports the idea that naturally occurring cell death in the neural tube and paraxial mesoderm may be controlled by SHH produced by the notochord and the floor plate.

We confirmed this hypothesis in a series of *in vitro* and *in vivo* experiments (Thibert *et al.*, 2003 and Fig. 4). Cells transfected with *Ptc* undergo massive apoptosis which is rescued by adding SHH to the culture medium. Electroporation of a *Ptc* construct in the neural tube of E2 chick embryo *in ovo,* induces cell death in the latero-dorsal neuroepithelial cells. These results suggest that the Patched receptor might work like a dependence receptor (Mehlen and Bredesen, 2000). In the presence of SHH, the Ptc ligand, the SHH pathway is started. When SHH is not available, an apoptosis pathway is engaged. In fact, molecular studies have shown that, in the absence of SHH, a pro-apoptotic site is exposed by caspase 3 cleavage in the intracellular domain of the transmembrane receptor. *In vitro* cell transfection with the cleaved *Ptc* induces apoptosis which cannot be rescued with SHH. A dominant negative Patched, unable to induce cell death when transfected in cells in culture, was constructed by mutating an aspartic acid in the cleavage site. Interestingly, this mutant *Ptc* electroporated into a neural tube deprived of midline cells was shown to rescue this tissue from death (Thibert *et al.*, 2003 and see Fig. 4 B-F). These results suggest that, during embryonic development, an anti-apoptotic SHH signal is required during a temporal window that goes from neurulation and specification of presomitic mesoderm to organogenesis of the neural tube and somites and then extends for at least 48 hours in the avian embryo. This period of time corresponds to a phase of active cell proliferation and early embryonic tissue patterning.

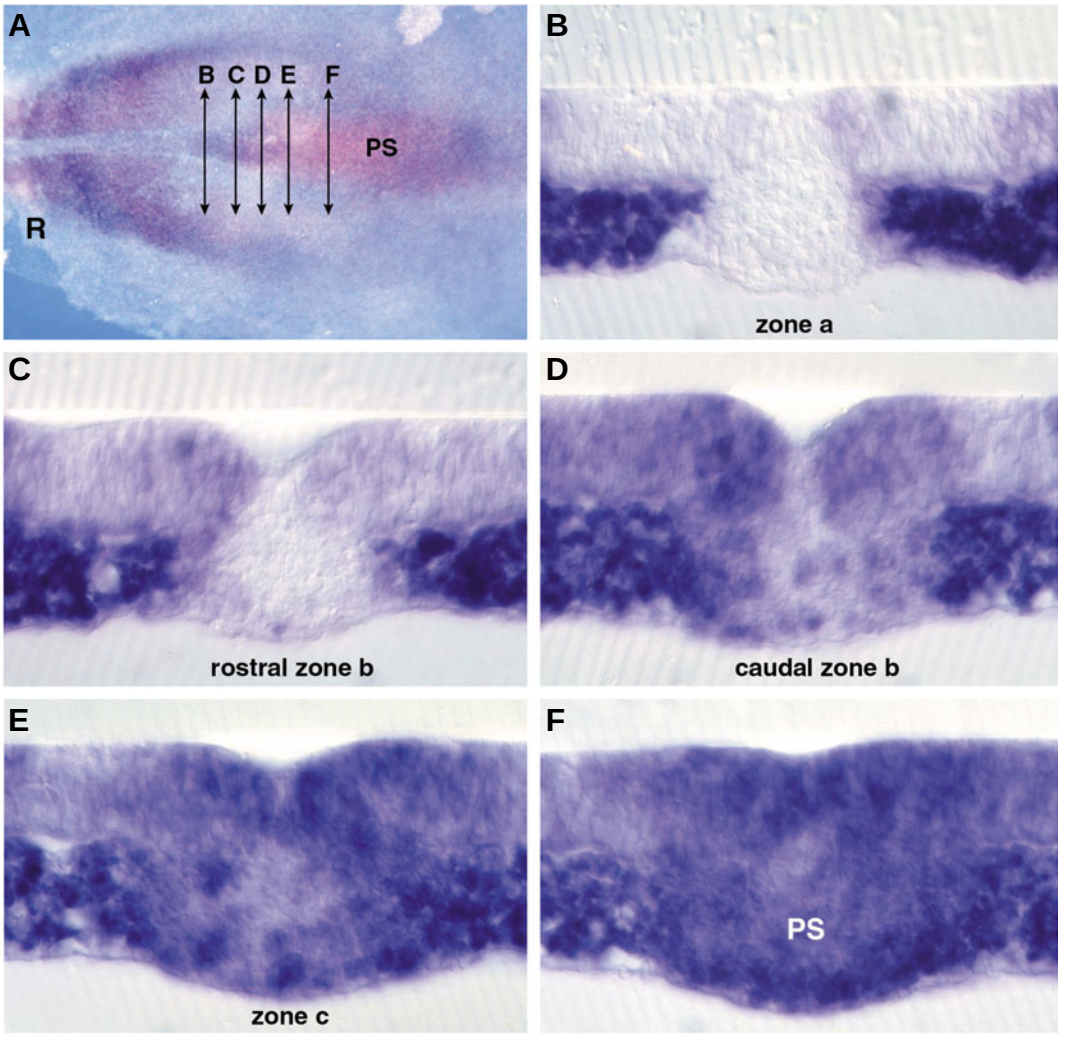


**Fig. 6. Delta/Notch expression pattern in Hensen's node and cell lineage choice. (A)** *Dorsal view of a 5-somite-stage chick embryo hybridized with* Delta *(red) and* Notch *(blue) probes. Rostral (R) is on the left.* **(B-F)** *Cross sections of the embryo show no expression of* Delta *and* Notch *at the level of Hensen's node zone a* **(B)** *and rostral zone b* **(C).** *Cells co-expressing* Delta *and* Notch *are present at the level of caudal zone b* **(D)** *and zone c* **(E)** *where notochord and floor plate progenitors are supposed to arise. A strong co-expression of both genes characterizes the primitive streak (PS) level* **(F)**.

## Dual origin of the floor plate of the neural tube

Molecular analysis of embryonic chimeras in which quail Hensen's node, or quail superficial epithelium caudal to Hensen's node, was substituted for its chick counterpart at the 5-6-ss, showed that the node-derived floor plate possesses the characteristic polarized epithelial organization with basal nuclei and expresses *HNF3β*, *Shh* and *chordin* with the exception of genes such as *Sox1* and *Sox2* which are expressed very early in the neural ectoderm (Charrier *et al.*, 2002). Thus, the node-derived floor plate that we have called Medial Floor Plate (MFP) by analogy with the zebrafish MFP (Odenthal *et al.*, 2000) is characterized by the same gene expression pattern as the node cells.

Later, *Sox1* positive neuroepithelial cells lateral to the MFP acquire molecular and functional characteristics that allow them to be considered as lateral floor plate (LFP) cells. In fact, LFP cells are induced by MFP cells to express *HNF3β* transiently and *Shh* and other floor-plate characteristic genes, such as *Netrin* and *Spondin,* continuously. Interestingly, LFP cells express other neural markers such as *Nkx2.2* and *Sim1* and maintain the pseudo-stratified structure of the neuroepithelium. This floor plate domain may represent the so-called p3 domain of the spinal

neural tube which is fated to form V3 interneurons (Briscoe *et al.*, 2000).

*HNF3β* and *Shh* are activated early in Hensen's node and are expressed by its derivatives, the notochord and floor plate cells. However, none of these genes are expressed by the neurectoderm prior to the time it becomes associated with the node-derived midline structures. Moreover, when insertion of the floor plate territory is inhibited by APH excision, the neural tube never expresses *HNF3β* nor *Shh*. Contact between the node-derived cells fated to become MFP and the adjacent neural ectoderm is necessary for the activation of the LFP genes to occur. The presence of *Sox1* transcripts distinguishes LFP from MFP which, in fact, does not display neural traits (Charrier *et al.*, 2002).

Interestingly, although MFP and LFP have distinct embryonic origins in normal development (the node and the neural plate respectively), a complete floor plate (MFP + LFP) can be induced in the neural epithelium under the action of either a notochord or a MFP. This was obtained either by grafting these embryonic structures close to a normal neural tube or by associating a notochord with a neural tube deprived of midline cells by APH excision. However, the full molecular characters of the MFP were present only after 5 days of exposure to the graft. Moreover, MFP could not be induced on the full length of the graft but only over a short length corresponding to a region of the host neural tube situated close to Hensen's node at the time of the graft. In contrast, grafting SHH-producing cells laterally to the neural tube only produced LFP characteristics. It was also the case when SHH-producing cells or MFP cells were grafted in contact with a neural tube previously deprived of midline cells by APH excision. Therefore, we concluded that SHH alone was not sufficient to fully transform neuroepithelial cells into MFP cells but could induce a LFP (Charrier *et al.*, 2002). It should be noted that, in the zebrafish embryo, SHH is not involved in specifying the MFP, but is essential for inducing the LFP (Schauerte *et al.*, 1998).

Interestingly, SHH-producing tissues like MFP or gut endoderm fragments grafted dorso-laterally to the neural tube did not induce MFP but only LFP, while they induced MFP characteristics when grafted more ventrally in the vicinity of the notochord (M.-A.T. unpublished results). These results suggest that other factor(s) produced by the notochord is(are) involved together with SHH to induce a MFP in the lateral neural tube (Fig. 5). As already suggested by *in vitro* experiments (Patten and Placzek, 2002), BMP antagonists like chordin or noggin present in the notochord may be good candidates to co-operate with SHH to induce a floor plate in the neuroepithelium.

## Floor plate development can occur independently of the notochord

Based on the results of notochord extirpation at early stages and on the possibility to induce a neo-floor plate by additional notochord grafts in the avian embryo (van Straaten *et al.*, 1985; van Straaten *et al.*, 1988; Placzek *et al.*, 1990; Placzek *et al.*, 1991; van Straaten and Hekking, 1991; Yamada *et al.*, 1991; Pourquie *et al.*, 1993), it has been postulated that the notochord is the natural inducer of the floor plate and is necessary for both its differentiation and its maintenance (Tanabe and Jessell, 1996). However, trunk MFP and notochord share a common origin from Hensen's node/CNH, i.e. from the organizer of the avian embryo (Catala *et al.*, 1995; Catala *et al.*, 1996). They become progressively separated from each other by the formation of a basement membrane between a dorsal epithelial structure which becomes incorporated into the neural ectoderm and a ventral notochord, both of which express the transcription factor *HNF3β* , as do Hensen's node/CNH cells (Charrier *et al.*, 1999 and Fig. 3 in this paper). In order to determine if the floor plate fate is acquired as soon as its territory is distinct from the notochord territory, we grafted quail Hensen's node into chick embryos at 5-6-ss and isolated the chimeric neural tube down to the level of the quail CNH at 20-25-ss. This chick neural tube with quail midline cells was back-grafted into a stage-matched chick embryo in the absence of notochord. A normal floor plate differentiated in the region where quail midline cells were present showing that self-differentiation of MFP can occur (Teillet *et al.*, 1998a). Moreover it should be noticed that in embryos in which the node was only partly excised (Charrier *et al.*, 1999), the floor plate alone differentiates in the lumbar region without an underlying notochord. It is only in the sacro-caudal region that the floor plate and notochord are co-localized. This observation confirms that floor plate differentiation after its insertion in the midline of the neural plate is independent of the presence of notochord.

## Hensen's node cell fate and differentiation

From these different studies, we propose that at 5-6-ss, corresponding to the mid-extension of the body axis of the avian embryo, different sub-regions of Hensen's node may be made up of differently committed pools of midline cell precursors. In the most rostral part of the node *(zone a)*, the dorsally located cells may be already committed to a floor plate fate while the ventrally located cells are fated to form a notochord. The medial part of the node *(Zone b)* gives rise to all the midline cells (floor plate and notochord) from the brachial level down to the end of the tail of the embryo and which are therefore endowed with a high proliferation potential. Gene expression patterns, cell organization (Charrier *et al.,* 1999) and results of node dorso-ventral inversion (M.-A.T. and J.-B.C. unpublished results) suggest that cell commitment is in progress in this region. In contrast, we hypothesized that cells of Hensen's node caudal region *(zone c)* may still be uncommitted. In fact, during normal development, *zone c* cells do not contribute massively to midline cell structures, as seen in quail/chick chimeras (Charrier *et al.*, 1999). In contrast, when challenged by the extirpation of the pool of highly proliferative precursors of *zone b*, more *zone c* cells are recruited than normal and a regulation process leads to the reappearance of a floor plate and a notochord in the caudal part of the embryo (Charrier *et al.*, 2001). Thus, *zone c* cells have the potential to compensate for the deficiency of *zone b* cells in experimental conditions. Moreover, they mediate caudalward movement of Hensen's node and thus insure the survival of caudal embryonic structures by midline cell deposition.

It was hypothesized that, at mid-gastrulation, *zone c* cells possess the multipotency and self-renewal capacity of stem cells (Le Douarin and Halpern, 2000). Pluripotency of early node cells was previously shown at the definitive primitive streak stage by means of intracellular cell staining (Selleck and Stern, 1991). Whether the choice of Hensen's node *zone c* cells to enter either the floor plate or the notochord differentiation pathway results from intrinsic cell fate decision and/or from extrinsic signals cannot be deduced from the previous data. However, recent

results in several animal models are in favor of a balance between notochord and floor plate cells by cell fate decision at the level of the organizer (Appel *et al.*, 1999; Lopez *et al.*, 2003; Przemeck *et al.*, 2003). Notch-Delta signaling is a good candidate for controlling this process. This signaling pathway is well known for its crucial role in lateral inhibition during neurogenesis (Artavanis-Tsakonas and Simpson, 1991 for a review). The hypothesis of multipotential progenitors located within the node is supported by the analysis of zebrafish mutants in which the proportion between notochord and floor plate cells can vary considerably (Appel *et al.*, 1999 and see Strähle *et al.,* 2004 for a review). It has been shown that *dlA* mutants have a reduced number of floor plate cells and an excess of notochord cells, whereas embryos that over-express *dlA* have a reduced number of trunk notochord cells and an excess of floor plate cells. More recently, the role of Notch signaling was analyzed during specification of the dorsal midline in *Xenopus* embryos (Lopez *et al.*, 2003). By activating or blocking Notch signaling, the authors found that Notch expands the floor plate domain of *Xenopus* embryos by stimulating *Shh* and *pintallavis (HNF3β)* and represses the notochordal markers *chordin* and *brachyury.* They propose that in *Xenopus*, as in Zebrafish, Notch may execute a binary decision, favoring the floor plate development at the expense of the notochord, preferentially before mid-gastrula stage. Moreover, they also showed that SHH down-regulates *chordin*, suggesting that SHH itself may be involved in reinforcing the binary decision executed by Notch. In a preliminary report, we performed *in toto* double hybridization with *Notch* and *Delta* probes in chick embryos during early development. Our observations are concordant with the previous observations and we showed co-expression of *Notch* and *Delta* within the *zones b* and *c* of the node at several stages including the mid-gastrula stage (Fig. 6). We are currently testing *Notch* and *Delta* over-expression using DNA electroporation in the node region of 5-6-ss chick embryos.

## Human caudal dysplasia recapitulates the phenotype of Hensen's node ablation

Caudal dysplasia is a rare human syndrome whose incidence varies between one to five per 100,000 (see Catala, 2002 for a review). One of the salient features of this syndrome is the abrupt truncation of the axis. For example, in the more severe cases reported (Frantz and Aitken, 1967; Ignelzi and Lehman, 1974), vertebrae develop down to L2 and T12 respectively. Actually, this syndrome is clinically very heterogeneous and severe cases resemble the effects observed after node X irradiation (Wolff, 1936) or node ablation (Charrier *et al.*, 1999). In the human cases, the hind-limbs develop even if they are abnormal due to congenital muscular paresis.

The syndrome of caudal dysplasia has been associated with maternal diabetes mellitus (see Nievelstein *et al.*, 1994 for a review). This maternal disease accounts for the occurrence of 16% of the cases. Moreover caudal dysplasia can be experimentally reproduced in mouse embryo by administration of retinoic acid (Shum *et al.*, 1999). It is interesting to note that maternal diabetes dramatically increases the prevalence of caudal dysplasia in pregnant mice treated by retinoic acid (Chan *et al.*, 2002) showing the effect of maternal diabetes on the development of the tail in the mouse.

Sometimes, caudal dysplasia can arise as an autosomal dominant disease that has been mapped to the region of chromosome 7q36 (Lynch *et al.*, 1995). *HLXB9* coding for the homeodomain-containing protein (HB9) corresponds to the major gene involved in this human malformation (Ross *et al.*, 1998). The role of HB9 in mice is crucial for the development of both the endocrine pancreas and the motor neurones and cannot explain the occurrence of such a syndrome in humans. Further molecular studies are thus needed to elucidate the role played by these human mutations in the genesis of caudal dysplasia.

## Conclusion

Growth and organization along the rostro-caudal axis in vertebrates is a continuous and complex process which takes place during gastrulation-neurulation and is achieved with final development of the trunk and tail. In the chick embryo, Hensen's node, the organizer, is essential for this development. When this structure is ablated, destructed or prevented from rostro-caudal movement, midline cells, notochord and floor plate of the neural tube, are lacking and neural and mesodermal cells rapidly die by apoptosis, showing the role of molecule(s) issued from node-derived cells in survival and differentiation of multiple tissues. One of these molecules is Sonic Hedgehog whose double role has been clearly demonstrated. Absence of SHH starts an apoptosis process in the neural tube and somites, while its presence insures growth and differentiation.

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