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The role of *vHnf1* and FGF signaling in caudal hindbrain patterning

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PhD Thesis supervised by
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Barcelona, June 2008

...coloca bolsas de té
debajo de las almohadas,
escribe imaginarios
en libretas deshojadas,...

A mons pares...

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Aragon, F., Vazquez-Echeverria, C., Ulloa, E., Reber, M., Cereghini, S., Alsina, B., Giraldez, F. and Pujades, C. (2005). *vHnf1* Regulates Specification of Caudal Rhombomere Identity in the Chick Hindbrain. *Dev. Dyn.* **234,** 567-576.

AGRAÏMENTS / ACKNOWLEDGMENTS

Abans que res vull agrair a la Cristina Pujades l'oportunitat que em va donar ja fa uns cinc anys d'iniciar-me en la vida científica. També li vull agrair totes les oportunitats d'assistir a congressos, cursos i l'estada que vaig fer al MRC de Londres. Però especialment li vull donar les gràcies per mostrar-se pacient en moments en que les coses no anaven com havien d'anar. Tant a ella com al Fernando Giráldez i la Berta Alsina els vull d'agrair també la generositat que han tingut a l'hora de compartir el seus coneixements i la seva dedicació a la nostra formació. Al Fernando, ara al final, li agraeixo la lectura crítica de la tesi, tot i que li podria agrair mil altres coses.

Gràcies també a tots el altres membres del laboratori. A la Gina que ha estat tant un mirall i com un horitzó durant tot el projecte, a més d'una gran amiga. A l'Enki per esenyar-me que jo també tinc polzes oposables i que també els puc fer servir i per intentar sense gaire èxit que deixés de ser un potiner. A la meva companyona Montse Coll i Lladó i també a la Montse Porta. A l'Andrés encara que fagi bromes una mica *cabronas* com per exemple amagar-me el portàtil el dia abans de dipositar la tesi. A la Citlali, la Safia, la Dora, la Marija i la Joana. Qui no pot ser feliç rodejat de tanta floreta. A l'Àlex, que es queda amb el jardí. Al nostre nou, flamant i *gambitero* tècnic, Miquel. I molt especialment a l'Eva J "la Guri", per haver fet una lectura crítica de la tesi tot i que els FGFs no són el seu rotllo i per ser una persona tan maca.

Altres companys de la UPF i el PRBB que m'han anat tirant cables, mans i alguna *colleja* durant aquests anys són Antonio Mas, Lola, Iván, Emma, Bea, Luisa, Katy, Mingui, Miquel "calvo", Xevi, Águeda, Yaniré, Anna, Ainhoa, Jimena, Filipe, Mariana, Ari, Isabel, Leo, Enri i Montse, Manuel Salmerón i tota la penya enrotllada de manteniment... I em deixo a molta gent...

I also wish to acknowledge Dr. Ivor Mason for giving me a place in his lab during six months in 2005-2006. It was a very constructive and interesting experience in both scientific and social ways. In London I met some of my favorite people in the world: the medicine student and guitar master Gregory Philp, the physician and blues guitar master Mariano Campoy, the doctor Hannes Petersen and my beloved Stefania Boscolo, la piu bella secretaria dil mondo.

Aquest treball també va dedicat a tota aquella gent fora de l'àmbit científic que m'han recolzat en els moments més durs i que han tingut una fe cega en les meves capacitats en els moments en que més feble m'he trobat. Als meus pares, Josep i Marian, ma germana Vane, la "tieta" Contxi, mi querida Lola otra vez, també als meus companys d'armes Efrén, Enric, Eulàlia i Leri, en altres paraules els PURI LOPEZ uuaaaaaaoooo, la Mercè, l'Alberto, l'Efrem, la Kampanilla, la Roci i a molts més que se'm queden en el tinter i també en el cor.

A tots vosaltres: Moltes Gràcies

Salut i Llibertat per a tots!.

SUMMARY

During early embryonic development of metazoans the tissues within the embryo become progressively patterned until they acquire specific fates. In vertebrates, the neural tube, which is the primordium of the central nervous system, is early regionalized along the anteroposterior (AP) axis in three brain vesicles and the spinal cord. The caudalmost brain vesicle, is called hindbrain or rhombencephalon. In the hindbrain, a further step of regionalization leads to a transient organization along the AP axis in a series of segments called rhombomeres (r). This segmental organization serves as scaffold for several structures that develop within the hindbrain in repeated patterns, for example the cranial nerves or the reticular neurons. Importantly, rhombomeres are not only morphological units but also constitute units of gene expression and cell lineage restriction. Rhombomeres are compartments, they are separated by boundaries, but even before the formation of morphological boundaries cell intermingling among them is restricted. In addition, transcription factors, genes related to different signaling pathways and genes related to adhesive properties of the cells are expressed within the hindbrain showing limits of expression that coincide with rhombomere boundaries. Each rhombomere has a molecular identity given by a specific combination of gene expression. Rhombomeric identity is the result of a progressively refined patterning that involves the interplay of different cell signaling pathways and rhombomere-specific transcription factors.

vHnf1 (*variant Hepatocyte Nuclear Factor 1)* is one of the earliest transcription factors expressed in the hindbrain. Initially, *vHnf1* was associated with different gut-derived structures including the liver, kidney and pancreas. In 2001 a mutational screening for genes involved in development in zebrafish linked *vhnf1* with hindbrain patterning (Sun and Hopkins, 2001). In this study two hypomorfic mutants for *vhnf1* that showed misspecification of the caudal hindbrain and hypoplastic otic vesicles were identified.

In the present project the role of *vHnf1* in the caudal hindbrain patterning and its interplay with FGF signaling has been analyzed during chick embryo development. The results show that *vHnf1* is expressed very early in the chick neuroepithelium with a sharp anterior boundary of expression coinciding with the presumptive r4/r5 interrhombomeric boundary. Gain-of-function experiments demonstrate that *vHnf1* is able to confer caudal character to rostral rhombomeres. This suggests that *vHnf1* is involved in conferring

caudal identity to r5 and r6 by inducing both *Krox20* and *MafB* and preventing *Hoxb1* expression, the latter in an indirect manner. Our results demonstrate that, in chick, *Fgf3* not only co-operates with *vHnf1* in inducing caudal markers but also its expression is induced by *vHnf1*. Moreover, RT-PCR semiquantitative analysis reveals that *Fgf3* transcription is rapidly activated upon *vHnf1* overexpression, suggesting that *Fgf3* is a direct target of *vHnf1*. We also analyze the expression of *MKP3*, *SPRY2* and *Pea3*, genes of the FGF synexpression group, in the caudal hindbrain. Upregulation of *MKP3* after *vHnf1* overexpression confirms that *vHnf1* is upstream FGF signaling. Finally, we show that the role of FGF signaling in regulating the caudal rhombomeric markers *Krox20* and *MafB* is mediated through the Ras-ERK1/2 intracellular pathway.

The results of this project provide new information about the molecular mechanisms involved in patterning the vertebrate caudal hindbrain. *vHnf1* is a crucial gene for early steps of hindbrain patterning. Interestingly, while requirement of *vHnf1* and FGF signaling for caudal hindbrain patterning is an evolutionary conserved feature, the ways by which FGF signals are regulated during this process differ across species.

INTRODUCTION

1. Regional specification and segmentation during embryogenesis

1.1 Embryonic patterning

Each multicellular organism is organized during its embryonic development by the coordinated function of a set of genes that instruct the cells to develop the organism's body plan. Surprisingly, the body plan of very distant metazoans, often with radically different forms, are constructed using the same sets of genes. These highly conserved groups of genes have been termed *developmental genetic toolkit* (Carroll *et al.*, 2004). The *toolkit* represents a relatively small fraction of the genome and comprises transcription factors and genes from signaling pathways. A crucial function of the *toolkit* is to regionalise the naive embryonic territories in order to generate the body parts at the right places, in other words, to pattern the embryo. Lewis Wolpert has defined the concept of patterning or regional specification as "the process by which a spatial and temporal pattern of cell activities is organized within the embryo so that a well-ordered structure develops" (Wolpert *et al.*, 2007). In triblastic metazoans, the primary patterning events occur during gastrulation. Gastrulation consists in a series of cell migrations and morphogenetic movements that lead to an organization of the embryo in the three germ layers (ectoderm, mesoderm and endoderm). The formation of the three germ layers is accompanied by the polarization of the embryonic body in the anteroposterior (AP), dorsoventral (DV) and left-right (LR) axes. After gastrulation, the germ layers undergo local specification processes along the AP, DV and LR axes. At the end of these patterning processes each territory within the embryo has been fated to a specific structure. Then, progressive steps of organogenesis, in which proliferation, apoptosis, migration and differentiation processes are coordinated, generate all the adult structures.

1.2 Organizers and morphogens

The concept of organizer refers to "a group of cells that has the ability both to induce a new fate in neighboring cells and to pattern the induced tissues and/or other neighboring tissues" (Stern *et al.*, 2006). In 1920's, Spemann and Mangold demonstrated that grafting the dorsal lip of the amphibian blastopore in the ventral side of a host embryo leads to the formation of a second neural axis and to the induction of an ectopic nervous system from

host cells originally not fated to do so. Because of its unique ability to induce such a patterned array of structures from a tissue not fated to do so, the dorsal lip of the blastopore became known as 'the organizer' (Spemann and Mangold, 2001). Similar organizers were later described in other vertebrate species: the Hensen's node in birds, the node in mammals and the shield in teleosts. In addition to these global organizers, some models proposed that secondary organizers or local signaling centers, established in the boundaries between different regions of the germ layers, may be involved in further patterning the tissues in their vicinity (Meinhardt, 1983; Meinhardt, 2008). In the vertebrate Central Nervous System (CNS), the best characterized example of local signaling center is the Isthmic Organizer (IsO) in the Midbrain/Hindbrain Boundary (MHB) (Raible and Brand, 2004), although several others are proposed (Kiecker and Lumsden, 2005; Maves *et al.*, 2002; Rhinn *et al.*, 2006).

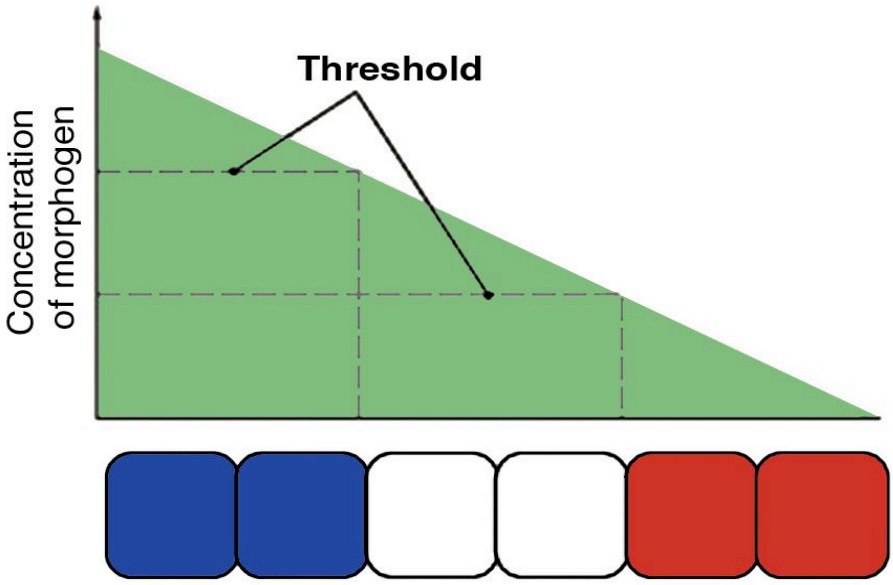


Figure 1. The French flag example. The diagram represents a theoretical regional specification process mediated by one morphogenetic gradient. The Y-axis represents the concentration of morphogen and the X-axis the distance from the source of morphogen. As distance increases concentration of morphogen decreases. The cells respond to different thresholds of morphogen concentration and acquire different positional identities and fates (represented by the three colours of the French flag). Modified from Kerszberg and Wolpert, 2007.

The signaling centers exert their organizing function by emitting morphogens. The diffusion of the morphogen from the signaling center generates a concentration gradient. Basically, different thresholds of morphogen concentration are interpreted by the cells that change gene expression and acquire positional identities according to their distance from the morphogenetic source (Fig. 1) (Ashe and Briscoe, 2006; Wolpert, 1996). This system is further complicated by the coordinated action of morphogenetic gradients from different sources (Diez del Corral and Storey, 2004; Goldbeter *et al.*, 2007; Olander *et al.*, 2006). The set of morphogens used during development include members of the FGF, WNT, RA, SHH and BMP signaling pathways (Bottcher and Niehrs, 2005; Goldbeter *et al.*, 2007; Kudoh *et al.*, 2002; Mason, 2007; Ulloa and Briscoe, 2007; Wilson *et al.*, 2000).

1.3 Segmentation

Segmentation is a developmental strategy that allows the generation of a variety of structures modifying one basic unit. It consists in dividing a territory in a series of segments or compartments along one body axis. Cells within a compartment behave under cell lineage restriction and do not intermingle with cells in the adjacent territories. A common combination of gene expression confers a specific identity to all the cells within a compartment. This identity is established by regional specification mechanisms and thus each compartment has a differentiated identity and fate from its neighbors. Segmentation coupled with regional specification was first described in the *Drosophila melanogaster* embryo (Akam, 1987). During early steps of *Drosophila* development the embryo becomes organized in a series of segments along the AP axis (Fig. 2A). Each segment gives rise to different parts of the abdomen, thorax and mouthparts of the head in the adult *Drosophila*. In vertebrates, segmentation can be transiently found only in three embryonic structures: the somites, the branchial arches and the hindbrain (Fig. 2B). It has been proposed that also the diencephalic part of the prosencephalon undergoes a segmentation process during the embryonic development (Larsen *et al.*, 2001; Puelles and Rubenstein, 2003; Shimamura *et al.*, 1995).

Segmentation is crucial to understand embryonic development. In segmental structures, regional units can be easily distinguishable by both morphological and molecular landmarks. Among them, the hindbrain has been object of intensive studies in the last twenty years. These studies led to a better understanding of how signaling pathways and

transcription factors interplay to confer positional identity to specific territories and thus organize the embryonic body plan.

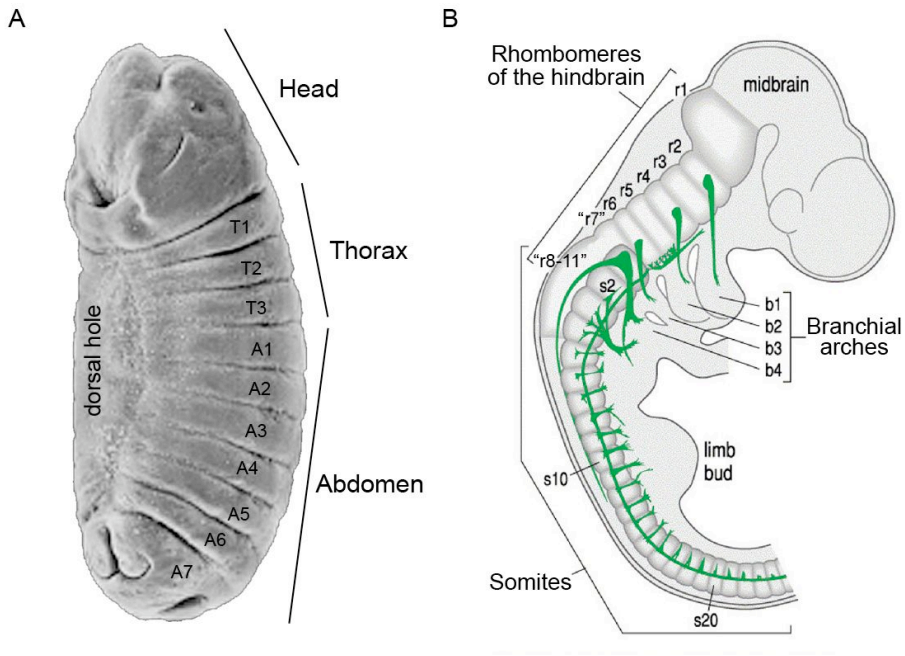


Figure 2. Segmentation in the *Drosophila* and vertebrate embryo. (**A**) Scanning microscopy image of a *Drosophila* embryo before dorsal closure. Segments that give rise to thoracic structures are marked with 'T' and segments that give rise to abdominal structures with 'A'. Modified from Parkhurst and S., 2008. (**B**) Schematic representation of a HH17 chick embryo in a lateral view. The three segmental embryonic structures are represented: hindbrain rhombomeres (r), somites (s) and branchial arches (b). Cranial nerves and dorsal root ganglions are shown in green. Modified from Wolpert L *et al.*, 2007. Lateral views with dorsal at left and anterior at top.

2. Introducing the model: the hindbrain

2.1 Anatomical and functional features

At the end of gastrulation the dorsal ectoderm thickens to form the neural plate in response to inductive signals from neighboring tissues. In amniotes, the neural plate folds giving rise to the neural tube, which is the primordium of the CNS. The neural tube divides along the AP axis into the primary brain vesicles: forebrain or prosencephalic vesicle that is later divided in telencephalon and diencephalon; midbrain or mesencephalic vesicle; and hindbrain or rhombencephalic vesicle that gives rise to the

metencephalon and the myelencephalon (Fig. 3A). Caudal to the hindbrain remains a narrow tube that is the spinal cord.

The hindbrain is the more posterior vesicle of the embryonic brain (Fig. 3A). Its most anterior area (metencephalon) gives rise to most part of the cerebellum structures. The cerebellum allocates essential functions for coordinating movements, position, and balance. The posterior hindbrain (myelencephalon) is constituted by the pons and medulla oblongata, in continuity with the spinal cord. The neurons located there generate nerve centers responsible for pain relay to the head and neck, auditory connections and balance control, tongue, neck and eye movement, as well as breathing, gastrointestinal and heart rate control (Fig. 3B) (reviewed in Netter *et al.*, 2002).

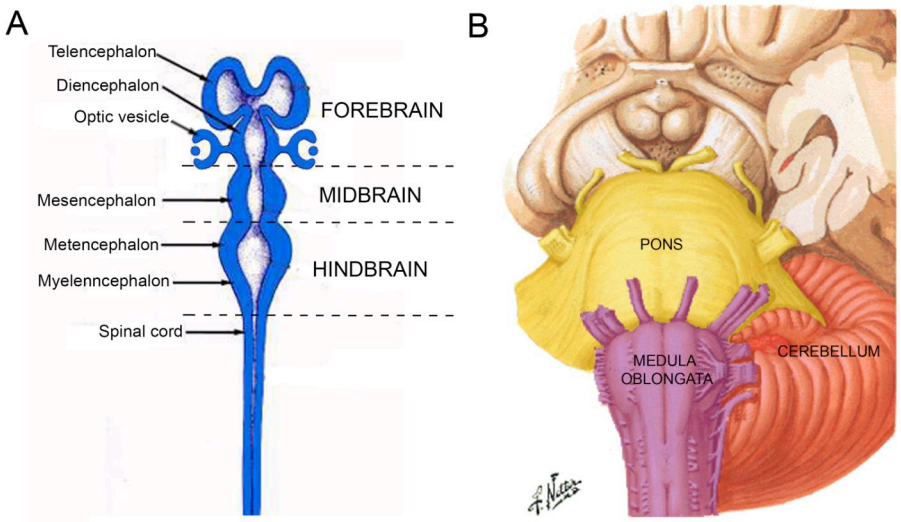


Figure 3. (A) Early regionalization of the neural tube along the AP axis. Schematic view of the early neural tube with the brain vesicles and the spinal cord. Anterior is at top. (**B) Encephalic trunk of an adult brain.** Illustration on a ventral view where anterior is at top. The three hindbrain-derived structures are highlighted with different colours: cerebellum in red, pons in yellow and medulla oblongata in violet. Modified from Ibarra *et al.*

The hindbrain is characterized by a comparatively small number of cell types disposed in an arrangement that is broadly similar to that of the spinal cord. Dorsal and lateral regions contain sensory projections and relay neurons whereas the ventral region contains the cell bodies of motor neurons. Motor and sensory neurons configure respectively the motor and sensory components of the cranial nerves (reviewed in Cordes, 2001). The hindbrain motor neurons are defined, by their arrangement in the neural tube and by their eventual synaptic targets, as visceral motor nuclei (vm), somatic motor nuclei (sm) or

branchial motor nuclei (bm) (Table 1). The nuclei of the motor neurons develop in the basal plate but the vm and bm axons converge on communal exit points in the alar plate, whereas the sm axons leave the neural tube ventrally in small clusters (with exception of the trochlear axons -mIV- that leave dorsally) (Fig. 4). The sensory components of the cranial nerves develop from placodal and neural crest cell-derived ganglions and their cell bodies are located outside the neural tube (Fig. 4). Hindbrain cranial nerves are listed in the Table 1, and their motor and sensory functions specified. Apart of the motor and sensory neurons, a population of interneurons constitutes the reticular formation in the hindbrain. The reticular neurons act to relay information along the anteroposterior (AP) axis of the neural tube. These neurons serve to integrate and coordinate complex behaviors, such as rhythmic breathing, arousal and modulation of pain sensation.

Cranial nerve	Class	Function/structures innervated
Motor nerves		
Trochlear (IV)	Somatic	Extraocular muscles
Trigeminal (V)	Branchiomotor	Muscles required for mastication
Abducens (VI)	Somatic motor	Extraocular musclesFacial (VII)
Facial (VII)	Branchiomotor	Muscles of facial expression
	Visceromotor	Tear glands, salivary glands
Glossopharyngeal (IX)	Branchiomotor	Muscles of jaw and neck for swallowing
	Visceromotor	Parotid gland
Vagus (X)	Branchiomotor	Muscles of larynx and pharynx; controls speech
	Visceromotor	Heart, blood vessels, trachea, bronchi, esophagus, stomach and intestines
Spinal accessory (XI)	Branchiomotor	Trapezius and sternocleidomastoid muscles
Hypoglossal (XII)	Somatic motor	Muscles of tongue
Sensory nerves		
Trigeminal (V)	General somatic	Sensation from skin, muscles and joints in face and mouth; sensory innervation of teeth
	General visceral	Proprioception from skin, muscles and joints in face and mouth
Facial (VII)	General somatic	Sensation of skin of external ear
	General visceral	Visceral sensation from anterior two-thirds of tongue and from external ear*
	Special visceral	Taste from anterior two-thirds of tongue
Acoustic (VIII)	Special somatic	Hearing and balance
Glossopharyngeal (IX)	General somatic	Sensations from skin of face and throat
	General visceral	Visceral sensation from palate and posterior third of tongue
	Special visceral	Taste from posterior third of tongue
Vagus (X)	General somatic	Sensation from skin of throat and abdomen
	General visceral	Visceral sensation from pharynx, larynx, thorax and abdomen
	Special visceral	Taste from epiglottus

*Visceral sensations include mechanical sensations, pain, temperature detection and proprioception

Table 1. Classification of the motor and sensory components of the hindbrain cranial nerves. Data from Cordes, 2001.

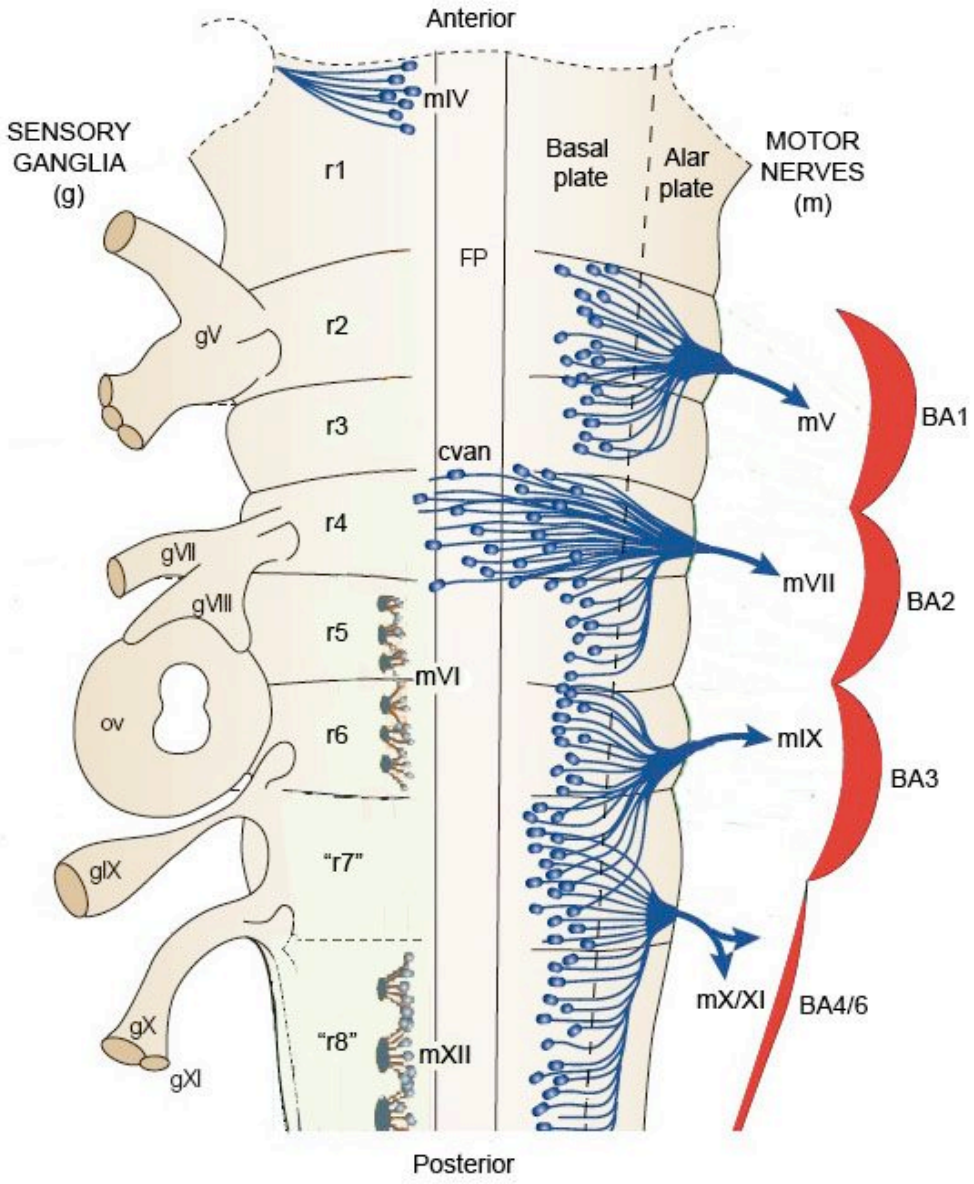


Figure 4. The cranial nerves in the segmented hindbrain. Schematic view of an HH20-24 hindbrain of a chick embryo representing a dorsal view where the roof plate has been removed and anterior is to the top. The hindbrain is segmented in a series of rhombomeres (**r1-r8**). Sensory and motor components of the cranial nerves are represented. At the left side the sensory ganglia (**gV-XI**), the otic vesicle (**ov**) associated with the vestibuloacustic ganglion (**gVIII**), and the somatic motor neurons of cranial nerves IV, VI and XII (**mIV,mVI,mXII**) are represented. At the right side the branchiomotor neurons which projections converge in dorsal exit points (**mV, mVII, mIX, mX/XI**) are depicted. mV, mVII and mIX innervate the branchial arches 1, 2 and 3 (**BA1-3**) respectively. mX and mXI innervate the 4th and 6th branchial arches (**BA4/6**). In r4, the contralaterally migrating efferent neurons (**cvan**) of the vestibuloacustic nerve (VIII) that share the exit point with mVII are represented. Dashed line separates basal and alar plates. FP: floor plate. Modified from Kiecker and Lumsden, 2005.

2.2 Segmental organization of the hindbrain during early development

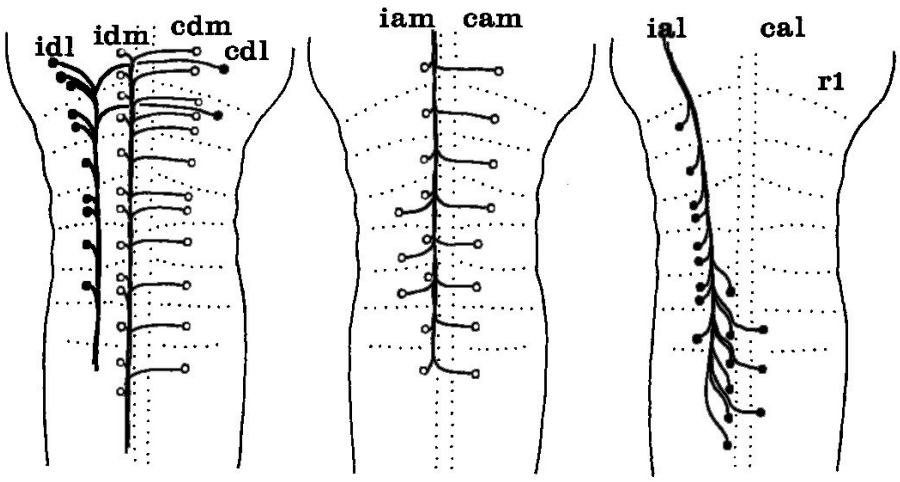
2.2.1 Segmental patterns within the hindbrain

During early embryonic development the hindbrain undergoes a transient segmentation process along the AP axis that leads to a series of compartments called rhombomeres (r) (Fig. 4). Vaage defined seven rhombomeres in the chick embryo, the last one being in continuity with the spinal cord (Vaage, 1969). Transient organization of the hindbrain in rhombomeres is a necessary step for the correct specification of all the neuronal types that the hindbrain will give rise to, the location of the cranial nerves exit points and the migration streams of the neural crest cells from the dorsal hindbrain to the branchial arches (Cordes, 2001; Lumsden and Guthrie, 1991; Lumsden, 2004; Lumsden and Keynes, 1989; Sechrist *et al.*, 1993; Trainor and Krumlauf, 2001).

Two patterns of metameric cellular organization are evident in the segmented hindbrain:

A) A repeated set of interneurons in each rhombomere. Up to HH20 stage in chick, the neuronal composition of each rhombomere in the hindbrain is remarkably similar, each generating similar axonal pathways and a set of eight similar basic reticular interneuron types (Fig. 5). Although there are regional differences, which increase in complexity as development proceeds, they appear to arise from variations on a basic theme rather than by abrupt phenotypic changes (Clarke and Lumsden, 1993). Similar observations were obtained in zebrafish where seven types of interneurons can be identified in the hindbrain at 5 days after fertilization. As it happens in chick, there is a basic set of these neurons in each rhombomere (Metcalfe *et al.*, 1986).

Figure 5. A pattern of eight interneuron types is repeated in each rhombomere. In 1993 Clarke and Lumsden classified the interneurons within the chick hindbrain in eight types. A basic set of these neuron types is found in each rhombomere. The diagram summarizes the interneuron populations within the hindbrain of a HH20 chick embryo. Neurons were classified according to three parameters: i) **i**psilateral or **c**ontralateral; ii) axons are **d**escendent or **a**scendent; iii) axonal pathways are located in the **l**ateral longitudinal tract or in the **m**edial longitudinal fasciculus. Thus the eight neuron types where: **idl, ial, idm, iam, cdl, cal, cdm** and **cam**. Unfilled cells have axons in the medial longitudinal fasciculus and filled cells have axons in the lateral longitudinal tract. Anterior at top. (Modified from Clarke and Lumsden, 1993)



B) A two-segment repeat pattern through alternate rhombomeres that involve the branchiomotor neurons (Fig. 6). The cell bodies of the branchiomotor neurons are born in the ventral side of the hindbrain during neurogenesis. In the even-numbered rhombomeres, r2 (mV), r4 (mVII) and r6 (mIX), these neurons send their axons ipsilaterally toward an exit point located within the same rhombomere. In the odd-numbered rhombomeres r3, r5 and r7 neurogenesis begins slightly later. Branchiomotor neurons in these rhombomeres send their projections to the anterior where they join the exit point of the neighboring even-numbered rhombomere contributing to the generation of the cranial nerves (Lumsden and Keynes, 1989). This alternating pattern between odd- and even- rhombomeres is also present in the migration of the neural crest cells from the hindbrain to the branchial arches. The neural crest cells are pluripotent cells that migrate from the dorsal part of the neural tube to the branchial arches (BA) where they generate nerves, ganglia, cartilage, bone and connective tissue of the head. Principally, three streams of neural crest cells migration are observed in the hindbrain: from r1-2 to branchial arch 1 (BA1), from r4 to BA2 and from r6-7 to BA3. Contribution of odd-numbered r3 and r5 to neural crest population is very small and are essentially considered neural crest cell-free territories (Lumsden and Guthrie, 1991; Sechrist *et al.*, 1993).

Although a basic repeated pattern is observed in each segment, clear segment-specific differences are recognized among rhombomeres. Neurons in each rhombomere show

different functional properties and specific axon navigation behaviors respect to their specific targets (see Table 1).

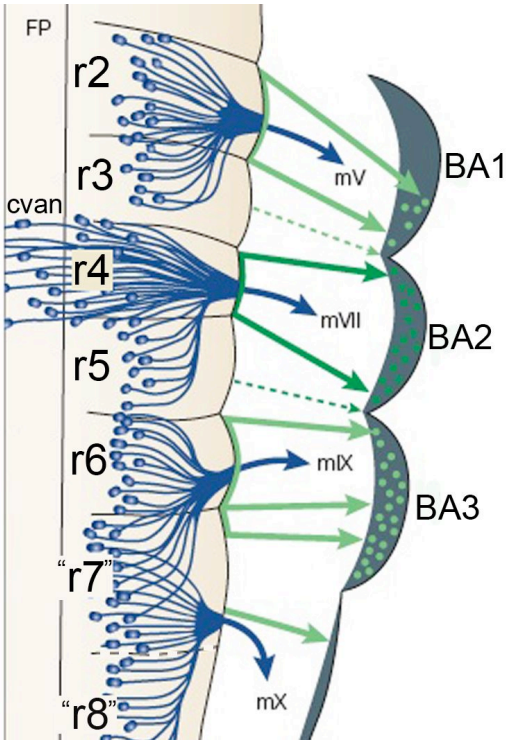


Figure 6. Branchiomotor axons and streams of neural crest cells migration follow a two-rhombomere pattern. Branchiomotor nerves **mV**, **mVII** and **mIX** (in blue) have their exit points in the even rhombomeres r2, r4 and r6 respectively. Motor neurons which bodies lie in even-numbered rhombomeres extend their axons laterally to reach the exit point; whereas axons of motor neurons which bodies lie in odd-numbered rhombomeres travel laterally and later rostrally to joint the exit point in even-numbered rhombomeres. The principal streams of **neural crest cell migration** (green lines) from the dorsal hindbrain to the branchial arches 1, 2 and 3 (BA1-3) are located in r2, r4 and r6 respectively, being r3 and r5 essentially neural crest cell-free territories. cvan: contralaterally migrating efferent neurons of the vestibuloacustic nerve; FP: Floor Plate. Anterior at top. Modified from Kiecker and Lumsden, 2005.

2.2.2 Rhombomeres are compartments with cell-lineage restriction

Rhombomeres are compartment-like units that observe cell-lineage restriction. Initial evidences of cell-lineage restriction among rhombomeres were obtained by clonal analysis (Fraser *et al.*, 1990). Experiments done in chick embryos where boundaries are ablated (Guthrie and Lumsden, 1991) or impeded to form by retinoic acid treatment (Nittenberg *et al.*, 1997), show that there is no mixing between adjacent rhombomeres

and no alteration of the segmental pattern of motor nuclei is detected. Moreover, after ablation of boundaries, if the tissue is allowed to grow again, and even-numbered rhombomere cells contact with odd-numbered rhombomere cells, boundaries are regenerated (Guthrie and Lumsden, 1991). The same happen when odd-identity and even-identity cells are experimentally juxtaposed (Guthrie *et al.*, 1993; Wizenmann and Lumsden, 1997), suggesting that cell-lineage restriction is established before the formation of the interrhombomeric boundaries.

Yet before the existence of any boundary, different rhombomere-restricted genes are expressed in the prospective rhombomeres. Those genes are involved in conferring AP positional identity to the cells within the rhombomeres. Initially those genes show ragged limits of expression, but those limits are progressively sharpened suggesting that there is a gradual mechanism that refines rhombomere territories before the appearance of morphologic boundaries. There are two different mechanisms not necessarily exclusive proposed for initial segregation of the rhombomere territories (Fig. 7) (reviewed in Cooke and Moens, 2002). The first one is based on cell plasticity and fate switching. In zebrafish and mouse it has been demonstrated that individual cells can change their AP identity if they are transplanted early enough from one rhombomere to another (Schilling *et al.*, 2001; Trainor and Krumlauf, 2000). The second model is based on cell sorting and proposes that some kind of interaction between even-identity and odd-identity cells, involving differential affinities or adhesion properties, promotes segregation of odd from even cells. First evidences for cell sorting in rhombomeres were obtained by experiments *in vitro*. Cells from even-numbered rhombomeres and odd-numbered rhombomeres were dissociated, labeled and cultured together. After a period of time, odd-identity cells segregated away from even-identity cells, a result that was not obtained when two odd or two even populations were mixed together (Wizenmann and Lumsden, 1997). The candidate effectors to mediate differential affinity states between odd and even rhombomeres are the receptor tyrosine kinases Ephs, and their membrane-bound ligands, the ephrins. The EphA receptors bind specifically to ephrin-A ligands while the EphB bind to ephrin-B ligands. The only known exception is EphA4 that binds ephrin-B2 and –B3 as well as ephrin-A ligands. Eph receptors and ephrins are expressed during rhombomere formation in complementary domains. The receptors *EphA4*, *EphB2* and *EphB3* are expressed in odd rhombomeres r3 and r5, and the ligands ephrin-B1, -B2 and –B3 are expressed in the even rhombomeres r2, r4 and r6 (reviewed in Xu *et al.*, 2000). EphA2 and –A7 are also expressed in the hindbrain but their ephrin-A ligands have not

been found. Some evidences for the Eph-ephrin involvement in rhombomeric cell sorting have been reported. When a construct containing *ephrin-B2* was microinjected in the hindbrain, cells expressing the construct in r3 or r5, where *Eph* genes are normally expressed, became displaced to the boundaries (Xu *et al.*, 1999). This result suggests that the activation of the signaling system Eph-ephrin is enough to produce cell sorting in the rhombomeres. Another set of experiments demonstrated that using antisense morpholinos against EphA4 in hindbrain cells resulted in disruption of the interrhombomeric boundaries (Cooke *et al.*, 2005). However, mutant mice for *EphA4* do not present any defect in boundary formation (Helmbacher *et al.*, 2000).

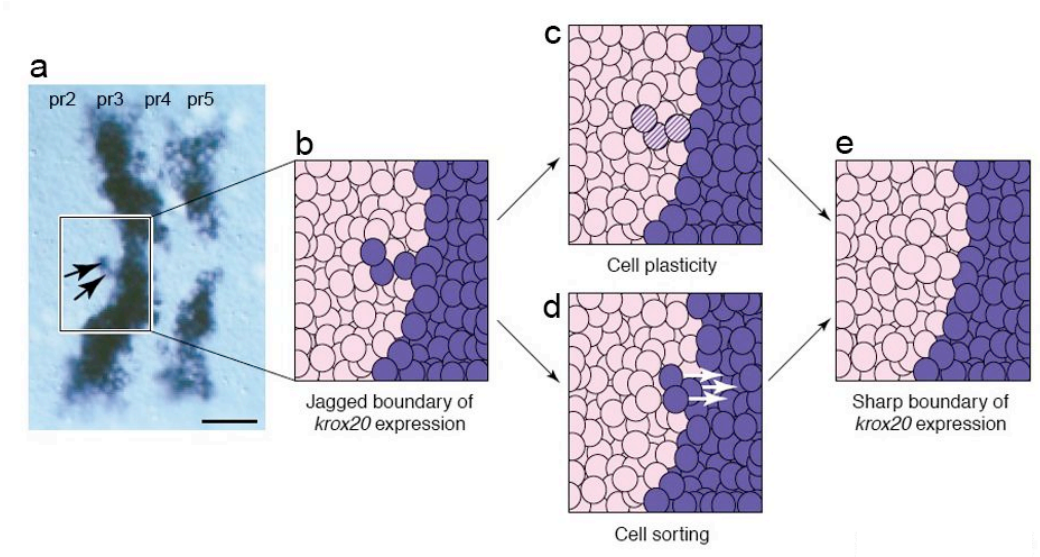


Figure 7. Two proposed models for refinement of rhombomere territories. (**a**) *In situ* hybridization for the r3 and r5 maker *krox20* is shown in a zebrafish embryo. Arrows point to r3-identity cells that are in an r2 context. Windowed part of the photo is schematized in (**b**). Blue cells are *krox20* positive and have r3 identity. Soft pink cells have r2 identity. (**c**) Cell plasticity model proposes that these r3-identity cells will be influenced by an r2 context and will change their identity to r2. (**d**) Cell sorting model proposes that these r3-identity cells will present different cell-affinity properties to those of r2 and will be repelled and excluded from the r2 territory. (**d**) Both models lead to a sharpening of the limits of expression of the rhombomere-restricted genes, thus a refinement of the rhombomere territories. pr, prospective rhombomere. Anterior is to the left. Modified from Cooke and Moens, 2002.

2.2.3 Interrhombomeric boundaries

The interplay between even and odd cells generates a new population of boundary cells, which their own markers (Amoyel *et al.*, 2005; Cheng *et al.*, 2004; Cook *et al.*, 1995; Heyman *et al.*, 1995; Mahmood *et al.*, 1995; Mechta-Grigoriou *et al.*, 2000; Qiu *et al.*,

2004). Some groups have proposed that interrhombomeric boundaries constitute not only morphological barriers but also signaling centers involved in regulating cell differentiation within the hindbrain (Amoyel *et al.*, 2005; Riley *et al.*, 2004). Boundaries are regions of low cell density and decreased proliferation if compared with rhombomeres (Guthrie *et al.*, 1991). At the onset of neurogenesis all the morphological interrhombomeric boundaries are established (HH12 in chick embryos) to disappear at later stages (HH25 in chick embryos) (Guthrie, 1996). The order by which rhombomere boundaries are established diverges among species (Guthrie, 1996; Moens and Prince, 2002).

3. AP patterning of the hindbrain

3.1 Neural induction and early AP regionalization of the neural plate

Neural induction is the process by which ectodermal cells are committed to the neural fate and the neural plate becomes differentiated from the rest of the ectoderm (epidermal or non-neural ectoderm). The first model proposed for neural induction, known as “default model”, was based on evidences in frog and proposed that ectodermal cells are originally committed to the neural fate but are redirected to an epidermal fate in response to BMP signaling (Fig. 8A,B) (Harland, 2000). In this context, neural induction proceeds when BMP antagonizing molecules are delivered from the organizer to the future neural plate. Accumulation of evidences has pictured more complex models that not only involve BMPs but also FGF and WNT signaling (Fuentealba *et al.*, 2007; Kuroda *et al.*, 2005; Pera *et al.*, 2003; Stern, 2005; Streit and Stern, 1999; Wilson *et al.*, 2000). In frog, De Robertis and colleagues have proposed a modification of the “default model” that integrates FGF and WNT signaling (Fig. 8C) (De Robertis and Kuroda, 2004; Fuentealba *et al.*, 2007; Pera *et al.*, 2003). The clue for this integration is the transcription factor Smad1. The Smad proteins are the transcriptional effectors of BMP signaling. These proteins are phosphorilated in their C-terminal region by the activated BMP receptor and translocated to the nucleus where they regulate gene expression (Baker and Harland, 1997). In the work by Pera *et al.* (2003) it is demonstrated that Smad1 can be inactivated by phosphorylation in a Mitogen Activated Protein Kinase (MAPK) specific site located in its linker (middle) region. The MAPK pathway is commonly activated by RTKs such as the FGFR (see section 5.3). Thus, FGFs and also IGFs antagonize BMP signaling and promote neural induction by inactivating Smad1 through the MAPK pathway. More recent

work by the same group suggests that WNT signals are also involved in the regulation of Smad1 (Fuentealba *et al.*, 2007). This work sustains that Smad1, once phosphorilated by both the BMP receptor and MAPK, is recognized by Glycogen-Syntase Kinase 3 (GSK3). GSK3 phosphorilates another Smad1 site and enhances its proteosomal degradation. GSK3 is inactivated by the canonical WNT pathway. This gives a molecular basis to previous observations that suggested that inactivation of the WNT pathway is required for neural induction (Heeg-Truesdell and LaBonne, 2006; Wilson *et al.*, 2001). On the other hand, studies in chick argue against the "default model" and propose that at least part of the FGF functions in neural induction are BMP-independent (Fig. 8D) (Stern, 2005; Wilson *et al.*, 2000; Wilson and Edlund, 2001). Indeed, Linker and Stern (2004) have proposed that BMP signaling is only involved in late stages of neural induction. In chick, while involvement of BMP signaling in neural induction results controversial, that of FGF signaling is well known. Blocking FGF signaling with chemical inhibitors abolishes neural induction (Streit *et al.*, 2000). Conversely, FGF8-beads implantation can mimic the Hensen's node in the induction of early neural induction markers (Streit *et al.*, 2000). However, induction by FGF8-beads is transient and late neural induction markers as *SOX2* are not induced. Similar conclusions have been reached for amphibians (Delaune *et al.*, 2005; Kuroda *et al.*, 2005). No combination of the known factors proposed to be involved in neural induction leads to the expression of late neural induction markers *in vitro* (Linker and Stern, 2004). Thus, it remains unknown which are the factors required for the stabilization of the neural character (Stern, 2005).

The early AP regionalization of the CNS is a process that occurs in close relation with neural induction. The most widely accepted hypothesis for early regionalization of the neural plate is based on the "activation-transformation model" by Nieuwkoop. This model proposes two steps for neural induction and early patterning in which neural tissue is first 'activated' to a general anterior neural character and later becomes progressively 'transformed' to a more posterior character (Stern, 2001). Stern has proposed a modification of the "activation-transformation" model in order to fit in the current knowledge in amniotes (Stern, 2001). This model involves three steps instead of two (Fig. 9): i) the epiblast is induced to a pre-forebrain state by underlying tissues (AVE/hypoblast); ii) this induction is stabilized by signals emitted from the node and prechordal mesoderm; iii) signals from the node subsequently promote caudalization of the posterior parts of the neural plate.

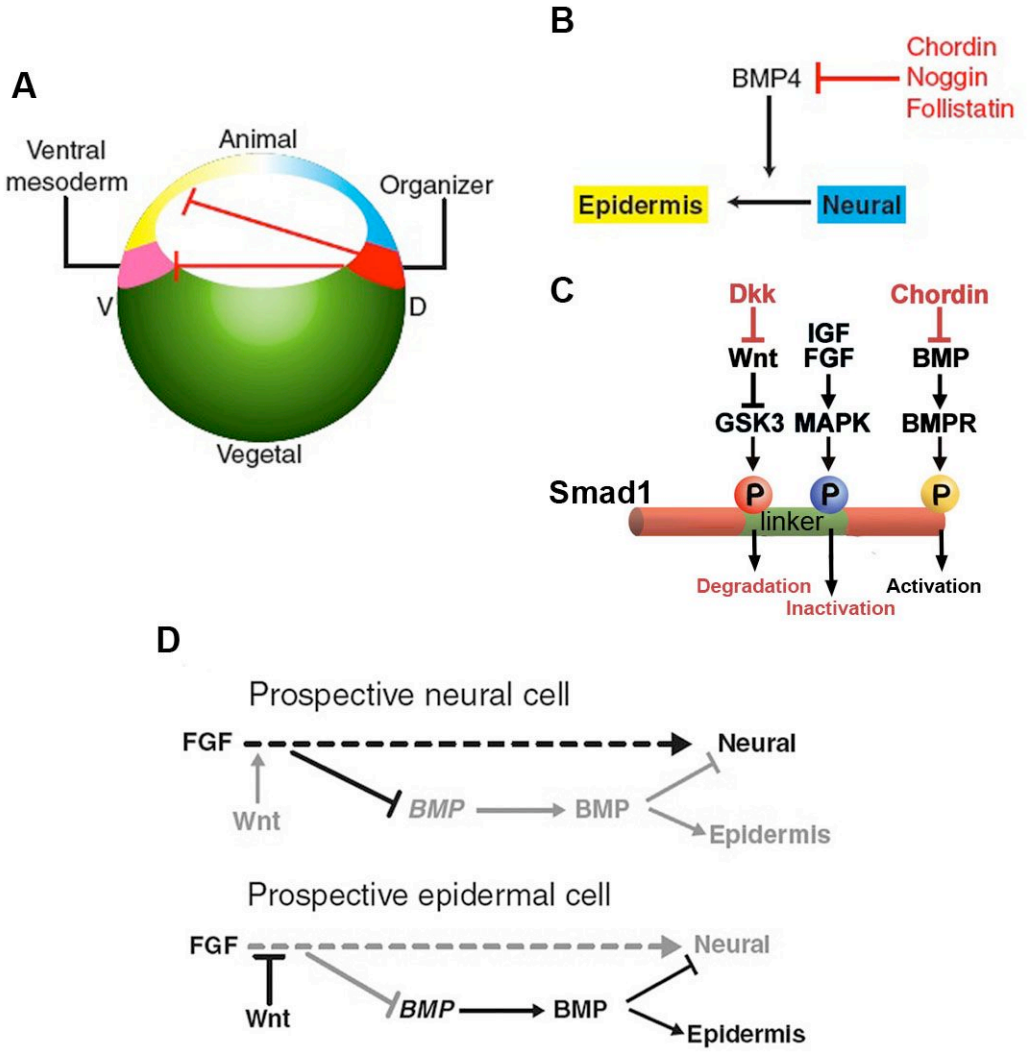


Figure 8. Models for neural induction. (**A**) "Default Model". Schematic representation of a frog early gastrula**.** Prospective territories are: the organizer in red, ventral mesoderm in pink, neural tissue in blue, epidermis in yellow and yolky endoderm in green. The red lines represent BMP antagonist activity emitted from the organizer. D, dorsal; V, ventral. (**B**) A diagram of the inductive interactions proposed by the "default model" (Modified from Stern, 2005). (**C**) Smad1 can integrate the functions of BMP, FGF and WNT signaling in neural induction. BMP activates Smad1 phosphorilating a specific site in the C-ter region. MAPK inactivates Smad1 phosphorilating a specific site in the linker region. GSK3 recognizes both phosphorylations and phosphorilates a third site. This promotes the proteosomic degradation of Smad1 (Modified from Fuentealba *et al.*, 2007). (**D**) A model for neural induction in chick. In prospective neural cells FGF signaling activates two distinct transduction pathways: first, the repression of BMP expression (solid line from FGF), and second, the promotion of a neural fate by a BMP-independent pathway (dashed line). In prospective epidermal cells, high levels of WNT signals block the response of epiblast cells to FGFs and thus BMP signaling promotes epidermal fate. Modified from Stern, 2005.

In addition to the node, the primitive strike and the newly formed axial and paraxial mesoderm have been proposed as sources for caudalizing factors (reviewed in Diez del Corral and Storey, 2004). Experiments in chick explants suggest that FGFs from the node and primitive strike in co-operation with WNTs from the paraxial mesoderm are required for instructing rostral forebrain-identity cells to caudal forebrain, midbrain and rostral hindbrain identities (Muhr *et al.*, 1997; Muhr *et al.*, 1999; Nordstrom *et al.*, 2002). On the other hand, RA synthesized by Raldh2 in the paraxial mesoderm is needed for caudal hindbrain and spinal cord identities (Muhr *et al.*, 1997; Muhr *et al.*, 1999). The role of FGF, RA and WNT signals as caudalizing factors has been proved in different models (Blumberg *et al.*, 1997; Chiba *et al.*, 2005; Erter *et al.*, 2001; Lamb and Harland, 1995; Lekven *et al.*, 2001; McGrew *et al.*, 1995; McGrew *et al.*, 1997). In addition to this, it has been proposed that the anterior neural plate is protected from the caudalizing factors by the action of anterior tissues such as the AVE/hypoblast and the prechordal mesoderm. These tissues are proposed to actively repress the caudalizing factors in anterior areas (Foley *et al.*, 2000; Kimura *et al.*, 2000). Additionally, the AVE/hypoblast directs the movement of at least some of the pre-forebrain cells anteriorly, moving them away from the influence of the caudalizing signals (Foley *et al.*, 2000).

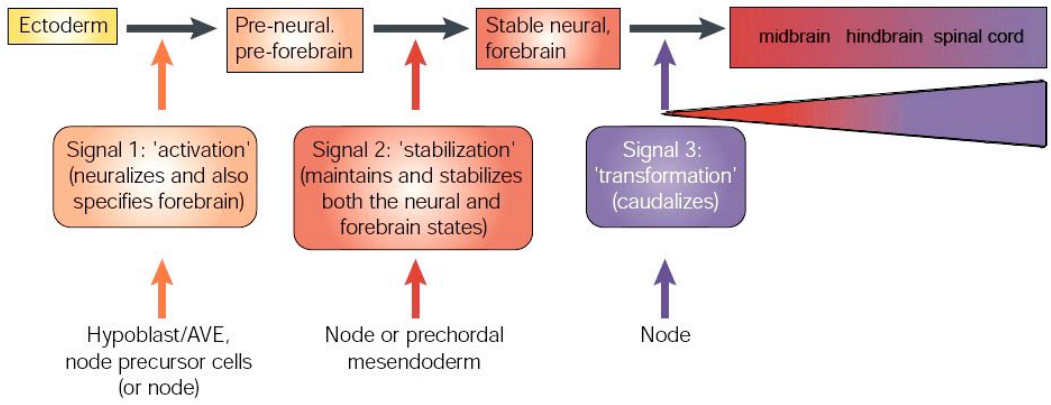


Figure 9. Stern's model for induction and early regionalization of the neural plate. A first step occurs before gastrulation, in which the hypoblast/AVE (perhaps together with organizer precursors) activates a labile, pre-neural and pre-forebrain state. To retain neural fate, cells must also receive stabilizing signals from the organizer and/or its descendants (prechordal mesendoderm, perhaps also the anterior head process/notochord). If they remain close to the organizer, however, they become progressively caudalized. Modified from Stern, 2001.

3.2 Local signaling centers within the hindbrain

A further refinement of the AP identities within the CNS is achieved through the establishment of local signaling centers, which emit signals that pattern territories in their vicinity. Two signaling centers are located within the hindbrain: the Isthmic Organizer (IsO), at the level of the Midbrain-Hindbrain Boundary (MHB), and the 'r4-FGF source' (Fig. 10) (Mason, 2007). RA provided by the somitic mesoderm is also involved in regional specification of the hindbrain (Gavalas and Krumlauf, 2000; Glover *et al.*, 2006). The progressive patterning of the hindbrain leads to the acquisition of specific positional identities by each rhombomere and to the generation of the rhombomere boundaries. This positional identity is conferred by a different combination of transcription factors expressed in each rhombomere (Lumsden, 2004; Moens and Prince, 2002; Schneider-Maunoury *et al.*, 1998).

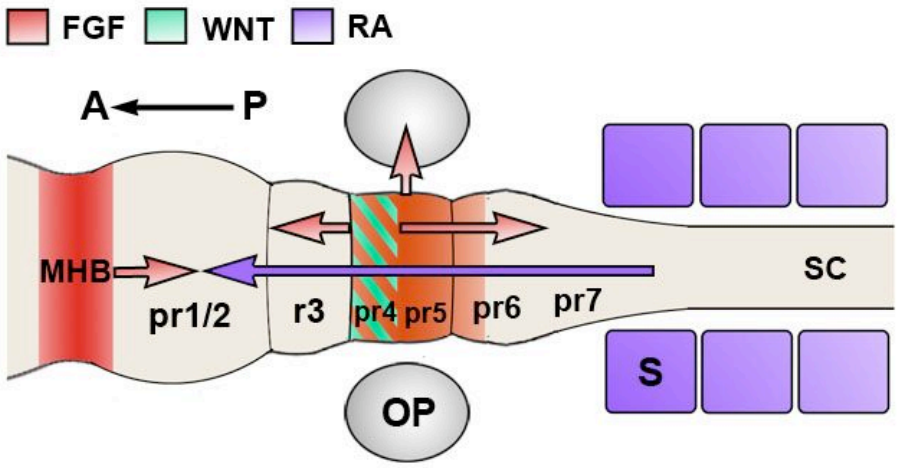


Figure 10. Different signaling sources are involved in regionalising the hindbrain. Schematic representation of a HH9 chick embryo in a dorsal view where anterior is at left. Red color marks FGF expression sites; green is used for WNT expression sites and violet for RA. Arrows represent in a simplified way the areas of influence of the different signaling sources. FGFs emitted from the MHB are involved in conferring cerebellar character to the anterior hindbrain. FGF3 from the central/caudal hindbrain is mainly involved in conferring r5 and r6 identities although certain influence in patterning the rostral hindbrain is also expected. This FGF3 source is also involved in the induction and patterning of the otic placode (OP). Finally, RA emitted from the somites (S) generates a gradient from the most caudal to the most anterior part of the hindbrain that is needed for the regional specification of the whole hindbrain.

3.2.1 The Isthmic Organizer (IsO)

The IsO is the best characterized local signaling center within the CNS. It is located at the level of the isthmus, the constriction that separates the midbrain from the hindbrain, also known as midbrain-hindbrain boundary (MHB). Grafting experiments in birds demonstrated that the IsO has the capability to organize both the midbrain and the anterior/cerebellar hindbrain (Liu and Joyner, 2001; Marin and Puelles, 1994; Martinez *et al.*, 1991). The IsO secretes signaling molecules from the WNT (mainly Wnt1) and the FGF (FGF8, FGF17, FGF18) families (Liu and Joyner, 2001). WNT signals are mostly associated with proliferative control, while FGFs, and particularly FGF8, are considered the principal molecules that exert patterning functions (Raible and Brand, 2004).

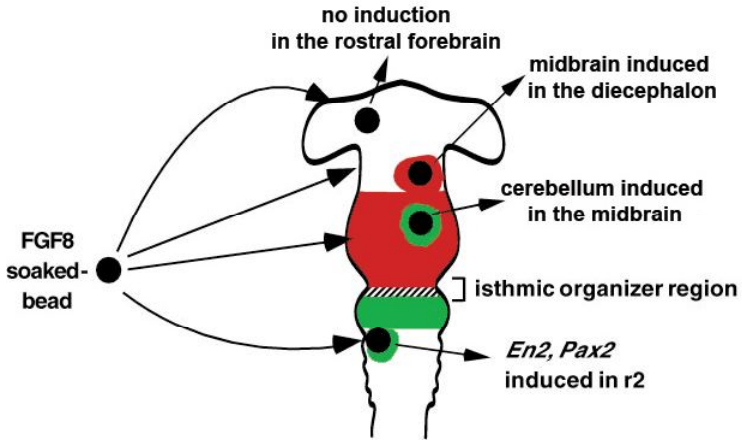


Figure 11. FGF8 mimics the organizing activity of the IsO. Red color represents midbrain character, green color cerebellar character and striped pattern the IsO. When FGF8 soaked-beads are implanted in the diencephalon or the midbrain, ectopic midbrain or cerebellum are respectively induced. Implantation in the non-cerebellar hindbrain induces the IsO genes *En2* and *Pax2* and implantation in the rostral forebrain has no inductive effects. Anterior at top. Modified from Liu and Joyner, 2001.

FGF8-coated beads can mimic the effects of isthmus grafting when they are implanted in the rostral mesencephalon or the diencephalon of chick embryos (Fig. 11) (Crossley *et al.*, 1996; Shamim and Mason, 1999). FGF8-beads in the rostral mesencephalon generate cerebellar structures and in the diencephalon generate mesencephalic structures. Moreover, FGF8-beads not only mimic the IsO function but also induce IsO-related genes (*En1*, *En2, Wnt1* and *Pax2*) just creating an ectopic IsO close to the bead (Crossley *et al.*, 1996; Shamim *et al.*, 1999). When implanted in the myelencephalon

(non-cerebellar hindbrain) FGF8-beads are not able to generate ectopic structures but they promote expression of the IsO-related genes *En2* and *Pax2* (Fig. 11). According to the essential function of FGF8 in the IsO, the zebrafish mutant for *fgf8*, *acerebellar* (*ace*), lacks isthmus and shows caudal extension of the tectum (mesencephalic structure) at expenses of the cerebellum that is totally absent (Reifers *et al.*, 1998). In mouse, conditional mutants for *Fgf8* in the isthmus show lack of both midbrain and cerebellum structures due to high rates of cell-death (Chi *et al.*, 2003). All these data indicate that FGF signaling elicits two different responses on either side of its source: midbrain development anteriorly and cerebellum formation posteriorly.

The IsO function requires the establishment of a complex network of gene expression. This network includes the homeodomain transcription factors *Otx2*, *Gbx2*, *En1,2* and *Pax2,5,8* and various secreted molecules of the WNT and FGF pathways. *Otx2* and *Gbx2* are very early expressed in the neuroepithelium. *Otx2* expression is already induced in the epiblast by the AVE/hypoblast at the very early steps of neural induction and confined to the anterior neural plate as gastrulation and caudalization of the neural plate proceeds (Bally-Cuif *et al.*, 1995; Simeone *et al.*, 1992). *Gbx2* is initially expressed in the posterior epiblast and is later restricted to the anterior hindbrain (Shamim and Mason, 1998; Wassarman *et al.*, 1997). The early expressions of *Otx2* and *Gbx2* divide the neuroectoderm into an *Otx2*-positive rostral and a *Gbx2*-positive caudal domain with a common border that lies at the MHB. After this, *En* and *Pax* genes and also *Wnt1* and *Fgf8* are activated in broad areas along the posterior midbrain and the rostral hindbrain (Liu and Joyner, 2001). Then *Fgf8* is restricted to a narrow domain in the *Gbx2*-positive region of the MHB whereas *Wnt1* occupies a complementary region in the *Otx2*-positive region of the MHB (Hidalgo-Sanchez *et al.*, 1999; Wurst and Bally-Cuif, 2001).

Studies in mouse and chick have demonstrated that mutual repression between *Otx2* and *Gbx2* is involved in the positioning of the IsO (Broccoli *et al.*, 1999; Katahira *et al.*, 2000; Millet *et al.*, 1999). However, the proper establishment of the isthmic organizer is defined not just by these two transcription factors but rather by a complex cascade of gene interactions that involves two phases. First, the IsO-specific genes are induced by planar and vertical interactions, which include signals from the underlying AVE/hypoblast and caudalizing factors from the node/organizer and the axial and paraxial mesoderm (Fig. 12A) (Rhinn and Brand, 2001; Rhinn *et al.*, 2005; Shamim *et al.*, 1999; Wurst and Bally-Cuif, 2001). Secondly, the IsO-specific genes maintain the IsO by establishing a network

of gene interactions among them (Li and Joyner, 2001; Raible and Brand, 2004; Wurst and Bally-Cuif, 2001). This network is summarized in Figure 12B.

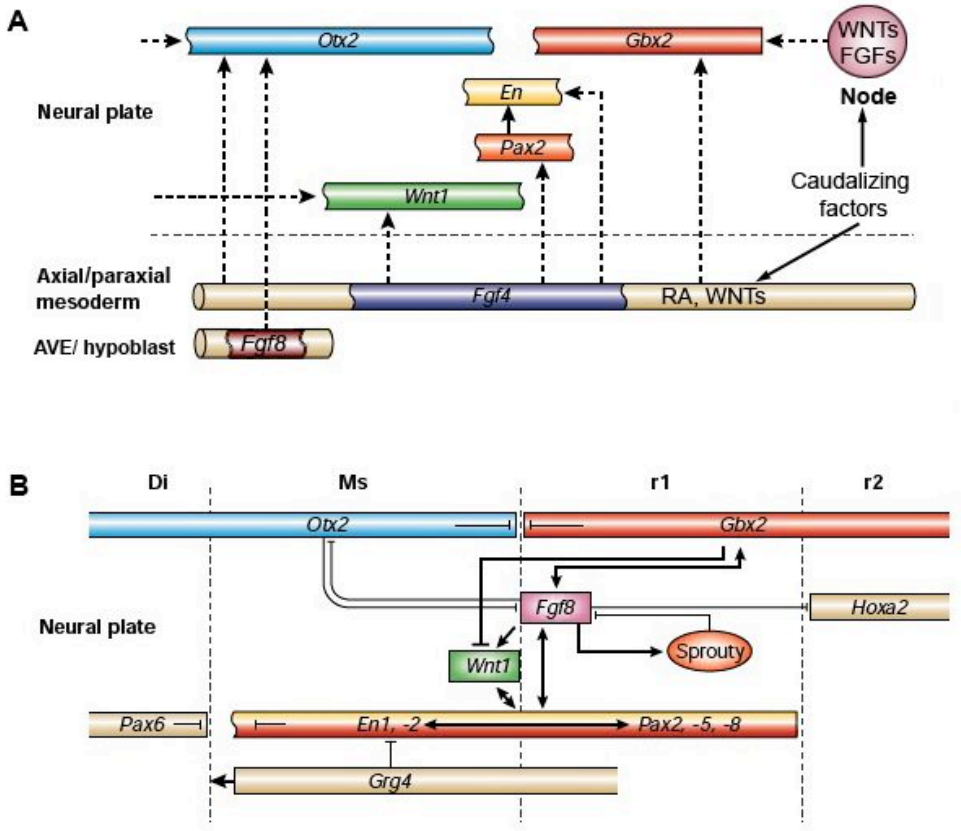


Figure 12. Establishment and maintenance of the IsO represent two different phases of gene expression. (A) Establishment phase. Gene expression patterns in the neural plate (top) or the underlying axial/paraxial mesoderm and AVE/hypoblast (bottom), anterior to the left. Vertical arrows represent vertical interactions, horizontal arrows depict planar interactions, and hatched arrows are hypothetical. All shown interactions are not necessarily direct. During gastrulation, the **anterior mesendoderm** and the **AVE/hypoblast** are involved in induction and/or maintaining of ***Otx2*** expression in the anterior neural plate. The mechanisms that regulate ***Gbx2*** expression are unknown, but posteriorizing influences might be involved. Candidate regulatory factors include members of the **FGF** and **WNT** families, and **retinoic acid (RA)**. In parallel, ***Fgf4*** expressed by the axial mesoderm might directly or indirectly induce ***En*** expression. ***En*** expression is also strictly dependent on ***Pax2*** function. Unknown factors initiate ***Wnt1*** expression independently. (**B**) **Maintaining phase.** Gene expression patterns within the neural plate during early somitogenesis, anterior to the left. After the initiation phase, ***Gbx2*** expression maintains ***Fgf8*** expression, whereas ***Otx2*** and ***Gbx2***/***Fgf8*** regulate each other negatively, leading to the establishment and maintenance of sharp expression borders. Concomitantly, the expression territories of ***Fgf8***, ***Wnt1***, ***En1*** and ***-2***, and ***Pax2***, ***-5*** and ***-8*** become interdependent and establish a positive regulatory loop that is necessary to maintain mid-hindbrain identity. At least two negative feedback mechanisms that involve ***Sprouty*** and ***Grg4*** restrict the expansion of the positive regulatory loop along the AP axis. The **mid-diencephalic border** is positioned in parallel by negative cross-regulations of ***En1, 2/Pax2***, ***-5***, ***-8*** and ***Pax6***, whereas ***Fgf8*** exerts a negative influence on the caudal expression of **Hox genes**, positioning the border between rhombomeres. This interaction scheme is based on alterations of gene expression patterns observed after gain- and loss-of-function experiments. Di, diencephalon; Ms, mesencephalon; r, rhombomeres. Modified from Wurst and Bally-Cuif, 2001.

3.2.2 The 'r4 FGF-source'

r4 is the first rhombomere morphologically discernible in zebrafish (Maves *et al.*, 2002). It has been identified in this species as a local organizing center that emits *fgf3* and *fgf8* and is involved in patterning the caudal hindbrain and in inducing and patterning the otic placode (Kwak *et al.*, 2002; Lecaudey *et al.*, 2007; Leger and Brand, 2002; Maroon *et al.*, 2002; Maves *et al.*, 2002; Walshe *et al.*, 2002). In presomitic stages (90% epiboly), *fgf3* is expressed in a domain corresponding to the prospective central hindbrain (Maves *et al.*, 2002) and it is maintained in r4 once the morphological boundaries are formed. Also in presomitic stages (70% epiboly), *fgf8* is initially induced in the whole rostral hindbrain and later is restricted to the MHB and rostral hindbrain and to r4 (Maves *et al.*, 2002; Reifers *et al.*, 1998).

In other vertebrate species, sources of FGF signals are also present in the central hindbrain. However, these sources are not constrained only to r4 (Lombardo *et al.*, 1998; Mahmood *et al.*, 1995; McKay *et al.*, 1996). For example the chick embryo has a dynamic pattern of *Fgf3* expression in the central and caudal hindbrain (Fig. 13) (Mahmood *et al.*, 1995). *Fgf3* is first detected in the presumptive hindbrain by HH7. By HH9, the first interrhombomeric boundaries, r3/4 and r5/6, are formed and *Fgf3* expression is confined to the pre-r4-r5 territory. Later, by HH10, *Fgf3* expression begins a progressive extension to r6 and fades in r4. At the beginning of neurogenesis *Fgf3* is restricted to r6 and to all the interrhombomeric boundaries. The chick central hindbrain also exhibits weak levels of *Fgf4* expression (Shamim and Mason, 1999). *Fgf3* is also dynamically expressed in the frog embryo in territories from r3 to r5 (Lombardo *et al.*, 1998) and in the mouse embryo from r4 to r6 (Mahmood *et al.*, 1996; McKay *et al.*, 1996).

In zebrafish, *fgf3* and *fgf8* from r4 have redundant functions in patterning the hindbrain. *ace* mutants (*fgf8*$^{-/-}$) or embryos treated with antisense *fgf3* morpholinos (MO) do not show gross defects in hindbrain patterning. However, when wild type embryos are co-injected with *fgf3*- and *fgf8*-MOs or *fgf3*-MOs are injected in *ace* mutants, the patterning of the whole hindbrain is affected, with especially dramatic effects in the caudal hindbrain (Maves *et al.*, 2002). Analysis of different markers for specific rhombomeres revealed that in these embryos, although r4 is maintained, r1-r3 are reduced while r5 and r6 are

completely absent, leaving r4 in continuity with r7. Rhombomeres that are not lost are misspecified (Walshe *et al.*, 2002). Consequently, neuronal populations of r5 and r6 are not present in these embryos (Maves *et al.*, 2002), whereas neuronal populations in r1-r3 and r7 are highly disorganized and frequently absent (Maves *et al.*, 2002).

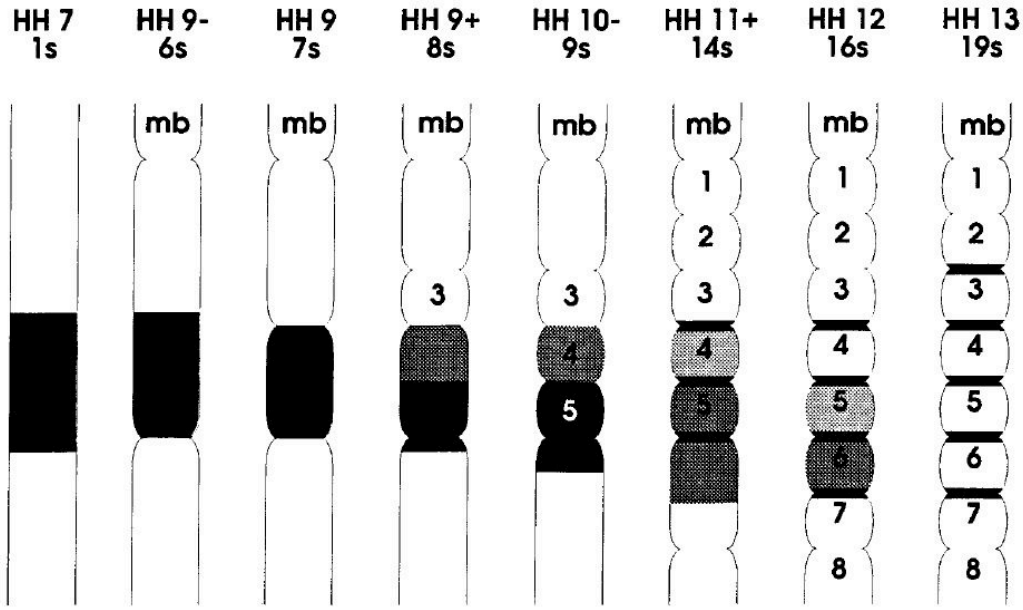


Figure 13. *Fgf3* is dynamically expressed in the chick hindbrain. Schematic representation of the *Fgf3* expression profile during chick hindbrain patterning. *Fgf3* is initially expressed in the prospective central hindbrain. By HH9 it is restricted to the pre-r4-r5 territory. Then, its expression begins to expand to r6 and is progressively downregulated in r4. At the end of segmentation *Fgf3* is restricted to the rhombomere boundaries. Modified from Mahmood *et al.*, 1995.

Missexpression of either *fgf3* or *fgf8* in zebrafish ectopically induces expression of *krox20*, a gene that prefigures r3 an r5, and *valentino* (*val*), a marker of r5 and r6. These ectopic inductions are restricted to the caudal hindbrain, suggesting that this is the only territory competent for expressing *krox20* and *val* in response to FGF signals (Maves *et al.*, 2002). The present work in chick and other studies in zebrafish demonstrate that this competence is conferred by the transcription factor *vHnf1* (Aragon *et al.*, 2005; Hernandez *et al.*, 2004; Wiellette and Sive, 2003).

3.3 Retinoic Acid (RA) in the AP patterning of the hindbrain

All-*trans* retinoic acid (RA) was one of the first morphogens identified (Eichele and Thaller, 1987). It is a metabolite of vitamin A synthesized from all-*trans* retinal by the RALDH enzymes (Lee *et al.*, 1991). The sources of RA within the developing embryo are the places were *RALDH1-4* genes are expressed (Duester *et al.*, 2003; Dupe *et al.*, 2003; McCaffery *et al.*, 1991; Niederreither *et al.*, 1997). Catabolism of RA is mediated by the CYP26 enzymes (Fujii *et al.*, 1997; Ray *et al.*, 1997; White *et al.*, 1997). RA is a low molecular weight lipophilic molecule, but it is also partly soluble in extra- and intra-cellular fluids (Blomhoff and Blomhoff, 2006). It passes across the plasma membrane and is detected by nuclear receptors termed RARs (RARα–γ) and RXRs (RXR α–γ) (Chambon, 2004; Giguere *et al.*, 1987; Petkovich *et al.*, 1987). RARs and RXRs are ligand-inducible transcription factors that form heterodimers and bind to short DNA motifs (RAREs and RXREs) to modulate transcription (Balmer and Blomhoff, 2005). Genes related to hindbrain patterning as 3' *Hox* genes and *vHnf1* contain RAREs in their regulatory regions (Dupe *et al.*, 1997; Marshall *et al.*, 1994; Pouilhe *et al.*, 2007).

Involvement of RA signaling in patterning the hindbrain was first demonstrated by exposing rat embryos to excess of vitamin A (Morriss, 1972). These rats presented teratogenic hindbrains. Subsequent experiments in mouse demonstrated that RA excess promotes a posteriorization of the anterior hindbrain with different phenotypes depending on the treatment stage (Fig. 14) (Morriss-Kay *et al.*, 1991; Wood *et al.*, 1994). The most severe phenotypes were obtained by treating embryos in presomitic stages. In these embryos the hindbrain was unsegmented and the territory corresponding to the anterior hindbrain (r1-r4) was contracted forming a rhombomere-like structure with r4 molecular identity (Morriss-Kay *et al.*, 1991; Wood *et al.*, 1994). Late treatment at early somitogenesis leads to a less severe phenotype in which r2 identity is changed to r4 (Wood *et al.*, 1994). Posteriorization of the anterior hindbrain by RA treatment has been documented in all the vertebrate models analyzed (Durston *et al.*, 1989; Holder and Hill, 1991; Papalopulu *et al.*, 1991). Conversely, RA deficiency has a complementary effect leading to rostralization of the caudal hindbrain (Gavalas and Krumlauf, 2000). The Vitamin A Deficient (VAD) quail shows no specification of the posterior hindbrain and, as a consequence, the cells of the posterior hindbrain contribute to an enlarged r3 that is in continuity with the spinal cord (Gale *et al.*, 1999) (Fig. 14). In the rat VAD model the posterior hindbrain is constricted and unsegmented and acquires r4 character (White *et al.*, 2000).

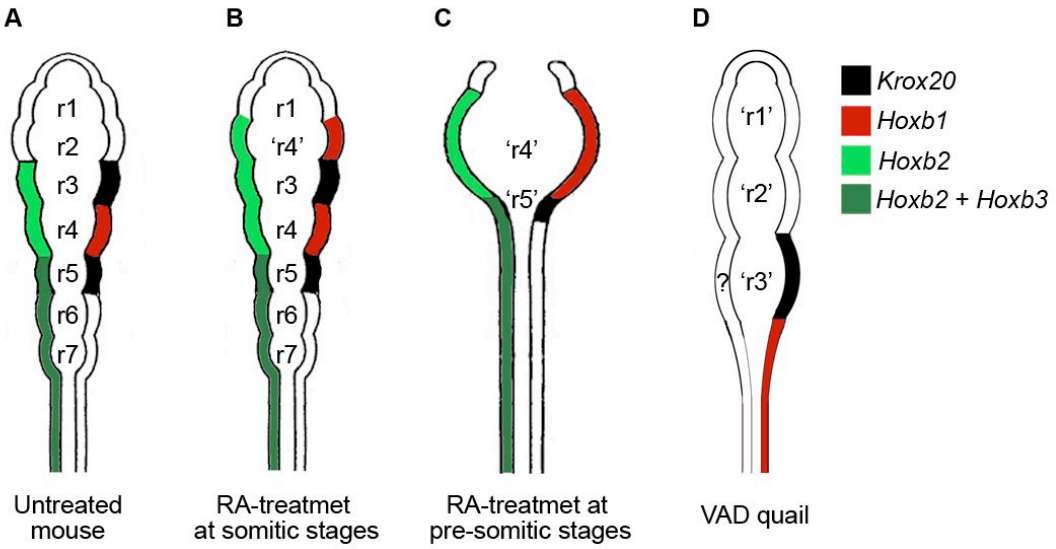


Figure 14. Effects of excess or deficiency of RA in hindbrain patterning. Schematic representations of mouse or quail hindbrains in a dorsal view with anterior at top. (**A**) To exemplify the molecular misspecification due to RA excess or deficiency the expression of four rhombomeric markers is followed: in light green ***Hoxb2*** which is normally expressed up to r2/r3 boundary; in dark green ***Hoxb3***, which is normally expressed up to r4/r5 boundary and is overlapped with *Hoxb2* expression; in black ***Krox20***, which is normally expressed in r3 and r5; in red ***Hoxb1*****,** which is normally expressed in r4. (**B**) Mice that are RA-treated at early somitic stages show a misspecification of r2 to r4. (**C**) Mice treated at presomitic stages show a much more severe posteriorization phenotype. The whole hindbrain is unsegmented and rostral hindbrain is transformed to a big rhombomere-like structure with r4 character. (**D**) Conversely, the Vitamin Acid Deficient (VAD) quail exhibits a rostralization phenotype and all the posterior hindbrain acquires r3 identity. '?' Indicates that *Hoxb2* and *Hoxb3* have not been analyzed in these embryos. Modified from Wood *et al.*, 1994 and data from Wood *et al.*, 1994 and Gale *et al.*, 1999.

Excess of RA disrupts the anterior hindbrain whereas RA deficiency disrupts the posterior hindbrain. This led to the concept that endogenous RA levels should be relatively high in the posterior hindbrain and relatively low in the anterior hindbrain. This is consistent with the expression patterns of *Raldh2* and *Cyp26* genes at early embryonic stages. Raldh2 is one of the major players in RA biosynthesis during embryogenesis (Gavalas and Krumlauf, 2000; Niederreither *et al.*, 1997). *Raldh2* is early expressed in the presomitic mesoderm (PSM), as soon as it ingresses the primitive strike (Begemann *et al.*, 2001; Berggren *et al.*, 1999; Niederreither *et al.*, 1997). Later, it remains in the PSM and in the somites. *Raldh2* is never expressed anterior to the first somite. The most caudal part of the hindbrain lies adjacent to the first somites, thus caudal hindbrain may be receiving relative high dosages of RA synthesized in the neighboring somites, whereas rostral hindbrain lies far from the RA source and must be receiving relative low dosages. Conversely, *Cyp26* genes are expressed in the anterior hindbrain, constituting an anterior

'sink' (de Roos *et al.*, 1999; Fujii *et al.*, 1997; Hollemann *et al.*, 1998). According to their function, abolishing expression of *Cyp26* genes leads to misspecification of the anterior hindbrain to a posterior character (Abu-Abed *et al.*, 2001; Emoto *et al.*, 2005; Hernandez *et al.*, 2007; Kudoh *et al.*, 2002; Sakai *et al.*, 2001), whereas overexpression of *Cyp26* genes causes anteriorization of the hindbrain similar to the effects of RA deficiency (Hollemann *et al.*, 1998; Kudoh *et al.*, 2002).

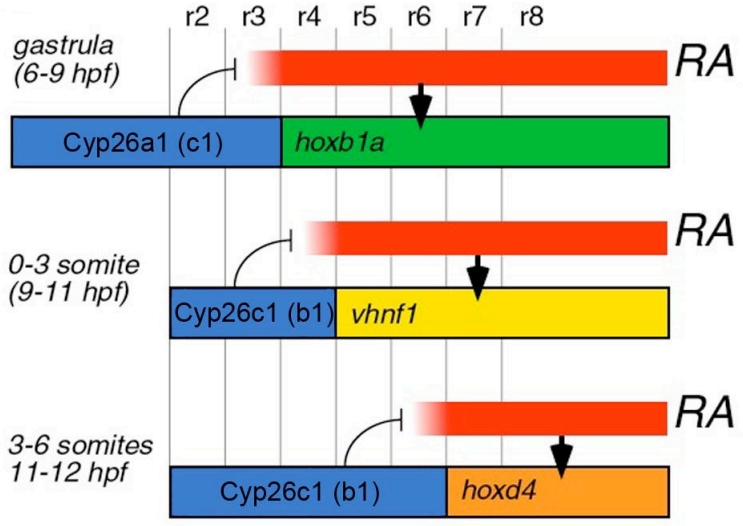


Figure 15. A 'gradient free' model for hindbrain patterning through spatio-temporally regulated RA inactivation. Dynamic patterns of *Cyp26* expression in the hindbrain (blue bars) antagonize RA-dependent gene expression by eliminating RA (red bars) first in the anterior hindbrain (6-9 hpf), then in r2-r4 (9-11 hpf), and then in r2-r6 (11-12 hpf). At each point, sequential RA-responsive genes (color red bars) are limited to progressively more posterior rhombomeres. Modified from Hernandez *et al.* 2007.

However, the concept that 'sources' and 'sinks' generate a morphogenetic gradient of RA necessary for hindbrain patterning has been challenged by certain observations. For example, embryos depleted of endogenous RA can be fully rescued by uniform concentration of RA (Begemann *et al.*, 2001; Begemann *et al.*, 2004; Gale *et al.*, 1999). When using RARE-LacZ reporters, no gradient of expression is detected in the hindbrain. Instead, distinct boundaries of reporter expression that shift over time are detected (Rossant *et al.*, 1991; Sirbu *et al.*, 2005). Recently, Hernandez *et al.* have proposed a "gradient-free" model based in their observations upon loss of function of *cyp26* genes in zebrafish (Fig. 15)(Hernandez *et al.*, 2007). In this model the dynamic expression of *cyp26* enzymes is responsible for sequentially limiting the extent of RA to certain

boundaries within the hindbrain. This spatio-temporal regulation of RA determines progressively more posterior limits of RA-dependent gene expression in a step-wise manner.

3.4 *Hox* genes and positional identity

The *Hox* transcription factors were first described in *Drosophila* embryogenesis and later identified in vertebrates. Most of the *Drosophila* body is formed by segmentation along the AP axis (Akam, 1987). *Hox* genes are involved in conferring AP positional identity to the cells within each segment. They work as selector genes regulating several downstream targets and ultimately give the correct AP position to different structures in the embryo. Missexpression of *Hox* genes in *Drosophila* leads to homeotic mutations, where certain body structures acquire the phenotypes of adjacent segments, anteriorizing if it is due to a loss of function, or posteriorizing if it is due to a gain of function. Thus, the AP plan of the *Drosophila* body depends on the establishment of a '*Hox* code'. This function has been conserved across species and the vertebrate embryonic structures that must be regionalized along the AP axis also exhibit a '*Hox* code' (Duboule and Dolle, 1989). Vertebrate structures that show a precise '*Hox* code' include the CNS, the branchial arches, the somitic mesoderm, and the gut (Dubrulle *et al.*, 2001; Dubrulle and Pourquie, 2002; Pitera *et al.*, 1999; Schneider-Maunoury *et al.*, 1998; Trainor and Krumlauf, 2001).

Structurally, *Hox* genes are characterized by having a short and highly conserved DNA motif called the homeobox, which codifies for the homeodomain. The *Hox* genes are grouped in clusters within the genome. In amniotes there are four *Hox* clusters, termed from a to d, which are thought to derive from a unique ancestral complex (Fig. 16) (Duboule and Dolle, 1989; Garcia-Fernandez and Holland, 1994). *Hox* genes can be classified in 13 paralogous groups according to sequence similarity and position within the cluster. Not all the paralogous groups are represented in all the clusters. A striking characteristic of the *Hox* genes is that they present spatial and temporal colinearity: the position that a *Hox* gene occupies within the cluster prefigures its expression pattern, being the most 3' genes expressed more anteriorly and earlier in development and the most 5' genes expressed more posteriorly and later (Fig. 16).

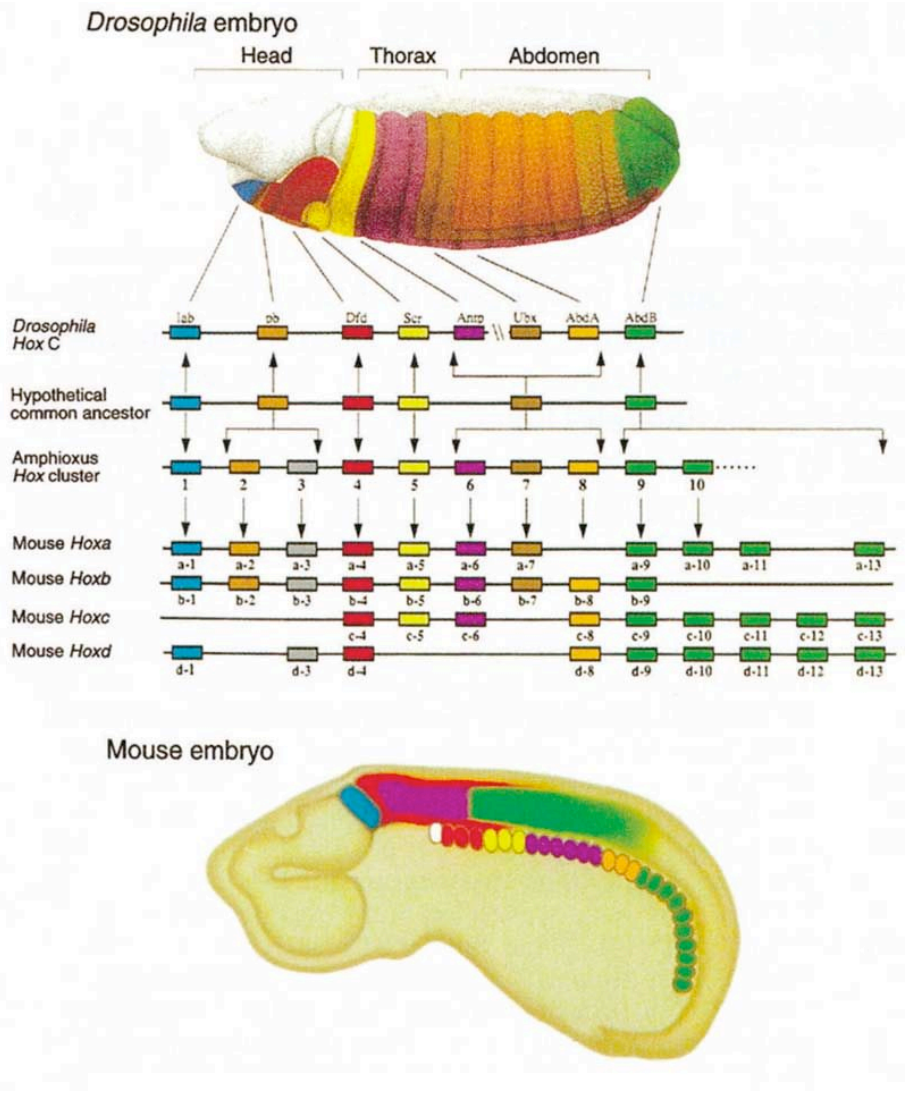


Figure 16. The arthropod and chordate *Hox* clusters derive from a common ancestor and present spatial and temporal colinearity. In the diagram each *Hox* paralogous group is represented with one color. From top to bottom: the AP expression of the *Hox* genes in the *Drosophila* embryo, the *Drosophila Hox* complex, the *Hox* complex of an hypothetical common ancestor for arthropods and chordates, the early vertebrate Amphioxus *Hox* cluster, the four mouse *Hox* clusters and the AP expression of the *Hox* genes in the mouse embryo. Arrows indicate evolutionary correlation between genes. Modified from Gee, 2000.

HOX factors alone bind weakly to DNA. This DNA-binding specificity is modified through interactions with other DNA-binding proteins (Mann and Chan, 1996), which act as cofactors, and that are necessary for the most part of *Hox* functions (Moens and Selleri, 2006). The *Hox* cofactors identified to date belong to two subfamilies of the TALE homeodomain proteins: i) the PBC subfamily that comprises *Drosophila Exd* and

vertebrate *Pbx1-4* (Monica *et al.*, 1991; Popperl *et al.*, 2000); ii) the MEIS subfamily that includes *Hth* in *Drosophila* and *Meis* and *Prep* in vertebrates (Kurant *et al.*, 1998; Moskow *et al.*, 1995; Rieckhof *et al.*, 1997). PBX proteins directly interact with the HOX proteins and enhance their DNA-binding specificity (Chang *et al.*, 1995), whereas MEIS/Prep regulates HOX activity both as a component of the DNA-bound HOX complex and by stabilizing and promoting nuclear localization of the PBX protein (Berthelsen *et al.*, 1998; Chang *et al.*, 1997; Knoepfler *et al.*, 1997; Waskiewicz *et al.*, 2001). Inactivation of *pbx* and *meis* genes in zebrafish mimics *Hox* loss of function phenotypes in the mouse (Popperl *et al.*, 2000; Waskiewicz *et al.*, 2001; Waskiewicz *et al.*, 2002).

3.4.1 *Hox* genes in hindbrain patterning

In the CNS, the *Hox* genes are expressed along the AP axis with sharp rostral limits of expression lying in the hindbrain and the spinal cord. The only *Hox* paralagous groups that are expressed up to the hindbrain are groups from 1 to 4. These genes show rostral limits of expression that coincide with rhombomere boundaries and some of them are restricted to specific rhombomeres (Fig. 17A). These *Hox* genes are involved in defining the AP identity of the rhombomeres. Missexpression or inactivation of *Hox* genes lead to partial homeotic changes in rhombomere identity in terms of gene expression and development of ulterior structures (Barrow and Capecchi, 1996; Bell *et al.*, 1999; Carpenter *et al.*, 1993; Chisaka and Capecchi, 1991; Chisaka *et al.*, 1992; Gavalas *et al.*, 1997; Gendron-Maguire *et al.*, 1993; Goddard *et al.*, 1996; Rijli *et al.*, 1993; Studer *et al.*, 1996). *Hox* inactivation leads to the acquisition of rostral features by more posterior rhombomeres and *Hox* overexpression leads to the opposite effect, thus the posteriorization of anterior rhombomeres (Fig. 17B). Differently to *Drosophila* homeotic changes, identity changes in vertebrate *Hox* mutants are not fully penetrant, the territories change part of their features but are not completely re-specified to another identity. The cause of this seems to be that *Hox* genes of the same paralogous groups overlap in part of their functions thus generating functional redundancy. Indeed, inactivation of two or more genes of the same paralogous group leads to more drastic phenotypes (Choe and Sagerstrom, 2004; Gaufo *et al.*, 2003; Greer *et al.*, 2000; Manley and Capecchi, 1997; McNulty *et al.*, 2005). The posteriorizing function of *Hox* genes is a general mechanism and the current thinking is that *Hox* genes operate by conferring caudal identity to a

rostral ground state. In the hindbrain, this rostral ground state is represented by r1-identity. Indeed in experiments in which the whole *Hox* system is inactivated by inhibiting its obligated Pbx cofactors, the entire hindbrain is re-specified to r1-identity (Waskiewicz *et al.*, 2002).

3.4.2 *Hoxa1* and hindbrain segmentation

Hoxa1 has been considered a special case among the *Hox* genes that are involved in the hindbrain patterning. This is because *Hoxa1* is the only *Hox* gene which inactivation leads not only to identity changes but also to loss of segments. *Hoxa1* is normally expressed up to r3/r4. Its inactivation causes reduction of r4 and severe reduction or complete loss of r5. The remnants of these rhombomeres do not form a boundary with r6, which results in a large rV rhombomere (Carpenter *et al.*, 1993; Dolle *et al.*, 1993). These mutants show defects in otic development as well as in cranial nerves VIIth, VIIIth and IXth (Chisaka *et al.*, 1992). As it has been said, paralogous *Hox* genes usually generate functional redundancy. A work by McNulty *et al.* demonstrated that silencing *Hoxa1*, *Hoxb1* and *Hoxd1* at the same time in frog leads to very drastic defects in hindbrain segmentation (McNulty *et al.*, 2005). In fact, the hindbrain is not segmented at all and it acquires r1-like identity.

3.4.3 Regulation of *Hox* genes in the hindbrain

Although now more complex models are considered, RA signaling was initially conceived as the overall mediator or modulator of *Hox* gene expression (Gavalas and Krumlauf, 2000). In the CNS, involvement of RA is clear in the regulation of the 3' *Hox* genes, which are expressed in the hindbrain, but not as clear in the regulation 5' *Hox* genes, which are expressed in the spinal cord. Several 3' *Hox* genes have been shown to contain RARE sequences: *Hoxa1* (Dupe *et al.*, 1997; Frasch *et al.*, 1995; Langston and Gudas, 1992), *Hoxb1* (Huang *et al.*, 1998; Marshall *et al.*, 1994; Morrison *et al.*, 1997; Studer *et al.*, 1994), *Hoxa4* (Packer *et al.*, 1998) *Hoxb4* (Gould *et al.*, 1998) and *Hoxd4* (Morrison *et al.*, 1996; Morrison *et al.*, 1997; Nolte *et al.*, 2003; Zhang *et al.*, 2000). Actually, establishment and maintenance of the '*Hox* code' in the hindbrain implies a complex network that involves signals from the RA and FGF pathways, transcription factors such as *Kreisler/MafB* and *Krox20* and auto- and cross-regulation of *Hox* genes themselves (Bel-Vialar *et al.*, 2002; Maconochie *et al.*, 2001; Manzanares *et al.*, 2002; Manzanares *et al.*, 2001; Oosterveen *et al.*, 2003; Pownall *et al.*, 1998; Pownall *et al.*, 1996; Theil *et al.*, 2002).

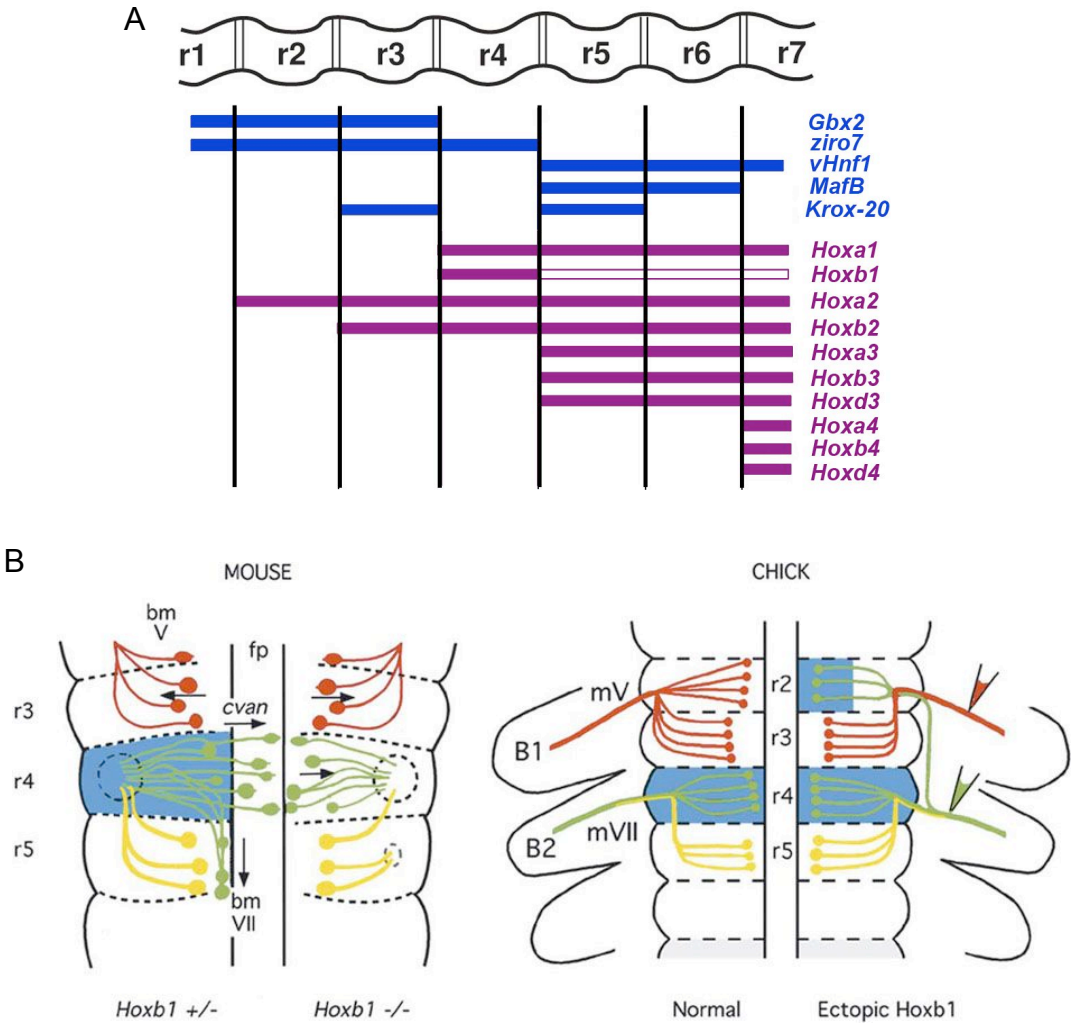


Figure 17. (**A**) **A combination of *Hox* and other transcription factors determinates the molecular identity of each rhombomere.** The diagram represents the expression profiles of *Hox* (violet) and non-*Hox* (blue) transcription factors expressed during hindbrain patterning. These genes are expressed showing sharp boundaries of expression coincident with rhombomere boundaries. The diagram is simplified because actually most of these expression profiles are very dynamic. For example, in the diagram *Krox20* and *vHnf1* are both presented in r5 but actually when *Kox20* is initiated in r5 *vHnf1* regresses caudally and they never coincide in the same domain. The empty bar in *Hoxb1* represents that *Hoxb1* is initially expressed in these rhombomeres but later is downregulated there. (Modified from Schneider-Maunoury *et al.*, 1998). (**B**) **Inactivation of *Hox* genes leads to acquisition of rostral identities by more posterior territories whereas *Hox* overexpression has the opposite effect.** In wild type and *Hoxb1* heterozygous mutant mice, motor neurons (green) arise in r4, the domain of *Hoxb1* expression (blue), and then migrate caudally (branchiomotor neurons of the VIIth cranial nerve) or across the floor plate (contralateral vestibulo-acoustic neurons). In the homozygous null animal, motor neurons develop in r4 but fail to acquire the specific identity of these motor neurons, instead they migrate laterally, like those in r2. When *Hoxb1* is overexpressed in the basal r2 of chick embryos, motor neurons extend their axons via a novel pathway to the second branchial arch, suggesting that their identity has changed from trigeminal (mV) to facial (mVII) although this is only seen in their pathfinding specificity. Axonal pathways have been traced by retrograde dye injection from the arch. (Modified from Lumsden, 2004).

In the hindbrain, current knowledge indicates that regulation of the 3' *Hox* genes depends on rhombomere-specific mechanisms that generally involve two phases (Studer *et al.*, 1998; Tumpel *et al.*, 2007). The first phase is an early activation step that involves RA signaling and rhombomere-specific transcription factors (*Krox20* and *Kreisler/MafB*) (Maconochie *et al.*, 2001; Manzanares *et al.*, 2002; Nonchev *et al.*, 1996; Sham *et al.*, 1993). The second phase is the maintenance of expression by *Hox* autoregulation, pararegulation (between paralogues) and/or cross-regulation (between non-paralogue members within or without the cluster) (Manzanares *et al.*, 2001; Popperl *et al.*, 1995). For example, in r4, *Hoxb1* and *Hoxa1* are induced by RA signaling (Dupe *et al.*, 1997; Marshall *et al.*, 1994; Studer *et al.*, 1994), later on *Hoxb1* is positively modulated by *Hoxa1* (pararegulation) and by itself (autoregulation) (Studer, 1998; Studer, 1996; Popperl 1995). *Hoxb1* also modulates *Hoxa2* and *Hoxb2* expression (cross-regulation) (Gavalas *et al.*, 2003; Maconochie *et al.*, 1997; Tumpel *et al.*, 2007). In r5 and r6, *Hoxa3* and *Hoxb3* are induced by *Kreisler/MafB* (Manzanares *et al.*, 1999). Later on, *Hoxa3* maintains its own expression by an autoregulatory loop while *Hoxb3* is downregulated (Manzanares *et al.*, 2001). In addition, *Krox20* co-operates with *Kreisler/MafB* in inducing *Hoxb3* in r5 (Manzanares *et al.*, 2002). Apart of these mechanisms, FGFs from the isthmus have been proposed to limit the rostral extension of Hox expression within the hindbrain. It is proposed that FGF8 arising from the isthmic organizer maintains r1 as a *Hox*-free territory and is involved in positioning the rhombomere boundary r1/2 (Irving and Mason, 2000; Trainor *et al.*, 2002). In chick embryos, when FGF8 is supplied in r1, the *Hoxa2* rostral limit of expression and the entire morphological r1/r2 boundary shifts caudally, while blocking FGF8 by a specific antiserum leads to rostral shift of *Hoxa2* expression and also r1/r2 and r2/r3 boundaries (Irving and Mason, 2000).

3.5 Non-*Hox* transcription factors involved in AP patterning within the hindbrain

In addition to *Hox* genes, other transcription factors are involved in conferring molecular identity to the hindbrain territories. Although these genes belong to different classes of transcription factors they have been commonly grouped in the functional term of "segmentation genes". This is because, in contrast with most of the *Hox* genes, their inactivation leads to the elimination of hindbrain territories or modification of their extension (Fig. 18) (Schneider-Maunoury *et al.*, 1998). This group includes the homeodomain-containing genes *Gbx2*, *Iro7* and *vHnf1*, the leucine-zipper gene *MafB* and

the zing-finger gene *Krox20*. All these genes are early expressed within the hindbrain in segment-restricted patterns and are involved in the initial steps of hindbrain patterning.

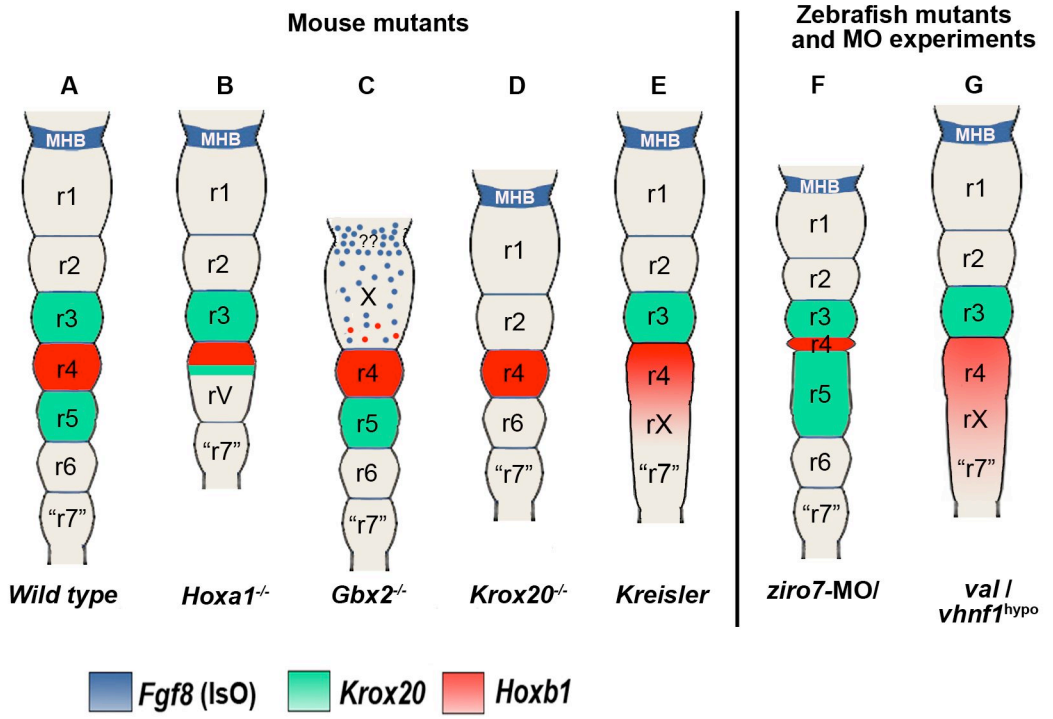


Figure 18. Inactivation of 'segmentation genes' not only promotes changes in rhombomere identity but also in size and number of rhombomeres. Schematic dorsal views of mouse (**A-E**) or zebrafish (**F-G**) hindbrains. The MHB is marked by *Fgf8* (blue), *Krox20* marks r3 and r5 (green) and *Hoxb1* marks r4 (red). (**A**) Wild type embryo. (**B**) *Hoxa1*$^{-/-}$ embryo; r4 is reduced and r5 almost lost. The remnants of these rhombomeres are fused with r6 in a territory termed rV (Dolle *et al.*, 1993). (**C**) *Gbx2*$^{-/-}$ embryo; the anterior hindbrain is unsegmented and severely reduced. Between the midbrain and r4 remains the zone X which displays aberrant expression of midbrain, MHB and r4 genes (Wassarman *et al.*, 1997). (**D**) *Krox20*$^{-/-}$ embryo. r3 and r5 are lost (Schneider-Maunoury *et al.*, 1997). (**E**) *Kreisler* mutant; the hindbrain is unsegmented from r3/r4 boundary. The r5/r6 territory is reduced and misspecified (rX). r5-*Krox20* expression is lost and *Hoxb1* is caudally expanded (Frohman *et al.*, 1993; McKay *et al.*, 1994). (**F**) Zebrafish injected with *iro7* antisense morpholinos (MO); r5 is expanded at expenses of r4. The anterior hindbrain is reduced in size but normally specified (Lecaudey *et al.*, 2004). (**G**) Zebrafish *val* or *vhnf1*^{hypo} mutations; r5 and r6 are reduced and misspecified (rX). r5-*Krox20* is lost and *Hoxb1* is caudally expanded (Moens *et al.*, 1996; Sun and Hopkins, 2001).

3.5.1 *Gbx2*

Gbx2 is a homeodomain-containing transcription factor (Chapman and Rathjen, 1995). As mentioned above, *Gbx2* is early expressed in the anterior hindbrain (r1-r3) showing a sharp anterior limit of expression that coincides with the posterior limit of the *Otx2* expression domain (Shamim and Mason, 1998; Wassarman *et al.*, 1997). *Gbx2* is

involved in positioning and maintenance of the IsO and also is essential for patterning the anterior hindbrain. Mice mutants for *Gbx2* show misspecification of the IsO and the anterior hindbrain (r1-r3) coupled with dramatic reduction of this territory (Wassarman *et al.*, 1997). Between the midbrain and r4 remains an area termed zone X which displays aberrant patterns of expression of midbrain genes (*Otx2*), IsO genes (*Fgf8*, *Wnt1*) and r4 genes (*Hoxb1*) (Wassarman *et al.*, 1997). Experiments in frog suggested that *Gbx2* is positively modulated by RA (von Bubnoff *et al.*, 1996).

3.5.2 *Irx genes*

Irx genes, the vertebrate orthologues of the selector *Iroquois* genes in *Drosophila*, constitute a family of TALE homeodomain transcription factors (Bosse *et al.*, 1997; Bosse *et al.*, 2000; Cohen *et al.*, 2000). Irx proteins share the highly conserved "iroquois-class homeodomain" and the IRO box, which can be found in all known Iroquois proteins (Bao *et al.*, 1999; Bellefroid *et al.*, 1998; Bosse *et al.*, 1997; Gomez-Skarmeta *et al.*, 1996; Gomez-Skarmeta *et al.*, 1998). *Irx* genes are expressed in the mouse neuroepithelium and have been related to neurogenesis (Bosse *et al.*, 1997; Cohen *et al.*, 2000). In zebrafish, *iro7* is very early expressed in the midbrain and anterior hindbrain. In the hindbrain it shows a caudal limit of expression that is coincident with the anterior limit of *vhnf1* expression. Mutual repression between *iro7* and *vhnf1* establishes the r4/r5 boundary (Lecaudey *et al.*, 2004). Injection of *iro7*-MOs leads to a reduction of midbrain and anterior hindbrain territories, anterior shifting of r5 at expenses of r4 and defects in otic development and neurogenesis in r2-r4 (Lecaudey *et al.*, 2004), suggesting that *iro7* expression is crucial for rostral hindbrain patterning. No orthologue of *iro7* exists in amniotes, however *Irx3* is early expressed in the neuroepithelium of chick and mouse embryos following an expression pattern similar to that of *iro7* in zebrafish. This suggests that *Irx3* could be the one involved in setting the r4/r5 boundary with *vHnf1* (Sirbu *et al.*, 2005; Sapede and Pujades, unpublished results).

3.5.3 *MafB*

MafB is a member of the MAF leucine zipper-containing transcriptional factors (Kataoka *et al.*, 1994). Its orthologue in zebrafish is called *valentino* (*val*) (Moens *et al.*, 1996) and its mutation in mouse is classically known as *kreisler* (Cordes and Barsh, 1994). *MafB* is

early expressed in the prospective r5-r6 territory and is maintained in r5 and r6 once these rhombomeres are established (Eichmann *et al.*, 1997).

The *Kreisler* mutation is generated by a submicroscopic inversion that does not disrupt the *Kreisler* transcription unit, but nonetheless abolishes *Kreisler* expression in r5 and r6 (Cordes and Barsh, 1994). It is characterized by the lack of morphological segmentation posterior to r3/r4 boundary (McKay *et al.*, 1996). The r5-r6 region is reduced in size and the expression pattern of several segment-restricted genes is affected (Cordes and Barsh, 1994; McKay *et al.*, 1994). Expression of the r5 marker *Krox20* is absent while *Hoxb1* is expanded caudally from r4 (Frohman *et al.*, 1993; McKay *et al.*, 1994). Expression of *Fgf3* in the caudal hindbrain is also absent (McKay *et al.*, 1996). Inactivation of *val* in zebrafish also leads to loss of segmentation posterior to r3/r4, defects in otic development and a reduced and misspecified r5-r6 territory (Moens *et al.*, 1996; Prince *et al.*, 1998; Kwak *et al.*, 2002). An interesting difference between *Kreisler* and *val* phenotypes is that while *Fgf3* expression is lost in the caudal hindbrain of the *Kreisler* mutants (McKay *et al.*, 1996; Vazquez-Ecevarria *et al.*, submitted), in *val* mutants it is caudally expanded from r4 to the misspecified r5-r6 territory (Kwak *et al.*, 2002).

MafB is involved in conferring caudal identity to r5 and r6 by regulating other AP positional identity genes. In mice, direct involvement of *MafB* in early expression of *Hoxa3* in the r5-r6 territory and *Hoxb3* in r5 has been demonstrated (Manzanares *et al.*, 1997; Manzanares *et al.*, 1999; Manzanares *et al.*, 2001), the latter in co-operation with *Krox20* (Manzanares *et al.*, 2002). In *Kreisler* and *val* mutants *Hoxb1* is expanded caudally; conversely, when *MafB* is overexpressed in r4 *Hoxb1* is downregulated, suggesting that in normal conditions *MafB* represses *Hoxb1* in r5 and r6 (Giudicelli *et al.*, 2003). Recently, functional analysis of the *vHnf1* regulatory regions in mouse suggested that this gene can be directly regulated by *MafB* (Pouilhe *et al.*, 2007).

MafB expression in the caudal hindbrain is dependent on FGF signals and *vHnf1* expression (Aragon *et al.*, 2005; Hernandez *et al.*, 2004; Kim *et al.*, 2005; Marin and Charnay, 2000; Wiellette and Sive, 2003). Ectopic expression of *MafB* in chick demonstrates that *MafB* is able to induce its own expression in a cell-autonomous manner in territories caudal to the r2/r3 boundary, suggesting that once induced *MafB* maintains its own expression in the caudal hindbrain (Giudicelli *et al.*, 2003).

3.5.4 *Krox20*

Krox20 encodes a zing finger transcription factor expressed within the hindbrain in a territory that initially prefigures and subsequently coincides with r3 and r5 territories (Wilkinson *et al.*, 1989). In mice, targeted inactivation of *Krox20* leads to a progressive elimination of r3 and r5, indicating that this gene is essential for the maintenance of these territories (Schneider-Maunoury *et al.*, 1993; Schneider-Maunoury *et al.*, 1997; Swiatek and Gridley, 1993; Voiculescu *et al.*, 2001). The lack of these territories has effects in the neighboring even-numbered rhombomeres and axonal projections from these rhombomeres are miss-routed (Schneider-Maunoury *et al.*, 1997). Surprisingly, the disappearance of r3 and r5 does not lead to fusion of even-rhombomeres and boundaries are conserved (discussed in Schneider-Maunoury *et al.*, 1997).

Krox20 is involved in conferring odd identity to hindbrain territories. *Krox20* directly regulates *Hoxb2*, *Hoxa2*, and *Hoxb3* (Maconochie *et al.*, 2001; Manzanares *et al.*, 2002; Nonchev *et al.*, 1996; Sham *et al.*, 1993; Vesque *et al.*, 1996). All of the former are genes expressed in r3 and r5, with the exception of *Hoxb3* that is only expressed in r5. Also the receptor tyrosine kinase *EphA4*, which is involved in differential adhesion properties of odd-numbered rhombomere cells, is directly regulated by *Krox20* (Theil *et al.*, 1998). *Krox20* ectopic expression in chick results in the acquisition of odd molecular character by even-rhombomeres (Giudicelli *et al.*, 2001).

Similarly to *MafB*, *Krox20* initiation depends on factors that include FGF signaling and *vHnf1* expression in r5 (Aragon *et al.*, 2005; Chomette *et al.*, 2006; Marin and Charnay, 2000; Wiellette and Sive, 2003). Some reports propose that initiation of *Krox20* in r5 is also dependent on *val/MafB* expression (Wiellette and Sive, 2003). After this, *Krox20* expression is maintained independently by an autoregulatory loop (Chomette *et al.*, 2006; Giudicelli *et al.*, 2001). Differently to *MafB*, this loop works in both cell and non cell-autonomous manners and promotes the recruitment of neighboring cells to r3 or r5-identities (Giudicelli *et al.*, 2001; Voiculescu *et al.* 2001). Expansion of *Krox20* expression is controlled by a negative autoregulatory loop with Nab proteins that are expressed in the rhombomere boundaries once these boundaries are established (Mechta-Grigoriou *et al.*, 2000).

4 A novel gene involved in hindbrain patterning: *vHnf1*

vHnf1 (*variant Hepatocyte nuclear factor 1*) also known as HNF1β, *TCF2* and *LF-B3*, is a transcription factor belonging to the HNF1 family. The HNF1 family uniquely encloses the *vHnf1* and *Hnf1* genes. These genes are highly conserved across species (Tronche and Yaniv, 1992). They contain two DNA binding domains: an homeodomain and a POU domain. *vHnf1* has two different splicing variants that show different DNA binding affinities and transactivation potencies (Ringeisen *et al.*, 1993). The HNF1 factors were originally found expressed in different gut-derived structures including the liver, kidney and pancreas (Coffinier *et al.*, 1999; Ott *et al.*, 1991; Rey-Campos *et al.*, 1991). In humans, mutations on *vHnf1* are associated with a kind of young onset diabetes called MODY5 and also to the glomerulocystic kidney disease (GCDK) (Horikawa *et al.*, 1997; Lindner *et al.*, 1999; Nishigori *et al.*, 1998).

In 2001, a zebrafish mutational screening for developmental genes identified *vhnf1* as a gene related to hindbrain patterning (Sun and Hopkins, 2001). The phenotype of three zebrafish hypomorfic mutants for *vHnf1* was analyzed. Two of these mutants showed misspecification of the caudal hindbrain and hypoplastic otic vesicles. These embryos lacked expression of *val* and r5-*krox20*. Conversely, *hoxb1* was caudally expanded from r4. This finding encouraged us to further study the role of *vHnf1* in the early steps of hindbrain patterning. The present project and other works have demonstrated that *vHnf1* is one of the earliest genes expressed in a segment-restricted manner in the hindbrain and that plays a crucial and early role in specifying the caudal hindbrain (Aragon *et al.*, 2005; Hernandez *et al.*, 2004; Lecaudey *et al.*, 2004; Wiellette and Sive, 2003). Studies in zebrafish proposed that *vhnf1* regulates *val* and *krox20* expression in synergy with FGFs from r4 (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). In addition to this, studies on the *MafB* regulatory regions in mice revealed that *vHnf1* can directly regulate the *MafB* enhancer (Kim *et al.*, 2005). In the present work we have demonstrated that in birds *vHnf1* also operates together with FGFs to confer caudal identity to the hindbrain, although differences in the mechanism by which this is done are proposed between zebrafish and chick (see results and discussion).

In the last years, some insights on how *vHnf1* is regulated in the caudal hindbrain have been reported. It has been demonstrated that *vHnf1* expression depends on RA signaling

(Hernandez *et al.*, 2004; Hernandez *et al.*, 2007; Maves and Kimmel, 2005; Sirbu *et al.*, 2005). Moreover, studies on the *vHnf1* regulatory regions of mouse suggest that RA directly regulates *vHnf1* (Pouilhe *et al.*, 2007). Other studies in zebrafish suggested the involvement of the *Hox* of the paralogous group 1 in inducing *vhnf1* in r5 and r6 (Choe and Sagerstrom, 2004). Finally, it has been proposed that *vHnf1* expression may be dependent on some MAF-type factors (Pouilhe *et al.*, 2007).

5. An overview of the FGF signaling system

5.1 Multiple roles of FGF signaling during early development

A surprisingly small number of growth factor and their receptor families orchestrate development by instructing different uncommitted cell types to proliferate, differentiate and/or organize into specific patterns. Among those, the FGF signaling system is involved in virtually all the early embryonic processes: body axis establishment and mesoderm formation, endoderm formation, neural induction and regionalization of the neural plate, somitogenesis and patterning of several embryonic structures (Bottcher and Niehrs, 2005; Mason, 2007).

Apart from their role in regionalizing the CNS (see sections 3.1 and 3.2), FGFs are involved in patterning several other embryonic structures. In the limb bud, FGFs from the Apical Ectodermal Ridge (AER) instruct the underlying mesenchime for a proper outgrowth (Capdevila and Izpisua Belmonte, 2001; Eblaghie *et al.*, 2003; Kawakami *et al.*, 2003). Similarly, FGFs from the ectoderm of the first branchial arch signal to the neural crest cell derivatives (Tucker *et al.*, 1999). FGF signals are also necessary for the induction and regionalization of the sensory placodes and further organogenesis of the sensory organs (Cvekl and Duncan, 2007; Maroon *et al.*, 2002; Mason, 2007; Schneider-Maunoury and Pujades, 2007; Streit, 2007; Torres and Giraldez, 1998); and different steps of chondrogenesis and skeletal (Brent and Tabin, 2004; Ornitz and Marie, 2002; Ornitz, 2005; Smith *et al.*, 2005), heart (Dell'Era *et al.*, 2003; Reifers *et al.*, 2000) and gut development (Serls *et al.*, 2005).

FGF signaling operates by the action of RTKs (FGFRs) that are specifically activated when they bind to diffusible FGF molecules secreted to the extracellular space. The

activation of the FGFRs leads to the recruitment of a signaling complex in the cytoplasmatic side of the cell membrane. This signaling complex is responsible for the activation of different intracellular pathways that mediate changes in gene expression and cell behavior. These pathways are the MAPK, the PI3K and PLCγ cascades. Finally, the whole system is autoregulated by specific negative and positive modulators that are induced by FGF signaling itself and which exert their actions at different steps of the signaling process. All this process is summarized in Figure 19.

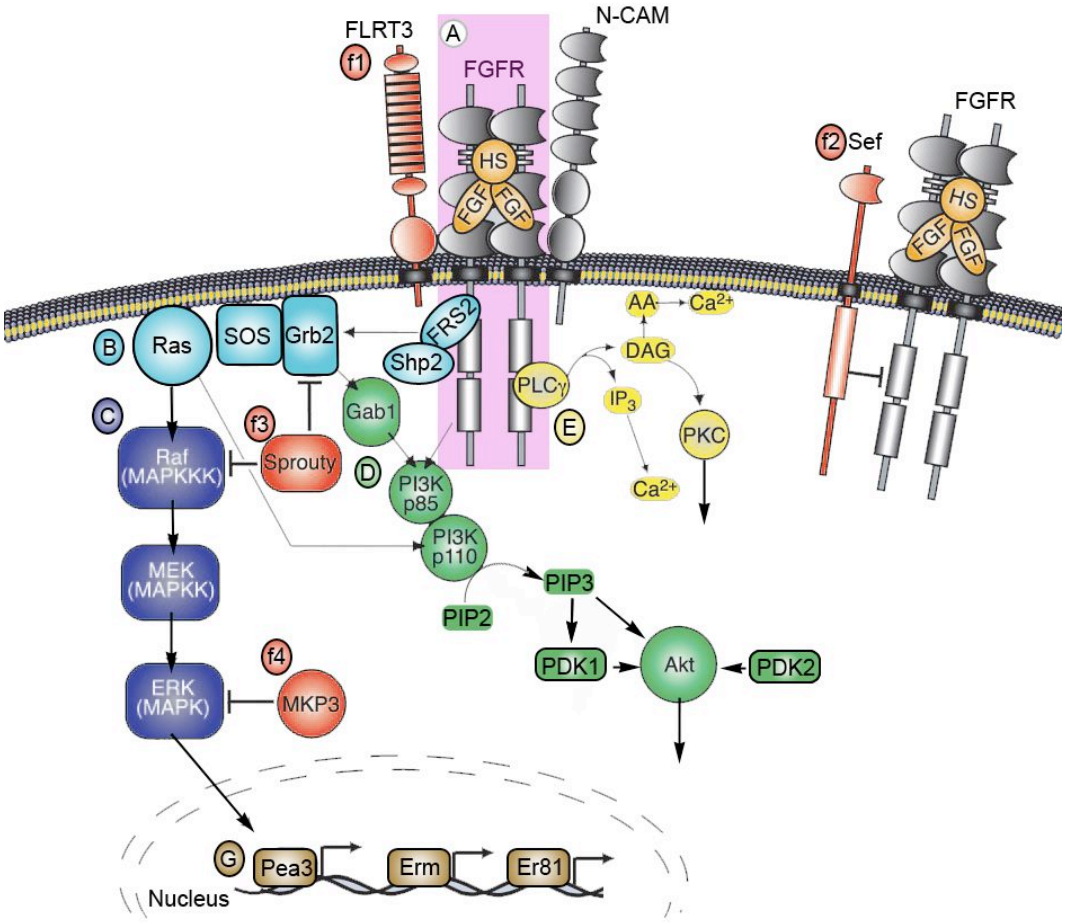


Figure 19. Schematic representation of the FGF signaling system with its downstream pathways and modulators. (**A**) Activated FGF transduction complex constituted by 2 FGFRs, 2 FGF molecules and 1 molecule of Heparan Sulfate (HS). (**B**) Signaling complex (light blue). (**C**) Ras-MAPK pathway (dark blue). (**D**) PI3K-Akt pathway (green). (**E**) PLCγ pathway (yellow). (**F**) Modulators of FGF activity (red): FLRT3 (**f1**), Sef (**f2**), Sprouty (**f3**) and MKP3 (**f4**). (**G**) Pea3 subfamily of Ets transcription factors (Erm, Pea3 and Er81) activated by ERK1/2. Modified from Bottcher and Niehrs, 2005.

5.2 Fibroblast Growth Factors (FGFs), FGF Receptors (FGFRs) and signal transduction

The FGFs were one of the first families of growth factors to be described. The first member of this family was identified by its ability to stimulate proliferation of mouse 3T3 fibroblasts (Armelin, 1973). To date 22 *FGF*s have been reported in the mouse and human genomes (Itoh, 2007). Orthologues for almost all of them have been also identified in the chick and zebrafish genomes, with the exception of *Fgf11*, *Fgf17* and *Fgf21* for chick, and *fgf9* for zebrafish (information based on v2.1 of the chick genome and v7 of the zebrafish genome). The zebrafish genome also contains paralogues for *fgf6 (6a,6b)*, *fgf8 (8*,*17a)*, *fgf10 (10a,10b)*, *fgf18 (18a,18b,24)* and *fgf20 (20a,20b)* (Itoh, 2007).

Most of the FGFs are constitutively secreted to the extracellular matrix (Ornitz and Itoh, 2001). However, FGFs 11–14 are not secreted and do not activate FGFRs, but instead localize to the cell nucleus (Goldfarb, 2005; Smallwood *et al.*, 1996); FGF1 and FGF2 are secreted but by non-canonical ways (Mignatti *et al.*, 1992); FGF2 and FGF3 can be translocated along both secretory and nuclear pathways (Antoine *et al.*, 1997; Arnaud *et al.*, 1999). Figure 20 shows the prototypical domain structure of a FGF molecule and the 3D conformation of FGF1.

*FGF*s are expressed during development in very diverse and dynamic patterns. Usually various *FGFs* are co-expressed in the same tissue. Examples are the AER of the limb bud that expresses *Fgf2*, *Fgf4*, *Fgf8* and *Fgf9* (Martin, 1998), the IsO that expresses *Fgf8*, *Fgf17*, *Fgf18* (Liu and Joyner, 2001) or r4 in zebrafish that expresses *fgf3* and *fgf8* (Walshe *et al.*, 2002). Co-expression of *FGFs* results in certain functional redundancy that is reflected when targeted knockouts for specific *FGFs* are generated. This is the case for *Fgf2* KO mice, which have no dramatic phenotypes although *Fgf2* is involved in processes such as mesoderm induction, limb development or angiogenesis (Ortega *et al.*, 1998). In zebrafish, both *fgf3* and *fgf8* are required for otic induction. When *fgf3* expression is blocked by antisense morpholinos in wild type embryos, moderated effects are observed in otic development. On the the other hand, when *fgf3* expression is targeted in a context of no *fgf8* expression (*ace* mutants), severe affectation or no otic development at all is observed (Léger *et al.*, 2002; Maroon *et al.*, 2002; Phillips *et al.*, 2001). It is likely that redundancy has been selected by evolution because it is a mechanism that confers robustness to a signaling system.

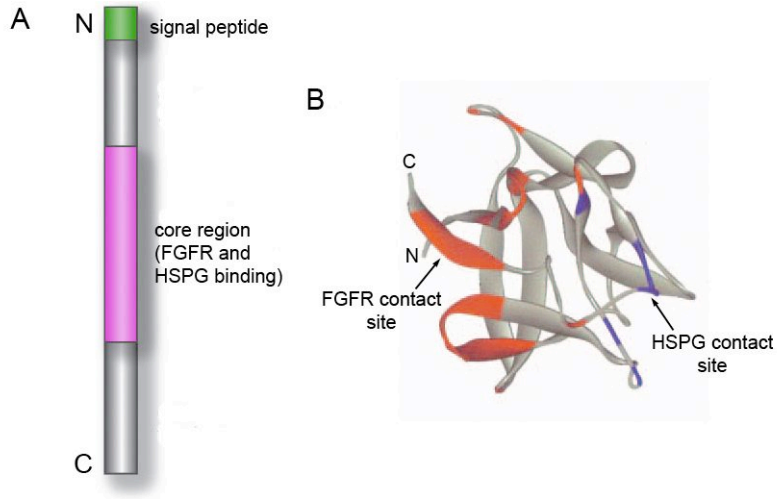


Figure 20. The FGF molecule. (**A**) Domain structure of a generic FGF with an exporting signal and the conserved core region that contains receptor- and HSPG-binding sites. Modified from Bottcher and Niehrs, 2005. (**B**) Ribbon model for FGF1 structure and folding. Receptor and HSPG contact sites are shown in red and blue respectively. Modified from Mason, 2007.

FGFRs are a subfamily of cell surface receptor tyrosine kinases (RTKs). The vertebrate *FGFR* gene family consists of four highly related genes, *FGFR1–4* (Johnson and Williams, 1993). FGFR are single-pass transmembrane proteins with an extracellular ligand-binding region and an intracellular domain harboring tyrosine kinase activity (Fig. 21) (Johnson *et al.*, 1990). The extracellular region is composed of three Ig-like domains (IgI, IgII, IgIII) that are required for FGF binding and that regulate binding affinity and ligand specificity. Located between Ig-like domains I and II there is a stretch of acidic amino acids (acidic box domain) followed by a heparin-binding region and a cell adhesion molecule homology domain (CHD). These domains are required for the interaction of the receptor with components of the extracellular matrix, in particular heparan sulfate proteoglycans (HSPGs), and cell adhesion molecules (CAMs) (Doherty and Walsh, 1996; Kan *et al.*, 1993). Continuous with the extracellular region there is a transmembrane domain followed by an intracellular region. The latter consists of a juxtamembrane domain, a tyrosine kinase domain splitted by a non-catalytic interkinase domain, and a short C-terminal tail (Johnson and Williams, 1993). In addition to the enzymatic activity, the intracellular domain harbors sites for protein binding and phosphorylation as well as several autophosphorylation sites. A range of FGFR isoforms are generated by alternative splicing. Some of these isoforms have been shown to generate receptors with different ligand binding specificities *in vitro* (Fig. 22) (Zhang *et al.*, 2006; Groth and Lardelli, 2002; Ornitz *et al.* 1996).

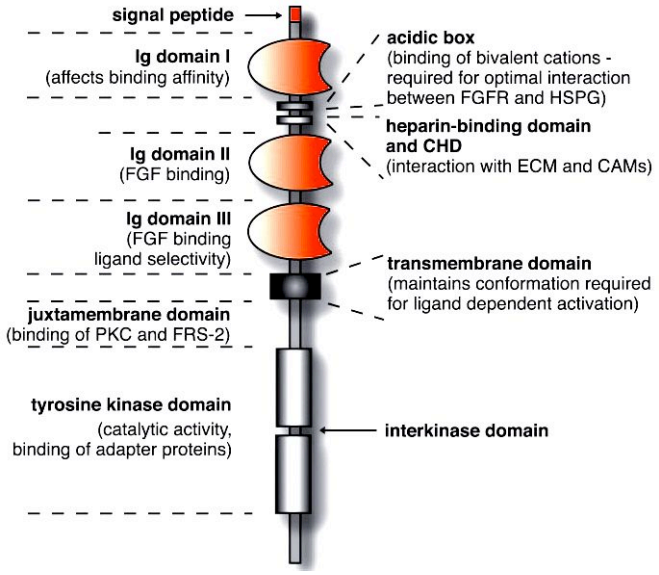


Figure 21. Domain structure of a generic FGFR. The main structural features of FGFRs include three Ig domains, acidic box, heparin-binding domain, CAM-homology domain (CHD), transmembrane domain, and a split tyrosine kinase domain. CAM, Cell adhesion molecule; ECM, extracellular matrix; PKC, protein kinase C**.** Modified from Bottcher and Niehrs, 2005.

FGF subfamily	FGF	FGFR isoforms
FGF1 subfamily	FGF1 FGF2	ALL FGFRs FGFR 1c, 3c > 2c, 1b, 4Δ
FGF4 subfamily	FGF4 FGF5 FGF6	FGFR 1c, 2c > 3c, 4Δ
FGF7 subfamily	FGF3 FGF7 FGF10 FGF22	FGFR 2b > 1b
FGF8 subfamily	FGF8 FGF17 FGF18	FGFR 3c > 4Δ > 2c > 1c >> 3b,
FGF9 subfamily	FGF9 FGF16 FGF20	FGFR 3c > 2c > 1c, 3b >> 4Δ
FGF19 subfamily	FGF19 FGF21 FGF23	FGFR 1c, 2c, 3c, 4Δ (weak activity)
FGF11 subfamily	FGF11 FGF12 FGF13 FGF14	No known activity

Figure 22. FGFR splicing isoforms. *In vitro* experiments revealed that FGF ligands have different binding affinities for different FGFRIII splicing isoforms. In the table, FGF ligands are grouped in subfamilies and isoforms are listed in relation to their affinities for members of the different subfamilies. Data from Ornitz, 2000 and Zhang *et al.*, 2006.

FGFRs show dynamic and differential spatio-temporal distribution suggesting that they have differentiated roles during development (Lunn *et al.*, 2007; Walshe and Mason, 2000). Some reports also suggest that FGFRs can activate preferentially different

intracellular signaling pathways. Specifically, that FGFR1 shows preference for ERK1/2 MAPK and FGFR4 for PLCγ (Umbhauer *et al.*, 2000).

The mechanism of FGFR activation is not yet clear. It is known that it requires heparin or heparan sulfate proteoglicans (HSPGs) (Ornitz, 2000). There are also evidences that HSPGs can differentially modulate FGF-FGFR specificity due to differential binding affinities (Knox *et al.*, 2002). Current data suggests that the FGF molecule may form an initial low affinity 1:1 complex with the FGFR (Hsu *et al.*, 1999; Pye and Gallagher, 1999). This minimal complex may allow transient receptor dimerization. In the presence of appropriate heparan sulfate molecules this complex becomes stabilized and activated (Pye and Gallagher, 1999). Subsequent binding of a second molecule of FGF may then lead to a more stable 2:2 FGF/FGFR signaling transducer (Fig. 23) (reviewed in Mohammadi *et al.*, 2005).

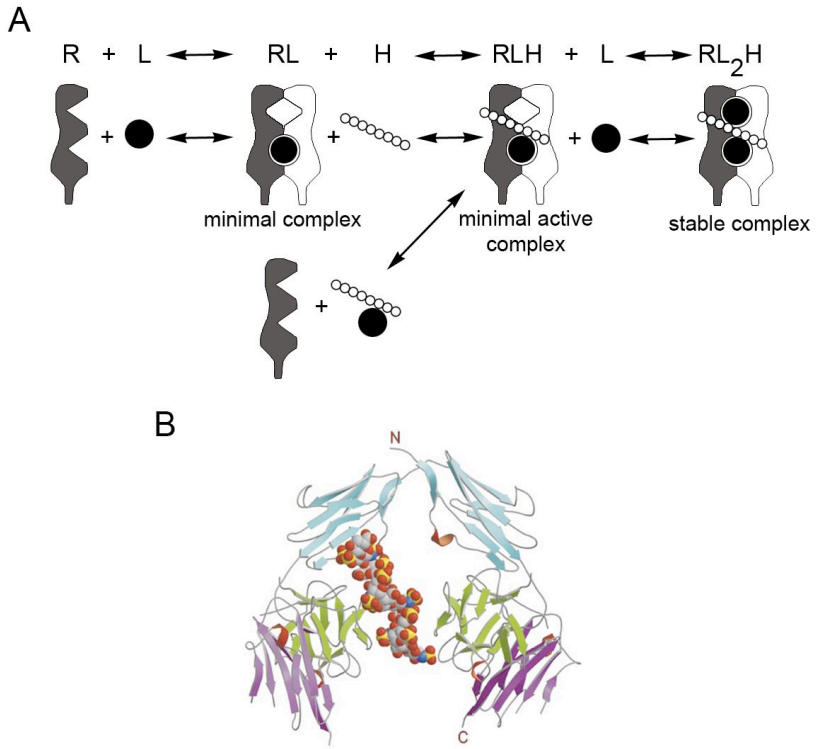


Figure 23. The FGF-FGFR complex. (**A**) Model showing the sequential formation of FGF/FGFR transduction complexes of increasing stability and activity. Initially, a minimal complex is formed. In the presence of apropriate heparan sulfate molecules this complex becomes stabilized and activated. Subsequent binding of a second molecule of FGF may then lead to a more stable 2:2 FGF/FGFR signaling transducer. R: FGFR, L: FGF; H: heparan sulfate. Modified from Ornitz, 2000. (**B**) Representation of the FGF1-FGFR2-heparin complex. FGFR2 domains IgII (D2) and IgIII (D3) are cyan and violet, respectively, and FGF1 is green. The heparin molecule is in the middle in CPK representation. Modified from Pellegrini *et al.*, 2000.

FGFR activation leads to tyrosine autophosphorylation in the intracellular domain of the receptor. These phosphorylations serve as docking sites for the recruitment of docking proteins as FRS2 and GAB1, adaptors as Grb2 and signaling enzymes as the tyrosine phosphatase Shp2. These proteins are recruited through their SH2 (src homology-2) and PTB (phosphotyrosine binding) domains. All these components are assembled in signaling complexes that initiate different intracellular signaling pathways and mediate changes in gene expression and cell behavior (Eswarakumar *et al.*, 2005). The known FGFR downstream pathways are the Ras/MAP kinase pathways (Ras-MAPK) which include ERK1/2, p38 and JNK cascades, the PI3 Kinase/Akt pathway (PI3K-Akt) and the PLCγ pathway.

5.3 Intracellular pathways downstream of the FGFR

5.3.1 Ras-MAPK pathway

The Ras-MAPK is the most common pathway employed by FGFs. It is activated through the FRS2-Shp2 signaling complex. Before activation of FGFR the adaptor protein Grb2 is in the cytoplasm forming a complex with the guanine nucleotide exchange factor Son of sevenless (SOS). Once the FGFR is activated, FRS2 is phosphorilated and Grb2-SOS complex is recruited and translocated to the plasma membrane. This allows SOS to activate the membrane-bound G protein Ras by GTP exchange. Once in the active GTP-bound state, Ras interacts with several effector proteins, including Raf and Rac leading to the activation of the different MAPK cascades (Fig. 24) (reviewed in Lee and McCubrey, 2002; McCubrey *et al.*, 2006). The endpoints of these cascades are the kinases ERK1 and ERK2 (ERK1/2), p38 and JNK/SAPK. Once activated, these kinases are translocated to the nucleus where they activate transcription factors and thus change gene expression. ERK1/2 kinases are classically associated to proliferation (Rossomando *et al.*, 1989) and have been also related to several developmental processes. p38 and JNK kinases are associated with inflammatory and stress-response processes usually in pro-apoptotic ways (Ichijo, 1999; New and Han, 1998). Contrary to the ERK1/2 pathway that seems to be common to all responses mediated by FGFRs, JNK and p38 appear to be specific of certain cell-types.

The ERK1/2 Ras-MAPK pathway is the most widely implicated pathway in FGF-required developmental processes. Examples are chick neural induction and limb development

(Eblaghie *et al.*, 2003), retinal and lens differentiation (Lovicu and McAvoy, 2001; McCabe *et al.*, 2006), sclerotome specification (Brent and Tabin, 2004), isthmic organizer function (Suzuki-Hirano *et al.*, 2005) and zebrafish DV patterning (Tsang *et al.*, 2004). Some reports and this work have shown that the activated forms of ERK1/2 (pERK1/2) co-localize with FGF-expressing regions during early embryonic development (Corson *et al.*, 2003; Lunn *et al.*, 2007).

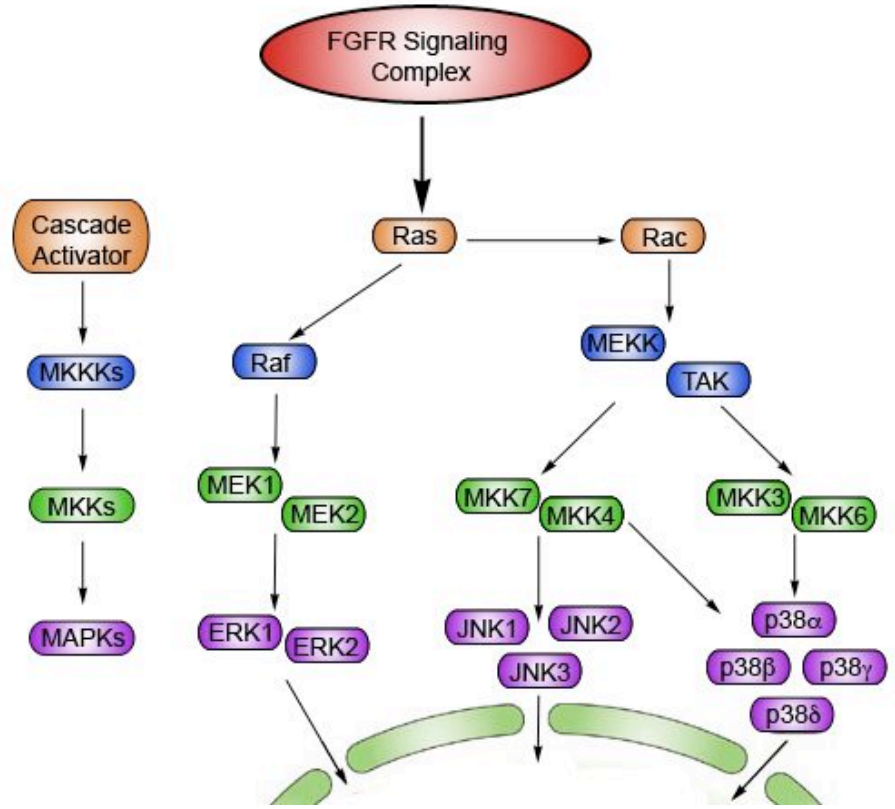


Figure 24. MAPK cascades downstream of FGF signaling. MAPKs cascades are initiated by activator kinases (orange) downstream of the FGFR signaling complex. These cascades consist in three phosphorylation steps in which MKKKs (in blue, also MEKKs) activate MKKs (in green, also MEKs) that activate MAPKs (in violet). MAPKs are translocated to the nucleus and activate transcription factors.

5.3.2 PI3K-Akt pathway

The PI3K-Akt pathway can be activated by three mechanisms upon FGFR activation: i) by binding of PI3K to the FGFR (Chang *et al.*, 2003); ii) through the FRS2-Shp2-Gbr2 signaling complex that recruits the docking protein Gab1 which activates PI3K (Holgado-

Madruga *et al.*, 1997); iii) through Ras kinase which also activates PI3K (Yan *et al.*, 1998). Activated PI3K converts phosphatidylinositol-4,5-diphosphate (PIP2) into phosphatydylinositol-3,4,5-triphosphate (PIP3). PIP3 recruits the kinases PDK1 and Akt to the plasma membrane. In the plasma membrane, Akt is activated by PDK1 and PDK2. Once activated, Akt regulate certain downstream effectors that include other kinases and also transcription factors, several of them involved in apoptosis and cell cycle regulation (reviewed in Hennessy *et al.*, 2005; McCubrey *et al.*, 2006).

Crosstalks of PI3K-Akt with the Ras-MAPK have been identified at several levels of both pathways. All these crosstalks are summarized in Figure 25. Additionally to this post-translational crosstalk, some reports also propose transcriptional crosstalk in which the PI3K pathway induces the expression of inhibitors for the MAPK pathway (Echevarria *et al.*, 2005; Kawakami *et al.*, 2003).

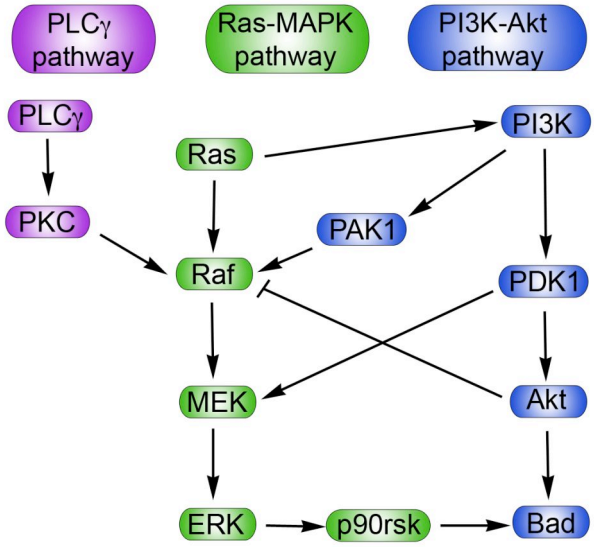


Figure 25. Crosstalk between intracellular signaling pathways. Diagram showing different crosstalkings between the PLCγ (violet), ERK1/2 Ras-MAPK (green) and PI3K-Akt (blue) pathways. PI3K can either activate Raf via the intermediate protein PAK-1 or prevent Raf activation via Akt (Chaudhary *et al.*, 2000; Moelling *et al.*, 2002). In addition to that, PDK1 is able to activate MEK (Koh *et al.*, 2006; Sato *et al.*, 2004). Conversely, ERK1/2 can regulate the Akt downstream effector Bad via p90rsk (Fang *et al.*, 1999).

The PI3K pathway is associated with anti-apoptotic responses and has been also implicated in developmental processes. In these developmental events PI3K-Akt pathway is usually proposed to act with the Ras-MAPK pathway in either synergistic or antagonistic manners. For example, PI3K and MAPK pathways synergistically induce

mesoderm formation in frog (Carballada *et al.*, 2001). On the other hand, Echevarria *et al.* propose a balanced action for the PI3K and MAPK pathways to maintain the integrity of the IsO (Echevarria *et al.*, 2005). This balance would be achieved by an antagonistic crosstalk between the two pathways in which the phosphatase *MKP3* would play a pivotal role. In limb development, the model proposed by Kawakami *et al.* also suggests an antagonistic crosstalk between PI3K and MAPK pathways with the involvement of *MKP3* (Kawakami *et al.*, 2003).

5.3.3 PLCγ pathway

PLCγ is directly recruited by the activated FGFR through its SH2 domain (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Activated PLCγ hydrolizes PIP2 to form diacylglycerol (DAG), and inositol-1,4,5-triphosphate (IP3). DAG activates protein kinase C (PKC), and IP3 stimulates calcium release and activation of calcium/calmodulin-dependent protein kinases (Schlessinger, 2000). Apart of activating several downstream effectors, PKC can create a crosstalk with the MAPK pathway by activating Raf (Fig. 25).

Only few FGF-dependent developmental processes mediated through this pathway have been reported. It has been demonstrated that PLCγ is necessary for the FGF2 and CAM-stimulated neurite outgrowth (Doherty and Walsh, 1996; Webber *et al.*, 2005). This pathway has been also implicated in the caudalization of neural tissue through FGFR4 in the frog (Umbhauer *et al.*, 2000).

5.4 Modulators of FGF activity

The FGF signaling system is tightly regulated by a series of modulators, most of them negative regulators, which exert their functions at different levels of the pathway. The expression of these genes is induced by FGF activity itself and regional and temporal variation in their levels of expression is though to tune FGF signaling to the appropriate levels for each particular event. Thus part of the variability in the outcomes that FGF signaling can generate in a context-dependent manner is thought to be dependent on the combinatorial action of these modulators. The group of FGF activity modulators includes transmembrane proteins that interact with the FGFR, such as *FLRT3* and *Sef*, and cytoplasmatic proteins such as *MKP3* and *Sprouty*.

The term “synexpression group” has been adopted to designate sets of genes that share complex spatio-temporal expression patterns and that function in the same processes (Niehrs and Pollet, 1999). Synexpression groups form expression cassettes that can be found at different times and places during development. The *FGFs* and *FGFRs*, together with the group of FGF activity modulators and the Pea3 subfamily of Ets transcription factors have been designated as the ‘FGF or FGF8 synexpression group’ (Bottcher and Niehrs, 2005).

5.4.1 MKP3

MKP3 belongs to the MKP (MAPK phosphatase) subfamily, the largest group of phosphatases specialized in the regulation of MAPKs (Dickinson and Keyse, 2006; Muda *et al.*, 1996; Muda *et al.*, 1997; Vasudevan *et al.*, 2005). MAPKs -ERK1/2, p38 and SAPK/JNK- are activated by phosphorylation on threonine and tyrosine residues within a conserved sequence. The MKPs constitute a subfamily of dual-specificity (Thr/Tyr) phosphatases (DUSP) that specifically inactivate phosphorilated forms of MAPKs. All MKPs share a common structure, comprising a C-terminal catalytic domain and a N-terminal MAPK-binding domain (Dickinson and Keyse, 2006).

MKP3 (also *DUSP6* or *Pyst1*) is the best studied regulator of the Ras-MAPK pathway downstream of FGF function. MKP3 exclusively inactivates ERK1 and ERK2. Analysis of *MKP3* expression during mouse development revealed expression in the limb bud, the isthmic organizer, the tail bud, the presomitic mesoderm and nasal, dental and mammary placodes (Dickinson *et al.*, 2002; Klock and Herrmann, 2002). All these sites are known places of *Fgf8* expression. *MKP3* also is expressed in FGF signaling regions during early zebrafish and chick development (Lunn *et al.*, 2007; Tsang *et al.*, 2004) and the present work). Functional studies in chick, mouse and zebrafish revealed that FGF signaling is necessary and sufficient for *MKP3* expression (Eblaghie *et al.*, 2003; Echevarria *et al.*, 2005; Kawakami *et al.*, 2003; Tsang *et al.*, 2004 and the present work).

Studies in a variety of patterning events lead to suggest that *MKP3* plays a pivotal role in tuning the activation levels of FGF signaling in these processes. However, it remains controversial by which mechanisms is *MKP3* expression regulated. While some groups propose that *MKP3* expression is under the control of the Ras-ERK1/2 pathway other groups propose PI3K-Akt pathway for this function leading to different and, in some

cases, contradictory models. It is well established that a key component for normal limb development is the signaling provided by FGF8 emanating from the AER to the underlying mesenchime (reviewed in Martin, 1998). Two groups analyzed the role of *MKP3* in limb development. Both noticed that *MKP3* is expressed in the mesenchime and excluded from the AER, and that this expression is dependent on FGF signaling from the AER. Both groups demonstrated that overexpression of *MKP3* results in limb missdevelopment (Eblaghie *et al.*, 2003; Kawakami *et al.*, 2003). However, they obtained divergent results in signaling localization and in loss- and gain-of-function analyses, leading them to generate contradictory hypotheses: i) Kawakami *et al.* propose that *MKP3* is induced in the mesenchime by FGF8 through the PI3K-Akt pathway. The role of MKP3 in the mesenchime would be to antagonize the Ras-MAPK pathway and prevent cell death. Thus, MKP3 would have an anti-apoptotic function in the mesenchime. Conversely, intact Ras-MAPK signaling would by necessary for the integrity of the AER (Fig. 27A) (Kawakami *et al.*, 2003). Eblaghie *et al.* (2003) defend a different model in which *MKP3* would be regulated by the Ras-MAPK pathway just creating an autoregulatory loop, in both limb development and neural induction (Fig. 27B). Reports from other groups support the idea that Ras-MAPK is involved in neural induction (Kuroda *et al.*, 2005). Recently, experiments using the murine *MKP3* in *vitro* and *in vivo* suggested that it is regulated by Ras-MAPK but not PI3K-Akt pathway (Ekerot *et al.*, 2008).

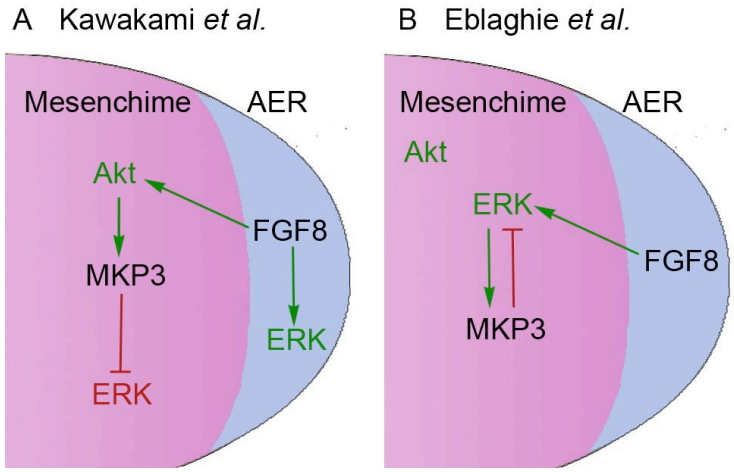


Figure 26. Models to explain the MKP3 role in limb development. (**A**) FGF8 from the AER activates the PI3K-Akt pathway in the mesenchime. This pathway induces *MKP3* and MKP3 dephosphorylates ERK1/2. The Ras-ERK1/2 pathway is silenced in the mesenchime. Conversely, in the AER FGF8 activates the Ras-ERK1/2 pathway. (**B**) FGF8 from the AER activates the Ras-ERK1/2 pathway and this pathway induces *MKP3*. MKP3 dephosphorylates ERK1/2 creating an autoregulatory loop. Data from Kawakami *et al.*, 2003; Eblaghie *et al.*, 2003.

It is known that FGF8 is the major organizing molecule in the isthmic organizer (reviewed in Nakamura *et al.*, 2005; Raible and Brand, 2004). Echevarria *et al.* propose that *MKP3* plays a fundamental role in the isthmic organizer by regulating both activity and expression of *Fgf8*. They propose that maintenance of the isthmic organizer requires self-regulation of FGF signaling through balanced action of both the Ras-MAPK and PI3K-Akt pathways. *MKP3* would be crucial for this balance being induced by the PI3K-Akt pathway and repressing the Ras-MAPK pathway (Echevarria *et al.*, 2005) .

5.4.2 Sprouty proteins

Sprouty proteins are ligand-inducible inhibitors of the ERK1/2 MAPK pathway upon activation by RTKs (EGFR, FGFR and VEGFR). There are four Sprouty proteins in vertebrates (*Spry1-4*). Sprouty proteins are activated both transcriptionally and postranslationally through the Ras-ERK1/2 pathway upon FGFR activation. Translocation of pERK1/2 to the nucleus activates transcription of *Spry* genes. Concurrently, FGFR activation leads to the translocation of Sprouty to the plasma membrane where it is activated. Once activated, SPRY inactivates FGF signaling by blocking that pathway at least at two different levels: i) blocking the formation of the signaling complex downstream the FGFR; ii) blocking Raf, the kinase which initiates the Ras-MAPK pathway (reviewed in Mason *et al.*, 2006).

The important role of *Spry* genes in containing the strength of FGF activity has been revealed by gain- and loss-of-function analyses. Missexpresion of *spry4* in the early zebrafish embryo results in a phenotype similar to *ace* embryos or more severe phenotypes with ventralization of the whole embryo (Furthauer *et al.*, 2001). Conversely, knockdown of *spry4* results in a dorsalization phenotype, enlarged telencephalic territory and the outgrowth of the facial ectoderm, which are the phenotypes expected of unrestrained FGF signaling (Furthauer *et al.*, 1997; Furthauer *et al.*, 2001). In addition to this, missexpression of *Spry2* in the midbrain and anterior hindbrain (cerebellum) of chick and mouse embryos resulted in fate changes due to defects in FGF signaling emanating from the isthmic organizer (Basson *et al.*, 2008; Suzuki-Hirano *et al.*, 2005).

Recently, a new group of proteins structurally and functionally related to sprouties has been found: the SPREDs (Sprouty-Related with an EVH1 Domain) (Wakioka *et al.*, 2001). Three *SPRED* genes have been identified in human and mouse, and two in frog

(Engelhardt *et al.*, 2004; Kato *et al.*, 2003; Sivak *et al.*, 2005). SPREDs, as sprouties, are membrane-associated negative regulators of the Ras-MAPK pathway (Bundschu *et al.*, 2007).

5.4.3 Transmembrane modulators of FGF signaling (*FLRT3* and *Sef*)

FLRT (fibronectin-leucine-rich transmembrane) proteins are single-pass transmembrane proteins. Their extracellular regions contain a conserved fibronectin type-III domain and multiple leucine-rich repeats. Differently to MKPs and Sprouties, FLRTs have uniquely been identified in vertebrates. *FLRT3* coincides in both chick and frog with *Fgf8* expression sites during development, including the limb bud and the MHB (Bottcher *et al.*, 2004; Smith and Tickle, 2006). Studies in frog demonstrated that *FLRT3* expression is FGF-dependent and that this protein works as positive modulator of FGF signaling (Bottcher *et al.*, 2004). XFLRT3 binds to FGFR1 and FGFR4a and its intracellular carboxy-terminal region is involved in Ras-MAPK activation (Bottcher *et al.*, 2004).

Sef, named for “similar expression to *FGF*s”, encodes a single-pass transmembrane protein that negatively modulates FGF signaling. Similarly to the rest of FGF modulators described above, its expression is FGF-dependent. Overexpression of *sef* in zebrafish results in a ventralized phenotype (Tsang *et al.*, 2002), similarly to what occurred after *mkp3* and *spry4* overexpression (Furthauer *et al.*, 2001; Tsang *et al.*, 2004), and contrary to the occurred after *fgf8* overexpression (Furthauer *et al.*, 1997). Mechanisms of Sef action are not clearly understood. Some reports propose that Sef acts at the level of MEK1 to inhibit the Ras-MAPK pathway (Furthauer *et al.*, 2002; Preger *et al.*, 2004; Yang *et al.*, 2003). However, other groups have shown that Sef associates to FGFR1 and FGFR2 and that the intracellular domain of Sef can prevent tyrosine phosphorylation of the FGFR, thus acting upstream of all the FGF-activated intracellular pathways (Kovalenko *et al.*, 2003; Kovalenko *et al.*, 2006; Tsang *et al.*, 2002).

5.5 Transcription factors downstream FGF signaling

Signaling pathways are usually associated with specific transcription factor families that mediate the response to those signals in all cell types. For example, Smads for BMP, LEF/TCF proteins for WNT or Gli proteins for Hedgehog signaling. In contrast, no comparable FGF-specific transcriptional mediators have been identified. In fact,

transcriptional mediators of FGF signaling are proteins from the Ets, AP-1 and CREB families that can be targeted by a number of other signaling pathways.

The AP-1 transcription factors are targets of MAPKs (reviewed in Lee and McCubrey, 2002). This family includes the classical oncogenes c-Jun and c-Fos. These genes are involved in the FGF-dependent induction of *Xbra* and mesoderm formation in frog embryos (Kim *et al.*, 1998). Both AP-1 and CREB proteins are involved in lens development being potentially regulated by FGF signals (reviewed in Cvekl and Duncan, 2007).

The Ets family is the most widely related to FGF-dependent developmental processes, specifically, the Pea3 subfamily. Ets factors are direct downstream targets for the MAPKs. This group of transcription factors is defined by having a conserved winged helix-turn-helix DNA binding motif, the Ets motif (Sharrocks, 2001; Wasylyk *et al.*, 1993; Wasylyk *et al.*, 1998). The Pea3 subfamily of Ets factors comprises *Pea3*, *Erm* and *Er81*. These genes show high homology in its Ets DNA-binding domain and in an acidic domain that is involved in transactivation properties (de Launoit *et al.*, 1997). Analysis of the expression of the Pea3 subfamily members in mouse, zebrafish, frog and chick early development revealed that they are expressed in FGF signaling areas (Chotteau-Lelievre *et al.*, 2001; Lunn *et al.*, 2007; Munchberg and Steinbeisser, 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001 and the present work). Location and intensity of expression of the Pea3 subfamily members not always coincide suggesting that they differ in part of their regulation mechanism (Lunn *et al.*, 2007; Roussigne and Blader, 2006). For example, in HH8 chick embryos while *Pea3* expression in the neural plate is restricted to the anterior forebrain and the caudal hindbrain, *Erm* is expressed throughout the neural axis from the forebrain to the hindbrain, and *Er81* is absent (Lunn *et al.*, 2007). Pea3 subfamily members are FGF-dependent. In zebrafish, the only Pea3 identified members, *pea3* and *erm* are regulated by *fgf8* and *fgf3* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). In *ace* mutants or SU5402 treated embryos both *pea3* and *erm* are downregulated (Roehl and Nusslein-Volhard, 2001).

AIMS OF THE PRESENT WORK

Hindbrain patterning during early embryonic development is a complex and gradual process that involves the interaction between signaling molecules and several transcription factors. Although several studies revealed many of these interactions we are still far from having a complete picture. The present work has been focused in studying the role of the transcription factor *vHnf1* and its interplay with FGF signaling in hindbrain patterning. We chose *vHnf1* for our studies because its hypomorphic mutants in zebrafish suggested a very early role for this gene in caudal hindbrain patterning. The role of *vHnf1* resulted to be tightly linked with the FGF signaling. In this way, our research in *vHnf1* led us to investigate the involvement of FGF signaling and its downstream pathways in mediating *vHnf1* functions in the hindbrain. As commented in the introduction FGF signaling is involved in several patterning processes during embryogenesis. FGF signaling mediates its function by activating different downstream intracellular pathways. The roles attributed to those downstream pathways differ between the processes analyzed and, in some cases, contradictory models are proposed. In this context, we were interested in identifying which of these downstream pathways were involved in the specification of caudal hindbrain.

Functional analysis of *vHnf1* in mice has been precluded because *vHnf1* null mutant dies before implantation due to abnormal extraembryonic visceral endoderm formation. Therefore, we selected another higher vertebrate for our functional analyses, the chick embryo. Despite of subtle differences in gene regulation, the networks that govern the early embryonic patterning are highly conserved in vertebrates. The chick model is very versatile and admits different kind of experimental manipulations *in vivo* and *in vitro*. Quick functional approaches can be undertaken by the mean of different techniques and their combination, such as electroporation *in ovo*, bead implantation or explant culturing *in vitro*.

The specific aims of the present work were:

1) To analyze the expression profile of *vHnf1* during early stages of the chick neural tube development using mRNA *in situ* hybridization (ISH) technique.

2) To explore the effects of *vHnf1* missexpression in the hindbrain patterning by the means of electroporation *in ovo*.

3) To study the involvement of FGF signaling in mediating *vHnf1* functions in the hindbrain combining gain- and loss-of-function techniques.

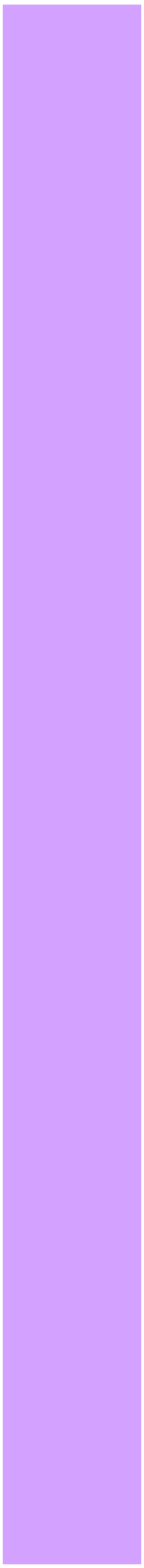
4) To analyze the kinetics of induction of *Fgf3* in the hindbrain upon *vHnf1* overexpression using RT-PCR semiquantitative technique.

5) To analyze the activity profiles of the Ras-ERK1/2 and PI3K-Akt pathways during hindbrain patterning using western blot and immunodetection *in toto*.

6) To check for readouts of FGF activity in the caudal hindbrain by exploring the expression profile of members of the FGF synexpression group using the mRNA ISH technique.

7) To determine which intracellular pathways downstream of FGF signaling are involved in the hindbrain patterning using specific chemical inhibitors for these pathways.

RESULTS



1. The caudalizing role of *vHnf1* in the hindbrain

1.1 *vHnf1* is expressed in the hindbrain in a segment-restricted manner

vHnf1 (*variant Hepatocyte nuclear factor 1*) is a transcription factor belonging to the HNF1 family that contains two DNA binding domains: a homeodomain and a POU domain. Sun and Hopkins (2001) analyzed the phenotype of zebrafish hypomorfic mutants for *vHnf1* and showed that the caudal hindbrain was misspecified and the otic vesicles were smaller than in wild type embryos. They described as well that *vHnf1* was transiently expressed in the caudal neural tube.

We extended these observations by undertaking a detailed analysis of *vHnf1* expression during early chick neurulation. *In situ* hybridization (ISH) experiments revealed a dynamic expression pattern of *vHnf1. vHnf1* was already expressed at the end of gastrulation, 0-1ss (HH7), in the posterior neural plate with a sharp anterior border of expression (Fig. 27a). During early hindbrain patterning stages, *vHnf1* expression persisted in the caudal most part of the hindbrain until 10-11ss (Fig. 27a-c,g). In those stages, *vHnf1* expression was always restricted to the neuroepithelium being absent in the notochord and floor plate (see transverse sections in Fig. 27d-f). Later, it was expressed in the intermediate mesoderm (Fig. 27h, arrowhead), and expression in the neuroepithelium ceased.

The sharp anterior limit of *vHnf1* expression suggested a segment-restricted expression profile. To accurately determine the position of the rostral limit of *vHnf1* expression, we performed double ISH with different hindbrain markers. First, we used probes for *vHnf1* and *Wnt8c*, a gene that is conventionally used to identify pre-r4 (Hume and Dodd, 1993). To check whether *Wnt8c* and *vHnf1* expression territories were actually adjacent, we performed single ISH for *Wnt8c* (Fig. 28a,d), and one-color (Fig. 28c,f) and two-color (Fig. 28b,e) double ISH for *Wnt8c* and *vHnf1* using NBT/BCIP (blue) and INT/BCIP (red) staining. No gap was observed between *Wnt8c* and *vHnf1* hindbrain expression domains before 3-4ss (Fig. 28b,c). However, by 5-6ss, the anterior border of expression of *vHnf1* was found to be posterior to *Wnt8c* (Fig. 28e,f). Note the gap that emerged between *Wnt8c* and *vHnf1* expression domains in 5-6ss embryos when compared with 3-4ss embryos (Fig. 28b,c,,e,f). Since *Wnt8c* is not expressed in r4 beyond 10ss (Hume and Dodd, 1993), and *Hoxb1* is not yet singularized in r4 at this stage (Studer *et al.*, 1994), it

was not possible to follow the precise correspondence between *vHnf1* expression and the posterior boundary of r4 at later stages. These results are consistent with the observations in zebrafish, where *vHnf1* is also expressed in the caudal neural plate at early somitic stages, with a rostral limit that lies at the prospective r4/r5 boundary (Lecaudey *et al.*, 2004).

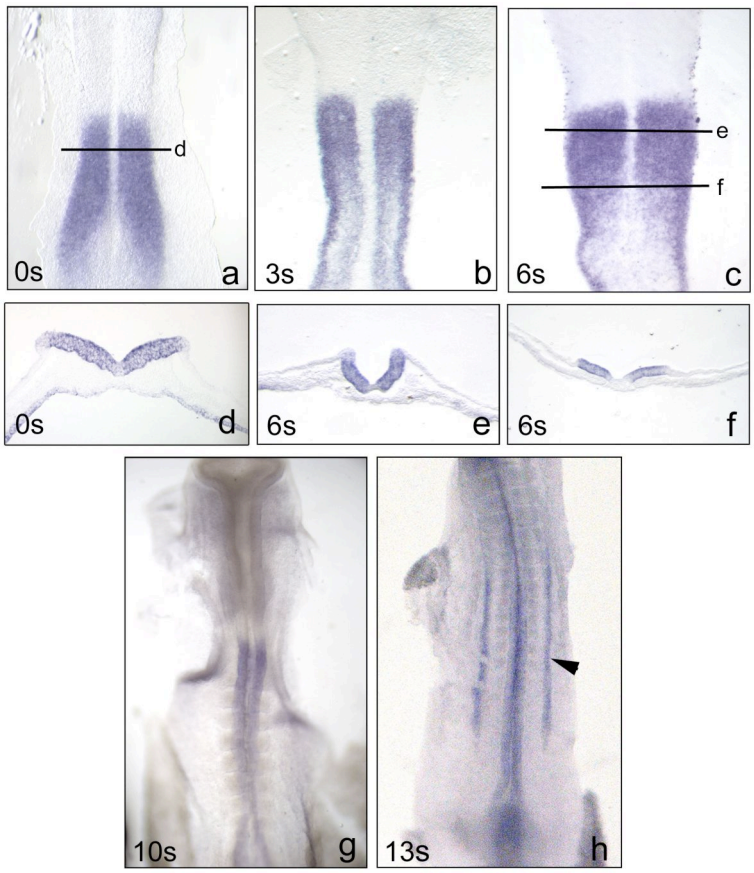


Figure 27. *vHnf1* is early expressed in the neuroepithelium. ISH for *cvHnf1* in embryos from 0 to 13ss. (**a-c**) flat-mounts. (**g,h**) whole-mounts. Anterior is to the top. (**d-f**) transverse sections from (**a**) and (**c**). Arrowhead in (**h**) points to expression in the intermediate mesoderm.

Further insight into the spatial regulation of *vHnf1* in r5 was obtained by comparing *vHnf1* and *Krox20* (Fig. 28g,h). *Krox20* is expressed at 4-5ss in pre-r3, and by 7ss a second more caudal band of sparse cells activate *Krox20* expression in r5 (Giudicelli *et al.*, 2001). We performed two-color double ISH with *Krox20* and *vHnf1* genes. At 5-6ss, *Krox20* expression was only detectable in r3 (Fig. 28g). By 10ss, as *Krox20* expanded in

r5, *vHnf1* expression decreased in this rhombomere (Fig. 28h), suggesting that *vHnf1* regressed from r5 as described in zebrafish (Lecaudey *et al.*, 2004).

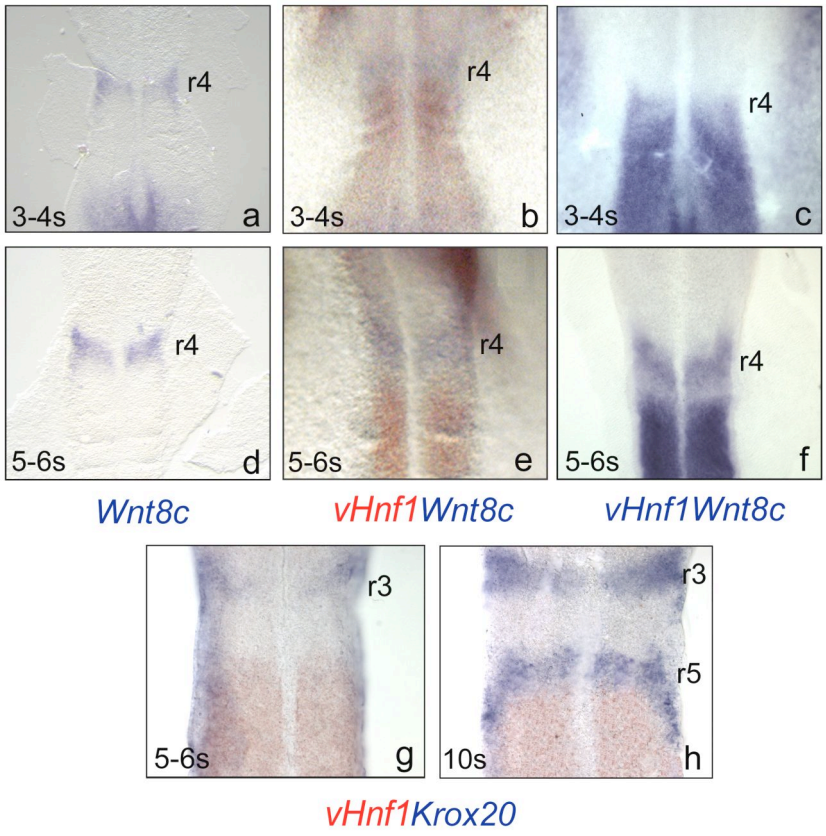


Figure 28. *vHnf1* is expressed in the caudal hindbrain up to the r4/5 boundary. (**a,d**) ISH for *Wnt8c*. (**b,e**) double ISH for *Wnt8c* (blue) and *vHnf1* (red). (**e,f**) double ISH for *Wnt8c* and *vHnf1* (both in blue). (**g,h**) double ISH for *Krox20* (blue) and *vHnf1* (red). Images shown are flat-mounted embryos in (**a-e**) and flat-mounted hindbrains in (**f-h**). Anterior is to the top.

In summary, *vHnf1* was expressed in the prospective hindbrain at early neurula stages, with an anterior limit of expression lying at the presumptive r4/r5 boundary. However, by the 9-10ss *vHnf1* expression regressed caudally and disappeared in r5 coincident with the onset of *Krox20* expression in r5, suggesting that the action of *vHnf1* on r5-cells may be transient and stage-specific.

1.2 Ectopic expression of *vHnf1* in the hindbrain leads to acquisition of caudal identity by rostral rhombomeric territories

In order to study the role of *vHnf1* in hindbrain patterning we performed gain-of-function studies. For this purpose we constructed a series of expression vectors that allowed the expression of a bicistronic mRNA under the control of the β-actin promoter. In the expression vector pIRES2-*vHnf1-GFP*, the first cistron encoded the mouse *vHnf1* cDNA and the second the Green Fluorescent Protein (GFP); an internal ribosome entry site (IRES) separated the two cistrons. Controls were performed using the pIRES2-GFP vector or a vector containing a form of *vHnf1* with a Q136E substitution in the POU-specific domain that completely impedes DNA-binding (Barbacci *et al.*, 1999). Embryos were electroporated just before the formation of the rhombomeres at 3-4ss (HH8) and analyzed after 16-22 hr, at approximately 16ss (HH12). Before analysis, electroporated embryos were screened for GFP expression under a fluorescence microscope. Since *vHnf1* is expressed in the caudal hindbrain up to the presumptive r4/r5 boundary, we assessed the consequences of *vHnf1* misexpression in more anterior rhombomeres. *Krox20* is necessary for the establishment and maintenance of r3 and r5 (Schneider-Maunoury *et al.*, 1993; Schneider-Maunoury *et al.*, 1997; Swiatek and Gridley, 1993) and is a direct regulator of *Hoxa3* and *Hoxb3* in r5 (Manzanares *et al.*, 2002; Manzanares *et al.*, 2001). Electroporation of the *vHnf1* expression construct induced the appearance of *Krox20*-positive cell patches in r4 (Fig. 29d-f, n=15/23, see arrowhead in f). Neither the vector alone nor the mutated *vHnf1* construct had any effect on *Krox20* expression (Fig. 29a-c, n=10). Surprisingly, *vHnf1*-electroporated embryos exhibited cell patches that did not express *Krox20* in r3 and r5 (Fig. 29d-f, see arrow in f), where it is normally expressed. We performed double ISH with *mvHnf1* and *Krox20* to elucidate whether the *Krox20*-negative patches were indeed expressing *mvHnf1*. As shown in Figure 29f, many *mvHnf1*-positive cells in r3 and r5 (Fig. 29f in red) did not express *Krox20* (Fig. 29f in blue, n = 6/6, see arrow). On the other hand, some of the ectopic *Krox20*-positive cell patches in r4 were negative for *vHnf1* (Fig. 29f, see arrowhead). Thus, misexpression of *vHnf1* resulted in: i) an ectopic cell- and non-cell-autonomous *Krox20* expression in r4, and ii) cell-autonomous downregulation of *Krox20* expression in r3 and r5. These results suggest a dual role of *vHnf1* in regulating *Krox20* expression.

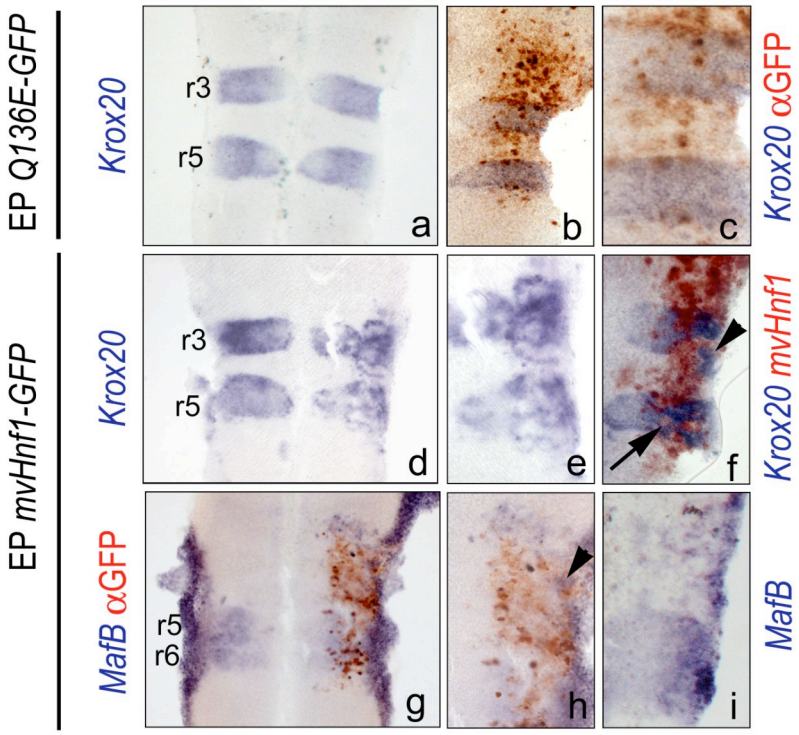


Figure 29. ***vHnf1*** **overexpression induces expression of caudal rhombomeric genes in anterior territories.** Embryos were electroporated with *mvHnf1* (**d-i**) or the control construct *mvHnf1-Q136E* (**a-c**) and analyzed by ISH for *cKrox20* (**a-f**) or *cMafB* (**g-h**). In (**b**), (**c**), (**g**) and (**h**) GFP was immunodetected and shown in red. (**f**) shows a double ISH with *cKrox20* in blue and *mvHnf1* in red. Arrowhead in (**f**) points to *cKrox20* non-cell-autonomous induction and arrow to cell-autonomous repression. Arrowhead in (**h**) points to non-cell-autonomous induction of *cMafB*. All pictures show flat-mounted electroporated hindbrains. Anterior is to the top and the electroporated side is the right one.

MafB is normally expressed in the caudal hindbrain from 5-6ss, in prospective r5 and r6, and it is known to be involved in caudal hindbrain segmentation and in the specification of AP regional identity (Cordes and Barsh, 1994; Eichmann *et al.*, 1997; Giudicelli *et al.*, 2003; Mechta-Grigoriou *et al.*, 2003). Missexpression of *vHnf1* caused a rostral expansion of *MafB* expression (Fig. 29g-i). Distinct *MafB*-positive patches were always observed within r3 and r4 (Fig. 29g-i, n = 16/20), and it is possible that other ectopic patches with low levels of *MafB* expression were masked by the high levels of electroporated *mvHnf1*. Ectopic *MafB* expression levels were always equivalent to those of the endogenous gene.

To further explore the disruption of rhombomere identity caused by *mvHnf1* misexpression, we analyzed the expression of *Hoxb1*, which is a major determinant of r4

identity (Studer *et al.*, 1994). At 16ss *Hoxb1* is evenly expressed at high levels in r4, as well as at lower levels in the spinal cord up to r7/r8 (Sundin and Eichele, 1990). *vHnf1* electroporation resulted in downregulation of *Hoxb1* in r4, as revealed by the appearance of patches of cells in r4 that did not express *Hoxb1* (Fig. 30a, n = 6/7), or more severe and homogeneous downregulation of *Hoxb1* expression (Fig. 30b,c). Importantly, *Hoxb1* was not repressed in all the cells that ectopically expressed *mvHnf1* (Fig. 30b,c, see arrowheads) and the extent of *Hoxb1* repression was much more restricted than the *mvHnf1*-electroporated region. These observations suggest that in chick *Hoxb1* is not directly repressed by *vHnf1* as proposed in zebrafish (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). *Hoxa3* is normally expressed in the caudal hindbrain up to the r4/r5 boundary (Grapin-Botton *et al.*, 1995). As shown in Figure 30d, *Hoxa3* expression was not significantly altered following misexpression of *vHnf1* (n = 8).

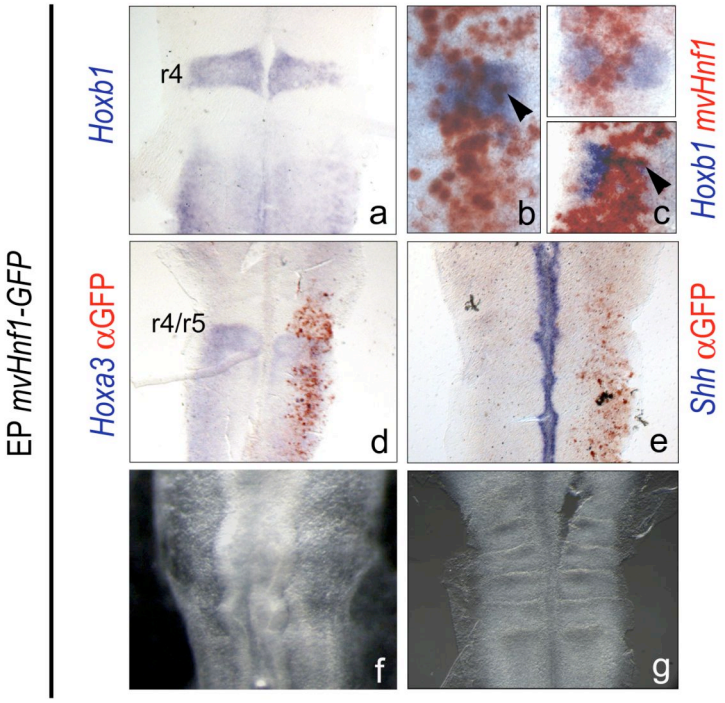


Figure 30. *vHnf1* overexpression downregulates *Hoxb1* whereas does not affect *Hoxa3* expression and does not disturb hindbrain segmentation. Embryos were electroporated with *mvHnf1* (**a-e**) and analyzed for *Hoxb1* (**a-c**), *Hoxa3* (**d**) or *Shh* (**e**). (**b**) and (**c**) show double ISH were *mvHnf1* was developed in red and *Hoxb1* in blue. Arrowheads point to cells that are positive for both *mvHnf1* and *Hoxb1*. In (**d**) and (**e**) GFP and therefore *mvHnf1* was immunodetected and shown in red. (**f,g**) Nomarsky analysis of whole-mounted (**f**) or flat-mounted (**p**) embryos after *mvHnf1* overexpression. In all cases anterior is to the top and electroporated side is the right one.

To assess whether the above changes in rhombomeric molecular markers were followed by disruption of the morphological segmentation process, we analyzed a marker that let us visualize morphological rhombomeric landmarks. ISH for *Shh* labels the floor plate and allows visualization of the rhombomeric swellings in the ventral part of the neural tube (Echelard *et al*., 1993). As shown in Figure 30e, no evident morphological effects were observed in response to *vHnf1* misexpression. Nomarski analysis of whole mount and flat-mounted hindbrains electroprated with *vHnf1* showed well-defined morphological rhombomeric boundaries as well (Fig. 30f,g).

In summary, ectopic expression of *vHnf1* leads to changes in rhombomere identity, which is reflected by the ectopic activation of *MafB* and *Krox20* in more anterior regions, and to the repression of *Hoxb1* in r4. It is worth noting that the effects described were observed when electroporation was performed between 3-4ss and 7ss. When electroporation was done after this period, no alteration in the expression of hindbrain genes was observed. This suggests a precise temporal window for *vHnf1* function, a possibility consistent with its fleeting expression in r5.

1.3 *vHnf1* operates in a non-cell-autonomous way

A common characteristic of ectopic inductions following overexpression of *vHnf1* was their occurrence in patches of cells. Because the electroporation is expected to hit isolated cells, the existence of such patches may be explained by clonal expansion of a single electroporated cell, by non-cell-autonomous modifications of gene expression around the transfected cells, or by cell movements. To address this issue more directly, we performed double-labeling experiments to detect both exogenous *vHnf1* and target genes. GFP or *mvHnf1* were detected with an anti-GFP antibody or *mvHnf1* riboprobe respectively, both developed in red, and *MafB* or *Krox20* were detected with a riboprobe developed with NBT/BCIP (blue). Double-labeling experiments in *vHnf1*-electroporated embryos indicated that the GFP (and therefore mouse vHNF1 protein) was not present in all cells ectopically expressing *MafB* or *Krox20*. GFP or *mvHnf1* were always present in cells that were within or bordering *Krox20* or *MafB* positive patches (see arrowheads in Fig. 29f,h). This reinforces the notion that ectopically expressed *vHnf1* could induce gene expression in both a cell- and non-cell-autonomous manner.

A possible explanation for these results is that ectopic *vHnf1* promotes its own expression due to an autoregulatory loop. To address this question, we overexpressed *mvHnf1* in 2-3ss embryos and performed double ISH with the chicken *vHnf1* probe in blue and the mouse *vHnf1* probe in red. As observed in Figure 31a, cells expressing the exogenous *mvHnf1* gene in the hindbrain did not activate the endogenous gene in that region (n = 5). The intermediate mesoderm that normally expresses *vHnf1* was a positive control for endogenous expression (data not shown). *vHnf1* expression does not behave in an autoregulatory loop and the explanation for the non-cell-autonomous effects must be found in other mechanisms.

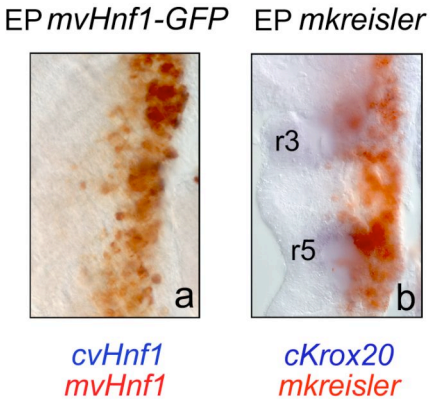


Figure 31. *vHnf1* cannot induce its own expression and *mKreisler* is not sufficient to induce *Krox20* expression. (**a**) Embryos were electroporated with *mvHnf1* and analyzed by double ISH for *cvHnf1* in blue and *mvHnf1* in red. (**b**) Embryos were electroporated with *mKreisler* and analyzed by double ISH for *cKrox20* in blue and *mKreisler* in red. All pictures show the electroporated halves of flat-mounted neural tubes where anterior is to the top.

In *kreisler* mouse and zebrafish *val* mutants, *Krox20* expression in r5 is absent (McKay *et al.*, 1994; Moens *et al.*, 1996), suggesting that *Krox20* is downstream of *Kreisler/val/MafB*. To assess whether the ectopic expression of *Krox20* after *vHnf1* misexpression was under the control of ectopic *MafB, mKreisler/MafB* was overexpressed and *Krox20* expression analyzed. Double ISH experiments showed that ectopic expression of *mkreisler* did not activate *Krox20* expression (Fig. 31b, n = 9), (Giudicelli *et al.*, 2003). This could mean that *Krox20* expression in the chick is controlled by *vHnf1* independently of *Kreisler/MafB* or maybe that *MafB* is necessary but not sufficient for *Krox20* expression as it is proposed in zebrafish (Wiellette and Sive, 2003).

Similarly, misexpression of *mKrox20* did not cause ectopic expression of *MafB* (Giudicelli *et al.*, 2001).

2. FGF signaling mediates the effects of *vHnf1* in the hindbrain

Since *vHnf1* does not work in autoregulatory loops it is possible to think that some kind of cell-to-cell signal is involved in mediating the non-cell-autonomous effects of *vHnf1*. In this section we propose FGF signaling as one of the mediators of *vHnf1* function in the caudal hindbrain.

2.1 *vHnf1* upregulates *Fgf3* expression throughout the hindbrain

Previous reports have addressed the importance of *fgf3* and *fgf8*, expressed in r4, in organizing the hindbrain in zebrafish (Maves *et al.*, 2002; Roy and Sagerstrom, 2004; Walshe *et al.*, 2002). In fact, two studies have proposed that *vhnf1* and FGFs from r4 synergize in regulating *val/MafB* and *krox20* expressions (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). This prompted us to examine whether FGF signaling could mediate the ectopic activation of *MafB* and *Krox20* upon *vHnf1* overexpression. In chick, the only FGFs expressed in the hindbrain at early stages are *Fgf3* (Karabagli *et al.*, 2002; Mahmood *et al.*, 1995) and, very weakly, *Fgf4* (Shamim and Mason, 1999). *Fgf19* is expressed in the hindbrain at later stages and in a very transient manner (Ladher *et al.*, 2000). Thus, we analyzed the expression of *Fgf3* and *Fgf4* upon overexpression of *mvHnf1*.

In the chick, *Fgf3* is normally expressed in the prospective hindbrain from late streak stage. By HH9 it is restricted to r4 and r5 and expands to r6 during subsequent stages (from HH9^{+} to HH11), later on becoming confined to rhombomere boundaries (HH12-13) (Mahmood *et al.*, 1995). Misexpression of *vHnf1* anterior to the r4/r5 boundary led to a widespread upregulation of *Fgf3* anterior to r4, up to the r1/r2 boundary (Fig. 32a-c). *Fgf3* expression became homogeneous within the electroporated rhombomeres (Fig. 32a-c, n = 18/21), its characteristic boundary-restricted pattern at that stage being masked by the high levels of ectopic *Fgf3* expression. Electroporation of the vector alone or the mutated form of *mvHnf1* did not have any effect on *Fgf3* expression (Fig. 32d). Similarly to the observed in *Krox20* and *MafB* ectopic inductions, activation of *Fgf3* upon *mvHnf1*

overexpression only occurred when electroporation was performed between 3 and 7ss. After this period it was not possible to ectopically induce *Fgf3*, suggesting a narrow time window for this induction.

We also analyzed the expression of *Fgf3* after *mvHnf1* overexpression by combination of ISH for *Fgf3* and immunodetection of GFP. As shown for *Krox20* and *MafB*, exogenous vHNF1 was always detected in cells that were either within or/and surrounding the *Fgf3*-positive domains (see arrow in Fig. 32c, n = 9/9,), suggesting cell- and non cell-autonomous activation of *Fgf3* upon *mvHnf1* ectopic expression. Experiments using double ISH for *Fgf3* and *mvHnf1* revealed similar results to the ones obtained with anti-GFP.

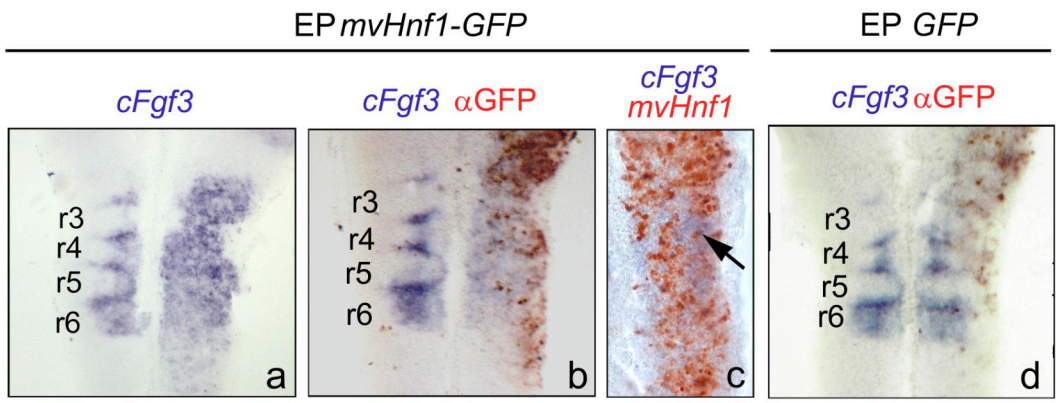


Figure 32. *Fgf3* is induced throughout the hindbrain after *mvHnf1* overexpression. Embryos were electroporated with *mvHnf1* (**a-c**) or *GFP* (**d**) and analyzed for *cFgf3*. In (**b**) and (**d**) GFP was immunodetected and developed in red. In (**c**) double ISH for *cFgf3* (blue) and *mvHnf1* (red) is shown. Arrow in (**c**) points to a *mvHnf1*-expressing cell neighbouring a patch of *cFgf3* ectopic expression.

Thus, misexpression of *vHnf1* resulted in an expansion of *Fgf3* along the hindbrain, with concomitant loss of its boundary-restricted expression pattern. This occurs in a non-cell-autonomus manner and in a specific temporal window. We analyzed as well the possible regulation of *Fgf4* by *vHnf1*. However, *Fgf4* expression was not affected after *mvHnf1* overexpression (data not shown).

2.2 FGF signaling is necessary but not sufficient to regulate caudal rhombomeric markers

In order to know whether FGF signaling was a mediator in the activation of *Krox20* and *MafB* by *vHnf1*, we took advantage of gain- and loss-of-function approaches.

First, to determine whether early hindbrain genes such *vHnf1, Krox20, MafB*, and *Hoxb1* were regulated by FGF signals, we explanted 1-4ss (HH7-8) embryos and cultured them during 6 hours in the presence or absence of SU5402 to block the FGFR activity (Mohammadi *et al*., 1997). A clear inhibition of *MafB* was observed in SU5402-treated explants (Fig. 33a,b, n = 9/9). Similar results were obtained for *Krox20* expression, which was dramatically reduced in response to the inhibition of FGF signaling (Fig. 33c,d, n= 9/9). In contrast, early expression of *Hoxb1* was unaffected (Fig. 33e,f, n = 3). Treatment with SU5402 also had no effects on *vHnf1* expression (Fig. 33g,h, n = 7) indicating that *vHnf1* expression does not depend on FGF signaling. Regarding *Fgf3*, blocking of FGF signaling did not have any effect on its expression (Fig. 33i,j, n = 10), suggesting that *Fgf3* regulation does not require a FGF positive feedback. Reinforcing this idea, gain of function of *mFgf3* in the hindbrain did not activate *cFgf3* expression (Fig. 33k,k', n=5).

Next, we investigated whether ectopic *MafB* and *Krox20* expressions induced by *vHnf1*-misexpression were dependent on FGF signals. Embryos were electroporated with *mvHnf1*, grown *in ovo* for 6 hours, explanted and incubated for another 6 hours with control medium or medium containing SU5402 and analyzed by ISH. In embryos incubated with SU5402, endogenous and ectopic *MafB* and *Krox20* expressions were absent (compare Fig. 34 a,c with Fig. 34b,d). This result demonstrates that FGF signaling mediates the ectopic induction of *MafB* and *Krox20* by *mvHnf1*.

To know whether *Fgf3* was sufficient to induce caudal rhombomeric genes, we overexpressed *mFgf3* in the hindbrain. Neither *MafB* (Fig. 34e, n=8) nor *Krox20* (Fig. 34g, n=5) were induced in the hindbrain upon *mFgf3* electroporation suggesting that *Fgf3* is not sufficient to induce the expression of these genes.

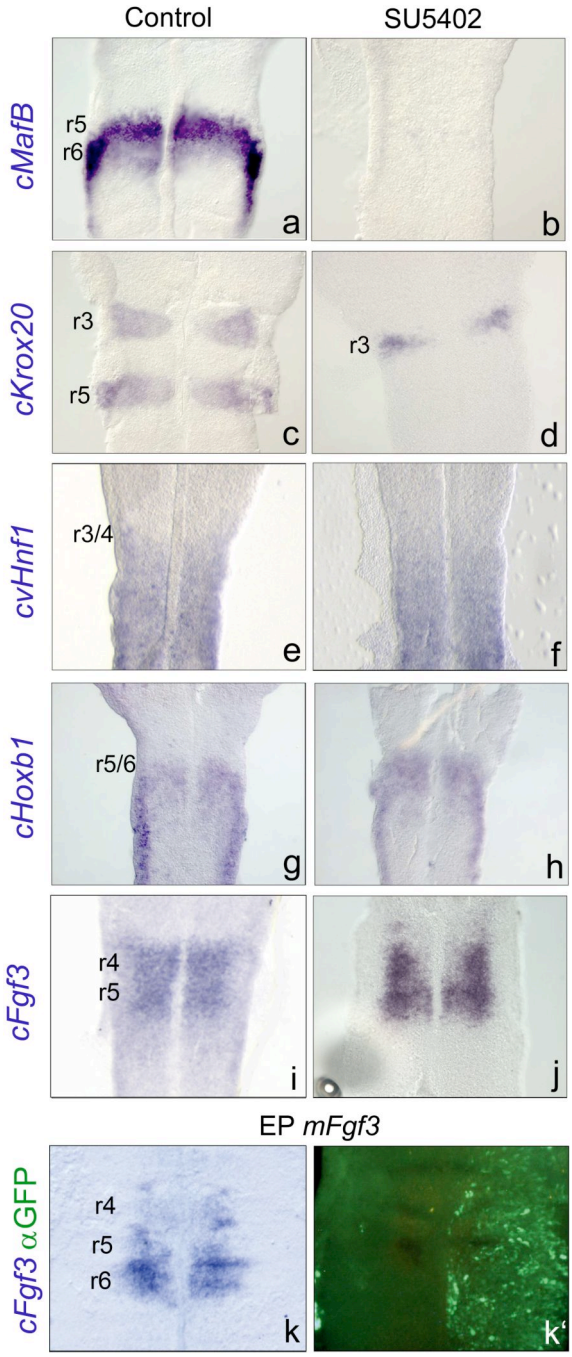


Figure 33. FGF signals are necessary for *Krox20* and *MafB* expression in the hindbrain. In (**a-j**) embryos were placed in culture during 6 hours in control medium or medium supplemented with 50µM SU5402 and analyzed for the expression of *cMafB* (**a,b**), *Krox20* (**c,d**), *cvHnf1* (**e,f**) and *cHoxb1* (**g,h**) and *cFgf3* (**i,j**). In (**k,k'**) Embryos were electroporated with *mFgf3* and analyzed for *cFgf3* expression. Images shown in (**k**) and (**k'**) correspond to the same electroporated embryo. In (**k**) *cFgf3* expression is shown in blue and in (**k'**) immunodetection of GFP and therefore *mFgf3* is shown in green fluorescence. All pictures show flat-mounted hindbrains. Anterior is to the top and electroporated side in (**k,k'**) is the right one.

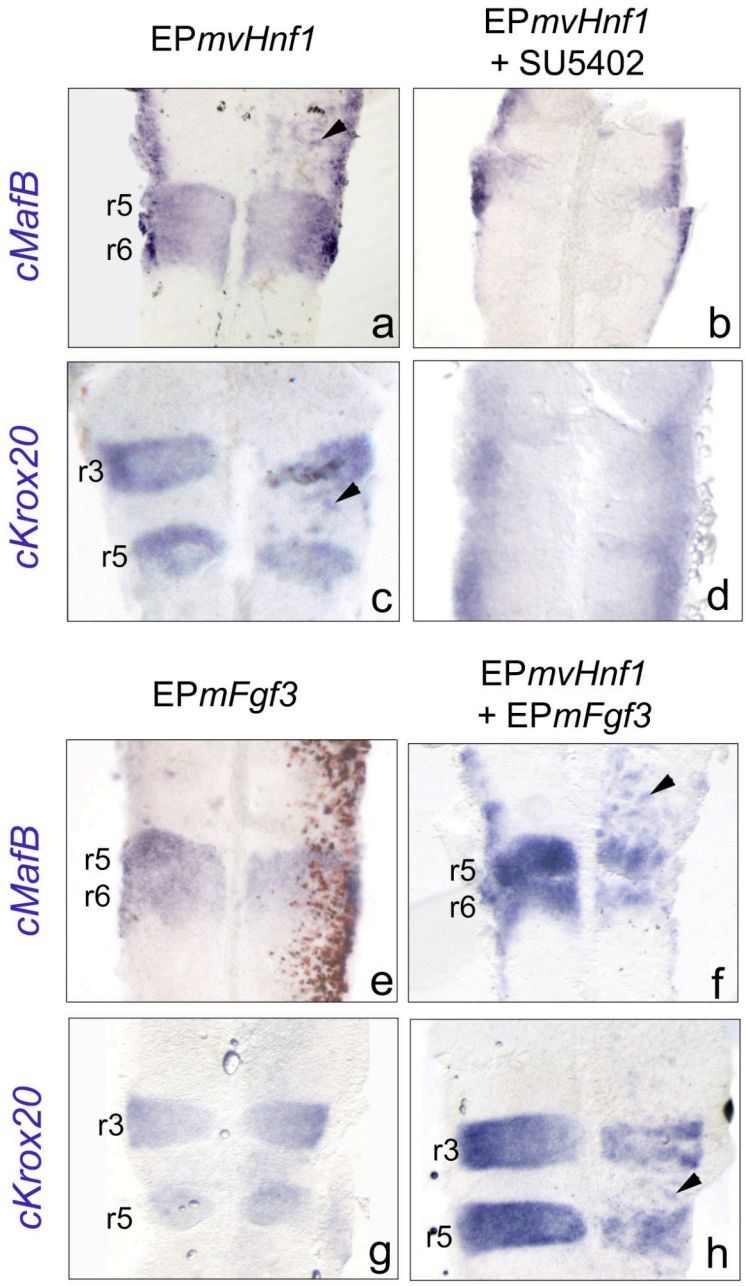


Figure 34. FGF signals are necessary but not sufficient to ectopically upregulate caudal rhombomeric genes. Embryos were electroporated with *mvHnf1* (**a-d**), with *mFgf3* (**e,g**) or with *mvHnf1* and *mFgf3* (**f,h**). Embryos in (**a-d**) were explanted after electroporation and cultured in the presence or absence of SU5402. (**a,b,e,f**) were analyzed for *cMafB* expression, (**c,d,g,h**) were analyzed for *cKrox20* expression. In (**e**) GFP immunodetection is shown in red. Anterior is to the top and electroporated side is the right one.

Finally, we wanted to study whether *Fgf3* overexpression potentiates the effects of *vHnf1* as shown in zebrafish (Hernandez *et al.*, 2004). For that purpose *mvHnf1* was co-electroporated with *mFgf3* in the rostral hindbrain of 3-4ss embryos. When electroporated embryos were analyzed, a similar phenotype to the obtained by overexpression of *vHnf1* alone was observed in terms of *MafB* and *Krox20* ectopic expression, (compare Fig. 34a,c with Fig. 34f,h, see arrowheads). These data suggest that co-expression with *Fgf3* does not substantially potentiate the effects of *vHnf1* overexpression.

Taken together, these results indicate that FGF signaling is necessary but not sufficient for the expression of *Krox20* and *MafB* in the hindbrain, suggesting that *vHnf1* patterns the hindbrain in part by inducing *Fgf3* but also other mechanisms downstream *vHnf1* must be involved.

2.3 *Fgf3* is rapidly induced after *vHnf1* overexpression

The results shown above suggested that *Fgf3* is downstream *vHnf1* in the induction of caudal rhombomeric markers. An important question to solve was to know whether *Fgf3* was a direct downstream target of *vHnf1*. To address this issue we studied the time course of *Fgf3* induction after *mvHnf1* overexpression. We designed a semiquantitative reverse transcription PCR approach (RT-PCR) to determine the kinetics of *Fgf3* induction. Embryos from 3-4ss (HH8) were electroporated with *mvHnf1* and incubated at 38ºC during different time periods (15min, 30min, 1h, 3h, 6h, 9h). After incubation, the hindbrain tissue was isolated and processed for RT-PCR amplification. 15min after electroporation, the RT-PCR revealed a 600bp band corresponding to *mvHnf1* expression at both 25 and 27 cycles of amplification (Fig. 36Aa, and see materials and methods). At the same time point, a weaker 450bp band corresponding to *cFgf3* expression was as well amplified by RT-PCR (Fig. 36Aa). As incubation time increased (30min and 1h), the intensity of the *cFgf3* band was progressively stronger as compared to *mvHnf1* band (Fig. 36Ab,c). From 1h onwards the intensity of the *cFgf3* band was relatively similar for all time points, suggesting that either the reaction reached saturation or that the induction of *cFgf3* was stabilized (Fig. 36Ac-f). Thus, already at 15min upon electroporation the expression of *Fgf3* was increased and by 1h a plateau was reached (Fig. 36B). As control, the *mvHnf1-Q136E* construct was overexpressed and embryos were incubated at 38ºC during 6h (Fig. 36Ag). *Fgf3* relative level of expression was much higher in experimental samples than in the control (Fig. 36C). These results showed a rapid *Fgf3* induction after *mvHnf1* overexpression suggesting a direct transcriptional regulation of *Fgf3* by *vHnf1*.

In summary, *Fgf3* is induced throughout the hindbrain after *mvHnf1* overexpression. This happens very fast after electroporation suggesting that *Fgf3* is a direct transcriptional target of *vHnf1*. In addition to this, FGF signaling is downstream *vHnf1* in regulating the expression of the caudal rhombomeric genes *Krox20* and *MafB*. However, although FGF signaling is necessary it is not sufficient to regulate these genes. This suggests the involvement of other *vHnf1* downstream mechanisms in these regulations. Finally, *vHnf1* operates in a specific time-window and is not able to induce *Fgf3*, *MafB* or *Krox20* at late stages of hindbrain patterning.

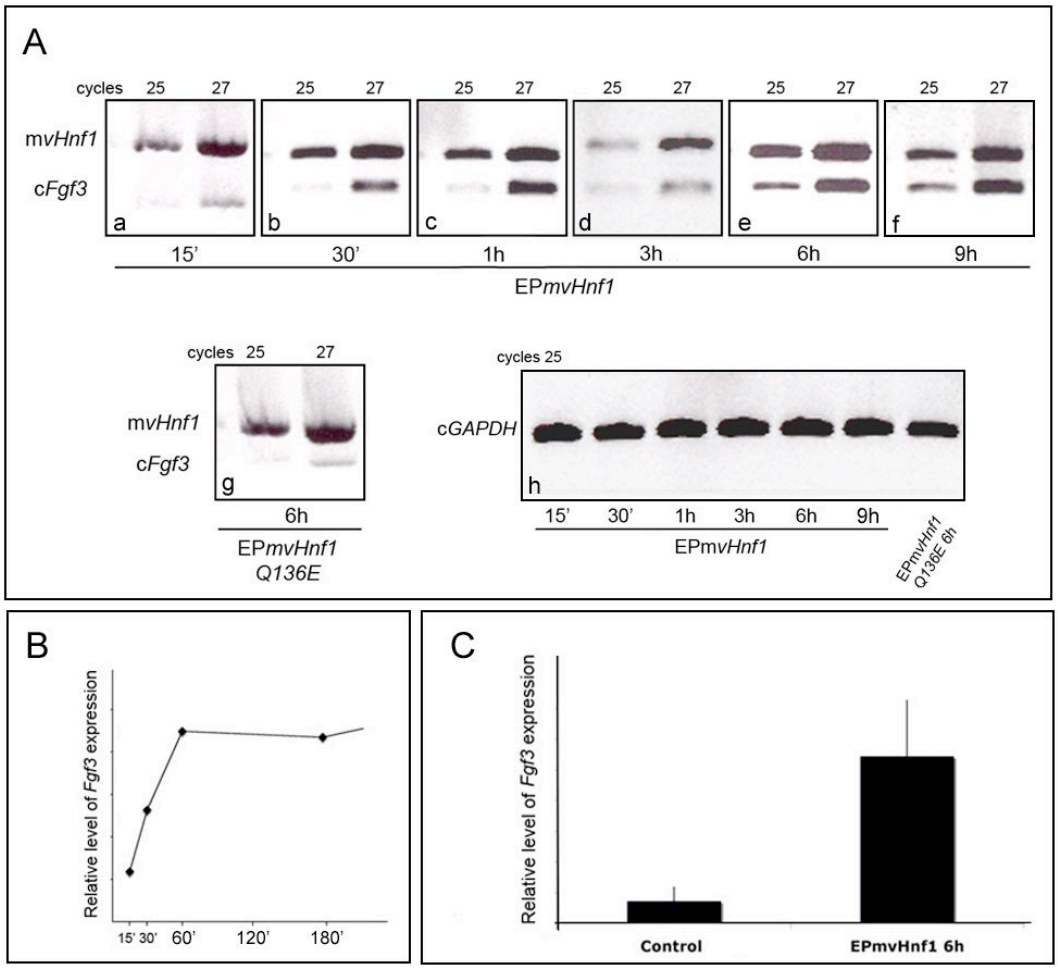


Figure 35. *Fgf3* is rapidly induced after *mvHnf1* overexpression. Embryos were electroporated with *mvHnf1-GFP* construct and incubated during different time periods. After the desired incubation period the hindbrain tissue was processed for total RNA extraction and one-step RT-PCR amplification for *cFgf3* and *mvHnf1*. (**A**)(**a-g**) Amplification of *cFgf3* and *mvHnf1*. Each sample amplification was checked after 25 and 27 cycles. Samples correspond to 15min (**a**), 30min (**b**), 1h (**c**), 3h (**d**), 6h (**e**). 9h (**f**) after *mvHnf1* overexpression and 6h after *mvHnf1-Q136E* (control) overexpression (**g**). Amplification of *GAPDH* was run in parallel to normalize sample values (**h**). (**B**) Plot of *cFgf3* expression normalized with *mvHnf1* at each time point. (**C**) Comparison of *Fgf3* relative level of expression after *mvHnf1* or *mvHnf1-Q136E* (control) overexpression.

3. Analysis of the FGF downstream pathways involved in caudal hindbrain patterning

The FGF signals exert their function by activating different intracellular pathways. Five intracellular pathways are known to be downstream of FGF signaling: the p38, JNK and ERK1/2 Ras-MAPK cascades, the PI3K-Akt pathway and the PLC_{γ} pathway. Among

them, Ras-ERK1/2 MAPK and, to a lesser extent, PI3k-Akt are those that have mostly been related to embryonic processes in different tissues and models (Echevarria *et al.*, 2005; Kobayashi *et al.*, 2007; McCabe *et al.*, 2006; Tsang *et al.*, 2004). The experiments that follow were designed to study the intracellular network that is activated downstream *Fgf3* in the caudal hindbrain. For that purpose we analyzed the activities of the PI3K-Akt and Ras-ERK1/2 pathways as well as the expression of FGF activity readouts in relation to *Fgf3* expression in the caudal hindbrain.

3.1 Ras-ERK1/2 MAPK and PI3K-Akt pathways are active in the caudal hindbrain

In order to analyze whether Ras-ERK1/2 and PI3K-Akt pathways were active during hindbrain patterning we analyzed the activated forms of their effectors within the hindbrain. We isolated caudal hindbrain tissue form HH8-HH9 embryos and performed western blot analysis of the Akt and ERK1/2 phosphorilated forms (pAkt and pERK1/2). Akt has an apparent molecular weight of 60kD. When antibodies against either the total or the phosphorilated forms of Akt were used in extracts of HH9 hindbrains, a 60kD band was obtained by western blot (Fig. 36), suggesting that the PI3K-Akt pathway is active at the stage when hindbrain patterning takes place. ERK1 and ERK2 proteins have apparent molecular weights of 44 and 42kD respectively. Western blot analysis using either the total or the phosphorilated forms of ERK1/2 revealed that Ras-ERK1/2 pathway is also active during hindbrain patterning (Fig. 36). As positive controls for ERK1/2 and Akt activation protein extractions of whole embryos were used (Fig. 36).

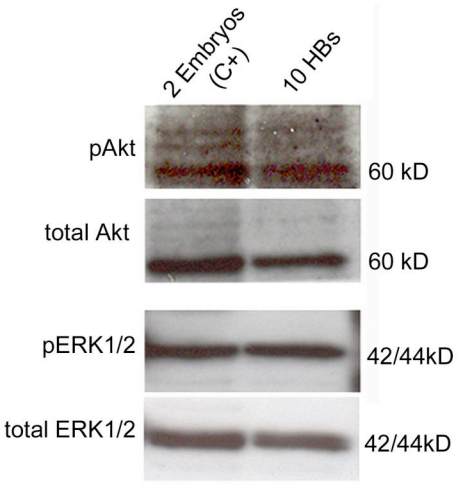


Figure 36. Phosphorilated forms of Akt and ERK are present in the caudal hindbrain. Two HH10 whole embryos and ten HH10 hindbrains were assayed by western blot for both the phosphorilated and the total forms of Akt and ERK protein kinases.

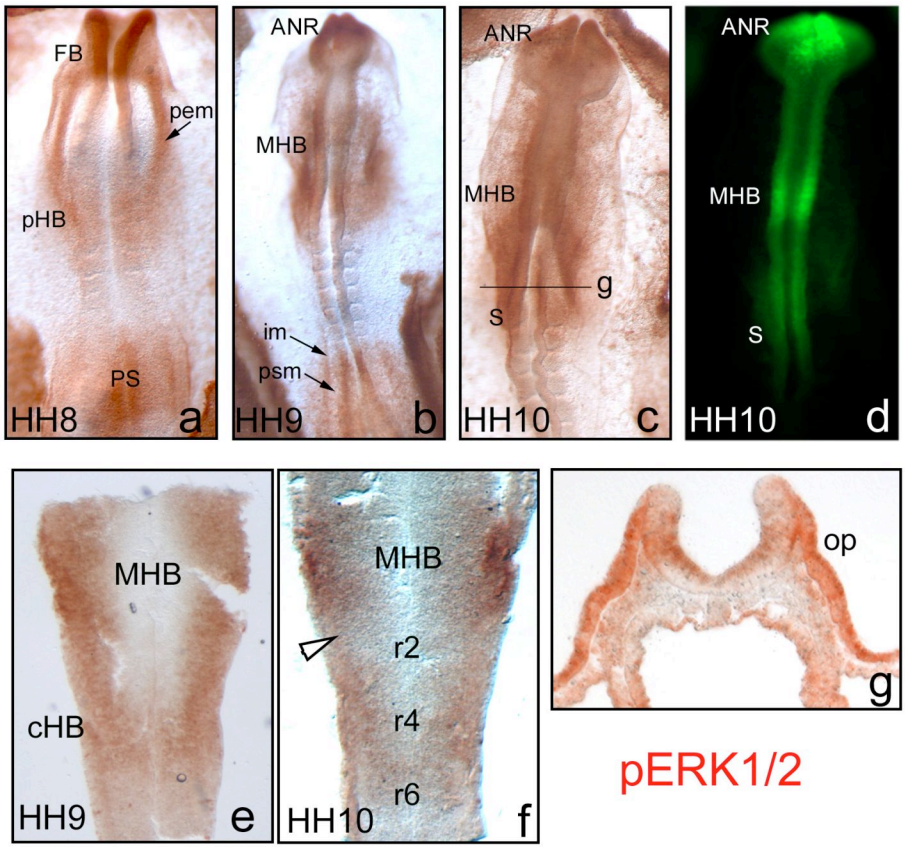


Figure 37. pERK1/2 is localized in the caudal hindbrain during its patterning. pERK1/2 immunodetection is shown in red or green fluorescence in whole-mount embryos (**a-d**) or flat-mounted hindbrains (**e,f**) from HH8 to HH10. White arrowhead in (**f**) points to gap in pERK1/2 immunostaining. In (**g**) transverse section from (**c**) is shown. ANR, anterior neural ridge; cHB, caudal hindbrain; FB, forebrain; HB, hindbrain; im, intermediate mesoderm; MHB, midbrain-hindbrain boundary; op, otic placode; pem, precardiac endo and mesoderm; pHB, prospective hindbrain; psm, presomitic mesoderm; r, rhombomere; s, somite.

Once demonstrated that MAPK and Akt pathways were active in the caudal hindbrain, we wanted to analyze the spatial distribution of these activities. With this purpose we performed whole-mount immunodetection of pERK1/2 at HH8-HH10 stages. At these stages pERK1/2 was detected in embryonic areas that are within or close to FGF sources: the forebrain, midbrain and hindbrain, the precardiac endo and mesoderm, the primitive strike, the caudal neural plate, and the presomitic and intermediate mesoderm (Fig. 37a-c). Between HH8 and HH10 *Fgf3* is expressed in a domain corresponding to r4 and r5 (Fig. 38c,d) (Mahmood *et al.*, 1995). Coincident with this expression domain, at 4ss (HH8) the Ras-ERK1/2 pathway was active in the prospective hindbrain (Fig. 37a). By HH9 pERK1/2 persisted in the caudal hindbrain and was extended to the rostral hindbrain, the MHB and the caudal midbrain (Fig. 37b). Flat-mounted hindbrain in Figure

37e shows that pERK1/2 was excluded from the ventral part of the tube; with the exception of the caudal hindbrain, where it was localized all over the dorsoventral axis. At HH10, Ras-ERK1/2 activity was still maintained in the hindbrain (Fig. 37c,d and flat-mount in f). The strongest activity was localized in the MHB (Fig. 37d,f). A faint gap in pERK1/2 distribution was noticed in the rostral hindbrain immediately posterior to the MHB (Fig. 37f, see white arrow). Transverse sections show that at HH10 Ras-ERK1/2 activity was along the DV axis of the hindbrain (Fig. 37g), with the exception of its most caudal part. The otic placodes and the rest of the non-neural ectoderm also showed strong Ras-ERK1/2 activity. For pAkt it was not possible to analyze its spatial distribution since available antibodies did not work in whole-mount immunostainings.

In summary, we showed that Akt/PI3K and ERK1/2 MAPK pathways are active during hindbrain patterning. pERK1/2 is present at early stages of development in embryonic areas that are within or close to FGF sources, such the forebrain influenced by FGF8/12/13 from the ANR (Karabagli *et al.*, 2002), the midbrain and the hindbrain influenced by FGF8 from the MHB and FGF3 from the caudal hindbrain, the precardiac endo and mesoderm influenced by FGF8 from the endoderm (Alsan and Schultheiss, 2002) and the otic placode under the influence of FGF3 from the hindbrain and FGF19 from the mesoderm (Ladher *et al.*, 2000). These observations are in agreement with recent work by Lunn *et al.* where pERK1/2 distribution is analyzed in early stages of chick development and compared with the expression of FGFRs and members of the FGF synexpression group (Lunn *et al.*, 2007).

3.2 Expression of readouts of FGF activity in the caudal hindbrain

To further investigate the FGF-activated intracellular pathways involved in caudal hindbrain patterning we checked for the expression of genes that could be readouts of FGF activity in the caudal hindbrain. The concept of synexpression group refers to groups of genes that are functionally related and commonly co-expressed (Niehrs and Pollet, 1999). The FGF synexpression group includes FGF factors such as *Fgf8* and *Fgf4*, the FGFRs (*FGFR1-4*), negative modulators of FGF signaling (*MKP3, SPRY2, Sef* and *Spred*), the positive modulator *FLRT3* and transcription factors such as the members of the Ets-type family *Pea3, Erm* and *Er81*. We have analyzed the expression of some of these genes in the caudal hindbrain between HH7 and HH10 in order to see whether they can be readouts of the FGF activity in the caudal hindbrain.

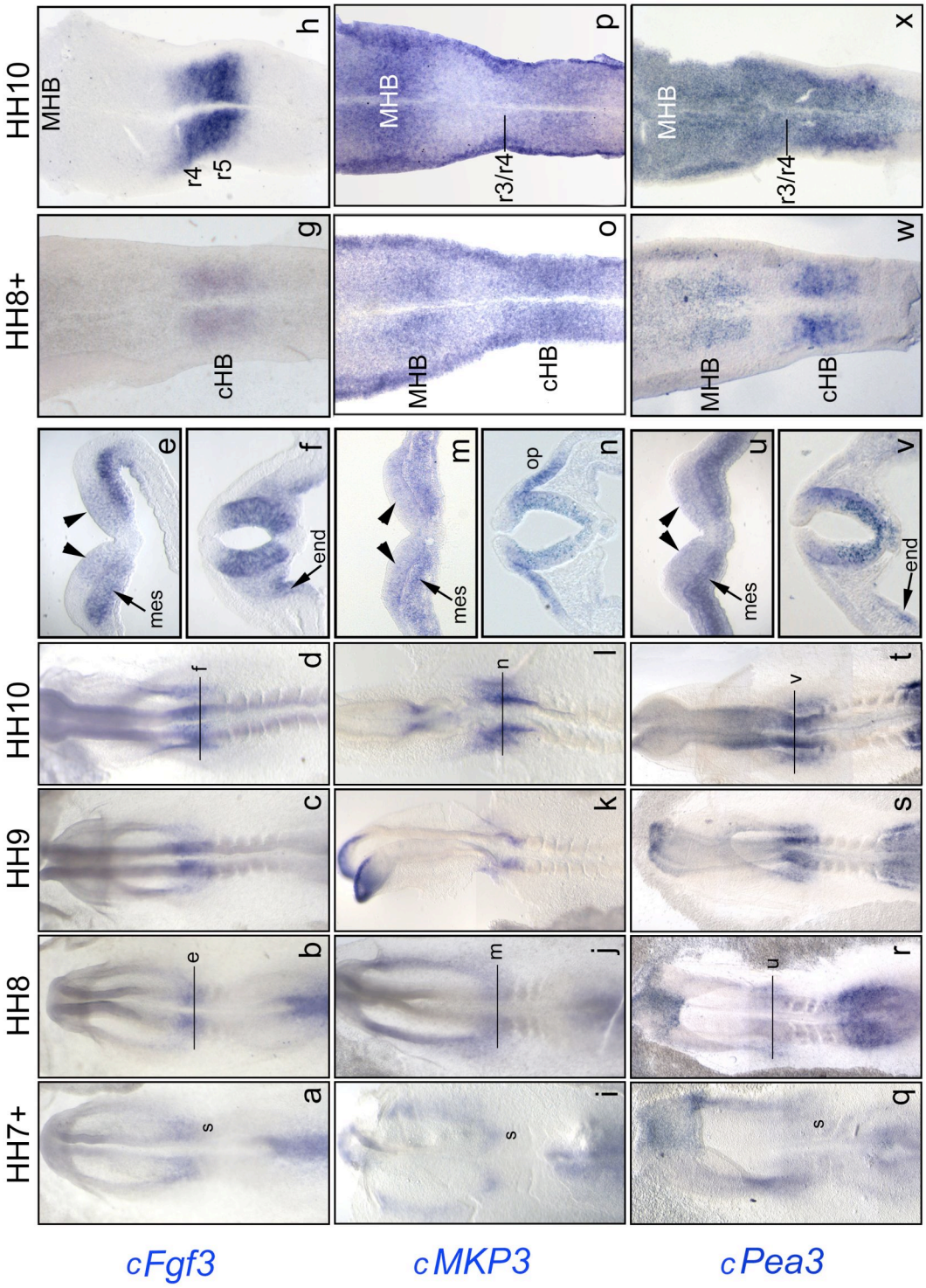
Fgf3 is the most strongly expressed *Fgf* during hindbrain patterning. As previously described by Mahmood *et al. Fgf3* has a dynamic pattern of expression in the hindbrain (Mahmood *et al.*, 1995). Figure 38a shows how at 2ss (HH7+) *Fgf3* transcripts were already present in the prospective hindbrain. At HH8 and HH9 *Fgf3* expression was maintained in the caudal hindbrain occupying a domain corresponding to the presumptive r4-r5 territory (Fig. 38b,c, and flat-mount in g). Transverse sections show that at HH8, *Fgf3* expression was restricted to the ventral part of the hindbrain, with exception of the floor plate (Fig. 38e, see arrowheads). At that stage *Fgf3* was also expressed in the hindbrain-underlying mesoderm (Fig. 38e, see arrow). At HH10 *Fgf3* was still expressed in r4 and r5 (Fig. 38d,h). Transverse sections show that *Fgf3* was expressed along the dorsoventral axis of the hindbrain with exception of the floor plate and the most dorsal part of the tube (Fig. 38f). At that stage *Fgf3* expression was reduced in the hindbrain-underlying mesoderm and appeared in the endoderm that gives rise to the pharyngeal pouches (Fig. 38f, see arrow).

MKP3 (MAPK Phosphatase 3) is a phosphatase belonging to the Erk-MAPK pathway. It is commonly regulated by FGF signaling (Eblaghie *et al.*, 2003; Tsang *et al.*, 2004) and works as negative modulator of the MAPK pathway by specifically dephosphorylating ERK1/2 (Camps *et al.*, 1998; Groom *et al.*, 1996). At 2ss (HH7+) *MKP3* was detected in the prospective caudal hindbrain (Fig. 38i). At HH8 and HH9, this expression became stronger and was maintained in the caudal hindbrain (Fig. 38j,k). The *MKP3* expression domain was broader than the *Fgf3*-expressing area (compare flat-mounts in Fig. 38o and g). *MKP3* transcripts were detected in the presumptive MHB territory (Fig. 38o), where *Fgf8* is already expressed (Crossley *et al.*, 1996). Transverse sections show that *MKP3* was expressed in the neural plate (Fig. 38m, see arrowheads), but also in the hindbrain-underlying mesoderm where *Fgf3* and *Fgf19* are both expressed (Fig. 38m, see arrow) (Ladher *et al.*, 2000). At HH10 *MKP3* expression was maintained in the caudal hindbrain up to r3/r4, and it increased in the MHB (Fig. 38n and flat-mount in p). Transverse sections show that at this stage *MKP3* was expressed along the DV axis of the neural tube, but that it was downregulated in the underlying mesoderm (Fig. 38n). The ectoderm corresponding to the otic placode was also positive for *MKP3* expression.

Pea3 (*Polyoma enhancer activator 3*) is a transcription factor belonging to the Pea3 subfamily of the Ets transcription factors. It is a common mediator of the FGF activity into the nucleus and its expression is also regulated by FGF signaling (Raible and Brand,

2001). *Pea3* was already detected in the prospective hindbrain by 2ss (HH7+) (Fig. 38q). At HH8 and HH9, *Pea3* expression was increased in the caudal hindbrain (Fig. 38r,s and flat-mount in w). Flat-mount in Figure 38w shows that also the MHB expressed *Pea3*. Transverse sections show that *Pea3* was not only expressed in the neural plate (Fig. 38u, see arrowheads), but also in the underlying mesoderm as shown for *MKP3* (Fig. 38u, see arrow). At HH10 *Pea3* was expressed in the hindbrain and in the midbrain (Fig. 38t and flat-mount in x). Transverse sections show that *Pea3* was expressed along the DV axis of the hindbrain but it was downregulated in the underlying mesoderm (Fig. 38n). Contrary to *MKP3*, *Pea3* was not expressed in the otic placode.

Sprouty2 (*Spry2*), as *MKP3*, is a negative modulator of the MAPK pathway regulated by FGF signaling. It is known to be expressed by HH10 in the MHB and r1 (Chambers and Mason, 2000) (Fig. 39a). Figure 39b shows a flat-mounted HH8+ hindbrain with an anterior patch of *Sprouty2* expression corresponding to the onset of this gene in the MHB and rostral hindbrain. A more posterior patch of expression was also appreciated within the hindbrain (Fig. 39b, red arrowhead). However, differently to *MKP3* and *Pea3*, this weak expression domain did not fully coincide with *Fgf3* expression but it was more anterior, as can be inferred by comparing its distance to the presumptive MHB. At HH10, *Sprouty2* expression in the MHB and r1 was highly increased (Fig. 39c) while the more posterior patch of expression within the hindbrain was weaker and narrowed (Fig. 39c, red arrowhead). We also analyzed the expression of the positive modulator of FGF signaling *FLRT3* but no expression in the caudal hindbrain was detected (results not shown).


HH7+
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MHB
r4
r5
m
n
op
r3/r4
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v
cFgf3
cMKP3
cPea3

Figure 38. Genes of the FGF synexpression group are expressed in the caudal hindbrain I. Embryos between HH7+ and HH10 were analysed by ISH for expression of *cFgf3* (**a-h**), *cMKP3* (**i-p**) and *cPea3* (**q-x**). Arrowheads in (**e**), (**m**) and (**u**) point to early expression in the neural plate. Pictures show whole-mount embryos (**a-d,i-l,q-t**), flat-mounted hindbrains (**g,h,o,p,w,x**) and transverse sections (**e,f,m,n,u,v**), which correspondences to whole-mounts are specified in the pictures. cHB, caudal hindbrain; end, endoderm; mes, mesoderm; MHB, midbrain hindbrain boundary; op, otic placode; r, rhombomere; s, somite.

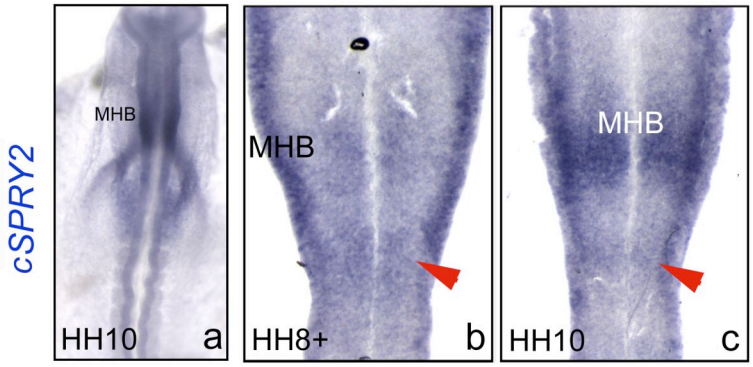


Figure 39. Genes of the FGF synexpression group are expressed in the caudal hindbrain II. Analysis of *cSpry2* expression shown in whole-mount embryo (**a**) or flat-mounted hindbrains (**b,c**). Red arrowhead points to expression within the hindbrain.

Our observations indicate that genes of the FGF synexpression group are expressed in the caudal hindbrain. At early stages of hindbrain patterning, HH7-HH9, *MKP3* and *Pea3* coincided with the *Fgf3* expression profile in the hindbrain. By HH10 *MKP3* was still maintained in the hindbrain in an area coincident with *Fgf3* expression. On the contrary *Pea3* was expanded in the hindbrain and in the midbrain. *SPRY2* expression in the caudal hindbrain was very transient and weak and not fully coincident with the *Fgf3* expression profile. Therefore, *MKP3* is the FGF readout that more closely recapitulates the *Fgf3* expression profile in the hindbrain.

3.3 *MKP3* is readout of FGF3 and *vHnf1* functions in the hindbrain

We have shown that early *MKP3* and *Pea3* expression in the caudal hindbrain coincided with *Fgf3* expression. In order to confirm that these genes are dependent on FGF signals within the caudal hindbrain we analyzed their expression after blocking FGF signaling. As it was expected, when FGF signaling was blocked in HH9 embryos by incubation with 25μM SU5402 during 2 hours, both *MKP3* and *Pea3* expression patterns were disrupted (Fig. 40b,d, n=5/5 and n=2/2 respectively). This is in agreement with several studies that show that the expression of both *MKP3* and *Pea3* is FGF-dependent (Brent and Tabin,

2004; Eblaghie *et al.*, 2003; Firnberg and Neubuser, 2002; Kawakami *et al.*, 2003; McCabe *et al.*, 2006; Raible and Brand, 2001).

Next we wanted to address whether *Fgf3* was sufficient to mediate *MKP3* induction. We overexpressed *mFgf3* in the neural tube of HH8-9 embryos and analyzed *MKP3* expression. As early as 3 hours after electroporation expression domains of *MKP3* in the hindbrain were expanded (Fig. 40e,f, arrowheads, n=5/6). By 6 hours after *mFgf3* electroporation, *MKP3* was ectopically expressed in the entire hindbrain (Fig. 40g, n=6/6). In addition, *mvHnf1* overexpression was also able to induce *MKP3* expression, although this effect was detected later, by 8 hours after electroporation (Fig. 40h, n=8/8). This observation suggests that *vHnf1* activates *MKP3* expression through *Fgf3*.

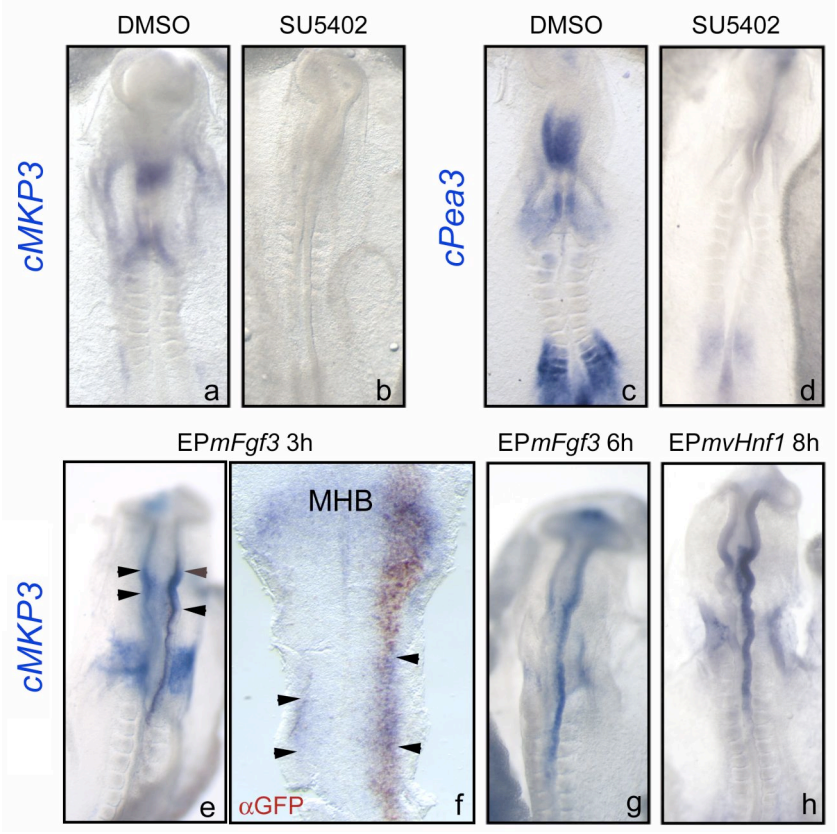


Figure 40. *Pea3* and *MKP3* expression are FGF-dependent. Explanted embryos were cultured during 2 hours in control medium (**a,c**) or medium supplemented with 25µM SU5402 (**b,d**) and assayed for expression of *cMKP3* (**a,b**) or *cPea3* (**c,d**). Embryos were electroporated with *mFgf3* (**e-f**) or *mvHnf1* (**h**), incubated during 3, 6 or 8 hours and then analyzed for *cMKP3*. In (**f**) GFP was immunodetected and developed in red. Electroporated side is the right one. Arrowheads in (**e**) mark the extension of the *MKP3* expression domain in the MHB in the control (left) and the electroporated side (right). Arrowheads in (**f**) mark the extension of the *MKP3* expression domain in the control (left) and the electroporated side (right) of the hindbrain. All the pictures show whole-mount embryos except (**f**) that shows a flat-mounted hindbrain.

In summary, the expression of *MKP3* and *Pea3* relays on FGF signaling. *Fgf3* is able to ectopically induce *MKP3* as soon as three hours after overexpression. *vHnf1* overexpression also induces *MKP3* but later than *Fgf3* suggesting an indirect induction through *Fgf3* and confirming *vHnf1* as a gene upstream of FGF signaling.

3.4 FGF activity involved in hindbrain patterning is mediated by the Ras-ERK1/2 pathway

Next, we wanted to dissect the intracellular pathways involved in hindbrain patterning. Different models are proposed for the involvement of Ras-ERK1/2 and PI3K-Akt pathways in patterning events. Most of them involve uniquely the ERK1/2 pathway that seems to be the common response mediated by FGF signaling (Eblaghie *et al.*, 2003; Suzuki-Hirano *et al.*, 2005; Tsang *et al.*, 2004). PI3K-Akt is usually proposed to crosstalk with Ras-ERK1/2 in either synergistic (Carballada *et al.*, 2001) or antagonistic manners (Echevarria *et al.*, 2005; Kawakami *et al.*, 2003). To understand how these pathways work in the caudal hindbrain we used a loss-of-function approach. We specifically blocked ERK1/2 activity with the chemical inhibitor PD184352 and PI3K function with LY2944002. For general FGF signaling blocking SU5402 was used (see Materials and Methods).

First of all, to validate the specificity and the efficiency of the pathway-specific inhibitors PD184352 and LY294002, we assayed their function in Jurkat T cells. Jurkat cells were treated and processed for protein extraction and western blot analysis for total and phosphorilated forms of Akt and ERK1/2. As expected, cells that were treated with PD184352 gave negative signal for pERK1/2 but not for pAkt; conversely cells that were treated with LY29402 did not phosphorylate Akt but did ERK1/2 (Fig. 41A). In all cases total forms of ERK1/2 and Akt were detected (Fig. 41A). It is worth noting that SU5402 was able to inhibit ERK1/2 but not Akt phosphorylation. As SU5402 is not a direct inhibitor of the PI3K pathway it is probable that FGF-independent pathways mediate the phosphorylation of Akt in Jurkat cells. Next, we tested the inhibitors in embryonic explants. $HH7^{+}$-HH8 embryos were explanted and incubated during 6 hours in the presence or absence of the inhibitors. Then, they were processed for protein extraction and western blotted for phosphorilated and total forms of Akt and ERK1/2. 20μM PD184352 treatment was able to specifically abolish ERK phosphorylation, while 40μM LY294002 treatment abolished Akt phosphorylation (Fig. 41B). Unexpectedly, no

changes in the phosphorylation states of ERK1/2 or Akt were detected after 25μM SU5402 treatment. It has been reported an FGF-independent activation of ERK1/2 upon tissue wounding and tissue dissociation (Christen and Slack, 1999; Lunn *et al.*, 2007; Kuroda *et al.* 2005). It is possible that mechanic dissociation of the tissue during protein extraction promoted an activation of ERK1/2 that, being independent of FGF signaling, was not blocked by SU5402.

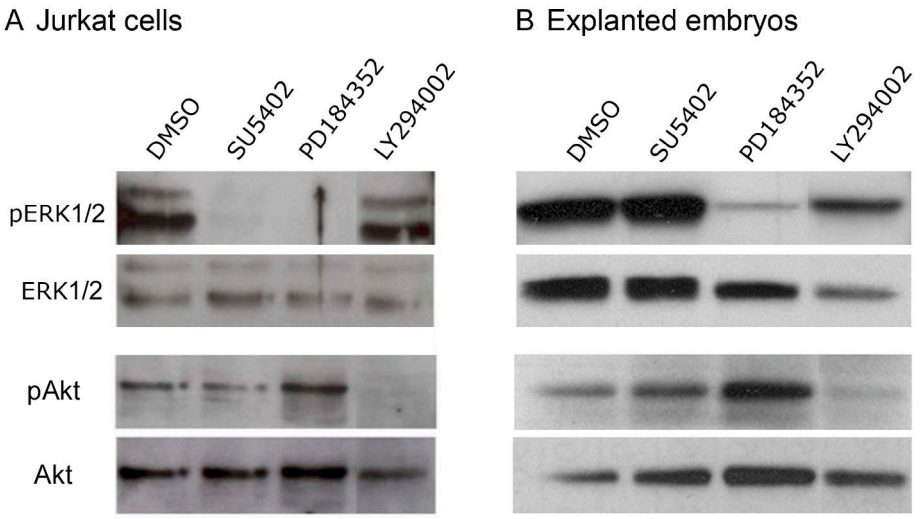


Figure 41. PD184352 and LY294002 specifically block ERK1/2 MAPK and Akt/PI3k pathways respectively. Jurkat cells (**A**) and HH7-8 explanted embryos (**B**) were treated with DMSO, 25μM SU5402, 20μM PD184352 or 40μM LY294002 and analyzed by western blot for total and phosphorilated forms of Akt and ERK1/2.

Once the specific inhibitors were tested, we analyzed how inhibition of ERK1/2 and PI3K pathways affected the expression of the readout of FGF activity *MKP3*. Formate beads were soaked in the specific inhibitors or in DMSO and then placed within the caudal hindbrain of HH7^{+}-HH8 explanted embryos. Explants were incubated during 6h at 38ºC and then analyzed for *MKP3* expression. When PD184252 coated beads were placed near the caudal hindbrain or the presumptive isthmus/MHB of HH8 embryos, *MKP3* expression was completely abolished in these territories (Fig. 42c, n=8/8). Conversely, *MKP3* expression was unaffected when LY294002 (Fig. 42d, n=4) or DMSO (Fig. 42a, n=5) coated beads were implanted. These results suggest that in the caudal hindbrain as well as in the early IsO/MHB, *MKP3* is regulated by Ras-ERK1/2 and not by PI3K-Akt pathway. MKP3 plays a crucial role in controlling Ras-ERK1/2 pathway by specifically dephosphorylating ERK1/2. Some models in chick limb development and neural induction (Eblaghie *et al.*, 2003; Smith *et al.*, 2006) and in zebrafish axial patterning (Tsang *et al.*,

2004) propose that *MKP3* expression is regulated by the Ras-ERK1/2 pathway generating an autoregulatory loop. However, other models in chick limb development and in the isthmic organizer propose *MKP3* expression to be regulated by the PI3K-Akt pathway (Kawakami *et al.*, 2003; Echevarria *et al.*, 2005), being *MKP3* a key molecule to establish a crosstalk between Ras-ERK1/2 and PI3K-Akt pathways. Our results in the caudal hindbrain are in agreement with the view that *MKP3* acts as a mediator of a negative feedback in the ERK1/2 MAPK pathway.

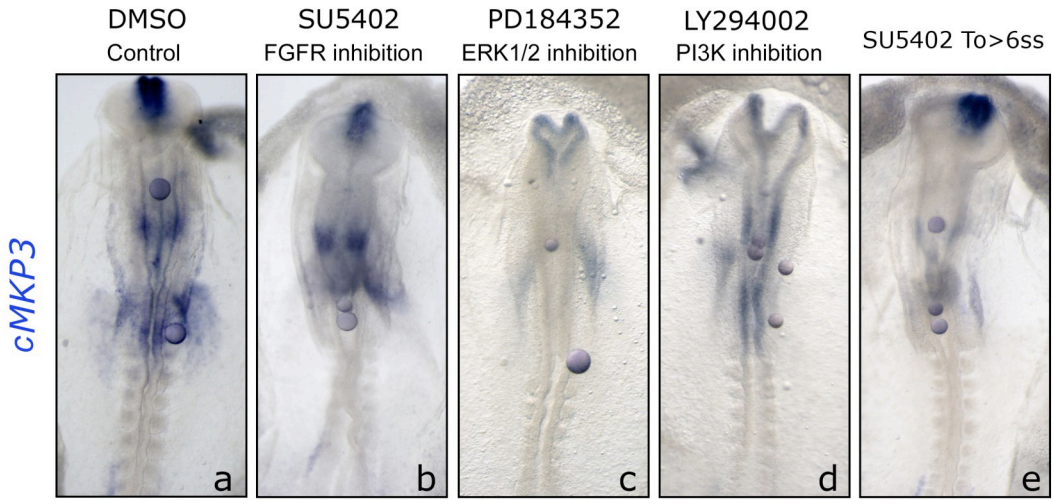


Figure 42. ***MKP3* is dependent on ERK1/2 MAPK but not PI3K-Akt.** HH8 (**a-d**) or HH9 (**e**) embryos were explanted and beads soaked in specific inhibitors were placed in the caudal hindbrain or in the pressumptive MHB. Explants were subjected to control treatment with DMSO (**a**), FGFR inhibition with SU5402 (**b,e**), ERK1/2 inhibition with PD184352 (**c**) or PI3K inhibition with LY294002 (**d**), incubated during 6h and analyzed for the expression of *cMKP3*.

Finally, we analyzed the FGF-activated intracellular pathways responsible for the expression of the caudal rhombomeric markers *MafB* and *Krox20*. *MafB* expression was downregulated when the ERK1/2 pathway was blocked with PD184352 (Fig. 43c, n=8/8). Neither DMSO (Fig. 43a, n=8) nor LY294002 (Fig. 43d, n=5) had this effect. Similarly, beads coated with PD184352 suppressed *Krox20* expression in r5 (Fig. 43h, n=6/6), while expression remained unaffected after treating with DMSO (Fig. 43f, n=7) or LY294002 (Fig. 43i, n=6). These results indicate FGF signaling mediates *MafB* and *Krox20* expression through the Ras-ERK1/2 MAPK pathway with no involvement of the PI3K-Akt pathway.

As previously shown with SU5402 in solution (Fig. 34), beads coated with SU5402 abolished *MafB* (Fig.43b, n=6/6) and *Krox20* (Fig. 43g, n=9/9) in the caudal hindbrain. Importantly, these effects were only observed when embryos were treated with SU5402 before the onset of these genes, 5ss for *MafB* and 6ss for *Krox20* in r5. Disruption of FGF signaling had no effect on *MafB* or *Krox20* expressions after this time window (Fig. 43e,x, n=6 and n=5 respectively), although still affected *MKP3* expression (Fig. 42e, n=8/8). These results suggest that FGF signaling is involved in the induction of *MafB* and *Krox20* in the caudal hindbrain, but not in their maintenance. As expected from previous results that demonstrated that *Fgf3* is not dependent on FGF signaling (Fig. 34), neither SU5402 (Fig. 43l, n=5) nor PD184352 (Fig. 43m, n=5) were able to inhibit *Fgf3* expression.

In summary, the expression of *MKP3*, our readout of FGF activity, as well as the induction of the caudal rhombomeric markers *MafB* and *Krox20* is dependent on Ras-ERK1/2 activity. All together these results suggest that the Ras-ERK1/2 pathway is involved in mediating FGF signaling during caudal hindbrain patterning with no apparent collaboration of the PI3K-Akt pathway, although both pathways are active during this process.

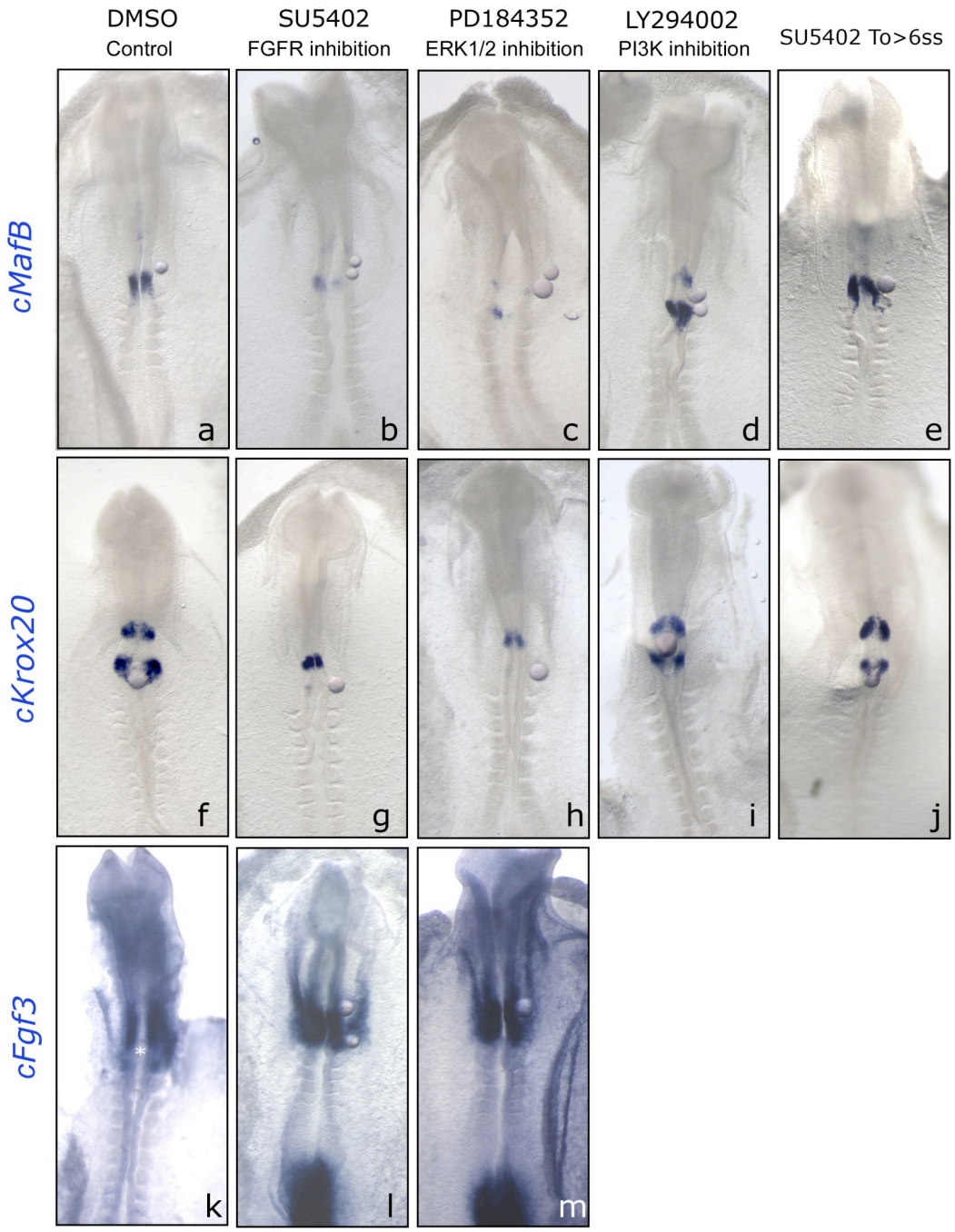


Figure 43. ***MafB* and *Krox20* are dependent on ERK1/2 MAPK pathway but not on Akt/PI3K.** HH8 (**a-d,f-i,k-m**) or HH9 (**e,j**) embryos were explanted and beads soaked in specific inhibitors were placed in the caudal hindbrain. Explants were subjected to control treatment with DMSO (**a,f,k**), FGFR inhibition with SU5402 (**b,e,g,j,l**), ERK1/2 inhibition with PD184352 (**c,h,m**) or PI3K inhibition with LY294002 (**d,i**), incubated during 6h and analyzed for the expression of *cMafB*, *cKrox20* or *cFgf3*.

DISCUSSION

In the present work we have studied the role of *vHnf1* and FGF signaling in the early steps of the hindbrain regionalization. We show that *vHnf1* is expressed in the hindbrain from very early stages of neural development. Gain-of-function experiments show that *vHnf1* is able to activate the caudal rhombomeric genes *Krox20* and *MafB* in anterior regions of the hindbrain, and to repress *Hoxb1* in r4. Interestingly, ectopic *vHnf1* induces an expansion of the *Fgf3* expression domain within the hindbrain. Blocking FGF signaling results in a selective loss of *MafB* and *Krox20* expression. The analysis by semiquantitative RT-PCR demonstrates that upregulation of *Fgf3* upon *vHnf1* overexpression is a rapid event suggesting that *vHnf1* directly induces *Fgf3* transcription. We also analyzed the expression profile of the readouts of FGF activity in the caudal hindbrain and show that they are induced by *vHnf1* overexpression, confirming the role of *vHnf1* upstream FGF signaling. In addition, we have demonstrated that FGF signaling in the hindbrain operates through the Ras-ERK1/2 pathway. Based on these observations, we propose that *vHnf1* together with FGF signals specify caudal hindbrain identity. These data demonstrate an early requirement for *vHnf1* expression and FGF signaling in chick hindbrain patterning and underlie the differences among distinct vertebrates in the regulatory network leading to caudal hindbrain patterning.

***vHnf1* in the specification of the caudal hindbrain**

We show that *vHnf1* is expressed in the neural tube in a segment-restricted manner, up to the r4/r5 boundary, at early stages of chick development. Coincident with the onset of *Krox20* expression in r5 cells, *vHnf1* expression regresses posteriorly, suggesting that the action of *vHnf1* in r5-cells is transient and stage-specific. These data are in agreement with observations in zebrafish (Lecaudey *et al.*, 2004; Sun and Hopkins, 2001). Later studies demonstrated that in mouse *vHnf1* is also early expressed up to the r4/r5 boundary to regress later on to more caudal regions (Kim *et al.*, 2005). Thus, the expression profile of *vHnf1* in the hindbrain is highly conserved across vertebrate species.

Using a gain-of-function approach, we demonstrate that ectopic expression of *vHnf1* in the hindbrain disrupts the molecular properties of rhombomeres rostral to the *vHnf1* expression domain (anterior to r5), forcing them to acquire some, but not all, the

molecular characteristics of r5 or r6. The expression of *Krox20* is ectopically activated in r4, and that of *MafB* in r2, r3, and r4. Evidences in mouse and zebrafish suggest that *val/Kreisler/MafB* expression is required for *Krox20* expression (Frohman *et al.*, 1993; McKay *et al.*, 1994; Moens *et al.*, 1996; Prince *et al.*, 1998), thus, there is the possibility that *Krox20* activation upon *mvHnf1* overexpression in chick is mediated through *MafB.* Indeed, in zebrafish it has been shown that *vhnf1* cannot induce *krox20* in a *val* mutant context suggesting that *val/MafB* is downstream *vhnf1* in the regulation of *krox20* in zebrafish (Wiellette and Sive, 2003). In chick, our results, in agreement with the results obtained by Giudicelli *et al.* (2003), suggest that *mKreisler/MafB* overexpression in the hindbrain does not induce *Krox20* expression, thus *MafB* is not sufficient to regulate *Krox20*. However, to answer whether *MafB* expression is necessary for *Krox20* expression in chick a loss of function approach would be required.

vHnf1 overexpression seems to have a dual action on *Krox20* expression: in r4 *vHnf1* induces ectopic expression of *Krox20* whereas in the normotopic *Krox20*-expression domains, r3 and r5, *vHnf1* downregulates this gene. The latter could suggest a mutual repression mechanism between these two genes. As mentioned above, caudal regression of *vHnf1* from r5 coincides with the onset of *Krox20* in this rhombomere. In addition, as will be further discussed, *vHnf1* is only involved in induction but not maintenance of *Krox20*. This raises the possibility that a sustained expression of *Krox20* needs the downregulation of *vHnf1* in r5.

Ectopic expression of *vHnf1* also leads to a repression of *Hoxb1* in r4. This is consistent with the zebrafish data (Choe and Sagerstrom, 2004; Hernandez *et al.*, 2004; Wiellette and Sive, 2003). However, in chick, repression *Hoxb1* occurs within a more restricted domain than the area in which *mvHnf1* is missexpressed. Consequently, and unlike the situation in zebrafish, the downregulation of *Hoxb1* does not seem to be due to a direct repression by *vHnf1*. A possibility is that the inhibition of *Hoxb1* expression in r4 is the result of the abnormal activation of *Krox20* and/or *MafB* in this rhombomere. Indeed, *Krox20* and *MafB* electroporation experiments in the chick neural tube showed that both genes independently can repress *Hoxb1* expression (Giudicelli *et al.*, 2001; Giudicelli *et al.*, 2003).

In mouse, *Hoxa3* is a direct transcriptional target of *MafB* in r5 and r6 (Manzanares *et al.*, 2001). Missexpression of *MafB* by electroporation in chick leads to a weak ectopic

activation of *Hoxa3* in r3 (Giudicelli *et al.*, 2003). In our experiments, ectopic expression of *vHnf1* does not lead to the ectopic activation of *Hoxa3*. This might be because the level of ectopic *MafB* in r3 following *vHnf1* missexpression is insufficient to activate *Hoxa3* transcription.

Taken together, these data show that in chick *vHnf1* is one of the earliest genes expressed in a rhombomere restricted manner and that it is involved in the acquisition of r5 and r6 identity in the caudal hindbrain.

An inductive-synergistic model for vHnf1 and FGF function

Interestingly, *vHnf1* overexpression in chick leads to strong expansion of *Fgf3* expression throughout the hindbrain. Previous work in chick and zebrafish demonstrated the involvement of FGF signaling in regulating *Krox20* and *MafB* in the caudal hindbrain (Marin and Charnay, 2000; Maves *et al.*, 2002; Walshe *et al.*, 2002). Furthermore, in zebrafish a synergy between FGFs and *vHnf1* to induce *val/MafB* in r5 and r6 and *Krox20* in r5 has been proposed (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). We treated embryos with an inhibitor of FGFR, SU5402, and demonstrated that in these embryos *MafB* and *Krox20* expression is abolished. Moreover, when FGF signaling is blocked in embryos where *vHnf1* is overexpressed, ectopic induction of *MafB* or *Krox20* is not observed. These results are in agreement with results in zebrafish and suggest that *vHnf1* and FGF signaling are required together for the expression of *Krox20* and *MafB* in the caudal hindbrain (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). However, important differences were found between zebrafish and chick in the way by which *vHnf1* and FGF signaling regulate *MafB* and *Krox20* expression. In zebrafish, overexpression of *vhnf1* leads to weak ectopic expression of *val/MafB* and no ectopic expression of *krox20*. In addition to this, ectopic administration of FGF signals does not lead to any ectopic induction of *val/MafB* or *krox20*. It is necessary to combine FGF ectopic activity with *vhnf1* overexpression to promote strong induction of *MafB* and *Krox20* (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). Thus, it is proposed that in zebrafish *vhnf1* and FGF signals operate synergistically to regulate *val* and *krox20* expression. On the other hand, in chick, overexpression of *vHnf1* alone is sufficient to upregulate *MafB* in the rostral hindbrain up to r2 and *Krox20* in r4, in spite of that FGF signaling is required for these inductions. These observations can fit in two different models (Fig. 44): i) *vHnf1* induces *Fgf3* that, in its turn, induces *MafB* and *Krox20*; ii) *vHnf1* induces *Fgf3* that in synergy with other *vHnf1*-dependent mechanisms induces *MafB* and *Krox20.* The latter involves a

synergistic mechanism as proposed in zebrafish. We favor this latter hypothesis since we observe that overexpression of *Fgf3* alone does not lead to any ectopic induction of *Krox20* or *MafB*, suggesting that, although being necessary, FGF signaling is not sufficient for *Krox20* and *MafB* induction. Thus, *vHnf1* is upstream the whole process and regulates FGF signaling and other downstream mechanisms to induce *MafB* and *Krox20* in the caudal hindbrain. These other mechanisms most probably are direct regulations of *vHnf1* over *Krox20* and *MafB.* Recent studies have demonstrated that both *Kreisler/MafB* and *Krox20* contain conserved *vHnf1* binding sites in their regulatory regions that mediate initiation of expression in the caudal hindbrain (Chomette *et al.*, 2006; Kim *et al.*, 2005). The model that we propose, in which *vHnf1* is upstream the whole process of initiation of *Krox20* and *MafB* expression, is supported by our observation that *vHnf1* alone is sufficient to induce these genes and that co-expression of *vHnf1* plus *Fgf3* in the rostral hindbrain leads to similar inductive effects to the observed when *vHnf1* alone is overexpressed. On the other side, as it was said before, overexpression of *vhnf1* alone in zebrafish has much less inductive capability than overexpression of *vhnf1* and *fgf3* together (Hernandez *et al.*, 2004). It would be interesting to analyse which of the models, synergy or induction plus synergy, have been conserved in rodents in order to see whether regulation of *vHnf1* over *Fgf3* constitutes an amniote-specific mechanism in the genetic network that governs hindbrain regionalization.

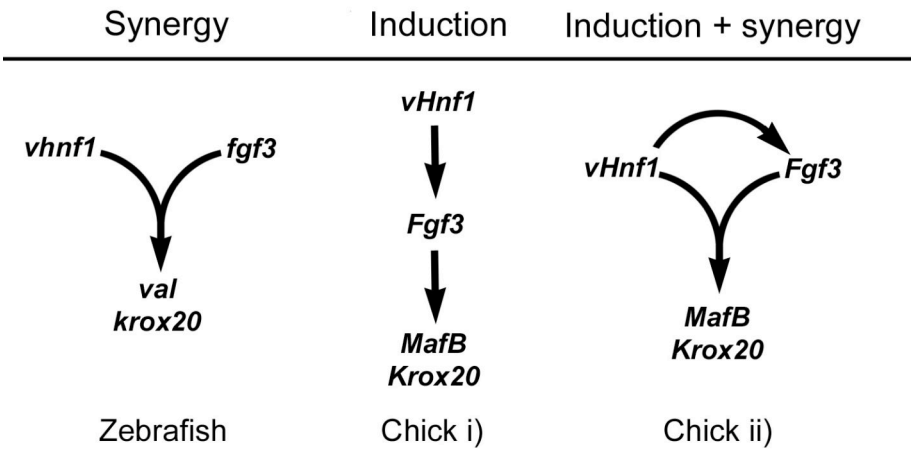


Figure 44. Different models for the regulation of *Krox20* and *MafB* by *vHnf1* and *Fgf3*. In zebrafish it is proposed that *vhnf1* and *fgf3* operate synergistically to initiate the expressions of *val* and *krox20*. We had two hypotheses for this regulation in chick. The first one involves a chain of inductions (*vHnf1* induces *Fgf3* that induces *Krox20* and *MafB*). However this is unlikely to happen because *Fgf3* is required but not sufficient to induce *Krox20* and *MafB*. Thus we favour a second model in which *vHnf1* induces *Fgf3* and then both co-operate to induce *Krox20* and *MafB*.

vHnf1 overexpression operates in cell- and non cell-autonomous manners and its effects are rhombomere-restricted

Interestingly, *vHnf1* overexpression promotes induction of *Krox20*, *MafB* and *Fgf3* in both cell- and non cell-autonomous manners. Giudicelli *et al.* (2001) also obtserved non cell-autonomous effects upon *Krox20* overexpression in chick. They demonstrated that this was due to an autoregulatory loop of *Krox20*. When we checked for this possibility in *vHnf1* we saw that exogenous *vHnf1* was not able to induce the endogenous gene and thus we discarded an autoregulatory *vHnf1* loop. Another possibility is that a secreted molecule induced by *vHnf1* mediates *vHnf1* function in non-electroporated cells. As *Fgf3* is strongly induced upon *vHnf1* overexpression and FGF signaling is necessary for *MafB* and *Krox20* expression, *Fgf3* became the candidate signal to mediate these effects. However, the non cell-autonomous effects of *vHnf1* overexpression cannot be explained only by the mediation of *Fgf3* because: i) overexpression experiments demonstrate that *Fgf3* is not able to induce *Krox20* or *MafB* in cells that are not expressing *vHnf1*; ii) neither *Fgf3* nor *vHnf1* are dependent on FGF signals and thus *Fgf3* cannot regulate its own expression. We can hypothesize that members of other signaling pathways could be mediating this effect. It has been demonstrated that in chick *Fgf19* from the hindbrain-underlying mesoderm induces *Wnt8c* in r4 (Ladher *et al.*, 2000), and that the expression of *wnt3a* and *wnt1* in the hindbrain is affected in *vhnf1* zebrafish mutants (Lecaudey *et al.*, 2007). Thus, there is the possibility that WNT signaling is a mediator of the non-cell autonomous effects of *mvHnf1* overexpression. We have checked for changes in *Wnt8c* and *Wnt1* expression upon *vHnf1* electroporation, but no differences were observed (unpublished). It would be interesting to check for changes in WNT activity, using a TCF reporter construct, upon overexpression of *vHnf1*.

Another interesting observation after *vHnf1* overexpression is that the effects are restricted to different territories of the hindbrain. *vHnf1* can ectopically induce *Krox20* only in r4 and *Fgf3* and *MafB* in territories caudal to the r1/r2 boundary. These kind of segment-restricted effects have been observed also when *Krox20* or *MafB* are overexpressed. For example, *MafB* activates ectopic *Hoxa3* expression restricted to r3 and *Krox20* can only induce *EphA4* and *Hoxa2* caudal to r1/r2 boundary (Giudicelli *et al.*, 2001; Giudicelli *et al.*, 2003). These observations lead to formulate two non-exclusive hypotheses: i) some unidentified factors that are distributed differentially within the hindbrain co-operate with *vHnf1*, *Krox20* and *MafB* to mediate their different inductive

effects; ii) some repressions factors prevent the function of these genes in determined areas of the hindbrain. The PBX-MEIS complexes are good candidates to co-operate with vHNF1 protein. It has been demonstrated that these Hox cofactors that are essential for hindbrain segmentation can also bind to non-Hox homeodomain proteins (Josephson *et al.*, 1998; Rave-Harel *et al.*, 2004). Consistently with the effects of *vHnf1* overexpression, MEIS proteins show segment-restricted expression profiles within the hindbrain between r2 and the spinal cord (Waskiewicz *et al.*, 2001). Thus, it would be interesting to analyse the putatives Pbx and Meis sites present in the regulatory regions of the *vHnf1* targets.

vHnf1 and FGFs are involved in induction but not maintenance of Krox20 and MafB

Another conclusion that can be extracted from our functional analyses is that *vHnf1* and *FGFs* operate within a narrow time window during early steps of caudal hindbrain patterning. *vHnf1* overexpression is only effective in inducing *Krox20*, *MafB* or *Fgf3* when embryos are electroporated before 7ss. Beyond this stage, hindbrain cells are not sensitive to this overexpression. Conversely, FGF blocking inhibits *Krox20* and *MafB* expression only if inhibitor treatment is done before the onset of these genes in the caudal hindbrain, this is 5ss for *MafB* and 7ss for *Krox20*. Thus, it seems that *vHnf1* and FGF signaling are needed for early establishment of *Krox20* and *MafB* expression rather that for maintenance of these expressions. This is consistent with the expression profile of *vHnf1* in the caudal hindbrain. While *vHnf1* is downregulated in the hindbrain by HH11, *Krox20* and *MafB* are expressed at least until HH14. Consistently, certain evidences suggest that maintenance of *Krox20* and *MafB* expression depends on autoregulatory mechanisms (Giudicelli *et al.*, 2001; Giudicelli *et al.*, 2003). Moreover, analysis of the *Krox20* regulatory regions in mouse revealed that one of the regulatory elements that is needed for *Krox20* initiation contains a *vHnf1* binding site, whereas another regulatory element that is involved in maintenance of *Krox20* expression contains a binding site for *Krox20* itself (Chomette *et al.*, 2006).

Regulation of FGFs within the hindbrain is highly divergent across species

Our results pointed out that transcriptional regulation of FGF signals is not conserved across species. The highly divergent expression profiles that *FGF* genes show in the hindbrain of different species may reflect this fact (Lombardo *et al.*, 1998; Mahmood *et al.*, 1995; Maves *et al.*, 2002; McKay *et al.*, 1996). In zebrafish, the expression of *fgf3* and *fgf8* is restricted to r4. On the contrary, in chick and mouse, *Fgf3* is expressed

dynamically in r4, r5 and r6. *vhnf1* and *val* zebrafish mutants show caudal expansion of *fgf3* suggesting that in wild type embryos *vhnf1* and *val/MafB* are involved in preventing expansion of *fgf3* expression to r5 and r6 (Hernandez *et al.*, 2004; Kwak *et al.*, 2002). On the contrary, mouse *kreisler* exhibits reduction in the levels of expression of *Fgf3* in the missexpressed r5-6 territory, suggesting that *MafB* is required for *Fgf3* expression in r5 and r6 (McKay *et al.*, 1996; Vazquez-Echeverria *et al.*, submitted). As mentioned above, the overexpression results suggest that *vHnf1* positively regulates *Fgf3* in the chick hindbrain. Moreover, RT-PCR semiquantitative analysis shows that *Fgf3* is rapidly induced after *vHnf1* overexpression, suggesting that this is the result of a direct transcriptional regulation. In summary, while the zebrafish genes *vhnf1* and *val* seem to be involved in preventing *fgf3* expression in the caudal hindbrain, their orthologues in amniotes seem to have opposite functions. Could an evolutionary switch have changed the role of these genes in *Fgf3* regulation? Detailed characterization of the *Fgf3* regulatory regions in different species would help to clarify this issue

A local gradient of ERK1/2 activity coincides with early expression of Fgf3 and FGF activity readouts in the caudal hindbrain

We further studied FGF signaling in the hindbrain patterning by analyzing the profile of the FGF-activated Ras-ERK1/2 pathway. We show that the activated form of ERK1/2, pERK1/2, is localized in the caudal hindbrain and other FGF activity areas during early embryonic development. This is in agreement with other works in mouse (Corson *et al.*, 2003) and chick (Lunn *et al.*, 2007). Particularly relevant is the recent work by Lunn *et al.* in which the localization of pERK1/2 is compared with the expression profile of *FGFRs1-4*, *MKP3* and the *Pea3* subfamily of Ets factors during early embryonic development. There are some differences between Lunn *et al.* and our observations which are worth mentioning. While Lunn *et al.* observed that by HH8-8+ ERK1/2 is more intensely activated in the presumptive anterior hindbrain than in the caudal hindbrain, in our hands, HH8 embryos display a pERK1/2 gradient extending rostrally and posteriorly from the caudal hindbrain (Fig. 45). This gradient is consistent with the expression of *Fgf3* in the caudal hindbrain. This discrepancy may be due to differences in staining astringency or to subtle differences in the embryo staging. At later stages, we observe that pERK1/2 is localized throughout the hindbrain, being more intense in the MHB. Therefore, our results suggest that the initial activation of pERK1/2 in the caudal hindbrain depends on a local FGF source within this area rather than being the tail of a gradient established in more

rostral areas. This idea is supported by our analysis of the FGF-activity readout *MKP3*. *MKP3* is expressed in two domains within the hindbrain, a more rostral one in the MHB and a more posterior one in the caudal hindbrain. Between these two domains there is a region of the rostral hindbrain that does not express *MKP3*, suggesting that this region has lower levels of ERK1/2 activity. On the contrary, although *Pea3* is also initiated in two different domains, at later stages is expressed throughout the hindbrain. This could reflect that *MKP3* and *Pea3* transcriptions require different thresholds of ERK1/2 activity to be induced. We have analysed the expression of *Spry2* and *FLRT3*, two other genes belonging to the FGF synexpression group. The negative modulator *Spry2* is highly expressed in the MHB and r1 but shows weak and very transient expression in the hindbrain. This expression does not fully coincide with the expressions of *Fgf3* or *MKP3* in the caudal hindbrain but it is slightly anterior to them. The positive modulator of FGF activity *FLRT3* was observed in the anterior part of the neural tube but not in the caudal hindbrain. This gene is later expressed in the MHB in a restricted manner (Smith and Tickle, 2006). A conclusion that can be extracted from the analysis of the genes of the FGF synexpression group is that their expression is dynamic and tissue-dependent. Since the role of these genes is to regulate FGF signaling (*MKP3*, *Spry2, FLRT3*) or to modulate gene expression in response to FGF signals (*Pea3*, *Erm*, *Er81*), it is likely that regional and temporal variation in the levels of expression of these genes during embryogenesis can tune FGF signaling in each particular event. How this context-specific tuning is achieved is largely unknown.

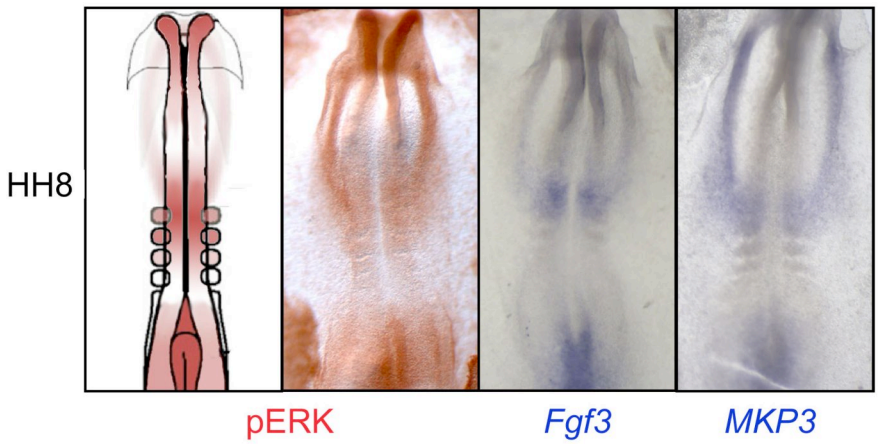


Figure 45. A gradient of pERK1/2 distribution is detected in the prospective hindbrain coincident with *Fgf3* and *MKP3* expression domains at HH8. Scheme modified from Lunn *et al.* 2007.

FGF activity in the caudal hindbrain is mediated by the Ras-ERK1/2 pathway with no involvement of the PI3K-Akt pathway

The ERK1/2 Ras-MAPK pathway is the most widely reported pathway in FGF-required developmental processes (Brent and Tabin, 2004; Eblaghie *et al.*, 2003; Lovicu and McAvoy, 2001; McCabe *et al.*, 2006; Suzuki-Hirano *et al.*, 2005; Tsang *et al.*, 2004). In certain cases PI3K-Akt pathway is proposed to act together with the Ras-MAPK pathway in either synergistic or antagonistic manners (Carballada *et al.*, 2001; Echevarria *et al.*, 2005; Kawakami *et al.*, 2003). We have analysed the involvement of the Ras-ERK1/2 and PI3K-Akt pathways in mediating FGF signaling during caudal hindbrain patterning. The result show that activated effectors of both pathways are present in protein extracts from hindbrains of HH8-9 embryos, suggesting that both pathways are active in this tissue. However loss-of-function studies of these pathways suggest that only the Ras-ERK1/2 pathway is involved in the hindbrain patterning.

First, we analyzed the regulation of the FGF activity readout *MKP3* in the caudal hindbrain. MKP3 has a very specific function in dephosphorylating ERK1/2 and thus inactivating the Ras-ERK1/2 pathway. Two models, one in the limb development and the other in the isthmic organizer propose *MKP3* as a pivotal molecule in mediating crosstalk between PI3K-Akt and Ras-ERK1/2 pathways (Kawakami *et al.*, 2003; Echevarria *et al.*, 2005). Both models propose that *MKP3* is induced by the PI3K-Akt pathway to turn off the Ras-ERK1/2. Our observations demonstrate that the expression of the *MKP3* is dependent on the Ras-ERK1/2 pathway but independent of the PI3K-Akt pathway in the hindbrain. Thus, in the caudal hindbrain, *MKP3* seems to be mainly involved in the autoregulation of the Ras-ERK1/2 pathway rather than in mediating crosstalk with the PI3K-Akt pathway. This is in agreement with the model proposed both in the limb development and neural induction (Eblaghie *et al.*, 2003; Smith *et al.*, 2006). Recent work by Ekerot *et al.* confers more consistency to the hypothesis that *MKP3* is the mediator of an autoregulatory loop within the Ras-ERK1/2 pathway (Ekerot *et al.*, 2008). These analyses show that the activation of the *MKP3* promoter by FGF signaling is ERK1/2 dependent and that it requires an intact Ets-binding site for its function. Other negative modulators of FGF signaling such as *Sprouty* genes and *Sef* are induced by activation of the Ras-ERK1/2 pathway and thus have been proposed to operate in negative feedback loops (Furthauer *et al.*, 2001; Furthauer *et al.*, 2002; Ozaki *et al.*, 2001; Tsang *et al.*,

2002). These feedback loops are thought to be crucial in control of timing and duration of MAPK activation and thus the final outcome of this activation.

Our results show that *MKP3* is early induced in response to FGF-signaling in the hindbrain and that it is rapidly downregulated with the suppression of FGF or ERK1/2 activities. *MKP3* induction is detected as soon as 3 hours after *Fgf3* overexpression and by 6 hours is expressed throughout the hindbrain. *MKP3* is also induced 8 hours after *vHnf1* overexpression suggesting that this gene is indirectly regulated by *vHnf1* through FGF signaling. Conversely, *MKP3* was downregulated in the caudal hindbrain as soon as 2 hours after blocking FGF signaling or the Ras-ERK1/2 pathway. These timings are consistent with experiments in which FGF-beads grafted in the chick epiblast induced *MKP3* within 1 and 4 hours (Eblaghie *et al.*, 2003). This induction was counteracted within 2 and 4 hours by adding a bead coated with the FGFR inhibitor SU5402 or the ERK1/2 inhibitor PD184352. These results indicate that *MKP3* is a highly sensitive FGF readout that quickly responds to variations in FGF signaling.

To further assess the role of the FGF downstream pathways in the caudal hindbrain patterning we analysed the expression of the rhombomeric markers *Krox20* and *MafB* after inhibition of Ras-ERK1/2 and PI3K-Akt pathways. Our results suggest that both genes are dependent on the Ras-ERK1/2 pathway and independent of the PI3K-Akt pathway. Thus, FGF signaling mediates caudal hindbrain patterning through the Ras-ERK1/2 pathway. Findings in zebrafish support this hypothesis. When *vhnf1* is co-expressed with a constitutively active form of ERK in the zebrafish embryo the *val/MafB* gene is ectopically induced (Hernandez *et al.*, 2004), just as co-expression of *vhnf1* and *fgf3* does. It is well established that FGFs are involved in cell survival and apoptosis through the PI3K-Akt pathway (Browaeys-Poly *et al.*, 2001; Chen *et al.*, 2000; Ong *et al.*, 2001). One possibility is that this pathway is involved in regulating these events in the caudal hindbrain rather than being involved on its patterning.

A model for the chick caudal hindbrain patterning

Taken together, the data presented in this work and previous knowledge lead us to propose a model in which *vHnf1* is the molecular switch that initiates the process of r5 and r6 specification (Fig. 46). *vHnf1* is very early expressed in the caudal neural plate with a sharp boundary laying in the prospective r4/r5 boundary. This expression may be

initiated very early in response to RA from the axial and paraxial mesoderm during mid/late stages of gastrulation (Hernandez *et al.*, 2004; Pouilhe *et al.*, 2007; Sirbu *et al.*, 2005). Anterior limit of expression of *vHnf1* may be established by mutual repression with an *irx* gene, probably *irx3* (Lecaudey *et al.*, 2004; Sirbu *et al.*, 2005; Sapede and Pujades, unpublished results). Some reports propose that *vHnf1* induction is also mediated by *Hox* PG1 genes, that at early stages are expressed in the neural tube up to pre r3/r4 boundary (Choe and Sagerstrom, 2004). *vHnf1* rapidly induces *Fgf3* in the caudal hindbrain, which activates the Ras-ERK1/2 pathway. *vHnf1* and *Fgf3-ERK1/2* co-operate for the induction of *MafB* expression in r5 and r6 at 4-5ss and *Krox20* in r5 at 6-7ss. *Krox20* induction is probably also dependent on *MafB* expression as suggested in mouse and zebrafish (Frohman *et al.*, 1993; McKay *et al.*, 1994; Moens *et al.*, 1996; Wiellette and Sive, 2003). *Krox20* is initially expressed in a narrow domain caudal to r4 and subsequently expands its expression area due to non-cell autonomous autoinduction. Coinciding with the onset of *Krox20* in r5, *vHnf1* progressively regresses to r6 between 7 and 10ss. Mutual repression between *vHnf1* and *Krox20* may prevent expansion of *Krox20* to r6. Later on, this expansion may be prevented by the Nab proteins that are activated by *Krox20* in the vicinity of the rhombomere boundaries (Mechta-Grigoriou *et al.*, 2000). By 10-11ss, *Hoxb1* is downregulated in the caudal hindbrain and gets restricted to r4. Both *Krox20* and *MafB* may be involved in that repression (Giudicelli *et al.*, 2001; Giudicelli *et al.*, 2003). On the other hand, *MafB* and *Krox20* enhance the expression of the *Hox* genes that confer positional identities to r5 and r6 (Maconochie *et al.*, 2001; Manzanares *et al.*, 1997; Manzanares *et al.*, 1999;; Manzanares *et al.*, 2002; Manzanares *et al.*, 2001;; Nonchev *et al.*, 1996; Sham *et al.*, 1993).

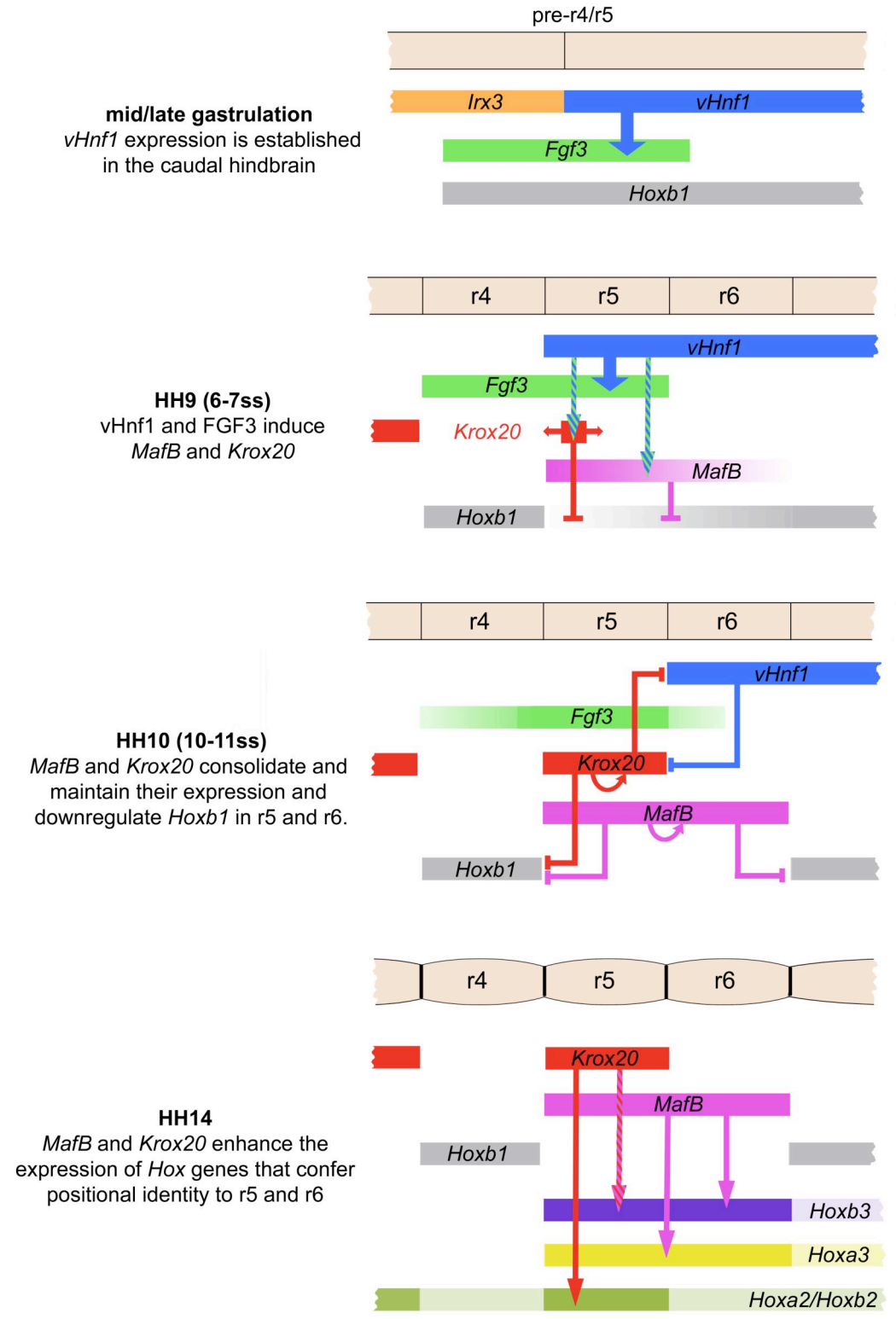


Figure 46. Model for early regionalization of the caudal hindbrain in the chick embryo.

Future directions

Our results unveiled some of the mechanisms involved in patterning the caudal hindbrain. Moreover, our research has opened some new questions on this issue that could constitute new interesting lines of investigation.

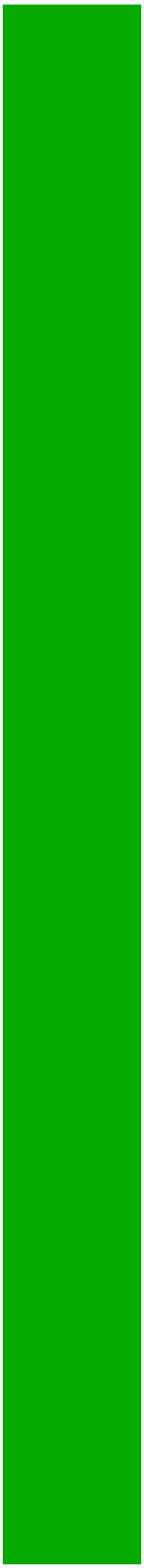
We demonstrated that *vHnf1* overexpression leads to non cell-autonomous induction of caudal rhombomeric genes. We assayed the involvement of FGF signaling in these inductions. However, our results suggest that other signaling pathways may also participate in mediating these effects. A candidate is WNT signaling. We observed no changes in *Wnt8c* and *Wnt1* expression upon *vHnf1* overexpression. Nonetheless, it would be interesting to use TCF reporters to analyse WNT activity during early steps of hindbrain development and see if this activity is affected by *vHnf1* or *Fgf3* overexpression or by blocking FGF signaling.

Our data indicates that, in chick, *Fgf3* is transcriptionally regulated by *vHnf1*, most likely in a direct manner. However, further investigation is required to fully understand which are the mechanisms that regulate *Fgf3* expression in the hindbrain. Analyzing the regulation of *Fgf3* expression is especially interesting because, in contrast to most of the hindbrain patterning genes, its expression profiles are highly divergent across species. Thus, it would be interesting to perform research *in silico* in order to find the putative *Fgf3* regulatory regions and subsequently analyse them *in vivo* by generating reporters that could be delivered into the chick hindbrain by electroporation *in ovo*. Understanding which kind of regulatory elements are involved in the expression of *Fgf3* would add an important piece of understanding to our knowledge of the hindbrain patterning.

Finally, the role of *vHnf1* in the chick caudal hindbrain will not be completely understood until loss-of-function approaches for this gene can be performed. Silencing *vHnf1* in the chick hindbrain will answer important questions as for example whether *vHnf1* expression is necessary and not only sufficient for *Fgf3* expression. During the development of this project we have undertaken serious attempts to silence *vHnf1* function by both siRNA and splicing morpholinos. Unfortunately these trials have not been successful. Probably, imprecision in the annotation of the chick genome has contributed to this. Chick *vHnf1* is a predicted gene (LOC427838) but its sequence has not been studied in detail. Indeed, when we cloned the 3' part of the gene we found a region of 500Kbs within the 3'UTRs

that was not annotated. Thus, maybe the initial step for silencing *vHnf1* function is to clone its 5' and 3' UTR regions and obtain reliable sequences to design interfering RNAs or morpholinos. Functional analysis of *vHnf1* in mouse has been precluded for many years due to the early lethality of the *vHnf1* mutation. Luckily, conditional mutants for *vHnf1* have been generated (Coffiner, 2002), although they are not widely available yet. The analyses of these Cre-lox mutants would be very helpful in the understanding of the role of *vHnf1* in early hindbrain specification.

CONCLUSIONS



1. *vHnf1* is very early expressed in the chick embryo neuroepithelium with a sharp anterior limit of expression that coincides with the presumptive r4/r5 boundary. This expression regresses caudally with the onset of *Krox20* in r5 and is downregulated at late stages of hindbrain patterning.

2. *vHnf1* overexpression confers caudal characters to rostral hindbrain territories by ectopically inducing *MafB* expression up to r2 and *Krox20* expression in r4, and by downregulating *Hoxb1* transcription in r4, the latter by an indirect mechanism.

3. Ectopic expression of *Krox20* and *MafB* promoted by *vHnf1* overexpression occurs in both cell- and non cell-autonomous manners, suggesting the involvement of secreted signals in mediating these inductions.

4. *vHnf1* overexpression in chick leads to a massive upregulation of *Fgf3* expression throughout the hindbrain up to r2. This is a very rapid induction, suggesting that *Fgf3* transcription is directly regulated by *vHnf1*.

5. Induction of *MafB* and *Krox20* expression in the caudal hindbrain is dependent on FGF signaling. However, FGF signaling is not sufficient to ectopically upregulate these genes. In addition, *vHnf1* requires FGF signaling to induce *Krox20* and *MafB* expression.

6. Therefore, *vHnf1* is upstream FGF signaling in patterning the caudal hindbrain. We propose that in the caudal hindbrain *vHnf1* induces the expression of *Fgf3* and both genes co-operate for the induction of *Krox20* in r5 and *MafB* in r5 and r6.

7. Acquisition of caudal characters only occurs in a narrow time window at very early stages of neural development, suggesting that *vHnf1* and *Fgf3* are involved in the induction but not the maintenance of these caudal rhombomeric genes

8. Neither *Fgf3* nor *vHnf1* expressions are dependent on FGF signaling. In addition, *vHnf1* cannot regulate its own expression.

9. The intracellular pathways downstream of FGF signaling Ras-ERK1/2 and PI3K-Akt are activated during hindbrain patterning. The activated forms of ERK1/2 are localized during early steps of embryonic development in FGF activity areas, including the caudal hindbrain.

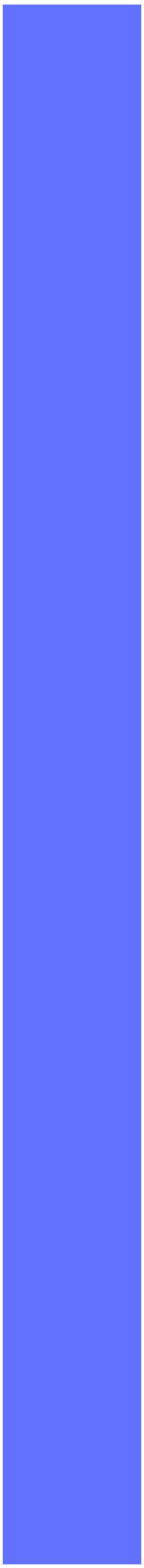
10. The genes of the FGF synexpression group *MKP3* and *Pea3* are expressed at early embryonic developmental stages in the caudal hindbrain in areas coinciding with the expression of *Fgf3*.

11. *MKP3* expression is upregulated throughout the hindbrain upon *Fgf3* or *vHnf1* overexpression. *MKP3* expression in the caudal hindbrain is dependent on the Ras-ERK1/2 but not the PI3K-Akt pathway.

12. *Krox20* and *MafB* inductions are dependent on the Ras-ERK1/2 but not the PI3K-Akt pathway, suggesting that FGF signaling is involved in hindbrain patterning through the Ras-ERK1/2 pathway.

Our results demonstrate an early requirement for *vHnf1* expression and FGF-ERK1/2 signaling in the chick hindbrain patterning and provide new information about the molecular mechanisms involved in patterning the vertebrate caudal hindbrain.

MATERIALS AND METHODS



1. Embryos and staging

Chick embryos were obtained from fertilized hens' eggs (Granja Gibert, Tarragona, Spain) and incubated in humidified atmosphere at 38ºC (Covattuto incubators). Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Stages used in the present work are shown in Table 2.

Hamburger and Hamilton (HH)	Somitic Stage (ss)	Hours
HH7	1ss	23-26h
HH8	4ss	26-29h
HH9	7ss	29-33h
HH10	10ss	33-40h
HH11	13ss	40-45h
HH12	16ss	45-49h
HH13	19ss	48-52h

Table 2. Chick embryonic stages used in this work.

2. Overexpression experiments by *in ovo* electroporation

Different constructs (Table 3) were overexpressed into the hindbrain of HH7-9 embryos by *in ovo* electroporation (Fig. 47). In order to access the embryo, a window in the shell was made. Fast green (approximately 10 times diluted from the stock solution 3mg/ml) was added to reveal the embryonic structures. A solution containing the construct (2µg/µl) was mixed 1:1 with Fast Green (1µg/µl). By using a micropipette (GC150-15 capillaries, Clark electromedical instruments, pulled with a Narishige Japan puller), this solution was introduced on the top of the neural plate or into the lumen of the neural tube. A platinum cathode was placed at the left side while the anode was placed at the right side of the embryo. 4 square pulses (5, 10 or 20V) were generated by an electroporator Square CUY-21 (BTX Co., Ltd, Tokiwasaiensu, Japan). This produced migration of the construct to the right wall of the neural tube and thus, the right side of the hindbrain overexpressed the construct while the left side remained as a control. Medium-199 (M199) (Gibco, #22350) was added immediately after electroporation to protect the embryo from dryness. The eggshell was closed with scotch tape and eggs were incubated in humidified atmosphere at 38ºC to the desired time period. After that,

embryos were collected in cold phosphate buffered saline (PBS pH7.4), selected for GFP fluorescence under the scope, and fixed overnight in 4%PFA/PBS for further analysis.

Construct	Plasmid	Obtained from
EGFP-Mut5	pCAß	I. Mason
mFgf3	pCS2	T. Schimmang
mKreisler	pAdRSV	F. Giudicelli
IRES-GFP	pIRES2-GFP	S. Cereghini
mvHnf1-GFP	pIRES2-GFP	S. Cereghini
mvHnf1Q136E-GFP	pIRES2-GFP	S. Cereghini

Table 3. Constructs used for *in ovo* electroporation.

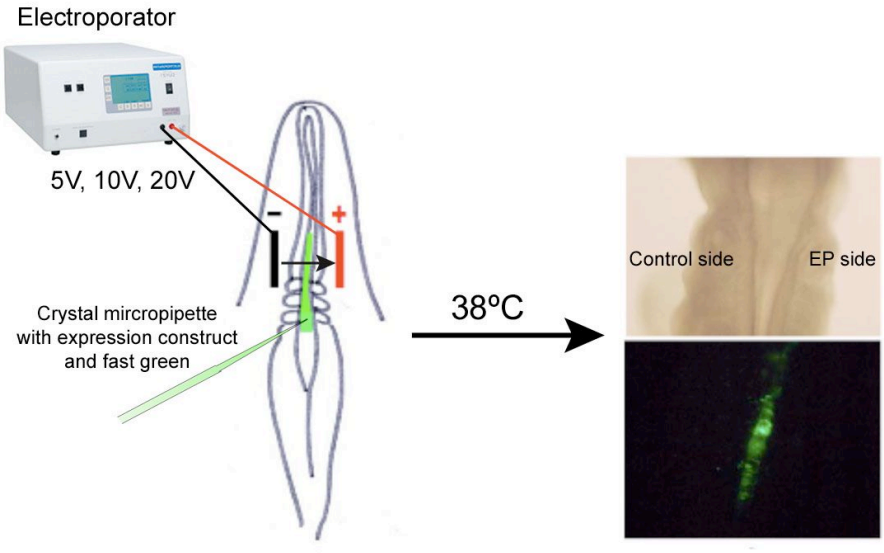


Figure 47. Electroporation *in ovo*. A crystal micropipette is used to introduce the construct of interest into the lumen of the neural tube. Platinum electrodes are placed near to both sides of the neural tube, the cathode to the left and the anode to the right. An electroporator is used to generate square pulses that open pores in the cell membranes and promote migration of the negatively charged DNA to the anode (+) in the right side of the embryo. Thus the right wall of the neural tube is electroporated and the left side remains as contralateral control. The construct of interest contains GFP within its sequence or is co-electroporated with a GFP construct and thus, after some hours of incubation *in ovo*, efficiency of electroporation can be checked under fluorescence scope.

3. Loss of function experiments in whole-embryo organotypic explants

3.1 Whole-embryo explant methodology

Collagen supports were prepared in 4-well dishes (Nunclon, #176740) by adding a drop of 10µl of Matrigel preparation (Invitrogen, #354234) in the center of each well. Matrigel should be defrosted on ice and once applied to the well allowed to jellify at room temperature (RT) during 10 minutes. Use cold tips to avoid jellification in the tip.

HH7-8 embryos were collected and dissected in M199 medium. Explants were prepared by cutting an area around the embryo that comprised the entire *area pellucida* and part of the *area opaca*. This kind of dissection ensures a good survival and development of the embryo and makes it easy to attach it to the matrigel support. Explants were transferred to the 4-well plates and positioned with the ventral side touching to the matrigel support. Then, M199 was removed and embryos were allowed to attach to the matrigel matrix for 5-10 minutes at RT. 200-250µl of DMEM medium was added to each well. Explants were incubated at 37,5ºC in a water-saturated atmosphere containing 5% CO_2 during 2, 4, 6 or 8 hours.

Culturing Medium

- 2%FBS
- 1X Antibiotic/antimicotic solution (Invitrogen, #15240)
- 4mM L-Glutamine
- D-MEM medium

3.2 Inhibitor treatment

Explants were treated with pathway-specific inhibitors of the FGF-dependent intracellular cascades whose characteristics are detailed in the Table 4 and Figure 48. Analysis of their function and specificity can be consulted at Bain *et al.*, 2007 and Davies *et al.*, 2000. Inhibitor treatment was performed in two different modalities: i) adding to the culturing medium described above the different inhibitors to the final concentrations specified in Table 3. As control medium, DMSO, the organic solvent in which the inhibitors were

dissolved, was added to the culturing medium in the same volume than the inhibitors. ii) bead implantation. AGI-X2 formate beads (Bio-Rad, #140-1434) were coated with the inhibitor stock solution for 1-2 hours at RT, protected from light and then washed in PBS before grafting. Beads were grafted next to the hindbrain region of the explanted embryos by using thin forceps.

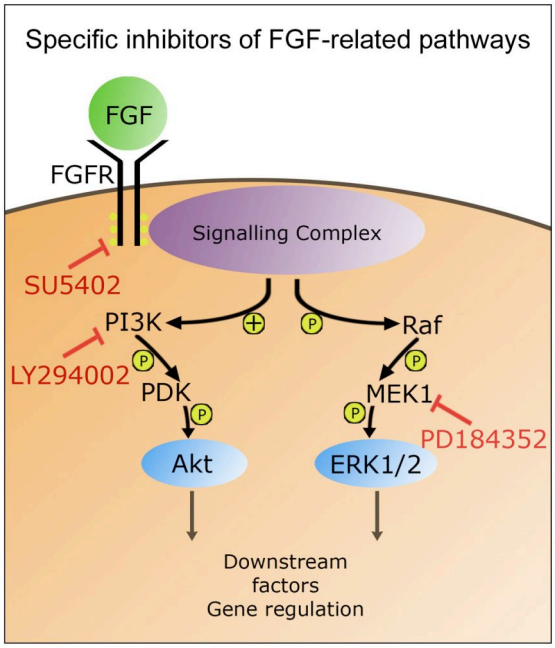


Figure 48. A simplified diagram of ERK1/2 MAPK and Akt/PI3K pathways. The pathway step that each inhibitor disrupts is indicated.

- Jurkat cells treatment: Cells from the immortalized line of T lymphocytes Jurkat were used as control for the inhibitors. These cells were stimulated via their Tcr/CD3 complex to highly activate both the Ras-MAPK and the PI3K pathways (Samstag and Nebl, 2005; von Willebrand *et al.*, 1994; Whitehurst *et al.*, 1992). 10^5 cells per sample were incubated during one hour with the inhibitors at the concentrations specified in Table 3 and processed for western blot.

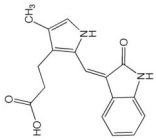
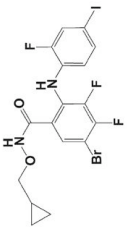
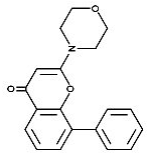
Inhibitor	Pathway	Action	Treatment	Stock	Solvent	Formulation	Supplier
SU5402	FGF Signaling	Impeding tyr-kinase activity of the FGFR	25-50µM	5mM	DMSO		Calbiochem #572630
PD184352	ERK1/2 MAPK	Impeding ERK1/2 phosphorylation	20µM	10mM	DMSO		University of Dundee (Dr.Philip Cohen)
LY294002	Akt/PI3K	Impeding PI3K activation	40µM	20mM	DMSO		Calbiochem #430202

Table 4. Pathway-specific inhibitors. References: SU5402 (Mohammadi *et al.*, 1997), PD184352 (Sebolt-Leopold *et al.*, 1999), LY29402 (Vlahos *et al.*, 1994).

4. Detection of gene expression by whole-mount *In situ* hybridization (ISH)

4.1 Antisense RNA probe synthesis

Complete or partial cDNAs of the genes of interest cloned into vectors and flanked by T7, T3 or SP6 RNA-polymerase promoter sequences were obtained from different sources (see Table 5). These vectors were amplified and stored in mQ-water at -20ºC in concentrations between 1 and 3µg/µl.

Gene	Vector	Linearization site And RNA-Polymerase
Chick		
Fgf3		NotI/T3
FLRT3	Blue Script KS-	NotI/T3
Hoxb1	Blue Script SK	XbaI/T7
Hoxa3	Blue Script KS+	BamHI/T7
Krox20	Blue Script KS-	StuI/T7
MafB		NotI/T3
MKP3		SalI/ T7
Pea3	Blue Script SK	NotI/T7
Shh		HindIII/T3
Sprouty2		SalI/ T7
vHnf1	pCR Script	SacII/T7
Wnt8c	pCRII	HindIII/T7
Mouse		
vHnf1	pGEM-Teasy	SalI/T7
Kreisler	Blue Script KS	EcoRV/T7

Table 5. Constructs used as templates for *in vitro* transcription reactions.

DNA linearization (V_f= 20µl), 2h at 37ºC. Add:

- 2-5µg of DNA
- 1X Restriction Enzyme buffer
- 1µl Restriction Enzyme (1U/µl approx.)
- H_2O to the final volume

Protein degradation 30min at 37ºC. Add:

- 1µl 20mg/ml proteinase K
- 1µl 10% SDS

Linearized DNA purification

- Add phenol (1V), vortex and centrifuge 5min at 13000rpm at 4ºC. Transfer upper phase to a new tube and discard lower phase.
- Add phenol (0.5V) and chloroform (0.5V), vortex and centrifuge 5min. at 13000rpm at 4ºC. Transfer upper phase to a new tube and discard lower phase.
- Add ethanol 100% (2.5V) and precipitate DNA 30 min at -20ºC.
- Centrifuge 15 min at 13000rpm at 4ºC. Discard supernatant.
- Add ethanol 70% (2.5V) and centrifuge 5min at 13000 rpm at 4ºC.
- Discard supernatant and dry at 37ºC 5min.
- Ressuspend linearized DNA in 20µl of sterile sterile mQ-H2O.

In Vitro Transcription (V_f: 20µl) 2h at 37ºC. Add

- 1µl linearized DNA
- 1X transcription buffer
- 1mM Digoxigenin-UTP mix (0.35mM DIG-UTP, 0.65mM UTP, 1mM ATP, 1mM GTP, 1mM CTP). Note: Fluo probes can be prepared by changing Digoxigenin-UTP mix by Fluorescein-UTP mix
- 40U RNAsine (RNAse inhibitor)
- 40U RNA-polymerase
- Sterile mQ-H_2O to final volume

Template DNA degradation. 30min at 37ºC. Add:

- 20U DNAseI-RNAse free

RNA precipitation. 30min at -20ºC. Add:

- 100µl sterile mQ-H2O
- 10µl LiCl 4M
- 300µl 100% Ethanol

- Centrifuge 10min at 13000rpm at 4ºC. Discard supernatant and add 500µl 70% Ethanol.
- Centrifuge at 13000rpm at 4ºC. Discard supernatant, dry for 5min at 37ºC.
- Ressuspend in 20µl sterile sterile mQ-H_2O.

4.2 Whole-mount *in situ* hybridization (ISH)

Pretreatments

Note: Each step rocking and at RT if no other specifications are given

- Collect embryos in cold PBS and fix 4%PFA overnight (O/N) at 4ºC.
- Wash 2X 5min PBT (0.1%Tween in PBS).
- Wash 1X 5min 50% MeOH/PBS (Methanol in PBS).
- Wash 2X 5min 100% MeOH. Keep 30min at -20ºC. Embryos can be stored at -20ºC at that point.
- Rehydratation through 75%, 50%, 25% MeOH/PBS (allow embryos to settle).
- Wash 2X 5min PBT.
- Add proteinase K to 10µg/µl. Time of incubation approximately according to the HH stage of the embryos (a.e, 8min for HH8 embryos). Without shaking.
- Rinse carefully with PBT.
- Fix for 20min in 4%PFA 0.1%Gluteraldehide in PBS.
- Wash 1X 5min PBT.
- Rinse with 1:1 PBT/hybridization buffer (pre-warmed at 70ºC). Allow embryos to settle. Embryos can be stored at -20ºC at this point.
- Rinse 2X with hybridization buffer (pre-warmed at 70ºC). Allow embryos to settle. Keep embryos at 70ºC in hybridization buffer during 30min-1h.

Hybridization

- Add DIG-labelled RNA probe (1µl/300µl).
- Incubate O/N at 70ºC.

Hybridization buffer

- ⌘ 50% Formamide
- ⌘ 1.3X SSC (Stock 20X pH4.5)
- ⌘ 5mM EDTA (Stock 0.5M pH8)
- ⌘ 50µg/ml yeast tRNA
- ⌘ 0.2% Tween20
- ⌘ 0.5% CHAPS
- ⌘ 100µg/ml Heparin
- ⌘ Sterile mQ-H_2O to final volume

Post-hybridization washes and DIG-antibody

- ➢ Rinse 2X with pre-warmed hybridization buffer.
- ➢ Wash 2X 30min in hybridization buffer at 70ºC.
- ➢ Wash 1X 20min in 50%hybridization buffer/50%MABT at 70ºC.
- ➢ Rinse 3X in MABT at RT.
- ➢ Pre-incubate in MABT+2%BBR+20%Goat Serum at RT for 1-2hs.
- ➢ Replace with MABT+2%BBR+20%Goat Serum containing 1/2000 dilution of anti-DIG-AP antibody (Roche, #11093174910) and incubate O/N at 4ºC.

MAB

- ⌘ 100mM maleic acid
- ⌘ 150mM NaCl
- ⌘ NaOH to pH7.5
- ⌘ Add 1%Tween20 to prepare MABT

BBR

- ⌘ Boehringer Blocking Reagent (Boehringer Mannheim # 1096176)
- ⌘ Make 10% stock solution in MAB by heating to dissolve, autoclave, aliquot and store at -20ºC

Goat Serum

- ⌘ Heat 55-60ºC for 30 mins and store at -20ºC

Post-antibody washes and histochemistry

- Rinse 3X MABT.
- Wash 5X 5min MABT.
- Wash 2X 10min NTMT.
- Incubate in developing solution (35µl BCIP Roche, #11403121 + 45 µl NBT Roche, #11403113 in 10ml NTMT) in a 4-well dish. Protect from light. Blue staining will be obtained.
- nce the desired staining is obtained, stop reaction with several NTMT rinses. Optionally: wash with NTMT O/N at 4ºC (to reduce background and improve contrast).
- Wash 3X 5 min PBS.
- Refix 4%PFA 1h.
- Keep in PBS, PBT or, for long term in PBT/glycerol.

NTMT

⌘ 100mM NaCl

⌘ 100mM Tris pH9.5

⌘ 50mM $MgCl_2$

⌘ 1%Tween20

⌘ H_2O to final volume

Make fresh on day of use

4.3 Two color double ISH. Double detection of DIG and Fluo-labeled RNA probes

Previous considerations

- Follow protocol detailed in 3.2 but in the hybridization step apply both DIG and Fluo labeled probes simultaneously.
- Do not fix after developing with NBT/BCIP and rinse several times with MABT.

Blocking and FITC-antibody

- Inactivate alkaline phosphatase (AP) by incubating embryos in MABT at 70ºC 30-40min.
- Wash 3X5min MABT.
- Pre-incubate in 2%BBR 20%Goat Serum/MABT at RT for 1-2hs.

- Replace with MABT+2%BBR+20%Goat Serum containing 1/2000 dilution of anti-Fluo-AP antibody (Roche, #11426340910) and incubate O/N at 4ºC.

Post-antibody washes and histochemistry

- Wash 3X 15min MABT.
- Change MABT hourly during 5h or longer.
- Wash 3X 15 NTMT.
- Incubate in developing solution (75µl INT/BCIP Roche, #11681460 in 10ml NTMT) in a 4-well dish. Protect from light. Red staining will be obtained.
- Wash briefly with NTMT.
- Refix in 4%PFA.
- Keep in PBT/glycerol.

4.4 Cryostat sectioning

- If sample was kept in PBT/glycerol wash several times in PBS.
- Keep in 15% sucrose O/N 4ºC.
- Change to 7.5% gelatin 15% sucrose in PBS. 2h 37ºC.
- Transfer embryos embedded in 15% sucrose/ 7.5%gelatine in PBS to a cryomould and orientate them for transverse sections.
- Dip block into -80ºC pre-cooled 2-methil-butane 1 min.
- Keep blocks at -20ºC until use.
- Before sectioning keep blocks in the cryostat chamber 15 min.
- Adhere blocks to the cryostat sectioning support with OCT compound (Tissue-Tek, #4583).
- Make 20µM cryostat sections.
- Collect sections in Superfrost slides.

5. Protein detection techniques

5.1 Whole-mount immunohistochemistry

GFP detection

Note: Each step with rocking and at RT if no other specifications are given

- Collect embryos in cold PBS and fix 4%PFA 2h-overnight (O/N) at 4ºC.
- Wash 3X 10min PBS.
- Block endogenous peroxidase (only necessary for HRP antibodies), with 6%H_2O_2 in PBTx (PBS + 1%Triton) 2h.
- Block 3X 1h in 10%Goat Serum/PBTx.
- Primary antibody (anti-GPF rabbit) 1:500 in 10%Goat Serum 0.02%Sodium Azide/PBTx. 3-4 days 4ºC.
- Wash 3X 1h 10%Goat Serum/PBTx.
- Secondary antibody (anti-rabbit-HRP or anti-rabbit-Alexa488) 1:200 in 1%Goat Serum/PBTx O/N 4ºC.
- Wash 3X 1h 10%Goat Serum/PBTx.
- Wash 2X 30min PBS.

Anti-rabbit Alexa488

- Observe under fluorescence scope (green channel) and stop washing when background level is satisfactory.
- Refix in 4%PFA/PBS 1h.
- Keep in PBS, PBT or glycerol/PBT (1:1).

Anti-rabbit HRP

- Transfer embryos to a 35mm dish or 4-well dish.
- Develop with AEC substrate system (Lab Vision, #TA-060-HA). Add 20µl of AEC chromogen to 1ml of AEC substrate and mix. Apply to the sample.
- Incubate 5-30min.
- Rinse with PBS or PBT. If there is too much background use PBTx but be careful because it can dissolve also the specific staining.
- Refix in 4%PFA/PBS 1h.
- Keep in PBS, PBT or glycerol/PBT (1:1).

pERK detection

- Collect embryos in cold PBS and rapidly fix 4%PFA overnight (O/N) at 4ºC.
- Wash 3X 5min PBS.
- Wash 1X 5min 50%MeOH/PBS.
- Wash 2X 5min 100%MeOH. Keep 30min in 100%MeOH.
- Rehydrate through 75%, 50%, 25% MeOH/PBS (allow embryos to settle).
- Wash 2X 5min PBS.
- Block endogenous peroxidase (only necessary for HRP antibodies). 6%H_2O_2 in PBTx (PBS + 1%Triton) 2h.
- Blocking 3X 1h in 10%Goat Serum/PBTx.
- Primary antibody (Anti-pERK rabbit) 1:100 in 10%Goat Serum 0.02%Sodium Azide/PBTx. 5 days 4ºC.
- Wash 3X 1h 10%Goat Serum/PBTx.
- Secondary antibody (Anti-rabbit biotinilated) 1:50 in 10%Goat Serum/PBTx O/N 4ºC.
- Wash 3X 1h 10%Goat Serum/PBTx.
- Biotin-Avidin reaction by using Vector ABC Kit (Vector, #PK6100). ABC reagent is prepared by adding 2 drops of reagent A and 2 drops of reagent 2 B in 5ml of blocking solution (10%Goat Serum/PBTx). Mix immediately and allow standing 30min before use. Incubate sample O/N 4ºC.
- Wash 3X 1h 10%GS/PBTx
- Transfer embryos to a 35mm dish or 4-well dish.
- Develop with AEC substrate system (Lab Vision, #TA-060-HA). Add 20µl of AEC chromogen to 1ml of AEC substrate and mix. Apply to the sample.
- Incubate 5-30min.
- Rinse with PBS or PBT. If there is too much background use PBTx but be careful because it can dissolve also the specific staining.
- Refix in 4%PFA/PBS 1h.
- Keep in PBS, PBT or glycerol/PBT (1:1).

5.2 Western blot

Protein extraction from chick embryonic tissue

- Collect fresh tissue in 1,5ml eppendorf tube.
- Dissociate tissue in 2mM EDTA/PBS 10-15 min 37ºC.
- Mechanical desegregation in a 35mm dish by multiple pippeting under the scope (until see isolated cells).
- Recover to a 1,5ml tube and centrifuge 290g 5 min. Discard supernatant (for protein extraction from RBL and Jurkat cells start protocol at this point).
- Solubilize by adding 10%SDS/Sample buffer (1:1). Pipette several times until obtaining a viscous solution.
- 100ºC 5 min.
- Keep at -80ºC.

Sample Buffer 5X

- 30% glycerol
- 40mM EDTA
- 0.1% SDS
- 0.5% Bromophenol blue and xylene cyanol.
- 25% ß-mercaptoethanol

Protein electrophoresis

Note: electrophoresis and transference were done with Mini Trans-Blot, Biorad, #170-3930

- Mount minigel units. Glasses must be clean.
- Prepare 10% separation gel and pour until 2cm from the top.
- Before it polymerizes add isopropanol to the top to get smooth surface.
- Wait for the stacking gel to polymerize (around 15 minutes). Pour isopropanol out by decantation and dry residual liquid with filter paper.
- Prepare 4% stacking gel and pour up to the top. Insert combs carefully to avoid bubbles.
- Wait for the stacking gel to polymerize (around 15 minutes).
- Fill the bottom of the gel tank with 1X Running Buffer avoiding bubble formation.

- Set up the gel gasket and put it in the gel tank avoiding bubble formation. Fill the inside of the gasket with 1X Running buffer until wells are covered.
- Fill the wells with 15µl of sample. If sample is too dense to pipette, warm it 5 min 100ºC and do a short spin down.
- Run electrophoresis at 100V. When the sample reaches the separation gel the voltage can be set to 150V.

4X Resolving buffer

- 1.5M Tris pH8.8

12.5% Resolving gel (12ml)

- 3,75ml 40% Acrilamide
- 3ml 4X Separation buffer
- 120µl 10% SDS
- 5ml H_2O
- 60µl 10% APS
- 6µl TEMED

4X Stacking buffer

- 0.5M Tris pH6.8

4% Separation gel (3,1ml)

- 300µl 40% Acrilamide
- 750µl 4X Stacking buffer
- 30µl 10% SDS
- 2ml H_2O
- 15µl 10% APS
- 3µl TEMED

10X Running Buffer (1L) pH8.3

- 20.2g Tris
- 143g Glycine
- 10g SDS
- H_2O to final volume

Transfer

- Prepare fresh 1X Transfer buffer and cool at 4ºC.
- Cut Immobilon-P membrane to the gel size (Millipore, #IPVH00010).
- Wet membrane in fresh MeOH to activate it.
- Incubate 30 min in 1X Transfer buffer.
- Set up the cassette on a tray (with some transfer buffer in it)
  - Cassette's black panel on the bottom of the tray
  - 2 pieces of 3MM paper
  - Electrophoresis gel
  - Membrane

- ⌘ 2 pieces of 3MM paper
- ⌘ Close cassette and hold firmly

- Put the cassette into the holder (black panel of the cassette facing the black wall of the holder) and the holder into the buffer tank.
- Add a magnetic bar and the cooling unit to the buffer tank.
- Fill the tank with 1X Transfer buffer
- Set to 30V (41mA 2 gels) O/N at 4ºC.

10XTG (2L) pH8-8.6

- ⌘ 288.2g Glycine
- ⌘ 60.6g Trizma base
- ⌘ mQ-H_2O to final volume

1X Transfer Buffer (2L)

- ⌘ 200ml 10XTG
- ⌘ 1400ml mQ-H_2O
- ⌘ 400ml MeOH

Ponceau staining

- Incubate membrane 5-10 min in Ponceau Red and wash with H_2O.
- Ponceau staining reveals the bands of proteins in the membrane and serves to check if the transfer has succeed
- At this step the membrane was cut at the level of 50kD, to separate Akt protein (60kD) and ERK protein (42/43 kD) in two different pieces membrane sheets.

Coomasie Brilliant Blue staining

- Incubate gel with staining solution 5-6 sec in a microwave oven
- Incubate for 2 min at RT.
- Replace staining solution for destaining solution.
- Coomasie staining reveals the bands of proteins in the gel. It can be used to compare with ponceau staining of the membrane in order to know the efficiency of the transference.

Antibody incubation

- Place membranes in a small tray.
- Wash with 3X 10 min TBST.
- Block with 5%milk/TBST 1h.
- In separate trays, incubate primary antibodies (pERK and pAkt, 1:1000 and 1:5000 respectively) in 1%milk/TBST. 1h.
- Wash 4X10 min TBST

- Incubate secondary antibody (rabbit-HRP, 1:2000) in 0.5%milk/TBST. 1h.
- Wash 4X TBST

TBST

- 25mM Tris·HCL pH7.5
- 150mM NaCl
- 5mM KCl
- 1% Tween20

Developing

- Put membranes on cling film after dry excess of TBST.
- Cover membranes with developing reagent (Pierce Laboratories, West Pico #34080 or West Femto #34095). Reagent is prepared mixing 1:1 the two components provided.
- Incubate 5min.
- Put membranes in an expositer cassette and apply photographic film (exposition time varies between 10sec and 15min).

Stripping and second incubation

- Put membranes in a small tray
- 1X 10min H_2O
- 3X 10min Stripping buffer
- 1X 10min H_2O
- 1X 10min TBST
- In separate trays incubate primary antibodies (total ERK and total Akt, 1:5000) in 1%Milk/TBST. Follow steps above for antibody incubation and developing.

Stripping buffer (pH2.4)

⌘ 50mM glycine

⌘ 1.5M NaCl

⌘ H_2O to final volume

	Origin	Supplier	Dilution	
Primary Antibodies			Whole-mount	Western-blot
Akt	rabbit polyclonal	Cell Signaling 9272	-	1:5000
pAkt	rabbit polyclonal	Cell Signaling 9271S	-	1:5000
ERK1/2	rabbit polyclonal	Promega 1761409	-	1:5000
pERK1/2	rabbit polyclonal	Cell Signaling 9101S	1:100	1:1000
GFP	rabbit polyclonal	Molecular Probes A11122	1:500	-
Secondary Antibodies				
rabbit IgG - alexa 488	goat polyclonal	Molecular Probes A11034	1:200	-
rabbit IgG - biotin	goat polyclonal	Vector BA-1000	1:50	-
rabbit IgG - HRP	donkey polyclonal	Amersham NA934V	1:200	1:2000

Table 6. Primary and secondary antibodies used for protein detection.

6. Amplification of mRNA/cDNA by Reverse Transcription-PCR

6.1 Objective of the experiment

The goal of this experiment was to know if induction of *Fgf3* upon *vHnf1* overexpression was a rapid event likely to be a direct regulation. For this purpose HH8 embryos were electroporated with *mvHnf1* and, after different incubation periods, hindbrain tissue was isolated for RNA extraction. RT-PCR was used to amplify *cFgf3* and *mvHnf1* transcripts. The relative levels of *cFgf3* expression after each time period were compared in order to determine the time-course of *cFgf3* induction upon *vHnf1* overexpression.

6.2 Samples

Samples for this experiment were obtained from:

- Embryos electroporated *in ovo* with *mvHnf1* and incubated at 40ºC during 15min, 30min, 1h, 3hs, 6hs and 9hs.

- Embryos electroporated *in ovo* with *mvHnf1-Q136E* (control) and incubated at 40ºC during 6hs.
- Mouse hepatic tissue (used as positive control for *mvHnf1*).

6.3 RNA isolation from fresh tissue

- Embryos were dissected in cold PBS, and hindbrain tissue was rapidly isolated and transferred to a 1.5ml tube with Trizol (Invitrogen, #18068-015) where it was immediately homogenized (5 hindbrains in 100µl Trizol).
- 5 min incubation RT
- Add 20ml chloroform. Incubate 3min RT.
- Centrifuge 12000g 15min 4ºC.
- Transfer supernatant to a new 1.5ml tube.
- Add 50µl 2-propanol. Incubate 10min RT.
- Centrifuge 12000g 10min 4ºC. Discard supernatant.
- Add 100µl ethanol 70%.
- Centrifuge 7500g 5min 4ºC. Discard supernatant.
- Dry pellet 10 min RT.
- Dissolve in 50µl sterile mQ-H_2O.
- Measure RNA concentration using a spectrophotometer.

6.4 One-step RT-PCR

One-step PCR kit (Qiagen, #210210) was used to amplify specific sequences of *mvHnf1*, *cFgf3* and *cGAPDH* genes (see Table 7).

Amplifying Solution (V_f: 20µl)

- ⌘ 1X Enzyme Buffer
- ⌘ 1X Enzyme Mix
- ⌘ 1X QSolution
- ⌘ 0.6µmol primer 5'
- ⌘ 0.6µmol primer 3'
- ⌘ 400µM dNTP mix (400µM ATP, 400µM TTP, 400µM CTP, 400µM GTP)
- ⌘ 100ng RNA

RT-PCR program

- Reverse transcription: 30 min 50°C
- Initial PCR activation step: 15 min 95°C
- 3-step cycling
  Denaturation: 1min 94°C
  Annealing: 1min 58°C
  Extension: 1min 72°C
  Number of cycles: 22, 25, 27 and 30.
- Final extension: 10 min 72°C

Primer	Sequence	TM	Product length
cFgf3 fw	CCTTGGAGAAAAACAGCGTC	59.4ºC	458bp
cFgf3 rv	AGCGTCCTCTCCTTCTCCTC	57.3ºC	
cGAPDH fw	TACTGGAATGGCTTTCCGTGT	65.4ºC	540bp
cGAPDH rv	ACTTTATTGATGTAAGGTGGTACAC	58.8ºC	
mvHnf1 fw	AGAGCTGCCCTGTACACTTG	58.1	616bp
mvHnf1 rv	CATGGTGACTGATTGTCGAA	58	

Table 7. Primers used for RT-PCR.

6.5 Electrophoresis and quantification

Amplification products were run in a 1.5% agarose gel (100V). Bands obtained were quantified using the software Quantity-one (Biorad).

Saturation levels

Because PCR reaction amplifies DNA strains in an exponential manner this reaction tends to reach a saturation point. When comparing relative amounts of PCR products it is important to ensure that these products are compared in a range before the saturation is reached. We determined that, for the PCR conditions exposed above, and by using an approximate initial amount of 100ng of RNA per sample, a range between 25 and 27 cycles was optimal for analyzing relative levels of *cFgf3*, *mvHnf1* and *cGAPDH* expressions. At 22 cycles, amplification of

cFgf3 was difficult to detect and by 30 cycles amplification of the three genes was near to saturation (Fig. 49).

GAPDH normalization

After the extraction, the concentration of RNA in each sample was estimated with a spectrophotometer. However, since having the same amounts of RNA in each sample was crucial for the experiment, a further step in estimating the relative RNA quantities was undertaken. *cGAPDH* was amplified 25 cycles and levels of amplification were compared between the different samples in order to ensure that all the samples had similar amount of total RNA (Fig. 50). An approximate amount of 100ng/µl of RNA was used in each sample.

Relative level of *Fgf3* expression

In each sample *mvHnf1* and *cFgf3* were amplified together. Relative level of *Fgf3* expression was estimated by calculating the ratio between *Fgf3* quantification value and *mvHnf1* quantification value.

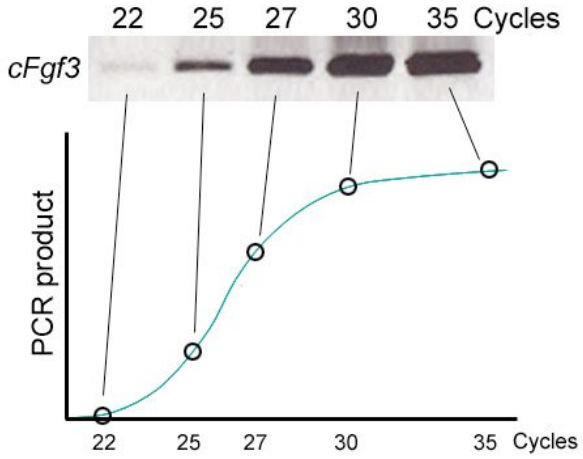


Figure 49. Saturation curve for *cFgf3* amplification using an initial amount of 100ng RNA/sample (approx.).

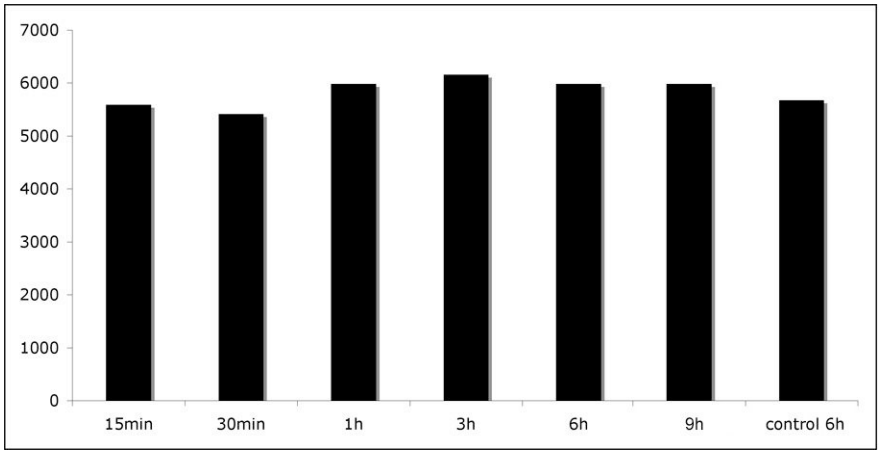


Figure 50. Relative quantity of *cGAPDH* in each sample. (Arbitrary units)

7. Photography and imaging

Whole, flat-mounted or sectioned embryos were photographed using LEICA DMR fluorescence microscope or LEICA, MZ FL III fluorescence scope both fitted with Leica DFC 300FX cameras. Images were captured with *Leica IM50 v4.0* and analyzed with *Adobe photoshop v7.0.1*.

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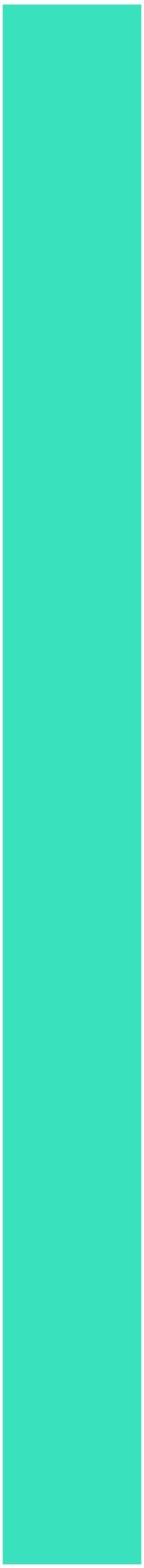

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ANNEX

Aragón F, Vázquez-Echeverría C, Ulloa E, Reber M, Cereghini S, Alsina B, Giradles F, Pujades C.
vHnf1 regulates specification of caudal rhombomere identity in the chick hindbrain.
Dev Dyn. 2005 Nov;234(3):567-76.

LIST OF ABBREVIATIONS

AER	Apical Ectodermal Ridge
ace	acerebellar (zebrafish mutant KO for *fgf8*)
ANR	Anterior Neural Ridge
AP	Anteroposterior
BA	Branchial Arch
bm	branchiomotor neurons
BMP	Bone Morphogenetic Protein
CAM	Cell Adhesion Molecule
CNS	Central Nervous System
cvan	contralateral vestibulo-acoustic neuron
DV	Dorso-Ventral
EP	Electroporation
ERK1/2	Extracellular Regulated Kinases 1 and 2
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FP	Floor Plate
GSK3	Glycogen-Syntase Kinase 3
g	sensory ganglia
HH	Hamburguer and Hamilton stage
HSPG	Heparan Sulfate Proteoglycan
ISH	In Situ Hybridization
IsO	Isthmic Organizer
LR	Left-Right
m	motor nerve
MAPK	Mitogen Activated Protein Kinase
MHB	Midbrain/Hindbrain Boundary
PG	Paralogous Group (referred to *Hox* genes)
pre-r	pre-rhombomeric territory
pERK	phosphorilated/activated forms of ERK1/2
O/N	overnight
r	rhombomere
RA	Retinoic Acid
RAR	Retinoic Acid Receptor

RARE	Retinoic Acid Response Element
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
RP	Roof Plate
s	somite
sm	somatic motor neurons
ss	somitic stage
VAD	Vitamin A Deficient
vm	visceromotor neurons