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The development of the neurosensory elements of the inner ear: the role of Sox2 and Notch signalling

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Para ser grande, sê inteiro:
Nada teu exagera ou exclui.
Sê todo em cada coisa.
Põe quanto és no mínimo que fazes.
Assim em cada lago a lua toda brilha,
porque alta vive.

Fernando Pessoa
Odes de Ricardo Reis

No one who studies development can fail to be filled with a sense of wonder and delight. Development of the embryo is a truly remarkable process. Understanding the process of development in no way removes that sense of wonder

Lewis Wolpert
The Triumph of the Embryo

To my parents, Cristina and Porfírio, who thought me the sense of wonder
To Pedro, for all the moments of wonder and delight

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...

I hope you all enjoy the “arrival” as much as I have enjoyed the journey

ABSTRACT

The experiments described in this thesis report were aimed at studying the functions of *Sox2* and *Serrate1* during the development of the neurosensory elements of the inner ear. First, we have described the expression pattern of *Sox2* during inner ear development and compared to that of *Sox3* and *Serate1*. Secondly, we have shown the results of plasmid based *in ovo* electroporation experiments, designed to manipulate gene expression exogenously, and to study the gain of function of *Sox2* and *Serrate1*. Effects on cell fate and downstream targets were assessed by in situ hybridization immunohistochemistry and quantitative real-time PCR (qRT-PCR).

The results show that *Sox2* is expressed in the neurosensory domain of the otic epithelium during the neurogenic period of otic development and, later on, during the development of the prosensory patches and sensory organs. As differentiation proceeds, *Sox2* is excluded from differentiated neurones and hair cells, but remains expressed in the supporting cells of the sensory organs. *Sox3* is co-expressed with *Sox2* in the neurogenic domain of the otic cup. But *Sox3* is then down-regulated and only *Sox2* expression persists in the sensory precursors, where it is co-expressed with the Notch ligand *Serrate1*. The expression domain of *Serrate1* is initially nested within *Sox2*, however, later in development *Sox2* becomes restricted within the boundaries of *Serrate1* expression, a process that is concomitant to the formation of the sensory patches. These expression patterns suggest: 1) that *Sox2* correlates with neurosensory fate in the otic placode, 2) that neurogenesis is associated with *Sox2* and *Sox3* and 3) that sensory development is associated with *Sox2* and *Serrate1.*

Gain of function studies show that *Serrate1* regulates prosensory fate and sensory organ development by maintaining *Sox2* expression in restricted domains of the otocyst, without affecting neurogenesis. *Serrate1* operates in a Notch-dependent manner, consistently with a mechanism of lateral induction that includes the induction of its own expression and downstream targets of the Notch signalling pathway *Hes1*, *Hey1* and *Hey2*. Similar studies on *Sox2* indicate that it specifies neurosensory fate in the otic epithelium. However, high concentrations of *Sox2* suppress sensory fate and promote neuronal fate. Besides, *Sox2* prevents cell differentiation though the cooperation with Notch and BMP signalling pathways.

We like to propose a model in which an extended neural competence is early established in the otic placode with the early expression of *Sox2* and *Sox3* genes. The cooperation between *Sox2* and *Sox3* then provides a high concentration of SoxB1 protein and promote neuronal fate. In parallel, *Serrate1* maintains *Sox2* expression in restricted domains, after Sox3 down-regulation. These domains retain the neurosensory competence and thereby develop as sensory patches.

RESUMEN

Los experimentos descritos en esta tesis tuvieron por objetivo estudiar la función de *Sox2* y *Serrate1* en el desarrollo de los elementos neurosensoriales del oído. En primer lugar describimos el patrón de expresión de *Sox2* durante el desarrollo del oído y lo comparamos con el de *Sox3* y *Serrate1*. En segundo lugar, mostramos los resultados de experimentos de electroporación *in ovo*, diseñados para manipular exógenamente la expresión génica y estudiar la ganancia de función de *Sox2* y *Serrate1*. Los efectos sobre el destino celular y las dianas moleculares se analizaron mediante hibridación in situ, inmunocitoquímica y *real-time PCR* (qRT-PCR).

Los resultados muestran que *Sox2* se expresa en el dominio neurosensoerial del epitelio ótico durante la fase de neurogénesis y, más adelante, durante el desarrollo de los parches prosensoriales y los órganos sensoriales. Con la diferenciación, *Sox2* es excluido de las neuronas diferenciadas y las células ciliadas, pero permanece expresado en las células de soporte. *Sox3* se coexpresa con *Sox2* en el dominio neurogénico de la copa ótica. Pero entonces, la expresión de Sox3 se reduce y sólo *Sox2* persiste en los precursores sensoriales, en donde se co-expresa con el ligando de Notch *Serrate1*. El dominio de expresión de Serrate1 está inicialmente contenido en el de *Sox2*, sin embargo, más adelante, *Sox2* se restringe dentro de los límites de Serrate1, un proceso que es concomitante con la formación de los parches sensoriales. Estos experimentos sugieren que : 1) *Sox2* se correlaciona con el destino neurosensorial de la placoda ótica, 2) la neurogénesis está asociada con *Sox2* y *Sox3*, y 3) el desarrollo sensorial está asociado a la expresión de *Sox2* y *Serrate1*

Los estudios de ganancia de función muestran que *Serrate1* regula el destino prosensorial y el desarrollo de los órganos sensoriales mediante el mantenimiento de la expresión de *Sox2* en dominios restringidos del otocisto, sin afectar a la neurogénesis. Serrate1 opera en un modo dependiente de Notch, consistente con un mecanismo de inducción lateral que comprende la inducción de su propia expresión y la de las dianas de Notch *Hes1*, *Hey1* and *Hey2*. Estudios similares sobre *Sox2* indican que *Sox2* especifica el destino neurosensorial en el epitelio ótico. Sin embargo, las concentraciones altas de *Sox2* suprimen el destino sensorial y promueven el destino neuronal. Además, *Sox2* previene la diferencoiación celular mediante la cooperación con Notch y Bmp.

Se propone un modelo en el cual la competencia neural se establece tempranamente en la placoda ótica mediante la expresión temprana de *Sox2* y *Sox3*. La cooperación entre *Sox2* y *Sox3* provee una alta concentración de factores SoxB1 que promueven el destino neuronal de los progenitores. En paralelo, Serrate1 mantiene la expresión de *Sox2* en dominios restringidos tras la supresión de *Sox3*. Estos dominios, retienen el potencial neurosensorial y, más adelante, se desarrollan como parches sensoriales.

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INTRODUCTION

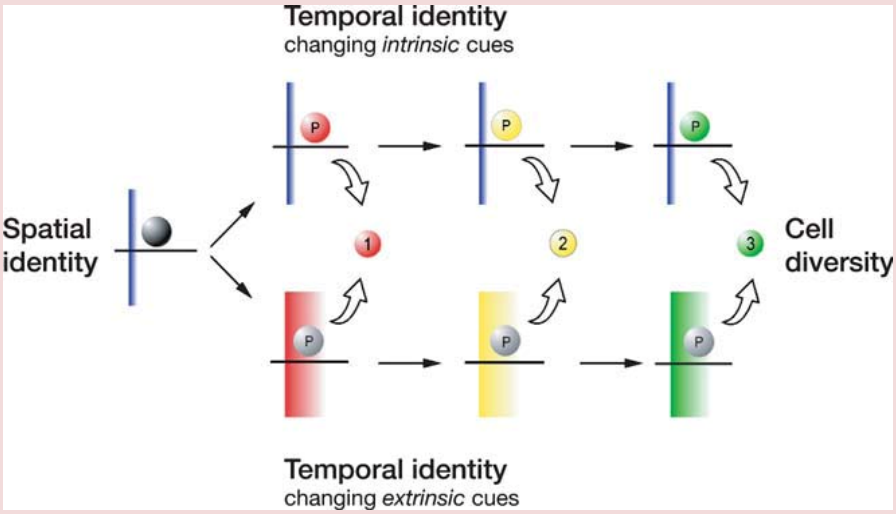
I. The development of multi-cellular organisms: an overview

Embryonic development is one of the most intriguing problems of biology. As stated by Lewis Wolpert in 1990, it deals with how "*From the single cell, the fertilized egg, come large numbers of cells – many millions in humans – that consistently give rise to the structures of the body.* […] *It is remarkable that a cell as overtly dull and structureless as the fertilized egg can give rise to such varied and complex forms. The answer lies in cell behaviour and how this behaviour is controlled by genes. Genes control development*". The way that leads to the generation of complex organs, like the inner ear, requires that cells are constantly able to make decisions on lineage commitment, differentiation, proliferation and death status. To keep uncommitted and with the ability of making decisions, cells needs to deal with the problem of avoiding cell cycle exit and differentiation, without falling into uncontrolled proliferation and tumour formation (Doe, 2008).

The problem of cell fate specification is central to development. How cell types with defined phenotypic characteristics can originate from a multipotent cell? The process usually follows the principle of potency restriction in which a multipotent cell– defined as **progenitor** – can give rise to different cells whose potential is restricted to fewer cell types generated upon terminal division – defined as **precursor**. The progenitor is a proliferating cell, capable of self-renewing through symmetric and asymmetric cell divisions. Symmetric cell divisions result in the expansion of the progenitor pool, whereas asymmetric cell divisions ensure the maintenance of a progenitor pool while generating specific lineages. The problem of cell fate specification is closely related to cell lineage. Lineage refers to the sequence of generation of the different cell types and the clonal relationship between them. The molecular basis of cell fate specification relies on the regulation of gene expression, so that fate commitment is associated with the up- or down-regulation of specific sets of genes, whose expression is responsible for the phenotypic treats that define a particular fate. This is why transcription factors and intercellular signalling pathways are crucial for cell fate specification (Pearson and Doe, 2004).

The study of human development makes use of model organisms, accessible for genetic manipulation, to study process and conditions that cannon be directly addressed in humans. The fruit fly (*Drosophila melanogaster)* has been one of the most powerful model organisms for the study of genetics in development. It has been used since the beginning of the 20th century in studies that led to the identification of many of the genes known today to control developmental processes in vertebrates. Among vertebrates, frog (*Xenopus laevis*), zebrafish (*Danio rerio*), chick (*Gallus gallus*) and mice (*mus musculus*) are the main model organisms used to study development. The chick remains one of the most powerful tools for studying gene function in development, because it allows the control of gene expression and tissue manipulations under defined temporal and spatial conditions.

BOX I.1: Time, space, intrinsic and extrinsic cues in cell fate



There are two major and interrelated questions associated with the problem of cell fate specification: First, that of time *versus* spatial cues and secondly that of intrinsic *versus* extrinsic factors. *Temporal specification* refers to the case in which the fate of the cell is specified with the basis on a temporal cue, the time at which the cell is born. *Spatial specification* refers to the case in which cell fate specification depends on the positional value of the cell in respect to the developing structure. *Intrinsic specification* refers to a process in which cell fate specification is dependent on autonomous properties of the cell resulting in birth order following an invariant cell lineage. *Extrinsic specification* refers to a process in which cell fate specification is dependent on external cues that modulate the fate producing variable cell lineages. Temporal *versus* spatial, and intrinsic *versus* extrinsic mechanisms of cell fate specification are not necessarily independent. (Pearson and Doe, 2004).

In this thesis I have addressed the problem of cell fate specification during the development of the neurosensory elements of the inner ear and, more specifically, I studied the function of two genes, *Sox2* and *Serrate1*. The chick embryo was used as a model organism because it allows precise manipulation of gene expression at specific times of development and in defined regions of the developing ear.

In the Introduction that follows, I will review the structure and development of the inner ear, with particular reference to the chick. Then, I shall discuss the state of the art on the function of SoxB1 gene group and, the knowledge on Serrate1 and the Notch pathway, with particular reference to neural development and ear development.

II. The vertebrate inner ear

The ear is a sensory organ with a complex three-dimensional structure. Functionally, the ear is the organ responsible for the perception of sound, balance and detection of acceleration. Evolutionarily, the external and middle ear are the solution found to the need of stimulation of hair cells in a liquid medium with sound waves produced in a less dense medium, the air. They are responsible for the collection, transmission and amplification of the acoustic waves produced in the environment and for directing and transforming them into mechanical vibration of the endolymphatic fluid contained in the inner ear. The inner ear contains the sensory organs that transform the mechanical perturbations generated by sound and accelerations into electrical nerve impulses [Mallo, 2003, Torres and Giraldez, 1998].

II.1 The structure of the vertebrate inner ear

The inner ear is composed of the bony labyrinth and membranous labyrinth. The bony labyrinth includes three different cavities, within the temporal bone cavity, which are the semicircular canals, the vestibule and the cochlea (the lagena in birds). It is filled with perilympha, a fluid, similar in composition to the cerebral spinal fluid and where the membranous labyrinth floats. The membranous labyrinth is filled with the endolympha and is lined by specialized epithelial tissues, which can be subdivided by its function in sensory and non-sensory [Romanoff, 1960; Condon, 2004; Bissonnette and Fekete, 1996].

The membranous labyrinth consists of an array of ducts and sacs and can be subdivided according to its morphology and function into a **vestibular** and an **auditory** part. The **vestibular** component of the inner ear is located dorsally and is further subdivided into the three *semicircular ducts* that are oriented in nearly orthogonal planes (anterior, lateral and posterior), two sacs hosted by the vestibule – the *saccule* and the *utricle* – *and* the *endolynphatic duct and sac* (EDS). Each of the three semicircular ducts is a tube shaped structure that ends in swollen structures called ampulla, which contains a specialized sensory epithelium known as crista. Two of the ampullae are located anteriorly, the superior and the lateral, and contain the superior and lateral cristae (superior crista is also referred to as anterior crista). The third ampulla is located posteriorly and hosts the posterior crista. The two connecting sacs, the utricle and the saccule, are each lined by specialized sensory epithelium known as maculae (macula utriculi and macula sacculi) [Condon, 2004; Bissonnette and Fekete, 1996]. Finally, the EDS is another tube-shaped structure located dorsally, lined solely by non-sensory epithelium that is connected to the endolymphatic compartment, which is filled with endolymph, a potassium-rich fluid that bathes the luminal side of the sensory epithelium. The functions of the EDS is the regulation of volume and pressure of the endolymph, the immune response of the inner ear and the elimination of endolymphatic waste products by phagocytosis [Couloingner et al., 2004]. The **auditory** component of the inner ear is restricted to the auditory duct, located ventrally. In birds, the lagena is a relatively straight tube that extends medially and ventrally. It hosts the auditory sensory epithelium, named *basilar papilla.* and a

vestibular sensory epithelium named macula lagena. In higher vertebrates, the cochlear duct is a coiled shape structure, with both proximal and distal ends pointing posteriorlly and forming an arc shape structure. The cochlear duct of higher vertebrates is coiled and it only hosts auditory sensory epithelium, known as the organ of Corti [Condon, 2004; Bissonnette and Fekete, 1996] (Fig.1A).

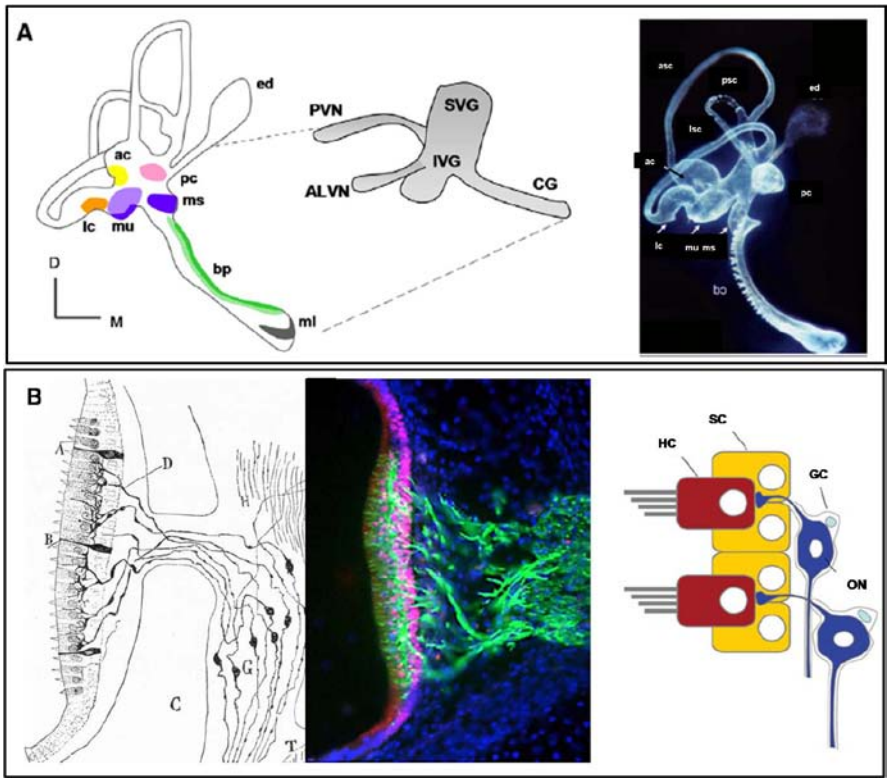


Figure 1: The vertebrate inner ear: Structure and functional unit. A: Schematic drawing of the inner ear and its innervations at HH30–31 (E7). (left) and corresponding structure of the membranous labyrinth of an E9 chick inner ear observed by paint filling (right). B: Functional sensory unit of the vertebrate inner ear. From left to right: Macula utriculi and vestibular ganglion in the four-day mouse from Ramon y Cajal and Tello (1928), "Elementos de Histología Normal y de Técnica Micrográfica"; Macula sacculi of an E7 chick embryo labelled with Sox2 (red), Tuj1 (green) and DAPI (blue); diagram representing the functional sensory unit of the inner ear with the four cell types: hair cells, supporting cells, otic neurons and glial cells. ac, anterior crista; lc, lateral crista; pc, posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ed, endolymphatic duct; ALVN, anterior-lateral vestibular nerve; IVG inferior vestibular ganglion; PVN posterior vestibular nerve; CG, cochlear ganglion; D, dorsal; M, medial; HC, hair cell; SC, supporting cell; GC, glial cell; ON, otic neuron. Adapted from Wu et al. (1998); Giráldez and Fritzsch (2007); Bell et al. (2008); Alsina et al. (2009).

II.2 The functional unit of the inner ear

The inner ear is the sensory organ responsible for the senses of motion, position and hearing, in response to sensory inputs of acceleration, balance and sound. The sense of motion and position are accomplished by its vestibular components in response to linear or angular accelerations, and the sense of hearing by the auditory component in response to sound [Purves et al., 2001]. All the sensory organs of the inner ear share a

common **functional sensory unit** composed of three main cell types: hair cells, supporting cells and otic sensory neurons. Supporting cells are located in the basal side of the sensory epithelium and their lateral membrane surrounds the hair cells which, in turn, project up to the luminal side. Sensory epithelia lie in between two extracellular structures, the basal lamina (or basal membrane), where supporting cells seat, and an extracellular structure associated with their luminal surface that varies in the different sensory organs: a cupula in the cristae, otolythic membrane in the maculae or tectorial membrane in the basilar papilla. Otic neurons innervate hair cells through the basal side, where they establish synaptic connections [Eatock and Newsome, 1999; Nayak et al., 2007] (Fig.1B).

Hair cells are highly specialized mechansotrasnducer sensory cell types that contain a group of derived microvilli, referred to as *stereocilia*. Hair cells vary between sensory epithelium and even within sensory epithelium, and can be distinguished by differences in their morphology, electrophysiology and innervations. Vestibular epithelium contains type I and type II hair cells. Auditory epithelium also contains two types of hair cell, tall and short hair cells in birds, that correspond to inner and outer hair cells in mammals [Eatok et al., 2006].

Supporting cells vary greatly in morphology and functional specialization and in vestibular sensory organs the population of supporting cells is largely homogenous. In the mammalian cochlea at least four unique types of supporting cells can be identified (Kelley, 2006). In addition to the scaffolding of the sensory epithelium, they maintain the correct ionic environment for the function of hair cells and they release factors that maintain the trophism and survival of hair cells (Bayly et al., 1999). Furthermore, they retain progenitor characteristics which enable them to regenerate hair cells after injury [Corwin and Cotanche, 1988; Ryals and Rubel, 1988; White et al., 2006; Stone and Cotanche, 2007].

Otic neurons are bipolar primary afferent neurons that connect the hair-cells with second order neurons in the vestibular and auditory nuclei in the brainstem. Their somas are intermingled with glial Schwann cells forming the cochlear and the vestibular ganglia (CVG, VIIIth cranial nerve) [D'Amico Matel, 1982; Rubel and Fritzsch, 2002].

II.3 The development of the vertebrate inner ear

The vertebrate ear develops from the **otic vesicle**, which in turn is the result of the proliferation, growth and invagination of the **otic placode** [Fekete, 1996; Torres and Giraldez, 1998; Kelley, 2006; Giraldez and Fritzsch, 2007; Alsina et al., 2009] (Fig.3). The otic placode is the first morphological evidence of the specification of the ear territory [Kupffer, 1895], and in some vertebrates, it signifies an irreversible commitment to generate the inner ear. The current view on the developmental origin of the otic placode is that it arises from a preplacodal territory which is competent to generate any placode but not yet specified to develop into any particular one. It is not only after a succession of inductive processes that one portion of the preplacodal ectoderm is

specified to become the otic placode. This process is frequently referred to as “otic induction”.

BOX II.1: Signal transduction by hair cells

The hair cells of the sensory epithelium use the hair bundle of steriocilli in their luminal surface to detect the mechanical stimuli generated by the movement of the endolymph, bending back and forth. Auditory stimuli induce a vibration in the basilar membrane while vestibular stimuli cause displacement of the extracellular structures overlaying hair cells. Linear acceleration stimuli are sensed and transmitted by otolythic membrane, overlaying the saccule and utricle, while rotational stimuli are sensed by the cupula of the cristae. The movements produced by the endolymphatic fluid in those structures cause excitatory bundle deflections that directly open transduction channels which admit cations and depolarize the hair cell. The endolymph is produced by a specialized region of the otic epithelium: the *stria vascularis* (*tegmentum vasculosum* in birds) and its composition with high K^+ concentration and low Na^+ concentration is essential for the process of transduction by hair cells. The ionic gradients established between the endolymph and the intracellular compartment generates an electromotive force that depolarizes the hair cell membrane upon opening of mechanosensitive K^+ channels. This depolarization of the membrane increases neurotransmitter release from the graded synapses on the baso-lateral surfaces of the hair cells. Post-synaptic afferent fibers of the VIIIth cranial nerve innervating hair cells transmit their signals to the large cochlear and vestibular nuclear complexes on the brainstem’s *medulla* and *pons*. The cochlear nuclei transmit the signal though the *pons*, midbrain and thalamus into the cerebral cortex. Auditory hair cells are also innervated by efferent fibbers that carry signals from the brain into the ear and thus influence cochlear function. The vestibular nuclei, send information to the cerebellum, oculomotor system and spinal cord [Eatock and Newsome, 1999; Condon, 2004; Eatock et al., 2006].

The otic placode is only morphological visible by 10 somite-stage in chicken embryos. However there is evidence of an earlier specification of the prospective otic field or otic primordium, as defined by the expression of molecular markers such as *Pax2* (4ss), *Sox3* (6ss) *Bmp7*(7ss), and *Notch1* (9-10ss) (Groves and Bronner-Fraser, 2000). The process of otic specification in the ectoderm is the result of inductive signals from the surrounding tissues operating in a coordinated fashion. The current vision postulates a three-step model for otic placode induction: pre-placodal induction followed by pre-otic character induction and finally otic placode induction [Ohyama et al., 2007] (Fig.2A).

The pre-placodal territory is initially specified as an ectodermal domain in between the neural plate and the epidermis, characterized by the expression of a set of genes – *Foxi*, *Msx*, *Dlx* and *Sox3*. As development proceeds, this pre-placodal domain is segregated from the neural crest domain and expresses a specific set of genes *Six/Eya/Dach* in a process that requires the interaction between the presumptive pre-placodal domain and the surrounding tissues, involving FGFs and BMP and Wnt antagonists (Streit, 2007). The pre-placodal region is competent to form all cranial placodes and specific inducing signals are responsible for the specification of otic identity in a multi-step process

[Ohyama et al, 2007]: First, the pre-otic region is induced by FGF signalling which is characterized by the expression of Pax2 [Martin and Groves, 2006; Ohyama et al., 2007]. After that, Wnts are responsible for the discrimination between **otic placode** and epidermis, through the repression of the epidermis-specific factor *Foxi2* [Ohyama et al, 2006]. An finally, Wnt signalling activates several components of the Notch signalling pathway which in turn augment the canonical Wnt signalling to specify the size of the otic placode (Jayasena et al., 2008) (Fig.2A).

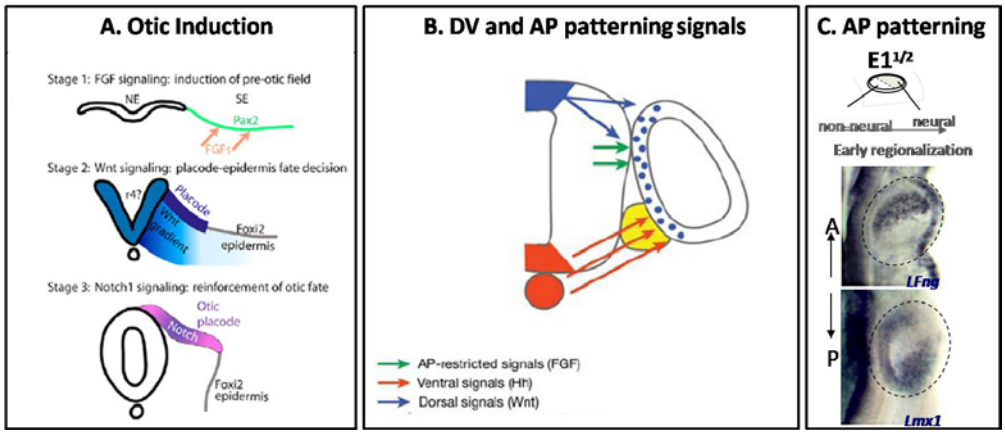


Figure 2: Otic inductions and otic patterning. A: three step model for otic induction consisting of induction of pre-otic field by FGF, placode-epidermis discrimination by Wnt and reinforcement of otic placode fate by Notch. B: DV and AP patterning of the otic vesicle by neural tube signals. FGFs specify AP axis. Hh ventral signals and Wnt dorsal signals specify DV axis. C: Evidenc of AP patterning in the chick otic placode by complementary expression of *LFng* and *Lmx1*. Adapted from Alsina et al. (2004) Schneider-Maunoury and Pujades (2007); Jayasena et al. (2008).

II.4 Otic patterning and morphogenesis

Axial patterning is an important step of inner ear development to provide the positional cues required for the formation of the individual inner ear components in the correct place and with the correct orientation. Inner ear patterning is evident along three axis: anterior-posterior (AP), dorsal-ventral (DV) and medial-lateral (ML) and is regulated by inductive signals from the surrounding tissues, that results in and/or maintains asymmetries in gene expression. In chick the specification of the AP axis precedes the specification of the DV axis, while the ML axis is the first one to be specified [Wu et al., 1998; Bok et al., 2007; Whitfield and Hammond, 2007]. The positional cues provided by the axial specification seem to segregate regions of the otic epithelium where the different sensory organs and corresponding neurons develop, as revealed by recent cell tracing studies (Bell et al., 2008).

DV axial patterning reflects the separation of the inner ear in two different parts, the dorsal vestibular component and ventral auditory component and is evident by the segregation of gene expression domains which are established before the commitment to a fixed DV axis (Wu et al., 1998). DV axial patterning is regulated by signals from the hindbrain, Wnts from the dorsal neural tube, and Sonic hedgehoc from the floor plate and notochord [Liu et al., 2002; Riccomagno et al., 2002; Bok et al., 2005; Ricommagno

et al., 2005, Bok et al., 2007]. A potential role of BMP and FGF signalling in this process has also been suggested [Bok et al., 2007; Whitfield and Hammond, 2007] (Fig.2B).

AP axial pattering is established prior to the DV axis and reflects the separation of two domains of gene expression, named the neurogenic (also called proneural) and non-neural domains. The neurogenic domain is located in the anterior part of the otic placode and projects ventrally as the otic vesicle invaginates, whereas the non-neural domain is located in the posterior-lateral and ridge areas, and extends dorsally upon formation of the otic vesicle. These two domains show limited cell intermingling [Abelló et al, 2007: Abelló and Alsina, 2007]. The anterior domain is characterized by the expression of *Sox3*, *Fgf10*, *LFng*, BEN. The non neural domain is characterized by the expression of *Irx1*, *Lmx1b*, HNK-1 and *Hairy1*. [Giraldez, 1998; Cole et al, 2000; Goodyear et al, 2001; Alsina et al, 2004; Abelló et al, 2007, Vazquez-Echeverria et al., 2008]. FGF, BMP and Notch signalling have been involved in the establishment of the AP patterning. FGFs and BMPs differentially regulate *Sox3* and *Lmx1*, and their restriction to the anterior and posterior domain in chick [Abelló et al., 2009]. In mice, FGF signalling from the hindbrain also influence AP patterning (Vazquez-Echeverria et al., 2008). The Notch signalling pathway is also involved in AP regionalization. The differential expression of Notch family members between the anterior and posterior domains suggests a differential activation of the Notch pathway in the two domains. Notch activation is required for the restriction of the posterior genes *Lmx1* and *Irx1* to the non-neural domain (Abello et al., 2007). *Tbx1* is also required for the correct AP patterning of the inner ear.(Raft et al., 2004; Bok et al., 2007a) (Figs.2B and C).

The axial patterning of the inner ear is translated into a morphogenetic process during development resulting in the formation of the adult sensory organs and associated non-sensory structures. Evidence suggests that a molecular interplay between the sensory and non-sensory components of the inner ear is important for their coordinate and correct formation (Pirvola et al., 2000; Bok et al., 2007b). The signalling mechanisms involved in the morphogenesis of the different sensory organs is complex and probably distinct for each type of sensory organ as discussed in Bok et al., (2007).

II.5 Cell fate specification during inner ear development

The different cell types of the epithelium of the membranous labyrinth are derived from otic progenitors residing in the otic placode, with the exception of the glial Schwann cells of the CVG which are of neural crest origin (reviewed in Alsina et al., 2009). The generation of hair cells, supporting cells, otic neurons and non sensory cells requires a process of cell fate commitment that is tightly regulated in space and time. The commitment of otic progenitors follows a stereotyped spatial and temporal sequence, with otic neurons being specified prior to hair cells and dorsal structures prior to ventral structures [Bell et al., 2008; Alsina et al., 2009] (Fig3).

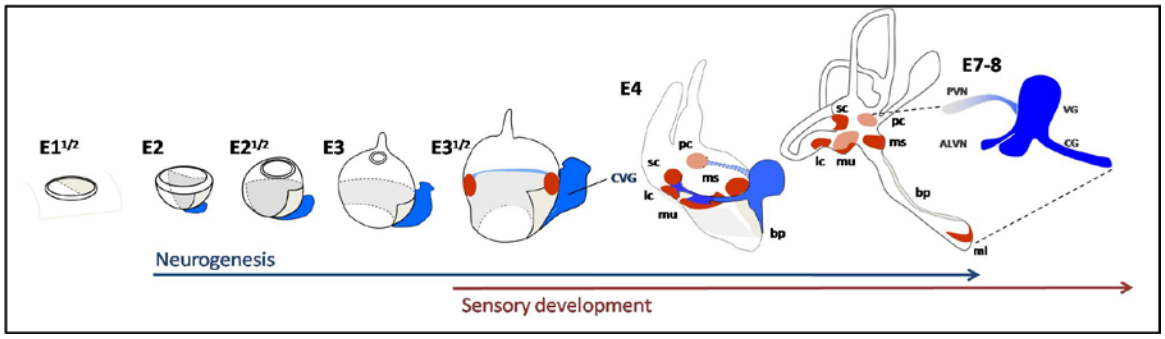


Figure 3: Overview of chick inner ear development. Otic placode invaginates to form the otic cup thta pintches off the ectoderm to from the otic vesicle. The otic vesicle goes though an intensive growth and morphogenetic period when the ducts and sacs develop to give rise to the mature organ Neurosensory domain (grey), cochleo-vestibular ganglion (blue), sensory organs (pink). Neurogenesis starts by day 2 in the chick otic cup and neuroblasts delaminate to form the neurogenic domain, to form the cochleo-vestibular ganglion. Delayed by 1,5 days, sensory patches emerge within the neurosensory domain. Adapted from Alsina et al. (2004), Neves et al. (2007) and Bell et al., 2008. ac, anterior crista; lc, lateral crista; pc, posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ed, endolymphatic duct; ALVN, anterior-lateral vestibular nerve; IVG inferior vestibular ganglion; PVN posterior vestibular nerve; CG, cochlear ganglion CVG, cochleo-vestibular ganglion.

Otic Neurogenesis

Otic neurons of the cochlear and vestibular ganglia derive from the otic placode, in contrast to what happens with most cranial ganglia in which neurons have a dual placodal and neural crest origin [D'Amico-Martel and Noden, 1983]. Neurogenesis in the ear starts at the otic placode, where neuronal fate is specified along with AP regionalization. As development proceeds neuroblasts delaminate from the anterior-ventral part of the otic vesicle to form the cochleo-vestibular ganglion. Delaminated neuroblasts proliferate to finally differentiate into vestibular and cochlear neurons that populate the corresponding vestibular and cochlear ganglia, and innervate back the sensory organs (Alsina et al., 2004). Neurogenesis in the ear follows similar principles as in the central nervous system. Neuronal fate is specified by the expression of proneural genes, like *Neurogenin1* (Henrique et al., 1995; Adam et al., 1998; Ma et al., 1998; Alsina et al., 2004). *Neurogenin1* is expressed in epithelial neuroblasts but not in delaminated neuroblasts, and it is the master gene for the specification of the neuronal fate. Neuroblasts are singled out through the Delta-Notch pathway and *NeuroD* and *NeuroM*, that are up-regulated in epithelial neuroblasts and drive delamination and neuronal differentiation. Later on, differentiation markers, like the LIM homeodomain transcription factor *Islet 1* and the neuron specific βIII-tubulin, Tuj1, are expressed in delaminated neuroblasts (Li et al., 2004). *Neurogenin1* and *NeuroD-M* are basic helix-loop-helix (bHLH) proteins involved in neuronal fate determination and neuronal differentiation and survival [Kim et al, 2001; Liu et al, 2000; Ma et al, 1998, 2000]. Notch signalling regulates neuronal production though the process of lateral inhibition (Abello et al., 2007; Daudet et al., 2007). FGF signalling has been shown to be important to neuronal fate specification and irreversible neuronal commitment. (Alsina et al., 2004) and IGF-1 is also important for survival, proliferation and differentiation of neuroblasts

(Camarero et al., 2003). Recent work has also shown that *Sox3* is crucial for the initial steps of otic neurogenesis: *Sox3* is expressed before proneural specification and accompanies the restriction of the neurogenic domain (Abello et al., 2007). Overexpression of *Sox3* results in ectopic *Delta1* and *Ngn1* expression and ectopic neurogenesis [Abelló et al., 2009; Safia Khatri, PhD Thesis] (Fig.4).

The development of sensory organs

During inner ear development, neurogenesis is followed by sensory development. Sensory fate specification occurs between days 4-8 of chicken development, and differentiation starts about day 5 and proceeds until hatching. Sensory progenitors develop within the prosensory patches. The current idea is that the prosensory patches emerge from a common domain of neurorosensory competence. Molecular markers for the sensory organs are *Bmp4* (Wu and Oh, 1996), *Ser1* (Adam et al., 1998) and *LFng* (Morsli et al.; Cole et al., 2000). *LFng* is not exclusively a sensory marker since it is also expressed in the neurogenic domain (see above). As to the role of *Bmp4*, it is probably not involved in sensory patch specification, but in the control of self-renewal and survival of sensory progenitors (Pujades et al., 2006). The function of *Sox2* and *Serrate1/Notch* will be reviewed in detail below (Fig.4)

One of the major morphogenetic events that precedes the individual development of each sensory organ is the division of the neurosensory competent region into various sensory patches, a process that is still not well understood, but in which Wnt signalling has been shown to take part (Stevens et al., 2003; Sienknecht and Fekete, 2008, 2009). The different sensory organs arise from specific locations in the otic epithelium, which are characterized by the expression of different sets of genes [Wu et al. 1998; reviewed in Bok et al., 2007; Bell et al., 2008].

The commitment to the sensory fate is associated with *Atho1*, a bHLH transcription factor, considered a master gene for hair cell fate specification. *Atho1* acts as proneural determination gene specifying cells committed to the sensory lineage, and the Notch signalling pathway is required for hair cell differentiation and lateral inhibition (Fig.4, see below).

The common origin of neurones and sensory cells

Available data suggest the existence of a neurosensory competent domain with neurogenic and prosensory competences, that gives rise first to neurones and then to sensory cells. Fate mapping studies in chick suggest that the sensory organs and neurons that innervate them arise from this common domain (Bell et al., 2008). Genetic studies in mouse have also demonstrated that neurones and hair cell originate from a common cell population, but according to that study that dual potency would only exist at the macular level that overlaps with the neurogenic domain (Raft et al., 2007). Using retroviral infection of otic vesicles and clonal analysis, Donna Fekete and co-workers had provided evidence that: 1) hair cells and supporting cells derive from a common precursor (Fekete et al., 1998), and 2) neurones and hair cells may also share a common lineage (Satoh and Fekete, 2005).

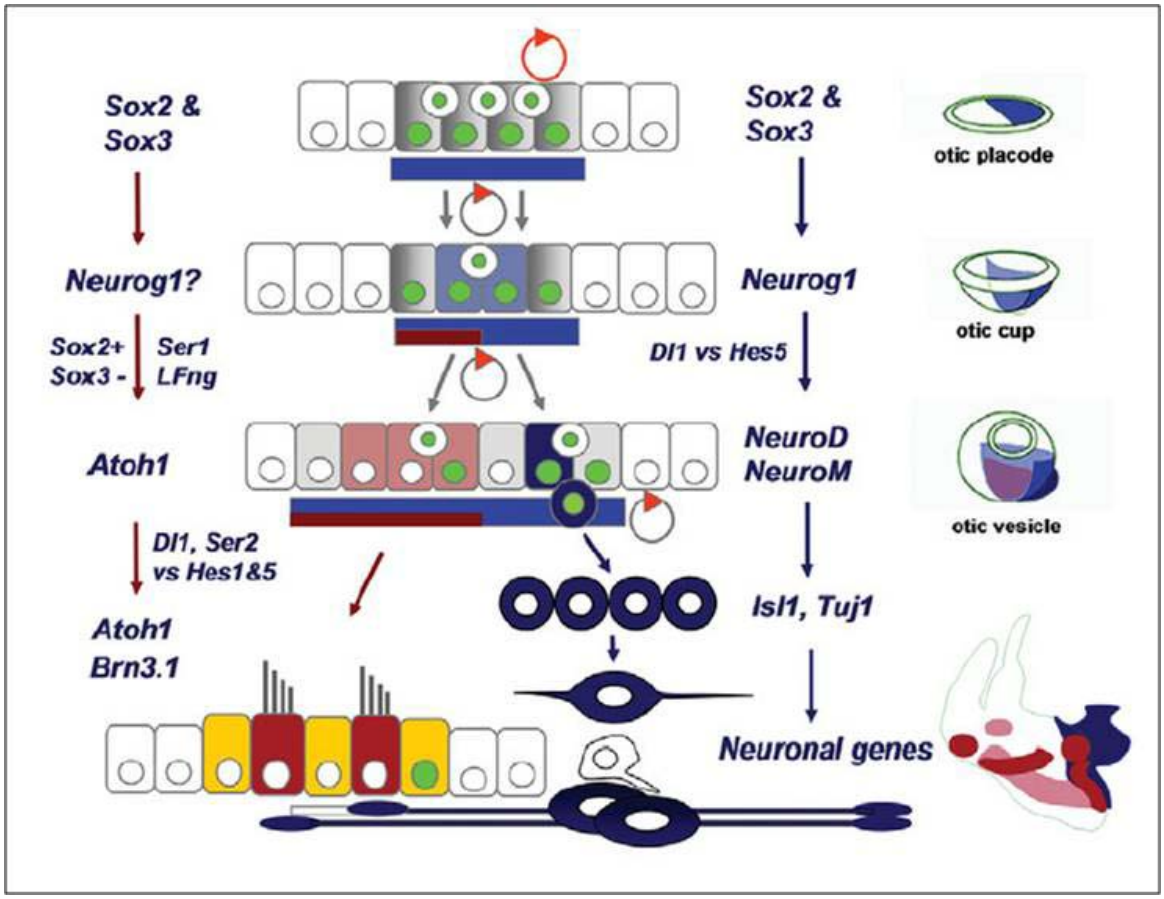


Figure 4: Cell fate specification during inner ear development. The diagram shows a model of hair cell and neurone specification during ear development in amniotes. The sequence of gene expression for sensory (left) and neuron development (right), is indicated. The neural competent domain is common for the two lineages and expresses genes of the *SoxB1* group. This domain is specified either by temporal and/or spatial cues to give rise to the two main lineages: sensory and neuronal. The bars under the epithelia indicate neural competence (blue) and prosensory specification (brown). Neuronal specification takes place by the enhanced expression of *Nng1* via the Delta-Notch pathway, and the subsequent expression of NeuroD and NeuroM proneural genes. The latter allow delamination and transient amplification of neuronal precursors within the ganglion. Hair cells are singled out within *Atoh1* clusters, under the sustained expression of *Dl1*/*Ser2*, thour the process of lateral induction. From Alsina et al. (2009).

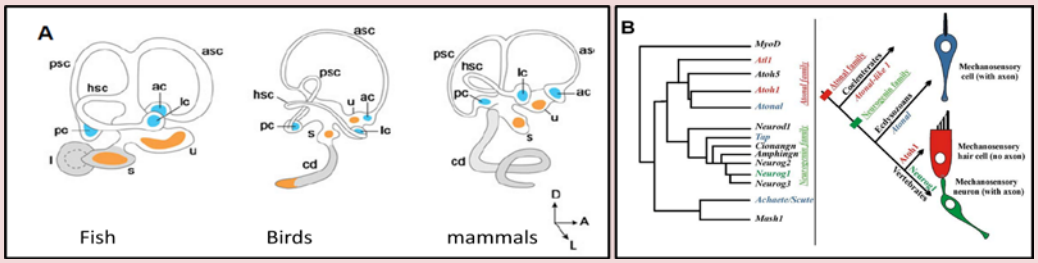
Time specification of the otic lineages

As mentioned above, the generation of the otic neural lineages follows a stereotyped time course (Bell et al., 2008). This is reminiscent of what occurs in other sensory organs like the retina where cell lineage is also established in a precise temporal sequence. In the retina this is the result, at least in part, of cell intrinsic mechanisms that dictate the sequence of cell-type generation from a common progenitor (Livesey and Cepko, 2001; Cayouette et al., 2003). Whether this is the case in the ear is not known. Otic axon guidance seems to depend on intrinsic cues and the bias to innervate specific targets of the ear occurs very early during neurogenesis. Studies in mouse showed that specification of vestibular and cochlear neuronal subtypes depends on their birth date

and are independent on their interactions with hair cells [Koundjakjin et al., 2007]. This temporal specification seems to be superimposed on sptial cues along the AP axes (Bell et al., 2008).

BOX II.2: Evolution of the vertebrate inner ear

The evolutionary origin of the ear is under discussion. Some evidence support its evolution from a pre-existing lateral-line-like system, while others suggest its independent evolution from a pre-existing structure of similar function (Fritzsch et al., 2007). There is a high conservation of the vestibular part of the ear among vertebrates that contrasts with the evolutionary modifications observed in the auditory part. Data suggests that the auditory organs evolved from vestibular organs (Fritzsch et al., 2002). Saccule and lagena which are auditory organs in fish, play a vestibular role in mammals and birds, with the lagena being absent in the former. The auditory cochlear portion observed in birds and mammals has no counterpart in fish and amphibians and it is thought to have evolved through embryonic transformation of parts of the saccule (Riley and Phillips, 2003) (A).



Modified from Alsina et al. (2009) and Fritzsch et al. (2006)

The evolution of the sensory functional unit containing a secondary mechnaosensory hair cell and sensory neuron from a primary mechanosensory hair cell with an axon has also been proposed. This evolution correlates temporally with the evolution of the *Atonal* and *Neurogenin* families of bHLH genes (see below). The *Atonal* family of bHLH genes is present in the ancestral primary mechanosensory hair cell and the emergence of the secondary mechanosensory cell and the sensory neuron is associated with the emergence of the *Neurogenin* family of bHLH genes (Fritzsch et al., 2006) (B).

III. SoxB1 transcription factors

III.1 Sox proteins: structure and function

The *Sox* gene family codes for a group of developmental transcription factors that belong to the group of High Mobility Group (HMG) box domain proteins. They are present only in the animal kingdom and must have evolved from canonical HMG domain proteins (Bowles et al., 2000). HMG box domain proteins subdivide into two families: the Sox family and the T-cell factor (TCF)/lymphoid enhancer binding factor (LEF-1) family. The HMG box domain shares only 20% similarity with the canonical HMG domain, but it conserves the amino acids responsible for the ability to alter DNA confirmation, with a few changes in residues that allow DNA bending at a flexible angle rather than a fixed one. Furthermore, HMG box domain binds DNA with some sequence specificity (Kiefer, 2007; Lefebvre et al., 2007).

The *Sox* gene family is named after the first *Sox* gene to be discovered, the *Sry* (sex-determining region on the Y chromosome) (Gubbay et al., 1990; Sinclair et al., 1990), and the acronym Sox stands for “Sry-related HMG box”. The *Sox* gene family harbours genes with a HMG box domain which shares ≥46% similarity with the HMG box domain of the *Sry* and contain the conserved motif, RPMNAFMVW. All Sox proteins bind DNA preferentially in the hexameric core sequence 5′-WWCAAW-3′, where W indicates A or T (Bowles et al., 2000; Kiefer, 2007). In vertebrates, there are twenty different *Sox* genes, classified in 8 groups A to H, with two B subgroups, B1 and B2, based on protein sequence comparison (Table1). Sox proteins within the same group share 70-95% degree of identity both within and outside the HMG box domain, whereas Sox proteins from different groups share partial identity(≥46%) in the HMG box domain and none outside this domain (Bowles et al., 2000; Kiefer, 2007; Lefebvre et al., 2007). *Drosophila melanogaster* and *Caenorhabditis elegans* have only five and eight *Sox* genes, respectively but, interestingly, each *Sox* gene in these species corresponds to a different vertebrate *Sox* group or subgroup (Bowles et al., 2000).

Most Sox proteins also feature other functional domains outside the HMG box. These domains have been highly conserved among orthologues as well as among members of the same group, and they are totally different between proteins from distinct groups. They include transactivation, transrepression and dimerization domains (Kamachi et al., 1998; Uchikawa et al., 1999; Lefebvre et al., 2007). The Sox B1, C, E and F proteins, which account for 12 of the 20 Sox proteins, feature a potent transactivation domain in their C-terminal region, and this domain in Sox2 and Sox9 physically interacts with the transcriptional co-activator CBP/p300 (Kamachi et al., 1998; Lefebvre et al., 2007). Sox B2 and Sox G act as transcriptional repressors [Uchikawa wt al, 1999; Beranger et al., 1999]. Sox D proteins may modulate transcription both as co-activators and as repressors (Table 1). Interestingly, Sox Neuro, the *Droshophila* ortholog of SoxB1 acts as a transcriptional repressor (Chao et al., 2007) a function that has also been observed in vertebrates Sox B proteins (Paul Scotting, personal communication). Sox proteins, therefore, must contribute to the activity of enhancersomes not only through architectural

roles, but also through direct interaction with partners of the transcriptional machinery. Furthermore, Sox D and Sox E proteins are also able to homodimerize and thereby to increase their DNA-binding affinity to pairs of adjacent binding sites (Lefebvre et al., 1998; Lefebvre, 2002).

Group	SoxA	SoxB1	SoxB2	SoxC	SoxD	SoxE	SoxF	SoxG	SoxH
Genes	*Sry*	*Sox1* *Sox2* *Sox3*	*Sox14* *Sox21*	*Sox4* *Sox11* *Sox12*	*Sox5* *Sox6* *Sox13*	*Sox8* *Sox9* *Sox10*	*Sox7* *Sox17* *Sox18*	*Sox15*	*Sox30*
C-terminal domain									

Table 1: Classification of Sox proteins and their function as activators (green) or repressors (red) of gene expression. Yellow stands for both activator and repressor functions.

Sox proteins play a central role in a large variety of developmental processes like testis development [Berta et al., 1990; Koopman et al., 1991; Vidal et al., 2001; Chaboissier et al., 2004], neurogenesis (Pevny and Placzek, 2005; Wegner and Stolt, 2005), oligodendrocyte development (Wegner and Stolt, 2005), chondrogenesis (Kiefer, 2007; Akiyama, 2008) and neural crest cell development (Kelsh, 2006; Kiefer, 2007). Although mechanisms of Sox function vary among cell types, there are some common features which can be generalized: 1) *Sox* genes regulate specification and differentiation of many cell types, 2) genes within Sox subgroups often share functional roles, 3) genes within one subgroup can counteract the function of genes in another subgroup, and 4) the same gene can mediate different stages of development in one cell type and/or developmental processes in more than one cell type (Kiefer, 2007).

III.2 Sox targets and partners

Sox proteins association with different partner transcription factors has been described for several Sox proteins (Kamachi et al., 2000; Wilson and Koopman, 2002). Sox partners include homeodomain, zinc finger, basic helix-loop-helix and leucine zipper proteins [Wismuller et al., 2006]. In all cases, the Sox and its partner interact through their DNA-binding domains and heterodimerization can also occur via the C-terminal part of the Sox HMG box domain and the DNA-binding domain of the other partner [Lefebvre et al, 2007; Kamachi et al, 2000; Wilson and Koopman, 2002] (Fig.1). Furthermore, the distribution of the partner factors is cell specific and may ascribe the cell specificity of the action of Sox proteins to their interaction with the cell-specific partner factors. In fact, protein fusion studies suggest that that the activation/repression activity can be mediated solely by the Sox protein when fused to a strong DNA binding domain, but that those fusion proteins exert their function without cell specificity, suggesting that the selectivity is conferred by the presence of the partner factor (Kamachi et al., 2000; Wilson and Koopman, 2002).

BOX III.1: The Regulation of Sox proteins function

Although Sox proteins exhibit some sequence specificity in respect to the DNA binding, several Sox proteins have been shown to bind DNA sequences in major target genes that only partially match the in vitro Sox consensus. In addition, Sox proteins alone bind DNA with a much lower affinity than other transcription factors (K_d ~ 10^{-7} to 10^{-9} M in contrast to K_d ~10^{-9} to 10^{-11}M) This suggests that DNA sequence is not the only factor that directs Sox proteins to their target genes in vivo (Kamachi et al., 2000; Lefebvre et al., 2007). Like their HMG domain relatives, Sox proteins may use the DNA configuration as one of several criteria to select their target genes, although this property remains to be demonstrated in vivo. Furthermore, Sox proteins have the ability to bend DNA at an angle that varies from as little as 30° up to as much as 110°, but surprisingly, even though their primary role could also be to assemble enhanceosomes, as described to his relative Lef-1, this role has not been demonstrated for any Sox protein yet (Lefebvre et al., 2007). In any case, individual HMG domains of the Sox proteins so far examined are similar to each other in their sequence preference and in their DNA-bending activity. Therefore, they alone cannot be the major determinant of the specific target site selection. In recent years, evidence has accumulated that Sox proteins form multi-protein complexes at gene promoters or enhancers, and that these complexes are likely to play a role in determining cell specificity of Sox proteins by accounting for its target selection (Kamachi et al., 2000; Wilson and Koopman, 2002) (see below).

Kamachi et al (2000) suggested a model for gene regulation by Sox proteins synergizing with their partners. According to that, in an *in vivo* situation where DNA is in a chromatin structure, the HMG domain of Sox is not sufficient to form a stable protein–DNA complex at a Sox site unless a partner factor is present, which interacts with the Sox protein and binds to a nearby DNA site. Transcriptional activation or repression by a Sox protein is achieved only under this condition. (Fig.1A) This model has been supported by recent data but, as suggested by Wilson and Koopman (2002), it does not explain the choice of partner where more than one is present. They suggest that, in addition to the function mediated by the HMG box domain, sequences outside that domain are required to stabilize protein binding and/or generate specificity (Fig.1B).

Although Sox proteins have been discovered more than twenty years ago, the number of genes identified as targets of Sox proteins is still limited. In agreement with the previous model, several target genes of Sox proteins have been identified in which a second site that can bind a partner factor is located nearby the Sox-binding site. This feature has been observed for different Sox proteins but I will center on *Sox2* gene, which is the subject of this work. *Fgf4*, *Nanog*, *Netsin*, UTF1, *Fbx5, Oct-4 and Sox2* have been identified as target genes of Sox2. In addition, the enhancer regions of those genes is regulated by the synergistic interaction between Sox2 and Oct-4 [Ambrosetti et al., 1999; Dailey et al., 1994; Johnson et al., 1997; Yuan et al., 1995; Nishimoto et al., 1999; Kuroda et al., 2005;Rodda et al., 2005; Tanaka et al., 2004; Tomioka et al., 2002; Okumura-Nakanishi et al., 2005; Tokuzawa et al., 2003]. Lefty1 is also regulated by the cooperative action of Sox2 and Oct-4, but in this case a third factor, Kfl4, synergizes with the previous two [Nakatake et al., 2006].

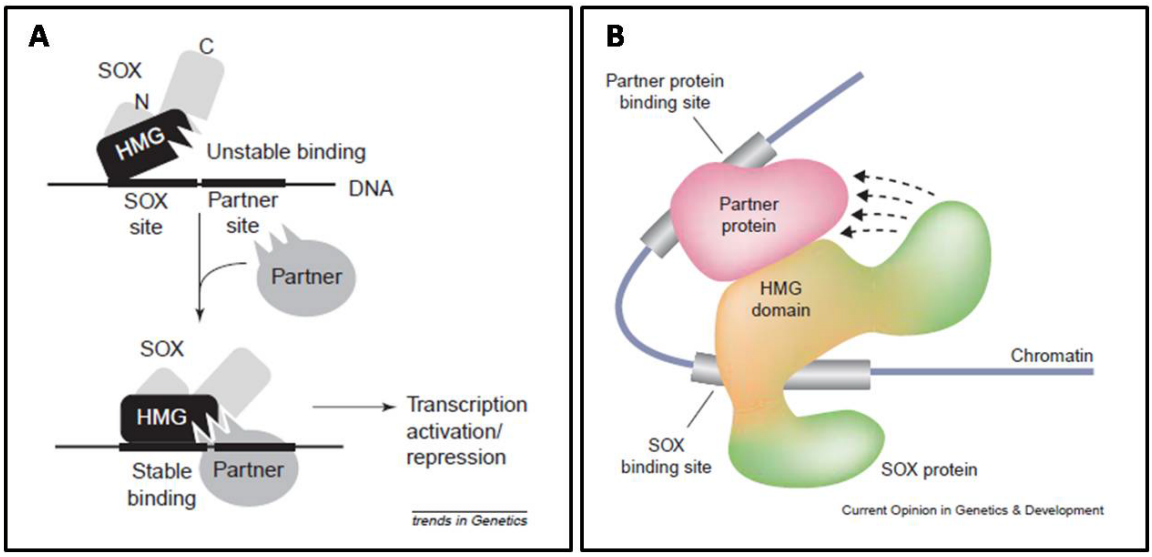


Figure 1: Model for the specificity of Sox transcription factor action: specific target sites on the chromatin are either activated or repressed via a three-step mechanism. A: First, the Sox factor HMG domain and a partner protein bind to adjacent binding sites on the chromatin, in a promoter or enhancer region of the target gene. Second, Sox–partner interaction stabilizes binding of the transcription factor complex to DNA B: . Third, non-HMG SOX domains may stabilize protein–protein interaction between the Sox and partner factors (dotted arrows). Non-HMG domains on the SOX protein may influence partner selection. Adapted from Kamachi et al. (2000) and Wilson and Koppman (2002)

δ-crystalin gene is another target of *Sox2*, but in this case, it is Pax2 who performs the synergistic interaction to activate the DC5 promoter (Kamachi et al., 1995; Kamachi et al., 1998). The synergistic interaction between Sox2 and Pax2 is responsible also for the regulation of enhancer N-3 of *Sox2* gene itself during eye development, both in the retina and in the lens (Inoue et al., 2007). A recent study has also reported *Notch1* as a target gene of *Sox2*, but no partner factor was identified yet, in this case (Taranova et al., 2006). More recently, *in silico* studies took advantage of the known synergistic interaction between Sox2 and other transcription factors to search for potential target genes, by looking for adjacent binding sites for Sox proteins and its known or candidate partners. A unifying transcriptional logic for a large set of CNS-expressed genes in which Sox and POU proteins act as generic promoters of transcription, while homeodomain proteins control the spatial expression of genes through active repression was recently described (Bailey et al., 2006). Similarly, Sox, POU and Nanog were described to form a core transcriptional regulatory circuit that allowed the identification of several target genes co-regulated by those proteins (Boyer et al., 2005). And more recently, an *in silico* analysis has expanded the number of genes identified as putative Sox2:Oct-3/4 targets, although many of them will still need experimental validation [Chakaravarthy et al., 2008].

III.3 SoxB1 proteins: neural commitment and progenitor state

SoxB1 proteins in neural development

In higher vertebrates, *SoxB1* subgroup of the *Sox* gene family is composed of three members: *Sox1*, *Sox2* and *Sox3* (*Sox1-3*). Sox1-3 share greater than 90% amino acid residue identity in the HMG box DNA binding domain and they are transcriptional activators with the activation domains located in the COO-terminal half, as revealed by their ability to bind and activate the DC5 minimal promoter of the chicken δ1-crystalin gene (Kamachi et al., 1995; Kamachi et al.; Uchikawa et al., 1999). However, recent data suggest that they can also act as repressors (Paul Scotting, personal communication). In addition to *SoxB1*, the SoxB group includes *Sox14* and *Sox21*, classified as the *SoxB2* subgroup. SoxB proteins homology is limited to the HMG box domain and its immediate C-proximal region, but within a subgroup similarities extend to the remaining portions of the protein. The COO-terminal of SoxB2 proteins harbours a repression domain, which makes them transcriptional repressors. So that, SoxB1 and B2 proteins regulate a similar set of target genes, but they have counteracting activities. (Rex et al., 1997; Uchikawa et al., 1999; Sandberg et al., 2005).

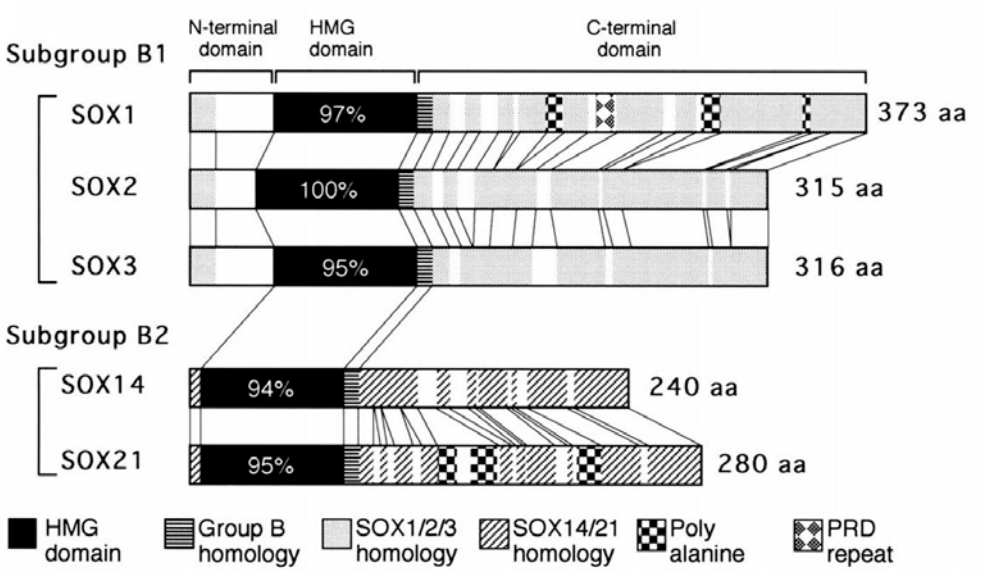


Figure 2: Comparison of the domain organization between the Group B Sox proteins, with indication of the conserved subdomains. Percent identity of the amino acid sequence of the HMG is shown in comparison with the Sox2 HMG domain. The regions and the domains conserved among all Group B Sox, among Sox1/2/3, and between Sox14 and Sox21 are indicated as respective `homologies'. From Unchikawa et al. (1999)

Throughout evolution, the expression of the *SoxB1* genes directly correlates first, with ectodermal neural competence, and second, with the commitment to neural fate (Pevny and Placzek, 2005). Although *SoxB1* genes are co-expressed in many tissues and their function is many times found to be redundant, they also exhibit a differential and dynamic temporal and spatial patterns, with one of the *SoxB1* genes taking the role of the so

called “panneural genes”, as master genes for neural specification. In amniotes, the pan-neural role is taken by *Sox2*, the hallmark of neural primordial cell state. *Sox2* is expressed throughout the development of the CNS. Its expression is first detected in the neural plate, following neural induction and later on becomes restricted to progenitor cells that reside in the ventricular zone of the developing neural tube and to neural stem cells. (Uchikawa et al., 1999; Wood and Episkopou, 1999; Zappone et al., 2000 2000; Pevny and Placzek, 2005) In all cases, *Sox2* is expressed in undifferentiated neural competent or neural committed cells, and its expression is down-regulated when progenitor cells differentiate as neurons (Bylund et al., 2003; Graham et al., 2003). Functional studies have demonstrated that SoxB1 proteins play important roles throughout the whole period of neural development: *SoxB1* genes are associated with neural induction and neural commitment, and later on with the regulation of neurogenesis (Pevny et al., 1998; Streit et al., 2000; Bylund et al., 2003; Graham et al., 2003; Dee et al., 2008). In contrast to the wide and redundant expression of SoxB1 proteins, SoxB2 proteins exhibit a restricted and non overlapping expression pattern in the CNS (Rex et al., 1997; Uchikawa et al., 1999; Hargrave et al., 2000).

Early neural development: neural induction

Sox2 expression in the early embryo is associated with the developing neural plate [Pevny et al., 1998; Wood and Episkopu, 1999; Pevny and Palczec, 2005, Rexet al., 1997; Uchikawa et al, 1999; 2003]. *Sox2* responds to neural inducing signals and its expression is regulated by an array of enhancers conserved among chicken and mammals (Streit et al., 2000; Uchikawa et al., 2003) Uchikawa and colleagues have identified several different small sequences of conserved non-coding regions (CNRs) responsible for *Sox2* expression in the CNS in different domains and developmental stages (enhancers N-1 to N-5 and SC-1 and 2). Activity of the enhancer N-1 is initiated around stage 4 in chick and it accounts for the Hensen’s node-induced *Sox2* expression. This enhancer is sequentially activated throughout the whole process of neural plate induction, from rostral to caudal. N-1 enhancer accounts mostly for posterior neural plate Sox2 expression. By stage 5 in chick, enhancer N-2 is activated similarly to enhancer N-1, and accounts for the *Sox2* expression in the anterior neural plate. Together, enhancers N-1 and N-2 recapitulate the process of neural plate induction. Other CNS associated enhancers are activated in later stages of development and account for *Sox2* expression in specific domains of the CNS (Uchikawa et al., 2003) (Fig.3). Enhancer N-1 exhibits a core element of 56bp which is synergistically activated by Wnt and FGF signalling, but not affected by BMP signalling. Two binding sites for Lef-1 account for the Wnt regulated activity of the enhancer. This core element also harbours a region that inhibits the activity of N-1 enhancer in mesodermal precursors (Takemoto et al., 2006) (Fig.3B). Enhancer N-2 activity is independent of Wnt signal and depends on epiblast Otx2 expression, induced by the hypoblast. In addition to OTX binding sites, POU and HMG binding sites were identified in the 73bp core region of the enhancer [Makkiko et al, communication]. N-2 derived expression of Sox2 is also regulated by the resulted interaction of three coiled-coil domain proteins ERNI, BERT and Geminin, two heterochromatin proteins HP1α and HP1γ acting as repressors, and the chromatin-remodeling enzyme Brm (Bramha) acting as activator. Brm is ubiquitously expressed in

the embryo and can activate *Sox2* through direct binding to enhancer N-2. However, HP1α can interact with Brm and repress this function, and this explains why *Sox2* is not prematurely expressed. In contrast, Geminin can interact with Brm, activating Sox2 expression. Geminin does not activate *Sox2* expression prematurely, due to its association with ERNI that recruits HP1γ. As neural induction takes place, BERT is upregulated and binds both Geminin and ERNI, and displaces ERNI- HP1γ from Brm-Geminin complex, thus activating *Sox2* expression (Papanayotou et al.). Both Geminin and ERNI are induced by FGF signaling, which might account for the FGF dependence of the neural induction process (Streit et al., 2000; Papanayotou et al., 2008) (Fig.3C)

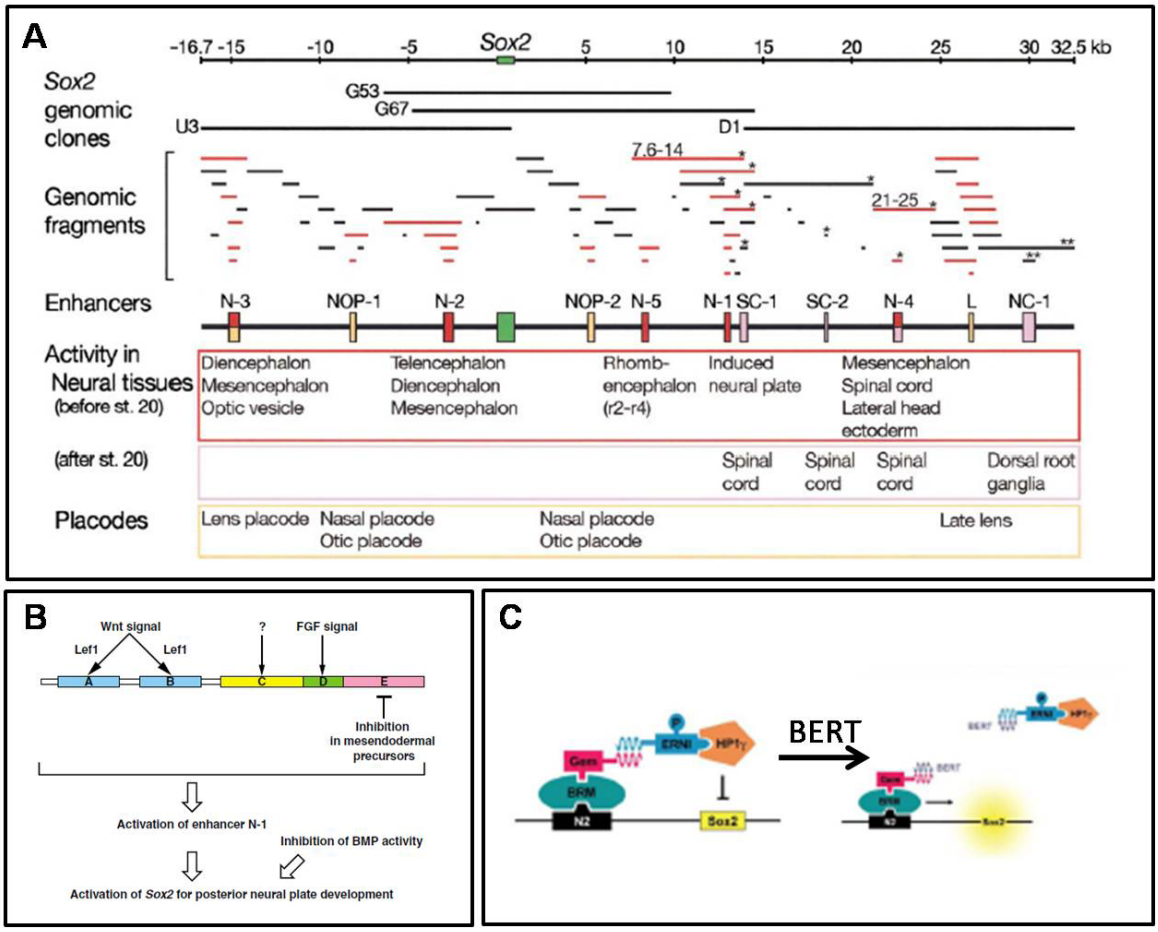


Figure 3: Regulation of *Sox2* expression during neural induction. A: Enhancers responsible for the expression of *Sox2* in various domains of CNS and sensory placodes. B: Regulation o enhancer N-1 and *Sox2* expression, By Wnt and FGF signalling, during neural plate induction. C: Regulation of enhancer N-2 and *Sox2* expression during neural plate induction by BERT. Adapted from Uchikawa et al. (2003); Takemoto et al. (2006) and Papanayotou et al. (2008).

In summary, Sox2 is expressed since early stages of neural development and the onset of its expression seems to be associated with the process of neural induction. The regulation of Sox2 expression is complex during this process and involves the multiple regulatory regions modulated by multiple signalling pathways.

Late neural development: neurogenesis

In addition to its expression in neural primordia, SoxB1 proteins are expressed in the progenitor cells of the developing CNS and in the neural stem cells of the adult CNS, suggesting a potential role in the maintenance of the neural progenitor sate (Zappone et al., 2000; Bylund et al., 2003; Graham et al., 2003; Ellis et al., 2004; Ferri et al., 2004; Bani-Yaghoub et al., 2006; Taranova et al., 2006; Qu and Shi, 2009). SoxB2 are expressed, along with SoxB1 proteins in neural progenitors in the developing CNS and they counteract SoxB1 function (Uchikawa et al., 1999; Sandberg et al., 2005).

Sox1-3 are expressed throughout the ventricular zone of the developing spinal cord in an overlapping manner. They are expressed in proliferating cells, co-express with Neurogenin2 and they are down-regulated as cells exit the cell cycle and start to differentiate. Overexpression of SoxB1 proteins hinders the generation of neurons, but it does not affect the expression of the proneural gene Ngn2. A proposed model for the regulation of neurogenesis by Sox B proteins postulates that the balance of Sox21 and Sox1–3 activities determines whether neural cells remain as progenitors or commit to differentiation, and that proneural genes regulate the relative expression levels of genes from both subgroups. As Sox B proteins do not regulate the expression of proneural genes, it is likely that the subset of target genes regulated by the counteracting activity of SoxB1 and SoxB2 genes are involved in the maintenance of progenitor features and in the repression the neurogenic activity of proneural proteins. Accordingly, the capacity of proneural proteins to upregulate Sox21 activity is likely to represent a mechanism that enables them to commit neuronal differentiation and suppress progenitor features (Bylund et al., 2003; Graham et al., 2003; Sandberg et al., 2005).

Notch signaling pathway uses a distinct mechanism to regulate neurogenesis (see below). Notch counteracts neurogenesis by repression of proneural gene expression in a Hes-dependent manner, and by the suppression of E-protein in a Hes-independent fashion. The capacity of Notch to maintain neural cells in an undifferentiated state requires the activity of SoxB1 proteins, whereas the mechanism by which SoxB1 block neurogenesis is independent of Notch signalling. The latter can be explained by the fact that in the absence of Notch, SoxB1 is sufficient to block progression of neuronal differentiation by blocking the activity of proneural proteins. The dependence of Notch on SoxB1 for the maintenance of the progenitor state suggests that SoxB1 factors, in addition to proneural proteins, regulate also a set of genes that contribute to the maintenance of the progenitor state (Holmberg et al., 2008) (Fig.4A).

In the neocortex and retina, *Sox2* is also involved in the regulation of neuronal differentiation. *Sox2* overexpression in neocortical progenitors prevents neurogenesis. However, *Sox2* does not block the subsequent steps of neurogenesis in neuronal precursors and neuroblasts, and exogenous Sox2 protein is degraded through the activity of serine protease activities in differentiating cells. Differently to the spinal cord, here *Sox2* cooperates with Notch in that process (Fig.4B). *Sox2* regulates the expression of *Notch1, RBP-J and Hes5*, but does not affect the expression of *Hes1* or *Jag1*. Furthermore, direct transcriptional regulation of *Notch1* by Sox2 was demonstrated in vivo by a chip assay, and the binding of Sox2 to *Notch1* by in vitro DNAse footprinting

and luciferase reporter assays. Together, this suggests that *Sox2* might use, at least in part, the regulation of Notch signalling to maintain retinal and neocortical progenitor identity (Bani-Yaghoub et al., 2006; Taranova et al., 2006). Furthermore, in the retina, *Sox2* dosage is associated with the regulation of retinal competence. Hypomorphic mice for *Sox2* eyes exhibit disrupted cell layering and aberrant ganglion cell differentiation (other cell types differentiated normally), suggesting that reduced levels of *Sox2* restricts retinal progenitor competence (Taranova et al., 2006). In the adult brain, in addition to its role in the regulation of the progenitor activity of adult neural stem cells, *Sox2* is necessary for neuron maintenance in selected areas of the brain (Ferri et al., 2004).
Sox2 conditional inactivation in neural progenitors reduces the number on neural progentiors but does not interfere with multipotency and self-renewal ability of the Sox2-deficient cells, as it does not alter the expression of Notch pathway components. This phenotype has been explained by the redundant activity of Sox3, which expression is up-regulated in the mutant cells (Miyagi et al., 2008).

Two mechanisms have been described for the regulation of Sox2 function in neurogenesis. In telencephlic neuroepithelial progentiors Sox2-Hes5 pathway is regulated by sonic hedgehog (Shh) signalling through its downstream effector, Gli2 (Takanaga et al., 2009). In *Xenopus* retina, Wnt dependent Sox2 activation inhibits neural differentiation through a mechanism that involves Notch activation. A feedback inhibition in which Sox2 inhibits Wnt and proneural inhibit Sox2 is responsible for the progression of the transition from progenitor to differentiation (Van Raay et al., 2005; Agathocleous et al., 2009) (Fig.4B).

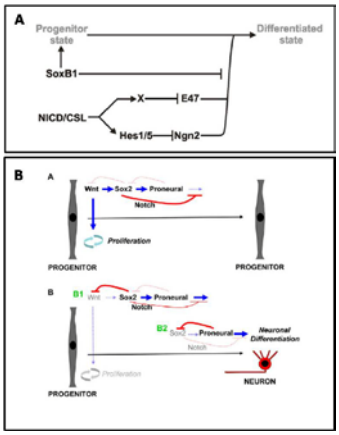


Figure 4: Regulation of neurogenesis by *Sox2* through mechanisms independent of Notch function (A) and cooperative with Notch (B). A: Sox2 acts downstream of Notch signalling and proneural expression in the regulation of neurogenesis, while Notch acts through different ways in the prevention of proneural and E-protein expression. Sox regulates the progression of neurogenesis in a Notch dependent manner, that prevents neuronal differentiation. A feedback loop of inhibition ensures the progression of differentiation. Adapted from Holmberg et al. (2008) and Agathocleous et al. (2009).

In summary, SoxB1 proteins regulate the progenitor state in the CNS through the repression of neurogenesis. Some mechanisms are independent of Notch function while others cooperate with Notch in the repression of proneural function.

BOX II.2: Sox B1 proteins in Embryonic Stem (ES) cells

Sox2 is expressed in Embyionic Stem cells (ES cells) and critical for specification of steamness (see REF). In the mouse embryo it is first detected in blastocyst stage, in the multipotent inner cell mass, but it is also expressed in the epiblast, trophoblast stem cells and in germ cells. Sox2 has an essential role maintaining stem cells of the mouse epiblast in an undifferentiated sate and Sox2-deficient mice die at implantation (Wood and Episkopou, 1999; Avilion et al.). Sox2, together with other transcription factors, is a key regulator of the stemness sate characterized by pluripotency and self renewing ability [Masui et al., 2007; Matoba et al., 2006]. In fact, Sox2 together with three other factors are enough to induce pluripotent cells from fibroblasts [Kazutoshi and Yamanaka, 2006]. The ability of Sox2 to maintain stemness is tightly coupled its expression levels and a high dosage of Sox2 in ES cells inhibits the expression of Sox2:Oct-3/4 target genes, prompts cells to differentiation [Boer et al., 2007; Kopp et al., 2008] . On the other hand, Notch signalling is not required for maintaining the undifferentiated state, since CSL-deficient ES cells can be preserved in a self-renewing and pluripotent state (Hitoshi et al., 2002; Lowell et al., 2006), however important for the regulation of the proliferative ability (Fox et al., 2008).

SoxB1 proteins in sensory development

In vertebrates, SoxB proteins are also expressed during development of PNS. The recruitment of SoxB proteins for PNS development is a novelty of craniates, suggested to be related with a rapid expansion of the ectodermal anlage (Fritzsch et al., 2006). Sox2 and Sox3 are expressed during the development of sensory cranial placodes and in the dorsal root ganglion, of neural crest origin. *Sox3* expression is initiated in placodal ectoderm prior to the specification of individual placodes and is then restricted to individual placodal areas. [Groves and Bronner-Fraser, 2000; Uchikawa et al., 1999; Ishii et al., 2001; Abu-Elmagd et al., 2001; Abello et al., 2007; 2009; Kamachi et al, 1998; Sun et al., 2007]. Functional studies in medaka demonstrated that Sox3 has the ability to induce placodal markers like *Pax6* and *Eya1* and to induce ectopic lens and otic vesicles (Koster et al., 2000), and in chick, *Sox3* is able to induce *Sox2* in the otic placode [Abelló et al., 2009]. *Sox2* expression is detected in lateral head ectoderm by stage 12 in chick and then it restricts to individual placodes (Kamachi et al., 1998; Uchikawa et al., 1999). Accordingly, Uchikawa and colleagues (2003) identified enhancers responsible for *Sox2* expression in sensory placodes. NOP-1 and 2 drive *Sox2* expression to otic and nasal placodes and L to the lens. The enhancer N-3, previously described for its role in CNS also accounts for *Sox2* expression in the lens. Finally, an enhancer responsible for driving *Sox2* expression in the neural crest derivatives of the dorsal root ganglia was also identified (NC-1) (Uchikawa et al., 2003). The regulation of these enhancers is yet to be explored, except N-3, which has been recently analysed (Inoue et al., 2007) (Fig.3A)

The involvement of SoxB proteins in lens development has been extensively studied, the lens gene δ1-crystallin being one of the first genes to be identified as a transcriptional target of Sox2 (Kamachi et al., 1998). *Sox2/3* expression occurs in the *Pax6*-positive

prospective lens placode, and it is activated when the optic vesicle comes into close proximity with the ectoderm. This is in turn dependent on the expression of *Pax6* and *SoxB1* in the optic vesicle [see above, Kamachi et al., 1998; 2001; Inoue et al., 2007].

The expression of *Sox3* in the epibarnchial placodes has been associated with the ectodermal thickening that anticipates placode formation. Furthermore, like in the CNS, it prevents the progression of neurogenesis and delamination of neuroblasts (Abu-Elmagd et al., 2001). In zebrafish and chick, functional studies have demonstrated the requirement of *Sox3* for epibranquial placode neurogenesis (Dee et al., 2008; Tripathi et al., 2009).

SoxB1 in inner ear development

Sox2 and *Sox3* are expressed during inner ear development. *Sox3* is initially expressed in a broad band that encompasses the otic and epibranchial territory and becomes progressively refined and enhanced in the more anterior part of the preotic territory to get restricted to the proneural (proneurosensory) region of the otic placode [Abelló et al.,2007; 2009; Sun et al., 2007]. Functional studies show that *Sox3* ectopic expression is able to induce *Dl1* and *NeuroD* and ectopic neuroblasts. This ability is restricted to a narrow time window and is no longer observed after the full establishment of the proneural vs non neural domains of the otic placode. This effects of *Sox3* is consistent with its ability to confer neural competence and suggests a role in the process of neural induction in the otic field, similar to that played by *SoxB1* genes in neural induction in the neural plate. In agreement with this, *Sox3* expression in the otic field is also dependent on FGF signalling. [Abelló et al, 2009; Abelló and Alsina, 2007]. As in neural plate development, *Sox3* expression precedes *Sox2* expression in the otic field. (Uchikawa et al., 1999; Neves et al., 2007)

The regulation and function of *Sox2* during inner ear development is still poorly understood. *Sox2* mutations in humans are reported to produce sensorineural hearing loss (Hagstrom et al., 2005). In mice, two studies have revealed the involvement of *Sox2* during sensory development of the ear. First, *Sox2* deficient mice *light coat and circling* (*Lcc*) and *yellow submarine* (*Ysb*), show hearing and balance impairment (Fig.5). These mice carry unique *Sox2* alleles in which complex chromosomal rearrangements have resulted in the loss of (*Lcc*), or interference with (*Ysb*) specific regulatory elements that direct expression of *Sox2* within the inner ear (Fig.5B). Paint filing analysis of these mice inner ears revealed abnormal inner ear morphology. *Lcc/Lcc* mice have absent ampullae, undercoiled cochlea and remarkably smaller sacule and utricule. *Ysb/Ysb* miss anterior and lateral ampullae and slightly undercoiled cochlea (Fig.5A). Histological analysis of the malformed cochlea revealed absence (*Lcc*) or disorganization and reduced number of hair cells (*Ysb*). These phenotypes were associated with a failure in the establishment of prosensory domains, as prosensory markers ($p27^{kip1}$ and *Lfng*) as well as hair cell specification (*Atoh1*) and early differentiation (*MyoVI, MyoVII*) markers expression were absent or reduced. (Fig. 5B) (Kiernan et al., 2005b). The second study used several mouse models to perform functional studies that suggest that, in addition to its involvement in prosensory specification, *Sox2* regulates hair cell formation (Dabdoub

et al., 2008). This work shows that *Sox2* overexpression in cochlear explants prevents hair cell formation by antagonizing *Atoh1* function. This same study supported previous observations on the involvement of Notch in the regulation of *Sox2* expression in the inner ear. *Sox2* expression is inhibited in cochlear explants treated with DAPT, and overexpression of NICD is sufficient to induce *Sox2*. The inner ear of *Jag1* conditional KO mice lose *Sox2* expression in the sensory domains of the otocyst (Kiernan et al., 2005b). Finally, functional studies in chick show that blocking Notch activity also down-regulates the expression of *Sox2* in the prosensory domains (Daudet et al., 2007). On the other hand, *Jagged1* and Nothch1 expression persist in the cochlea of *Lcc/Lcc* mice. These experiments, suggest that the Notch pathway is upstream *Sox2*, however, they do not exclude that Notch signalling operates *also* downstream of *Sox2*.

In summary, the data available so far suggests that *Sox2* plays an important role in the development of the sensory elements of the inner ear. A parallel may be traced with its function in the development of the CNS. First, *Sox3* seems to be involved in the process of induction of neural competence of the otic territory, similarly to the function played by SoxB1 proteins in neural induction. Secondly, *Sox2* is required throughout the expansion of all neural cell phenotypes. Finally, *Sox2* is down-regulated during the transition between progenitor and differentiated cell sates. This poses interesting questions on the role of *Sox2* in the specification of cell fate and in the regulation of differentiation of the neurosensory elements of the inner ear: 1) Is *Sox2* sufficient for sensory specification? 2) Is *Sox2* also involved in otic neuronal development? Is it necessary and/or sufficient for neuronal fate specification? 3) What are the downstream targets of *Sox2* during inner ear development and which mechanism does it use to regulate the generation of hair cells and /or neurons? 4) How does *Sox2* interact with the Notch signalling pathway during the process of Sensory specification (specifically its interactions with the ligand *Jag1*) and sensory differentiation? In this thesis, I propose to answer some of these questions.

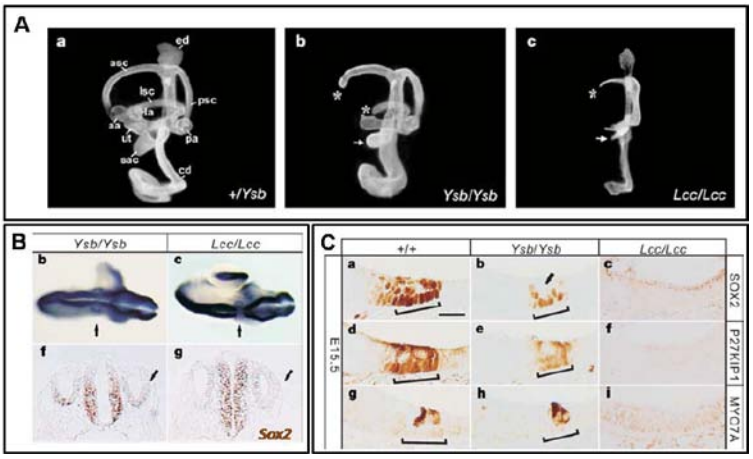


Figure 5: Phenotype of Sox2 mutant mice *Ysb* and *Lcc*. A: Abnormal inner ear morphology in *Ysb* and *Lcc* mice. B: specific ablation of Sox2 expression in the inner ear in *Ysb* and *Lcc* mice (note normotopic expression in the neural tube) C: Reduction or absence of p27 and MyoVII expression in *Ysb* and *Lcc* mice, suggesting the absence of sensory progenitors. Adapted from Kiernan et al. (2005)

VI. Serrate1 and the Notch signalling pathway

IV.1 The Notch pathway

The Notch signalling pathway is a juxtacrine short-range communication transducer that it is involved in the regulation of multiple cellular processes, such as cell proliferation, cell death, stem cell niche maintenance, cell fate specification and differentiation. The core pathway consists in the interaction between a transmembrane Notch receptor anchored in one cell, with a transmembrane ligand (Delta or Serrate) in a neighbouring cell. Once bound by a ligand, the intracellular subunit of the receptor is cleaved (NICD) and translocated to the nucleus where it cooperates with CSL and its co-activator Mastermind (Mam) to activate the transcription of target genes (Artavanis-Tsakonas et al., 1999; Kadesch, 2004; Schweisguth, 2004; Bray; Fior and Henrique, 2008; Fortini, 2009) (Fig.1).

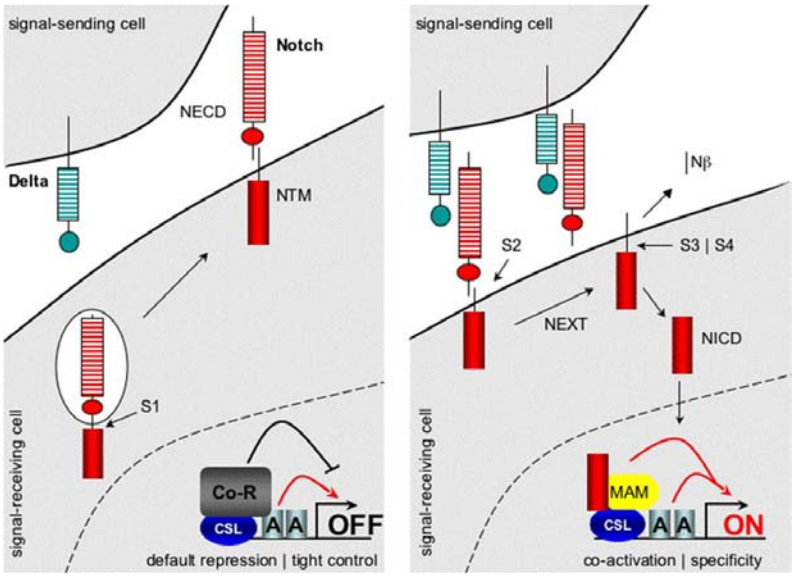


Figure 1: The Notch signalling pathway. The mechanisms that lead to Notch activity in the receiving cell are depicted, including the cleavage of Notch at the cell membrane and the assembly of a tripartite nuclear complex with the transcription factors CSL (CBF-1, Drosophila Suppressor of Hairless and C. elegans Lag-1) and MAM (mastermind co-activator) that releases the default repression. From Fior and Henrique (2008)

The core pathway

Notch receptors are a type-I transmembrane proteins composed of and extracellular domain (NECD, Notch Extracellular Domain) with multiple EGF-like repeats, three cystein rich NLRs (Notch/Lin-12 repeats) and a transmembrane-intracellular domain (NTM, Notch Transmembrane Domain), that includes six tandem ankyrin repeats, one or two nuclear localization signals, a glutamine-rich domain (opa) and a PEST domain. These two subunits are the result of a proteolytic cleavage mediated by furin protease at s1 site and then form heterodimers held together by non covalent interactions. Notch

ligands are also type-I transmembrane proteins which contain a N-terminal unique motif DSL (for the ligand names Delta, Serrate and Lag-2) and multiple EGF-like repeats, similar to the ones found in the receptor, which compose the extracellular domain of the ligand and a transmembrane domain with a truncated intracellular domain (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). The molecular structure of the Notch pathway and its components are evolutionary conserved in the metazoan *phyla*, but the number of paralogues for each element of the pathway is variable in different species(see Table 1) [Artavanis-Tsakonas et al., 1999; Bray, 2006, Fior and Henrique, 2008].

	Drosophila	*C. elegans*	Chick	Mammals
Notch Receptor	*Notch*	*lin-12* *glp-1*	*Notch1* *Notch2*	*Notch1* *Notch2* *Notch3* *Notch4*
LIGAND	*Delta* *Serrate*	*lag-2* *apx-1* *arg-2* *f16b12.2*	*Delta1* *Delta4* *Jagged1* *Jagged2*	*Dll-1* *Dll-3* *Dll-4* *Jagged1* *Jagged2*
CSL	*Su(H)*	*Lag-1*	*CBF1/RBPJK*	*CBF1/RBPJK*
MAM	*Mam*	*Lag-3*	*Mam1* *Mam2* *Mam3*	*Mam1* *Mam2* *Mam3*

Table1: Components of the Notch signalling pathway are evolutionary conserved. From Fior and Henrique (2008).

The binding of the ligand to the receptor occurs through the conserved DSL domain and one or more EGF-like repeats (Rebay et al., 1991) and results in a series of three proteolytic cleavages that lead to the release of the NICD. The first cleavage, at site S2, is catalyzed by ADAM-family metalloproteases and generates an activated membrane-bound form of Notch. The third and fourth cleavages at S3 and S4 are catalyzed by γ-secretase (enzymatic complex containing presenilin, nicastrin, PEN2 and APH1) and release the NICD into the cytoplasm, which is then translocated to the nucleus (through its nuclear localization signals) where it acts as a co-activator of the CSL transcription factor, together with the co-activator Mastermind (MAM). CSL is named after its designation in mammals (CBF-1), Drosophila (Suppressor of Hairless, SuH) and C. elegans (Lag-1). In mammals, it is also referred to as RBP-Jk (for recombination signal sequence binding protein for Jk genes) (Kadesch; Bray, 2006). In the absence of NICD, Notch target genes are repressed by CSL, which forms a repressor complex that bind cis-regulatory regions named Su(H) or S binding boxes in those target genes, inactivating their transcription. When Notch signalling is initiated, NICD is translocated to the nucleolus, binds CSL and recruits HAT (Histone Acetylase) and displaces the co-repressor complexes to release repression. Finally, MAM binds to CSL-NICD forming the active tertiary complex that is able to activate transcription of the Notch target genes (Kadesch, 2004) (Fig.1). There are many binding sites for CSL throughout the genome (Rebeiz et al., 2002), but it is not clear which of them are actually Notch targets. The best

characterized Notch targets are the Hes (Hairy–Enhancer of Split) and Hrt (Hes related type, also known as Hey, Hesr, CHF, Herp, gridlock) family genes (Tatsuya et al., 2003). The function of these genes is responsible for most of the roles ascribed to the Notch signalling pathway (see below chapter V).

Modulation of Notch activity

The signalling through Notch depends on the interaction between receptor and ligand and each receptor can signal only once, so that, the availability of both receptors and ligands at the cell surface may be a limiting step to control the strength and directionality of the signal. There are several mechanisms to control this availability that include both transcriptional regulation and post translational modifications of both receptor and ligands. In addition, other pot-translational modifications can modulate the interactions between the receptors and DSL ligands (Schweisguth, 2004; Bray, 2006; D'Souza et al., 2008; Borggrefe and Oswald, 2009; Fortini, 2009) .

Notch Receptor

A model in which Notch activation in one cell induces its own expression is a positive feedback mechanism for transcriptional regulation of Notch in *C. elegans* (Greenwald, 1998). Endocytosis and trafficking of Notch protein is yet another mechanism to regulate the levels of the protein in the cell and its localization. In this case, E3-utiquitin ligases (Itch/Nedd4/Su(Dx) have been shown to interact with membrane bound Notch, targeting it to the endocytic pathway and subsequent degradation by the lysosome. Another E3-ubiquitin ligase, Deltex, can counteract this effect by deviating Notch from the lysosomal degrading route and stabilizing the protein. Numb is yet another protein described to interact with Notch, in vertebrates, and target it for the degradation through the endocytic pathway (In *Drosophila*, it also exerts a negative regulation but in this case by targeting Sanpodo, a positive regulator that interacts with Notch in the membrane, for endocytosis) (Schweisguth, 2004; Bray, 2006). Furthermore, NICD turnover is regulated by MAM, that in addition to associate with NICD in gene promoters is able to target it to poly-ubiquitination and proteosome degradation in a PEST-dependent manner by Sel-10, regulating its availability in the nucleous (Hubbard et al., 1997; Wu et al., 2001).

DSL ligands

The activation of Notch signalling pathway can regulate the expression of DSL ligands both by activating or repressing its expression in the signal receiving cell. This are two different ways of Notch exerting its function: lateral inhibition and lateral induction (see below). Endocytosis also regulates ligand activity and there are at least 3 E3-ubiquitin-ligases that can interact with DSL ligands: Neur, Mib-1 and Mib-2. The role of these proteins seems to have evolved differently in different species. In *Drosophila*, their function is mostly found to be redundant and they ubiquitinate DSL ligands to stimuli endocytosis and enhance signalling activity. In vertebrates, Neur and Mib do not have equivalent functions. Studies in mammalian cells suggested that Mib is responsible for DSL ligand endocytosis, with consequences in the enhancement of ligand activity while Neur functions downstream to direct lysosomal degradation of internalized ligands (D'Souza et al., 2008).

Notch receptor- DSL ligands interaction

Upon translation, Notch protein is fucosylated by O-fut (o-fucosyl trasnferase), a protein with both enzymatic and chaperone activity. O-fuct adds fucose to the EGF like repeats of the receptor, a process that is absolutely essential to the generation of a functional receptor. In addition, O-fut promotes the proper folding and transport of the Notch receptor from the endoplasmic reticulum to the membrane (Haines and Irvine, 2003; Schweisguth, 2004; Bray et al., 2008; Fortini, 2009). After the addition of the first fucose, the carbohydrate chain can be extended by addition of N- acetilglucosamine in a reaction catalyzed by N-acetilglucosaminyl trasnferases of the Fringe family (lunatic fringe, radical fringe and maniac fringe in mammals). Fringe modifications seem to act at the molecular level of ligand binding. It increases binding of Delta-like ligands to Notch, whereas Serrate/Jagged binding is perturbed [Panin et al., 2007; Haines and Irvine, 2003; Schweisguth, 2004; Bray, 2008; Fortini, 2009]

In addition to ligand and receptor availability and to ligand-receptor effective binding, the activity of the Notch pathway can also be regulated by photolytic cleavages that elicit the release of NICD or at the level of its targets, which are subject to negative feedback and oscillation of transcriptional regulation, as well as post-transcriptional regulation mediated by microRNAs. Such a complex regulatory mechanisms imply that there are various checkpoints at which the pathway can me modulated and that the expression of both ligands and receptors do not necessarily imply that the pathway is active or reflect the degree of activation [Schweisguth, 2004; Bray, 2006; D'Souza, 2009; Borggrefe, 2009; Fortini, 2009] .

IV.2 The mechanisms of action of Notch: Lateral Inhibition vs Lateral Induction

Regardless of the complexity of its regulation and its pleiotropic effects, Notch signalling pathway mode of action is rather simple and falls into two types of mechanism: lateral inhibition and lateral induction. By definition, lateral inhibition refers to the case in which Notch activation in one cell inhibits the expression of the Notch-activating ligand, while lateral induction refers to the opposite case, in which Notch activation in one cell induces the expression of the Notch-activating ligand in that cell. The lateral inhibition mostly mediates binary cell fate decisions in which the two signalling cells are driven to differ from one other. The mechanism of lateral induction drives cells to adopt a similar fate, and is associated with boundary and cooperative formation of cell clusters (Bray, 1998; Lewis, 1998; Schweisguth, 2004; Fior and Henrique, 2008).

Lateral inhibition and the regulation of neurogenesis

The classical view of Notch-mediated lateral inhibition is largely based on studies of *D. melanogaster* neurogenesis, sensory organ formation and vulval development in *C. elegans* [reviewed in Artavanis-Tsakonas et al., 1999]. But lateral inhibition has been shown to operate also during vertebrate CNS neurogenesis, and in the generation of hair

cells in the inner ear sensory organ [Henrique et al., 1995; Adam et al., 1998; Lanford et al., 1999; reviewed in Lewis, 1998]. According to the model, neurogenesis is initiated within a population of progenitors that are provided with neural competence and that are equivalent (equipotent), they all express proneural proteins and both DSL ligands (*Dll1*) at similar levels. Then, stochastic variations of product levels within the cells lead some cells to express the ligand *Dll1* at higher levels and thus, to activate Notch in the neighbouring cells more efficiently. Consequently, the activated cells start to express higher levels of *Hes/Hey* genes that repress the expression of proneural genes and consequently Notch ligands. As the signal receiving cells express ligand at lower levels, it is less effective in activating the signal sending cell, which results in the release of the repression on proneural genes and Notch ligands, with the consequent up-regulation of their expression. The subset of selected cells that end up expressing higher levels of proneural genes and ligand will differentiate into a neuron that activates Notch in its neighbours and forces them to retain the progenitor fate. Thus, the mechanism of lateral inhibition amplifies stochastic variations between neighbouring cells and creates “salt-and-pepper” patterns in gene expression that ultimately results in the adoption of two different fates by the neighboring cells [Artavanis-Tsakonas et al., 1999; Shweisguth, 2004; Bray, 2006; Kageyama et al., 2008] (Fig.2Aa and Fig. 2B). Although the model is based on a pure random choice, there is also evidence for mechanisms that bias the selection and are mediated by both extrinsic and intrinsic cues. For instance, in the development of the *Drosophila* sensory organ, intrinsic cues like the localization of Numb and Neur biases the asymmetry of the division, with one cell keeping Notch activated and another repressed [Artavanis-Tsakonas et al., 1999]. Also cis-inhibition of Notch by DSL ligands occurring within the same cell has been shown to cooperate with the negative feedback loop associated with lateral inhibition to ensure the directionality of the signal [del Alamo and Schweisguth, 2009; Miller et al, 2009].

However, recent studies have proposed a revision for the classical view of Notch-mediated lateral inhibition in mammals (Fig.2Ab). According to Kageyama, the salt-and-pepper pattern of expression of proneural and Delta genes originates from oscillations in their expression, and not through the amplification of stochastic differences [Kageyama et al., 2008]. The model suggests that *Hes1* expression oscillates in neural progenitors because of a negative feedback in which Hes1 protein represses *Hes1* transcription. The oscillations in *Hes1* expression result in an oscillatory pattern of expression of proneural genes, but in opposite phase. When *Hes1* levels are low enough, the repression exerted over proneural genes is released and their expression is upregulated along with the levels of *Delta1*. As *Hes1* expression is restored, proneural genes and *Delta* levels are also decreased. In this situation, the Notch-mediated lateral inhibition operates alternatively and reciprocally between neural progenitors and it does not result in neuronal selection. On the contrary, it keeps an equipotent population of proliferating neural progenitors [Shimojo et al., 2008; Kageyama et al., 2008]. The maintenance of the progenitor state is ensured by the fact that proneural expression is never sustained long enough to allow the expression of slow responding genes associated with neuronal differentiation. The authors suggest that the sustained repression of *Hes1* is responsible for the sustained expression of proneural genes and *Dl* (Kageyama et al., 2008).

Neuronal selection is determined by asymmetrical cell division, wherein one progenitor divides to give rise to one neuron and another progenitor, or by a symmetric neurogenic division wherein one progenitor divides to originate two neurons. Both types of cell division are likely to be regulated by Numb, which is accumulated in neuron fated cells and functions as a Notch repressor [Cayouette and raff, 2002; Kageyama et al., 2008]. It is not know what controls the timing of transition from proliferation to asymmetric or symmetric neurogenic division, but is has been suggested that cyclic expression of proneural genes may lead to the accumulation of factors required for cell cycle exit, so that after a certain *rounds of oscillation*, those factor reach a critical value and cell cycle exit is promoted (Politis et al., 2007). Once neuronal precursors are selected, the mechanism of lateral inhibition operates from neurons to neuronal progenitors, and reinforces the fate of each daughter cell [Kageyama et al., 2008].

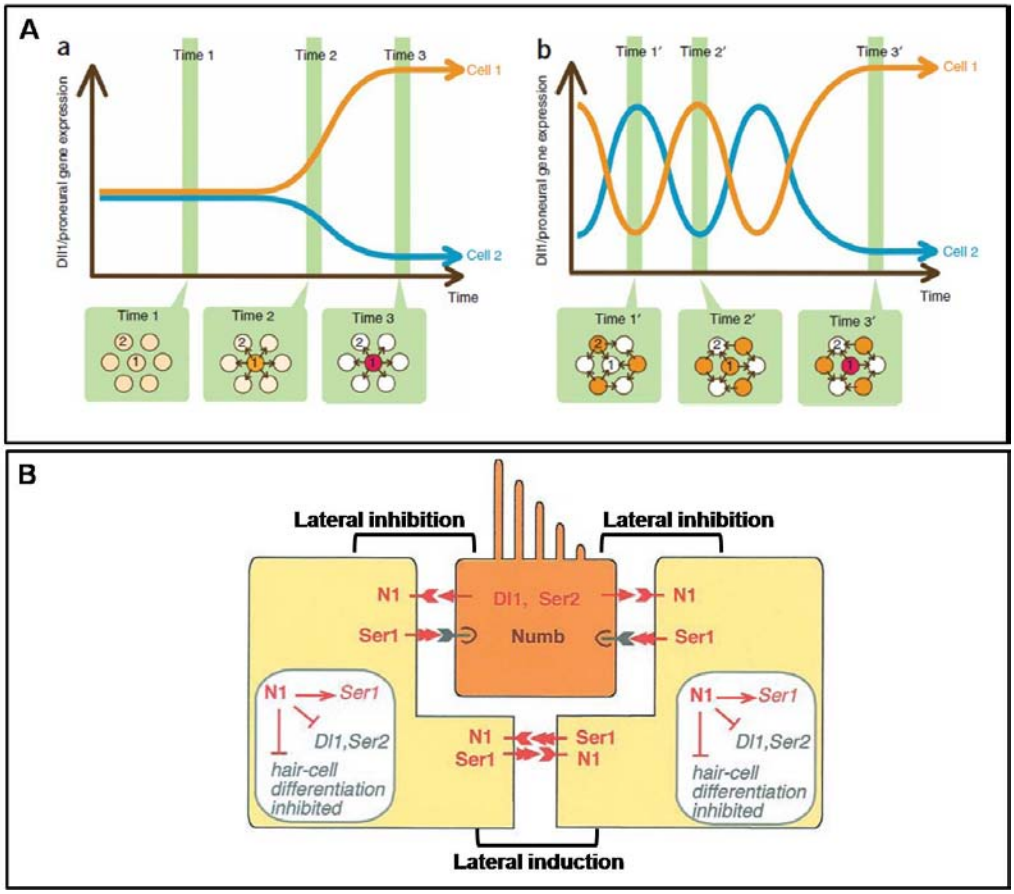


Figure 2: Lateral inhibition and lateral induction. A: Lateral inhibition the neural progenitors (a) classical view of lateral inhibition driven by stochastic variations. (b) Revised view of lateral inhibition bases on Hes1 oscillations. B: Lateral induction and lateral inhibition mechanisms proposed to operate in the sensory organs of the inner ear between supporting cells and hair cells and supporting cells, respectively. Modified from Kageyama et al. (2008) and Eddison et al. (2000).

Lateral induction and boundary formation

The mechanism of lateral induction is a well established mechanism for some juxtacrine signals like TGFα/EGF binding to EGF receptors, in which ligand or receptor expression are positively regulated by the signalling pathway [Clark et al., 1985; Coffey et al., 1987].

Notch–mediated lateral induction functions in such a way that a cell expressing a DSL ligand activates Notch in the neighbouring cell which results in the up regulation of the ligand in the signal receiving cell. So that, lateral induction promotes cooperation between neighbours instead of competition, and results in the establishment of a territory with a common identity (Lewis, 1998). A mathematical model developed in 2000 by Maskus et al. demonstrates that such positive feedback, in combination with juxtacrine communication, provides and effective mechanism for the formation of spatial patterns. The "wavelength" of the pattern is determined by the relative strength in the production of ligand and receptor induced by the feedback mechanisms. If the expression of the receptor is strong, then the pattern range is short because it is not possible for one cell with high levels of receptor to have more than two neighbours with similar high expression. However, if the effect on the production of ligand is stronger than on the receptor, then it can be propagated through a large number of cells [Markus et al., 2000]. Such a feedback loop mechanism has been described during the formation of morphological boundaries in vertebrate somitogenesis, the development of the *Drosophila* wing margin, and the rhombomeric boundaries (de Celis and Bray, 1997; Bray, 1998; Cheng et al., 2004; Baek et al., 2006). The process of Notch-mediated lateral induction has also been proposed to regulate the specification of sensory patch formation during inner ear development (Fig.2Bsee below) (Eddison et al., 2000).

BOX IV.1: Lateral induction in the D/V wing margin of *Drosophila*

In the *Drosophila* wing the mechanism of lateral induction operates as follows: *Notch* receptor is expressed throughout the wing disc, while *Serrate* and *LFng* are expressed in the dorsal part of the disc, and *Delta* in the ventral region. Since *Lfng* promotes Notch binding to Delta, Serrate can only activate Notch in cells that do not express *Lfng*, thus in the ventral side of the D/V interface. Delta will only signal cells in the dorsal margin of the D/V interface, which express LFng and it is inhibited from signalling to cells in the ventral compartment by Delta-Notch mediated cis-inhibition [Kim et al, 1995; Doherty et al., 1996; Fleming et al., 1997, de Celis and Bray, 1997]. The first key feature of Notch activation at boundaries therefore is that it has a positive outcome in which the boundary cells acquire a new organizer character, coordinating growth and patterning and activating the expression of specific target genes, such as wingless. For this reason, this type of signalling by Notch has been called inductive [Artavanis-Tsakonas et al., 1999; Bray, 1998]. However, more recently it has been demonstrated that the major role of Notch activation at the boundary cells is to relieve the repression on target genes mediated by Su(H) (Koelzer and Klein, 2006), which suggests that instead of inductive, Notch activity plays a rather permissive role. The second key feature is that border cells that receive the signal need themselves to upregulate the production of the ligand to activate the cells on the opposite side, which is the basis of the positive feedback mechanism that underlies lateral induction (Bray, 1998).

IV.3 Serrate1 during development: function and regulation

Serrate is one of the two known DSL ligands that interact with the Notch receptor in *Drosophila*. *Serrate* orthologs have been identified in frog, fish, chick, mouse, rat and human. Chick has two *Serrate* paralogs, *c-Serrate1* and *c-Serrate2* (from now on referred to simply as *Serrate1* and *Serrate2*) (Hayashi et al., 1996; Myat et al., 1996), which are orthologs of mammalian *Jagged1* and *Jagged 2* (Lindsell et al., 1995; Shawber et al., 1996; Luo et al., 1997; Mitsiadis et al., 1997; Oda et al., 1997; Valsecchi et al., 1997). In *Xenopus*, it was identified one *Serrate* ortholog, *X-Serrate1* (Kiyota et al., 2001). In zebrafish, three *Serrate* paralogs were identified: *Jagged 1a* and *Jagged1b* are paralogs of mammalian *Jagged1* and *Jagged2,* ortholog of mammalian *Jagged2* (Zecchin et al., 2005). *Serrate1/Jagged1* is expressed in a wide variety of tissues during development, where it is co-expressed with different Notch receptors and complementary to *Dl1*. *Serrate 1* is expressed in all cranial placodes (except the trigeminal), in the somites and the nephrogenic mesoderm and, later on, in the metanephric kidney. It is also expressed in the vascular system, developing heart and the developing limb buds. In the CNS, *Serrate1/Jagged1* is expressed in restricted domains of all subdivisions of the developing brain, and in the adult CNS. (Lindsell et al., 1996; Myat et al., 1996; Mitsiadis et al., 1997; Jones et al., 2000; Kiyota et al., 2001; Stump et al., 2002; Irvin et al., 2004; Zecchin et al., 2005).

In humans, mutations in *Jag1* gene are responsible for Alagille syndrome, an autosomal-dominant disorder characterized by intrahepatic cholestasis and abnormalities of heart, eye and vertebrae, as well as a characteristic facial appearance [Oda et al., 1997; Jones et al., 2000; Marchetti et al., 2009]. *Jag1* homozygus null mice die from haemorrhage early during embryogenesis (E11.5), exhibiting defects in the remodelling of the embryonic and yolk sac vasculature. Mice heterozygous for *Jag1* null allele exhibit eye dismorphology (Xue et al., 1999). A recent study in *Jag1* null mice has revealed that it is required for normal embryonic hematopoiesis, where it regulates *Gata2* expression in a Notch-dependent manner (Robert-Moreno et al., 2008). Three other mouse models, carrying mutations associated with the *Jag1* gene have been described: Slalom, headturner and coloboma. Homozygous mice for those mutations exhibit embryonic lethality and similar defects to the ones described for *Jag1* null mice (Xue et al., 1999; Kiernan et al., 2001; Tsai et al., 2001). Heterozygous mice for *Jag1* mutations as well as *Jag1* ear conditional null mice also reveal defects in the development of the inner ear (Fig.3A, see below) (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006). Functional studies in rat revealed a role of *Jag1* during lens fibber differentiation.

The pattern of expression of *Serrate1* in the developing CNS is consistent with a role during neural development. In chick and rat, *Delta* and *Serrate* are expressed in the ventricular zone of the developing hindbrain and spinal cord. Both ligands are expressed in different subsets of cells departing from the ventricular zone, suggesting that they are involved in the regulation of neurogenesis. (Lindsell et al., 1996; Myat et al., 1996). Functional studies in *Xenopus* revealed that *X-Serrate-1* regulates primary neurogenesis in a complementary way to *X-Delta-1*. When over-expressed, both genes lead to a

reduction in the production of primary neurons and, although they do not influence each other expression, they are able to rescue effects due to their reciprocal loss of function (Kiyota et al., 2001). In the post-natal brain, *Jagged1* and *Notch1* are necessary for the maintenance of the neural stem cell population of the SVZ. Reduction in *Jagged1* expression reduces proliferation and self renewal ability, but it does not affect the differentiation potential of NSC. In addition, soluble Jagged1 promotes self-renewal and neurogenic potential of multipotent neural progenitors *in vitro*, and it is able to replace growth factors in the maintenance of NSC (Nyfeler et al., 2005).

As to its regulation, *Jag1* has been identified as an evolutionary conserved target of canonical Wnt signalling in progenitor cells. *Jag1 Wnt*-dependent Notch signalling activation is proposed as a key mechanism in maintaining the homeostasis of stem and progenitor cells (Katoh, 2006). *Jag1* regulation by Wnt signalling has also been described in hair follicle formation in adult epidermis (Estrach et al., 2006), colorectal cancer (Pannequin et al., 2009; Rodilla et al., 2009), and in the developing inner ear (see below) (Jayasena et al., 2008). Some reports also indicate the regulation of *Serrate1* by FGF signalling. During tooth development, *Jag1* expression is regulated by epithelial FGFs, in a process dependent on epithelial-mesenchimal interactions (Mitsiadis et al., 1997). Finally, during secondary lens fiber differentiation FGF activated MAPK/ERK pathway induces *Jag1* expression. In this model system, *Jag1* regulates its own expression through a mechanism of lateral induction that requires Notch signalling (Saravanamuthu et al., 2009).

IV.4 The Notch pathway during inner ear development: Different roles of Delta and Serrate ligands.

The expression of Notch pathway elements during otic development

Several elements and targets of the Notch signalling pathway are expressed throughout the development of the inner ear with a highly dynamic temporal and spatial pattern. In chick *Notch1* is expressed from the otic placode stage until late stages of otocyst development. It is initially expressed throughout the otic placode, but then is absent from the dorsal regions that form the semicircular canals and non-sensory parts of the utricle and saccule. *Notch2* is not expressed in the chick or mouse otic placode/vesicle. In the mouse, *Notch1* and *Notch3* receptors are expressed in the otic vesicle (Weinmaster et al., 1991; Williams et al., 1995; Lindsell et al., 1996; Adam et al., 1998; Lanford et al., 1999; Abello et al., 2007).

Delta 1 expression is detected in scattered cells that occupy the anterior half of the otic placode. Expression is maintained in the neurogenic and in delaminated neuroblasts. By embryonic day E3.5 and up to at least E12, *Delta1* is expressed in scattered cells in the sensory patches. The timing of expression differs between patches, according to their different time courses of hair-cell production. Thus, in the chick, *Delta1* expression correlates with nascent hair cells and neurons, and its expression is switched-off upon cell differentiation (Adam et al., 1998; Abello et al., 2007). In mouse, the pattern of

expression of *Delta1* is similar [Bettenhousen et al., 1995; Morrison et al, 1999; Vazquez-Echeveria, et al., 2008]. In both animal species, therefore, *Delta1* labels nascent neuroblasts and hair cells, and becomes silent upon cell differentiation. In mouse, in addition to *Delta1*, *Jagged2* is also expressed in nascent hair-cells (Lanford et al., 1999; Shailam et al., 1999).

Serrate1 expression is first detected in the chick otic placode by E2 and, in contrast with *Delta1*, it is expressed in compact domains rather than in a speckled pattern. *Serrate1* is initially expressed in the posterior-medial aspect of the otic placode. This initial pattern of expression rapidly changes and resolves into two poles of strong expression, one anterior and another posterior. They are connected by a medial domain of weak intensity that encompasses the dorsal-ventral part of the otocyst. In the otic vesicle, *Serrate1* is expressed in the dorsal-posterior aspect and shows a weak expression in the neurogenic domain. As sensory patches emerge *Serrate1* expression gets restricted to those domains that foreshadow the future sensory organs, where it is expressed throughout the period of sensory development. Upon cell differentiation, hair cells do not express *Serrate1*, which is retained by supporting cells. (Myat et al., 1996; Adam et al., 1998; Cole et al., 2000; Abello et al., 2007). Likewise, in mouse, *Jagged1* is expressed in the prosensory domains and gets restricted to the supporting cell layer as hair cells differentiate (Lewis, 1998; Morrison et al., 1999).

Lunatic Fringe (*Lng*), a modulator of the Notch signalling pathway (see above), is also dynamically expressed during otic development. In chick, *Lfng* is first detected by E2, in the neurogenic region. This domain expands ventrally as the otic cup invaginates, overlapping with *Delta1*. By otocyst stage *Lfng* expression overlaps with *Serrate1* in the medial region, but it is stronger than Serrate1 in the anterior-ventral aspect of the otocyst. Similarly to *Serrate1*, *Lfng* expression becomes restricted to the developing sensory patches and, later on, to the supporting cell layer of the nascent sensory organs. *Lfng* is also expressed in the CVG. This pattern of expression is very similar in mouse (Morsli et al., 1998; Cole et al., 2000; Abello et al., 2007)

Notch target genes from the *Hes/Hey* family of transcription factors are differentially expressed throughout otic development. *Hes5*, *Hes1* (*Hairy-1*), *Hes6, Hey1*, *Hey2* and *HeyL* expression patterns have been reported in either or both chick and mouse (see below, chapter V, *Hes/Hey* genes in ear development).

The complex expression pattern of ligands, receptor and targets of the Notch pathway anticipates the diverse roles that Notch plays during inner ear development. In early stages, Notch is required for the induction and patterning of the otic placode (Abello and Alsina, 2007; Abello et al., 2007; Jayasena et al., 2008). Later on in development, the Notch pathway exerts apparently contrasting functions: Early blockage of Notch activity results in a decrease in the number of hair cells, while a late blockade results in the generation of supernumerary hair cells (Takebayashi et al., 2007; Hayashi et al., 2008). This is believed to result from a dual function of Notch, on one hand, in the regulation of neuron and hair cell formation by the process of lateral inhibition and, on the other, in the specification of the sensory patches (Daudet and Lewis, 2005; Brooker et al., 2006).

The role of Notch signalling in the early induction and patterning of the otic placode

Notch signalling is involved in the process of otic placode induction. First, Notch regulates the size of the otic placode, and the inactivation of *Notch1* reduces the size of the otic placode. Notch signalling regulates also the expression of otic markers like Pax8, the thickening of the otic placode and the repression of the epidermal marker Foxig2 (Jayasena et al., 2008). Although Notch signalling does not regulate the onset of its own expression and activation in the otic placode, once that happens, it is able to augment Wnt activity to maintain it.(Jayasena et al., 2008). Thus, there is a loop by which Wnt promotes Notch that, in turn, promotes Wnt activity, which cooperates to specify the otic placode (Jayasena et al., 2008). Notch signalling is required also for the early patterning of the otic placode into a neural-competent and a non-neural domain. Notch blockade results in the expansion of non-neural genes *Lmx1b* and *Irox1* into the anterior aspect of the placode, where they are normally not expressed. This expansion is not due to cell migration, but to the lack of repression of these genes (Abello et al., 2007). Therefore, Notch is required for induction and early patterning of the otic placode.

The role of Delta/Jagged2 ligand during otic development: lateral inhibition mediates neurogenesis and hair cell differentiation

In chick, mammals and zebrafish, *Delta1* expression foreshadows the differentiation of otic neurons and hair cells, and *Jagged2* that of hair cells. (Adam et al., 1998; Haddon et al., 1998; Lanford et al., 1999; Daudet and Lewis, 2005; Brooker et al., 2006; Abello et al., 2007; Daudet et al., 2007). The role of *Delta1* in the inner ear was first elucidated by studies in zebrafish (Haddon et al., 1998). *Mib* zebrafish mutants carry a mutation in the *mindbomb* gene, which leads to the loss of function of *Delta* and the disruption Notch pathway. The expression of *Delta1* is increased and disrupted from its normal salt-and-pepper pattern consistently with the disruption of Notch pathway activity. *Mib* mutant fish exhibit supernumerary otic neurons and hair cells, suggesting strongly that the process of lateral inhibition mediated by Notch pathway regulates the development of those cell types (Haddon et al., 1998). These observations were further confirmed in chick and mice. In chick, the γ-Secretase inhibitor DAPT or the electroporation of a dominant negative form of MAM result also in neurone or hair cell overproduction, without the disruption of the neural competence of the domain (Abello et al., 2007; Daudet et al., 2007). Forced activation of Notch1 prevents hair cell differentiation (Daudet and Lewis, 2005), but it also induced ectopic hair cells (see below). More recently, the occurrence of lateral inhibition has been shown to occur in the auditory epithelium during the regeneration of hair-cells. The blockade of the Notch signalling results in the overproduction of hair cells at the expense of supporting cells (Daudet et al., 2009).

In *Delta1* ear conditional knockout mice, maculae are severely affected (Fig.3C) and the volume of the CVG increased, suggesting that the loss of lateral inhibition caused an excessive number of vestibular epithelial cells to adopt a neuronal fate, exhausting the pool of sensory progenitors (Brooker et al., 2006). Conditional knockout mice for *Delta1*

and *Jagged2* produce supernumerary hair cells. The effects of *Jag2* predominate in the inner hair cell layer, while those of *Delta1* in the outer hair cell layers (Lanford et al., 1999; Kiernan et al., 2005a; Brooker et al., 2006).

The loss of function of several Notch target genes also help to illustrate the role played by Notch in the process of lateral inhibition, and they will be discussed below. Taken together, the evidence strongly suggests that the generation of neurons and the mosaic pattern of hair/supporting cells of sensory organs are generated by sequential rounds of Delta-Notch mediated lateral inhibition.

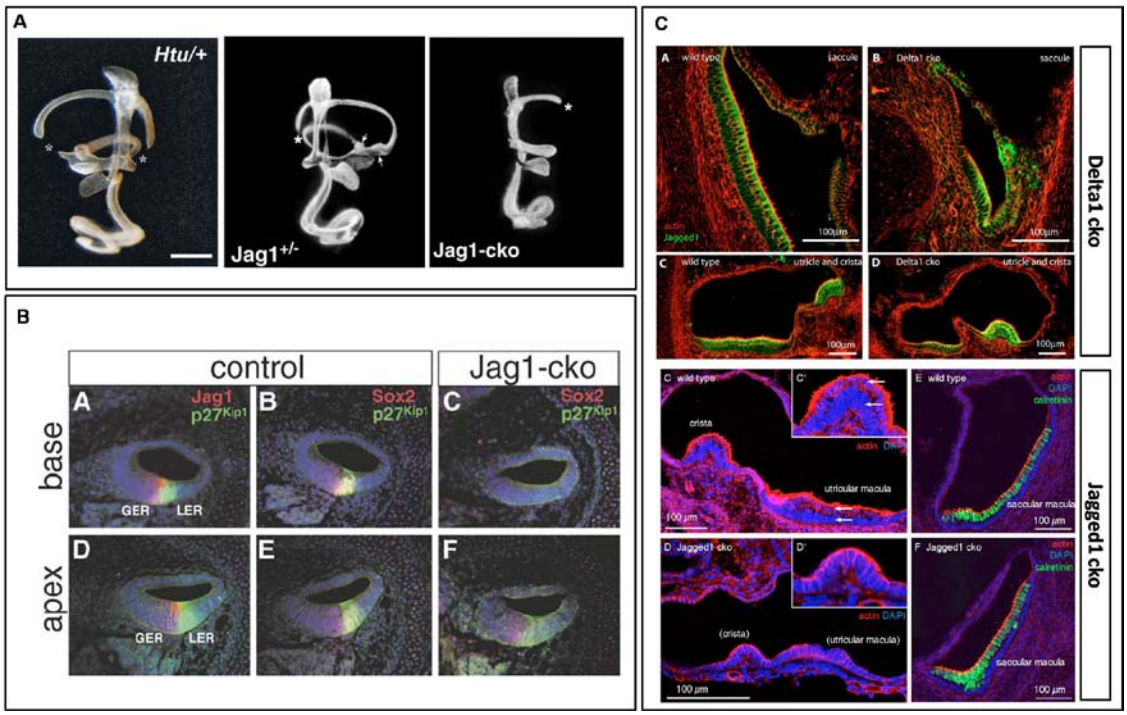


Figure 3: The functions of *Delta1* and Serrate*1* in the inner ear . A: Paintfilling of inner hears of headturner and *Jag1* cko mutant mice, showing truncated semi-circular canals and deformed cochlea. B: Expression of *Sox2* and *p27* in the cochlea of *Jag1* cko mice. *Sox2* and *p27* expression is absent the basal turns of the cochlea and low in the apical ones. C: comparison of the effects of *Delta1* and *Jagged1* conditional deletion in the epithelium of the cristae and macula sacculi. *Delat1* cko mice have normal cristae but abnormal sensory epithelium in the macula sacculi. *Jag1* cko show impaired sensory development in the cristae but normal epithelium morphology in the macula sacculi. Modified from Kiernan et al. (2001, 2006) and Brooker et al. (2006).

The functions of Serrate1 during inner ear development: prosensory specification

The observation that the expression of *Serrate1* foreshadows the emergence of the sensory organs soon suggested its association with the mechanisms that specify the prosensory domains (Adam et al., 1998; Lewis et al., 1998; Morrison et al., 1999; Cole et al., 2000). Several functional studies in chick and mouse have shed light on the function of *Serrate1* during inner ear development, although the information is still incomplete.

The onset of *Serrate1* expression during otic development is not dependent on Notch (Abello et al., 2007; Daudet et al., 2007), but rather on Wnt signaling (Jayasena et al., 2008). The maintenance of *Serrate1* expression, however, requires Notch signalling and this may be dependent on the process of lateral induction as first suggested by Eddison et al. (2000). This mechanism is supported by the comparison of *Jag1* expression and the expression of the activated form of Nocth1 ligand (actN1) during inner ear development, which suggests a positive cooperation between *Jag1* and actN1 (Murata et al., 2006). These observations suggest also that Notch may be involved in the establishment of the boundaries of the prosensory domains, but this possibility has not been tested directly. However, there are no reports in the gain or loss of function of *Serrate1*, which function is inferred from gain and loss of function studies on Notch (Eddison et al., 2000; Daudet and Lewis; Daudet et al., 2007).

Several mutant mice have been used as loss of function models to elucidate the function of *Serrate1*: slalom, coloboma and headturner (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006). These mutations lead to embryonic lethality, so that heterozygous mice for these mutations were used as loss of function models to study inner ear development. All three mutants exhibit defective inner ears and abnormal development of the sensory organs suggesting that Jagged1 is required for normal sensory development. Slalom and headturner mouse mutant show truncated anterior and/or posterior semicircular canals with missing ampullae (Fig.3A). In the organ of Corti, the number of outer hair cells is significantly reduced and the number of inner hair cells slightly increases, with sometimes duplication of the inner hair cell row and the appearance of atypical outer-hair-cell-shaped cells residing in the inner row (Kiernan et al., 2001; Tsai et al., 2001). Coloboma mutants exhibit similar, but milder phenotype in the cristae. The phenotype in the organ of Corti it is more complex: There are supernumerary inner hair cells throughout all the turns of the cochlea, but the phenotype for outer hair cells diverge from the basal and middle regions to the apex. In the middle-base, like in the other mutants, the number of outer hair cells is reduced, although the phenotype is milder. In the apical regions, however, coloboma mutants show increased number of outer hair cells (Kiernan et al., 2001).

Two conditional null alleles for *Jag1* in the ear exhibit defects similar to the ones described above. Both mice exhibit truncated semicircular canals and missing anterior and posterior cristae. Lateral crista and macula utriculi are present but with reduced number of hair cells, while the macula sacculi is mostly unaffected (Figs.3A and C) (Brooker et al., 2006; Kiernan et al., 2006). Similarly to the mutants, the patterning of outer and inner hair cells in the organ of Corti is altered, but differently in both KO mouse. Kiernan and colleagues report that hair cell and supporting cell formation is suppressed in the most basal turns of the cochlea. In the midbasal and apical regions, hair cells are present, but in reduced numbers and disorganized patterns. In the apical region, only inner hair cells form and associated inner phalangeal cells, and no outer hair cells or Deiter's cells develop (Kiernan et al., 2006). Brooker and collegues report a phenotype that is more similar to the one described for the mutants, with reduced number of outer hair cells and supernumerary inner hair cells. Both studies demonstrate that the reduced number of hair cells is not due to defects in differentiation, which does

not occur prematurely in these mice, or to degeneration (Brooker et al., 2006; Kiernan et al., 2006). In *Jag1* mutants, the expression of *Sox2* and *p27^{kip}* is disrupted. Consistently with the defects in hair cell formation, the basal turns of the cochlea do not express *Sox2* or *p27^{kip}*, and the most apical turns exhibit reduced expression (Fig.3B). In the vestibular organs, Sox2 expression in the cristae and macula utriculi is absent or reduced, but mostly unaffected in the macula sacculi (Brooker et al., 2006; Kiernan et al., 2006). Similarly, in chick, blockade of Notch signalling down regulates the expression of *Sox2* and *Bmp4* (Daudet et al., 2007). Together, these results strongly suggest an early role of *Serrate1* and Notch in the specification of the prosensory patches.

But *Jagged1/Serrate1* play another role during late stages of sensory development. Antisense *Jag1* in cultured cochlea leads to an overproduction of hair cells, which is in contrast with the cochlear phenotype of *Jag1* mutant mice described above. One explanation is that, during hair cell differentiation, *Jag1* cooperates with *Delta1* in the process of lateral inhibition. This would explain the effect contradictory effect observed in the inner and outer hair cells of *Jag1* mutants. The gain of supernumerary cells would result from the disruption of lateral inhibition, whereas the loss of outer hair cells to the loss of prosensory progenitors (Zine et al., 2000). The fact that the macula saculli is not affected in the *Jag1* mutants, but it is in *Delta1* mutants (Fig.3C), together with the observation that the CVG is enlarged in these mice, strongly suggests that the lateral inhibition mediated by Delta during the formation of neuroblasts is required for the regulation of the number of sensory progenitors that remain in the epithelium and that will probably give rise to the macula sacculi (see also the paragraph on neurosensory fate and multipotent progenitor).

These studies suggest multiple roles of *Jag1* during inner ear development: 1) in the induction and patterning of the otic placode, 2) in the specification of prosensory domains and the regulation of the extension and boundaries of those domains and, 3) in the process of lateral inhibition during hair cell differentiation that maintains Notch activity in the supporting cells. The mechanism by which *Serrate1* mediates those functions is largely unexplored. There is no information on whether *Serrate1* is able to activate Notch in the sensory patches, what are their targets or how lateral induction works. Neither it is known whether *Serrate1* has an inductive or permissive role in the process of prosensory specification, or if it sufficient for the induction of the prosensory fate. I shall address some of these questions in this thesis work.

V. Helix-loop-helix proteins: function and regulation

Helix-loop-helix (HLH) family of proteins comprises more than two-hundred members and it is present in organisms ranging from yeast to humans. In metazoa, HLH proteins function in the coordinated regulation of gene expression, through the regulation of cell cycle, cell lineage commitment and cell differentiation during haemopoietic, myogenic, pancreatic and neurogenic development [Murre et al.,1994; Littlewood and Evan, 2005; Massari and Murre, 2000]. The common feature among the members of the family is the highly conserved HLH domain that contains two amphipathic α-helices separated by a shorter intervening loop that has a more variable length and sequence. The HLH domain primarily mediates homo- or hetero-dimerization, which is essential for DNA binding and transcriptional regulation mediated by HLH proteins (Massari and Murre, 2000). In addition to the HLH domain, several sufbfamilies of HLH proteins contain an additional domain, composed of highly basic residues adjacent to the HLH domain, known as the basic domain. The proteins containing this domain are known as basic-helix-loop-helix (bHLH) and differ from their relatives in the ability to bind DNA containing the canonical 'E Box' recognition sequence (CANNTG). Some HLH proteins can also bind the related 'N box' sequence (CACNAG). HLH lacking the basic domain are not able to bind DNA (Massari and Murre, 2000).

V.1 Proneural bHLH proteins

The concept of '*proneural gene'* was first defined in Drosophila to characterize genes that are both required and sufficient to drive neuronal commitment in the neuroectoderm [Garcia-Bellido, 1979; Ghysen et al., 1989; Romani et al., 1989; Ghysen et al., 1993]. Proneural proteins are bHLH proteins that bind the class A subtype of 'E box' recognition sites and generally act as transcriptional activators (Fig.1c). Like other tissue-specific bHLH proteins, proneural proteins bind DNA as heterodimeric complexes that they form with ubiquitously expressed bHLH proteins, the E proteins (Atchley and Fitch, 1997; Bertrand et al., 2002). Proneural genes are conserved from flies to vertebrates and can be classified into two subfamilies, according to their proteins similarities with the two *Drosophila* proneural subfamilies: the *Achaete-scute* (*as*) and *Atona*l (*ato*) (Bertrand et al., 2002) (Fig.1a).

Owing to the large number of HLH proteins that have been described, several classification schemes were proposed based upon tissue distribution, dimerization capabilities, and DNA-binding specificities (Murre et al., 1994; Atchley and Fitch, 1997). I shall briefly describe three families of HLH proteins whose functions are relevant to the work described in this report, the proneural, Hes/Hey and Id HLH protein families. The Achaete-scute gene family contains four Drosophila genes – achaete (ac), scute (sc), lethal of scute (lsc) and asense (ase) – and three vertebrate representatives involved in neural development. The as vertebrate homologues (Ash genes), include Ash1 (mouse Mash1, chick Cash1, zebrafish Zash1 and Xenopus Xash1) and the Mash2, Xash3 and Cash4, each identified in only one class of vertebrate (Villares and Cabrera, 1987; Bertrand et al., 2002). The Atonal-related family (ato) includes four genes in Drosophila –

ato, cato, tap and amos – which specify subtypes of sense organs (Bertrand et al., 2002; Gibert and Simpson, 2003).

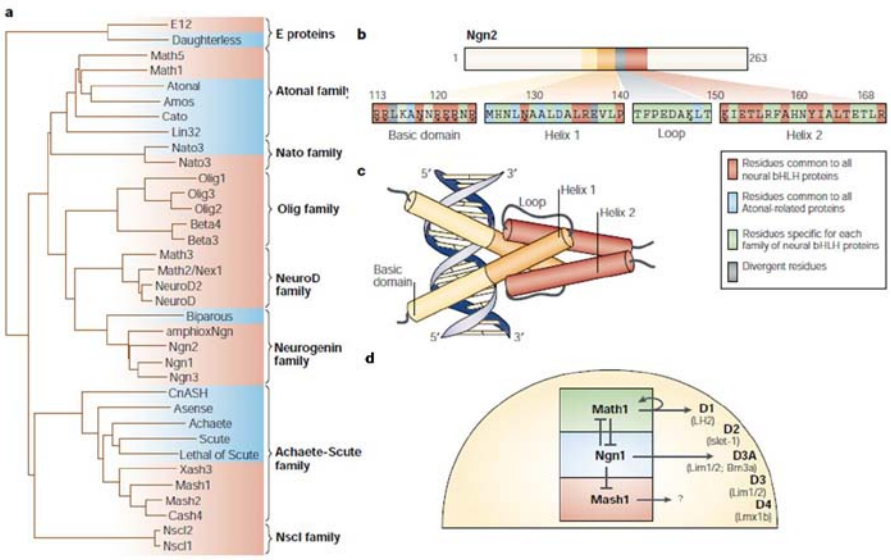


Figure 1: Proneural proteins. a: Dendrogram of the sequence of the basic helix–loop–hélix (bHLH) domain of invertebrate (blue) and vertebrate (red) neural bHLH proteins. Proteins have been grouped in distinct families on the basis of closer sequence similarities in the bHLH domain. b: Sequence of the bHLH domain of the mouse proneural protein neurogenin 2 (Ngn2). A colour code indicates the degree of amino-acid conservation between neural bHLH proteins at each position. c: Schematic representation of the structure of a bHLH dimer that is complexed to DNA The basic region fits in the main groove of the DNA, and many residues in this region make direct contact with the E-box sequence. The two α-helices of both partners together form a four-helix bundle. d: Regulatory relationships between basic helix–loop–helix (bHLH) proteins and neuronal populations in the dorsal spinal cord. Different neural bHLH proteins, expressed in distinct dorso-ventral progenitor domains of the dorsal spinal cord, control the specification of different interneuron subtypes. The domains of neural bHLH gene expression are established and/or maintained by cross-repression. Adapted from Bertrand et al. (2002)

In vertebrates, the Atonal gene family is greatly expanded and can be roughly subdivided into four groups – Atonal (Atoh), Neurogenin (Ngn), NueroD and Olig – according to sequence similarities within the bHLH domain. From those, only Atoh1 and Atoh5 (the members of the Atoh group) are considered ato orthologues, while the others are referred to as ato-related genes, due to the presence of family-specific residues in their bHLH domains and common biochemical properties (Bertrand et al., 2002) (Fig.1a and b). Proneural genes are key regulators of vertebrate neurogenesis as they regulate all features inherent to the process of neuronal differentiation. First, they modulate the transition from a proliferating neural progenitors to a post-mitotic neuron generally by activating the expression of cyclin-dependent kinase inhibitors and promoting cell cycle exit (Farah et al., 2000; Ohnuma et al., 2001; Bertrand et al., 2002; Kageyama et al., 2005; Nguyen et al., 2006). Proneural proteins also coordinate the acquisition of both generic and specific neuronal characters, as they trigger the expression of other transcription factors that regulate pan-neural and subtype specific characters (Bertrand et al., 2002). Neurogenins have a similar proneural function to that of their Drosophila

counterparts, whereas other proneural bHLH, such as NeuroD are involved in the specification of neuronal subtypes, and in neuronal differentiation and survival (Bertrand et al., 2002; Cau et al., 2002).

One important characteristic of proneural proteins is their ability to positively regulate their expression though positive feedback loops. This process is required to the progression of neurogenesis, which is regulated by levels of these proneural proteins. Initially, neural competent progenitors express low levels of proneural proteins but they are not yet committed to differentiation, and it is not until proneural proteins reach a certain high level that irreversible commitment to differentiation occurs. This requires additional mechanisms to increase and maintain proneural gene expression, even when the inductive signal is switched off. *as and ato* genes in Dorsophila (Gilbert et al., 2003) and *Atoh1* in vertebrates (Helms et al., 2000) are known to positively regulate their own expression. Interestingly, this type of mechanism does not seem to regulate *Mash1* or *Ngn* genes in vertebrates (Bertrand et al., 2002)

Another central characteristic of proneural genes is the cross-regulatory interactions that occur between members of the family leading to cascades of proneural activity (Bertrand et al., 2002) (Fig.1d). In vertebrates, *Mash1* and *Neurognenins* are expressed earlier in neural progenitors and *NeuroD* family members are expressed later in immature neurons (reviewed in Bertrand et al., 2002). *Ngn1* is necessary for *Math3* and *NeuroD* expression in cranial sensory neurons, and *Mash1* acts upstream of *Ngn1* and *NeuroD* in the olfactory sensory system (Fode et al., 1998; Ma et al., 1998; Cau et al., 2002). In addition, *Ngn1* can inhibit *Mash1* expression (Gowan et al., 2001) and *Ngn1* and *Atoh1* cross-inhibit each other expression, segregating different cell lineages in the brain and ear (Fode et al., 2000; Raft et al., 2007).

Proneural proteins in inner ear development

During inner ear development different proneural genes are associated with the development of neurons and hair cells: *Ngn1* and *NeuroD*, play critical roles during inner ear neurogenesis, while *Atoh1* (*Cath1, Math1*) is a key regulator in hair cell development.

Ngn1 and NeuroD are expressed in the neurogenic domain of chick and mouse in a sequential manner, *Ngn1* preceding *NeuroD* (Alsina et al., 2004; Vazquez-Echeverria et al., 2008). *Ngn1* is necessary to specify neuronal fate as mice lacking *Ngn1*, lack all sensory neurons in the inner ear (Ma et al., 1998; Ma et al., 2000). *NeuroD* function is associated with neuronal differentiation and survival (Liu et al., 2000; Kim et al., 2001). Mice lacking *NeuroD* exhibit a near-complete loss of cochlear ganglia and a significant loss of vestibular ganglia. The surviving vestibular ganglion display disorganized fibber projection onto the vestibular sensory epithelium (Kim et al., 2001). In summary, *Ngn1* is required for determination of neuronal fate, while downstream transcription factors such as *NeuroD* play various roles in differentiation and perhaps neuronal identity specification (Liu et al., 2000; Kim et al., 2001) (See Fig.4, chapter II).

Atoh1 is expressed in the ear sensory epithelia at developmental stages coincident with hair cell differentiation (Bermingham et al., 1999; Lanford et al., 1999; Lanford et al., 2000). Deletion of *Atoh1* leads to a complete loss of hair cells (Bermingham et al., 1999), whereas overexpression of *Atoh1* is sufficient to induce sensory epithelium to develop as hair cells in both embryonic and post-natal stages (Zheng and Gao, 2000; Jones et al., 2006). Ectopic expression of *Atoh1* is also sufficient to induce hair cell formation in non-sensory regions located near to the organ of Corti in embryonic and postnatal inner ears (Zheng and Gao, 2000; Woods et al., 2004). Recenty, Gubbels et al. (2008) were able to introduce *Atoh1* into precursor hair cells in the inner of mice at day 11.5 of embryonic development in utero. The result was that by embryonic day 18.5 the transfected cells had differentiated into hair cells and were integrated among untransfected, normal hair cels. Transfected cells formed the correct neural contacts with afferent nerve fibbers and to be capable of mechano-electrical transduction (Gubbels et al., 2008). Taken together, existing data suggest strongly that *Atoh1* behaves as a master gene for hair cell development (See Fig.4, Chapter II).

V.2 Hes and Hey bHLH proteins

Hes and *Hey* genes are best know to their function as downstream targets of Notch signalling and counteracting activity of the proeneural genes [Davis and Turner, 2001, Iso et al., 2003; Fischer and Gessler, 2007]. They belong to class C bHLH proteins and most of them function as transcriptional repressors. All members bind class C E-Box like sites (CACGNG) as well as N-box sequences (CACNAG), and some of the members also can bind class B E-Box sites to some degree, but not class A sites. Hes and Hey products can function as homodimers to achieve their transcriptional function. But also heterodimerisation between Hes ahd Hey proteins has been described, and heterodimers seem to display stronger effects than homodimers [Ohsako et al., 1994; Van Doren et al., 1994; Fisher and Caudy, 1998, Jennings et al., 1999, Iso et al., 2001; Iso et al., 2003].

Hes are the mammalian counterparts of the *Hairy* and *Enhancer-of-Split* (*E(spl)*) type of genes in *Drosophila.* In *Drosophila* there is one *hairy* gene and seven clustered *E(spl)* genes that control crucial developmental processes like segmentation, myogenesis and neurogenesis (reviewed in Fischer and Gessler, 2007). In mammals, seven *Hes* genes have been identified (*Hes1-7*), among which one is a *Hairy*-like gene, *Hes1* (*Hairy1/2 in chick*) and the others are *E(Spl)*-like genes, *Hes2-7*, (Akazawa et al., 1992; Sasai et al., 1992; Ishibashi et al., 1993; Bae et al., 2000; Hirata et al., 2000; Koyano-Nakagawa et al., 2000; Pissarra et al., 2000; Bessho et al., 2001). Some *Hes* genes are Notch-responsive (*Hes1*, *Hes5* and *Hes7*, Fig.2A), while others are not (*Hes2*, *Hes3* and *Hes6*). Only some of them have been described to take part in neural development (*Hes1*, *Hes3*, *Hes5* and *Hes6*) [Bessho et al., 2001; Jarrialut et al., 1995; Nishimura et al., 1998; Ohtsuka et al., 1999]. *Hes* genes encode bHLH proteins, characterized by an invariant proline residue in the basic domain and a carboxy-terminal tetrapeptide motif WRPW, conserved from fly to mammals (Fig.2A). The function of this carboxy-terminal domain is also conserved and it is related with the recruitment of co-repressors, Groucho in *Dorosphila* and TLE1-4 in mammals (Fig.2Ba). Hes proteins contain another helix-helix

domain, known as the Orange domain, which is thought to function as an additional interface for protein interactions that confer protein specificity [Fisher and Caudy, 1998; Iso et al., 2003 Fischer and Gessler, 2007].

Hey are strongly conserved during evolution and *Hey* genes in vertebrates have a counterpart in *Drosophila* with a still unknown function. There are three *Hey* genes in vertebrates (*Hey1*, *Hey2* and *HeyL*, also known as *Hrt1-3*, *Hesr1-3*, *Herp2,1* and *Chf2,1*). So far only *Hey1* and *Hey2* have been associated with neural development [Kokubo et al., 1999; Leimeister et al., 1999; Leimeister et al., 2000; Maier and Gessler, 2000; Nakagawa et al., 2000; Iso et al., 2001; Iso et al., 2002; Masami et al., 2002; Iso et al., 2003; Fischer and Gessler, 2007]. Hey proteins are structurally similar to Hes proteins in their bHLH and orange domains, with the main difference being the replacement of the invariant proline residue in the basic domain by a glycine that results in the inability of Hey proteins to bind N-box sequences (Fig.2A). Hey proteins preferentially bind E-box-like sequences, which are also recognized by Hes1 and Hes6. The most striking difference between Hes and Hey proteins is the lack of the WRPW tetrapeptide, which is replaced by a related YRPW peptide or a further degenerated YXXW (HeyL) sequence that cannot bind TLE co-repressors (Fig.2A). This sequence is followed by a conserved peptide of unknown function. Although Hey proteins cannot recruit TLE co-repressors, they are strong transcriptional repressors and the recruitment of co-repressors is mediated mainly by the bHLH domain and the repressor function associated with histone deacetylase activity (Iso et al., 2001; Fischer et al., 2002).

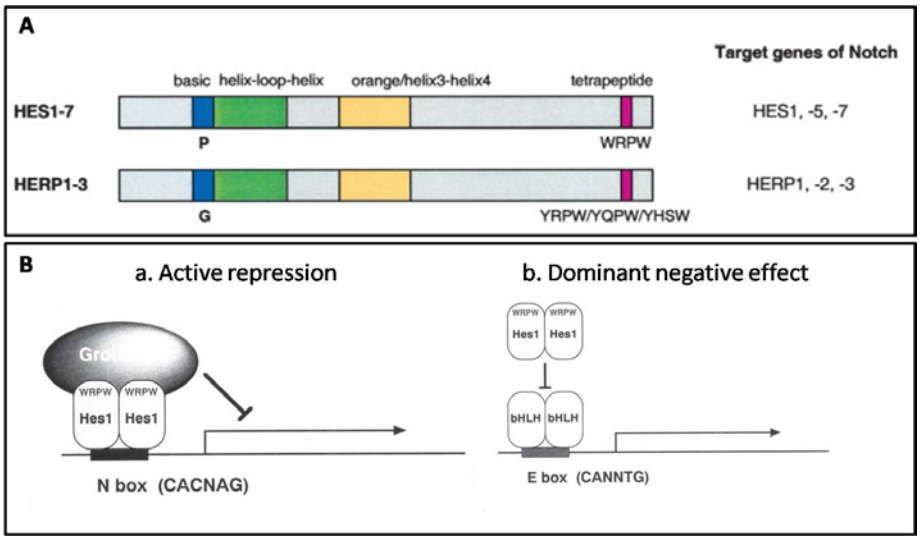


Figure 2: A: Schematic diagram of HES and HERP proteins. Conserved domains are marked by distinct colours: Blue for the basic domain, green for the helix-loop-helix domain, orange for the Orange domain, and pink for the tetrapeptide motif. Potential target genes of Notch are listed on the right. B: mechanisms of transcriptional repression by Hes1. (a) Active repression. Hes1 forms a dimer and binds to the N box. The co-repressor Groucho interacts with the carboxy-terminal WRPW and mediates active repression. (b) Dominant-negative effect. Most bHLH factors bind to the E box and activate gene expression. Hes1 shows a dominant-negative effect on these bHLH activators by forming a non-functional heterodimer complex.. Adapted from Kageyama and Ohtsuka (1999) and Iso et al. (2003)

Hes/Hey proteins play an important role in multiple developmental processes such as angiogenesis, somitogenesis, myogenesis, gliogenesis and neurogenesis. During neurogenesis, Hes/Hey proteins counteract neurogenesis by repressing proneural gene expression [Ishibashi et al., 1994; Otsuka et al., 1999; Ohtsuka et al., 2001; Masami et al., 2002; Sakamoto. et al., 2003; Fior and Henrique, 2005; Holmberg et al., 2008] (Fig.2Ba). Alternatively, they also form non-functional pairs with proneural proteins or E-proteins that block proneural activity (Fig.2Bb) (Sasai et al., 1992; Fischer and Gessler, 2007).

In vertebrates, *Hes1* and Hes7 negatively regulate their own expression [Hirata et al., 2002; Bessho et al., 2003; Shimojo et al., 2008], which results in an oscillatory pattern of expression. This appears to play a central role in the segmentation clock during somitogenesis (Dubrulle and Pourquie, 2004; Giudicelli and Lewis, 2004), and in the regulation of neurogenesis [Shimojo et al., 2001; Kageyama et al., 2008]. *Hes5* genes have also been shown to cross-inhibit their own expression which is suggested to be important to the *Hes5/Hes6* circuit regulating the switch off of Notch activity during neurogenesis (Fior and Henrique, 2005). *Hes5* and *Hes6* negatively cross-regulate each other expression and this mechanism is through to regulate the ability of neural progenitors to go through several rounds of Notch activity (Fior and Henrique, 2005). Hes6 protein has also been shown to modulate Hes1 function by forming heterodimers and blocking its ability to induce proneural activity (Bae et al., 2000).

Hes/Hey genes in inner ear development

Hes1 and Hes5: In chick, *Hes5* is initially expressed in the neurogenic domain of the otic placode. The expression is complementary to *Delta* and depends on Notch activation (Abello et al., 2007; Daudet et al., 2007). *Hes5* is not expressed in prosensory patches, but it is expressed in the supporting cells of the vestibular and cochlear organs (Shailam et al., 1999; Lanford et al., 2000; Zine et al., 2001; Doetzlhofer et al., 2009). It is likely that *Hes5* plays a role in otic neurogenesis similar to the CNS neurogenesis, through the negative regulation of *Ngn1* and *NeuroD* proneural gene expression, although this has not been directly demonstrated.

Hes1 is expressed in the posterior aspect of the chick otic placode, overlapping with *Serrate1*, and high levels of *Hes1* expression occur at the interface between neurogenic and non-neural territories at late otic cup stage. This domain does not express proneural genes so one likely function of *Hes1* is to prevent their expression, however, this has not been demonstrated with functional studies. ((Abello et al., 2007), see also (Jayasena et al., 2008) for the mouse). *Hes1* is also detected in the prosensory domain of the cochlea by E12.5 along with activated Notch protein. Thereafter, it becomes down-regulated as hair cells emerge and becoming restricted to the lateral edges of the prosensory domains. As hair cells differentiate, *Hes1* is again detected with activated Notch in the supporting cell layer (Zine et al., 2001; Doetzlhofer et al., 2009; Murata et al., 2009). This expression pattern suggests that *Hes1* is a likely target of *Serrate1*, but there is no information on this respect.

In a recent study Murata and colleagues have analyzed the prosensory regions of *Hes1* mutant mice, and observed an accelerated expression of $p27^{Kip1}$, along with a reduction in the cell proliferation rate. This suggests that the Notch-Hes1 pathway contributes to the adequate proliferation of sensory precursor cells through the transcriptional repression of $p27^{kip}$ (Murata et al., 2009). Interestingly, the positive feedback loop between the expression of *Jag1* and actN1 expression was conserved in *Hes1* deficient mice, suggesting that the mechanism of lateral induction was not disrupted in these animals. Furthermore, and similarly to the *Jag1* mutants, no precocious differentiation of hair cells was observed, which further argues that Notch alone is not the responsible for keeping cells from differentiating prematurely (Zine and de Ribaupierre, 2002; Murata et al., 2009).

Hes1 and *Hes5* mutant mice exhibit an increased number of hair cells in the macula utriculi and sacculi (Zheng and Gao, 2000; Zine et al., 2001). *Hes1* mutants exhibits also increased number of inner hair cells in the organ of Corti, while that of *Hes5* show increased number of outer hair cells. In *Hes1* mutants, additional ectopic hair cells are detected in the non sensory regions of GER and LER where *Hes1* is normally expressed, while *Hes5* mutants exhibit the same phenotype in the LER, where *Hes5* is normally expressed. Compound mutants exhibit an enhanced effect in both outer and inner hair cells (Zheng et al., 2000; Zine et al., 2001). *Atoh1* is up-regulated in these mutants, and it is expressed in the supernumerary outer, inner and ectopic hair cells (Zine et al., 2001; Zine and de Ribaupierre, 2002). Furthermore, the expression of *Jag2* is also up-regulated in the supernumerary hair cells of *Hes1*-deficient mice (Lanford et al., 1999; Zine and de Ribaupierre, 2002). These observations further suggest that lateral inhibition underlies the process of hair cell differentiation and that *Hes* genes may mediate the repression of *Atoh1* and DSL ligands. In fact, co-expression of *Hes1* with *Atoh1* in the GER, prevents the induction of hair cell fate that is driven when *Atoh1* is transfected alone (Zheng and Gao, 2000; Zheng et al., 2000). *Hes6* expression has been only reported in the developing hair cells of the auditory and vestibular system [Quian et al., 2006].

Hey1 and Hey2: *Hey1* and *Hey2* expression patterns during ear development have been recently described in the mouse, and there is still no information in the chick. *Hey1* is expressed in the vestibular and auditory prosensory regions by E12.5, and it is expressed in sensory organs until birth (Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2009). *Hey2* is first detected at E11.5 in the medial region of the otic epithelium, outside the neurogenic domain (Li et al., 2008). Later on, *Hey2* is expressed in the prosensory domain of the cochlea, the expression becomes stronger by E14.5 when it extends to all turns of the cochlea, overlapping with *Hey1*. As hair cell differentiation occurs, *Hey2* becomes restricted to pillar cells (Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2009). Contradictory reports describe that *Hey2* expression in the cochlea after E18.5 (Hayashi et al., 2008; Li et al., 2008; Doetzlhofer et al., 2009). *Hey2* expression has not been detected during the development of the vestibular system (Hayashi et al., 2008). *HeyL* is not expressed in the cochlea until the initiation of hair cell differentiation and it is expressed along with *Hey1* during the development of the vestibular organs (Hayashi et al., 2008; Doetzlhofer et al., 2009).

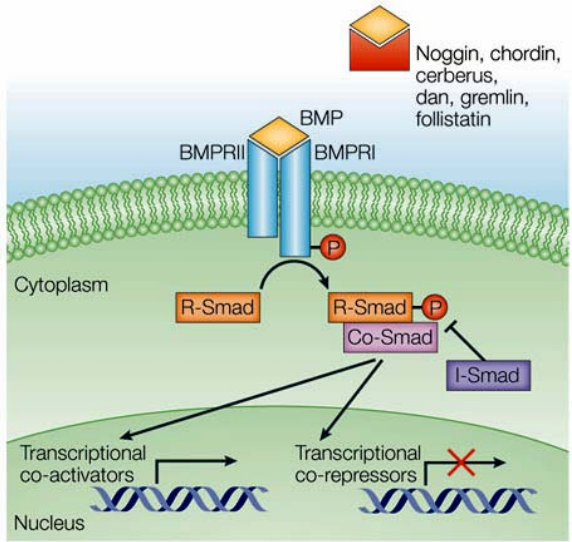
The expression patterns of *Hey1* and *Hey2* place them as good candidates to mediate the function of *Jag1* and Notch. Mice deficient for both *Hey1* and *Hey2* die between E9.5 and E11.5 due to cardiac arrest and vascular defects (Fischer et al., 2004; Kokubo et al., 2005; Hayashi et al., 2008). The phenotype of the double mutation is very similar to that of *Jagged1* mutants, suggesting an association between these downstream targets and *Jag1* function [Xue et al.1999, Fishcer et al., 2004; Kokubo et al., 2005b]. *Hey2* mutants exhibit a significant increase in the number of outer hair cells which is enhanced in *Hey2/Hes5* compound mutants. The increase in the number of inner hair cells is not significant in *Hey2* deficient mice, but compound *Hey2/Hes1* mutants show increased number of inner hair cells in comparison with *Hes1* single mutants, suggeststing a synergistic interaction between the two genes (Li et al., 2008). The increase in the number of hair cells is accompanied by an increase in the number of supporting cells, similarly to what has been observed in ligand mutants (see above). However, contrary to *Jag2/Dl1* compound mutants, no alterations in cell proliferation are detected in this case (Li et al., 2008). Furthermore, *Hey2* and *Atoh1* co-transfection in cochlear explants prevent the hair-cell-promoting activity of *Atoh1* (Doetzlhofer et al., 2009). A recent study has shown that *Hey2* expression is regulated by FGF in pilar cells, and this expression is necessary to maintain pillar cell identity in the absence of Notch signalling (Doetzlhofer et al., 2009). However, the blockade of FGF signalling alone does not prevent *Hey2* expression, unless Notch signalling pathway is also blocked. Thus, FGF signalling is sufficient to induce *Hey2,* but not required to maintain its expression, which can be maintained by Notch in the absence of FGF. Similarly, Notch signalling *per se* is not required for *Hey2* expression and in the presence of FGF, *Hey2* is deaf to Notch signalling. This is an example Notch-independent regulation of a Hes-related gene, and brings evidence on the still unexplored field of the cross-regulation among signalling pathways (Doetzlhofer et al., 2009).

V. 3 .Id HLH proteins

Id proteins are a particular subfamily of the HLH family of proteins, characterized by sharing the HLH domain, but lacking the basic domain, responsible for DNA binding (Reviewed in Norton, 2000). As a consequence, Id proteins physically interact with other bHLH proteins, but the heterodimers fail to bind DNA. Id proteins act, therefore, as dominant negative regulators of bHLH proteins [Benezra et al., 1990; Garrell and Mondolell, 1990; Ellis et al., 1990]. Since Id proteins interact with bHLH that are transcriptional activators and promote cell differentiation, the term Id referred to the ability of these proteins to inhibit both DNA binding and differentiation (Norton, 2000). Genes encoding Id proteins have been isolated from several metazoan species, but they have been most studied in *Drosophila*, in which a single *Id*-like locus, *extramacrochaetae (emc*), encodes an Id-like protein [Garrell and Mondolell, 1990; Ellis et al., 1990] and Mammals, mouse and human, which possess four *Id* family members (*Id1-Id4*) [Benezra et al., 1990; Sun et al., 1991; Christy et al., 1991; Biggs et al., 1992; Riechmann et al., 1994].

In *Drosophila* and mouse, functional studies have demonstrated the ability of Id proteins to counteract differentiation in different systems [Campuzano et al., 2001; Lsorella et al.,2001; Ruzinova and Benezra, 2003]. In addition, Id proteins regulate the cell cycle by modulating the transcription of several known target genes, both by interacting with bHLH and non bHLH proteins (reviewed in Norton, 2000). Id proteins also play important roles during development, which is illustrated by the widespread expression of *Id1-3* throughout the developing organism from early gestation to birth in mouse, frog and chick (Kee and Bronner-Fraser, 2001; Yokota, 2001). Indeed, double null mice for *Id3* and *Id1* are embryonic lethal with aberrant neuronal differentiation and angiogenesis (Lyden et al., 1999; Perk et al., 2005).

BOX V.1: BMP signalling pathway



Bone morphogenetic protein (BMP) ligands bind to the BMP receptors BMPRI and BMPRII, and BMPRII then phosphorylates and activates BMPRI. Phosphorylated BMPRI subsequently phosphorylates receptor-activated Smad proteins (R-Smads), which associate with common mediator-Smad (co-Smad) and enter the nucleus, where they regulate gene expression. The Smad proteins regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression. The BMP signal can be blocked by extracellular antagonists, such as noggin, which bind BMP ligands and prevent their association with the BMP receptors, as well as by intracellular proteins, such as inhibitory Smads (I-Smads), which prevent the association between R-Smads and co-Smads.

The regulation of Id proteins can be mediated by both transcriptional and posttranslational mechanisms. Post-translational mechanisms include the regulation of Id protein degradation and subcellular localization (Deed et al., 1996; Bounpheng et al., 1999). At the transcriptional level, BMPs (See BoxV.1 for an overview of the BMP pathway) appear as the most important regulators of *Id* gene transcription. Induction of

Id1-3 expression in response to BMP has been shown in a range of diverse cell lines and ES cells (Miyazono and Miyazawa, 2002; Ruzinova and Benezra, 2003). *Id1* is a direct BMP target gene whose expression can be upregulated by BMPs in the absence of *de novo* protein synthesis, and requires Smad1 or Smad5 [Korchynskyi and Ten Dijke, 2002; Lopez-Rovira et al., 2002; Katagiri et al., 2002]. Accordingly, elements conferring BMP responsiveness to the *Id1* promoter have been identified as Smad-binding elements (SBEs) and a GC-rich region, which bind BMP-associated Smad1 and Smad5 (ten Dijke et al., 2003). There is also evidence that *Id2* and *Id3* are regulated by BMPs and sequence analysis of *Id3* promoter revealed potential BMP-responsive elements (Ruzinova and Benezra, 2003). During embryogenesis, the expression of *Id* genes and *Bmp2* and *Bmp4* overlap in many sites and misexpression of *Bmp4* during embryonic development induces *Id3* expression [Yanagisawa et al., 2001]. In vitro, neural progenitors exposed even briefly to BMP2 fail to undergo neurogenesis and upregulate the expression of *Id1*, *Id3* and *Hes5* (Nakashima et al., 2001). Finally, *Id* genes have shown to be direct targets of BMP in ES cells where they suppress differentiation (Hollnagel et al., 1999; Ying et al., 2003).

Finally, ZEB and SIP genes modulate BMP signalling. The *Zfhx1* family of transcription factors, *Zfhx1a* (δEF1, ZEB1 – delta crystallin enhancer binding factor 1) and *Zfhx1b* (SIP1, ZEB2, Smad interacting protein) can act as co-factors of Smad proteins and modulate their activity through the recruitment of co-repressors and co-activators (Verschueren et al., 1999; Postigo, 2003; Postigo et al., 2003). Different studies suggest that these genes may mediate both redundant and antagonistic functions. The expression of ZEB proteins during embryonic development partially overlaps and mutant mouse for *Zfhx1a/b* show up-regulation of the functional genes, when its relative is knocked down (Miyoshi et al., 2006). However, ZEB proteins have also been described to have opposing effects on BMP pathway regulation Both ZEB proteins bind to Smads, but while ZEB1 synergizes with Smad proteins to actívate transcription, promote osteoblastic differentiation and induce cell growth arrest, the highly related ZEB-2/SIP1 protein has the opposite effect. These antagonistic effects by the ZEB proteins arise from the differential recruitment of transcriptional co-activators (p300 and P/CAF) and co-repressors (CtBP) to the Smads (Postigo, 2003; Postigo et al., 2003).

Id proteins during inner ear development

In mammals *Id1, Id2*, and *Id3* are expressed in the otic vesicle of mice and rats (Jen et al., 1997; Lin et al., 2003), and in the developing mouse and rat cochlea (Ozeki et al., 2005; Jones et al., 2006). In the chick otocysts, *Id1-3* are expressed in the otic vesicle in broad regions that include the prosensory patches, and precede the onset of expression of *Atoh1*. But, further in development, *Id*s are down-regulated from sensory patches in parallel to the onset of *Atoh1* expression [Kamaid et al., 2009]. *Id* expression in the otocyst is regulated by BMP-induced Smad signalling. BMP4 induces *Id1-3* expression in the otic epithelium in parallel to an increase in P-Smad expression, while Noggin and Dorsomorphin rapidly reduce *Id* expression, suggesting that BMP-induced Smad signalling is necessary to sustain *Id* expression [Kamaid et al., 2009]. Id function during ear development has been associated with the negative regulation of hair cell

differentiation. *Id3* sustained expression in the mouse cochlea results in the forced adoption of the supporting cell fate (Jones et al., 2006). Its forced expression in prospective cristae of the chick reduces the expression of *Atoh1*, prevents differentiation into hair cells, and maintains progenitor/supporting cell fate. [Kamaid et al., 2009]. Thus, *Id* genes are important regulators of the differentiation of hair cells in the developing inner ear.

INTRODUCTION – CHAPTER V

AIMS

The neurosensory elements of the inner ear, neurones and sensory cells, derive from a common population of progenitors that reside in the otic placode. Their development is tightly controlled to ensure that neurones and hair cells are born at the correct moment and location. The mechanisms that regulate the production of neurones and sensory cells are still not completely understood. At the beginning of this thesis, several studies suggested the requirement of *Sox2* and *Serrate1* for the correct development of the sensory elements of the inner ear [Kiernan et al., 2005; Daudet and Lewis, 2005; Kiernan et al., 2006; Brooker et al., 2006, Daudet et al., 2007]. They showed that both *Sox2* and *Jag1* are necessary for the development of sensory organs, and that both the gain and loss of function of Notch result in profound changes in the development of neurones and hair cells. These studies also suggested a possible cross-regulation between *Serrate1* and *Sox2*. However, some basic questions regarding the function of *Serrate1* and *Sox2* remained unanswered. Sox2 expression pattern during inner ear development was not known, as it was whether *Sox2* and *Serrate1* are sufficient to specify sensory fate or what are their roles during otic neurogenesis. Furthermore, it was also not known whether and how these pathways regulate the maintenance of the progenitor state during otic development, or which were their downstream targets.

This thesis was aimed at studying the function of *Sox2* and *Serrate1* in the neurosensory development of the inner from two perspectives: the specification of cell fate, and the regulation of progenitor state. Specifically I wanted to explore possible interactions between *Sox2* and the Notch signalling pathway, as well as the possible cross-talk with other signalling pathways. Specific aims were:

1. To characterize the expression pattern of *Sox2* during inner ear development.
2. To compare *Sox2* expression with that of other *SoxB1* genes (*Sox3*) in order to analyse possible redundant and/or differential functions.
3. To compare the expression of *Sox2* and *Serrate1*, and to test their interactions during neurosensory development.
4. To understand the mechanism of function of *Serrate1* and the downstream targets of the pathway.
5. To test whether *Sox2* and *Serrate1* are sufficient to specify neurosensory commitment in the otic epithelium.
6. To investigate whether *Sox2* and *Serrate1* regulate neuronal fate in the otic epithelium.
7. To test whether *Sox2* and *Serrate1* regulate the maintenance of the progenitor state in the otic epithelium

Part of this work has been published already and is included as Appendix I.

In the course of this work, I had also the opportunity to collaborate on a parallel and complementary project designed to study the regulation and function of the *Id* genes during hair cell differentiation. The results of this work have been sent for publication and the manuscript is under editorial consideration. It is included as Appendix II.

RESULTS

RESULTS – CHAPTER I

Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick

The results presented in this chapter have been published in a paper in Journal of Comparative Neurology, in August 2007 (see appendix 1)

I. Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick

At the beginning of this thesis, little was known about the expression and function of Sox2 during inner ear development. However, some studies suggested that *Sox2* may be important for the development of the sensory elements of the inner ear. *Sox2*-deficient mice show hearing and balance impairment, they fail to establish a prosensory domain, and are unable to differentiate hair cells or supporting cells differentiate (Kiernan et al., 2005b). Mutations of *Sox2* in humans cause anophthalmia, sensorineural hearing loss and global brain defects (Hagstrom et al., 2005). *Sox2* and *Sox3* are expressed in the otic placode and the *Sox2* regulatory region contains two enhancer sequences that drive its expression to the inner ear (Uchikawa et al., 1999; Uchikawa et al., 2003). In order to study the function of *Sox2*, we performed first a detailed characterization of *Sox2* expression during inner ear development.

I.1 Sox2 is expressed in the neurogenic domain of the otic vesicle

The experiments that follow show the expression of the Sox2 protein throughout the development of the inner ear, between early otic vesicle and pre-hatching stages. The neurogenic domain of the inner ear is specified early in development, during initial stages of the formation of the otic primordium. It is located in the anterior aspect of the otic cup and otic vesicle, and it is complementary to the posterior non-neural region of the otic cup. The latter, is characterized by the expression of patterning genes and the carbohydrate epitope HNK1 (Alsina et al., 2004; Abello et al., 2007). The expression of Sox2 in an E2.5 (HH16) otic vesicle is shown in Fig.1. Parasagittal sections of otic vesicles were double stained for Sox2 and HNK1, red and green, respectively. Figure 1b shows that Sox2 was abundantly expressed in cell nuclei, but only in cells residing in the anterior domain of the otic vesicle, a domain corresponding to the neurogenic domain The expression of Sox2 was complementary to HNK1, as shown by comparing Fig.1b and Fig.1c, and in the merged images (Fig.1a and Fig.1d). The relation between Sox2 expression and neuroblast generation was also explored by double-labelling otic vesicles with Sox2, and Islet1 or Tuj1 (Figs.1e-l). Islet1 is a Lim-homeodomain protein expressed in young neuroblasts during delamination, and in auditory and vestibular neurons (Adam et al., 1998; Li et al., 2004). Figures 1e-h show that Sox2 expression overlapped with the domain of Islet1 expression and the region of generation of the cochleo-vestibular neurons. Only few scattered cells in the otic epithelium showed co-expression of both Sox2 and Islet1 proteins, indicating that initiation of Islet1 expression can occur before Sox2 protein is down-regulated (Fig.1h, orange, and see also below). Sox2, however, was never expressed in delaminated neuroblasts in the cochleo-vestibular ganglion. This is also illustrated by double labelling otic vesicles for Sox2 and Tuj1, which showed that early neuroblasts were generated from the Sox2-positive domain, and that Sox2 was not expressed by Tuj1-positive neuroblasts (Figs.1i-l). Sox3 was also expressed in the neurogenic domain of the otic cup, overlapping Sox2 expression and complementary to HNK1 (see below).

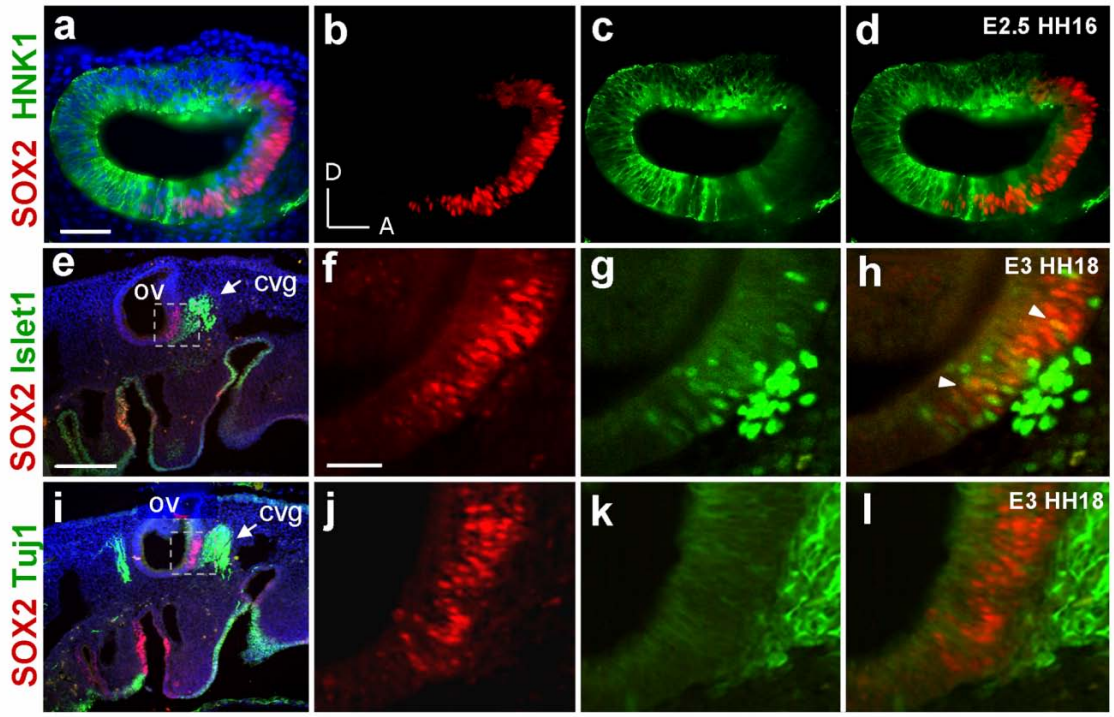


Figure 1: Sox2 is expressed in the neurogenic domain of chick E2.5 and E3 otic vesicles. (a-d): Double immunostaining of parasagittal sections of HH16 otic vesicles for Sox2 protein (red), and the HNK1 epitope (green) Sox2 expression was detected in the nuclei of the cells located in the anterior domain (b), and HNK1 expression in a complementary fashion in the posterior domain (c). (e-h): Double immunostaining of parasagittal sections of HH18 otic vesicles against Sox2 protein (red), and Islet1 (green). Islet1 is expressed in the cochleo-vestibular ganglion (CVG, arrow in e). The scarce yellow nuclei corresponded to cells positive to Islet1 and Sox2 (h, arrowheads). (i-l): Expression of Sox2 and Tuj1. Tuj1 expression was restricted to the cochleo-vestibular ganglion (i, k and l). No overlapping between Tuj1 and Sox2 positive cells was detected (l). DAPI is shown in a, e and i. Dotted frames indicate the neurogenic domain enlarged at the right. D, dorsal; A, anterior; CVG, cochleo-vestibular ganglion; OV, otic vesicle. Scale bars: 50μm for a-d; 100μm for e and i; 25μm in f, applies to f-h and j-l.

I.2 Sox2 is expressed in the prosensory patches of the otocyst

By E3.5-E4 (HH22-24) ear prosensory domains become specified, first vestibular and then auditory, and by E5 (HH26) the different sensory organs can be clearly identified [Wu et al., 1998; Cole et al., 2000; Sanchez-Calderon et al., 2005]. Figure 2 shows serial coronal sections of an E4 and E5 embryo that were stained for Sox2 and Tuj1. Tuj1 recognises a neuron-specific βIII tubulin epitope that is also expressed in nascent hair cells (Stone et al., 1996; Molea et al., 1999). At E4, the prosensory patches can be detected by gene expression domains that anticipate future sensory organs (see diagram of Fig.2a and Cole et al., 2000; Sanchez-Calderon et al., 2005). The experiment shown in Figs.2b-f, illustrates the expression of Sox2 in the prospective cristae (Fig.2b), macula utriculi (Figs.2c,d), macula sacculi (Fig.2e) and basilar papilla (Fig.2f). The correspondence between Sox2 expression and the sensory patches continued in E5, as illustrated in Figs.2h-l, which shows an experiment processed as above for Sox2 and Tuj1. Again, the expression of Sox2 was restricted to the sensory patches. From dorsal

to ventral, Sox2 expression is shown in cristae (Figs.2h,l), maculae (Figs.2j,k) and basilar papilla (Fig.2l). The diagrams in Fig.2a and Fig. 2g show the location of sensory patches as drawn from gene expression patterns and were modified from Sanchez-Calderón et al (2005).

To better analyze the correspondence between Sox2 expression and cell differentiation in sensory patches, we performed a comparison between Sox2 expression and MyoVIIa, which labels the incipient differentiation of hair cells (Sahly et al., 1997; Wolfrum et al., 1998). Serial alternate sections of E5 embryos were immunostained in parallel for Sox2/Tuj1 (Figs.3a-c) and MyoVIIa/Tuj1 (Figs.3d-f). The experiment illustrates that the expression of Sox2 and MyoVIIa overlapped in sensory patches. To further analyse this correspondence at the cellular level, selected patches were examined at higher resolution and shown in Figs.3g-h. As shown, Sox2 was concentrated in the basal cell layer, whereas MyoVIIa was restricted to the luminal layer occupied by hair cells. At these early stages of hair cell production, Sox2 was sometimes detected in the hair cell layer, but later in development, Sox2 was not expressed in hair cells (see below).

Interestingly, and in contrast to what was observed in previous stages, Sox2 was now expressed in the cochleo-vestibular ganglion. In E5 embryos, Sox2 was distinctly expressed in a spotted pattern that intermingled with neurons (Fig.3i). The staining was nuclear as confirmed at high magnification with DAPI counterstaining (Fig.3j). These cells corresponded well to Schwann glial cells that populate the ganglion and that surround the neurons and nerve fibbers. Note that the large nuclei of Tuj1-positive neurons (Fig.3j) were negative to Sox2, suggesting that Sox2 was not expressed in post-mitotic otic neurons.

Islet1 is known to be expressed in nascent hair and supporting cells and it persists until later stages of development in differentiating sensory cells (Li et al., 2004). The correspondence between Sox2 and Islet1 expression in E5 otocysts is shown in Figs.4a-c (superior and posterior cristae, a, and basilar papilla, b). Both in cristae and basilar papilla there was a restricted and overlapping expression of Sox2 and Islet1. At cellular level, sensory patches showed cells that were positive for Sox2, Islet1 and for both (Fig.4c). Generally, the Sox2 domain was contained within the Islet1 patch (Fig.4b). Taking Islet1 as an early marker for sensory cell commitment, this suggests that Sox2 expression persisted transiently during early stages of differentiation (see below). Double labelling experiments with Sox2 and the proliferation marker PCNA showed that most Sox2 expression in sensory patches of the E5 otocyst was related to proliferating cells (Figs.4d-f). Double labelling with Sox2 and PCNA showed a very intense proliferative activity in the otocyst at this stage, and a great extent of overlap (Figs.4d-f). At the cellular level, Fig.4f illustrates that most Sox2-positive cells also expressed PCNA. This indicates that there is a transition state in which sensory progenitors are committed but still proliferative (Doetzlhofer et al., 2006).

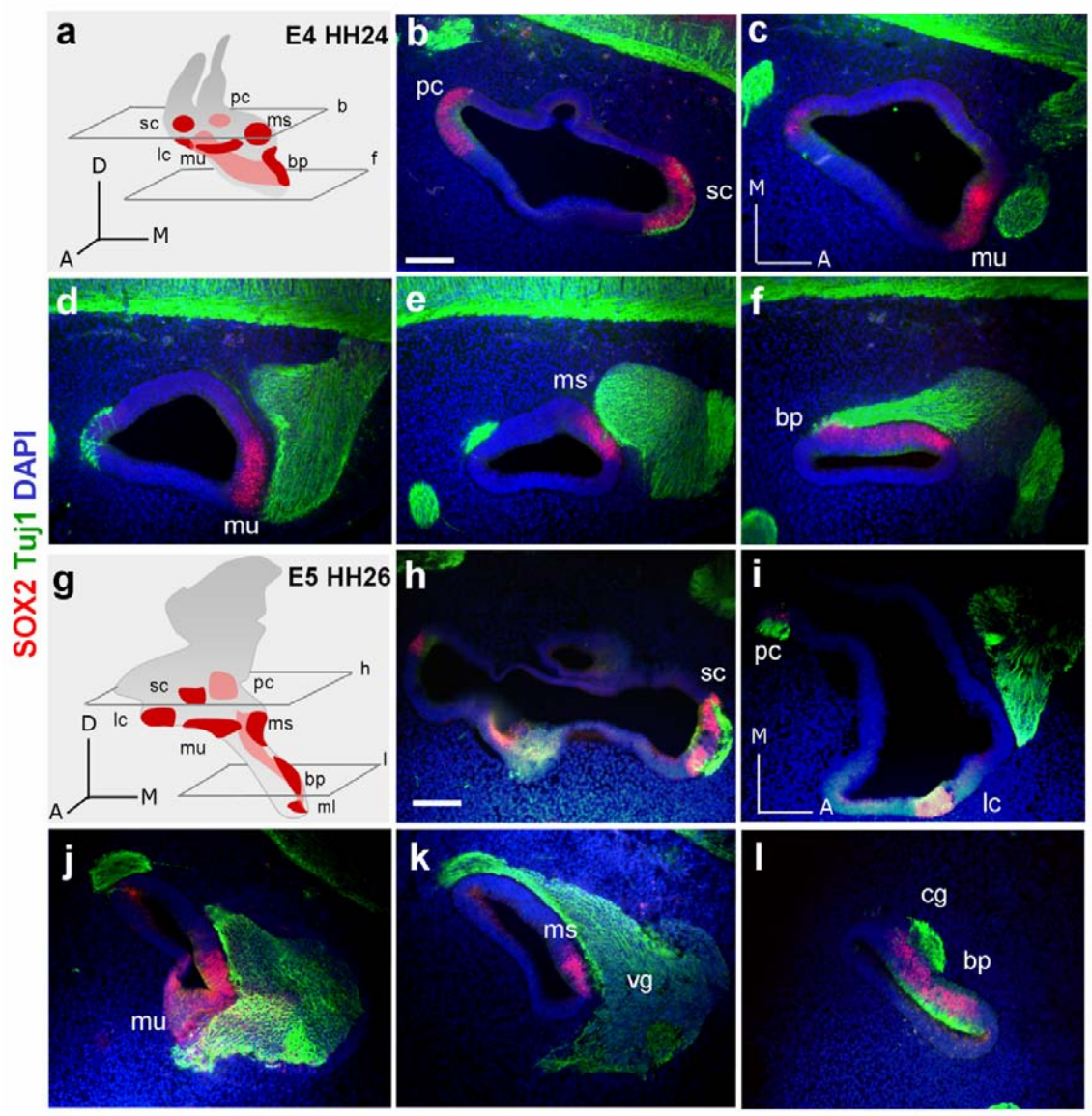


Figure 2: Sox2 expression is restricted to the prosensory patches of otocysts in the E4 and E5 embryo. Diagrams represent otocysts from E4 (a) and E5 (g) stage embryos. Sensory patches are represented in red, as defined by gene expression and were modified from Sánchez-Calderón et al. 2005. Planes represent the extreme levels of the sections shown for each stage. All sections were immunostained for Sox2 (red), Tuj1 (green) and DAPI (blue). (b-f): Serial coronal sections of an E4 otocyst, in a dorsal-ventral sequence. Sox2 expression in vestibular (b-e) and auditory (f) prosensory patches. (h-l): Serial coronal sections of an E5 otocyst in a dorsal to ventral sequence. Sox2 expression in vestibular (h-k) and auditory (l) sensory patches. Tuj1 labelled neurons of the vestibular (vg) and cochlear ganglion (cg), and the fibbers innervating the prosensory domains (d-f and i-l). Sox2 in posteior crista (b and i), superior crista (b and h), lateral crista (i), macula utriculi (c, d and j), maucla sacculi (e and k) and basilar papilla (f and l). pc, posterior crista; sc, superior crista; lc, lateral crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ml, macula lagena; vg, vestibular ganglion; cg, cochlear ganglion. Orientation: D, dorsal; M, medial; A, anterior. Scale bars: 100μm for b-f; 100μm in h, for h-l.

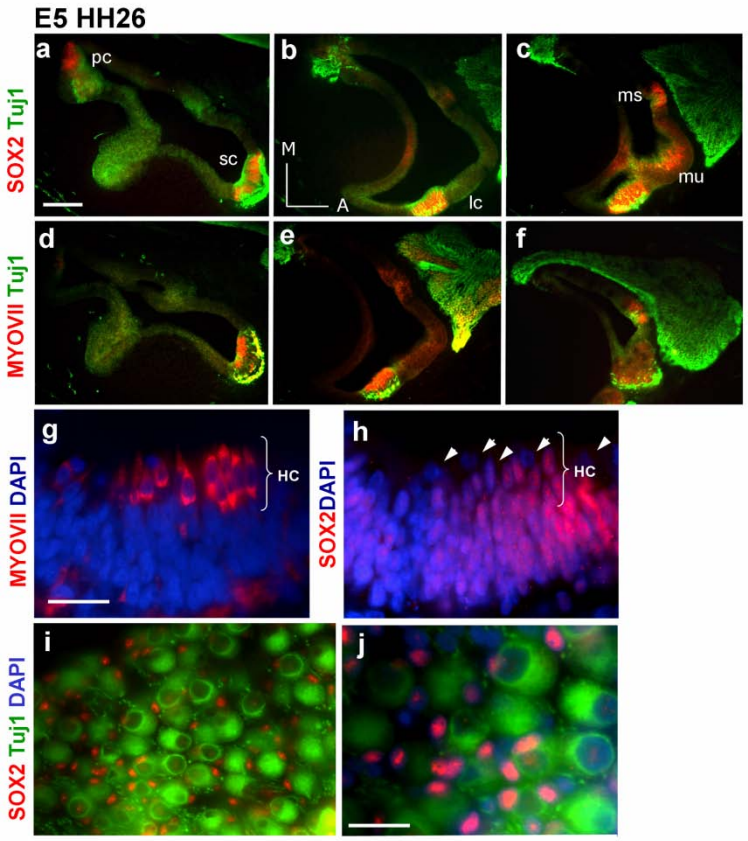


Figure 3: Expression of Sox2 and MyoVIIa in E5 embryos and Sox2 expression in Schwann cells. (a-f): Alternate coronal sections of an E5 otocyst were immunostained for Sox2 (red) and Tuj1 (green) (a-c) or MyoVIIa (red) and Tuj1 (green) (d-f), respectively. Sox2 and MyoVIIa staining in vestibular prosensory patches, in a dorsal-ventral sequence: posterior crista and superior crista (a and d), lateral crista (b and e), macula utriculi and macula sacculi (c and f). High magnifications of superior crista show MyoVIIa (g) and Sox2 (h) in alternate sections. Note that Sox2 was expressed in some cells corresponding to the hair cell (HC) layer, but not in the most apical ones (arrowheads). (i and j): Sox2 expression in Schwann cells of the cochleo-vestibular ganglion. Sox2 was expressed in small nuclei typical of Schwann cells and not in large nuclei of Tuj1-positive neurons. pc, posterior crista; sc, superior crista; lc, lateral crista; mu, macula utriculi; ms, macula sacculi; vg, vestibular ganglion. Brackets in g, h, j and k indicate the position of the row of hair cells. A, anterior; M, medial. Scale bars: 100μm in a, applies to a-f; 20μm for g-i, and 10μm for j.

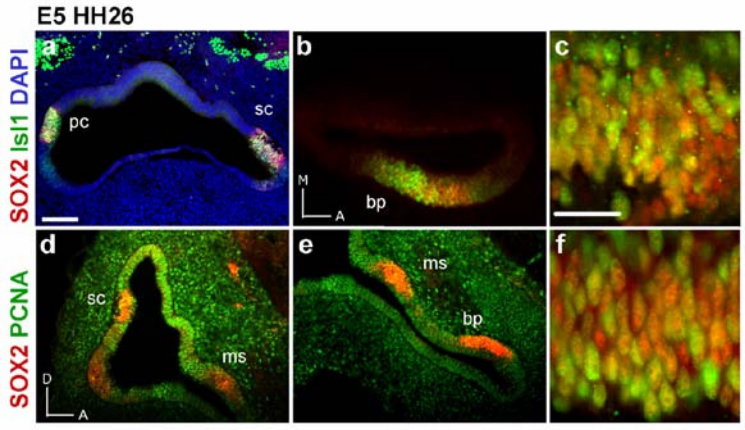


Figure 4: Sox2, Islet1 and PCNA expression in developing inner ear of the E5 embryo. (a-c): Coronal sections were immunostained for Sox2 (red) and Islet 1 (green) and (d-e): parasagittal sections were immunostained for Sox2 (red) and PCNA (green). DAPI staining (blue) is shown in (a). Vestibular (a) and auditory (b) sensory patches of an E5 embryo otocyst labelled with Sox2 and Islet1. Sox2-positive domains overlapped with Islet1. In (c), a high magnification detail of the basilar papilla shown in (b) with nuclei only labelled with Islet1 (green), with both Islet1 and Sox2 (yellow) and only with Sox2 (red). Vestibular (d) and auditory (e) sensory patches of an E5 embryo otocyst labelled with Sox2 and PCNA. PCNA expression was widely spread through the otocyst and Sox2 labelling was mostly overlapping PCNA positive cells at their restricted domains. (f) shows a high magnification detail of the basilar papilla represented in (e), where it can be observed the high number of yellow nuclei, showing that most Sox2 positive labelled cells were also PCNA-positive. pc, posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla. Orientation: D, dorsal; M, medial; A, anterior. Scale bars: 100μm in a, also applies to a, b, d and e; 50μm in c, applies to c and f.

I.3 Sox2 is expressed in supporting cells during differentiation stages

Expression of Sox2 was followed further in development during stages E8 and E17, well beyond the period of cell specification and when hair and supporting cells are in the process of differentiation. The results in Fig.5 show the expression of Sox2 and Tuj1 in a stage E8 otocyst. Sox2 was, as before, restricted to the sensory organs, which were now clearly identified by morphological criteria and by the expression of Tuj1 that labelled both hair cells and the innervating fibbers. Sox2 expression was intense in all sensory organs, some of which are shown as examples in Fig.5: posterior crista (Fig.5b), macula utriculi (Fig.5c), macula sacculi (Fig.5d) and basilar papilla (Fig.5e). A detailed analysis with cellular resolution is shown in Fig.5f for the macula sacculi and in Fig.5g for the basilar papilla. It can be seen that Sox2 expression was low or absent in hair cells, but remained intensely expressed in supporting cells (Figs.5f-g).

The results obtained from stage E17 otocysts are shown in Fig.6. Again Sox2 was expressed in all sensory organs. Examples are shown of the posterior cristae (Figs.6a-b), macula utriculi (Fig.6c), macula sacculi (Fig.6d) and basilar papilla (Fig.6e -f, the inset shows the macula lagena). High magnification in Fig.6b and Fig.6e show that Sox2 was not expressed in hair cells of vestibular and auditory organs, but it persisted at high levels in the layer of supporting cells. This was clearly distinguishable by the ordered position of nuclei and the negative staining for Tuj1. Further confirmation of this pattern of expression was obtained by double labelling E17 otocysts with Sox2 and Islet1. The latter is typically expressed in supporting cells at this stage (Figs.6g and h, and Li et al., 2004). This is seen at high resolution in Figs.6i-k. Islet1 (green) was co-expressed with Sox2 in the supporting cells of macula utriculi (Fig.6i) and in the basilar papilla (Figs.6j, k). Note also that hair cells in the macula (Fig.6i) still expressed Islet1, whereas it was down-regulated in the papilla (Figs.6j, k see also Li et al., 2004). Sox2 was absent from hair cells in both sensory organs.

Examination of E17 otocysts with Sox2 and PCNA revealed that some, but not all of the supporting cells that were positive to Sox2 were actively proliferating. As shown in Figs.6l-m, Sox2 labelling concentrated in the ordered supporting cell layer, which was spotted by PCNA-positive cells (yellow-green, arrows). At higher magnification, double labelled cells were clearly visible in the supporting cell layer. Some of them are pointed with arrows in the examples in Figs.6o-q.

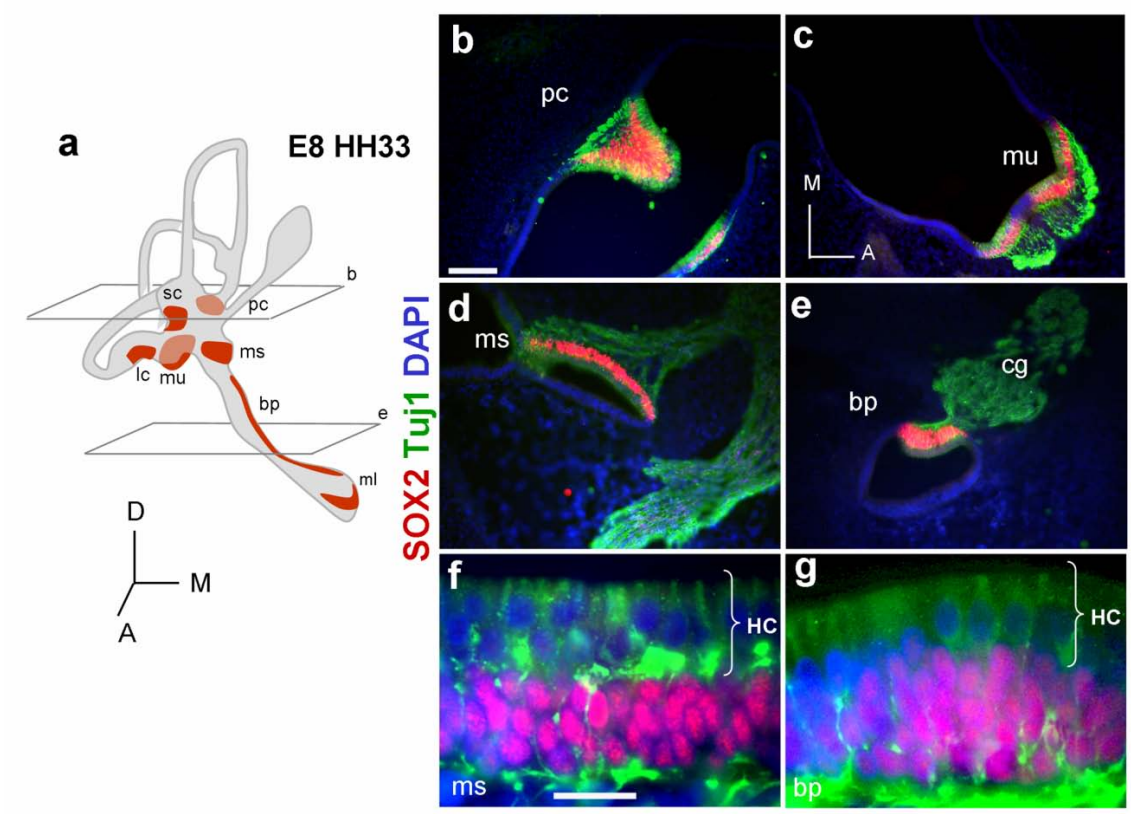


Figure 5: Sox2 expression in the sensory patches of the E8 embryo. (a): Diagram representing an otocyst from E8 chicken embryo. Sensory patches are represented in red (adapted from Sánchez-Calderón et al. 2005). (b-e): Sections were immunostained for Sox2 (red), Tuj1 (green) and DAPI (blue). Serial coronal sections of an E8 otocyst are shown in a dorsal-ventral sequence. Sox2 expression was detected in vestibular (b-d) and auditory (e) sensory organs (pink for double staining with Sox2 and DAPI). Sox2 labelled cells in posterior crista (b), macula utriculi (c) macula sacculi (d) and basilar papilla (e). (f): High magnification of macula sacculi. In this sensory patch, hair cells, indicated with arrows, did not express Sox2, that was detected in supporting cells (pink nuclei). (e): High magnification of basilar papilla illustrating Sox2 staining in hair cells (g). pc, posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; cg, cochlear ganglion. Orientation in a: D, dorsal; M, medial; A, anterior. Orientation in b applies to b-e: M, medial; A, anterior. Scale bars: 100μm in b applies to b-e; 50μm in f, applies to f and g.

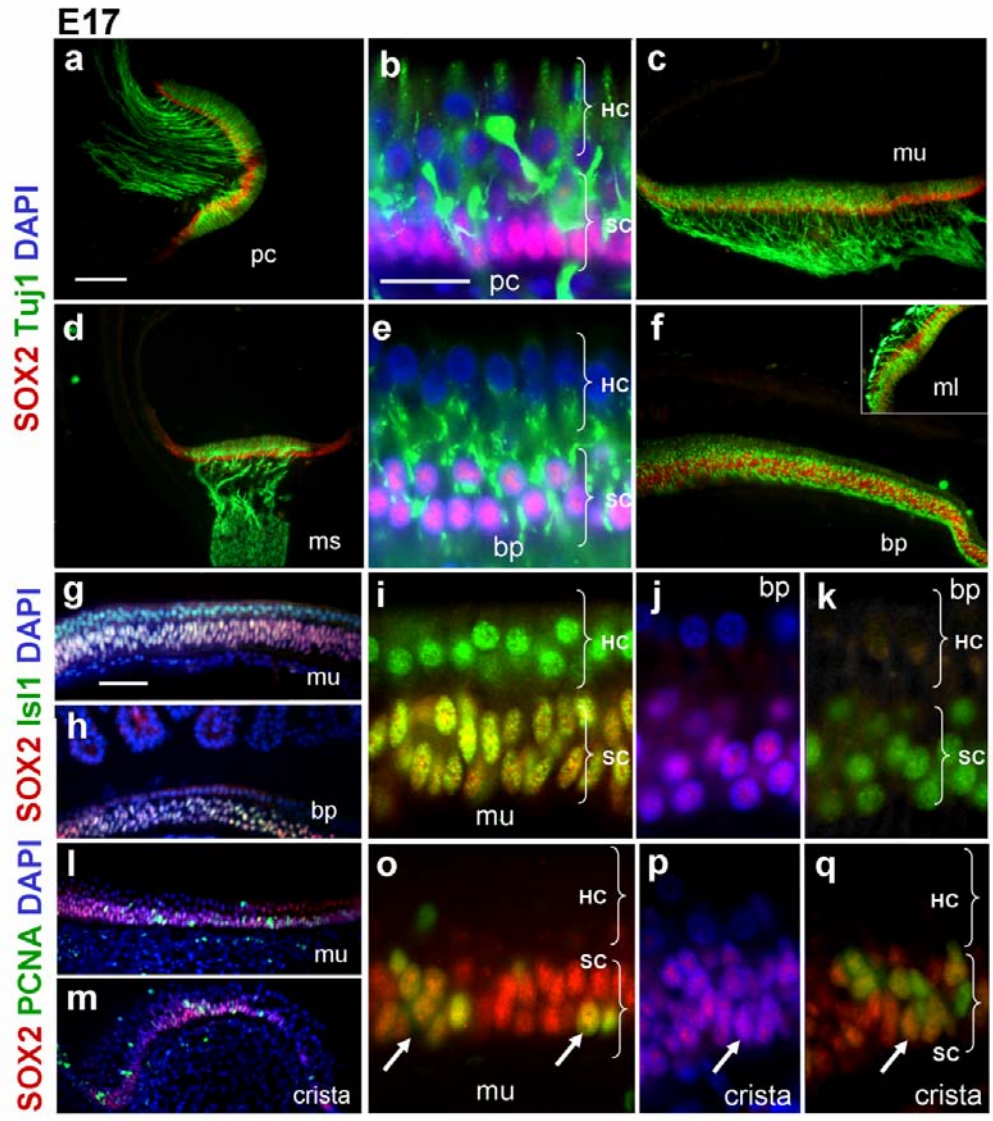


Figure 6: Sox2 expression is detected at post-differentiation stages in supporting cells of E17 sensory organs. (a-f): Sections were immunostained for Sox2 (red) and Tuj1 (green). (g-k): Sections were immunostained for Sox2 (red) and Islet 1 (green) and (l-q): sections were immunostained for Sox2 (red) and PCNA (green). DAPI staining is shown in (b), (e), (g), (h), (j), (l), (m) and (p). Transverse sections of E17 otocysts showing vestibular (a,c,d,) and auditory (f) sensory organs. Sox2 labelled cells in posterior crista (a), macula utriculi (c), macula sacculi (d), and basilar papilla (f). High magnification details of posterior crista and basilar papilla (b and e). Sox2 was restricted to the supporting cells (red nuclei) and it was excluded from the hair cell layer (blue nuclei), that was also labelled with Tuj1. Vestibular (g, macula utriculi) and auditory (h, basilar papilla) sensory patches of an E17 embryo otocyst double labelled for Sox2 and Islet1. High magnification of macula utriculi (i), and basilar papilla (j,k). Sox2 expression occurred in the supporting cell row. Islet1 expression in supporting cells in vestibular (i) and auditory (j and k) sensory organs, and in hair cells of the macula utriculi (i). Macula utriculi (l), and cristae (m) organs of an E17 embryo otocyst labelled with Sox2 and PCNA. (o-q) High magnification detail of macula utriculi (o) and crista (p-q) Sox2 expression was restricted to the supporting cell layer. Proliferating cells were detected with PCNA (yellow nuclei with arrows). No PCNA positive cells were detected in the hair cell layer with the exception of one cell in the macula utriculi (l,o, green nucleus). pc, posterior crista; mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; ml, macula lagena; HC, hair cells, SC, supporting cells. Brackets in b, e, i, k, o and q indicate rows of hair and supporting cells. Scale bars: 100μm for a, c, d, and f; 20 μm for b, e, i-k and o-q; 50 μm for g, h l and m.

I.4 Sox3 is expressed the neurogenic domain of the otic vesicle, but not in the prosensory patches of the otocyst

Sox3 is expressed in the neurogenic placodes (Abu-Elmagd et al., 2001), and there is evidence that it is crucial for early steps in the specification of the neurogenic domain of the otic placode [Abelló et al., 2007; Abelló et al., 2009]. We compared the expression of Sox2 and Sox3 in the neurogenic domain of the early otic cup and in the sensory patches of E5 otocysts. Figures 7a-d show the expression of Sox3 in the otic cup of an E2 (HH14) embryo. Similarly to Sox2, Sox3 was expressed in the neurogenic domain of the otic cup (Figs.7a-b,d), in a complementary manner to HNK1 (Figs.7a,c-d). Further in development, however, Sox3 protein was lost from the prosensory patches, while Sox2 expression persisted (Figs.7e-j). Analysis was carried out on parallel equivalent sections that were analysed for Sox2 and Sox3 expression in vestibular (Figs.7e,h) and in the auditory (Figs.7f,i) domains. The positive control of the Sox3 detection was the strong positive reaction in the ventricular zone of the neural tube (Fig.7j), which was very similar to that of Sox2 (Fig.7g). A similar difference between *Sox2* and *Sox3* expression patterns was observed when comparing the mRNA expression in situ (Figs.7k-n, in situ hybridisation is shown as a blue precipitate and immunostaining for Tuj1 in brown). These results suggest the possibility that the neurogenic potential of otic progenitors may require the expression of both *Sox2* and *Sox3*, whereas the prosensory potential would be associated with that of *Sox2*.

Sox2 and Sox3 were co-expressed in the neurogenic domain as shown by the double labelling for Sox2 and Sox3 (Fig.8A, HH14). Sox3-positive cells were contained within Sox2 expression domain and were located in the anterior-ventral aspect of the otic vesicle (arrowheads in Figs.8Aa''- d''). In the most posterior-ventral aspect of the otic vesicle, Sox2 staining was also detected but at lower levels than in the anterior ventral part (arrows in Figs.8Aa-d). There were cells located in the anterior-dorsal aspect of the otic vesicle that expressed Sox3, but not Sox2 (asterisks in Figs.8Aa'- d'). A detailed analysis of co-expression domains revealed that not all cells expressed Sox2 and Sox3. Rather, double labelled cells (arrowheads in Figs.8Ae-e'') intermingled with cells expressing Sox2 but not Sox3 (arrows in Figs.8Ae-e''). The co-expression of Sox2 and Sox3 in the neurogenic domain was transient and by HH20, there were almost no cells expressing Sox3 (Figs.8Ba'-d'), while Sox2 was still widely expressed in the neurosensory progenitors of the otic vesicle (Figs.8Ba-d). At this stage, only few cells co-expressed Sox2 and Sox3 in the dorsal-posterior aspect of the otic vesicle (asterisk in Figs.8Ba-a'') and in the anterior-medial-ventral domain (arrowheads in Figs.8Bd-d''). In contrast, Sox2 and Sox3 were intensely co-expressed in the neural tube (nt in Figs.8Ba-a'' and 8Bb-b''). This results show that Sox2 and Sox3 are co-expressed transiently in the otic epithelium during otic cup stages. The spatio-temporal pattern of this co-expression correlates in time and space with the neurogenic period and domain of otic development.

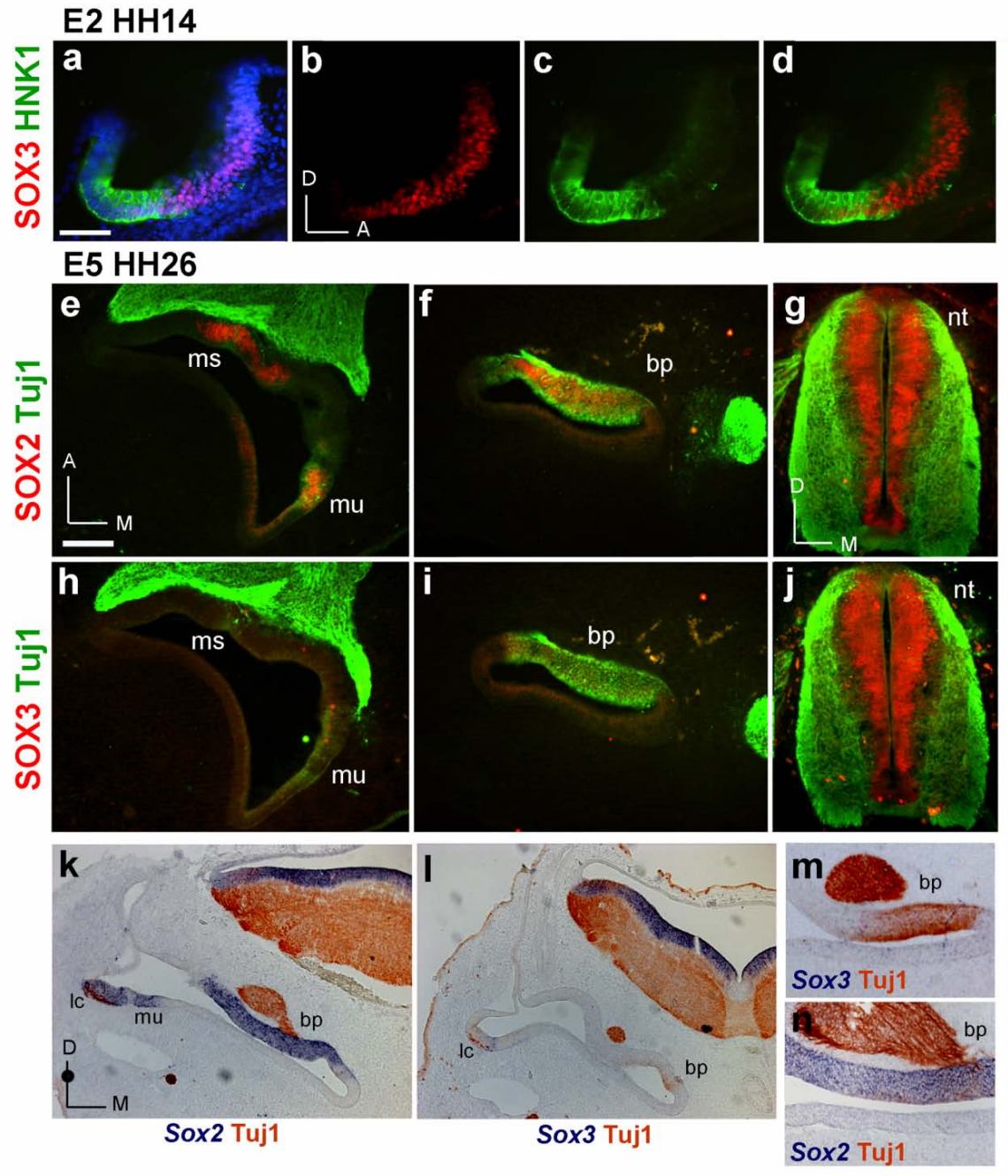


Figure 7: Sox2 and Sox3 differential expression during ear development. (a-d): Sox3 expression in the E2 otic cup. Double immunostaining of parasagittal sections of E2, HH14, otic cup for Sox3 protein (red) and the HNK1 epitope (green). Sox3 expression was detected in the nuclei of the cells located in the anterior domain (b and d), and HNK1 expression in a complementary fashion in the posterior domain (c and d). (e-j): Sox2 and Sox3 expression in sensory organs of E5 embryos. (e-g): Coronal sections of an E5 otocyst, processed for Sox2 expression (red) and Tuj1 (green). Sox2 expression in the macula sacculi and macula utriculi (e) basilar papilla (f) and in the ventricular zone of the neural tube (g). (h-j): Corresponding alternate sections, but processed for Sox3 expression. No Sox3 expression was detected in the macula sacculi, macula utriculi (h) and basilar papilla (i) but it was detected in the neural tube (j). (k-n): In situ hybridization for mRNA of *Sox2* and *Sox3* in sensory organs of E5 embryos. Sections were counterstained for Tuj1. mu, macula utriculi; ms, macula sacculi; bp, basilar papilla; nt, neural tube. D, dorsal; M, medial; A, anterior. Scale bars: 50μm in a, 100μm in a-f.

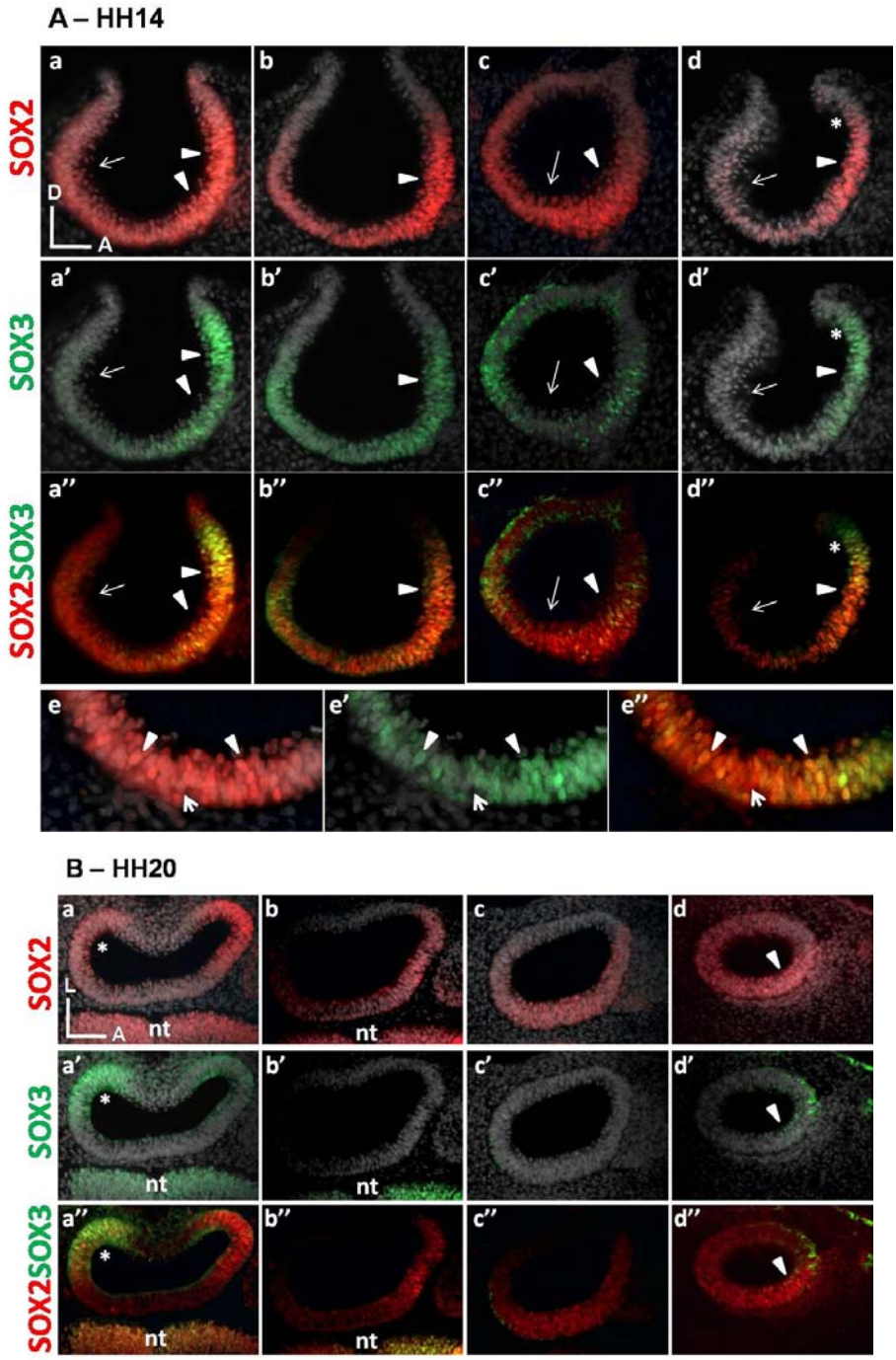


Figure 8: Sox2 and Sox3 are transiently co-expressed in the neurogenic domain of the otic vesicle. A: serial parasagittal sections of HH14 otic cup stained for Sox2 (red) (a-d) and Sox3 (green) (a'-d'), merged double labelling is shown in (a''-d''). Sox2 and Sox3 were co-expressed in the neurogenic domain of the otic cup (arrowheads) but Sox2 expression domain (arrows) was bigger than Sox3 expression domain. (e-e'): detail of the neurogenic domain at high magnification stained for Sox2 (e), Sox3 (e') and merged (e''). Within the neurogenic domain it was possible to identify cells co-expressing Sox2 and Sox3 (yellow, arrowheads) and cells only expressing Sox2 (red, arrows). B: serial coronal sections of HH20 otic vesicles stained for Sox2 (red) (a-d) and Sox3 (green) (a'-d'), merged double labelling is shown in (a''-d''). Sox2 was expressed in a wide domain excluding the dorsal-posterior-lateral-wall of the otic vesicle, while Sox3 expression in the otic vesicle was almost absent. Both genes were strongly expressed in the neural tube (nt, a-, a'-b' and a''-b''). nt, neural tube, A, anterior, D, dorsal, L, lateral.

RESULTS – CHAPTER II

Sox2 and Serrate1 co-expression and cross-regulation

The results presented in this chapter are unpublished, were obtained in collaboration with Dr. Carolina Parada and will be part of a manuscript in preparation.

II. Sox2 and Serrate1 co-expression and cross-regulation

Genetic studies have shown that *Sox2* and *Serrate1* are required for correct development of the ear sensory organs (Kiernan et al., 2005b; Brooker et al., 2006; Kiernan et al., 2006). Some evidence suggests also that these two genes interact during ear development, since *Sox2* expression is down-regulated in *Jag1* mutant mice and after the pharmacological blockade of Notch activity (Kiernan et al., 2006; Daudet et al., 2007; Dabdoub et al., 2008). In the present work, we have explored further the interactions between *Serrate1* and *Sox2* during the development of the neurosensory elements of the ear. First, we analyzed in detail the expression of *Serrate1* and *Sox2* during the development of neurones and sensory organs of the inner ear, and then we performed functional studies to study possible their effects and interactions.

II.1 Sox2 and Serrate1 are co-expressed during neurosensory development

The expression patterns of *Sox2* and *Serrate1* during inner ear development have been already described separately in chick and mouse embryos, and it was shown that both foreshadow the development of sensory organs (see Introduction and previous chapter, (Morrison et al., 1999; Cole et al., 2000; Hume et al., 2007; Neves et al., 2007; Mak et al., 2009). We wanted to analyze in detail the temporal and spatial correspondence between these two genes throughout ear development. For this purpose, we used double immunohistochemistry on cryostat sections of chick otic vesicles, at different developmental stages (Figs.1B-C and Fig.2). Serrate1 protein was detected using a rabbit-anti-hJag1 antibody that recognized a membrane localized protein(Materials and Methods). Sox2 was detected using a goat-anti-hSox2 that recognizes Sox2 protein in chick (see Materials and Methods). Serrate1 and Sox2 staining was detected in the green and red channel, respectively.

Figure 1B shows serial parasagittal sections of a HH14 chick otic cup from lateral to medial, and Fig.1Aa shows a three-dimensional reconstruction of the corresponding expression domains. In the most lateral section (Fig.1Ba), Sox2 was expressed throughout the whole otic vesicle, while Serrate 1 was only expressed in an anterior domain that was contained within that of Sox2. The intermediate sections (Figs.1Bb-c) show the well characterized anterior and posterior poles of Serrate1 expression, which overlapped with Sox2 in the ventral part of the otic vesicle. Medial sections show that Sox2 and Serrate1 were expressed in the ventral domain of the otic vesicle, but not in the dorsal aspect (Figs.1Bc-d). Serrate1 expression was weaker in the medial wall (Fig.1Bd), but always overlapped with that of Sox2, which was always broader than Serate1.

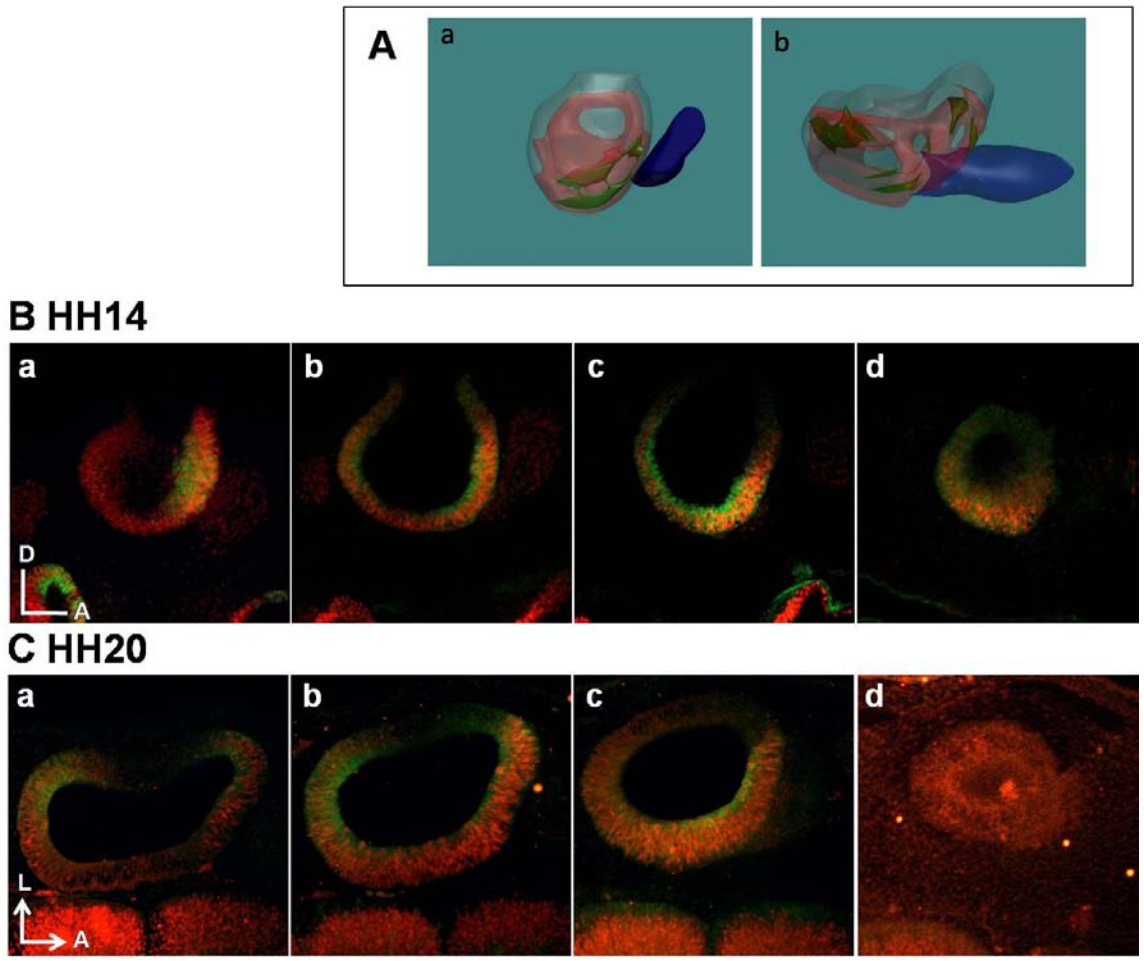


Figure 1: Sox2 and Serrate1 co-expression during early stages of otic development. AB: 3D reconstructions of Sox2 (red) and Serrate1 (green) expression domains in HH14 otic cups (a) and HH20 otic vesicles (b). 3D models were obtained from the serial section showed in (B) and (C). Cochleo-vestibular ganglion is shown in blue. Anteriorto the right. Lateral views. B: Serial parasagittal sections of HH14 otic cup, from lateral to medial (a-d), stained for Sox2 (red) and Serrate 1(green). Sox2 and Serrate1 co-expressed but Sox2 expression domain was broader in the most lateral aspect of the otic vesicle (a-b). C: Serial coronal sections of HH14 otic cup, from dorsal to ventral (a-d), stained for Sox2 (red) and Serrate 1(green). Sox2 and Serrate1 co-expressed but Sox2 expression domain was broader in the most dorsal (a) and ventral-posterior-lateral (c) aspects of the otic vesicle. Sox2 and Serrate1 were not expressed in the lateral wall (a-c). A, anterior; D, dorsal; L, lateral

Expression patterns in the otic vesicle stage are shown in Fig.1C, which displays coronal sections of HH20 chick otic vesicles (from dorsal to ventral). As before, Serrate1 and Sox2 were strongly expressed in the anterior and posterior poles of the otic vesicle, but not in the lateral wall. Sox2 expression domain remained always broader than that of Serrate1 In the dorsal aspect of the otic vesicle (Figs.1Ca-b), Sox2 was expressed in the medial part of the otic vesicle, but Serrate1 expression was very low or absent. In contrast in the most ventral domains (Figs.1Cc-d), the expression of both genes overlapped in the medial domain of the otic vesicle, with Sox2 expression extending laterally. In conclusion, during the stages of otic cup and otic vesicle, when the

prosensory patches are not yet specified, Sox2 and Serrate1 were co-expressed in broad regions of the anterior and posterior poles and in the most medial-ventral part of the otic vesicle. Sox2 expression domain was always broader than that of Serrate1, which was always contained within the former. A summary of this analysis is shown in the 3D reconstruction of Fig.1Ab.

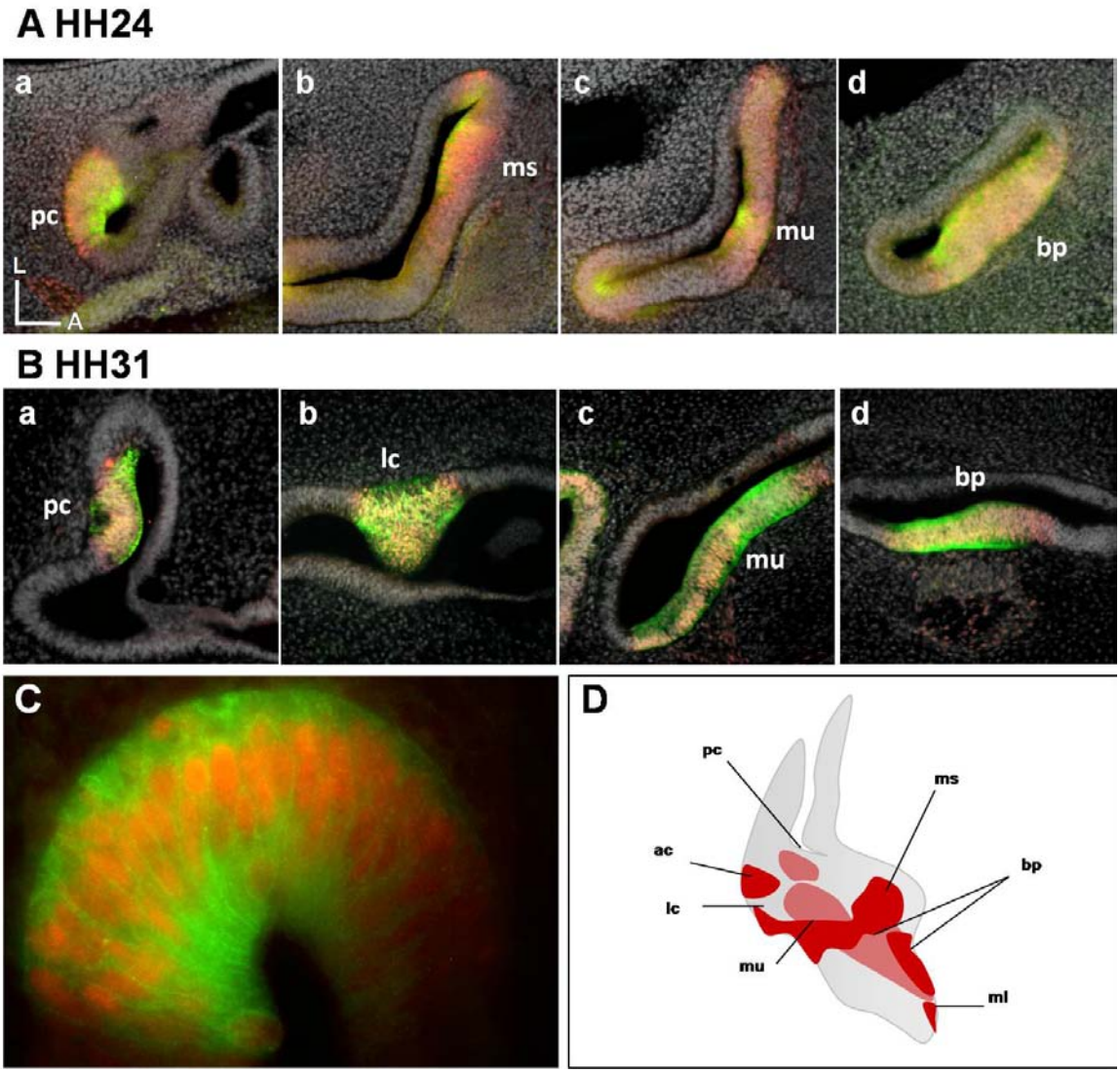


Figure 2: Sox2 and Serrate1 co-expression in the prosensory domains of the otocyst. A: Coronal sections of HH24 otocyst, form dorsal to ventral (a-d) double stained for Sox2 (red) and Serrate1 (green). Sox2 and Serrate1 expression in the vestibular (a-c) and auditory (c-d) prosensory patches, Sox2 and Serrate1 in the posterior crista (a), macula sacculi (b), macula utriculi (c) and basilar papilla (c-d). B: Coronal sections of HH31 otocyst, form dorsal to ventral (a-d) double stained for Sox2 (red) and Serrate1 (green). Sox2 and Serrate1 expression in the vestibular (a-c) and auditory (d) prosensory patches, Sox2 and Serrate1 in the posterior crista (a), lateral crista (b), macula utriculi (c) and basilar papilla (d). C: High magnification of posterior crista of HH24 otocyst, showing the sharp correspondence of the borders of expression of Sox2 and Serrate1. Sox2 expression was restricted within the borders of Serrate1 domain in the posterior crista by HH24 but in the maculae only by HH31. D: model of an HH24 otocyst showing the location of the different sensory organs swoh in the coronal section in (A) and (B). ac, anterior crista; pc, posterior crista; lc, lateral crista; ms, macula sacculi; mu, macula utriculi; bp, basilar papilla; A, anterior; L, lateral.

The expression of Sox2 and Serrate1 was also analysed during the emergence of the prosensory patches and sensory differentiation (Fig.2). Coronal sections of HH24 and HH31 otic vesicles double stained for Sox2 and Serrate1 are shown from dorsal to ventral in Fig.2A and Fig.2B, respectively. At this stage, both Sox2 and Serrate1 proteins were detected in all the prospective sensory domains of the otocyst. Strikingly, in the cristae, Sox2 became restricted within distinct borders that were defined by Serrate1 expression (Fig.2Aa). A high magnification of the posterior crista is shown in Fig.2C, where the boundaries of Sox2 and Ser1 expression domains were seen to coincide sharply. Sox2 expression remained broader than that of Serrate1 in the maculae (Figs.2Ab-c) and basilar papilla (Fig.2Ad). However, further in development (stage HH31), the expression of Sox2 became also restricted to the Serrate1 domain in the maculae (Fig.2Bc), although not yet in the basilar papilla (Fig.2Bd). This spatial and temporal sequence of Sox2 restriction to Serrate1 domains is reminiscent of that followed by the generation of the sensory organs, which develop from dorsal (vestibular) to ventral (auditory) [Wu and Oh, 1996; Bell et al., 2008].

In summary, as prosensory patches are specified, *Sox2* expression gets restricted within the boundaries of *Serrate1* expression, following a dorsal to ventral order, which is characteristic of the order of differentiation of the sensory organs. This suggests that the interaction between *Serrate1* and *Sox2* may be instrumental for the specification of the sensory organs and, moreover, that the persistence of Sox2 expression in the prosensory domains may depend on *Serrate1*.

II.2 Serrate1 and Sox2 cross-regulation

Previous experiments suggested that there may be a hierarchical relationship between *Serrate1* and *Sox2*, since in the mouse cochlea *Jagged1* is required for *Sox2* expression, but *Jag1* expression is not affected in *Sox2* mutant mice [Kiernan et al., 2005, Kiernan et al, 2006; Dabdoub et al., 2008]. In this connection, it is important to know whether *Serrate1* is sufficient to induce *Sox2* expression, and whether *Sox2* is able to regulate back *Serrate1* in the sensory patches. With this purpose, we performed gain of function studies using in ovo electroporation (see Materials and Methods) to overexpress *cSox2* and *hJag1* in chick otic vesicles. The effects on gene expression were analyzed by in situ hybridization (ISH), quantitative real-time polymerase chain reaction (qRT-PCR) and fluorescent immunohistochemistry (IHC) (See Materials and Methods).

First I shall describe and validate the methods used for the gain of function experiments to then describe the functional studies on the interactions between *Serrate1* and *Sox2*.

2.1 c*Sox2* and h*Jag1* transient expression in the chick otic vesicle

Plasmid based in ovo electroporation is a well established transfection method to overexpress a gene of interest in chick (Muramatsu et al., 1997; Nakamura and Funahashi, 2001). The transgene is incorporated into a plasmid DNA which is

transfected into the cells by in ovo electroporation. The expression of transgene is transient in time, decreasing due to the decrease in the concentration of plasmid in the progeny of transfected cells.

Full length human *Jagged1* gene cloned into pCIG vector, and full length chick *Sox2* cloned into pIRES-EGFP or pCMV/SV1 vectors were used to overexpress *Serrate1* and *Sox2*, respectively. DNA solutions containing the corresponding plasmid vectors were injected into the right otic cupand electroporated as described in the Methods section. Left untransfected otic cupswere used as a control.A plasmid containing *Sox2* gene with a deletion in the region coding for HMG box domain (pCMV/SV1 - cSox2ΔHMG), and the pCIG empty vector were also electroporated as control experiments. Embryos were electroporated before sensory specification (stages HH12-14), and allowed to develop in ovo for 20h, 48h or 72 hours. Transfected embryos were selected by the green fluorescence exhibited in the transfected otic vesicle due to GFP protein. GFP was used as a cell tracer of electroporated cells and their progeny (Fig.3).

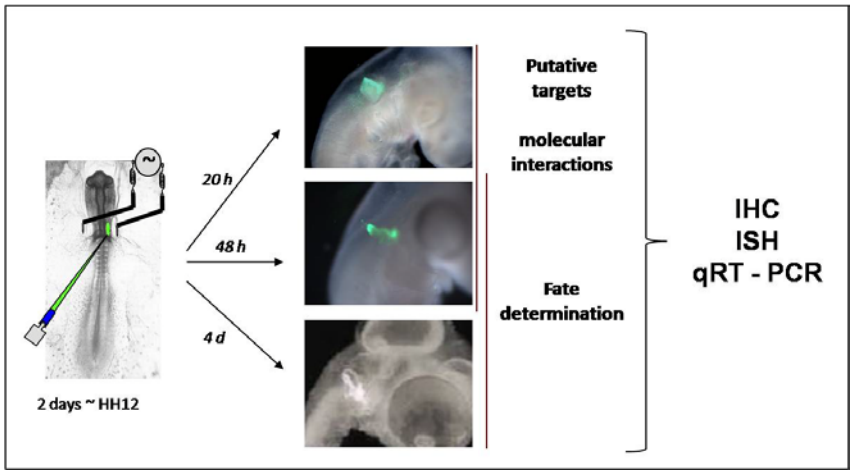


Figure 3: Diagram of experimental procedures. HH12 embryos are electroporated with injected plasmids and allowed to develop 20h, 48h or 3/4days in ovo, after which embryos are selected by green fluorescence emoted by GFP Embryos positive for GFP in the otic vesicle are collected and processed for ISH, IHC or qRT-PCR 20h and 48h embryos are used to analyse molecular interaction and putative targets while 48h and 3/days embryos are used to analyse effects in fate determination. ISH, in situ hybridization; RT-PCR, quantitative real-time polymerase chain reaction; IHC, fluorescent immunohistochemistry.

The following experiments show that the electroporation technique can be effectively used to overexpress and ectopically express *Sox2* and *Serrate1* proteins in the chick otic vesicle. Transgene expression was efficient but transient, being maximal at 20h post electroporation and collapsing to very low levels after 48h. GFP protein expression, however, was much more stable and was still detected four days after electroporation, allowing it to be used a cell tracer for longer periods of observation.

The ectopic expression of *Sox2* is illustrated in Figs.4A(a-e). Sox2 protein extend throughout the electroporated epithelium (arrowheads in Figs.4Ab and c), and outside the normal expression domains (compare Figs.4Aa and b). Electroporation frequently

extended to the periotic ectoderm (asterisk in Figs.4Ab and c, and see below). The left otic vesicles did not receive DNA and were used as a control. Those otic vesicles stained for Sox2 exhibited the typical endogenous expression domains (Fig.4Aa). Note that the image of the left untransfected otic vesicle was flipped horizontally, so that control otic vesicles are shown with the same orientation as the electroporated ones. Electroporation with the control vector pCMV/SV1 - *cSox2*ΔHMG did not result in Sox2 ectopic expression (Figs.4Ad-e). A similar set of experiments is shown in Figs.4A(f-j) for *hJag1* transfection. The expression of the transgene corresponded with the electroporated cells as revealed by GFP expression (Fig.4Ag and h endogenous expression domains are indicated by arrows). Also here, hJag1 protein was expressed in the electroporated periotic ectoderm (asterisk in Figs.4Ag-h). GFP expression driven by pCIG transfection alone did not co-localize with Jag1 expression out of the normal expression domains (compare Figs.4Ai and j)

To fully describe our experimental conditions, we further characterized the temporal profile of the transgene expression. For that purpose we extracted RNA from electroporated and control otic vesicles, 20h and 48h after electroporation, and analyzed transgene expression by qRT-PCR. The bar diagrams in Fig.4B (a-b) show the result of that analysis. *cSox2* mRNA levels showed a twenty-fold increase in pIRES-EGFP-*cSox2* electroporated samples, 20h after electroporation (Fig.4Ba, left blue bar). However, the relative levels of expression significantly decreased after 48h (Fig.4Ba, right blue bar). A similar transient profile was observed after the transfection of hJag1-pCIG (Fig.4Bb). The values for *hJag1* transgene relative expression are arbitrary and cannot be compared those of *Sox2*, because there is no endogenous expression of human Jag1 in control otic vesicles. In summary, pIRES-EGFP-*cSox2* and *hJag1*-pCIG plasmids efficiently drive the expression of *Sox2* and *Serrate1* in the otic vesicle, transgene expression being maximal at 20h post-electroporation and significantly reduced by 48h after electroporation.

Finally we wanted to validate the co-electroporation of pCMV/SV1-*cSox2*ΔHMG and pCIG as a reliable control condition for the gain of function studies. For this purpose, we analyzed mRNA relative expression levels of the transgene (*cSox2* with deleted HMG box coding region) and the endogenous *cSox2* and *cSerrate1* in electroporated otic vesicles, in respect to untransfected otic vesicles. In order to distinguish the transgene from the endogenous *Sox2* expression we designed two different sets of primers within the *Sox2* coding region (see Materials and Methods). Figure 4Bc shows that after co-electroporation of pCMV/SV1-*cSox2*ΔHMG and pCIG vectors, the transgene mRNA levels were increased by approximately fifty-fold with respect to control. However, the mRNA levels of endogenous *Sox2* and *Serrate1* were not increased in respect to control levels. Therefore, pCMV/SV1-*cSox2*ΔHMG and pCIG co-electroporation constitutes a reliable control condition for *Sox2* and *Serrate1* gain of function.

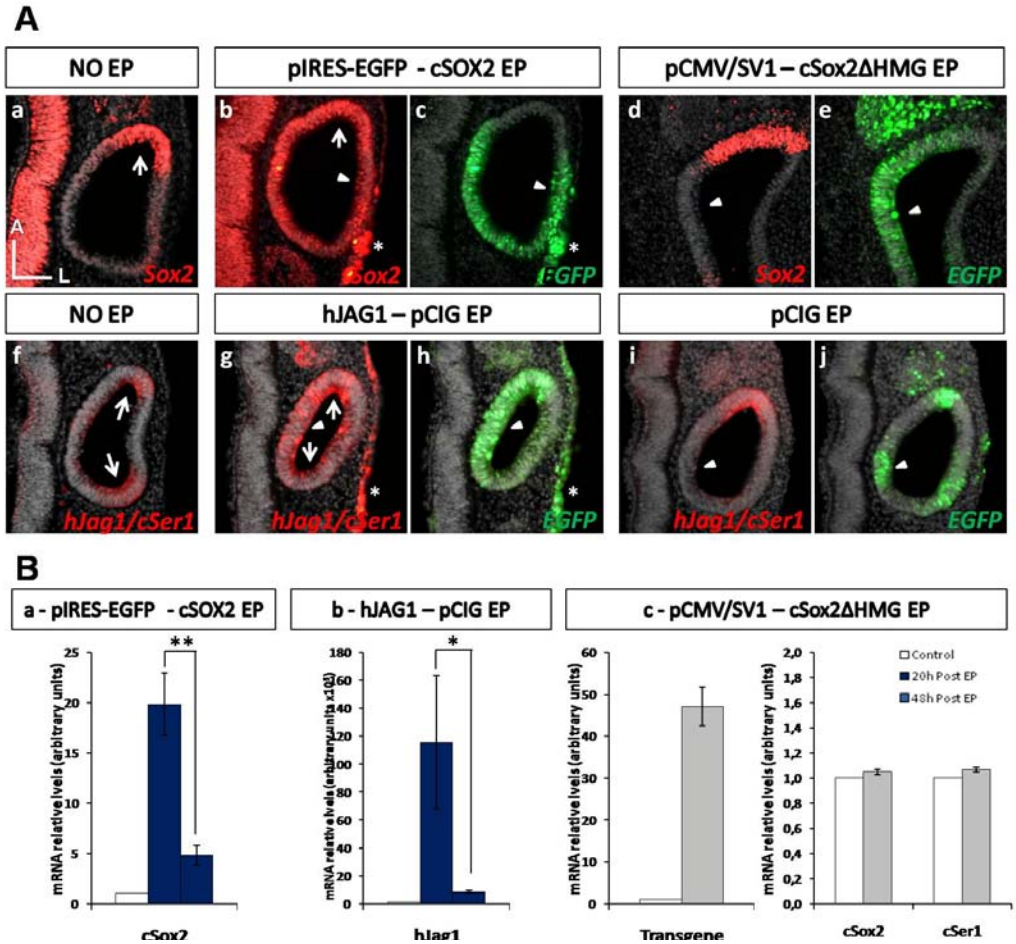


Figure 4: Characterization of transgene expression in *cSox2* and *hJag1* gain of function and control experiments. A (a-e): coronal sections of untransfected (a), transfected with pIRES-EGFP-*cSox2* (b-c) and transfected with pCMV/SV1 – *cSox2*ΔHMG (d-e), stained for Sox2 (red) (a, b, d) and GFP (c and e). Otic vesicles transfected with *cSox2*, but not the ones transfect with *cSox2*ΔHMG, extended Sox2 protein expression throughout the electroporated domains (arrowheads, b-c and d-e), A (f-j): coronal sections of untransfected (e), transfected with *hJag1*-pCIG (g-h) and transfected with pCIG empty vector (i-j), stained for Serrate1 (red) (a, b, d) and GFP (c and e). Otic vesicles transfected with *hJag1*, but not the ones transfect with empty pCIG vector, extended Serrate1 protein expression throughout the electroporated domains (arrowheads, g-h and i-j), Sox2 and Serrae1 endogenous expression was detected in both transfected and control otic vesicles (arrows, a-b and f-g) which were flipped to be shown with the same orientation as electroporated ones. B (a and b): Bar diagrams showing the relative increase in the levels of *cSox2* and *hJag1* (blue bars) after *cSox2* (a) and *hJag1* (b) transfection respectively, in respect to control untransfected otic vesicles (white bar). The levels of transgene expression were highly increased 20h after electroporation and significantly decayed 48h after electroporation (*cSox2*, p<0,005 *hJag1*, p<0,05). B (c): Bar diagrams showing the relative increase in the levels of *cSox2*ΔHMG transgene (left) and *cSox2* and *cSer1* (right) after *cSox2*ΔHMG transfection, in respect to control untransfected otic vesicles (white bar). The levels of transgene expression were highly increased 20h after electroporation but the endogenous levels of cSox2 and cSerrate1 endogenous expression were not affected in the control experiment. A, anterior; L, lateral

2.2 Unidirectional regulation of Sox2 by Serrate1

The experiments that follow addressed the question of the cross-regulation between Sox2-Serrate1 using a gain of function approach. The results show that *Sox2* does not regulate *Serrate1* expression, but *Serrate1* is sufficient to maintain *Sox2* expression in the otic epithelium. This effect is both cell autonomous and non-cell autonomous and it is dependent on the activation of the Notch signalling pathway.

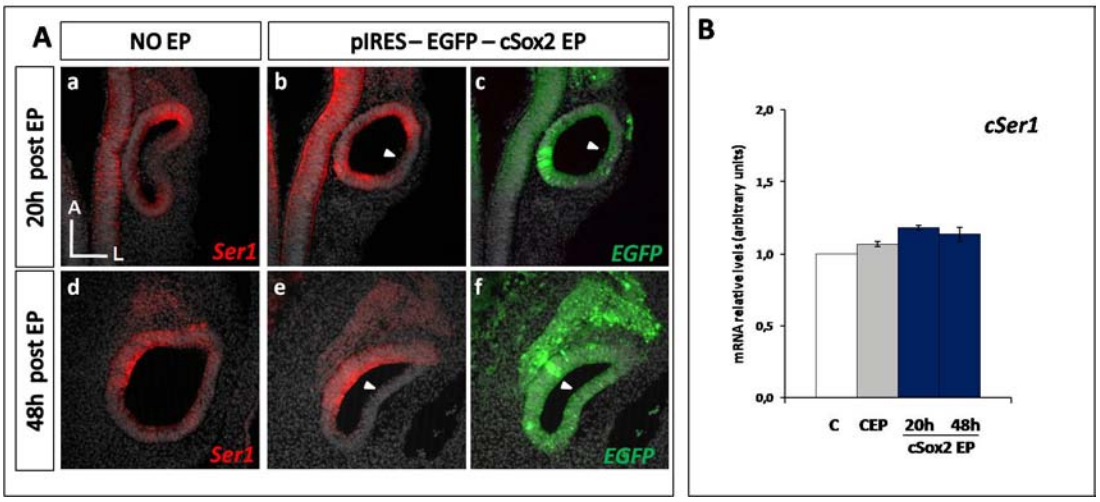


Figure 5: *Sox2* does not regulate *Serrate1* expression in the otic epithelium. A: Coronal sections of otic vesicles untransfected (a and d) and transfected with pIRES-EGFP –*cSox2* (b-c and e-f), 20h (a-c) and 48h (d-f) after electroporation, stained for Serrate1 (red) and GFP (green). Serrate1 protein was not detected in electroporated regions (arrowheads, b-c and e-f) but was present in endogenous expression domains (a-b and d-e). B: Bar diagram showing the relative mRNA levels of *cSerrate1* in otic vesicles transfected with control plasmids (grey bar) and with pIRES-EGFP-*cSox2* (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). The differences in *cSerrate1* mRNA levels were not significantly different between control electroporation and *cSox2* electroporation (compare grey bar and blue bars, p=0,266 and p=0,327 for 20h and 48h, respectively). A,anterior; L, lateral

Figure 5A shows examples of otic vesicles transfected with *cSox2* and analyzed for cSerrate1 expression, 20h (Figs.5Ab-c) and 48h (Figs.5Ae-f) after electroporation, along with the corresponding untransfected sides (Figs.5Aa,d). After 20h, the two poles of Serrate1 expression were clearly observed in control otic vesicles (Fig.5Aa). The ectopic expression of *cSox2* in the lateral wall of the otocyst (arrowheads in Fig.5Ac) did not result in ectopic expression of Serrate1 (arrowheads in Fig.5Ab, n=4/4). Similarly, forty-eight hours after electroporation, Serrate1 expression remained restricted to the prosensory domains (compare maculae in Figs.5Ad and e), and cells transfected with *cSox2* outside those domains did not express Serrate1 (arrowheads in Figs.5Ae-f, n=4/4). Similar results were obtained by in situ hybridization using a *cSerrate1* probe (not shown, n=8/8). These results were also confirmed by qRT-PCR analysis. mRNA levels were not different in *cSox2* transfected otic vesicles (blue bars) and in otic vesicles electroporated with pCMV/SV1-cSox2ΔHMG (grey bar) (p=0,266 and p=0,327 for 20 and 48h, respectively). These results indicate that Sox2 does not regulate Serrate1 expression in the otic epithelium.

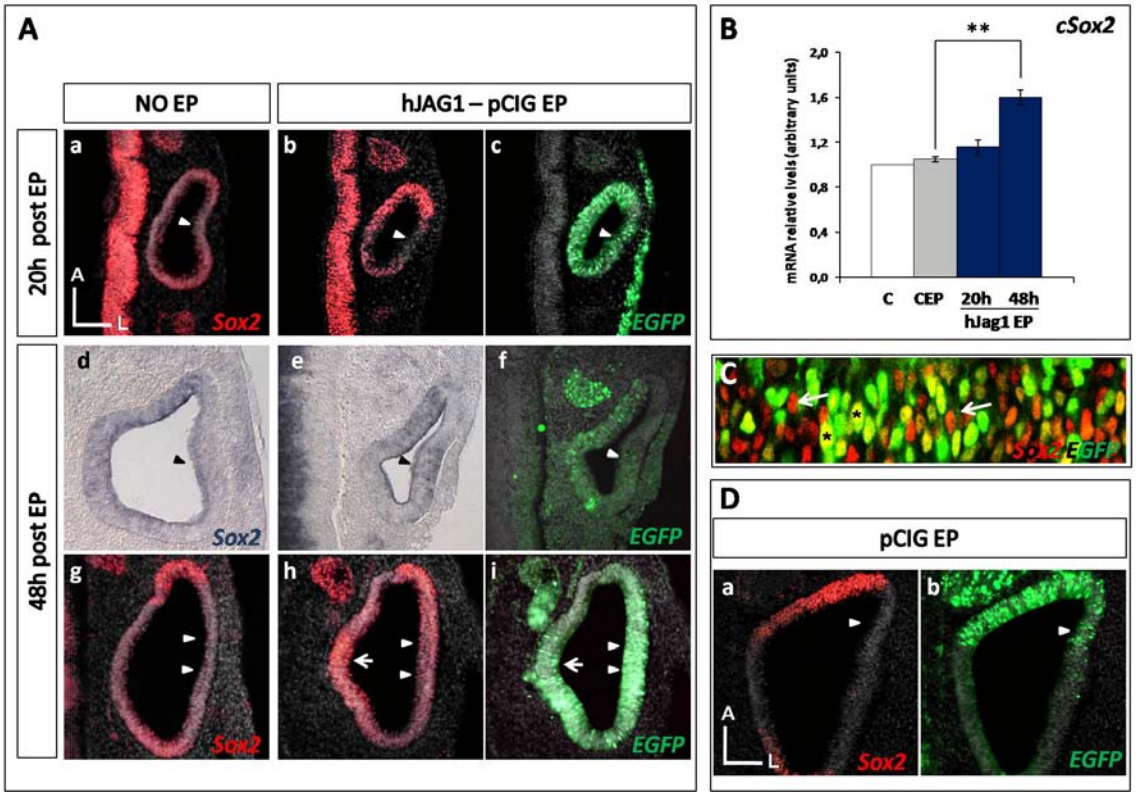


Figure 6: *Serrate1* regulates *Sox2* expression in the prosensory domains of the otic vesicle. A: Coronal sections of otic vesicles untransfected (a, d and g) and transfected with *hJag1*-pCIG (b-c, e-f and h-i), 20h (a-c) and 48h (d-i) after electroporation, stained for Sox2 protein expression (red, a-b and g-h), *Sox2* mRNA expression (d-e) and GFP (green). Sox2 protein was not detected in electroporated regions 20h after transfection (arrowheads, b-c) but *Sox2* expression was induced 48h after electroporation (arrowheads and arrows, e-f and h-i). B: Bar diagram showing the relative mRNA levels of *Sox2* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). The differences in *cSox2* mRNA levels were not significantly different between control electroporation and *hJag1* electroporation 20h after transfection (compare grey bar and left blue bars, p=0,318), but *cSox2* mRNA levels were significantly increased 48h after electroporation (compare grey and right blue bar, p<0,005) C: Confocal image of a detail of a domain of Sox2 ectopic expression stained for Sox2 and GFP where cells double stained (yellow, asterisk) and expressing Sox2 but not GFP (red, arrow) can be observed, indicating that the effect of *hJag1* in *Sox2* is both cell autonomous and non cell autonomous. D: Coronal sections of otic vesicles transfected with pCIG 48h after electroporation, stained for Sox2 protein expression (red, a) and GFP (green, b). Sox2 protein was not detected in electroporated regions 48h after transfection (arrowheads) A, anterior; L, lateral

In order to analyze the effects of *Serrate1* on *Sox2*, we carried out the gain of function experiments of *Serrate1* and looked at possible changes in *Sox2* expression. HH12-14 otic cups were electroporated with a *hJag1*-pCIG plasmid, and embryos allowed developing in ovo for 20h and 48h. *Sox2* expression was analysed by ISH, IHC and qRT-PCR. After 20h, *Sox2* expression in electroporated otic vesicles was not different from controls (n=8/8, 2 analyzed by IHC and 6 analyzed by ISH). Figures 6Aa-c show one example one of those experiments. As illustrated, the lateral aspect of the otic vesicle was negative to Sox2 in either the control (Fig.6Aa) or the electroporated otic

vesicle (Fig.6Ab), where the transgene was expressed (arrowheads in Fig.6Ac). These reslts were confirmed by qRT-PCR analysis, using the method described above. *Sox2* mRNA levels were not changed with respect to control otic vesicles (Fig.6B, compare the blue bar labelled 20h with the white or grey bars, that were untransfected or transfected with the control plasmid, 1,06 ± 0,06, mean ± SE, p=0,318).

However, 48h after electroporation of *hJag1*, *Sox2* was strongly induced. Figures 6Ad-i show two examples of otic vesicles transfected with *hJag1* and analyzed for *Sox2* expression by ISH (Figs.6Ad-f) and IHC (Figs.6Ag-i), 48h after electroporation. GFP expression (arrowheads in Fig.6Af and Fig.6Ai) showed electroporated cells in the lateral wall of the otocyst that corresponded well to ectopic *Sox2* expression in the same domains (arrowheads in Fig.6Ae and Fig.6Ah, n=8/11, 4 analyzed by IHC and 7 analyzed by ISH). GFP expression in the medial domain of the otic vesicle (arrows in Fig.6Ai) also corresponded to an ectopic expression of Sox2 in this domain (arrows in Fig.6Ah). Careful analysis of the electroporation sites revealed that ectopic expression of *Sox2* occurred only when the electroporated domains were adjacent to *Serrate1*/*Sox2* normotopic expression domains (Figs.6Ah-i). Transfection in ectopic isolated domains did not induce *Sox2* expression (see below). These results were confirmed using qRT-PCR. *Sox2* mRNA levels, 48h after electroporation, were increased by 1,6-fold in transfected with respect to control otic vesicles (Fig. 6B, compare the blue bar labelled 48h, 1,60 ± 0,07, mean ± SE, with the untransfected side, white bar). The increase was also significant when compared with otic vesicles electroporated with control plasmids (grey bar, p<0,005). This quantification probably underestimates the magnitude of the induction at cellular level, because analysis is carried out with mRNA extracted from the whole otic vesicle and not only from the electroporated domain. A coronal section of an otic vesicle electroporated with pCIG and analyzed for Sox2 expression (Figs.6Da-b) showed that the effect was specific for *hJag1* gene, since electroporation with a pCIG plasmid did not induce this effect (arrowheads in Figs.6Da-b).

Analysis with confocal microscopy of the domains of ectopic expression of Sox2 revealed that the effect of *hJag1* was both cell autonomous and non cell autonomous (Fig.6C). It was possible to identify Sox2-positive cells that were either electroporated (yellow, asterisk) or non electroporated (red, arrow). The former, expressed Sox2 ectopically but did not receive the transgene, suggesting that Serrate1 can exert its effect in both cell autonomous and non cell autonomous manner.

The observations indicate that *Serrate1* is not able to induce *Sox2* expression *de novo,* but it is sufficient to expand the *Sox2* expression domain. This suggests that one function of *Serrate1* may be to maintain *Sox2* expression in the sensory patches while it may be switched off outside *Serrate1* expression domains (see discussion).

2.3 Serrate1 regulation of Sox2 expression is dependent on active Notch signalling

The experiments above show that the effects of *Serrate1* on *Sox2* expression are complex. They cannot be explained by direct transcriptional regulation, but must involve cell to cell communication. Serrate1 is a ligand of Notch receptor and Notch signalling is active in the prosensory patches [Edisson et al, 2000; Daudet et al., 2005; Abelló et al., 2007; Daudet et al., 2007, Murata et al., 2006]. We investigated whether the activity of the Notch signalling pathway was required for the effects of *Serrate1* on *Sox2* expression. For this purpose we combined the in ovo electroporation of *hJag1* with in vitro culture of explanted otic vesicles in the presence of DAPT. DAPT blocks Notch activation by inhibition of γ-secretase activity that is required for the S3/S4 cleavage of NICD (Dovey et al., 2001; Geling et al., 2002).

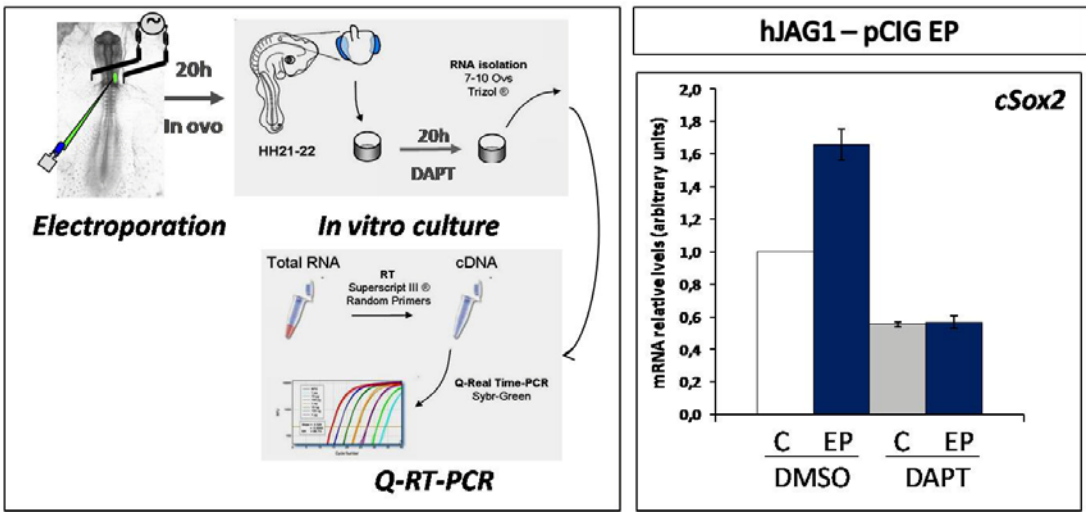


Figure 7: *Sox2* regulation by *Serrate1* is dependent on Notch signalling. A: Scheme exemplifying the experimental procedure used in the experiment combining *hJag1* electroporation and Notch signalling blockade with DAPT. HH12-13 chick embryos were electroporated with *hJag1*-pCIG and allowed to develop in ovo for 20h. After that, otic vesicles were microdissected and cultured in the presence or absence of DAPT for additional 24h. RNA was extracted from cultured otic vesicle and used in qRT-PCR analysis. B: bar diagram sumarazing the results obtained from the experiment represented in (A). Bar diagram shows the relative mRNA levels of *Sox2* in otic vesicles transfected with with *hJag1*-pCIG (blue bars), in the absence (left blue bar) or presence of DAPT (right blue bar), and untransfected otic vesicles cultured in the presence of DAPT (grey bar) in respect to untransfected otic vesicles cultured in control conditions (white bar). *cSox2* mRNA levels were increased after *hJag1* trasnfection (left blue bar, 1,65 ± 0,09, mean ± SD) and decreased after DAPT treatment (grey bar, 0,55 ± 0,01, mean ± SD) but *hJag1* trasnfection did not produce an increase in *cSox2* mRNA levels when the otic vesicles were cultured in the presence of DAPT (right blue bar, 0,56 ± 0,04, mean ± SD).

HH12-14 otic vesicles were electroporated with *hJag1*-pCIG and allowed to develop in ovo for 20h. Then, electroporated otic vesicles that exhibited green fluorescence, and the corresponding contra-lateral otic vesicles were isolated by microdissection and further cultured in presence or absence of DAPT for 24h. RNA extracted from cultured otic vesicles was analysed by qRT-PCR for *Sox2* expression (Fig.7A). The results show that *Sox2* mRNA levels increased by 1,6-fold in otic vesicles cultured in normal medium (left

blue bar) with respect to the untransfected side (white bar), what mimicked the results obtained in ovo 48h after *hJag1* electroporation (see above). However, *Sox2* expression in the presence of DAPT, did not increase after *hJag1* electroporation and, on the contrary, *Sox2* levels decreased to about one half the control values (Fig.7B, 0,57 ± 0,04, mean ± SD, right blue bar). Sox2 mRNA levels were also reduced in the presence of DAPT in the absence of hJag1 (grey bar, 0,55 ± 0,01, mean ± SD). This suggests that the mechanism involved in the up-regulation of *Sox2* expression by *Serrate1* requires active Notch signalling.

Taken together the results suggests that Notch signalling is placed up-stream of Sox2 during neurosensory formation, probably acting though Serrate1 and mediating the maintenance of Sox2 expression in the prosensory patches. This does not exclude another possible function of Notch signalling downstream Sox2, as it will be shown below (chapter IV).

RESULTS – CHAPTER III

The functions of Serrate1 in inner ear neurosensory development

The results presented in this chapter are unpublished, were obtained in collaboration with Dr. Carolina Parada and will be part of a manuscript in preparation.

III. The functions of Serrate1 in inner ear neurosensory development

During neurosensory development, early blockade of the Notch pathway results in a decrease in the number of hair cells, while a late blockade results in the generation of supernumerary hair cells (Takebayashi et al., 2007; Hayashi et al., 2008). The later effect is due to the well documented role played by Notch in the process of lateral inhibition and it is mediated by *Delta1* and *Jagged2* (Haddon et al., 1999; Lanford et al., 1999; Daudet and Lewis, 2005; Kiernan et al., 2005a; Brooker et al., 2006). The former effect has been attributed to a potential role of Notch in the maintenance of the prosensory domains in the inner ear, which is thought to be mediated by *Serrate1* (Kiernan et al., 2006; Daudet et al., 2007; Takebayashi et al., 2007; Hayashi et al., 2008). Yet, the mechanism of action underlying *Serrate1* function, the downstream targets or the cellular processes affected are unknown. We have addressed these questions by carrying out gain of function studies on *Serrate1*

III.1 Serrate1 acts through a lateral induction mechanism mediated by Notch signalling

The cellular expression pattern of *Serrate1* in the prosensory patches of the inner ear is ubiquitous and uniform in all the cells of the patch, as opposed to the salt-and-pepper pattern displayed by *Delta1* or *Serrate2* (Adam et al., 1998; Cole et al., 2000). Furthermore, the gain of function of Notch is able to upregulate *Serrate1* expression (Eddison et al., 2000; Daudet and Lewis, 2005), and Notch blockade results in its downregulation (Daudet et al., 2007). This suggests that *Serrate1* depends on a positive feedback loop of Notch signalling, what has been described as lateral induction (Bray, 1998; Lewis, 1998). We have explored this model by analyzing the ability of *Serrate1* to activate Notch and to induce its own expression. The results show that *Serrate1* can activate Notch in a non cell autonomous manner, and regulate its own expression, both in a cell autonomous and non autonomous manner.

1.1 Serrate1 activates Notch signalling in the otic epithelium

In order to study the ability of Serrate1 to activate Notch signalling in the otic epithelium we used a fluorescent reporter assay of Notch activity in situ. The reporter construct is composed of twelve CSL-binding motifs linked to DsRedExpress, a stable red fluorescent protein that reflects the activation status of Notch signalling with single-cell resolution (Hansson et al., 2006).

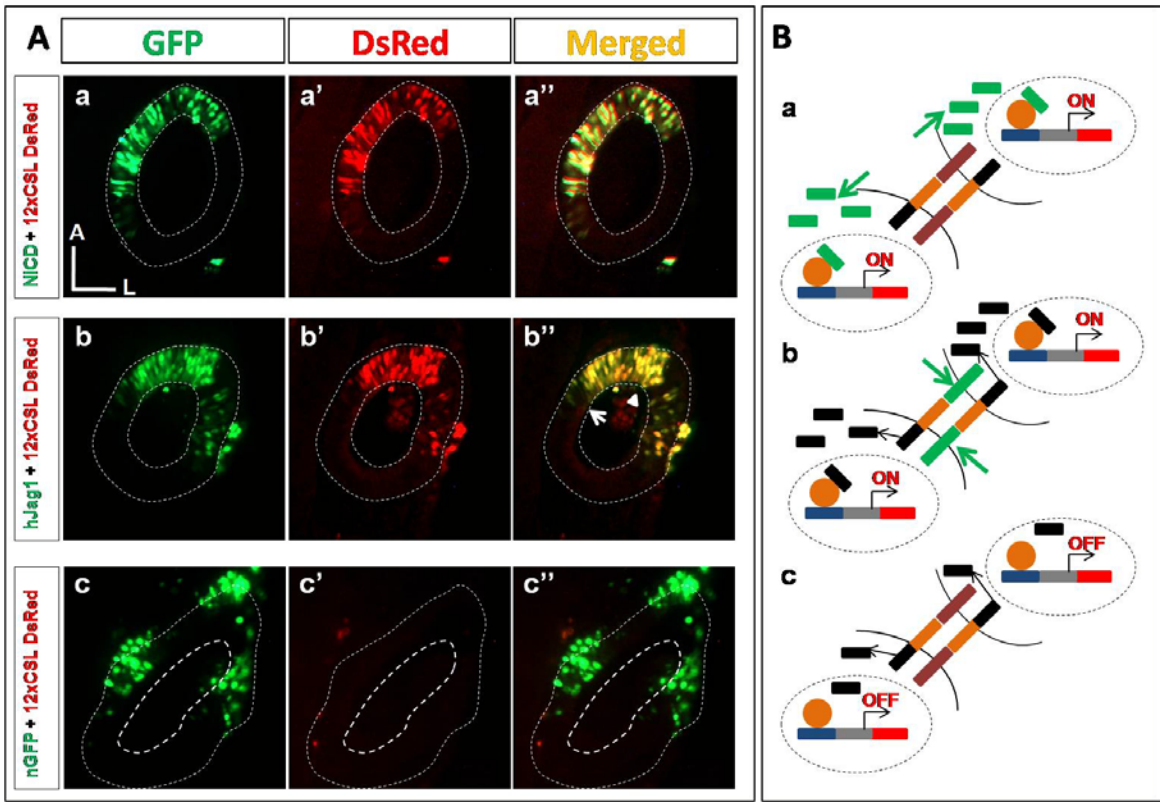


Figure 1: *Serrate1* activates Notch in the otic epithelium. A: coronal sections co-electroporated with 12xCSL Ds Red and NICD (a-a''), *hJag1*-pCIG (b-b''), and pCIG (c-c''), analysed for GFP (a-c) and DsRed expression (a'-c'). Merged images shown in (a''-c''). NICD activated DsRed expression from the reporter in all electroporated cells (a-a') while hJag1 ability to activate Dsred expression seem to compact domains of electroporation (b-b''). pCIG did not induce DsRed expression from the reporter, that in turn was not actiated by endogenous Notch activity. B: Diagram summarizing the cellular events occurring in each case of electroporation. When the reporter vector was co-electroporated with N*EGFP (a), NICD* is expressed in the transfected cells (green rectangles) and binds to CSL proteins (orange circle) in the 12xCSL binding sites (blue rectangle) inducing the expression of DsRed. Similarly, overexpression of hJag1 (b) over-activates the Notch receptor in the neighbouring cells and results in the overproduction of NICD (black rectangles) neighbouring cells with similar consequences in the activation of DsRed protein expression. Finally, when the DsRed reporter vector is electroporated alone (c) the endogenous levels of NICD (black rectangles), produced by endogenous receptor and ligands, is not enough to activate the expression of DsRed (Emil Hansson, personal communication; see materials and methods).A, anterior; L, lateral

The experiments in Fig.1A show coronal cryostat sections of otic vesicles that were co-electroporated with the reporter *plus* a constitutive active form of Notch, N*EGFP (Fig.1Ba-a''), *hJag1*-pCIG (Fig.1Bb-b'') or pCIG alone as a control (Fig1Bc-c''). N*EGFP induced the activation of the reporter in all electroporated cells (compare Fig. 1Ba and a', and the merged picture a''). The co-transfection of *hJag1*-pCIG and the reporter vector resulted also in the up-regulation of DsRed expression in the same domains where GFP expression was detected (compare Fig.1Bb with Fig.1Bb'). However, careful analysis of GFP and DsRed expression in the merged image (Fig.1Bb''), revealed two different situations. First, when clusters of cells were co-transfected with *hJag1* and the reporter plasmid, both GFP and DsRed were expressed, and cells appeared as yellow in the merged image (arrowheads). However, when individual cells were co-electroporated,

only GFP expression was detected in those cells (arrows). The simplest interpretation of these results is that Serrate1 activates Notch in a non cell autonomous manner. If so, when cells are co-electroporated in clusters, adjacent cells receive both the transgene and the reporter, so that, the transgene in one cell will activate the expression of DsRed in the neighbouring cell and *vice versa*. Consequently all cells become yellow. However, when transfection is speckled and only single cells receive the transgene, although they are also able to activate Notch in their neighbours, those adjacent cells cannot activate DsRed expression, because they did not receive the reporter vector. Conversely, adjacent cells that did not receive the transgene cannot activate Notch in the transfected cells that only express GFP. The co-transfection with the control vector was unable to turn on the expression of DsRed protein form the reporter plasmid (no red staining in Fig.1Bc'). Note that although Notch activity is likely to be not negligible in the otic vesicle during this stages (Murata et al., 2006), it was not enough to turn on the expression of DsRed. Figure 1B shows a schematic representation of the cellular events taking place in the experiment, where green is used to represent the electroporated transgene and red the fluorescent protein expression driven by the reporter vector.

Taken together, these results show that *Serrate1* is able to activate the Notch pathway in the otic epithelium in a non cell autonomous manner.

1.2 Serrate1 induces its own expression the otic epithelium

The mechanism of lateral induction relies on the ability of *Serrate1* to induce its own expression (Bray, 1998; Lewis, 1998). In order to analyse this, we electroporated otic vesicles of HH12-14 chick embryos with *hJag1* transgene, and analysed the expression of *Serrate1* with a specific probe for *cSerrate1*, or with primers that detected *cSer1*, but not *Jag1* transcripts. Relative levels of *cSerrate1* mRNA expression, 20h and 48h after *hJag1* transfection, and after electroporation of control vectors, are shown in Fig.2A. (non transfected, white; control transfection, grey; *hJag1* transfection, blue bars). Transcript levels of *cSerrate1* increased by about 1,5-fold with respect to control 20h after transfection (1,47 ± 0,05, mean ± SE). This increase was significant when compared with mRNA levels of otic vesicles electroporated with the control plasmid ($p<0,005$). After 48h, *cSerrate1* mRNA levels returned to control values (1,06 ± 0,06, mean ± SE; $p=0,998$). These results show that *hJag1* transfection induced *cSer1* expression in the otic epithelium. The effect was transient and parallels the temporal profile of transgene expression (see above).

The induction of *cSerrate1* by *hJag1* was further confirmed with ISH analysis using a specific chick probe to detect *cSerrate1* expression in electroporated otic vesicles. *cSerrate1* was induced 20h after transfection with *hJag1* (n = 3/3). One example of a coronal section of an otic vesicle electroporated with *hJag1*-pCIG is shown in Figs.2Bb-c), along with the corresponding control section (Fig.2Ba). *cSerrate1* expression was induced in transfected cells located in the medial anterior and lateral aspect of the otic vesicle (arrowheads in Fig.2Bb and c). The posterior signal corresponds to endogenous expression that can also be detected in controls (Fig.2Ba).

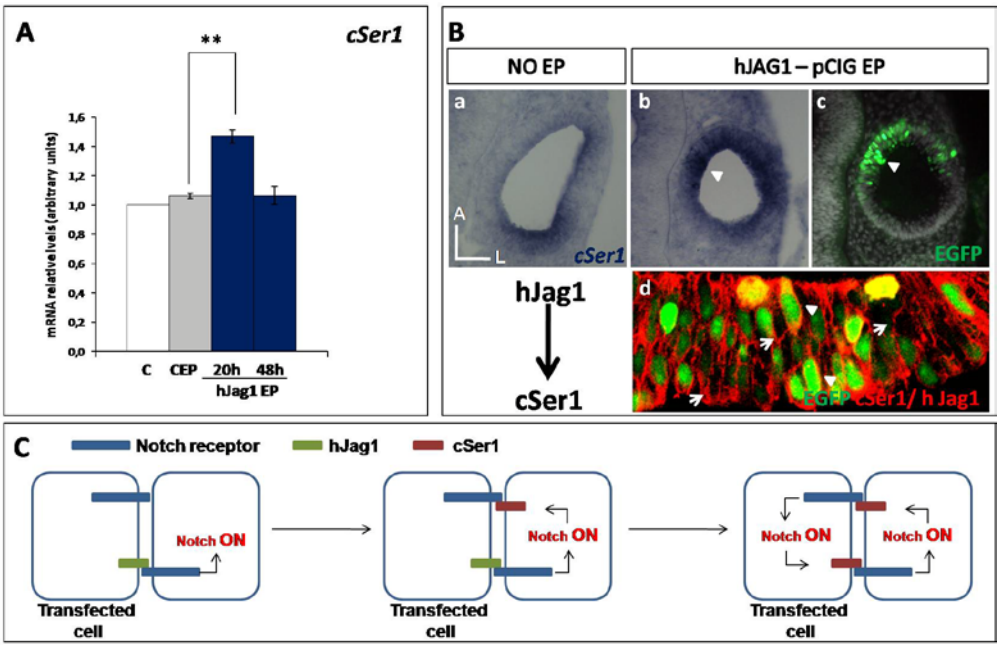


Figure 2: *Serrate1* induces its own expression in the otic epithelium. A: Bar diagram showing the relative mRNA levels of *cSerrate1* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *cSerrate1* mRNA levels significantly increased between control electroporation and *hJag1* electroporation 20h after transfection (compare grey bar and left blue bars, p=0,<0,005), but no longer 48h after electroporation (compare grey and right blue bar, p=0,998). B: Coronal sections of otic vesicles untransfected (a) and transfected with *hJag1*-pCIG (b-c), 20h after electroporation, where *cSerrate1* mRNA was detected by ISH. *cSerate1* mRNA was detected in electroporated regions 20h after transfection (arrowheads, b-c). Confocal image of a detail of a domain of Serrate1 ectopic expression (d) stained for hJag1/cSerrate1 (red) and GFP (green) where cells double stained (yellow) and expressing Serrate1 but not GFP (red membrane signal) can be observed, indicating that the effect of *hJag1* in *cSerrate1* can be non cell autonomous. C: Notch receptor (blue rectangle) is expressed ubiquitously in the otic vesicle. If Serrate1 (green rectangle) is transfected into one cell, it will activate the Notch receptor in the neigbouring cell. Notch activity will result in the up-regulation of Serrate1 (pink rectangle) in the non trasnfected cell. The induced Serrate1 then activates Notch in transfected cell that will further induce Serrate1 expression and reinforce the effect of transfection. A, anterior, L, lateral.

We took advantage of the fact that the anti-Jag1 antibody used in this work recognizes both the transgene and the endogenous products, to analyze the induction of *Serrate1* by h*Jag1* at a cellular level. Figure 2Bd shows a detail of the confocal analysis of a Serrate1 ectopic domain of otic epithelium, which was induced by *hJag1* and analyzed with the hJag1 antibody. Green staining corresponds to GFP expression and labels all the transfected cells, while red staining corresponds to hJag1 or cSer1 expression. Detailed analysis showed two types of cells in the electroporated area: a) cells that were double labelled (arrowheads), b) cells that exhibited only red staining (arrows). The red staining in double labelled cells were attributed to hJag1 expression driven by the transgene. However, the red staining in cells that did not express GFP were interpreted as *cSerrate1* expression that was induced by their adjacent neighbouring cells. A schematic representation of the experiment is shown in Fig.2C. This experiment demonstrates that *hJag1* induced *Serrate1* expression in a non cell autonomous manner

III.2 Serrate1 targets in the otic epithelium: Hey1, Hey2 and Hes1

The identification of the target genes regulated by *Serrate1* is of relevance to understand its function. *Hes/Hey* genes are well known direct transcriptional targets of the Notch signalling pathway, *Hes1*, *Hey1* and *Hey2* are expressed in the mouse prosensory patches however their expression in the chick otocyst is unknown, as it is whether they are downstream effectors of *Serrate1* function. For this purpose, we analysed the effects of the gain of function of *hJag1* on the expression of *Hes1*, *Hes5*, *Hey1* and *Hey2*, by qRT-PCR and ISH. The results show that *Hes1*, *Hey1* and *Hey2*, but not *Hes5*, are upregulated in response to *hJag1* overexpression and that the effect is transient and follows the temporal pattern of *hJag1* expression.

The relative mRNA levels of the four *Hes/Hey* genes were analyzed by qRT-PCR, 20h and 48h after *hJag1* transfection and compared with those from untransfected or transfected with control plasmids. The results are summarized in the bar diagram of Fig.3A. *Hes1*, *Hey1* and *Hey2* mRNA levels increased, 20h after overexpression of *hJag1*, (*Hes1*: 1,59 ± 0,02, p<0,005; ; *Hey1*: 1,95 ± 0,15, p<0,001; *Hey2*: 1,64 ± 0,06, for p<0,005). On the contrary, *Hes5* mRNA levels were not significantly different from control levels. After 48h, however, all those effects diminished and mRNA levels were not significantly different from control values (compare the right blue bar with the grey bar in Fig.3A for each gene).

In order to further confirm the effects of *Serrate1* on Notch targets, we analysed the in situ expression of *Hes1*, *Hes5* and *Hey1* 20h after *hJag1* transfection. Coronal sections of otic vesicles probed for *Hes1*, *Hes5* and *Hey1* by ISH, 20h after *hJag1* transfection, are shown in Fig.3B. *Hes1* (Fig.3Ba'-a''') and *Hey1*(Fig.3Bc'-c''') expression in electroporated otic vesicles corresponded well with the domains of GFP expression, indicating that *Hes1* and *Hey1* expression was induced in electroporated cells. *Hes1* is normally expressed in the posterior and lateral aspect of the otic vesicle (Fig.3Ba). This domain was expanded after electroporation (arrowheads in Figs.3Ba'-a''', n=6/6). Similarly, *Hey1* expression, which is normally detected in the lateral aspect of the otic vesicle was expanded to the medial wall of the otic vesicle after *hJag1* electroporation (arrowheads in Figs.3Bc'-c''', n=3/3). In contrast, *hJag1* overexpression did not produce any visible effect on *Hes5* (n=3/3, arrowheads in Figs.3Bb'-b'''). After 48h, the effects were negligible (n = 3/3 for *Hes1*; n=4/4 for *Hes5*, results not shown), and only some otic vesicles still exhibited a weak increased *Hey1* signal when compared to controls (n=2/4, not shown).

These results show that *Serrate1* is able to induce the expression of *Hes1*, *Hey1* and *Hey2*. Since the time course of induction parallels *hJag1* expression, the suggestion is that this is probably due to the direct activation of Notch by *Serrate1*. *Hes1* and *Hey1* expression was extended to domains where these genes are usually not expressed which is probably due to the ubiquitous expression of Notch1 receptor.

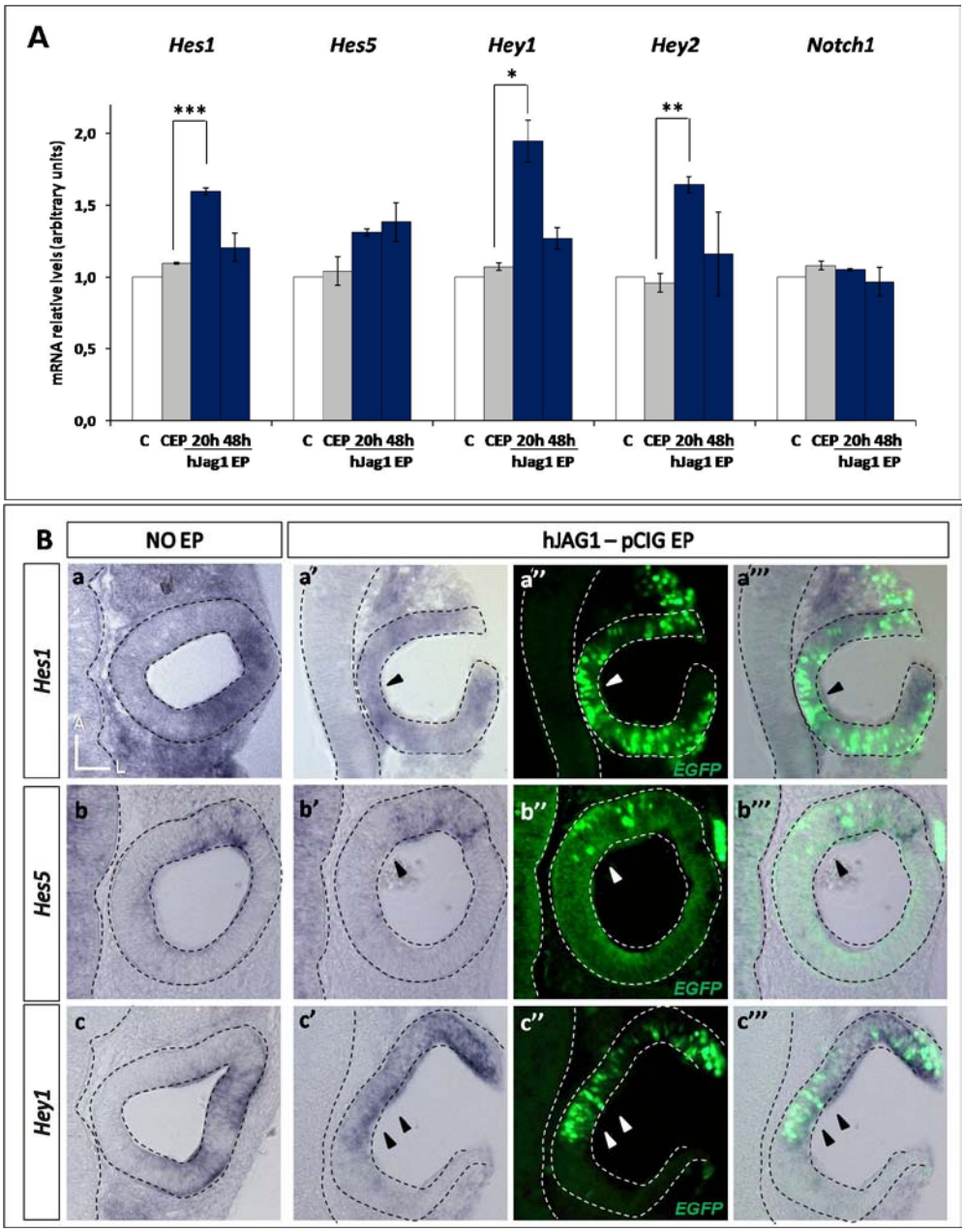


Figure 3: *Serrate1* induces *Hes1*, *Hey1* and *Hey12*expression in the otic vesicle. A: Bar diagram showing the relative mRNA levels of *Hes1, Hes5, Hey1* and *Hey2* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *Hes1, Hey1 and Hey2* mRNA levels significantly increased between control electroporation and *hJag1* electroporation 20h after transfection (compare grey bar and left blue bars, *Hes1*: 1,59 ± 0,02, p<0,005; ; *Hey1*: 1,95 ± 0,15, p<0,001; *Hey2*: 1,64 ± 0,06, for p<0,005), but no longer 48h after electroporation (*Hes1*: p=0,384; *Hey1*: p= 0,094; *Hey2*: p= 0,597). *Hes5* and *Notch1* mRNA levels were not significantly different between otic vesicles electroporated with *hJag1*-pCIG and control plasmids, 20h or 48 h after electroporation (*Hes5*: p=0,093, 20h; p=0,148; 48h; *Notch1*: p=0,671, 20h; p= 0,389, 48h). B: Coronal sections of otic vesicles untransfected (a-c) and transfected with *hJag1*-pCIG (a'-c', a''-c'', a'''-c'''), 20h after electroporation, where *Hes1, Hes5 and Hey1* mRNA was detected by ISH. *Hes1* (a- a''') and *Hey1* (c-c''') mRNA was detected in electroporated regions 20h after transfection (arrowheads), but not *Hes5* (b-b'''). A, anterior; L, lateral.

We analyzed also the effects of hJag1 transfection on the transcript levels of *Notch1* receptor. *Notch1* mRNA levels were not affected after *hJag1* electroporation, when compared to transcript levels in otic vesicles transfected with control plasmids (Fig.3A, differences are not significant). This indicates, first, that lateral induction relies exclusively on a feedback positive loop provided by ligand induction, and does not involve induction of the receptor. Secondly, that the effect on Notch targets is solely due to a ligand-mediated overactivation of the pathway, and not to an increase in receptor availability.

III.3 Serrate1 regulates cell proliferation in the otic epithelium

Cell proliferation and cell death are biological processes involved in the regulation tissue growth. We analyzed the effects of *Serrate1* on cell proliferation rate in the otic epithelium by immunochemistry against phospho-Histone H3 (pH3) and short-pulses of bromo-deoxyuridine (BrdU) incorporation. qRT-PCR analysis of mRNA levels of the cell cycle regulator *cyclin-dependent kinase 1* (*cdk1*) was also used to access proliferation rate. These methods reflect only a fraction of mitotic cells going thought specific phases of the cell cycle. Our objective was to compare the cell proliferation in electroporated otic vesicles with respect to controls. Since the faction of cells going through each phase of cell cycle is proportional to the total amount of proliferating cells, this methods can be effectively used for the purpose of this work.

Otic vesicles were transfected with *hJag1* or with control plasmids, and analysed after 20h or 48h for pH3 immunorectivity or BrdU incorporation. After 20h, the number of pH3-positive cells increased by 32% in electroporated otic vesicles with respect to the corresponding controls (Fig.4A, left bar diagram, 1,32 ± 0,14; mean ± SE; $p<0,05$; n=3 otic vesicles/ condition; approximately 8-12 sections/embryo). This effect increased to 44% after 48h (Fig.4A, right bar diagram, 1,44 ± 0,23; mean ± SE; $p<0,05$). These figures may be probably an overestimation since we observed that electroporation with the control vector also produced an increase in pH3.positive cells of about 10% (not shown). Figure 4B shows examples of sections stained for pH3 and GFP used in the counting summarized in the bar diagram. pH3 positive cells were counted within the electroporated domain as defined by GFP expression, and in an equivalent domain in the control vesicle (arrowheads in Fig.4B).

Similarly, BrdU incorporation was 36% higher in otic vesicles that were electroporated with hJag1, when compared with control otic vesicles. This increase was significant. (Fig.4C,top, 1,36 ± 0,24; mean ± SE; $p<0,05$). BrdU positive cell counting was performed as described for pH3. Fig.4C (bottom) shows a detail of otic epithelium electroporated with *hJag1*-pCIG and treated with BrdU for 30 min, and analysed for BrdU incorporation. The corresponding control section is shown on the left.

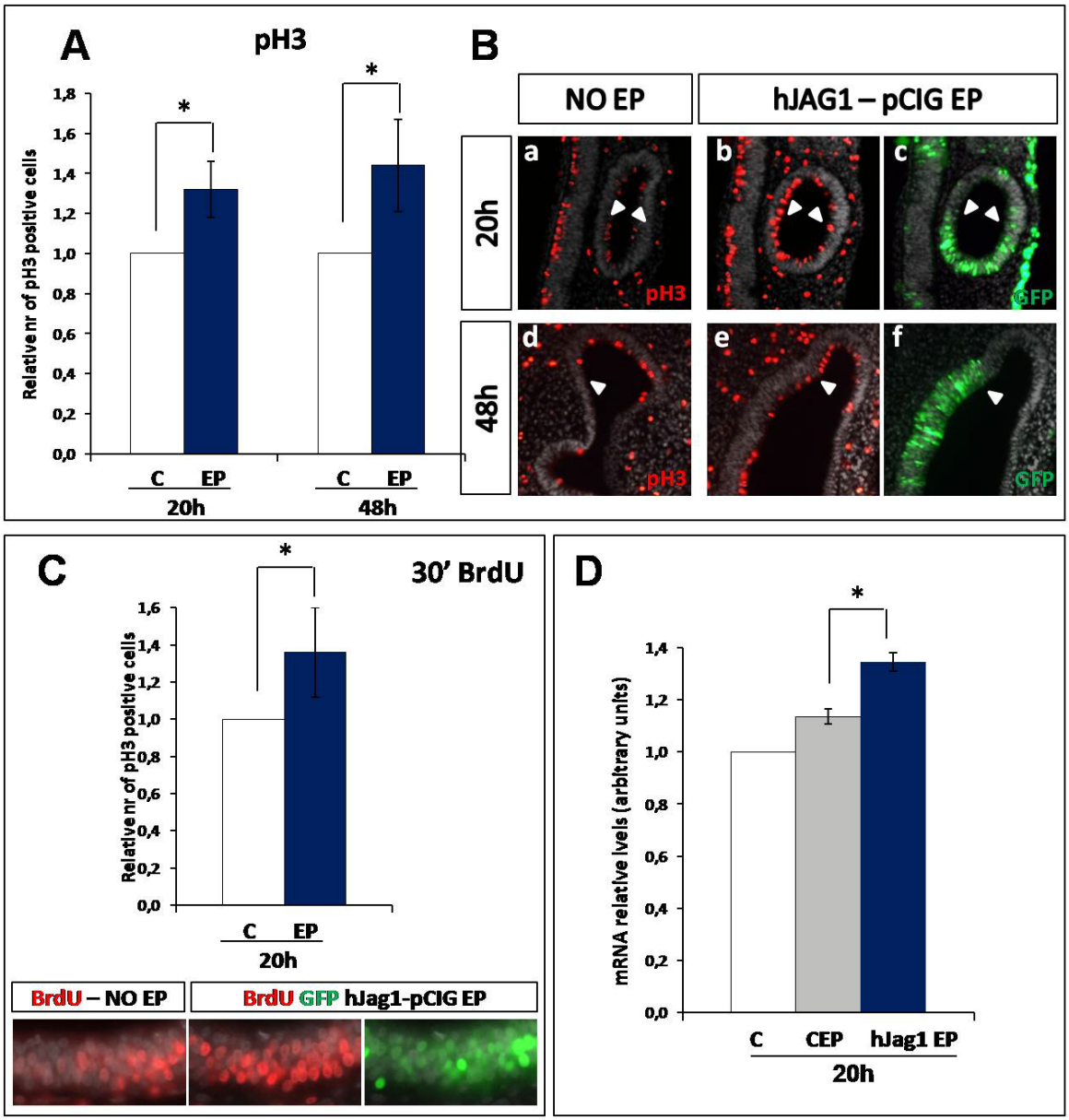


Figure 4: Serrate1 regulates proliferation in the otic epithelium. A: Bar diagram showing the relative number of pH3 positive cells in otic vesicles electroporated with *hJag1* compared to untransfected otic vesicles 20h (left) and 48h (right) after transfection. The number of pH3 positive cells was increased by 32% (p<0,05) 20h after transfection and 44% (p<0,05) 48h after transfection. B: Examples of coronal sections of otic vesicles untransfected (a and d) and transfected with *hJag1*-pCIG (b-c and e-f), 20h (a-c) and 48h (d-f) after electroporation, stained for pH3 protein (red) and GFP (green). Arrowheads indicate the limits of GFP expression domains in transfected otic vesicles (b-c and e-f) and the correspondent domain in controls (a and d) used for the counting. C: top, bar diagram showing the relative number of BrdU positive cells in otic vesicles electroporated with *hJag1* compared to untransfected otic vesicles, both incubated for 30min in the presence of BrdU, 20h after transfection. The number of BrdU positive cells was increased by 36% (p<0,05) 20h after transfection. Bottom, examples of otic epithelium of untransfected (left) and *hJag1* transfected otic vesicles (right) double stained for BrdU (red) and GFP (green). D: Bar diagram showing the relative mRNA levels of *cdk1* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h after transfection, in respect to untransfected otic vesicles (white bar). *cdk1* mRNA levels significantly increased between control electroporation and *hJag1* electroporation 20h after transfection (1,34 ± 0,03, mean ± SE, p=0,<0,05),

Proliferation is regulated by an array of proteins that regulate cell cycle progression and cell cycle exit. Cyclins, cyclin-dependent kinases and cyclin-dependent kinases inhibitors are some of these molecules. We analysed the relative mRNA levels of *cdk1* in *hJag1* transfected otic vesicles by qRT-PCR (Fig.4D). *Cdk1* increased in electroporated otic vesicles when compared to controls, and also when compared with the control plasmid electroporation (1.35 ± 0,04; mean ± SE; p<0,05).

We also investigated a potential role of Serrate1 in the regulation of cell death, by caspase3 expression. However, no differences were observed in the expression of caspase3 between *hJag1*-transfected and non transfected otic vesicles (not shown), suggesting that *Serrate1* does not play a role in the regulation of apoptosis.

Taken together, these results indicate that *Serrate1* regulates cell proliferation but not cell survival in the otic epithelium. This is consistent with the increase in the size of the otocyst induced by the forced expression of hJag1 after 4 days of development (see below).

III.4 Serrate1 regulates the size of the sensory domains of the otic vesicle, but does not affect otic neurogenesis

Serrate1 loss of function in mice results in the abnormal development of the sensory organs of the inner ear, with an overall reduction in the number of hair cells, and this defects are not due to altered differentiation or to cell degeneration (see introduction (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006)).The results described above show that *Serrate1* is able to expand *Sox2* expression domain. This suggests that *Serrate1* may regulate the formation of prosensory patches in the inner ear. To further analyze this possibility, we analysed the effects *Serrate1* overexpression on the differentiation of the sensory patches and the production of hair cells.

4.1 Effects of Serrate1 on prosensory genes

We studied the effects of *hJag1* overexpression on two genes expressed in the prosensory domains: *Bone morphogenic protein 4* (*Bmp4*) and *Lunatic Fringe* (*LFng*). Bmp4 is a signalling molecule that regulates hair cell specification and the size of the sensory patches [Pujades et al., 2006; Chen et al., 2008; Kamaid et al., 2009]. LFng is a modulator of Notch signalling and its function during inner ear neurosensory development is yet unknown. Transfection with *hJag1* induced a 2,3-fold increase in *Bmp4* transcript levels with respect to controls 20h after transfection. This increase was significant when compared to mRNA levels in otic vesicles electroporated with control plasmids (Fig.5A, left blue bar, 2,2 ± 0,07; mean ± SE; p<0,005). This effect was transient, and 48h after transfection, *Bmp4* mRNA levels were not significantly different from control (Fig.5A, right blue bar, 0,92 ± 0,09; mean ± SE; p=0,217).

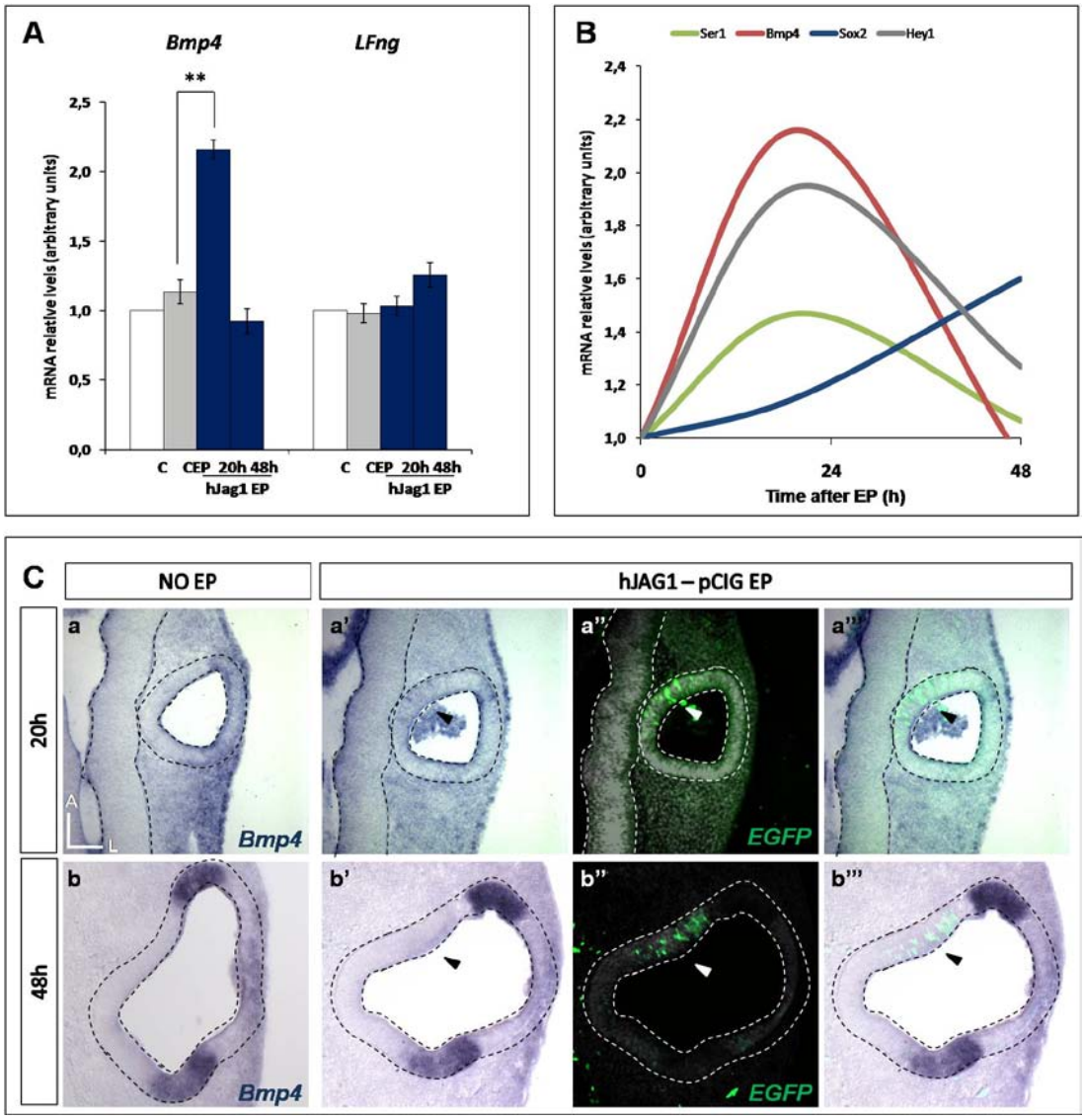


Figure 5: *Serrate1* induce *Bmp4* expression transiently in the otic epithelium. A: Bar diagram showing the relative mRNA levels of *Bmp4* and *LFng* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *Bmp4* mRNA levels significantly increased between control electroporation and *hJag1* electroporation 20h after transfection (compare grey bar and left blue bars, 2,16 ± 0,07; mean ± SE; p<0,005) but no longer 48h after electroporation (p=0,217). *LFng* mRNA levels were not significantly different between otic vesicles electroporated with *hJag1*-pCIG and control plasmids, 20h or 48 h after electroporation (p=0,398, 20h; p= 0,183, 48h). B: temporal profile of the relative levels prosensory genes in response to *hJag1* transfection. *Serrate1* (green), *Bmp4* (red) and *Hes/Hey* genes (grey) followed the profile of transgene expression, while *Sox2* (blue) was only upregulated when transgene levels are already decreasing. C: Coronal sections of otic vesicles untransfected (a-b) and transfected with *hJag1*-pCIG (a'-a''', b'-b'''), 20h (a-a''') and 48h (b-b''') after electroporation, where *Bmp4* mRNA was detected by ISH. 20h after electroporation (a'-a''') *Bmp4* mRNA was detected in electroporateddomain (arrowheads), but not 48h after. A, anterior; L, lateral.

These results were confirmed by ISH (Fig.5C n=3/3 for each time point). Figure 5C shows examples of coronal sections of otic vesicles electroporated with *hJag1* in the anterior medial aspect of the otic vesicle (where *Bmp4* is not expressed, see controls in Figs. 5Ca and 5Cb), and probed for *Bmp4* mRNA expression after 20h (Fig.5Ca'-a''') and 48h (Fig.5Cb'-b'''). *Bmp4* signal was detected in the electroporated region in the 20h, but not in the 48h specimens (arrowheads in Fig.5Ca'-a''' and Fig.5Cb'-b'''). *LFng* expression levels were not significantly affected after *hJag1* transfection (Fig.5A, right bar diagram, for 20h: 1,03 ± 0,04, mean ± SE, p=0,398; for 48h: 1,26 ± 0,15, mean ± SE, p=0,183).

Figure 5B summarizes the effects of *Serrate1* gain of function on the expression of prosensory genes Serrate1 (green), Bmp4 (red) and Hey1 (grey), which followed the temporal profile of transgene expression (see above). Expression was high by 20h after *hJag1* transfection, when transgene expression was maximal, and it faded down with transgene downregulation 48h after electroporation. This suggests that *Serrate1*, *Bmp4* and *Hey1* (like *Hes1* and *Hey2*, not shown) may be targets of *Serrate1* and mediate by early Notch signalling functions. Conversely, the response of *Sox2* (blue) was delayed with respect to *hJag1* expression, and *Sox2* expression increased only after 48h, when transgene and the rapidly responding genes were down-regulated.

4.2 Serrate1 is not sufficient to specify the sensory fate, but it regulates the size of sensory domains

Since *Serrate1* is able to upregulate prosensory genes we sought to explore the effects of *Serrate1* on the development of the sensory patches. For that purpose, otic vesicles were transfected with *hJag1* and analysed after 4 days of development in ovo. This corresponds to day 6 of development (E6), when sensory organs are differentiated, hair express differentiation markers like *MyoVII*, and supporting cells express *Sox2*. Embryos were serially sectioned and stained by IHC for MyoVII, analysed by confocal microscopy and otic vesicles were reconstructed in 3D to analyse the size and position of sensory patches.

Figure 6A shows a dorsal view of a 3D reconstruction of an otocyst that was electroporated in the region of the maculae (right), and the corresponding control otocyst (left). Red dots represent MyoVII-expressing cells and green dots those expressing GFP. As shown, the maculae were expanded in the transfected otic vesicle and the expansion corresponded well to the GFP expressing domain. Calculation of the surface area occupied by the maculae in the electroporated otic vesicle and in control showed that the former increased by approximately two-fold, the cristae (1,2 and 3, in Fig.6A) remaining unaffected.

Confocal analysis of sections containing the maculae revealed that transfected cells differentiated as either hair or supporting cells, as revealed by MyoVII staining (hair cells, Figs.6Bb-d, arrowheads, supporting cells, Figs.6Bb-d arrows). However, differentiation of hair and supporting cells occurred only within the expanded sensory domains, and GFP-positive cells occurring outside those domains (asterisk in Fig 6A) did not express MyoVII or Sox2 (not shown).

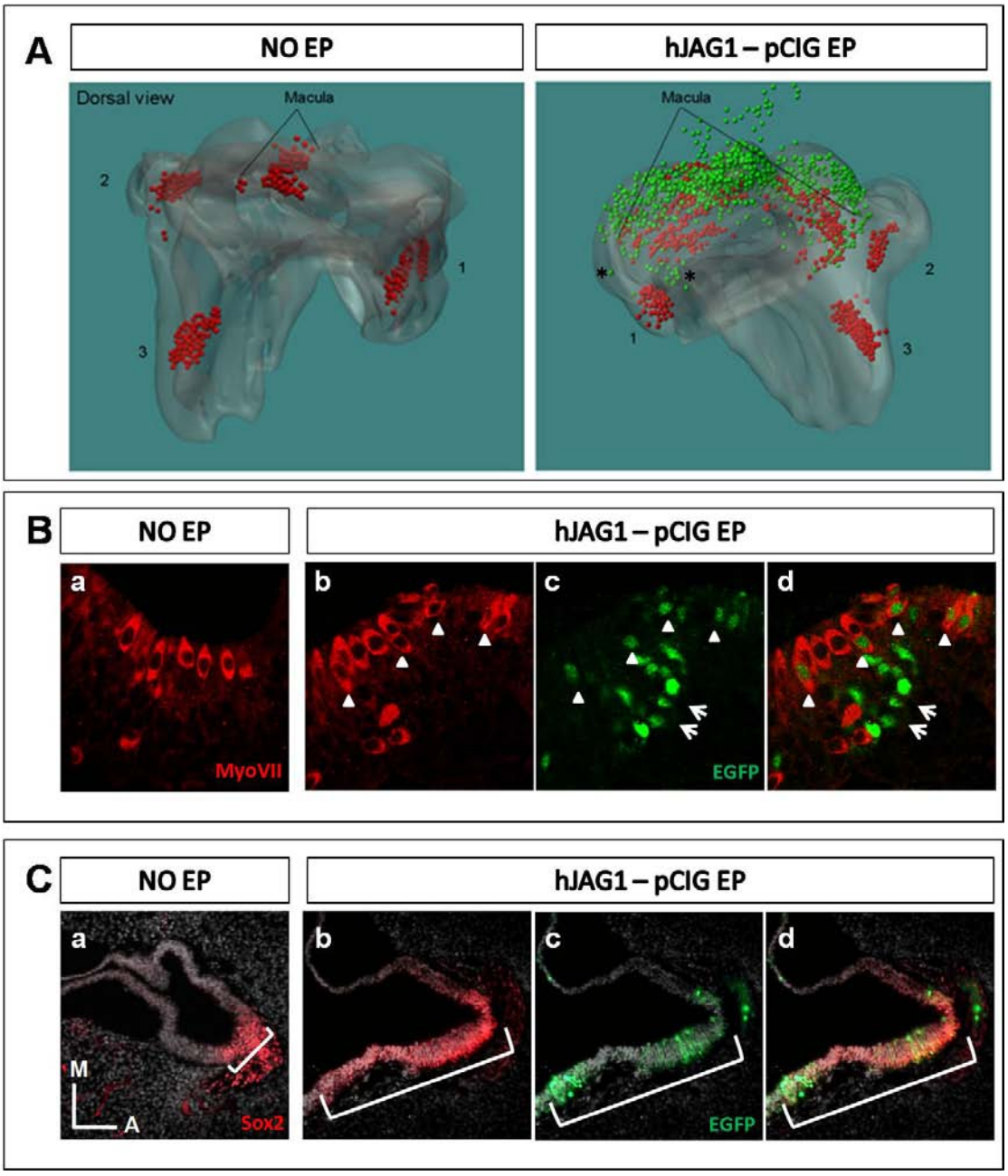


Figure 6: ***Serrate1*** **regulates sensory organ formation. A: Dorsal views of 3D reconstruction of otocysts transfected with hJag1 (right) and corresponding contralateral otocyst (left) from serial coronal section double stained with GFP (green dots) and MyoVII (red dots), 4 days after transfection. 1, 2 and 3 represent the cristae and maculae domain is also delimited. Maculae domain was expanded in transfected otic vesicles and the expansion corresponded to GFP positive domain. GFP cells outside the expanded sensory domain were not MyoVII positive. B: confocal analysis of a detail of the macular otic epithelium of untransfected (a) and** ***hJag1*** **transfected (b-d) otocyst, 4days after electroporation, stained for MyoVII (red) and GFP (green). GFP cells were detected both in the hail cell layer (double labelling, arrowheads) and in the supporting cell layer (arrows). C: coronal sections of otic epithelium of untransfected (a) and** ***hJag1*** **transfected (b-d) otocyst, 4 days after electroporation, stained for Sox2 (red) and GFP (green). Sox2 expression was still expanded 4 days after transfection in a domain adjacent to the anterior crista. A, anterior, M, medial.**

The expanded expression of *Sox2* observed 48h after electroporation with *hJahg1* (see above) was maintained during development, for at least another 48h, well beyond transgene exhaustion (Fig.6C, brackets, compare control side with electroporated side). This suggests that the induced *Sox2* expression was rather stable and persisted after transgene downregulation.

Taken together, the results indicate that *Serrate1* is not sufficient for specification of sensory fate, but it is able to expand the normal competent domains. This, along with the fact that *Serrate1* expands Sox2 expression and that during development *Sox2* expression is originally broad, but then restricts to *Ser1*-positive domains suggests that one function of *Serrate1* would be to maintain *Sox2* expression and thus prosensory fate in specific domains derived from an originally broader competent region (see discussion).

4.3 Serrate1 does not affect otic neurogenesis

The expression pattern of *Serrate1* revealed that it was expressed also in delaminating neuroblasts (see Fig.1Dc, chapter II). Therefore it was interesting to know whether *Serrate1* was also involved in the regulation of otic neurogenesis. For this purpose, we analysed the effects of *hJag1* transfection on the expression of neuronal markers and the size of the cochleo-vestibular ganglion (CVG). *hJag1*-transfected cells were able to differentiate as neurons and populate the CVG as judged from the presence of delaminating neuroblasts that expressed the transgene (Fig.7A). This suggests that Serrate1 did not perturb normal delamination and neuronal production. 3D reconstructions from serial coronal sections of transfected, and the corresponding control, otic vesicles (Fig.7B) were used to calculate volume of the CGV and otocysts Transfected otic vesicles showed an increase of about 50% in volume with respect to control, but the volume of the CGV changed by only 5%.

Ngn1 and *NeuroD* are expressed in neuronal precursors during the phases of neuronal fate commitment and neuronal differentiation, respectively (see introduction). The analysis of *Ngn1* expression by qRT-PCR, 20 and 48h after electroporation showed no significant differences between transfected and control otic vesicles (Fig7.Ca; 20h: 0,92 ± 0,03, mean ± SE, p=0,614; 48h: 1,05 ± 0,04, mean ± SE, p= 0,152). ISH analysis of *NeuroD* expression 48h after electroporation gave similar results (n =3/3, Fig.7Cb-b'''). Therefore, Serrate1 was able to increase the size of the otic vesicles and the sensory patches, but had no effect on neuronal development.

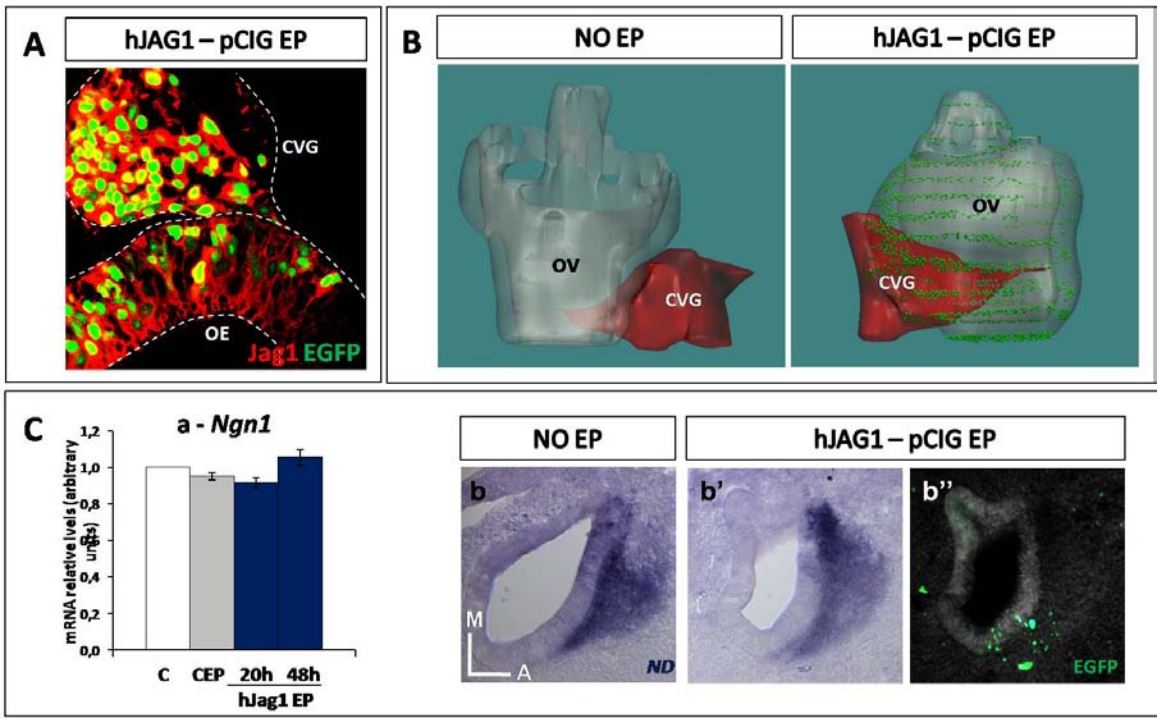


Figure 7: *Serrate1* does not regulate otic neurogenesis. A: Confocal analysis of a coronal section of an otic vesicle and associated cochleo-vestibular ganglion transfected with *hJag1*, 20h after transfection and double stained for Jag1 (red) and GFP (green). Transfected cells were detected in the cochleo-vestibular ganglion. B: lateral view of 3D reconstruction of otic vesicles transfected with *hJag1* (right) and corresponding control (left), from serial coronal sections stained for Islet1 (red) and GFP (green dots). C: Bar diagram (a) showing the relative mRNA levels of *Ngn1* in otic vesicles transfected with control plasmids (grey bar) and with *hJag1*-pCIG (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *Ngn1* mRNA levels were not significantly different between otic vesicles electroporated with *hJag1*-pCIG and control plasmids, 20h or 48 h after electroporation (p=0,614, 20h; p= 0,152, 48h). (b-b''): Coronal sections of otic vesicles untransfected (b) and transfected with *hJag1*-pCIG (b'-b''), 48h after electroporation, where *NeuroD* mRNA was detected by ISH. *NeuroD* mRNA was not detected in electroporated domains. CVG, cochleo-vestibular ganglion; OE, otic epithelium; OV, otic vesicle; A, anterior; M, medial.

RESULTS – CHAPTER IV

The functions of Sox2 in inner ear neurosensory development

The results presented in this chapter are unpublished and will be part of a manuscript in preparation.

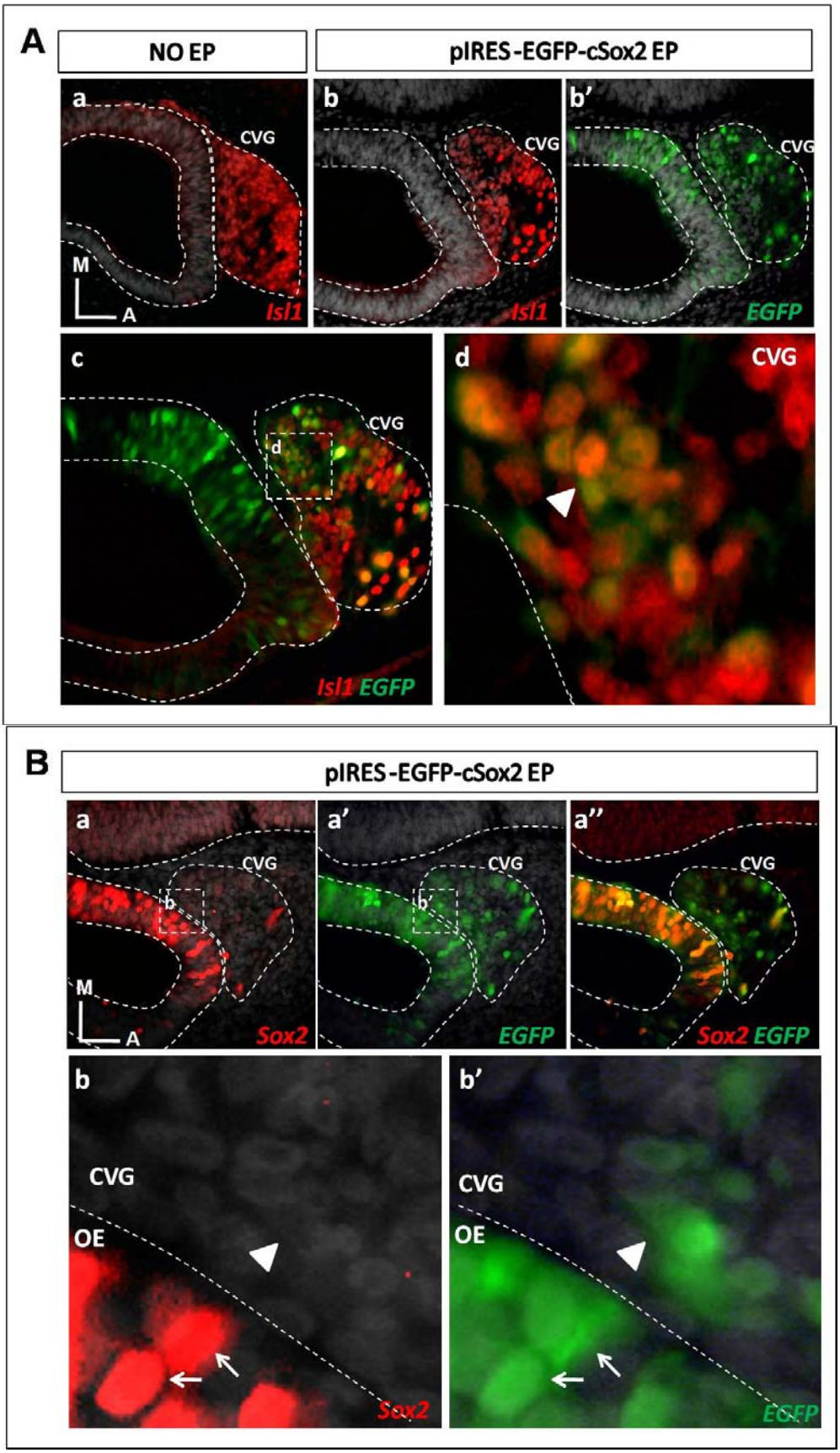
IV. The functions of Sox2 in inner ear neurosensory development

In the CNS, *Sox2* has been associated with neural commitment and regulation of neurogenesis (Bylund et al., 2003; Graham et al., 2003; Pevny and Placzek, 2005). *Sox2* conditional loss of function in the mouse inner ear results in impaired sensory development with many sensory organs exhibiting a reduced number or absence of hair cells (Kiernan et al., 2005b). Functional studies in the mouse cochlea have shown that *Sox2* counteracts hair cells differentiation by antagonizing *Atoh1* function [Dabdoub et al., 2008]. In the present work, we performed gain of function studies that served to investigate the function of *Sox2* in the development of the neurosensory elements of the ear. Specifically, we wanted to explore the role of *Sox2* in the regulation of the fate and differentiation of otic neurosensory progenitors.

IV.1 Sox2 regulates neurosensory differentiation in the otic epithelium

In the following experiments we have analysed the effects of the ectopic and normotopic overexpression of *Sox2* on the differentiation of neurons and sensory cells. First, we shall describe the effects on neurogenesis and then those on the development of the sensory patches.

HH12-14 otic cups were electroporated with a *cSox2* plasmid and analysed for Islet1 expression after 20h or 48h of development in ovo. At the time of electroporation, the anterior-ventral aspect of the otic cup does express *NeuroD* and *Islet1* in the neuronal precursors [Adam et al., 1998; Alsina et al., 2003, Li et al., 2004]. Twenty hours after transfection, embryos reached stage HH18-19, when the cochleo-vestibular ganglion (CVG) is already formed. The sustained expression of *Sox2* did not prevent the delamination of neuroblasts as judged from the ability of GFP positive cells to delaminate and express neuroblast markers (Fig.1A). GFP-positive cells carrying the transgene were detected in the CVG, indicating that they had delaminated from the neurogenic epithelium (Fig.1Ab', n=8/8, note that only the otic epithelium is electroporated at time zero). GFP-positive cells co-expressed Islet1, which is a marker of early neuronal differentiation during normal otic development (Figs.1Ac-d, arrowhead in Fig.1Ad, n=8/8). As it will be discussed later, these GFP-positive neuroblasts probably corresponded to precursors that were already specified in the epithelium at the time of electroporation and that were insensitive to *Sox2* overexpression. Delaminated neuroblasts fully differentiated into neurons, as judged from the expression of Tuj1 (not shown), irrespectively of transgene expression.


A
NO EP
pIRES-EGFP-cSox2 EP
a
CVG
M
A
Isl1
b
CVG
Isl1
b'
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Sox2 protein is normally downregulated form delaminated neuroblasts and it is absent in differentiated neurons [chapter I, Neves et al., 2007]. Similarly, the Sox2 protein driven by the transgene was down-regulated in the neuroblasts populating the CVG (Figs.1Ba-a'', n=6/6). Neuroblasts that expressed GFP did not express Sox2 within the CVG (Figs.1Bb-b', arrowheads), whereas GFP-positive cells in the otic epithelium did express high levels of Sox2 (Fig.1Bb-b', arrows). This suggests that the downregulation of both exogenous and endogenous Sox2 protein takes place as neuroblasts delaminate and differentiate within the CVG. Since Sox2 expression driven by the transgene is regulated by a constitutive promoter, which is not subject to transcriptional regulation, the results suggest that there is at least one post-transcriptional mechanism that regulates Sox2 protein levels in mature neuroblasts and neurones.

In another set of experiments, Islet1 expression was analysed 48h after electroporation with c*Sox2* and control plasmids. Otic vesicles reached stage 24-25 (E4), when Islet1 is expressed in the prosensory patches (Li et al., 2004). After 48h, transgene expression was already low (see Fig.4, chapter II), but Sox2 protein was still expressed at high levels in transfected cells. The results show that *Sox2* overexpression hindered Islet1 expression in the prosensory otic epithelium in both vestibular and auditory prosensory patches (Figs.2Aa-a''', Figs.2Ab-b''', n=8/8 for vestibular, Figs.2Ac-c''', n=6/6, for auditory). GFP-expressing cells within Islet1-positive domains did not express Islet1 as revealed by the green colour in the detailed merged images (arrowheads in Fig.2Ba). This was not the case when pCMV/SV1- cSox2ΔHMG was electroporated, in which transfected cells expressed Islet1 and the transgene, as revealed by the yellow colour of the nuclei (arrowheads in Fig.2Bb, see supplementary information 1, Fig.2, for the effects of control transfection in the different sensory organs). The fraction of double-labelled Islet1/GFP cells within the prosensory patches were only 11% and 13% in the maculae and basilar papilla, respectively, and 36% in the cristae (Fig.2C, blue bars). In contrast, 93-96% of the cells electroporated with the control plasmid co-expressed GFP and Islet1 (Fig.2C, grey bars).

Figure1: *Sox2* does not prevent neuroblast delamination and it is downregulated in the CVG. A: coronal sections of otocysts untransfected (a) and transfected with *cSox2* (b-d), 20h after electroporation, stained for Islet1 (red) and GFP (green). GFP cells were detected both in the otic epithelium and in the cochleo-vestibular ganglion (b'). Islet expression was detected in the cochleo-vestibular ganglion of untransfected (a) and transfected otic vesicles (b-d), where it co-expressed with GFP (yellow, c-d). B: coronal sections of otocysts transfected with *cSox2* (a-a''), 20h after electroporation, stained for Sox2 (red) and GFP (green). GFP cells were detected both in the otic epithelium and in the cochleo-vestibular ganglion (a'-a'' and arrowhead in b'). Sox2 expression was detected in the otic epithelium (arrows in b) but not in the cochleo-vestibular ganglion (arrowheads in b). GFP positive cells in the cochleo-vestibular ganglion (arrowheads in b') did not express Sox2 (arrowhead in b). CVG, cochleo-vestibular ganglion; OE, otic epithelium; A, anterior; M, medial. (Previous page)

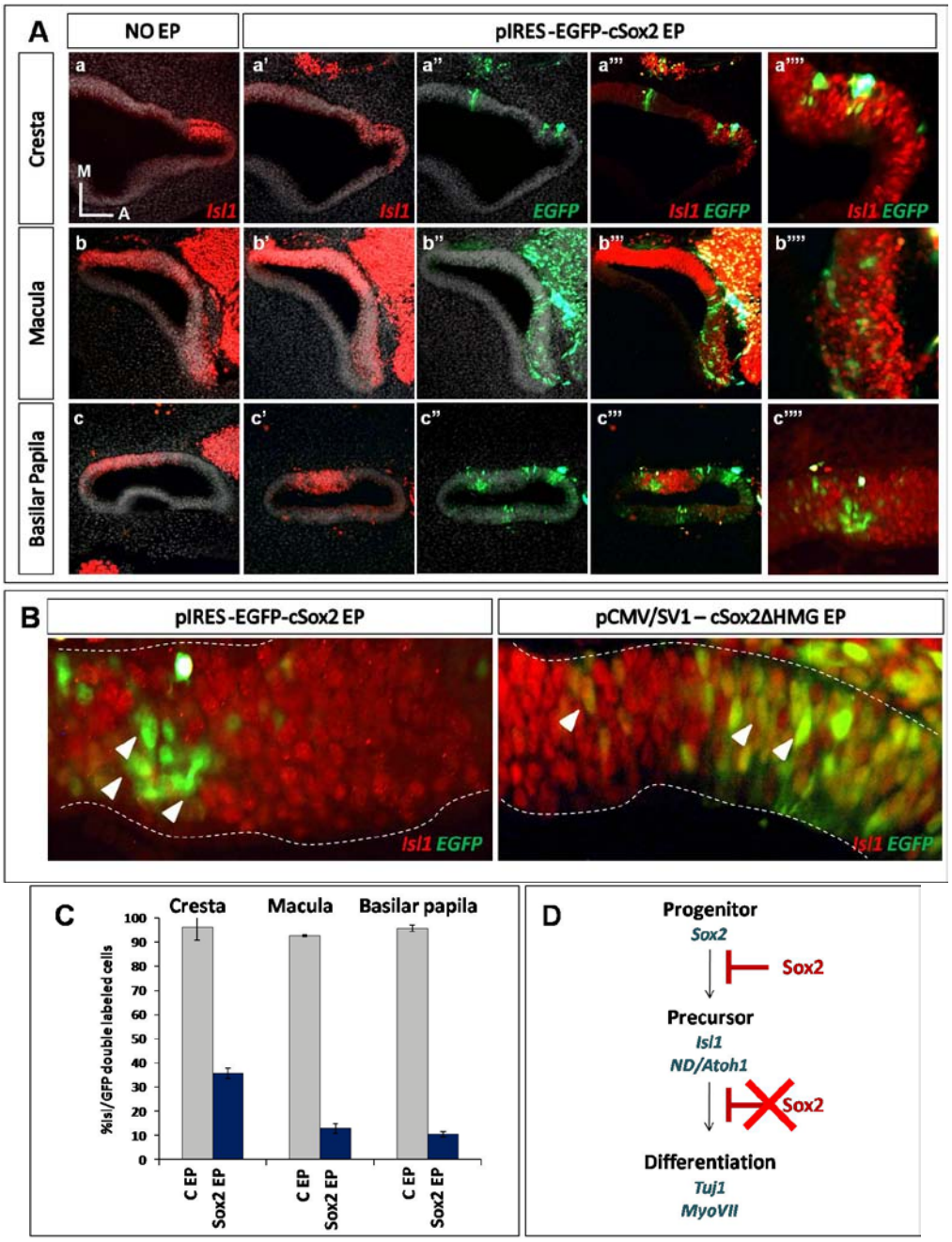

Figure 2: ***Sox2*** **prevents Islet1 expression in the sensory precursors. A: coronal sections of otocysts untransfected (a-c) and transfected with** ***cSox2*** **(a'-a'''', b'-b'''', c'-c''''), 48h after electroporation, stained for Islet1 (red) and GFP (green). GFP cells were detected both in the otic epithelium and in the cochleo-vestibular ganglion (b'-b''''). Islet expression was detected in the cochleo-vestibular ganglion, where it co-expressed with GFP (yellow, b''') but it was not expressed in GFP positive cells in the otic epithelium, within the prosensory domains (a'''', b'''' and c''''). B: High magnification of the basilar papilla epithelium transfected with** ***cSox2*** **(a) or** ***cSox2*****ΔHMG (b) 48h after electroporation, stained for Islet1 (red) and GFP (green). GFP positive cells transfected with** ***cSox2*** **(a) do not co-express Islet1 (a, arrowheads, green) but** ***cSox2*****ΔHMG transfected cells do (b, arrowheads, yellow). C: Bar diagram with the fraction of double labelled cells in the different sensory organs, 48h after** ***cSox2*** **(blue bar) or** ***cSox2*****ΔHMG (grey bar) transfection. D: Diagram representing the interpretation of results from Fig.1 and Fig.2.** ***Sox2*** **can prevent Iselt1 expression in uncommitted progenitors but cannot reverse its expression in committed precursors. CVG, cochleo-vestibular ganglion; OE, otic epithelium; A, anterior; M, medial.**

This suggests that *Sox2* is able to prevent Islet1 expression in the sensory precursors. The observation that this effect was weaker in the cristae than in the other sensory organs suggests that the ability of *Sox2* to prevent the progression of differentiation may depend on the state of commitment of the progenitor/precursor cells. The cristae are the first sensory organs to differentiate therefore it is likely that a fraction of sensory precursors were already specified at the time of maximal transgene expression. Thus, *Sox2* was not able to hinder Islet1 expression in the cristae, similarly to what was observed for the neuronal precursors (See Fig.1). In summary, the results support the notion that *Sox2* prevents the differentiation of sensory progenitors. However, once the differentiation program is initiated, the sustained expression of *Sox2* is not able to reverse the process (Fig.2D).

IV.2 Sox2 cooperates with Notch during neurosensory development

The Notch signalling pathway plays also a critical function during neurogenesis by maintaining neural cells in an undifferentiated state (Kageyama and Ohtsuka, 1999). We sought to explore the possibility that *Sox2* regulates the Notch pathway as part of the mechanism by which it prevents the expression of Islet1. We analysed the effects of *Sox2* gain of function on *Hes/Hey* genes, which are downstream targets of Notch.
Notch targets (*Hes1*, *Hes5*, *Hey1* and *Hey2*), Notch receptor (*Notch1*) and Notch ligand (*Dl1*) expression were analyzed by qRT-PCR, 20h and 48h after electroporation. As illustrated in Fig.3A, *Hes5*, *Hey1* and *Hey2* transcript levels increased in response to *Sox2* overexpression, 20h after electroporation (*Hey1*: 1,62 ± 0,10; *Hey2*: 1,54 ± 0,10; *Hes5*: 1,43 ± 0,03, mean ± SE). The increase was significant when compared with embryos electroporated with the control transgene coding for non functional Sox2 ($p<0,05$ for *Hey1*, *Hey2* and *Hes5*).These results were confirmed by ISH analysis of electroporated otic vesicles, using riboprobes specific for *Hes5* and *Hey1* (Fig.3Ba-a'' and b-b''). *Hes5* and *Hey1* signal was increased in electroporated otic vesicles when compared to controls in equivalent sections (for *Hes5* n = 5/8 with a strong effect and n=3/8 with a moderate effect; for *Hey1* n= 3/3). The induction corresponded to domains where transfected cells were detected by GFP expression (compare Fig.3Ba' and Fig.3Ba'' for *Hes5* and Fig.3Bb' and Fig.3Bb'' for *Hey1*). This effect was not observed in otic vesicles electroporated with control plasmids (see supplementary information 1, Fig.1). The effects of Sox2 on Notch targets were transient and 48h after electroporation transcript levels of *Hes5*, *Hey1* and *Hey2* were no longer significantly different from control (Fig.3A). Therefore, the effect of *Sox2* on these Notch targets followed the time profile of transgene expression. *Hes1* expression was not significantly affected by *Sox2* gain of function (Fig.3A)

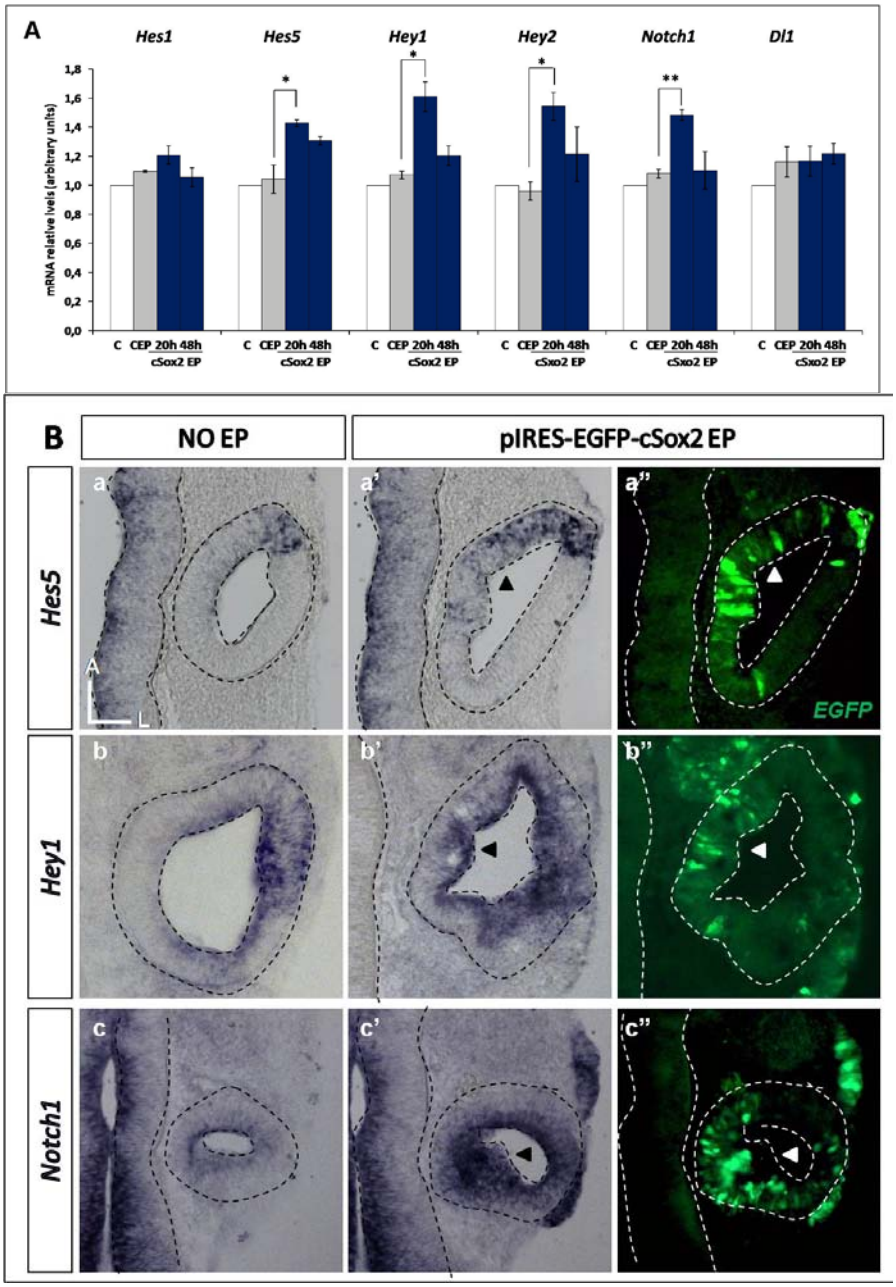

Figure 3: *Sox2* regulates *Notch1* and Notch targets in the otic epithelium. A: Bar diagram showing the relative mRNA levels of *Hes1, Hes5, Hey1, Hey2, Notch1* and *Delta1* in otic vesicles transfected with control plasmids (grey bar) and with *cSox2* (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *Hes5, Hey1, Hey2* and *Notch1* mRNA levels significantly increased between control electroporation and *cSox2* electroporation 20h after transfection (compare grey bar and left blue bars, *Hes5*: 1,43 ± 0,03; *Hey1*: 1,62 ± 0,10; *Hey2*: 1,54 ± 0,10; *Notch1*: 1,48 ± 0,03; mean ± SE; p<0,05 for *Hes5, Hey1* and *Hey2* and p<0,005 for *Notch1*), but no longer 48h after electroporation (*Hes5*: p=0,087; *Hey1*: p= 0,190; *Hey2*: p= 0,327; *Notch1*: p=0,891). *Hes1* and *Delta1* mRNA levels were not significantly different between otic vesicles electroporated with *cSox2* and control plasmids, 20h or 48 h after electroporation (*Hes1*: p=0,424, 20h; p=0,618; 48h; *Delta1*: p=0,976, 20h; p= 0,712, 48h). B: Coronal sections of otic vesicles untransfected (a-c) and transfected with *cSox2* (a'-c', a''-c'') 20h after electroporation, where *Hes5, Hey1* and *Notch1* mRNA was detected by ISH. *Hes5* (a- a''), *Hey1* (b-b'') and *Notch1* (c-c'') mRNA was detected in electroporated regions 20h after transfection (arrowheads). A, anterior; L, lateral.

We also explored whether *Sox2* was able to regulate the expression of *Notch1* and/or Notch ligands. Figure 3A shows that *Sox2* was able to induce *Notch1* expression in the otic epithelium. *Notch1* transcript levels increased by 1,5-fold with respect to controls, and the effect was significant when compared with the transfection with the non-functional transgene (Fig3.A, 1,48 ± 0,03, mean ± SE, p<0,005). Like for Notch targets, the effect of *Sox2* on *Notch1* was transient, and no significant differences were detected 48h after electroporation (Fig.3A). The induction of *Notch1* after 20h was further confirmed by ISH analysis. *Notch1* hybridization signal was enhanced in otic vesicles electroporated with *Sox2* when compared to controls (Figs.3Bc and c'; n = 3/4 with strong effect; n=1/4 with a moderate effect). This effect was not observed with control plasmids (see supplementary information 1, Fig.1). *Sox2* transfection in the ectoderm also resulted in the up-regulation of *Notch1* expression (asterisk in Figs.3Bc'-c'', n=4/4) which suggests that the transcriptional regulation is independent of the cellular context. Note, that the ectodermal domains electroporated with *Sox2* exhibited a thickened morphology that reminiscent of placodal structures (See supplementary information 2 for further details of the effects of *Sox2* gain of function in the ectoderm and other morphological effects).

Notch ligands were not affected by *Sox2* gain of function. *Dl1* transcript levels, like shown above for *Serrate1,* were not significantly different after *Sox2* electroporation (Fig.3A). These results suggest that the effect of Sox2 gain of function on the activation of the Notch targets may be, at least in part, the result of an increased in amount of receptor available and not to changes in ligand expression.

IV.3 Sox2 cooperates with BMP signalling during neurosensory development

Progression though differentiation relies on the function of proneural transcription factors that have the ability to regulate the transcription of other downstream genes involved in cell differentiation. In addition to the regulationby *Hes/Hey* bHLH transcription factors, Id proteins are negative regulators of differentiation and proneural protein function. They heterodimerize with proneural proteins and prevent their ability to bind and activate the transcription of target genes (Norton, 2000). Id proteins are expressed in the otic epithelium during inner ear sensory development and counteract hair cell differentiation [Jones et al., 2006; Kamaid et al., 2009]. In the inner ear, *Id* expression is regulated by BMP signalling [Kamaid et al., 2009]. We explored the effects of *Sox2* on the expression of *Bmp4*, *Id* genes and other modulators of the BMP pathway. Expression levels of *Id1*, *Id2* and *Id3* expression were measured by qRT-PCR 20h and 48h after *Sox2* electroporation. Transcript levels of all three *Id* genes were increased in response to *Sox2* overexpression after 20h (Fig.4A, *Id1*: 1,94 ± 0,04, p<0,05; *Id2*: 1,49 ± 0,03, p<0,001; *Id3*: 1,56 ± 0,06, p<0,005, mean ± SE). Like with Notch targets, this effect was transient and not detectable after 48h (Fig4A). These results were further confirmed by ISH using specific riboprobes for *Id2* (Fig.4B). *Id2* expression was induced in the medial region of the otic vesicle, along with the occurrence of GFP-positive cells (*Id2*: arrowheads in Figs.4Ba'-a'', n= 3/4, strong effect, n=1/4, mild effect). Arrows in Fig.4Ba

show the limits of normotopic *Id* expression in the lateral wall of the otic vesicle. Similar effects were observed with an *Id3* probe (n=6/7, strong effect, n =1/7 mild effect, not shown). This effect was not observed in otic vesicles electroporated with control plasmids (see supplementary information 1, Fig.1) Interestingly, *Bmp4* transcript levels were not affected after *Sox2* overexpression (not shown, p=0,340, for 20h; p=0,068 for 48h).

The ability of *Sox2* to activate the BMP pathway was analysed with BRE-GFP, a construct where GFP expression is driven by an enhancer containing the BMP response element isolated from *Id* promoter region (kindly provided by Dr. Elisa Martí). pDsRed alone or with pCMV/SV1- *cSox2* were co-electroporated with BRE-GFP and transfected otic vesicles were analyzed in coronal sections by fluorescent microscopy. When BRE-GFP was co-transfected with DsRed alone, GFP expression was only detected in domains that corresponded to the endogenous *Id* expression (Figs.5Aa-a', n=3/3). Transfected cells in the medial wall of the otic vesicle did not express GFP (arrowheads in Figs.5Aa-a') in agreement with the absence of *Id* expression in those domains (see Fig.4B). Conversely, GFP expression was detected in the lateral and anterior limit of the electroporated domain (arrows in Figs.5Aa-a'), where *Ids* are expressed endogenously. When BRE-GFP was co-transfected with DsRed + *cSox2*, GFP expression was detected throughout the electroporated domain (Fig.5Ab-b', n=3/4). In this case, transfected cells in the medial wall of the otocyst expressed GFP, driven by BRE (arrowheads in Fig.5Ab-b'). This suggests that *Sox2* is able to activate BMP-dependent *Id* expression.

BMP signalling acts though the Smad intracellular signalling pathway. BMP receptor activation results in the phosphorylation of Smad1, 5 and 8. Phsophorylated Smad (P-Smad) proteins form heterodimer complexes with Smad4 and translocate to the nucleus where they regulate the transcription of target genes, like *Id* genes (Massague et al., 2005; Ross and Hill, 2008). We studied the effects of Sox2 on the expression of P-Smad. No differences were detected in the expression of P-Smad protein in *cSox2*-transfected otic vesicles when compared with controls (Fig.5B, n=3/3). *Sox2* was not able to induce P-Smad ectopically (green nuclei, arrows in Figs.5Bb-b''), nor to enhance or prevent its expression (yellow nuclei, arrowheads in Figs.5Bb-b''). This suggests that *Sox2* does not regulate *Id* expression through the regulation of Smad intracellular pathway. Note that P-Smad expression was ubiquitously distributed in the whole otic vesicle (Fig.5Ba), even in those domains where *Id* was not expressed. This suggests that P-Smad expression is not sufficient to drive *Id* expression and that other factors might play a role in its regulation.

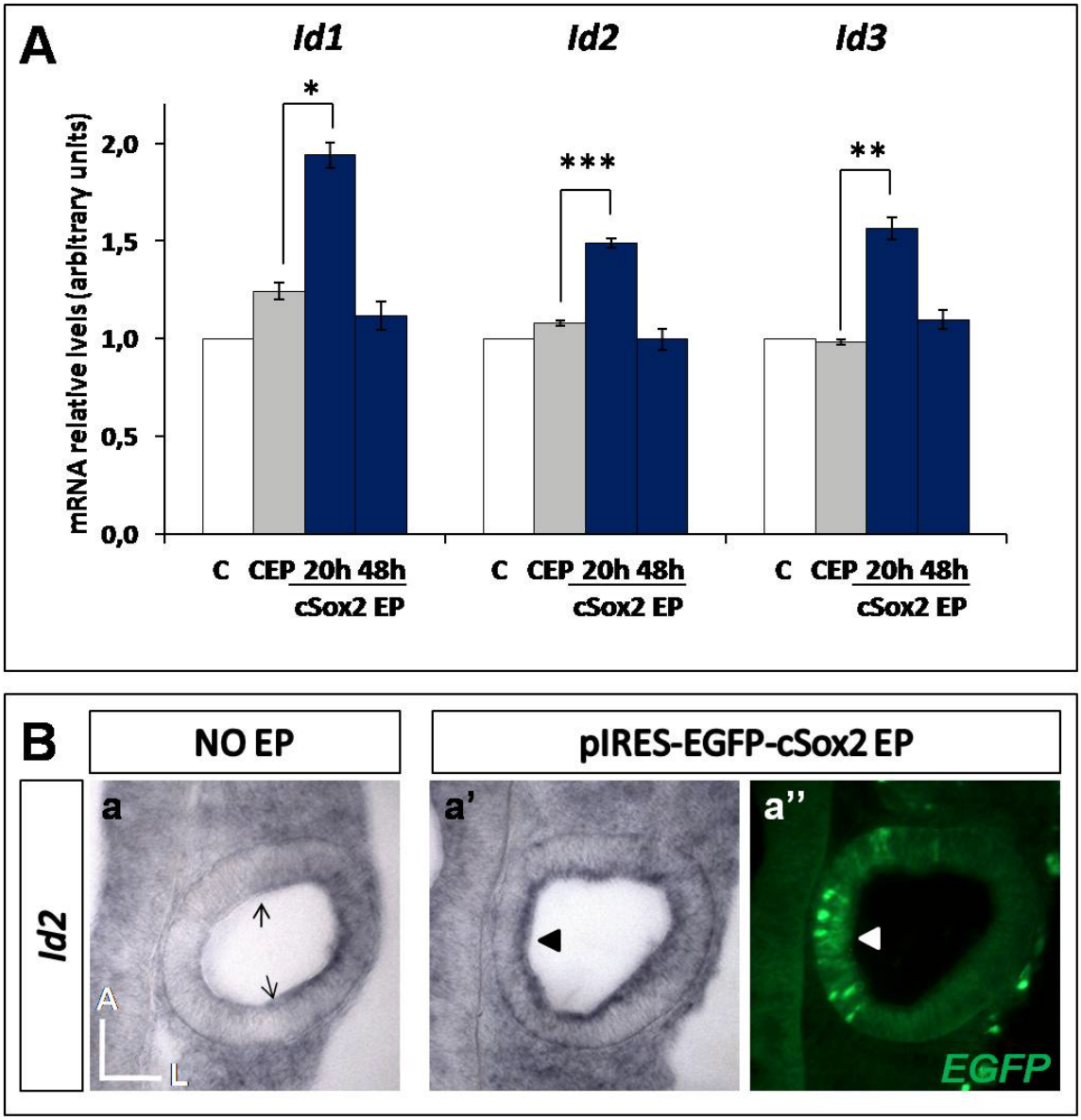

Figure 4: ***Sox2*** **regulates** ***Id*** **expression in the otic epithelium. A: Bar diagram showing the relative mRNA levels of** ***Id1, Id2*** **and** ***Id3*** **in otic vesicles transfected with control plasmids (grey bar) and with** ***cSox2*** **(blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar).** ***Id1, Id2, Id3*** **mRNA levels significantly increased between control electroporation and** ***cSox2*** **electroporation 20h after transfection (compare grey bar and left blue bars,** ***Id1*****: 1,94 ± 0,04, p<0,05;** ***Id2*****: 1,49 ± 0,03, p<0,001;** ***Id3*****: 1,56 ± 0,06, p<0,005, mean ± SE), but no longer 48h after electroporation (***Id1***: p=0,270;** ***Id2***: p= 0,289;** ***Id3:*** **p= 0,118). B: Coronal sections of otic vesicles untransfected (a) and transfected with** ***cSox2*** **(a'-a'') 20h after electroporation, where** ***Id2*** **mRNA was detected by ISH.** ***Id2*** **(a- a''), mRNA was detected in ectopic electroporated regions 20h after transfection (arrowheads). A, anterior; L, lateral.**

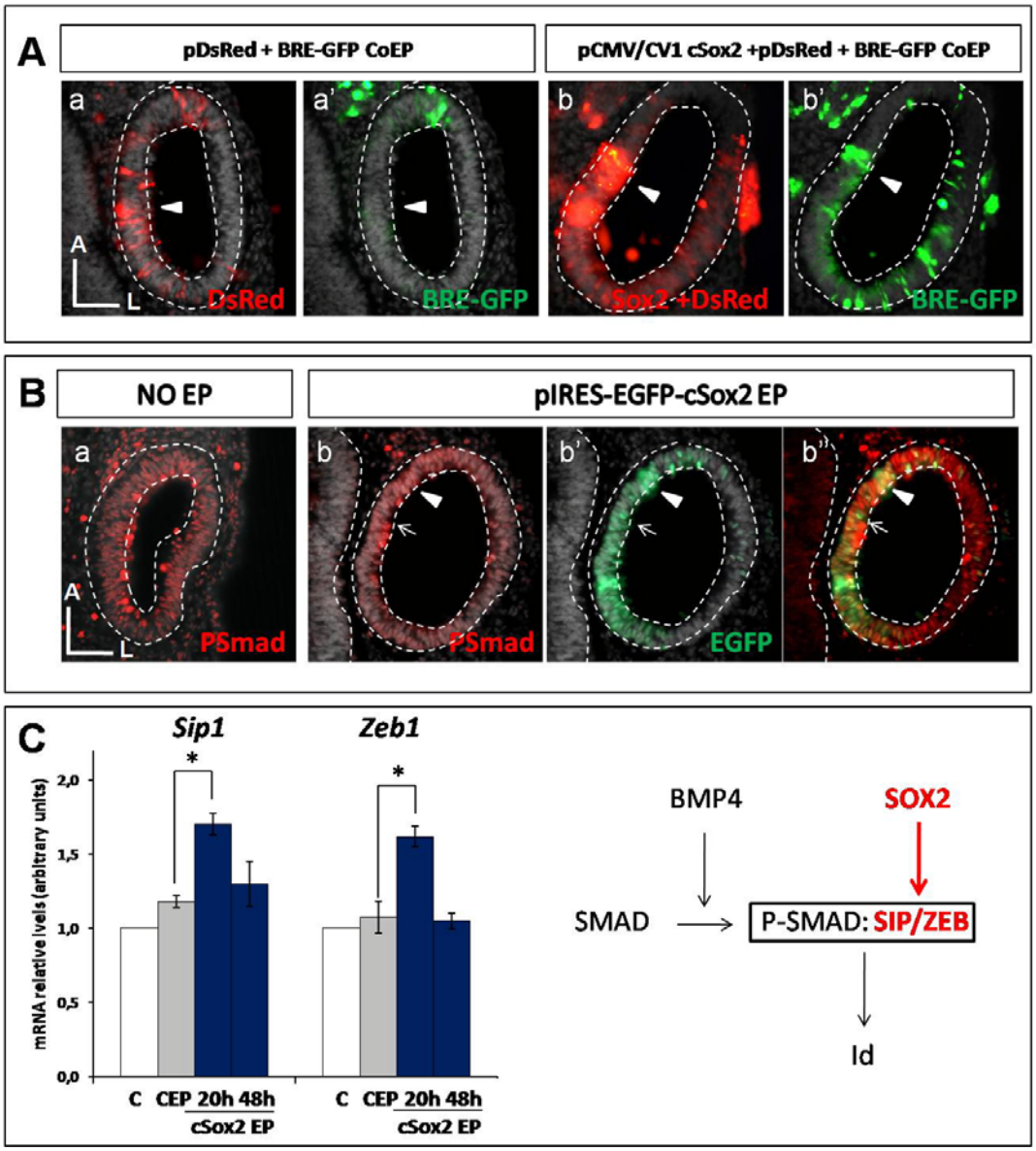

Figure 5: ***Sox2*** **cooperates with BMP signalling in the otic epithelium. A: coronal sections of otic vesicles transfected with pDsRed + BRE-GFP (a-a') or with pDsRed +** ***cSox2*** **+ BRE-GFP(b-b') analysed for DsRed (red) and GFP (green) signals. GFP expression driven by BRE was only activated in endogenous domains of** ***Id*** **expression, when co-electroporated with pDsRed (arrow, a-a'). When co-electroporated with** ***cSox2***, **GFP expression driven by BRE extended to the whole electroporated epithelium (arrowhead, b-b'). B: Coronal sections of untransfected (a) and** ***cSox2*** **transfected (b-b''), 20h after electroporation, double stained for P-Smad 1,5,8 (red) and GFP (green). P-Smad expression was not altered in transfected otic vesicles (compare a and b) and cells expressing only GFP (arrows in b-b'') and double stained (yellow, arrowheads in b-b'') could be identified. C. left: Bar diagram showing the relative mRNA levels of** ***Sip1*** **and** ***Zeb1*** **in otic vesicles transfected with control plasmids (grey bar) and with** ***cSox2*** **(blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar).** ***Sip1*** **and*****Zeb1*** **mRNA levels significantly increased between control electroporation and** ***cSox2*** **electroporation 20h after transfection (compare grey bar and left blue bars,** ***Sip1*****: 1,70 ± 0,07, p<0,05;** ***Zeb1*****: 1,62 ±0,07, p<0,05, mean ± SE), but no longer 48h after electroporation (*****Sip1*****: p=0,543;** ***Zeb1*****: p= 0,877). C, right: Arrow diagram showing the proposed cooperation between** ***Sox2*** **and the BMP pathway in the regulation of** ***Id*** **expression. A, anterior; L, lateral.**

Smad regulation of gene expression is modulated by the recruitment of co-activators and/or co-repressors. ZEB proteins are two-handed zinc finger/homeodomain transcription factors which interact with Smad proteins and modulate their activity, by differential recruitment of co-activator and co-repressors (Verschueren et al., 1999; Postigo, 2003; Postigo et al., 2003; Yoshimoto et al., 2005). *Sip1* and *Zeb1* expression levels were up regulated by 1,6-1,7-fold in otic vesicles electroporated with *cSox2* and this increase was significant when compared with control plasmids (Fig.5C, bar diagram, *Sip1*: 1,70 ± 0,07, p<0,05; *Zeb1*: 1,62 ±0,07, p<0,05, mean ± SE,). This effect was transient and 48h after electroporation mRNA levels of *Sip1* and *Zeb1* were not significantly different from otic vesicles transfected with control plasmids (Fig.5C, bar diagram).

Taken together, the results show that *cSox2* was able to induce *Id* gene expression along with Smad interacting proteins, but not *Bmp4* or P-Smad, This suggests that *Sox2* may enhance the response of *Id* genes not by increasing the activation of the pathway, but through the regulation of the transcriptional activity of active Smad complexes (Fig.5C, diagram).

IV.4 Sox2 specifies neurosensory cell fate in a dose-dependent manner

The above results indicate that *Sox2* is able to prevent cell differentiation while transgene levels are high but, what happens when transgene is downregulated? Does the transient expression of *Sox2* have an effect on the fate of transfected cells, during the development of the sensory organs? Hair cells, supporting cells and the otic neurons arise from common population of cells [Raft et al., 2008; Bell et al., 2008], and there is evidence that sensory elements and neurons might share a common progenitor (Satoh and Fekete, 2005). Furthermore, hair cell generation and neurogenesis overlap temporally in the maculae (Raft et al., 2007) and Sox2 expression in time and space is associated with both processes (see above, chapter I and (Neves et al., 2007)). Thus we wanted to investigate wether *Sox2* was able to specify neurosensory fate in the otic epithelium.

The fate of transfected cells was analysed three and four days after transfection. Embryos reached E5-6 (HH22-23), a stage when hair cells start to differentiate and neurones project to sensory patches. MyoVIIA was used to assess hair cell differentiation, and the neuron-specific βIII tubulin Tuj1 or Isl1 to identify otic neurons (Stone et al., 1996; Sahly et al., 1997; Wolfrum et al., 1998; Molea et al., 1999).

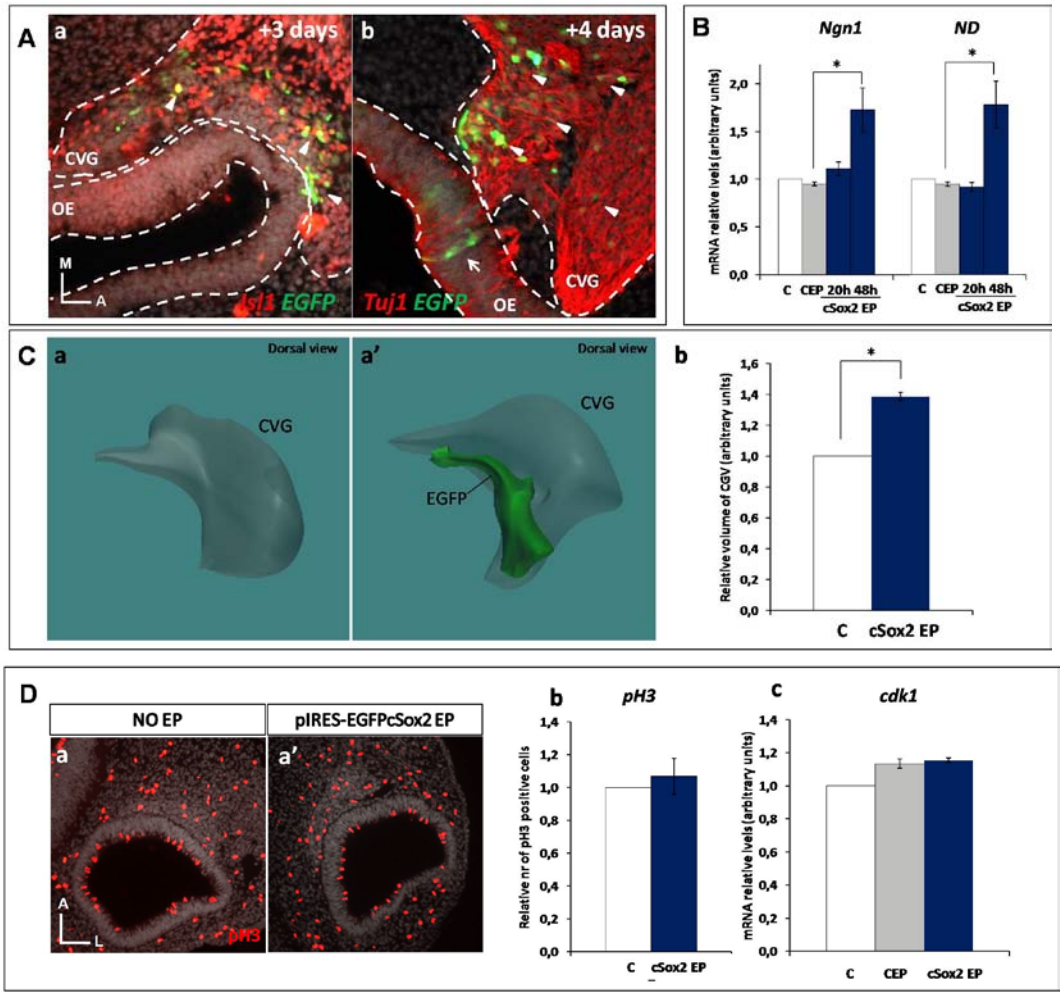

Figure 6: Increased levels of *Sox2* induce neuronal fate in the otic epithelium. A: coronal section of the CVG of and otic vesicle transfected with *cSox2* double stained for Islet1 (red, a) and GFP (green, a), three days after transfection or for Tuj1 (red, b) and GFP (green, b) four days after transfection. GFP positive cells were mostly detected in the cochleo-vestibular ganglion (a-b) where they co-expressed Islet 1(arrowheads, a) and Tuj1 (arrowheads, b). Some low expressing GFP cells remains epithelial (arrows, b). B: Bar diagram showing the relative mRNA levels of *Ngn1* and *NeuroD* in otic vesicles transfected with control plasmids (grey bar) and with *cSox2* (blue bars), 20h (left blue bar) and 48h (right blue bar) after transfection, in respect to untransfected otic vesicles (white bar). *Ngn1* and *NeuroD* mRNA levels only significantly increased between control electroporation and *cSox2* electroporation 48h after transfection (compare grey bar and right blue bars, *Ngn1*: 1,72 ± 0,23, p<0,05; *NeuroD*: 1,78 ±0,24, p<0,05, mean ± SE), but not 20h after transfection (*Ngn1*: p=0,202; *NeuroD*: p=0,684). C: 3D reconstruction of CVG associated with otocyst transfected with *cSox2* (a') and corresponding control (a), from serial coronal section stained with Islet1 and Tuj1. In green it is shown the domain of the CVG corresponding to electroporated cells. (b), bar diagram showing the the relative increase in the volume of the CVG in transfected otic vesicles, in respect to controls, normalized to the degree of electroporation. CVG was increased by 38% of its volume, in average. D: pH3 staining in sections of otic vesicles untransfected (a) and transfected with *Sox2* (a') 48h after transfection (a') used for cell counting to access relative proliferation rate. (b) Bar diagram showing the relative number of pH3 positive cells in the otic epithelium of transfected (blue bar) and untransfected (white bar) otic vesicles, 48h after transfection. (c): Bar diagram showing the relative mRNA levels of *cdk1* in otic vesicles transfected with control plasmids (grey bar) and with *cSox2* (blue bars), 48h after electroporation. *Cdk1*transcript levels were not significantly different between otic vesicles transfected with *Sox2* and control plasmids (p=0,626). CVG, cochleo-vestibular ganglion; OE, otic epithelium.

Transfected cells were identified by GFP expression. Note that GFP was used here as a tracer for those cells that had received the *Sox2* transgene, although they did not express *Sox2* anymore. One example of this experiment is illustrated in Fig.6A. Strikingly, the majority of the transfected cells were located in the CVG, where they co-expressed GFP and Islet1 or Tuj1 (arrowheads in Figs.6Aa-b), and only very few resided in the otic epithelium (arrows in Fig.6Ab). This suggested that the early overexpression of *Sox2* induced in the long run an increased production of neurones. Indeed, 3D reconstructions of CVGs revealed that the CVG was larger in *cSox2* electroporated otocysts (Figs.6Ca-b). Three-D reconstructions of the CVG from control (Fig.6Ca) and electroporated otic vesicles (Fig.6Ca') were made from serial coronal sections stained with Iset1 and Tuj1. As illustrated above, the volume of the cochleo-vestibular ganglion was increased in the electroporated side, a change that was proportional to the degree of electroporation. Figure 6Cb shows the relative increase in the volume of the ganglion, normalized to the intensity of the electroporation (n=3/3, 1,38 ± 0,03, mean ± SE, $p<0,05$). The increase in the size of the CVG induced by Sox2 was not associated with an increase in the rate of cell proliferation occurring during the activity of the transgene as measured by the number of pH3 positive cells (Fig.6Da-b), or the transcription of *Cdk1* (Fig.6Dc, right bar diagram).

We analysed the possibility that the increase in the size of the CVG was anticipated by an increase in epithelial expression of *Ngn1* and *NeuroD*. The analysis of *Ngn1* and *NeuroD* expression by qRT-PCR. Twenty hours after electroporation there were no significant differences between transfected and control otic vesicles (Fig6B). However, after 48h, the transcript levels of *Ngn1* and *NeuroD* were significantly increased in otic vesicles electroporated with *Sox2* when compared to otic vesicles that were transfected with control plasmids (Fig.6B, *Ngn1*: 1,72 ± 0,23, $p<0,05$; *NeuroD*: 1,78 ±0,24, $p<0,05$, mean ± SE). This suggests that 48h after electroporation, when transgene is down-regulated, transfected cells start to be specified as neuronal progenitors.

In these experiments, we were able to analyse also the behaviour of transfected cells both inside and outside the prosensory patches. Within the normotopic domains, only a minor fraction of transfected cells remained within the epithelium. Those that did so, expressed low levels of GFP and developed into either hair or supporting cells (not shown). Ectopic expression of Sox2 resulted in ectopic patches that contained all neurosensory cell types. Sox2 induced Isl1 and Tuj1-positive cells that formed small groups of delaminated neuroblasts (arrowhead in Figs7a-a'', n=5/7). Transfected cells that remained in the otic epithelium tended to expressed low levels of GFP. Some of them expressed MyoVII and were associated with ectopic neuroblasts (arrows in Fig.7Ab-b'' and Fig.7c,), from which they received innervations (arrows in Figs.7Aa-a'', see fibers extending towards low GFP expressing cells n =2/3). Some others did not express MyoVII and, although not labelled with specific markers, their position and morphology indicated that were supporting cells (asterisk in Fig.7c and Fig.7a'').

Taken together, these results suggest that 1) the overexpression of *Sox2* promotes the generation of neurones within the neurosensory domain, 2) ectopic *Sox2* is sufficient to induce neurons, hair cells and supporting cells, that is to induce neurosensory fate, and 3) the resulting cell fate (neuron vs. sensory) correlated with the levels of GFP expression.

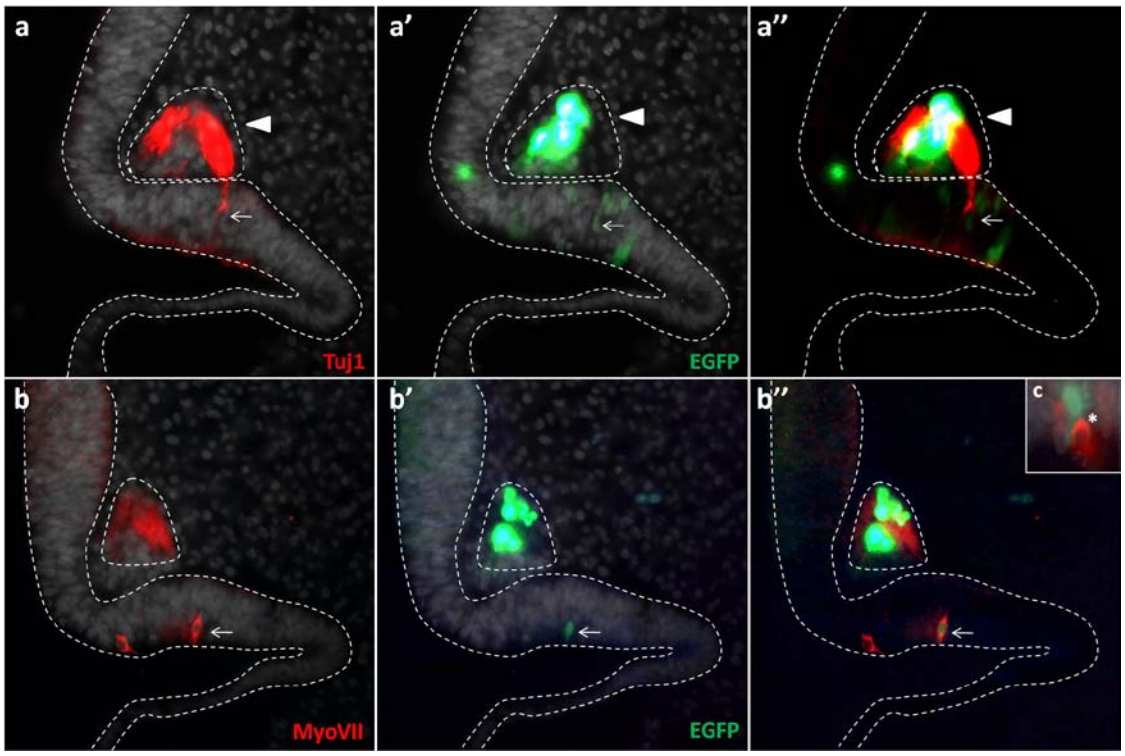

Figure 7: *Sox2* induces neurosensory fate in the otic epithelium. (a-a'') coronal section of an otocyst of transfected with *cSox2*, 4 days after transfection, double stained for Tuj1 (red) and GFP (green). (b-b'' and c): coronal section of an otocyst of transfected with *cSox2*, 4 days after transfection, double stained for MyoVII (red) and GFP (green). GFP positive cells with high levels of GFP expression were detected adjacently to the otic epithelium, as delaminated neuroblasts (arrowhead, a') where they co-expressed Tuj1 (arrowheads a and a''). GFP positive cells with low levels of GFP were detected in the otic epithelium both apically and basally (a' and b') where some co-expressed MyoVII (b''). Tuj1 positive fibbers innervate this ectopic sensory cells (arrow a'' and b'').

Is *Sox2* overexpression ability to promote neuronal fate related with the stage of commitment of otic progenitors, or is it able to induce neuronal fate at stages beyond the neurogenic period of otic development? Is it able to determine hair cell vs supporting cell fate? To investigate these questions we electroporated *cSox2* in the prospective developing anterior crista of E3 (HH20-21) embryos, and otocysts were analyzed after $2^{1/2}$ days (Fig.8A). Analysis of MyoVII and GFP expression within the sensory organs showed that transfected cells were either hair or supporting cells (Figs.8Ba-a'', arrowheads point to hair cells, and asterisks to supporting cells). This suggests that transfected cells can differentiate both as hair cells and supporting cells. In these experiments, it was also prominent that there were cells that expressed high levels of GFP, which had delaminated after electroporation (Fig.8Ca-b). Those GFP-positive cells co-expressed Islet1 (yellow, arrowheads in Fig.8Cb), indicating that they developed as

neurons. By the time of electroporation (HH19-20), otic neurosensory progenitors have gone through most of their neurogenic period. Therefore, this observation suggests that the increased expression of *cSox2* is able to switch the cellular fate of otic progenitors towards the neuronal lineage.

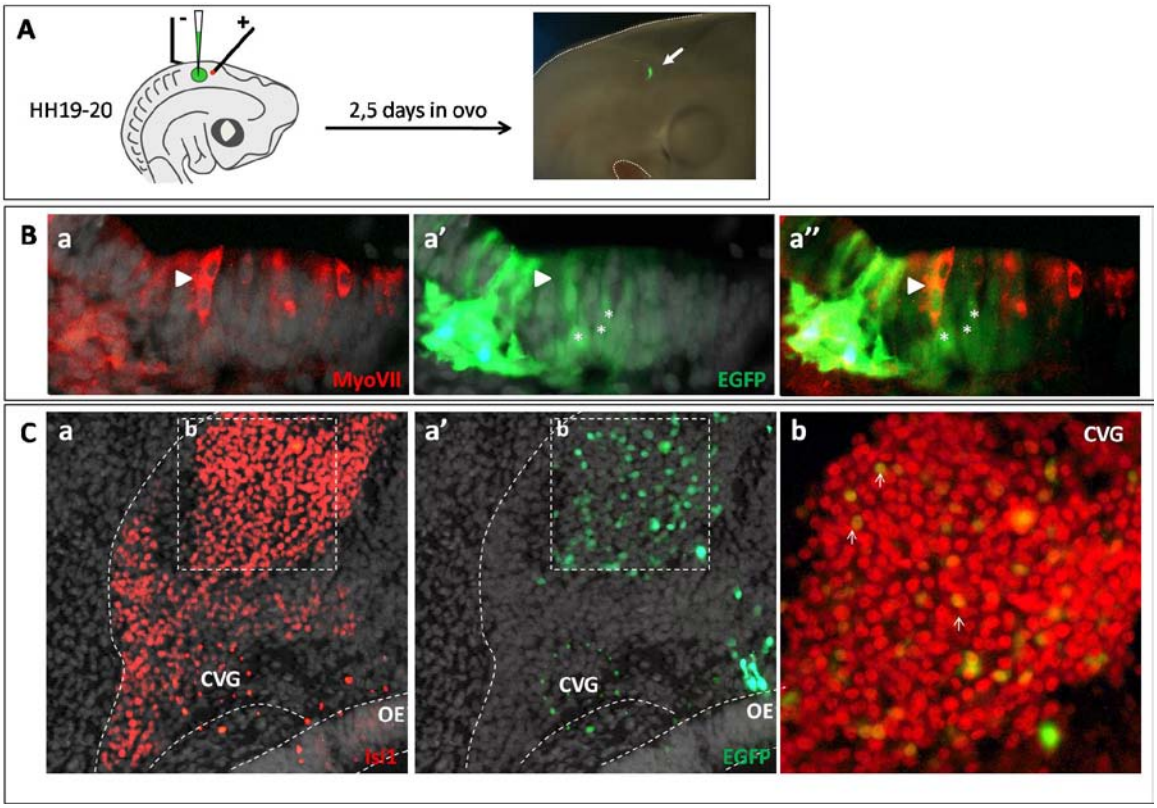

Figure 8: *Sox2* did not regulate hair cell vs supporting cell fate and still induced neurons after the neurogenic period. A: Diagram showing the experimental assemble for electroporation of cSox2 into the prospective anterior cristae in E3 chick embryos. DNA was injected in the otic vesicle and current was applied along the posterior-anterior axis of the embryo. Two and a half days after electroporation, transfected domains were identified by GFP expression. B (a-a''): detail of otic epithelium transfected with *cSox2* as described in (A) and double stained for MyoVII (red) and GFP (green), 2,5 days after transfection. GFP positive cells were identified in the hair cell layer (arrowheads) and in the supporting cell layer (asterisks). C: coronal section of the CVG of and otic vesicle transfected with *cSox2* as described in (A) and double stained for Islet1 (red) and GFP (green). b represent the inset in a-a'. GFP positive cells were still detected in the cochleo-vestibular ganglion (a') where they co-expressed Islet 1(arrows, yellow in b). CVG, cochleo-vestibular ganglion; OE, otic epithelium.

DISCUSSION

The experiments described in this thesis report were aimed at studying the functions of *Sox2* and *Serrate1* during the development of the neurosensory elements of the inner ear. First, we have described the expression pattern of *Sox2* during inner ear development and compared to that of *Sox3* and *Serate1*. Secondly, we have shown the results of plasmid based in ovo electroporation experiments, designed to manipulate gene expression exogenously, and to study the gain of function of *Sox2* and *Serrate1*. Effects on cell fate and downstream targets were assessed by in situ hybridization immunohistochemistry and quantitative real-time PCR (qRT-PCR).

The results show that *Sox2* is expressed in the neurosensory domain of the otic epithelium during the neurogenic period of otic development and, later on, during the development of the prosensory patches and sensory organs. As differentiation proceeds, *Sox2* is excluded from differentiated neurones and hair cells, but remains expressed in the supporting cells of the sensory organs. *Sox3* is co-expressed with *Sox2* in a sub-domain of the *Sox2* positive region that corresponds in space and time with the neurogenic domain of the otic cup. But *Sox3* is then downregulated and only *Sox2* expression persists in the sensory precursors, where it is co-expressed with the Notch ligand *Serrate1*. The expression domain of *Serrate1* is initially nested within *Sox2*, however, later in development *Sox2* becomes restricted within the boundaries of *Serrate1* expression, a process that is concomitant to the formation of the sensory patches. These expression patterns suggest 1) that *Sox2* correlates with neurosensory fate in the otic placode, 2) that neurogenesis is associated with *Sox2* and *Sox3* expression and 3) that sensory development is associated with *Sox2* and *Serrate1* (Fig.1)

Gain of function studies show that *Serrate1* regulates prosensory fate and sensory organ development by maintaining *Sox2* expression in restricted domains of the otocyst, without affecting neurogenesis. *Serrate1* operates in a Notch-dependent manner, consistently with a mechanism of lateral induction that includes the induction of its own expression and downstream targets of the Notch signalling pathway *Hes1*, *Hey1* and *Hey2*. Similar studies on *Sox2* indicate that it specifies neurosensory fate in the otic epithelium. However, high concentrations of *Sox2* suppress sensory fate and promote neuronal fate. Besides, *Sox2* prevents cell differentiation though the cooperation with Notch and BMP signalling pathways.

We like to propose a model in which an extended neural competence is early established in the otic placode with the expression of *Sox2* and *Sox3* genes. The cooperation between *Sox2* and *Sox3* would then provide a high concentration of SoxB1 protein to promote neuronal fate. In parallel, *Serrate1* would maintain *Sox2* expression in restricted domains, after Sox3 down-regulation. These domains would retain the neurosensory competence and thereafter develop as sensory patches.

I. Serrate1 and neurosensory development

The transition between the neurosensory domain of the otic vesicle and the prosensory patches identifiable by E3.5-E4) has not yet been resolved unambiguously. This occurs between E3 and E4, after the neurogenic period of otic development, and it is not clear whether it is the result of the development of a common domain, or the emergence of different, perhaps overlapping independent regions. Some genes expressed in the neurogenic domain, like *Lfng* and *Fgf10*, persist in the prospective sensory domains, during the stages of sensory organ development (Cole et al., 2000; Pujades et al., 2006). Other genes, like *Bmp4* are exclusively associated with the sensory domains and its expression foreshadow the emergence of the sensory organs (Cole et al., 2000), but it is not until E4 when *Atho1* (*Cath1*)-positive cells are clearly identifiable at the sensory patches (Pujades et al., 2006).

Serrate1 expression during ear development foreshadows the emergence of the sensory organs, and this soon led to the suggestion that Serrate-mediated Notch signalling is important for sensory development (Adam et al., 1998; Cole et al., 2000). Several groups have addressed this question using the gain and loss of function of Notch as an experimental approach. The pharmacological blockade of Notch activity in the chick suggests that Notch signalling is necessary to maintain, but not to initiate, *Serrate1* expression in the otic epithelium. This blockade results in the parallel loss of other prosensory markers like *Sox2* and *Bmp4* (Eddison et al., 2000; Daudet and Lewis, 2005; Daudet et al., 2007). Similarly, early Notch blockade in the mouse cochlea leads to a reduction in the number of sensory progenitors, *Sox2* expression and hair cells. Conversely, the over-expression of a constitutively active form of Notch is sufficient to induce Sox2 expression ectopically in the mouse cochlea [Takebayashi et al., 2007; Hayashi et al., 2008; Dabdoub et al., 2008]. Taken together, these studies suggest that Notch plays a crucial role in the specification of the sensory domains.

The specific function of *Serrate1* during inner ear development has been addressed directly in a series of genetic studies on mouse models carrying *Jag1* mutations or deletions. These studies show that *Jag1* is indeed required for sensory development, as *Jag1* mouse mutants show impaired sensory development with an overall reduction in the number of hair cells. Furthermore, *Sox2* is not expressed in *Jag1* null mice (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006). Although these studies implicate *Serrate1* in the development of the sensory organs, they do not demonstrate the specific functions of *Serrate1* and its downstream targets. We have worked with the idea that Serrate1-mediated Notch activation is, along with *Sox2*, a key event in the specification of the prosensory patches and we used a gain of function approach to address this question.

The combined expression pattern of *Sox2* and *Serrate1* described in the present report shows that *Serrate1* expression domains were initially contained within *Sox2* positive regions. The association of *Sox2* and *Serrate1* expression correlates well with the sensory competence of the otic epithelium. *Sox2* and *Serrate1* co-expressed since early

stages of otic development in the anterior lateral pole and posterior medial pole of the otic vesicle (Fig.1), which correspond to the prospective cristae and basilar papilla [Cole et al., 2000; Bell et al., 2008]. *Serrate1* expression is present, but initially weak, in the anterior-medial aspect of the otic epithelium, which harbours the neurogenic domain. After the neurogenic period, *Serrate1* expression is enhanced in the anterior-medial domain of the otic vesicle, which now defines the prospective maculae [Cole et al., 2000; Bell et al., 2008; Raft et al., 2008]. In conclusion, our results suggest that the co-expression of *Sox2* and *Serrate1* is associated with the prospective sensory domains since early stages of otic development.

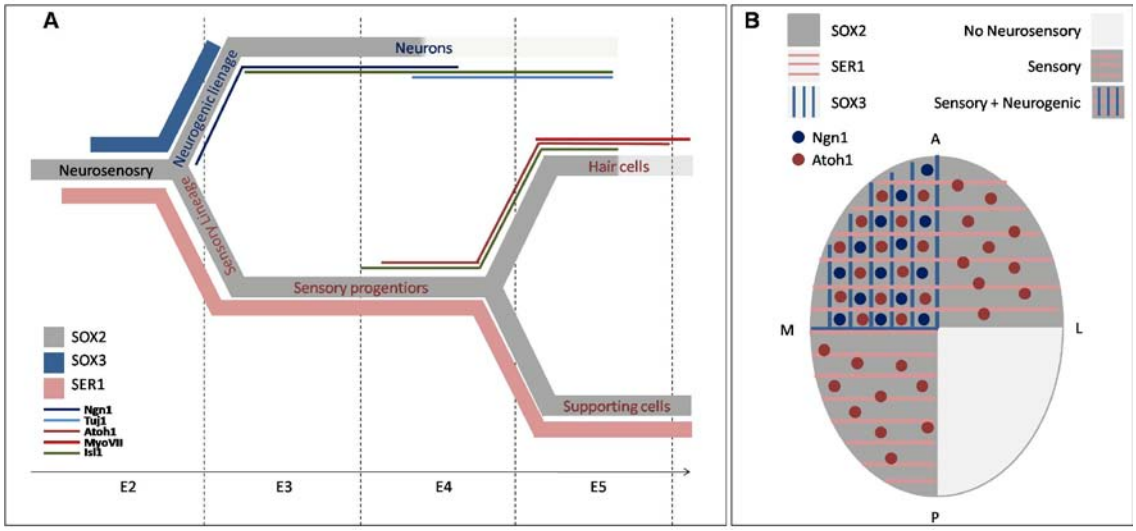

Figure 1: *Sox2*, *Sox3* and *Serrate1* temporal and spatial profiles of expression during otic neurosensory development. Schematic representation of the temporal (A) and spatial (B) profiles of expression of *Sox2* (grey), *Sox3* (blue) and *Serrate1* (pink) in the otic epithelium as described in the results section of this report. Thin lines (A) and dots (B) show the temporal and spatial profiles of expression of other relevant genes in the development of the neurosensory elements of the inner ear, as described in the literature, for comparison.

I.1 Sensory organ development and the restriction of Sox2 expression

One interesting feature of the expression patterns of *Serrate1* and *Sox2* is that the one of *Sox2* is initially broader than *Serrate1*, but when sensory patches emerge, *Sox2* expression becomes confined to within the boundaries of *Serrate1*. This spatial restriction follows a temporal profile that parallels the development of the different sensory organs [Bartolami et al., 1991; Oh et al., 1996; Wu and Oh, 1996; Fritzsch et al., 2002; Bell et al., 2008]. It is tempting to suggests that the restriction of *Sox2* expression to the discrete domains of *Serrate1* expression may be on the basis of the transition from a common neurosensory domain to discrete prosensory domains that foreshadow the future sensory organs. One possible function of *Serrate1* may be to regulate the confinement of *Sox2* expression to those domains. Several studies show that *Serrate1* and Notch signalling are indeed necessary to maintain *Sox2* expression in the otic epithelium [Kiernan et al., 2006; Daudet et al., 2007; Dabdoub et al., 2008]. Our results show that *Serrate1* is not sufficient to induce *Sox2* expression ectopically, but it is

sufficient to maintain its expression in regions adjacent to normal sensory patches, where *Sox2* expression is normally switched off. The regulation of *Sox2* expression by *Serrate1* is probably permissive rather instructive, and relies on the maintenance of *Sox2* expression in domains that otherwise would loose its expression. One possible mechanism would be that Serrate1-mediated Notch activity prevents the repression of *Sox2* in the prosensory patches. How this would take place is unknown (Fig.2).

During the formation of the dorsal/ventral border in the *Drosophila* wing, the binding of hairless to Su(H) (homolog of vertebrate CSL) results in the formation of an repressor complex in the absence of Notch signaling. When Notch signaling is activated this repressor function is released and the transcription complex is transformed in an activator (Koelzer and Klein, 2006). The regulation of *Sox2* expression by *Serarte1* may rely on a similar mechanism. In this model, CSL binding to the *Sox2* promoter region would result in ubiquitous repression. However, in the prosensory domains, Notch would be activated by Serrate1, by a process of lateral induction (see below), which would form a coherent domain of Notch activity where CSL repression on *Sox2* gene is released. Conversely, outside the prosensory domains, *Serrate1* is not expressed, Notch is not active (Murata et al., 2006), and CSL would down-regulate Sox2 expression. This model is consistent with the observation that the effect of *Serrate1* on *Sox2* expression was both cell autonomous and non cell autonomous, and dependent on Notch activation. If Sox2 depends on the release of the repression by CSL, then it is expected that it happens in every cell where Notch is activated. Since we have demonstrated that Serrate1 activates Notch and its own expression through the process of lateral induction, Notch signaling is activated both in transfected and untransfected cells, which results in the release of the repression on Sox2 expression in both kind of cells.

In parallel with the effects on *Sox2*, *Serrate1* was able to increase the number of hair cells and to expand the size of the sensory organs, but not to induce ectopic hair cells. Since *Sox2* is able to induce the sensory fate (see below), it is possible that the mechanism by which *Serrate1* promotes sensory specification depends on its ability to maintain *Sox2* expression. The results also suggest that, at least during early developmental stages, *Serrate1* does not dictate a specific sensory cell fate, since the forced expression of *Jag1* gives rise to either hair- or supporting cells. We have no information on the effects of *Serrate1* in late differentiation stages.

I.2 Serrate1, sensory fate and the regulation of Bmp4 expression

Bmp4 is also induced by *Serrate1*. This is in agreement with previous data showing that the expression of *Bmp4* in the prosensory patches is dependent on Notch (Daudet and Lewis, 2005; Daudet et al., 2007). Our results indicate further that this effect is mediated by *Serrate1*. Moreover, they suggest that the effects of *Serrate1* on *Bmp4* and *Sox2* are sequential. *Bmp4* is induced before *Sox2*. Thus, 1) the regulation of *Sox2* and *Bmp4* may originate from different mechanisms and/or 2) that *Bmp4* induction may be a step for Sox2 de-repression. The observations on the expression patterns of *Bmp4* and *Serrate1* (Cole et al., 2000) allow to discard that *Bmp4* regulation by *Serrate1* is like the one described for *Sox2*. Indeed, *Bmp4* expression in the otic epithelium follows *Serrate1*

expression in the different prospective sensory organs from very early in development, and the expression of both genes mainly overlaps (Wu and Oh, 1996; Cole et al., 2000). This, along with the fast time course of the induction of *Bmp4* by *Serrate1*, suggests that it may rather depend on an inductive mechanism. Further experiments are required to substantiate this hypothesis.

What can then be the function of *Serrate1*-induced *Bmp4* expression in the otic epithelium? Regulation of *Bmp* genes by Notch signalling has been demonstrated in other systems. During neural crest development, Notch signalling regulates *Bmp4* expression and neural crest fate in frog and chick, although the mechanisms underlying this regulation are different between species (reviewed in (Cornell and Eisen, 2005)). During the development of the trabecular myocardium, *Bmp10* expression is regulated by Notch signalling, and is associated with the regulation of proliferation (Grego-Bessa et al., 2007). These two cases represent Notch-mediated regulation of *Bmp* expression associated with either cell fate acquisition (neural crest) or regulation of proliferation (heart). In the inner ear, we have shown that *Serrate1* regulates also these two processes. The regulation of proliferation by *Serrate1* and the possible involvement of *Bmp4* in that process will be further discussed bellow. The induction of *Bmp4* by *Serrate1* prior to the detection of the effects on *Sox2* expression suggested that the regulation of Sox2 by *Serrate1* could be also dependent on BMP signalling. However, *Bmp4* conditional deletion in the mouse and chick inner ear shows that *Bmp4* does not regulate *Sox2* expression (Chang et al., 2008). Instead, *Bmp4* regulates *msx1* and *Lmo4* expression in the prosensory domains, and that those genes may account for a possible prosensory function of *Bmp4* (Chang et al., 2008).

One other possibility is that prosensory function of *Bmp4* were to switch off *Sox3* expression in the sensory progenitors (Fig.2). *Bmp4* is initially expressed within *Sox2* neurosensory domain, but outside the neurogenic region, in the domains that will give rise to the cristae and where Serate1 is also expressed. As development proceeds by E3.5, *Bmp4* expression emerges in the prospective maculae, after this epithelium had gone trough its neurogenic period, in parallel to the up-regulation of *Serrate1* in that domain [Wu et al., 1998; Cole et al., 2000]. Thus, the onset of *Bmp4* expression in the otic epithelium coincides both spatially and temporally with *Sox3* downregulation and could be responsible for *Sox3* switch off in the remaining sensory progneitors. In this way *Bmp4* would be an effector of the prosensory function of *Serrate1* by favouring sensory fate against neuronal fate. BMP signalling does not impede the early induction of *Sox3* in the otic placode [Abelló et al., 2009], but this does not exclude the possibility that this may occur later in development.

We would like to propose a model in which, as hypothesized by Chang and colleagues (2008), *Serrate1* has two parallel functions in the regulation of prosensory function. On one hand, *Serrate1* sustains *Sox2* expression in the prosensory patches through a mechanism that depends on Notch. On the other, *Serrate1* prevents neurogenesis through a mechanism that requires *Bmp4* to repress *Sox3* expression in the prosensory domains (Fig.2).

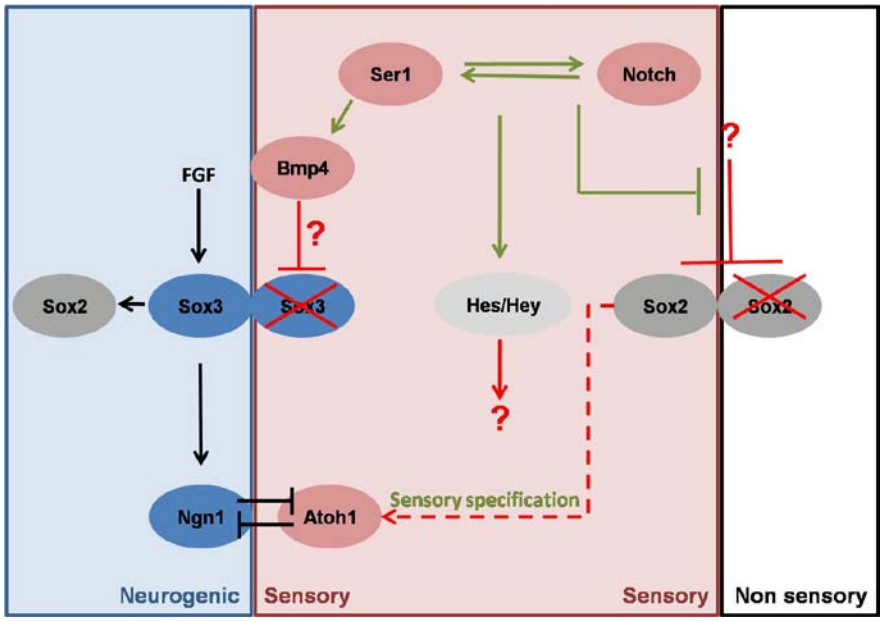

Figure 2: The role of *Sox2* and *Serrate1* in specification of cell fate in the otic epithelium. Diagaram represents the gene interaction in and between three cell populations: Neurogenic (blue, left), sensory (pink, middle) and non-sensory (white, right). Black arrows represent gene interactions described in the literature. Green arrows represent gene interactions suggested by our results. Red arrows represent hypothesized gene interactions still untested. *Sox2* (grey) and *Sox3* (blue) are expressed in the neurosensory compartment. *Serrate1* (pink) and *Sox2* (grey) are expressed in the sensory compartment. *Serrate1* expression in the sensory compartment is maintained by the mechanism of lateral induction mediated by Notch which is also responsible by the un-repression of *Sox2* expression in the sensory compartment. *Serrate1* also induces *Hes/Hey* genes in parallel with *Bmp4* which possibly cooperates in the maintenance of *Hes/Hey* gene expression [Kamaid et al., 2009]. *Sox3* in the neurognic compartment specifies neuronal fate, together with *Sox2,* thought the induction of *Ngn1* [S. Khatri PhD Thesis]. Sox2 expression in the sensory compartment specifies sensory fate though unknown mechanisms. We hypothesize that *Bmp4* induced by *Serrate1* could cooperate in the induction of sensory fate through the blockade of *Sox3* expression and thus the repression of neuronal fate.

I.3 Serrate1 and neurogenesis

Although *Serrate1* expression is weak in the neurogenic domain of the otic cup [Cole et al., 2000, our results], it transiently overlaps with *Delta1*, which is expressed in the neuroblasts during the neurogenic period of otic development (Alsina et al., 2004; Abello et al., 2007). However, the gain of function studies on *Serrate1* produced no effect on neurogenesis. One possible explanation is that *LFng* is co-expressed with Delta and Serrate1 in this domain. *LFng* is a modulator of Notch signalling pathway that favours Notch signalling though *Delta* ligands. Thus, *LFng*,may be responsible for favouring Notch signalling though *Delta* ligand, and ensuring the dominant effect of Delta during the neurogenic period, in which *Serrate1* would take no part. On another hand, *Serrate1* does not induce *Sox3* expression (not shown), and the levels of SoxB1 protein in the expanded prosensory patches in *Jag1* transfected embryos are not increased. The fact that SoxB1 concentration is not altered by Serrate1 is consistent with its lack of effect on neuronal production (see below).

I.4 Lateral induction and the regulation of Serrate1

As discussed above, the ability of *Serrate1* to maintain *Sox2* expression in the prosensory patches is dependent on its capacity to activate Notch signalling uniformly. Such a uniform pattern of activation is inconsistent with a mechanism of lateral inhibition, where Notch activation prevents the activation of neighbouring cells. On the contrary, it can only be explained if receptor and ligand are expressed and active in all the cells, each cell being simultaneously a signal-sender and a signal-receiver. This mechanism has been referred to as lateral induction (Bray, 1998; Lewis, 1998). Several studies based on gain and loss of function studies of Notch in the chick otic vesicle had suggested that *Serrate1* expression in the otic epithelium was maintained by a process of lateral induction, (Eddison et al., 2000; Daudet and Lewis, 2005; Daudet et al., 2007). However, these studies have addressed the problem from the point of view of the requirement of Nocth signalling, but none of them actually demonstrated that the mechanism operates in a Serrate1-dependent manner. Our results show that *Serrate1* activates Notch signalling and induces its own expression. Furthermore, we show that *Serrate1* induction by *Serrate/Jag1* is non cell-autonomous, but the result of the activation of Notch in a neighbouring cells. This mechanism of lateral induction is likely to be basis of the regulation of the maintenance of *Serrate1* expression in the prosensory patches described by Daudet et al. (2007) (Fig.2).

Lateral induction mechanisms mediated by Notch have been implicated in boundary formation in several model systems (de Celis and Bray, 1997; Bray, 1998; Cheng et al., 2004; Baek et al., 2006). Thus, it is tempting to propose that in the inner ear, Serrate1 also maintains the defined borders of the sensory patches separating sensory territories from non sensory ones. However, it remains to be explored which mechanism restricts Serrate1 expression. The onset of Serrate1 expression in the otic epithelium is not dependent on Notch (Daudet et al., 2007) and Serrate1 expression in early otic epithelium is regulated by Wnt signaling (Jayasena et al., 2008). The expression of Wnts and Wnt receptors (frizzled proteins) is segregated between adjacent non sensory and sensory territories. Wnt gain of function in the otic epithelium results in ectopic and fused sensory patches, which has led to the hypothesis that Wnt signaling may regulate sensory vs nonsensory boundaries in the otic epithelium (Stevens et al., 2003; Sienknecht and Fekete, 2008). In the context of our observations this data can be interpreted in a hierarchical cascade: Wnt signalling would regulate the expression of *Serrate1*, which then would activate Notch in the sensory domains which, in turn, would result in the maintenance of *Sox2* and the commitment to sensory fate.

I.5 Serrate1 and Notch targets

Notch activation by *Serrate1* results in the induction of some but not all Notch targets. *Hes1*, *Hey1* and *Hey2* are induced in response to *Serrate1*, but not *Hes5*. Instead, *Hes5* is tightly associated with the process of lateral inhibition mediated by *Delta1*, during the

differentiation of neurons and hair cells. Furthermore, the *Hes/Hey* genes induced by *Serrate1* are those expressed in the prosensory domains of the mouse otocyst (Hayashi et al., 2008; Murata et al., 2009), which suggests that their expression in those domains may depend on *Serrate1*, and actually part of its effector mechanism. However, it is worth noting that some of the *Hes/Hey* genes are not dependent on Notch activity but, as recently shown, they may be regulated by BMP [Doetzelfe et al., 2008; Kamaid et al., 2009]. This suggests that *Bmp4* expression induced by Serrate1 may also cooperate to the regulation of these genes in the prosensory patches (Figs.2 and 3).
Different target genes activated by *Serrate1* may be responsible for different functions. *Hes1* has been associated with the regulation of cell proliferation (Murata et al., 2009), a function of Serrate1 that will be discussed in the next section (Fig.3). The functions of *Hey1* and *Hey2* in the prosensory domain remain to be studied. The positive feedback loop between the expression of *Jag1* and *actN1* is essentially unaffected in *Hes1* deficient mice, suggesting that lateral induction is not disrupted in these mutants, and that *Hes1* does not take part in the maintenance of this mechanism, which could be accounted by *Hey1*and/or *Hey2*.

I.6 Serrate1 and the progenitor state

6.1 Serrate1 does not regulate the timing of differentiation

The progenitor state is characterized by the ability of cells to self-renew and prevent differentiation. A well characterized function of *Hes/Hey* genes is the prevention of differentiation though the repression of proneural gene expression (Bertrand et al., 2002; Fischer and Gessler, 2007). Our results do not suggest that *Serrate1* downstream targets regulate the maintenance of the progenitor state in this way. If that were the case, the expression of genes associated with the initiation of differentiation, such as *Islet1*, should be hindered in response to the over-expression of *Serrate1*. However, and in contrast to *Sox2*, *Jag1* does not prevent Islet1 expression (not shown), suggesting that *Serrate1* and its targets do not regulate the timing of differentiation. This is in agreement with the observation that in *Jag1* mutant mice precocious differentiation does not occur (Brooker et al., 2006). Similarly, no precocious differentiation of hair cells is observed in *Hes1* mutant mice (Murata et al., 2009). But nevertheless, these observations do not exclude the possibility that Notch may play a role in the maintenance of progenitor state, by preventing proneural expression through *Hes/Hey* function. It just indicates that *Serrate1* does not mediate that function. One interesting possibility is that the levels of Notch activation maintained by *Serrate1* are low, possibly through the presence of *Lunatic Fringe* expressed in the sensory domains, which disfavours Notch activation by this ligand, and thus is not sufficient to prevent proneural expression. Besides, *Serrate1* was revealed to play a role in the regulation of proliferation, which may be one way to cooperate in the maintenance of the progenitor state.

6.2 Serrate1 regulates cell proliferation in the otic epithelium

Cell proliferation is a biological processes involved in the regulation tissue growth and self renewal of progenitor cells. *Serrate1* has been shown to regulate cell proliferation and self-renewal of neural stem cell populations, like the subventricular zone of the adult brain (Nyfeler et al., 2005). On the other side, *Sox2* conditional inactivation in neural progenitors does not interfere with self-renewal (Miyagi et al., 2008), and *Sox2* over-expression does not affect cell cycle (Bylund et al., 2003). In our experiments we did not detect any effect of *Sox2* on the rate of cell proliferation which was, however increased by *Serrate1*. This suggests that the function of Serrate1 in the maintenance of the progenitor state may be related to its ability to regulate cell proliferation, while *Sox2* function may be to counteract cell differentiation (Fig.3, see also next chapter).

Otic neurosensory progenitors are continuously proliferating, and cell cycle exit occurs upon cell differentiation into neurones or hair cells. $p27^{kip1}$ (cyclin-dependent kinase inhibitor - CKI) and *Retinoblastoma* (*pRb,* a tumor suppressor gene) regulate cell cycle exit in sensory progenitors of the mouse inner ear. In the $p27^{kip1}$ knockout mouse, there is an expansion of the sensory progenitor cells, and cell proliferation is kept in supporting cells in early postnatal stages, but not in hair cells. *Rb* mutant mice show continuous proliferation in sensory progenitors and supporting cells, without effects on cell survival, fate or differentiation (Chen and Segil, 1999; Mantela et al., 2005; Sage et al., 2005; Sage et al., 2006). Thus, cell cycle control is critical for the regulation of the maintenance of progenitor state and of the expansion of sensory domains. Is there a mechanism that links *Serrate1* and cell cycle? A recent study indicates that *Hes1* regulates normal cell proliferation of sensory precursors via the transcriptional repression of $p27^{kip}$ (Murata et al., 2009). This is of interest because in the present study, we showed that Serrate1 regulates *Hes1* expression in the otic epithelium. It is possible, therefore, that the regulation of cell proliferation by *Serrate1* may be mediated by the regulation of *Hes1* expression. But this does not exclude that other Serrate1 targets, *Hey1* and *Hey2*, also cooperate in this process.

BMP signaling regulates proliferation of cardiomyocites during cardiogenesis [Chen et al., 2004], the expression of *Bmp10* depending on Notch (Grego-Bessa et al., 2007). We have shown that *Serrate1* regulates *Bmp4* expression and that both *Serrate1* and *Bmp4* regulate *Hes1* and *Hey2* expression (present work and Kamaid et al., 2009, see appendix 2). One interesting possibility is that *Serate1* and *Bmp4* cooperate to regulate cell proliferation in otic sensory epithelium. This possibility provides an interesting interpretation to the results reported in Pujades et al. (2006). They show that BMP blockade results in an increased differentiation of hair cells, and the proposed mechanism was that *Bmp4* prevents cell cycle withdrawal. One possibility is that *Serrate1* and *Bmp4* cooperate in the induction of *Hes1* which, in turn, prevents $p27^{kip1}$ expression. This would be an interesting example of cross-talk between two signalling pathways that cooperate to the maintenance of the appropriate rate of cell proliferation and the regulation of cell cycle exit of otic progenitors (Fig.3).

II. Sox2 and neurosensory development

II.1Sox2 and the neurosensory fate

During neural development Sox2 expression is associated with neural competence and neural commitment of ectodermal progentior cells (Pevny and Placzek, 2005) and it is regulated by neural inducing signals (REF:). This suggests Sox2 plays a role in the early specification of neural fate. As shown in the results, in the developing otic epithelium *Sox2* is associated with neuronal and sensory progenitors. As discussed by Fritzsch et al. (2006) the vertebrate sensory organ requires a mechanism for rapidly expanding the basic sensory unit, so that placodal epithelial cells bear characteristics of stem cells. Therefore, it is expected that they express typical genes of the stem cell cassette (Takahashi and Yamanaka, 2006). Our experiments show that the expression of *Sox2* in the inner ear is reminiscent of this general stem cell function, but restricted to neurosensory committed progenitors. This restriction suggests that *Sox2* may be crucial for the acquisition of the neurosensory competence ot the otic epithelium. The gain of function of Sox2 show that the ectopic expression of *Sox2* results in the generation of ectopic neurons, hair cells and supporting cells, suggesting that *Sox2* is sufficient to specify neurosensory fate in the otic epithelium (Fig.4, left).

Recent work showed that *Sox2* can be induced by *Sox3* in the otic epithelium and that *Sox3* expression depends on inductive FGF signalling from the ectoderm [Abelló et al., 2009]. This is similar to the situation described in the neural tube during CNS development, in which *SoxB1* expression is associated with neural commitment and regulated by FGFs (Uchikawa et al., 1999; Streit et al., 2000; Takemoto et al., 2006). It is possible that otic expression of *Sox2* were also directly dependent on FGF signalling like during neural plate induction (Takemoto et al., 2006). Furthermore, some reports have placed Wnt and Shh signalling upstream of *Sox2* in retinal progenitors and neural stem cells, respectively, and Wnt responsive elements were identified in N-1 *Sox2* enhancer (Van Raay et al., 2005; Takemoto et al., 2006; Agathocleous et al., 2009; Takanaga et al., 2009). Notch signalling is necessary for the maintenance of *Sox2* expression in the otic epithelium but not for its induction (Daudet et al., 2007) and this regulatory pathway was discussed above. In summary, we know still very little about the signalling factors/pathways that regulate the onset and the maintenance of *Sox2* expression in the inner ear.

II.2 SoxB1 dosage and cell fate

2.1 The transient co-expression of Sox2 and Sox3 and neurogenesis

Sox3 and *Sox1* are co-expressed with *Sox2* in many tissues during embryonic development (Uchikawa et al., 1999), and they play redundant roles in CNS neurogenesis (Bylund et al., 2003). However, there are also places where the expression of those genes is unique (Uchikawa et al., 1999). *Sox1* is not expressed in the otic placode and its expression is very weak or absent in the sensory epithelium other then

the cristae, suggesting that *Sox1* does not play a central role in neurosensory development of the inner ear (Uchikawa et al., 1999). Conversely, *Sox3* is expressed along with *Sox2* since early stages of otic development (Uchikawa et al., 1999; Abello et al., 2007). *Sox3* is known to be expressed in the neurogenic placodes (Abu-Elmagd et al., 2001), and it appears to be essential for the neurogenic capacity of epibranchial placodes (Tripathi et al., 2009). Recent work has shown that *Sox3* is a key player in the specification of the neurogenic domain of the otic placode, it is sufficient the neurogenic potential, and it is able to induce Sox2 (Abello et al., 2007).

In this study we show that *Sox3* and *Sox2* co-expression is restricted both temporally and spatially to cells associated with neurogenic potential and that only *Sox2* expression persists in sensory progenitors. This suggests that the association between *Sox2* and *Sox3* may drive neurogenesis, whereas *Sox2* alone would promote sensory development (Fig.2). In principle, this may result from the specificity of the combination of *Sox2* and *Sox3* or, alternatively, from the high concentration of *SoxB1* activity produced by otherwise redundant factors. The functional studies discussed in the following section favour the latter possibility.

2.2 SoxB1 dosage as a possible regulator of cell fate

In our experiments, co-expression of *Sox2* and *Sox3* is associated with neuronal fate, while *Sox2* expression alone is associated with sensory fate. Alternatively, neuronal vs. sensory fate may depend on *SoxB1* dosage rather than on a specific function of *Sox3*. In this case, cells that co-express *Sox2 and Sox3* would receive high dosages of *SoxB1* genes and would be determined to the neuronal lineage while cells that only express *Sox2*, would experience a lower SoxB1 activity and commit to the sensory lineage (Fig.1). *Sox3* down-regulation would be responsible for determining *SoxB1* dosage in the otic epithelium and would function as the molecular switch for the transition between a neurogenic and sensory sates. A relationship between dosage and cell fate has been described during retinal development, where *Sox2* regulates the competence of the retinal progenitors to differentiate into the different cell lineages (Cormier et al., 2006).

Our results show that a large fraction of cells carrying the *Sox2* transgene delaminated from the otic epithelium and developed as neurones. In fact, the expression of *Ngn1* and *NeuroD* increased, after the transient increase in the transgene, and the size of the CVG was larger in transfected embryos. This effect was not due to an increase in the rate of cell proliferation and the expansion of the progenitor population. In addition, there is a correlation between the levels GFP expressed by the cells and the fate that they acquired during development. Cells that expressed high levels of *Sox2* delaminated as neuroblasts, while those that expressed low levels of GFP remained within the epithelium and differentiated as hair or supporting cells. We like to propose two possible actions of *Sox2* to explain these results: 1) *Sox2* expression hinders differentiation of neurosensory progenitors during the specification of the early (neuronal) fate in otic development. Thereafter, when the effect of *Sox2* is released progenitors re-start differentiation by generating early fate lineages, and thus producing neurones. This implies that cell fate specification in the otic epithelium is strongly dependent on intrinsic

temporal cues. 2) Alternatively, *Sox2* high dosage may be instructive for neurosensory progenitors and result in the active induction of neuronal fate. The results of transfection at later stages of development suggest that *Sox2* induced neurone overproduction with independence of the state of commitment of the progenitor, and favour that Sox2 promotes neuronal fate at high dosage.

What may be the significance of the fact that high concentrations of *Sox2* induce neuronal fate, whereas endogenous levels of *Sox2* are necessary for sensory fate? We would like to speculate that the association of high *Sox2* dosage with neuronal fate is an experimental condition that mimics the high concentration of *SoxB1* genes experienced by otic neurosensory progenitors undergoing neurogenesis during normal development. As described above, neurogenic otic progenitors express both *Sox2* and *Sox3*, while sensory progenitors express only *Sox2*. One possibility is that the combined expression of *Sox2* and *Sox3* is an efficient mechanism to regulate *SoxB1* levels in otic neurosensory progenitors. *Sox2* would specify neurosensory fate and its association with *Sox3* would determine the dosage of SoxB1 activity. Cells expressing both genes would have high concentrations of *SoxB1* and therefore differentiate as neurones, while otic progenitors that only express *Sox2* will have low SoxB1 activity and would stay as sensory precursors. The result would be that during normal development, both *Sox2* and *Sox3* are required to specify the neuronal fate, whereas *Sox2* alone is sufficient to specify the sensory fate. This is only possible provided that both genes regulate the same set of target genes. The differential use of two functionally similar *SoxB1* genes is probably a simple mechanism that allows regulation of *SoxB1* levels through the independent regulation of two different regulatory regions associated with two different genes. The study on the regulation of the enhancer regions of *Sox2* and *Sox3* that drive their expression in the otic territory will help to clarify these questions.

2.3 The temporal regulation of SoxB1 dosage/ Sox3 expression and the neurone-sensory phenotypic switch

The possibility of a phenotypic switch of cycling neurosensory progenitors from a neurone to sensory fate has been suggested by experiments with *Ngn1* and *Atoh1* null mice [Matei et al., 2005, Raft et al., 2008]. The cross-regulation between *Ngn1* and *Atoh1* has been proposed to regulate the transition between neurogenesis and sensory development in the otic epithelium (Raft et al., 2007). The authors propose that *Atho1* and *Ngn1* cross-inhibit each other in a common neurosensory domain. The process takes place sequentially: first a multipotent, *Ngn1*-positive domain is established from where neurons are generated and, thereafter, *Atoh1* expression displaces *Ngn1* and generates sensory cells. *Atoh1* has an inhibitory effect on *Ngn1,* resulting in the replacement of a neurogenic epithelium for a sensory epithelium (Raft et al., 2007). The spatial and temporal overlap between the *Sox3* turn-off and *Atho1-Ngn1* cross-inhibition, suggests that *Sox3* function may be associated with this phenotypic switch. Its function may be associated with the maintenance of *Ngn1* expression. In that case, only when *Sox3* were lost (or *SoxB1* dosage lowered), *Atoh1* inhibition over *Ngn1* would be allowed and sensory fate promoted. Recently, functional studies in chick have demonstrated that *Sox3* can re-specify non neurogenic otic epithelium and induce *Ngn1* and *Delta1* positive

neuroblasts [Abelló et al., 2009, Katri PhD Thesis]. This suggests that *Sox3* may be responsible for sustaining the expression of *Ngn1* in neuronal progenitors and thus for the initial imbalance of the inhibitory loop in the direction of neurogenesis. Cells that do not express *Sox3*, on the contrary, would be efficiently converted in sensory precursors upon *Atoh1* expression (Fig.2).

The question that the dosage of *SoxB1* results in a switch of cell fate is relevant to the discussion lineage of neurons and hair cells Retroviral tracing studies suggested that macular progenitors give rise to both hair cells and neurons (Matei et al., 2005; Satoh and Fekete, 2005). Two other studies based on cell and genetic tracing also support the idea that they derive from a common cell population [Bell et al., 2008; Raft et al., 2008]. The fact that the expression of *Sox2* is sufficient to specify all neurosensory fates, suggests that multipotent/neurosensory progenitor cell may occur in normal development and depend on the expression of *SoxB1 genes*. The expression of *SoxB1* genes would provide with an extended competence to generate both neurones and hair cells. However, this may be projected in time, because the co-expression of *Sox2* and *Sox3* would provide a high *SoxB1* concentration and promote the neuronal fate. But later on, the loss of *Sox3* and the maintenance of *Sox2* would commit precursors to the sensory fate (Fig4, left).

II.3 Sox2 and the progenitor state

3.1 Sox2 expression is downregulated in neurones and hair cells

As shown in the results, *Sox2* is downregulated when neurons and hair cell differentiate (Fig.1), similarly to what occurs in CNS development. In the developing neural tube, *Sox2* negatively regulates neurogenesis by repressing proneural activity until *SoxB2* group genes are expressed. *SoxB2* genes counteract Sox2 function and allow the progression of neurogenesis, which results in the downregulation of *Sox2* by the activity of proneural genes (Bylund et al., 2003; Graham et al., 2003; Sandberg et al., 2005). Otic neurons and hair cells are neural cell types in a strict sense, and both are specified upon the activation of proneural basic helix-loop-helix (bHLH) genes, into neurones by *Ngn1* and into sensory cells by *Atoh1.* Thus it is possible that *Sox2* plays a similar function in the inner ear, maintaining the state of proneural commitment, but preventing also the precocious differentiation of neurones and hair cells. The observation that *Ngn1* and *Atoh1* expression emerge within Sox2 expression domains (Fig.1B), and that expression of *Ngn1/Sox2* and *Atoh1/Sox2* overlaps temporally [Alsina et al., 2004; Pujades et al., 2006; Neves et al., 2007; Raft et al., 2008; Bell et al., 2008] (Fig.1A), further suggests that a mechanism of cross regulation between proneural genes and *Sox2* might also take place in the inner ear. If this were the case, *Sox2* should be co-expressed with *Ngn1* in neuronal precursors and with Atoh1 in sensory precursors. The expression of Sox2 with low levels of Ngn1 and *Atoh1* in sensory precursors would prevent their activity and the progression to differentiation. However, when high levels of expression of proneural genes were triggered, and the differentiation program is started, products of proneural gene activity would downregulate *Sox2* expression (Bylund et al., 2003). *Sox21*, a *SoxB2* group gene, is also expressed in the otic epithelium in

overlapping domains with *Sox2*, however, the temporal profile of expression has not been detailed (Uchikawa et al., 1999). It will be interesting to explore whether *SoxB2* genes take part in the development of the neural elements of the ear with a similar role to the one they played in the CNS.

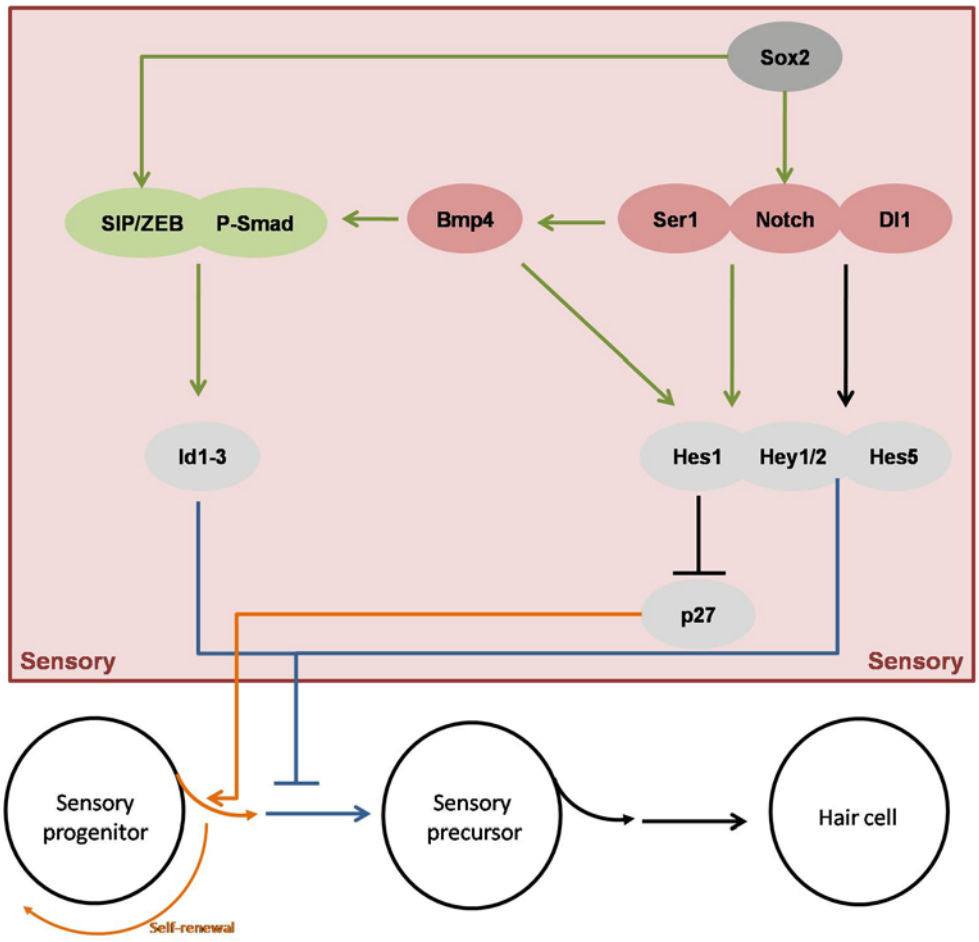

Figure 3: The role of *Sox2* and *Serrate1* in the maintenance of the progenitor state. *Sox2* function is to prevent differentiation (Blue pathway) while *Serrate1* regulates the maintenance of self-renewal (orange pathway). *Sox2* cooperates with the Notch signalling pathway through the induction of *Notch1* receptor and with BMP pathway through the induction of *Sip1/Zeb1* co-activators in the induction of *Hes/Hey* and *Id* genes respectively. Those genes prevent the transition to a committed precursor state, possibly through the regulation of proneural protein expression and activity (Norton, 2000; Fischer and Gessler, 2007). *Serrate1* in cooperation with *Bmp4* induce *Hes1* which is thought to function in the maintenance of self-renewal through the prevention of cell cycle exit, by hindering p27 expression (Murata et al., 2009).

Gain of function experiments showed that the exogenous Sox2 protein was also downregulated in delaminated neuroblasts. GFP expression in the CVG 20h after transfection allowed us to trace the cells that were transfected with *Sox2*, whether expressing *Sox2* or not. The observation was that Sox2 protein was not detected in transfected delaminated neuroblasts at a time where transgene expression should be maximal, while Sox2 levels were high in the transfected cells that remained in the epithelium, Since the exogenous protein is the product of a transcription of the transgene

from a constitutive promoter, it was not subject to the transcriptional regulatory mechanisms that control *Sox2* expression in vivo. Therefore, it is likely that a post-transcriptional mechanism is responsible for this down-regulation. During CNS development, post-transcriptional mechanisms down-regulate Sox2 in neural precursors and neuroblasts, and they involve protein degradation via the activity of serine proteases (Bani-Yaghoub et al., 2006). Several miRNA are expressed during inner ear development, some of them specific to neuroblast and hair cell lineages and the analysis of conditional Dicer mutants revealed that miRNA regulation is important for inner ear development (Weston et al., 2006; Friedman et al., 2009; Soukup et al., 2009). miR-124, a neuron-specific miR is expressed in otic neuroblasts and interestingly, it has been shown to regulate the differentiation of neural stem cell in the SVZ of the adult brain and Sox9 was identified as one of its target genes [Soukup et al., 2009, Chen et al., 2009].

3.2 Sox2 prevents the differentiation of neurosensory elements of the inner ear

We have used Islet1, as an early marker of the differentiated state of neurons and sensory cells, to analyze the role played by *Sox2* in the regulation of differentiation. The observation was that *Sox2* over-expression hindered *Islet1* expression in the otic epithelium. However, the ability of *Sox2* to counteract differentiation was associated with time and the state of commitment of the progenitor cells. The over-expression of *Sox2* did not prevent *Islet1* expression after 20h, at maximal transgene expression, but it did so after 48h. This suggests that either the effect of *Sox2* has a long latency, or that the ability of *Sox2* to prevent differentiation is related to its ability to block the very first steps of differentiation, in which case, once this is initiated *Sox2* can no longer counteract differentiation. The current evidence favours the latter possibility.

In the CNS, *Sox2* regulates neurogenesis, and *Sox2* over-expression prevents neuronal differentiation, by inhibiting the neurogenic *activity* of proneural genes (Bylund et al., 2003; Graham et al., 2003; Bani-Yaghoub et al., 2006). A similar function has been attributed during the development of the cochlea of the mouse, where *Sox2* antagonizes *Atoh1* function and inhibits hair cell differentiation [Dabdoub et al., 2008]. From this mechanism, one would expect that *Sox2* had no effect on cells that had already started their commitment to neuronal or sensory fates. The experiments show that neurones and cristae were most resistant to the forced expression of *Sox2*, whereas regions that developed later were not. As mentioned above, the development of the neural elements of the ear follows a stereotyped temporal profile: neurones are specified and differentiate between E2 and E3, the dorsalmost elements of the vestibular system, the cristae, start their specification by E3, and the most ventral domains by E5 (Adam et al., 1998; Cole et al., 2000; Alsina et al., 2004; Pujades et al., 2006; Bell et al., 2008). In agreement with this view, *Sox2* had little effect on *Ngn1* and *NeuroD* after 20h, but the effect was strong on genes associated with the inhibition of differentiation, like bHLH Notch and BMP downstream targets (see below)

3.3 Sox2 cooperates with the Notch signalling pathway in the regulation of differentiation

The Notch pathway plays a crucial role in vertebrate neurogenesis. *Hes/Hey* genes are direct targets of Notch and their products counteract neurogenesis by repressing proneural gene expression [Ishibashi et al., 1994; Otsuka et al., 1999; Ohtsuka et al., 2001; Sakamoto. et al., 2003; Fior and Henrique, 2005; Holmberg et al., 2008]. *Hes* transcription factors may also antagonise neurogenesis by forming non-functional pairs with proneural proteins or E-proteins (Sasai et al., 1992; Fischer and Gessler, 2007). *Sox2* regulation of neurogenesis in the CNS is related also to its capacity to counteract differentiation. Studies in the spinal cord suggest that *Sox2* acts down-stream and independently of Notch signalling (Holmberg et al., 2008). In contrast, two other studies in the developing retina and neocortex, suggest that *Sox2* regulation of neurogenesis is, at least in part, mediated by the regulation of Notch signalling (Bani-Yaghoub et al., 2006; Taranova et al., 2006). These observations may seem contradictory, however they probably reflect that *Sox2* may act both dependent and independently of Notch.

As discussed above, during otic development Serrate1-mediated Notch signalling acts upstream of *Sox2,* to regulate its expression. This interaction is unidirectional and *Sox2* is unable to modify *Serrate1* expression. On the other hand, Sox2 over-expression results in the up-regulation of *Hes5*, *Hey1* and *Hey2*, suggesting that *Sox2* can regulate the Notch pathway. In the mouse cochlea, *Hes5*, *Hes1* and *Hey2* regulate hair cell and supporting cell differentiation, and gain of function studies suggest that they act as negative regulators of Atoh1 function in the cochlear epithelium [Zheng et al., 2000; Zine et al., 2001; Liet al., 2008; Doetzlhofer et al., 2009]. It is reasonable to think that. at lest in part, *Sox2* prevents differentiation in the otic epithelium through the regulation of Notch (Fig.3).

The ability of *Sox2* to induce Notch targets seems related to its ability to induce the transcription of *Notch1* receptor, and not of its ligands. *Delta1* and *Serrate1* expression was not affected by *Sox2*. However, *Notch1* transcription was up-regulated in response to *Sox2* overexpression in the otic epithelium. Up-regulation *Notch1* in response to *Sox2* gain of function has been also observed in neocortical progenitors (Bani-Yaghoub et al., 2006). This is agreement with the presence of Sox binding sites in the promoter region of *Notch1*, and with the binding of Sox2 to the *Notch1* promoter region in retinal progenitors (Taranova et al., 2006). Specific inactivation of *Sox2* in the developing mouse brain, however, does not result in decreased levels of *Notch1* or *Hes5* transcripts, and this has been attributed to the concomitant up-regulation of *Sox3* in these mice (Miyagi et al., 2008). A mechanism based on the regulation of *Notch1* transcription has the consequence that *Sox2* function does not result in the activation of Notch pathway, but in the augmentation of an endogenous Notch activity through the regulation of the amount of receptor. Such a mechanism has been described in other model systems, where the strength of the Notch activity is controlled by a positive feedback activation of Notch receptor transcription (de Celis and Bray, 1997; Del Monte et al., 2007) However, it is also possible that *Sox2* regulates the transcription of Hes/Hey genes through a mechanism that is independent of the induction transcription of Notch receptor.

In summary, our results support the hypothesis that the interaction between *Sox2* and the Notch signalling pathway occurs in multiple levels, *Sox2* being downstream *Serrate1*, but being also able to induce *Notch* receptor and targets. This provides a route by which *Sox2* may maintain its own expression and promote the maintenance of the undifferentiated state of neurosensory progenitors (Figs.2 and 3).

3.4 Sox2 cooperates with BMP signalling pathway in the regulation of differentiation

Progression through differentiation relies on the function of proneural transcription factors that have the ability to regulate other downstream genes involved in differentiation. Id proteins (Inhibitors of Differentiation and DNA binding) inhibit differentiation and proneural protein function. They hetero-dimerise with proneural proteins and prevent their ability to bind and activate the transcription of target genes (Norton, 2000). *SoxB1* regulation of neurogenesis in the CNS also relies in Notch independent mechanisms, as demonstrated by Holmberg et al. (2008), who showed that SoxB1 proteins counteract neurogenesis, even in the presence of Notch inhibition. The authors suggest that this function may be downstream of proneural gene transcription and associated with the regulation of the activity of proneural proteins (Holmberg et al., 2008). Since *Id* genes are able to prevent proneural protein activity, they are good candidate to be the mediators of this function.

Our results show that Sox2 over-expression results in the induction of *Id1-3* transcripts. The induced *Id* expression is not dependent on Notch signalling (Supplementary Information 3), indicating that the effect of *Sox2* must occur through an alternative mechanism (Fig.3). Id proteins are expressed in the otic epithelium during sensory development and counteract hair cell differentiation [Jones et al., 2006; Kamaid et al.,2009]. Furthermore, their expression in the otic epithelium is positively regulated by BMP signalling through a mechanism dependent on SMAD phosphorylation [Kamaid et al., 2009].

The induction of *Id* expression by *Sox2* was not caused by the activation of the BMP pathway, since *Sox2* did not induce an increase in the levels of P-Smad protein. However, *Sox2* was able to induce GFP expression driven by the BMP-response element derived from the promoter region of an *Id* gene. This suggests that the regulation by *Sox2* was cooperative with BMP signalling, but downstream of Smad phosphorylation. Smad regulation of gene expression is modulated by the recruitment of co-activators and/or co-repressors. ZEB proteins are two-handed zinc finger/homeodomain transcription factors which interact with Smad proteins and modulate their activity, by differential recruitment of co-activator and co-repressors (Verschueren et al., 1999; Postigo, 2003; Postigo et al., 2003; Yoshimoto et al., 2005). *Zfhx1b* (gene coding for SIP1 protein) has been recently identified as a putative *Sox2* target gene, in an in silico study (Chakravarthy et al., 2008), and *Sip1* deficient mice exhibit reduced *Sox2* expression (Miyoshi et al., 2006), suggesting that this genes might cross-regulate each other during embryogenesis. Our results show that Sox2 induces

Sip1 and *Zeb1* expression in the otic epithelium. Therefore, it is possible that the regulation of *Id* expression by *Sox2* could rely on the regulation of *Sip1*/Zeb1 expression that would cooperate with Smad signaling to induce *Id* expression (Fig.3).

II.4 Sox2 in supporting cells

Sox2 expression is maintained in differentiated supporting cells (Fig.1). Unlike mammals, birds and other vertebrates have the ability to regenerate auditory hair cells at the expenses of supporting cells. Upon damage, supporting cells leave the quiescent state, activate *Atho1* expression and single out hair cells by the Delta-Notch pathway (Cotanche and Lee, 1994; Stone and Rubel, 2000; Bermingham-McDonogh and Rubel, 2003). Notch signalling is not required to maintain quiescent state or to initiate hair cell regeneration, but rather to regulate the correct number of hair cells that are generated after damage (Daudet et al., 2009). *Sox2* maintenance in supporting cells may suggest a mechanism that enables them to maintain the potential to differentiate into hair cells. However, *Sox2* expression is also retained by the supporting cells of the mammalian embryonic and adult cochlea, where hair cells do not regenerate. Besides, *Sox2* expression is not affected in drug-damaged cochlear epithelium (Hume et al., 2007; Oesterle et al., 2008), suggesting that *Sox2* may function as a permissive factor required for regeneration, but that it is not sufficient alone to confer that potential. In addition to *Sox2*, at least three other genes, *c-myc*, *kfl4* and *Oct4* have been associated with the pluripotent state of stem cells and the four genes have been demonstrated to be sufficient to induce pluripotent cells (Takahashi and Yamanaka, 2006). In this context, it would be interesting to explore whether any of these factors function together with *Sox2* to maintain the potential for hair cell regeneration.

II.5 Sox2 in Schwann glial cells

The results show also the expression of *Sox2* in Schwann glial cells of the cochleo-vestibular ganglion. Expression of *Sox2* is down-regulated in the neural plate when neural crest segregates from dorsal neural tube and remains low during crest cell migration. *Sox2* expression is subsequently up-regulated in some crest-derived cells in the developing peripheral nervous system and is later restricted to glial sublineages. The expression of *Sox2* fits very well with this pattern given the neural crest origin of the Schwann cells of the cochleo-vestibular ganglion as described by the classical work of D'Amico-Martel and Noden [D'Amico-Martel and Noden, 1983; Matei et al., 2005]. *Sox2* expression in Schwann cells of the auditory and vestibular ganglion, therefore, may reflect the neural crest origin of the Schwann cells.

III. Summary

The results of this thesis report provide new insights into long lasting questions on the mechanisms underlying the development of the neurosensory elements of the inner ear and the pathways that regulate the cell fate decisions. Specifically, we disclose some of the specific roles played by *Sox2* and *Serrate1* in that process. Our experiments provided evidence that *Sox2* and *Serrate1* are both involved in the regulation of cell fate in otic epithelium and in the maintenance of the progenitor state of epithelial cells, and that they use different mechanisms to achieve their function (Fig.4). As to the mechanism of cell fate specification, our results are consistent with a model in which SoxB1 proteins specify the neurosensory competence of otic progenitors, and further regulate cell fate decisions in a dose-dependent manner. High levels of SoxB1 proteins associated with the co-expression of *Sox2* and *Sox3* drive progenitors into a neuronal fate. Low levels of SoxB1 proteins associated with the down-regulation of Sox3 and the expression of *Sox2* alone drive progenitors into the sensory fate. Besides, our results support the idea that *Serrate1* is necessary and sufficient to maintain *Sox2* expression in the otic progenitors of the sensory domains. This process is mediated by Notch through the mechanism of lateral induction (Fig.4, left). As to the maintenance of the progenitor state we like to propose that *Sox2* and *Serrate1/Notch* cooperate in the maintenance of the progenitor sate through independent mechanisms. *Serrate1* promotes self-renewal through the activation of *Hes1* and *Sox2* prevents differentiation through the activation of *Hes/Hey/Id* genes (Fig.4, right).

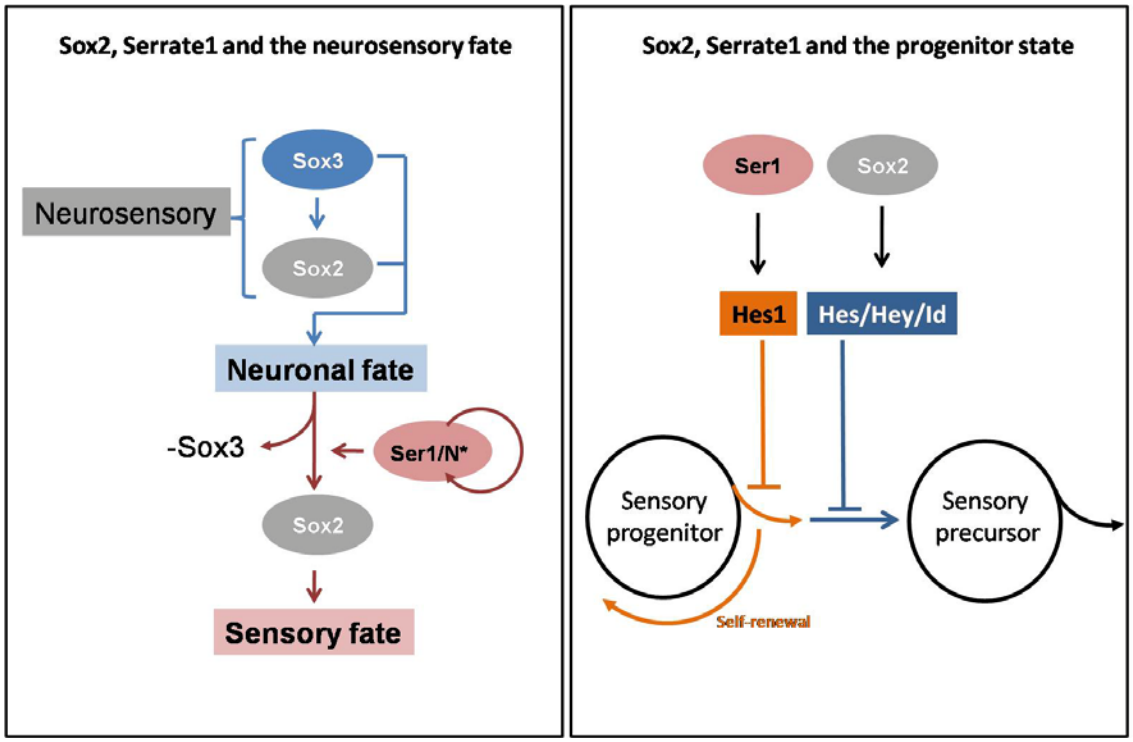

Figure 4: Summary of Sox2 and Serrate1 functions in the specification of cell fate (left) and regulation of progenitor state (right) in the otic neurosensory progenitors.

CONCLUSIONS

1. *Sox2* is expressed in the neurosensory domain of the otic epithelium, first, during the neurogenic period of otic development and, later on, during the development of the prosensory patches and sensory organs. As differentiation proceeds, *Sox2* is excluded from differentiated neurons and hair cells, but it remains expressed in the supporting cells of the sensory organs.
2. *Sox3* is co-expressed along with *Sox2*-positive in the neurogenic domain of the otic cup, but *Sox3* is downregulated after the neurogenesis. Only *Sox2* expression persists in the sensory precursors, where it is co-expressed with the Notch ligand *Serrate1*.
3. *Serrate1* expression is initially nested within *Sox2*, but later on *Sox2* becomes restricted within the boundaries of *Serrate1* expression. *Sox2* correlates with neurosensory fate, neurogenesis with the association between *Sox2* and *Sox3* expression, and sensory development with that of *Sox2* and *Serrate1.*
4. *Serrate1* regulates prosensory fate and sensory organ development by promoting *Sox2* expression, without affecting neurogenesis. We propose that the induction of *Bmp4* cooperates in this prosensory function through a mechanism that is independent of *Sox2.*
5. *Serrate1* operates in a Notch-dependent manner, and consistently with a mechanism of lateral induction. This results in the maintenance of a cluster of cells with active Notch signalling that activates down-stream targets of the Notch signalling pathway *Hes1*, *Hey1* and *Hey2.*
6. *Serrate1* regulates the rate of cell proliferation in the otic epithelium. We propose that this function is mediated in cooperation with *Bmp4*, trough their common target *Hes1*.
7. *Sox2* regulates the differentiation of the neurosensory elements of the inner ear in cooperation with Notch and BMP signalling pathways.
8. *Sox2* promotes neurosensory fate. However, high concentrations of *Sox2* suppress sensory fate and promote neuronal fate.
9. We propose a model in which an extended neural competence is early established in the otic placode by the expression of *Sox2* and *Sox3* genes. The cooperation between *Sox2* and *Sox3* would provide a high concentration of SoxB1 protein to promote neuronal fate. In parallel, *Serrate1* would maintain *Sox2* expression in restricted domains that retain the neurosensory competence and thereby develop as sensory patches.

CONCLUSIONS

MATERIALS AND METHODS

I. In ovo electroporation

In ovo electroporation is a well established method for transgenesis in chick that allows a tight control in space and time. The site of electroporation can be controlled by manipulation of the electrodes assembling given that DNA moves towards the anode. In addition, this method allows a tight control of the time of onset of transgene expression, which starts roughly two hours after electroporation, and has its peak twenty hours after electroporation. In addition, the duration of transgene expression is also limited as the concentration of the plasmid in the transfected cells decreases with time (Nakamura and Funahashi, 2001). This method has been initially applied for transgenesis in the chick neural tube but it can be easily applied to the inner ear system, as during the invagination and closure process, the otic vesicle can be easily filled with plasmid DNA solution, which can then be transfected into the cells by electroporation. The positioning of the electrodes allows targeting for electroporation specific domains of the otic vesicle. Since only one of the otic vesicles is filled with DNA solution, the other one remains untransfected and can be used as control

HH12-14 embryos

The constructs of interest (see below) were electroporated into the otic cup of HH12-14 embryos. A small hole was made into the vitelline membrane to expose the otic cup. The cathode platinium electrode was placed next to the right otic cup and anode electrode parallel to it, on the other side of the embryo. The desired vector (Tabel1) mixed with fast green (0.4 μg/μl) was injected onto the otic placode by gentle air pressure through a fine micropipette. Square pulses (8 pulses of 10 V, 50Hz, 250 ms) were generated by an electroporator Square CUY-21 (BEX Co., LTd, Tokiwasaiensu, Japan). Medium-199 (Gibco) was added immediately after each electroporation. Eggs were sealed and incubated for designated times after which embryos were collected as descibed.
incubated for designated times after which embryos were collected as descibed.

vector	concentration	Source
pIRES-EGFP-cSox2	1μg/μl	Hisato Kondo Lab
pCMV/SV1-cSox2	1μg/μl	Hisato Kondo Lab
pCMV/SV1-cSox2ΔHMG	1μg/μl	Hisato Kondo Lab
hJag1-PCIG	1μg/μ	Elisa Marti Lab
pCIG	1μg/μll	Elisa Marti Lab
12xCLS-DsRed	2μg/μl	Jonas Muhr
BRE-GFP	0,5μg/μl	Elisa Marti Lab
N*EGFP	1μg/μll	Ana Bigas Lab

Table 1: Vectors used for electroporation

HH20 embryos

The constructs of interest (see below) were electroporated focally into HH20-21 otic vesicles following a method modified from Chang et al. (2008). The cathode consisted of a 0.3 mm diameter Pt tip attached to a handle placed on top of the otic vesicle and the

anode was a 0.5 mm diameter Pt electrode that was placed underneath the embryo. DNA (pIRES EGFP-cSox2) was injected into the otic vesicle at a concentration of 6-8 µg/ml and electroporation Square pulses (8 pulses of 10 V, 50Hz, 250 ms) were generated by an electroporator Square CUY-21 (BEX Co., LTd, Tokiwasaiensu, Japan). Medium-199 (Gibco) was added immediately after each electroporation. Eggs were sealed and

II. Embryo collection and manipulation

Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38ºC for designated times and embryos were staged according to [Hamburger and Hamilton, 1951].

Embryo collection

- Embryos were dissected in cold PBS and fixed for 4h (0 to 15ss embryos) or overnight (more than 15ss embryos) in 4% Paraformaldehyde/PBS (4%PFA/PBS), at 4°C.
- Wash 3x5min in PBT (PBS-0.1% Tween20)
- Dehydrate in successive solutions of increasing Methanol concentrations (in PBT):
  10min in 25% Methanol/PBT
  10min in 50% Methanol/PBT
  10min in 75% Methanol/PBT
  10min in 100% Methanol/PBT
- Store in cold 100% Methanol at -20°C

Preparation of cryostat sections

Embryos stained for analysis of gene or protein expression were cryosectioned at 16-20 µm using a Leica cryostat.

- Rehydrate and Wash 3x10min PBS at RT
- Cryoprotect in 15% Sucrose at 4°C
- Embed in pre-warmed 30% gelatine/15% sucrose at 37°C rocking
- Prepare the blocks with pre-warmed 30% gelatine/15% sucrose at 37°C
- Freeze the blocks in pre-cold 2-Methylbutane at -80°C
- To improve tissue preservation, thin section at 20µm thickness using Superfrost Plus Slides (Fisher, Pittsburg) and store at − 20°C. The cryostat Leica CM 1510-1 was used for sectioning. Sections were used for immunohistochemistry

III. Immunohistochemistry in sections

- Hydrate the cryostat sections with PBS 15min
- Block the sections with 10% goat serum or horse serum in PBT for 1h at RT. Use a humid atmosphere.

- Incubate with the primary antibody (Table 2) in 10% goat serum or horse serum/PBT, overnight at 4°C in a humid atmosphere
- Wash 4x15min with PBT
- Incubate with secondary antibody (alexa-fluor conjugated, molecular probes) in 10% goat serum or horse serum/PBT at RT in a humid atmosphere for 2h.
- Wash 6x15min with PBT
- Mount the sections with Mowiol

Antibody	Species	Source	Dilution
α-Sox2	Rabbit polyclonal	Abcam (ab15830)	1:400
α-Sox2	Goat polyclonal	Santa Cruz (Y-17)	1:400
α-Sox3	Rabbit polyclonal	Thomas Edlund (Gift)	1:300
α-Jag1	Rabbit polyclonal	Santa Cruz (H-114)	1:50
α- CD57(HNK1)	Mouse monoclonal	B-D	1:2
α-TUJ1	Mouse monoclonal	Babco	1:400
α-Islet1	Mouse monoclona	Hybridoma Bank (39.4D5)	1:400
α-MyoVIIa	Rabbit polyclonal	T.Hasson lab.	1:5000
α-PCNA	Mouse monoclonal	Abcam	1:200
α-PH3	Rabbit polyclonal	Milipore	1:400
α-BrdU	Mouse monoclonal	Roche	1:200
α-caspase 3		BD Biosciences	1:400
α-GFP	Rabbit polyclonal	Clonethec	1:400
α-GFP	Mouse monoclonal	Invitrogen	1:200

Table 2; Antibodies used for immunohistochemistry.

We have characterized the anibody α-Sox2 rabbit polyclonal for its specificity (See Supplementay information 4)

IV. Whole mount In situ Hybridization

Embryos were processed according to Wilkinson and Nieto (Wilkinson and Nieto. 1993). The procedure is described below:

- Rehydrate embryos:

  10min in 75% Methanol/PBT
  10min in 50% Methanol/PBT
  10min in 25% Methanol/PBT

- 3x5min in PBT
- Treat with proteinase K 10□g/ml in PBT (stock: 10mg/ml)
- The time of treatment depends on the size of the embryo:
- Post-fix with 4%Paraformaldehyde 0.2% Glutaraldehyde in PBT, 20min room temperature (RT)
- 3x5min washes in PBT
- Prehybridize in hybridization buffer at 70°C for 1h

- Dilute the probe in hybridization buffer (2□l/300ml)
- Denature the probe in hybridization buffer for 10min at 80°C
- Replace the prehybridization buffer for the denatured probe in hybridization buffer
- Hybridize overnight at 70°C (See probes used in Table 4)
- (Pre-warm wash buffers at 70°C)
- Wash with: 3x30min wash buffer I at 70°C
  2x30min wash buffer II at 65°C
  2x30min TBST at RT (TBST-0.1% Tween20)

Immunostaining

- Block for 30min at RT with 10%NGS in TBST at RT
- Incubate for 2h to overnight with anti-DIG-AP or anti-FLUO-AP in blocking solution [1:2000] at RT or 4ºC
- 2x10min TBST at RT and overnight at 4°C

Developing (It is performed in the dark)

- Preincubate in NTMT 4x15min at RT
- Incubate in staining solution at RT in the dark until the staining is developed. Change the solution frequently, if the colour of the solution turns to red change it and keep the embryos at 4°C in a fresh staining solution.
- Stop staining by several washes in PBT. It is recommended to wash for 1 or 2 days in PBT at 4°C.
- Embed in 50% Glycerol /PBT
- Store in 100% Glycerol at 4°C

This protocol was performed using the automated system from InsituPro VS (Intavis AG, Bioanalytical systems).

Solutions

<table>
<tr><td><u>Hybridization buffer</u>
50% Deionized Formamide (FAD)
5xSSC pH 4.5
1% SDS</td><td><u>Wash I</u>
50% FAD
5x SSC pH4.5
1% SDS</td><td><u>Wash II</u>
50% FAD
2x SSC pH4.5</td></tr>
<tr><td><u>NTMT</u>
100mM NaCl
100mM TRIS pH9.5
50mM $MgCl_2$
0.1% Tween20</td><td colspan="2"><u>Developing solution</u>
0.3mg/ml NBT 0.175mg/ml BCIP in NTMT
<u>For 10ml NTNT:</u>
45µl NBT 75mg/ml in DMF
5µl BCIP 50mg/ml in DMF</td></tr>
</table>

Table 3: Solutions for in situ hybridization

Gene	Vector source	Vector	Lin and Pol	Ref.
cSox2	P.Scotting Lab	pB SK	BamH1/T7	Rex et al. (1997)
cSer1	D. Henrique Lab	pB KSI-	HindIII/T7	Henrique et al. (1995)
cHes1	D. Henrique Lab	pB KSII+	HindIII/T7	Palmeirim et al. (1997)
cHes5	D. Henrique Lab	EST	Not1/T3	Fior and Henrique (2005)
cHey1	D. Henrique Lab	pB KS	BamHI/T3	-
cNotch1	D. Henrique Lab	pB KSII+	BamHI/T3	Henrique et al. (1995)
cId2	M. Bronner-Fraser Lab	-	NcoI/SP6	*Kee and Bronne-Fraser*
cId3	M. Bronner-Fraser Lab	-	AflII/T7	*(2001)*
cBmp4	M. Ross Lab	pB SK	BamHI/T3	-
cNeuroD	D. Henrique Lab	pB KS+	EcoRI/T3	Laufer et al. (1997)

Table 4: Riboprobes used for in situ hybridization

V. Organotypic cultures of otic vesicles

Electroporated and control otic vesicles were were dissected from electroporated embryos and transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37°C in a water-saturated atmosphere containing 5% CO_2 as described (Leon et al., 1995) unless otherwise stated. Additions were 1% fetal bovine serum (Bio Whittaker Europe) and DAPT (Calbiochem) at 20µM

VI. qRT-PCR

Otic vesicles were dissected out and total RNA was isolated using RNeasy Mini kit (Qiagen). The method used for RNA extraction excludes t-RNAs and small ribosomal RNAs, enriching the sample at about 15% in mRNA. Purified mRNA was retrotranscribed with the Superscritpt III DNA polymerase (Invitrogen) using random primers (invitrogen). Real time PCR was carried out using SybrGreen master mix (Roche). For each qRTPCR run, cDNA generated from 15 ng of RNA was used. Amplification was performed using an LightCycler (Roche). Primer sets (Invitrogen, Table 5) were designed to have comparable melt curves (Tm = 60 °C, pearl primer), and, when possible, spaming exon-exon juntions.. GAPDH was used as calibrator gene. Although 18s rRNA is also represented in the RNA sample it revealed not to be a reliable calibrator gene for these experiments as its levels of expression were too high when compared to the endogenous levels of expression of the genes of interest (so that, for the total amounts of RNA used, its calibration curve falls out of its linear part, data not shown). For each relative quantification three lots of 5-10 otic vesicles (depending on the stage), generated in three impendent experiments were used. Each of these samples was retrotranscribed three times and each retrotrasncription was used as template for each pair of primers in a triplicated PCR reaction. Relative expression levels in the electroporated otic vesicles were referred to the levels of expression in control otic vesicles which were arbitrarily set to 1. Results are shown as averages ± standard erros

of the three independent experiments and t-students test was applied to access statistical significance.

Gene	Forward primer	Reverse primer
GAPDH	TTGGCATTGTGGAGGGTCTT	GTGGACGCTGGGATGATGTT
cSox2	AAGAGACCCTTCATTGACGA	CGTGTACTTATCCTTCTTCATCAG
ΔHMG	ACAACGCGGCGCAGAT	GTCCCTTGCTGGGAGTACGA
cSer1	TGCCAGACGGTGCTAAGTG	TCGAGGACCACACCAAACC
hJag1	GTTCTCCTAATAACTGTTCCCA,	CCATTAACCAAATCCCGACAG
Dl1	TAACTCCGATAAAAACGCCTACAA	GCCATGCTCCTCTTTCACAG
Hes1	GGCGCGCATCAACGA	TGGAGGTTCCTCAGGTGCTT,
Hes5	GAAATCCTGACACCCAAAGAG	TCAATGCTGCTGTTAATCCT
Hey1	CGGAGGGAAAGGTTATTTCG	CAGCAATGGGTGAGATATGTG
Hey2	CAACCACAACATCTCAGATTATG	CAACTTCAGTCAAGCACTCC,
Notch1	GAACAATAAGGAGGAGACCC	TTTCGTAGCTCCCTTCTCTG
Id1	GCACCGGAGGGTCTCTAAAGT	CCAGCTGCAGGTCCCAGAT
Id2	ACAGACATCAGCATCCTCTC	CACTCGCCATTAGTTCTGAG
Id3	CCCACCCCACCATTATGA	GCCTCGTAACAGCTCCTGAC
Sip1	CTACACCTACCCAACTGGAG	AGCAAGTCTCCCTGAAATCC
Zeb1	GTTATTTACCCTGAAGCACCT	GTCACATGTCTCTGATCTCGT
Bmp4	TGATTCCTGGTAACCGAATGC	GATCCGGCTTGTCCCTGAA
LFng	GAAGAGCTGCGGGAGGAAG	GCTCCACCATGAGCACCAG
Ngn1	AGCGGAACCGCATGCA	AGGGCCCAGATGTAGTTGTAAGC
NeuroD	GCTACTCCAAGACGCAGAAACTC	CACAGCGTCTGCACGAATG
Cdk1	CTTCAGAATCTTCAGAGCTTTAGG	TTTAGACAACAGATCGAGTCC

Table 5: Primers used for qRT-PCR

VII. Proliferation and cell counting

We have accessed cell proliferation by immunodetection of PH3 expression and BrdU incorporation. Phospho-Histone3, pH3 can be recognized with aspecific antibody that is specific for the phosphorylated form of the amino-terminus of histone H3 (Ser10). Mitotic phosphorylation of histone H3 initiates non-randomly in late G2 interphase and is maximal just prior to the formation of prophase chromosomes. Dephosphorylation of H3 begins in anaphase and is complete immediately prior to detectable chromosome decondensation in telophase cells. Thus, pH3 antibody labels the subset of mitotic cells that are between profase and anaphase of cell cycle (Hendzel et al., 1997). BrdU is a thymidine analogue that is specifically incorporated into DNA during DNA synthesis in the S phase of the interphase of cell cycle. Electroporated embryos were incubated in the presence of BrdU (Roche, 280879) for 30min, after in ovo development with the transgene. This results in the incorporation of the analogue in the cells going through S phase during the time of treatment. The cells that incorporate BrdU can be then detected using an antibody against bromodeoxyuridine (Campana et al., 1988). Both methods label only a fraction of mitotic cells, going thought specific phases of the cell cycle, which makes them inappropriate for absolute quantification of mitotic cells. Our objective is to

compare the number of mitotic cells in electroporated otic vesicles in respect to controls. Since the faction of cells going through each phase of cell cycle is proportional to the total amount of proliferating cells, this methods can be effectively used for a our relative quantification.

Cryostat sections of electroporated embryos were double stained for pH3/BrdU (after incorporation) and GFP and positive cells were counted within the electroporated domains and equivalente dominas in control embryos. Relative in increase in pH3 expression or BrdU incorporation is shown in respect to the values in controls arbitrarily set to 1. Results are shown as averages ± standard errors of three cell counting in three different embryos and t-students test was applied to access statistical significance.

VIII. Imaging and 3D reconstructions

Sectioned embryos processed for ISH or IHC were photographed using LEICA DMR conventional fluorescence microscope fitted with Leica DFC 300FX camera. Confocal images were obtained with a LEICA DM IRBE confocal microscope. Images were captured with Leica IM50 v4.0 and analyzed with Adobe photoshop v7.0.1.

3D reconstructions and volume calculations were made from serial 20µm sections using BIOVIS 3D (http://www.biovis3d.com/index.html)

MATERIALS AND METHODS

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SUPPLEMENTARY INFORMATION

Supplementary Information 1: Effects on gene expression produced by the electroporation of control vectors.

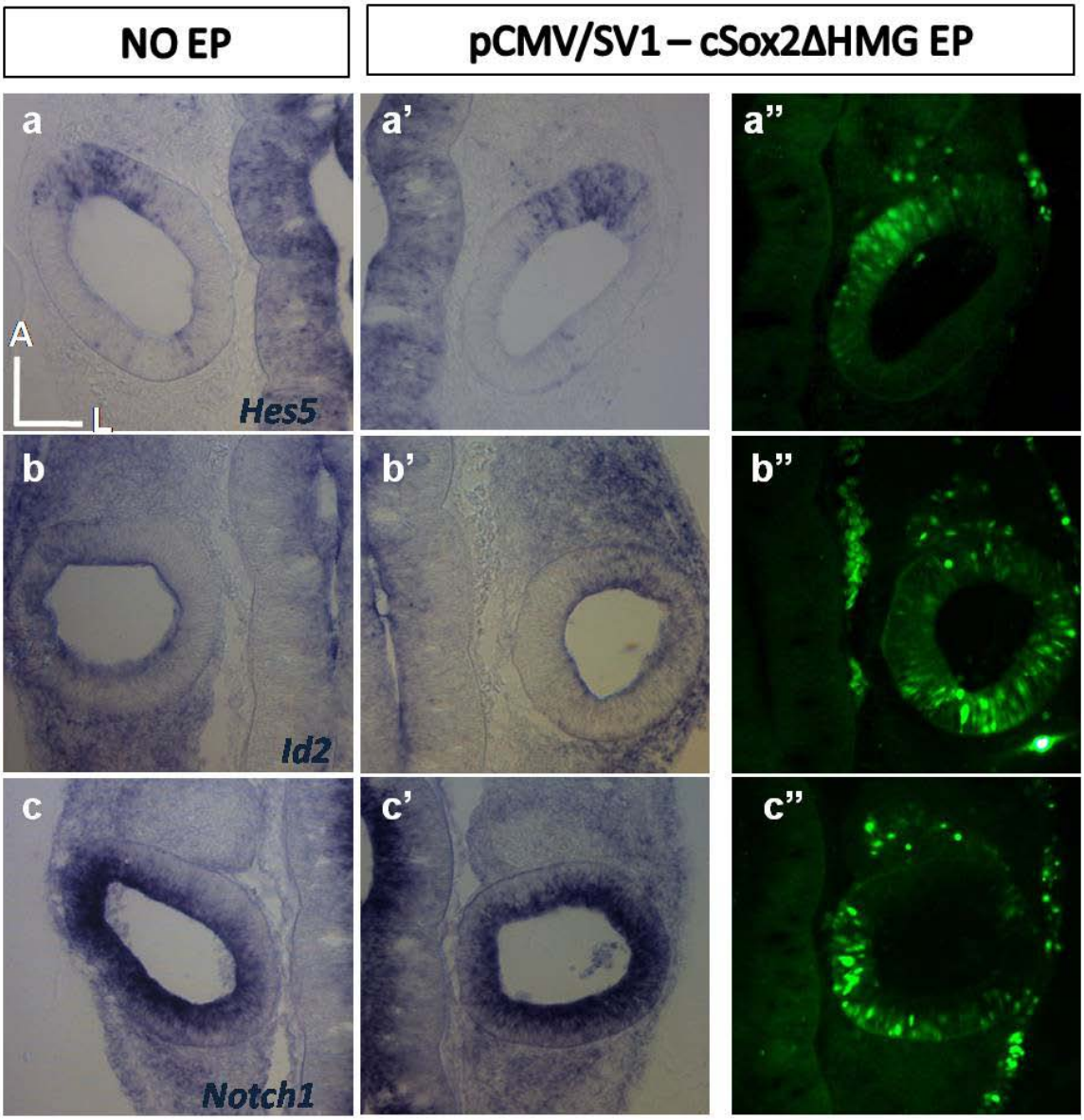


Figure 1: Effects of *Sox2 ΔHMG* transfection on *Hes5, Id2* and *Notch1* expression detected by ISH. Coronal sections of otic vesicles untransfected (a-c) and transfected with cSox2 ΔHMG (a'-c', a''-c'') 20h after electroporation, where *Hes5, Id2* and *Notch1* mRNA was detected by ISH. *Hes5* (a- a''), *Id2* (b-b'') and *Notch1* (c-c'') mRNA expression patter was not altered in electroporated regions 20h after transfection when compared to controls. A, anterior; L, lateral.

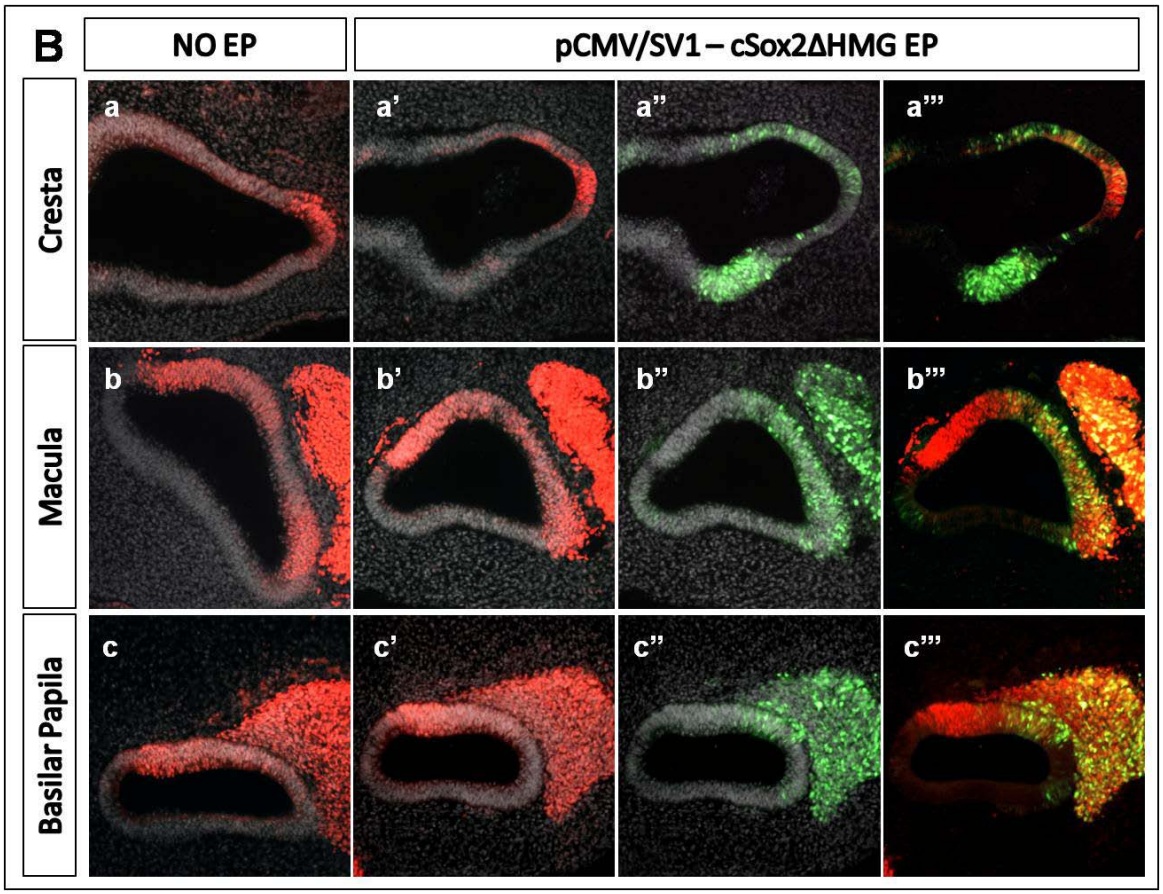
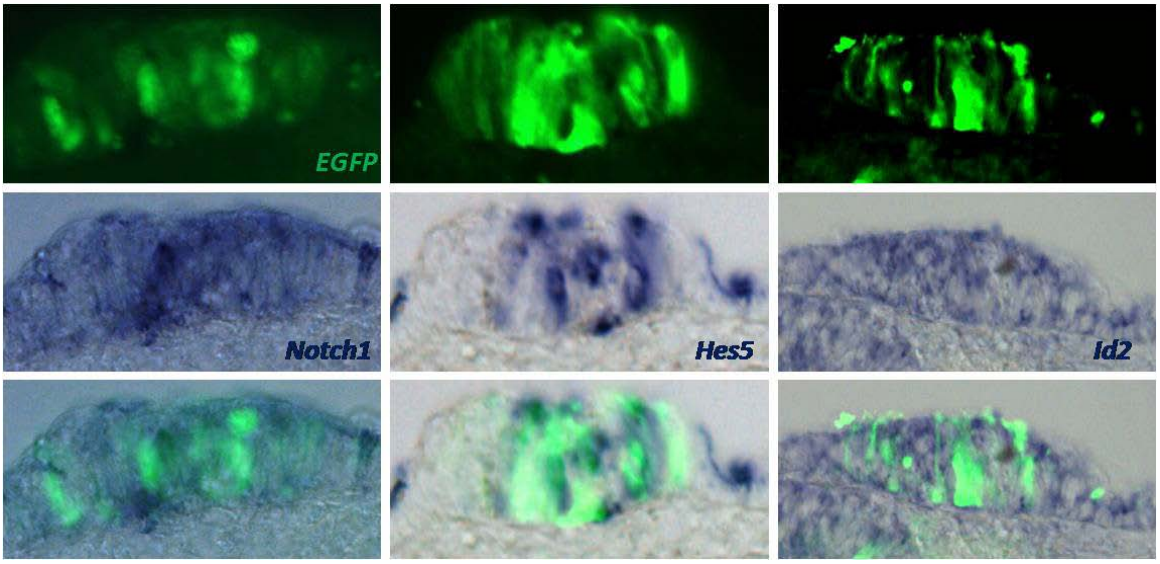


Figure 2: Effects of *Sox2 ΔHMG* transfection on Islet1 expression. Coronal sections of otocysts untransfected (a-c) and transfected with *cSox2ΔHMG* (a'-a''', b'-b''', c'-c'''), 48h after electroporation, stained for Islet1 (red) and GFP (green). GFP cells were detected both in the otic epithelium and in the cochleo-vestibular ganglion (b'-b''' and c'-c'''), supporting our hypothesis that many of the neuronal progneitors are already sppecified at the time of electroporation. Islet1 expression was detected in the cochleo-vestibular ganglion, where it co-expressed with GFP (yellow, b''' and c''') and also in GFP positive cells in the otic epithelium, within the prosensory domains (yellow, a''', b''' and c'''). This demonstrates that the effect observed upon Sox2 trasnfection is actually due to the function of the protein and not an artefact of the experimental conditions.

Supplementary Information 2: Morphological effects induced by Sox2 overexpression



The elctroporated embryos with pIRES-EGFP-cSox2 exhibited morphological alterations in the otic vesicle. Electroporated vesicles showed an incomplete closure of the otic pore as well as an altered orientation of the pore (27/29 embryos). This effect was observed with variable intensities, which correlated with the extent and strength of the electroporation. It was not observed in embryos electroporated with the empty vector (pIRES-EGFP) (10/11 embryos), nor in embryos where Sox2 was ectopically expressed outside the otic vesicle (4/4 embryos), suggesting that the altered morphogenesis was due to the over expression of Sox2..

The ectopic expression of Sox2 in the ectoderm adjacent to the otic placode also resulted in the induction of ectodermal thicknings similar to the ones descibed for Sox3 ectopic expression in the same terrytory 23/25 embryos electroporated with Sox2 exhibited those morphological thicknings which was not observed in controls (n=0/10). This suggests that Sox2 and Sox3 may play

Supplementary Information 3: Notch pathway does not regulate Id genes

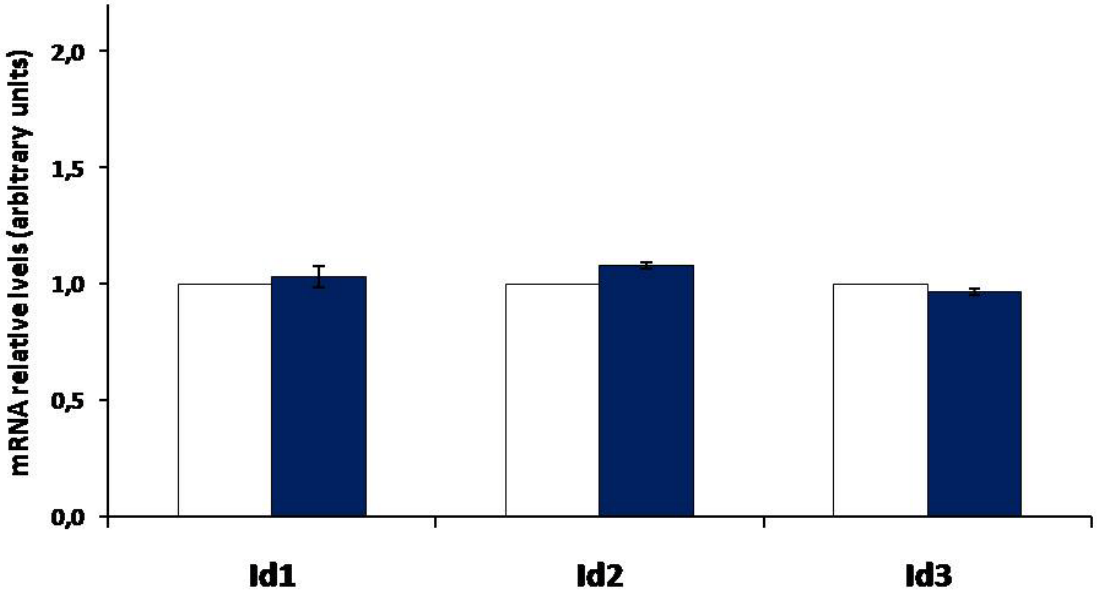


Figure 1: Notch signalling does not regulate *Id* genes expression in the otic epithelium. Bar diagram showing the relative mRNA levels of *Id1*, *Id2* and *Id3* in otic vesicles transfected treated with DAPT (Blue bars) in respect to controls (white bars) *Id1, Id2, Id3* mRNA levels were not significantly increased between control condition and treated otic vesicles

Supplementary Information 4: Characterization of Rabbit polyclonal anti- Sox2 antibody.

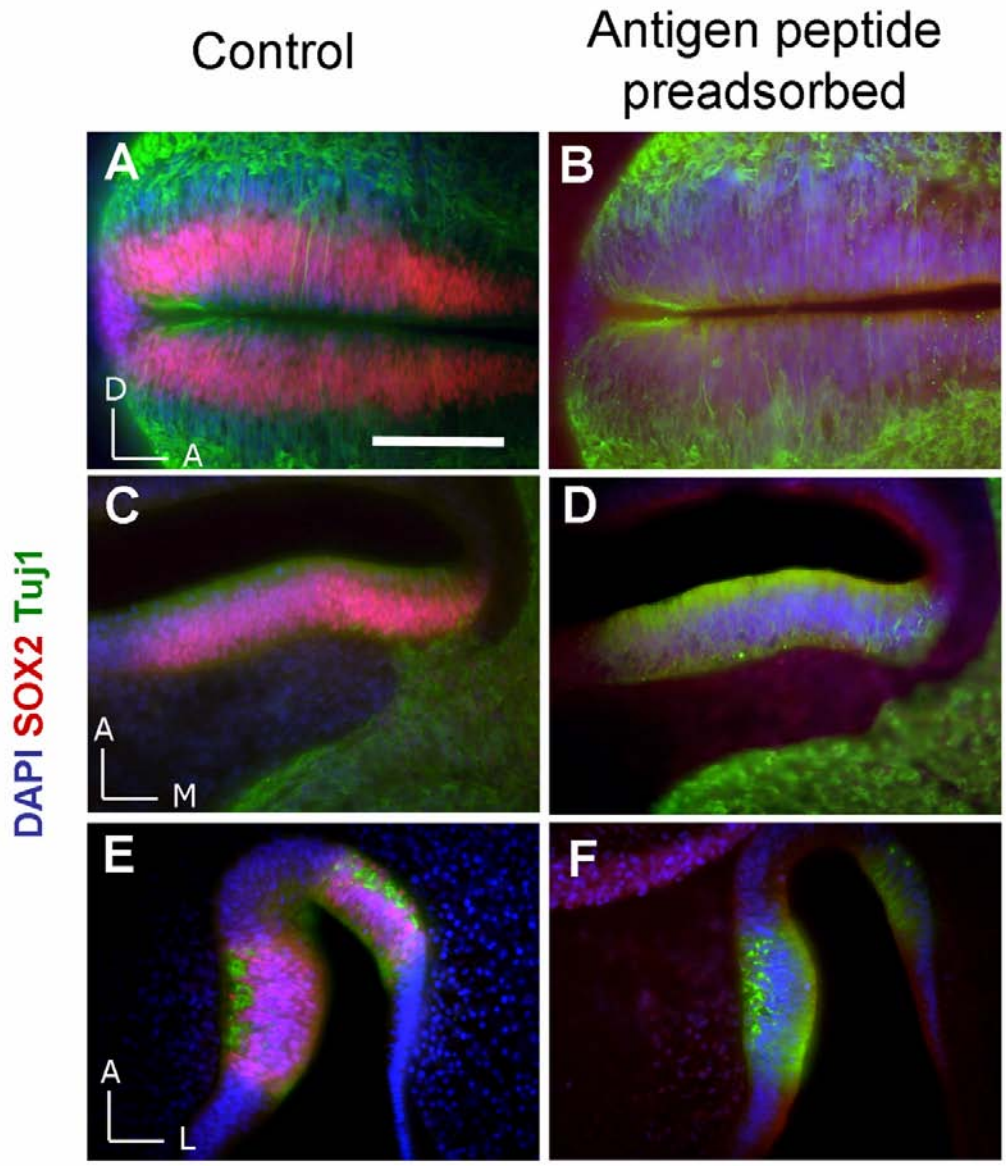


Figure 1: SOX2 nuclear staining is displaced by antigen peptide. Alternate coronal sections of an E5 otocyst were immunostained using antiSOX2 antibody Ab15830 (red) and antiTuj1 antibody (green). The left column shows the conventional immunofluorescence procedure. The right column shows equivalent alternate sections in which the primary antibody was preadsorbed with the peptide Ab15831. The antigen peptide displaced the SOX2 antibody binding to the ventricular zone of the neural tube (B), basilar papilla (D) and superior and lateral cristae (F). Scale bar= 10µm. Methods:1ml of rabbit polyclonal antiSOX2 antibody was incubated in parallel with 2,5 ml of PBS and 2,5 ml of SOX2 antigen peptide used to raise the antibody (Abcam, ab15831) at 37ºC. After incubation the mixture was centrifuged at maximum speed for 15min to spin down the complexes. Volume was made up to 400µl in each tube and both preadsorbed and non-preadsorbed solutions were used as described in the immunostaining protocol.

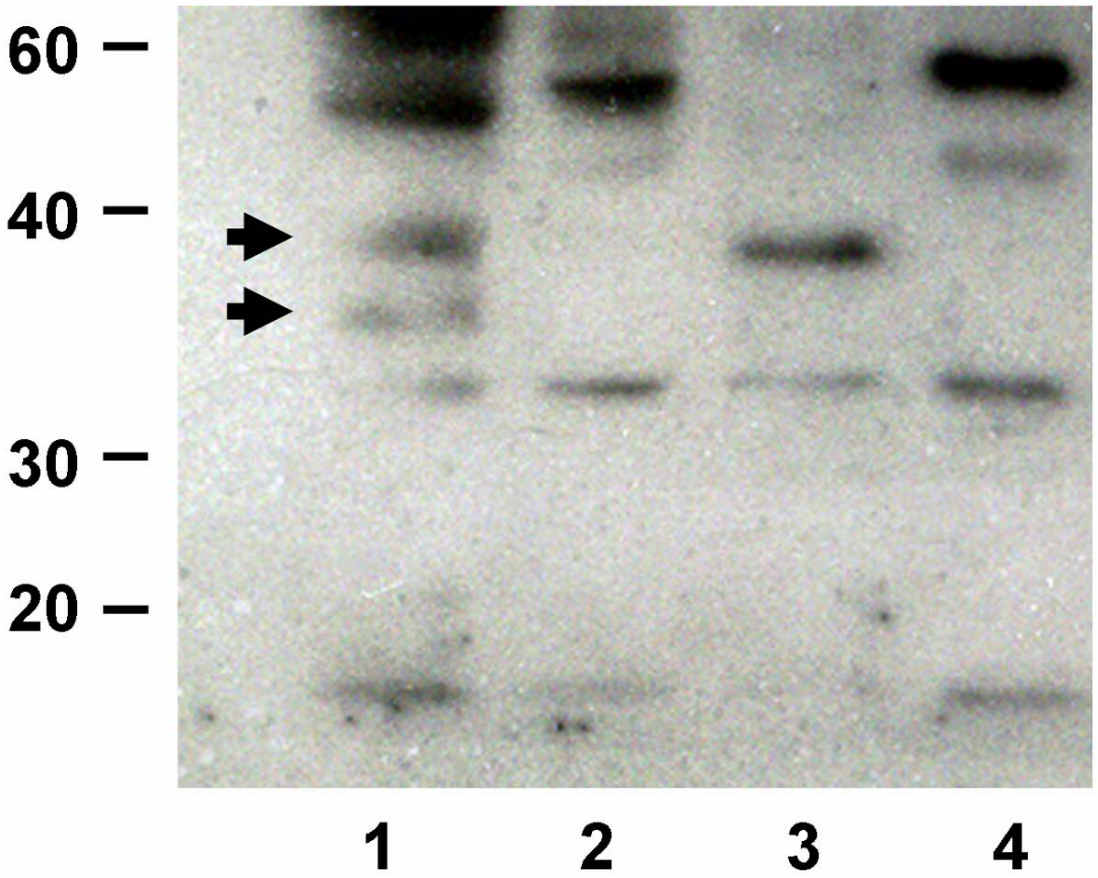


Figure 2. Western blot analysis of chicken embryo extracts of E3.5 with SOX2 antibody Ab15830. Immunodetection of SOX2 protein extracts blotted to a filter membrane after electrophoresis. Lane 1: nuclear extract of neuroectoderm from E3.5 chicken embryos. Lane 2: extraembryonic tissue of the same embryos. Lane3: mouse embryonic stem cells. Lane 4: cytoplasmic extract of E3.5 chick neuroectoderm. The antiSOX2 antibody detected two bands of 38 and 35kDa (upper and lower arrows, respectively), that were not present in other extracts. The 38kDa band corresponded well to the band detected in the nuclear extracts of mouse ES cells in lane3. The lower band corresponded to the predicted mw of SOX2. Neither extraembryonic membranes (lane 3) or cytoplasmic embryonic extracts of neuroectoderm (lane 4) showed those two bands. Methods: Protein extracts from 26mg of E3.5 chicken neuroectoderm tissue and 126 mg of E3.5 chicken extra-embryonic tissue were obtained using a modified protocol from Schreiber et al. (1989) Nucleic Acid Res. 17:6419. Samples were sonicated with the first lysate buffer and additional protease inhibitors. Protein extracts from ES CGR8 cells were obtained using RIPA buffer. 13,6 µl of protein extracts were mixed with 3x sample loading buffer and boiled for 5 min at 95ºC. 20 µl was loaded in each well of a 12,5%polyacrilamide gel and run at 150V for 2h. Transfer to a PVDF membrane (Immobilon Transfer membranes, Millipore) was preformed overnight at 4ºC and 30mA. Membrane was blocked with 5% milk in TBST and incubated with antiSOX2 antibody diluted 1:4000 in 1%milk in TBST at room temperature for 1h. Membrane was washed 3 times with TBST and incubated with the secondary antibody (ECL anti-rabbit IgG, peroxidise-linked species-specific whole antibody, from donkey- Amersham Biosciences), diluted 1:3000 in 1%milk in TBST, for 30 min at room temperature. SuperSignal West Pico Chemiluminescent Substract (Pierce) was used for development.

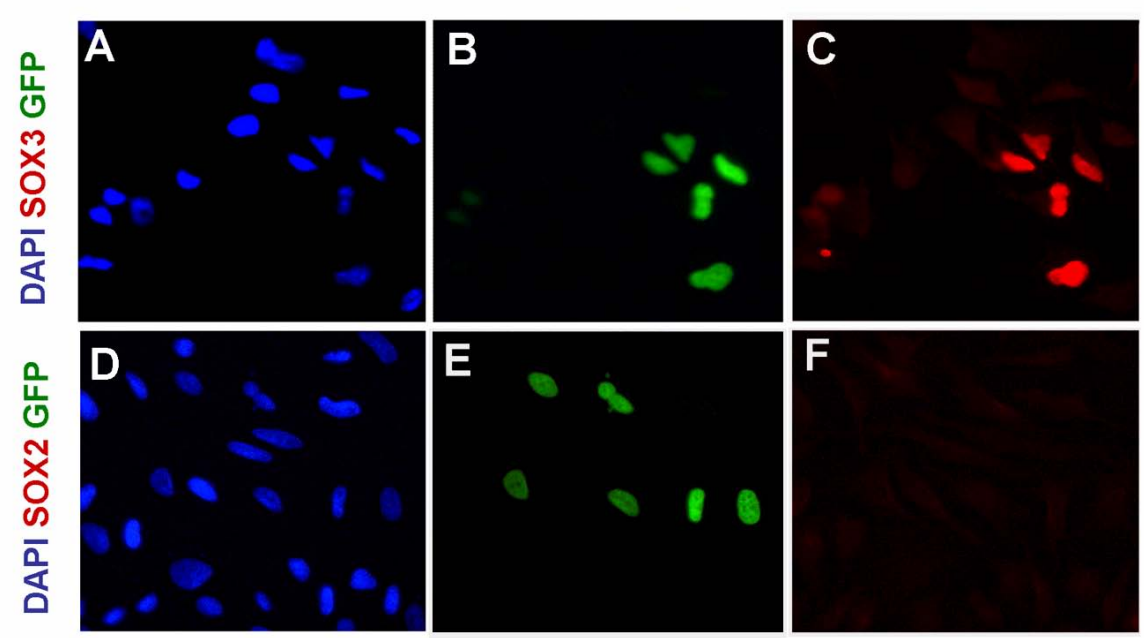


Figure 3: SOX3-GFP fusion protein expressed in HeLa cells is recognized by antiSOX3 antibody, but not by antiSOX2 antibody. HeLa cells were transfected with cSOX3-pEGFPC1 and immunostained 24 hours after transfection using either antiSOX3 (Ab from Edlund & Muhr, upper row) or antiSOX2 antibody (Ab15830, lower row). All nuclei were counter-stained with DAPI (Blue) (A and D). Transfected cells were observed in green, corresponding to the green fluorescent protein expressed in their nuclei (B and E). AntiSOX3 antibody stained the nuclei of only those cells that were GFP-positive (C). AntiSOX2 antibody did not react with HeLa cells, either transfected or not (F). Scale bar=20 µm. Methods: Human HeLa cells from cell line CCL-2 were cultured over ethanol sterilized cover slips at a 30% confluence, in 500µl of DMEM medium and let grow for 24 hours at 37ºC. FuGene 6 reagent (Roche) was complexed with cSOX3-PEGFPC1 construct DNA (Professor J David Brook, School of Biology, The University of Nottingham) in a proportion 3:1,5 (µl FuGene : µg DNA) in 100µl OptiMEM medium and approximately 25 µl of the complex were added the cultured cells. Cells were allowed to rest at 37ºC for 24h, washed with PBS and fixed with 4%PFA in PBS for 20 min on ice. Fixed cells were washed twice with PBST and blocked with 10%Goat Serum in PBST solution for one hour. Primary antibody incubation was performed overnight at 4ºC in water saturated chamber, and secondary antibody incubation for 1h at room temperature, in water saturated chamber. Antibodies were used as follow: rabbit polyclonal anitSOX2 antibody, diluted 1:400 in blocking solution and rabbit polyclonal antiSOX3 antibody, diluted 1:300 in blocking solution. Secondary antibody was Alexa-594 conjugated goat antirabbit diluted 1:200 in blocking solution. Cells were examined under a conventional fluorescence microscope.

SUPPLEMENTARY INFORMATION

APPENDIX

APPENDIX 1

Neves J, Kamaid A, Alsina B, Giraldez F.
Differential expression of Sox2 and Sox3 in neuronal and sensory progenitors of the developing inner ear of the chick.
J Comp Neurol. 2007 Aug 1;503(4):487-500.

APPENDIX 2

Journal Section: Development/Plasticity/Repair

Title: *Id* genes mediate the regulation of *Atoh1* by BMP in the sensory epithelium of the inner ear

Abbreviated Title: BMPs, *Id* genes and hair cell development

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Number words: Abstract: 234/250, Introduction: 477/500, Materials & Methods: 717, Results: 2986, Discussion: 1510/1500, References:

Key words: hair cells, inner ear, neural development, sensory progenitor, Hes, Hey, Inhibitor of differentiation, Bone Morphogenetic Protein (BMP), SMAD

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Abstract

Hair cells are responsible for the initial step in the neural processing of sound. Their differentiation depends on the expression of *Atoh1*, a basic helix-loop-helix gene (bHLH) which regulation is not yet understood. BMP signaling regulates the generation of hair cells (Pujades et al., 2006) and *Id* genes regulate bHLH activity inhibiting neural differentiation and hair cell development (Jones et al., 2006). The purpose of this study was to explore the possible link between BMP signaling, *Id* genes and the regulation of hair cell production. The results show that BMP4 down-regulates *Atoh1* expression, whereas Noggin or Dorsomorphin induce the opposite effect, revealing that an endogenous BMP activity normally prevents the expression of *Atoh1.* In parallel, BMP4 induces the expression of *Id1-3* genes, and Noggin or Dorsomorphin suppress *Id* expression. *Id* genes are expressed along with BMP4 in the prosensory domains, but they are down-regulated from sensory patches during hair cell differentiation. Forced expression of *Id3* is sufficient to suppress *Atoh1* expression, suggesting that *Id*s behave as a molecular switch for hair cell differentiation. In addition, BMP4 results in the activation of the *Hes*-related gene *Hey2* in a Notch-independent manner, but this effect is not mediated by *Id.* These experiments suggest that BMP signaling prevents premature differentiation of the sensory organs of the inner ear through the regulation of the expression of *Id* genes, which are in turn sufficient for blocking *Atoh1* expression and hair cell differentiation.

Introduction

Hair cells (HC) of the inner ear are responsible for the initial step in the neural processing of sound and balance in vertebrates. The cellular elements of the ear originate from the otic placode, and their commitment to a specific lineage follows a stereotyped spatial and temporal sequence (Fritzsch et al., 2006; Jones et al., 2006; Abello and Alsina, 2007; Bell et al., 2008). The proneural gene *Atoh1* behaves as a master gene for hair cell specification, *Atoh1* being necessary and sufficient for hair cell generation (Bermingham et al., 1999; Zheng and Gao, 2000). However, little is known about the mechanism that triggers the expression of *Atoh1* in sensory patches.

Bone morphogenic proteins (BMPs) regulate essential processes in development (Hogan, 1996; Massague et al., 2005). BMP4 is expressed in the prosensory domains of the developing inner ear (Oh et al., 1996; Wu and Oh, 1996; Morsli et al., 1998), and several studies have revealed multiple functions in the formation of sensory and non-

sensory structures of the vestibular apparatus (Chang et al., 1999; Gerlach et al., 2000; Chang et al., 2002; Li et al., 2005; Pujades et al., 2006; Chang et al., 2008). One particular function of BMP4 may be the maintenance of self-renewal of otic progenitors (Pujades et al., 2006), a function that is reminiscent to the role played by BMPs in stem-cells (Chen and Panchision, 2007)

Inhibitor of Differentiation and DNA binding (Id) proteins are dominant negative regulators of bHLH proteins that promote self renewal and inhibit differentiation in several cell types (Benezra et al., 1990; Norton, 2000). Recently, *Id3* has been shown to regulate hair cell development in mouse cochlear explants (Jones et al., 2006), suggesting that Ids may regulate *Atoh1* expression in the ear. However, little is known about how are *Ids* regulated in the sensory epithelium. Since BMPs regulate Id expression in diverse cell lines and embryonic stem cells (Ruzinova and Benezra, 2003), we hypothesized that *Id* genes are directly regulated by BMP signalling in sensory progenitors, and this regulation is at the basis of the balance between self-renewal and hair cell generation.

Hes-related genes (*Hes*, *Hey* genes) are bHLH transcriptional repressors that maintain the undifferentiated state of embryonic progenitors (Fischer and Gessler, 2007; Kageyama et al., 2008). Several members are expressed throughout the development of the ear sensory organs (Hayashi et al., 2008; Doetzlhofer et al., 2009). Most, but not all *Hes*-related genes are induced upon Notch activation under certain cellular contexts (Hatakeyama et al., 2004; Kawamura et al., 2005). This opens the possibility that Notch and BMP signaling cooperate to regulate *Atoh1* expression and the transition between prosensory precursors and hair cells.

The present work explores these problems and shows that BMP4 regulates *Id* gene expression in the prosensory patches and, in turn, *Atoh1* and hair cell differentiation. This supports the notion that one critical function of BMP in the development of the sensory organs of the ear is to regulate the pool of sensory progenitors and to prevent precocious differentiation.

MATERIALS AND METHODS

Embryos Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38ºC for designated times and embryos were staged according to Hamburger and Hamilton, 1951. Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phospate-buffered saline (4%PFA/PBS) overnight at 4ºC.

Whole mount In situ Hybridization: For *in situ* hybridization (ISH), embryos were dissected in phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde, and processed according to Wilkinson and Nieto (Wilkinson and Nieto. 1993). Riboprobes utilized were: *Atoh1* (BSRC chick EST) Bmp4 (M.Ross), *Id*1, 2 and 3 (M. Bronner Fraser) and *Id*4 (E. Marti).

Electroporation: Two types of electroporation were carried out. The vector expressing a constitutively active form of the BMP receptor ALK3 (pCIG-actALK3-IRES-GFP, Thomas Schultheiss, Harvard, USA) was electroporated in the otic vesicle of HH14 chick embryos, by placing two parallel Pt electrodes at both sides of the embryo, and applying 8 pulses of 10V, 250ms, 50Hz. Embryos were allowed to develop *in ovo* for 6 hours. DNA (1µg/ml) was applied locally on the otic placode with a micropipette. The second procedure consisted of focal electroporations of HH20-HH21 otic vesicles with a vector expressing *mId3* (pCIG-m*Id*3-IRES-GFP, Elisa Martí, Barcelona) following a method modified from Chang et al. (2008). The cathode consisted of a 0.3 mm diameter Pt tip attached to a handle and the anode was a 0.5 mm diameter Pt electrode that was placed underneath the embryo. DNA was injected into the otic vesicle at a concentration of 6-8 µg/ml and electroporation conditions were 8 pulses of 10-12V, 250ms, 50Hz.

Organotypic cultures of otic vesicles: Otic vesicles were dissected from E3.5-4 embryos corresponding to stage HH20-23, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37ºC in a water-saturated atmosphere containing 5% CO_2 as described by Leon et al., 1995; Pujades et al., 2006 Additions were 1% fetal bovine serum (Bio Whittaker Europe), recombinant human BMP4 (R&D) was used in culture at concentrations of 20 and 100 ng/ml, Noggin (R&D) at 1µg/ml, Dorsomorphin (6-[4-[2-(1-Piperidinyl) ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine dihydrochloride (Biomol) at 10µM, and (N-[N-(3,5-diuorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, DAPT (Calbiochem) at 20µM.

Immunohistochemistry: 20 µm cryostat sections of whole mount-ISH treated, or naive embryos fixed over night with 4% paraformaldehyde, were processed for immunohistochemistry as described (Pujades et al., 2006) Antibodies and dilutions used were as follows: □-TUJ1, mouse monoclonal (Babco, 1:1000); □-PROX1 rabbit polyclonal (Abcam, 1:200); □-MyoVIIa rabbit polyclonal (provided by T.Hasson, 1:5000); □-Islet-1and

□-3A10 mouse monoclonal (developed by Tom Jessell, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242."Hybridoma Bank, both at 1:600), □-GFP mouse monoclonal (Invitrogen, 1:400), □-P-SMAD 1-5-8 rabbit polyclonal (Cell Signaling Technology, 1:400). Fluorescent detection was performed using secondary Alexa-conjugated goat antibodies (Molecular Probes, 1:2000).

Quantitation of results: In order to quantify changes in gene expression in the epithelium and in the mesenchyme separately, we adapted a method by Matkowskyj et al 2000. Briefly, several otic vesicles were serially sectioned and reconstructed (one per group are shown in Fig.1Cg). Digitalized photomicrographs were acquired with the same conditions of magnification, illumination and capture settings and imported into ImageJ software (Rasband, W.S., ImageJ, U.S.-National Institutes of Health, http://rsb.info.nih.gov/ij/, 1997-2008). An empty area situated at a position of the section that is not affected by the experimental treatment was used for reference and allowed comparison between different slides (Paizs et al., 2009) . Images were converted to binary and set up an intensity threshold (control epithelia without ISH signal). Either the entire otic epithelium or the surrounding mesenchymal tissue was selected on each section, and measured above the threshold level for each compartment. At least 3 entire otic vesicles were processed in each condition (control, BMP4, Noggin), and the results were plotted in a bar diagram in which epithelial and mesenchymal area values of Id expression are shown in different columns (Fig. 1Ch). The total area of epithelium (or mesenchyme) in each slide was considered as 100%. Values are expressed as mean ± SD.

Quantitative real-time polymerase chain reaction (qRTPCR): Otic vesicles were isolated from HH21-22 embryos, and cultured either in control conditions or in the presence of different concentrations of BMP4, Noggin or Dorsomorphin. RNA was isolated using RNeasy Mini kit (Qiagen) and purified mRNA was retrotranscribed with the Superscritpt III DNA polymerase (Invitrogen) using random primers (invitrogen).. Real time PCR was carried out using SybrGreen master mix (Roche) For each qRTPCR run, cDNA generated from 15 ng of RNA was used. Amplification was performed using an LightCycler (Roche). Primer sets (Invitrogen, Table 1) were designed to have comparable melt curves (Tm = 60 °C), and, when possible, spaming exon-exon juntions.. GAPDH was used as calibrator gene. mRNA relative levels are the difference between each treatment and the control, calculated using the ΔΔCt method. Results are the mean of 3 PCR quantifications for each gene, for 3 independent retrotranscriptions, averaged from 3 different experiments (5 otic vesicles per condition and experiment). Error bars represent S.E. about the mean. One asterisk indicates $p<0.05$ and double asterisk $p<0.01$, levels of

significance of the difference with respect to control values calculated by the Student's t-test

Table 1

Gene	5′ Sequence	3′ Sequence
GAPDH	TTGGCATTGTGGAGGGTCTT	GTGGACGCTGGGATGATGTT
Atoh1	AACCACGCCTTCGACCAG	TGCAGCGTCTCGTACTTGGA
Hes5	GAAATCCTGACACCCAAAGAG	TCAATGCTGCTGTTAATCCT
Sox2	AAGAGACCCTTCATTGACGA	CGTGTACTTATCCTTCTTCATCAG
Lfg	GAAGAGCTGCGGGAGGAAG	GCTCCACCATGAGCACCAG
Id1	GCACCGGAGGGTCTCTAAAGT	CCAGCTGCAGGTCCCAGAT
Id2	ACAGACATCAGCATCCTCTC	CACTCGCCATTAGTTCTGAG
Id3	CCCACCCCACCATTATGA	GCCTCGTAACAGCTCCTGAC
Hes1	GGCGCGCATCAACGA	TGGAGGTTCCTCAGGTGCTT
Hey1	CGGAGGGAAAGGTTATTTCG	CAGCAATGGGTGAGATATGTG
Hey2	CAACCACAACATCTCAGATTATGG	CAACTTCAGTCAAGCACTCC

Results

Bmp signaling regulates Atoh1 transcription

BMP4 regulates the generation of hair cells in the chick otic vesicle (Pujades et al., 2006), and we sought to explore the molecular mechanisms involved in this process. First, we measured the changes in *Atoh1* and *Hes5* mRNA levels upon activation or inhibition of BMP signaling (Fig. 1A). Otic vesicles from HH20 embryos were isolated by microsurgery, grown in culture and then analyzed for gene expression by quantitative-Real-Time-Polymerase-Chain-Reaction (qRT-PCR) using specific oligonucleotide primers (Fig. 1Aa). Incubation medium contained BMP4 (20 ng/ml), or Dorsomorphin (10µM), as an inhibitor of the BMP pathway (DM, (Yu et al., 2008)) *Atoh1* mRNA levels increased after incubation with Dorsomorphin, and they were reduced by BMP4 (Fig. 1Ab). The increase in *Atoh1* mRNA with Dorsomorphin indicates that under normal conditions there is an *endogenous* BMP activity that prevents the expression of *Atoh1.* In parallel, *Hes5* expression was also induced by Dorsomorphin and reduced by BMP4 (Fig. 1Ab), suggesting that the attenuation of BMP signaling promotes hair cell determination, and the consequent process of Notch-mediated lateral inhibition (Pujades et al., 2006). Together, these results indicate that during normal development, BMP activity prevents the expression of *Atoh1* and the initiation of hair cell differentiation.

Comentario [s1]: Fig. 1 near here

BMP signaling regulates Id expression in the inner ear

Id genes interfere with proneural function and are direct targets of BMPs in a variety of model systems (see introduction). We hypothesized that BMP signalling may inhibit *Atoh1* expression through the direct regulation of *Id* gene(s). In order to study this question we studied, first, the regulation of *Id*s by BMP4 in the otic vesicle and, secondly, the effects of the gain of function of *Id*s on *Atoh1* expression.

BMP4 induced the expression of *Id*1-3 after in isolated HH20 otic vesicles (Fig. 1Ba, blue bars). Increase in mRNA was about 2,5-fold for BMP4 20 ng/ml (and about 5-fold for 100 ng/ml, not shown). The induction of *Id* genes by BMP4 was very rapid, and increased levels of *Id*1-3 transcripts were detected already at 1h of incubation (Fig. 1Bb upper diagram). The addition of Dorsomorphin (10µM) or Noggin (1µg/ml, not shown) resulted in a strong and also rapid reduction of endogenous *Id* expression (Fig. 1Ba, red and light red bars, and 1Bb, lower diagram), suggesting that *Ids* have a fast turnover rate and that most if not all *Id* expression in otic vesicles is dependent on sustained BMP signaling.

The regulation of *Id* genes by BMP4 in the otocyst was further demonstrated by in situ hybridisation. A typical experiment is shown in Fig. 1C for *Id2*. When otic vesicles were grown in control medium, *Id2* expression was concentrated in two regions of the otocyst, one anterior and another posterior (Fig. 1Ca, see also the in vivo expression pattern below). BMP4 (5 hours) produced a strong and ubiquitous induction of *Id2* expression (Fig. 1Cb, n=10/10, 4 different experiments). On the contrary, the incubation in this occasion with Noggin during the same period abolished *Id2* expression (Fig. 1Cc, n=10/10, 4 experiments). The same results were obtained for *Id1* and *Id3* (n=10/10, at least two experiments for each gene, not shown). Cryostat sections of cultured otic vesicles showed that *Id* expression was regulated by BMP both in the epithelium and the surrounding mesenchyme (Figs. 1Cd-f). Again, BMP4 induced *Id2* expression in both the epithelium and mesenchyme (Fig. 1Ce), and the incubation with Noggin caused an almost complete downregulation of *Id2* (Fig. 1Cf). Only a few vesicles (n=2/10) retained a weak residual signal for *Id2* after incubation with Noggin, and this was mostly located in the mesenchyme adjacent to the epithelium (arrows in Fig. 1Ce and f). To quantify these effects separately in the epithelium and in the mesenchyme, we reconstructed the area of *Id* expression (bar diagram in Fig. 1Ch, see methods)

BMP induced Id expression in parallel with SMAD1,5,8 phosphorylation

Upon BMP binding to its receptors, a series of phosphorylation events transduce this signal to the receiving cell. Although BMP may activate several intracellular pathways, the main mechanism involved in the cellular response to BMPs is the phosphorylation of SMAD1-5-8 proteins, which results in binding to SMAD4, translocation of the complex to the nucleus and regulation of gene expression (Massague et al., 2005). The observation that Noggin and Dorsomorphin show similar effects on *Id* expression (Nogging impeding BMP receptor binding and DM the phosphorylation of R-SMADs) suggest that the effects of BMP on *Id* expression indeed depend on the activation of the SMAD pathway (Cuny et al., 2008; Yu et al., 2008). We studied that further by comparing SMAD phosphorylation and *Id* expression during normal development and after BMP4 stimulation. Otic vesicles of E3 embryos were cryosectioned and analysed by ISH for the expression of *Ids*, and by immunochemistry for the phosphorylated form of SMAD 1-5-8 (P-SMAD1-5-8). Immunoreactivity was restricted to regions of the otic epithelium that corresponded to the domains of *Id2* expression (Fig 2Aa-a'). Incubation of otic vesicles with BMP4 for 4 hours, increased *Id2* expression, together with P-SMAD immunoreactivity all throughout the epithelium (Fig 2Ab-b'). Further, the overexpression of a constitutively active form of

Comentario [s2]: Fig.2 near here

BMPRI-a (act-ALK3) in the otic epithelium produced a parallel activation of P-SMAD1-5-8 and *Id* expression (Fig. 2Ba-c and a'-c'). Electroporated otic vesicles exhibited a strong upregulation of the expression of *Id*1, *Id2* and *Id*3 (compare Figs. 2Ba and a' and b and b' for *Id3*, n=3 for each gene, two different experiments). This was paralleled by the upregulation of P-SMAD1-5-8 immunoreactivity (Fig. 2c, c'). The same vector containing only GFP produced no differences (n=10 at least three experiments, not shown).

In summary, over-activation of the BMP signalling pathway either with exogenous BMP4 or by overexpression of the constitutively activated receptor ALK3, provoked a rapid increase in P-SMAD1-5-8 and *Id1-3* mRNA. The fact that Bmp4 induced *Id* expression so rapidly in all regions of the otocyst implies that BMP receptors must be present ubiquitously in the otic epithelium, making all the tissue competent to respond to the BMP signal. Conversely, blockade of BMP signalling with Noggin or Dorsomorphin was sufficient to rapidly downregulate the expression *Id*1-3 genes demonstrating that the endogenous activation of the BMP pathway is strictly required for the normal expression of *Id*1-3 genes in the otic vesicle. This indicates that *Id* mRNAs must have a high turnover so they are rapidly degraded in the absence of BMP signal, making *Id*1-3 expression dependent on the continuous activation of the pathway.

Id genes are initially expressed in prosensory regions of the otic epithelium

Since *Id* genes behave as reliable readouts of BMP activity, we wanted to know the relation between *Id* and hair cell differentiation so to test the idea that BMP signaling is attenuated in vivo during hair cell differentiation. The expression of *Id*1-3 genes was analysed by in situ hybridisation during the stages of sensory organ formation, between embryonic day 3 (E3, HH18-20) and day 7 (E7, HH29-30). We shall describe first the expression pattern of the *Id*1-4 genes during the period of sensory patch formation (E3-4) and then during the differentiation of the sensory organs (E5 and E7). *Bmp4* is expressed in the prosensory and sensory domains throughout the observed periods (Wu and Oh, 1996; Pujades et al., 2006), but only after E5 *Atoh1* expression begins in the cristae, and, later on, in the maculae and basilar papilla.

Figure 3A shows the expression of all four *Id* genes in HH19 embryos, where *Bmp4* and *Atoh1* are shown for comparison. *Bmp4* was expressed as two distinct patches in the otic epithelium at the equator of the otocyst (Fig. 3Aa-a''), which was paralleled by *Id*1-3 (Figs. 3Ab-d'). *Id*4 was not detected in whole mount preparations (Fig.3Ae-e'). Cryosectioning of these embryos revealed that *Ids* were expressed in the otic epithelium,

Comentario [s3]: Fig. 3 near here

including the prosensory patches, and the non-sensory epithelium of the lateral wall of the otocyst (Fig. 3Ab''-e''). The medio-ventral wall of the otocyst was consistently devoid of *Id* expression (see Fig. 3Ac'-e'). *Id1* and *Id2* expression was high in the mesenchyme adjacent to the prosensory patches and surrounding the cochleo-vestibular ganglion (CVG) (Fig. 3Ab', c'). *Id2* was expressed in some cells of the CVG, but not *Id1* (Fig. 3Ab'', c''). As mentioned above, *Atoh1* expression was not yet detected at this stage.

Expression of *Id2* in a HH23 otocyst is shown in Fig. 3B. Coronal sections were probed for *Id2* and then processed for double immunohistochemistry using antibodies against Prox1 and TUJ1. The in situ hybridisation signal is shown in red and nuclei were counterstained with DAPI. A schematic diagram exemplifying the analysis of *Id* gene expression pattern is shown in Suppl. Fig. 1. *Id* extended to two discrete regions that anticipated the location of the anterior and posterior cristae (Fig. 3Ba-c). The posterior domain was continuous from dorsal to ventral (Fig. 3Bc-f, and Suppl Fig. 1). *Id2* expression was absent from the anterior ventral region, where Prox1 positive cells were still observed (Fig. 3Bd). Those cells expressing Prox1, but not *Id2* are likely to be neuronal progenitors rather than prosensory precursors (see Stone et al., 2003). In the ventral tip of the otic vesicle *Id2* was highly expressed in the posterior anlagen of the auditory sensory epithelium, as identified by the TUJ1 positive signal (Fig. 3Be, f). *Id2* was also expressed in the periotic mesenchyme in the dorsal regions of the otocyst (Fig. 3Ba-d, and g). A similar pattern was observed for *Id3* and is illustrated in Suppl Fig. 2. In summary, *Id* genes were expressed in the otic epithelium at the initial steps of prosensory patch formation, including the areas defined by the expression of *Bmp4* Sox2, Prox1 (Oh et al., 1996; Stone et al., 2003; Neves et al., 2007). *Id* expression domains were broader than the prosensory patches, and included the adjacent non sensory epithelium (Oh et al., 1996). Moreover, strong expression of *Id*1-3 genes was found in the periotic mesenchyme adjacent to the sensory patches.

Id genes are down-regulated during differentiation of sensory patches

Between days 3 and 5, new prosensory patches emerge and become distinguishable at specific times and locations (Wu and Oh, 1996; Neves et al., 2007). The first hair cells are generated at this stage, and the order of hair cell formation in the chick inner ear is well defined: vestibular organs mature before the auditory basilar papilla (Bartolami et al., 1991; Oh et al., 1996; Wu and Oh, 1996; Fritzsch et al., 2002; Bell et al., 2008).

Double ISH hybridization experiments with *Atoh1* and *Id2*-3 probes showed that the expression of *Id* genes was downregulated in the regions were *Atoh1* was expressed. This is illustrated by transversal sections at stage HH27 showing the absence of *Id3* expression in the lateral crista and sacular maculae (lc, ms, Fig. 4A). A high magnification view of the lateral cristae showed also that the non-sensory epithelium surrounding the crista retained high levels of *Id3* mRNA (Fig. 4B), while, in contrast, it was down-regulated from the dome-shaped sensory tissue that expressed *Atoh1* (Fig. 4B'). Similar results were obtained after staining the same sections with an antibody against MyoVIIa to reveal the presence of differentiated hair cells in the macula sacularis (Fig.4C, D and D'). However, at this stage *Id3* was still expressed in the elongated cochlear duct which, as mentioned above, differentiates later in development (Fig. 4C, bp). Identical pattern was observed for *Id2*, as illustrated by the proximal region of the cochlear duct (Fig. 4F-F''). The area positive for *Id3* and *Id2* was identified as the prosensory auditory region with Islet-1 antibody (Fig. 4F). Merged images, in which *Id2* ISH was pseudocoloured in blue showed that *Id2* was still expressed in auditory progenitors during this stage (Fig.4F''). Examination of the basilar papilla at E7 also revealed the withdrawal of *Id3* during the initiation of hair cell differentiation (Suppl. Fig.3).

Comentario [s4]: Fig. 4 near here

These results show that *in vivo*, the sequence of *Id* attenuation from the sensory patches followed the dorsal/vestibular to ventral/auditory sequence of hair cell generation. Note that BMP4 was continuously expressed in the sensory patches throughout this period (Suppl Fig.3). These observations, along with previous results ((Jones et al., 2006; Pujades et al., 2006) suggest that BMP-dependent *Id* expression contributes to prevent neurosensory progenitors from differentiation.

Id genes suppress Atoh1 expression

The question arising at this stage was how BMP signaling prevents *Atoh1* expression and hair cell differentiation, and whether *Id*s are part of this mechanism, i.e.: are *Id* genes sufficient to prevent *Atoh1* expression? Otic vesicles (HH20) were electroporated with a vector expressing mouse *Id3 (*m*Id3),* and analysed after 20h for MyoVII and *Atoh1* expression by immunochemistry, in situ hybridisation and qRTPCR. Electroporations were performed focally, in the anterior aspect of the otic vesicle (Fig. 5A a-c). Cells overexpressing m*Id3* did not express MyoVII or *Atoh1* and concentrated in the basal aspect of the epithelium (Figs. 5Ba and b, respectively). Note that outside the sensory region, *mId3*-positive cells resided throughout the depth of the epithelium, but within the

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sensory patch they remained concentrated at its basal aspect. This indicates that *mId3* either maintains cells in the progenitor state, it directs supporting vs. hair cell fate, or both. However, whether or not *mId3* had an effect on *Atoh1* expression was difficult to quantify from in situ preparations. In order to analyze this question, *Atoh1* mRNA levels were estimated by qRT-PCR. Otic vesicles were electroporated as described above and the targeted domain was dissected for qRT-PCR analysis (Fig. 5Bc). The results show that *mId3* was sufficient to induce a decrease of *Atoh1* mRNA below control levels (Fig. 5C). The suppression of *Atoh1* was paralleled by the reduced expression of *Hes5* (Fig.5Cc), as expected from the concomitant reduction of lateral inhibition.

Bmp4 and Hes/Hey-related genes

Hes and *Hey* genes are vertebrate homologues of *Drosophila hairy* and *Enhancer of split*, which encode bHLH transcriptional repressors. *Hes* genes are effectors of the Notch signaling pathway, and are able to regulate proneural bHLH activity, thus maintaining the undifferentiated state of neural progenitors (Fischer and Gessler, 2007; Kageyama et al., 2008). This parallel between the functions of BMP and Notch pathway in the maintenance of the undifferentiated state, suggested us to study the possible regulation of *Hes*-related genes by BMP4, and their relation to *Id* activation. For this purpose we screened the effects of BMP4 on the expression of the four major *Hes*-related genes expressed in the ear. Fig. 6A shows the results of the qRTPCR analysis of isolated otic vesicles that were treated with BMP4 or DM for 20h. The results show that BMP4 induced a somehow paradoxical effect on *Hes*-related genes, increasing the expression of *Hey2 and Hes1,* but suppressing that of *Hes5*, *Hey1* remaining unaltered. On the other hand, DM produced a mirror image of BMP4, suppressing *Hey2* and increasing *Hes5* expression levels. Both effects were also present after 5h incubation, but with less intensity (not shown)

Comentario [s6]: Fig. 6 near here

The opposing effects of BMP4 and DM on *Hes5* and *Hey2* were further explored by examining whether or not they were dependent on the activation of Notch. Fig. 6B shows the effects of BMP4 and DM on *Hey2* (left bar diagram) and *Hes5* (middle bar diagram), both in standard medium or in the presence of the γ-secretase inhibitor DAPT (Dovey et al., 2001; Geling et al., 2002). The effects on *Atoh1* are shown in the bar diagram at the right for comparison. The results show that *Hey2* expression was only partially dependent on Notch activity and, moreover, that the BMP-induced expression of *Hey2* was independent on Notch (Fig. 6B, left diagram). Therefore, BMP activation regulates *Hey2* expression and provides an alternative mechanism by which BMP4 may prevent the

expression of *Atoh1* and hair cell differentiation. On the other hand, *Hes5* expression was totally suppressed by DAPT, as it was the increase in *Hes5* mRNA after BMP blockade with DM (Fig. 6B, middle diagram). The effects of DAPT on *Hes5* expression indicate that *Hes5* expression is tightly coupled to Notch activation, in which absence *Hes5* it was deaf to changes in BMP signaling. The divergent responses of *Hey2* and *Hes5* to BMP and DM may be better understood when compared to the behavior of *Atoh1* in the same otic vesicles. *Atoh1* was induced by DM and by DAPT (Fig, 6B, right diagram), as expected after massive Notch blockade and the consequent release of repressor activity on *Atoh1* exerted by BMP and Notch down-stream targets. However, in the presence of DAPT, the expression of *Atoh1* was respectively reduced or increased by BMP4 or DM (BMP: blue and grey bars, DM: red and grey bars).

Given that BMP4 induces both *Hey2* and *Id* genes, one interesting possibility would be that *Hey2* expression may results from the induction of *Id* by BMP4 (See Bai et al, 2007). With this in mind, we tested the effects of *mId3* overexpression on *Hey2* and other *Hes*-related genes (Fig. 6C). In our hands, however, the forced expression of *mId3* did not induce substantial effects on *Hey2* or other *Hes*-related genes (Fig. 6C), suggesting that *Ids* and *Hey2* are activated in parallel, as part of the BMP4 gene synexpression group.

DISCUSSION

The experiments described in this paper were aimed at studying the mechanisms by which BMP signalling regulates the cell fate of sensory progenitors of the inner ear. In particular, we focused on the transcriptional targets of BMPs that can regulate Atoh1 expression and therefore HC generation.

Id genes are regulated by BMP in the prosensory patches

The direct regulation of *Id* gene transcription by BMP has been extensively studied in several model systems (Yokota et al.; Ruzinova and Benezra, 2003). We show that *Id*1-3 genes are induced by BMP4, in parallel to P-SMAD1-5-8 expression. The initiation of the response is very fast, suggesting that *Id*1-3 are direct transcriptional targets of BMPs in the otic epithelium (Hollnagel et al., 1999). BMP blockade rapidly reduces *Id* expression in the prosensory epithelium, indicating that the endogenous BMP signaling is required to maintain *Id* expression in vivo. This agrees well with the fast turnover of the *Id* mRNA and protein that have reported half-lives of 20-60 min, depending on the cell type (Deed et al., 1996; Bounpheng et al., 1999; Norton, 2000). Furthermore, Dorsomorphin phenocopied the effects of Noggin. Dorsomorphin has been shown to specifically block the phosphorylation of R-Smad mediated by type I BMP-receptors without affecting other signaling pathways that can be activated by BMPs (Anderson and Darshan, 2008; Cuny et al., 2008; Hao et al., 2008; Yu et al., 2008)*Id* expression in the otocyst, therefore, depends strictly on the steady activation of the BMP-induced SMAD signaling pathway.

Id1-3 genes are expressed in the prosensory patches, but they are down-regulated during hair cell differentiation

Recent studies have reported the expression pattern of *Id* genes at early stages of development in the chick, but they did not reach the stages of sensory organ formation (Kee and Bronner-Fraser, 2001a, b, c). In mammals *Id1, Id2*, and *Id3* are expressed in the otic vesicle of mice and rats (Jen et al., 1997; Lin et al., 2003) and in the developing mouse cochlea ((Jones et al., 2006). We provide here a detailed description of *Id* genes in the chick otocyst during the initial stages of sensory organ formation in the auditory and the vestibular otic epithelia. The results show that *Id1-3* are expressed in the otic vesicle in broad regions that include the prosensory patches, and precede the onset of expression of *Atoh1*. But, further in development, *Id*s are down-regulated from

sensory patches in parallel to the onset of *Atoh1* expression, and they follow the characteristic dorsal/vestibular to ventral/auditory sequence of molecular differentiation.

Given that *Id1-3* expression strictly depends upon BMP signalling it is reasonable to think that *Id* transcription reflects the local activity of the pathway *in situ*. Then, the spatial and temporal pattern of *Id* expression *in vivo* strongly suggest that local attenuation of the BMP *activity* in the prosensory patches is required for hair cell generation, rather than the regulation of *Bmp4* expression. Indeed, downregulation of *Id* occurs in regions where *Bmp4* is still expressed (Wu and Oh, 1996, and this work, suppl fig 3). This is interesting in connection with the model proposed by Chang et al. (2008), where a sequential action of BMP4 is proposed, first to promote prosensory development, and secondly for specification of non-sensory elements. We like to suggest that proper BMP signaling is necessary for maintaining a high expression of *Id* and the proliferative state of prosensory progenitors and to prevent the from premature differentiation. Further in development, however, BMP activity is silenced within the sensory patches, allowing *Id* suppression and hair cell-determination. But its persistence in the surrounding domains would promote the expression of genes like *Msx* and *Lmo* that dictate the non-neural fate within sensory domains (Chang et al., 2008).

How is BMP activity silenced within the sensory patches? There are several BMP inhibitors whose gain or loss of function induce impairement of ear development. However, none of them has been shown to be expressed in the prosensory regions at these critical stages of hair cell differentiation. One exception is DAN, which has been shown to be expressed in the dorsal-lateral otic epithelium, but its inhibition does not seem to affect sensory development (Gerlach-Bank et al., 2004). Intracellular inhibitors can also modulate BMP activity (Massague et al., 2005), but although the forced expression of Smad6 effectivelly inhibits BMP4 activity in the inner ear (Chang et al., 2008), there is little information about its expression pattern and function in the otic epithelia. Whether TGFb signaling can modulate regulation of *Id*s in hair cells remains to be determined (Paradies et al., 1998). Other signaling pathways modulate BMP activity including IGF, FGF and Wnt (Massague, 2003; Eivers et al., 2008). This cross-talk occurs at the level of Smad1-5-8 proteins, and relies in different phosphorylation sites within the Smad proteins (Massague, 2003). FGF signals may reduce the intensity of the BMP activity and contribute to silence BMP target genes (Massague, 2003; Eivers et al., 2008). Several FGF ligands are expressed in the prosensory regions, including Fgf-10 and Fgf19, and may account for such function (Sanchez-Calderon et al., 2002; Alsina et al., 2004; Sanchez-Calderon et al., 2007).

BMP signaling regulates Atoh1

Our results show that *Id* genes are sufficient to suppress *Atoh1* expression and hair cell differentiation. The generation of hair cells requires the activity of the bHLH factor *Atoh1* (Bermingham et al., 1999; Zheng and Gao, 2000), but the factors that regulate *Atoh1* transcription and activity during inner ear development are essentially unknown. *Ids* are BMP-induced-immediate early genes in ES cells, where they also function as inhibitors of cell differentiation through the functional inactivation of bHLH transcription factors (Hollnagel et al., 1999). Moreover, in some model systems the activity of bHLH genes such as *Atoh1* must be actively inhibited to prevent premature differentiation (Bertrand et al., 2002; Grimmer and Weiss, 2008) . Therefore, BMP-regulated *Id*s are good candidates to dictate the commitment of hair cells through the inhibition of Atoh1.

Id proteins act primarily as dominant-negative regulators of tissue specific bHLH transcription factors, such as Atoh1, and prevent them from forming functional complexes with transcriptional activity (Yokota, 2001). However, this does not explain the reduction in *Atoh1* mRNA levels after *Id* overexpression (present work), or that the overexpression of E47 does not block *Id* function (Jones et al., 2006). Alternatively, Id proteins may negatively regulate the activity of Atoh1 by increasing the rate of degradation of this proneural protein (Vinals et al., 2004; Vinals and Ventura, 2004). In addition, the *Atoh1* gene contains an E-box consensus binding site in its enhancer region that is related to its autoregulation (Helms et al., 2000), this autoregulatory enhancer being active in developing hair cells(Woods et al., 2004). Hence, the Id-induced destabilization of Atoh1 may result in a negative regulation of *Atoh1* mRNA levels (Zhao et al., 2008). In summary, BMP-mediated *Id* expression within the prosensory patches, may function as a molecular switch by functionally blocking the expression and activity of Atoh1.

BMP4 and Hes-related bHLH factors

The parallel between BMP and Notch pathway in the maintenance of the undifferentiated state, suggested us to study the possible regulation of *Hes*-related genes by BMP4, and their relation to *Id* activation. BMP4 induce two apparently paradoxical effects on *Hes5* and *Hey2*. *Hes5* is down-regulated by BMP4, it is dependent on Notch activation and secondary to lateral inhibition. On the contrary, *Hey2* is shown to be induced by BMP4 in a Notch-independent manner, which provides an additional molecular mechanism by which BMP4 may control the activity of prosensory progenitors. In the mouse cochlea,

Doetzlhofer et al. 2009 have shown that *Hey2* may act through a FGF-dependent regulation, independently of Notch signaling, in pillar cell fate determination. This suggests an interesting convergence between BMP, Notch and FGF pathways on the regulation of *Hes*-related genes.

Is there a direct interaction between *Ids* and Hey2? Recently, Bai et al. (2007) showed that *Id* genes prevent *Hes1* autoregulation, which in turn suppress neuron differentiation. But this seems not to be the case for *Hey2* in the inner ear, because *Hey2* expression is not enhanced by m*Id3* overexpression, The regulation of *Id*s and *Hey2* (and perhaps *Hes1*) appear as parallel events triggered by BMP as part of a gene synexpression group. Further work is required to analyse the interactions between the BMP pathway and Notch signaling pathways.

In summary, we provide here a mechanism by which BMPs regulate hair cell generation. This takes place through the transcriptional regulation of *Id* genes, which, in turn, prevents hair cell differentiation and maintains prosensory progenitors in an undifferentiated state. Only when BMP activity is locally attenuated *Id* expression is reduced, and hair cell development permitted. In addition, BMP4 may use alternative pathways to prevent hair cell differentiation including the regulation of *Hes*-related genes.

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LEGENDS TO FIGURES

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Figure 2. *Id* expression is associated with BMP pathway activation. A. BMP4 addition to cultured otic vesicles induces phosphorylation of SMAD1-5-8 proteins and *Id2* expression. **a-b'':** Cryostat sections of HH21 otic vesicles cultured during 4 hours in control conditions (a-a'') or in the presence of 100 ng/ml BMP4 (b-b''). Otic vesicles were processed for

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Supplemental figure 2. *Id*3 expression at at E3-5. A: representative section throught the otocyst showing high expression in vestibular (mu and ms) and auditory (bp) prosensory regions. **B**: Section through the presumptive basilar papilla probed for *Id*3 ISH. **C-C'**: adjacent section to that shown in B processed for IHC with antibodies against Sox2 (B') and Islet1 (D) to reveal the presence of sensory progenitors. White arrows (B-C'') indicate the prosensory region where *Id*3, SOX2 and Islet coincide. **D**: Schematic representation

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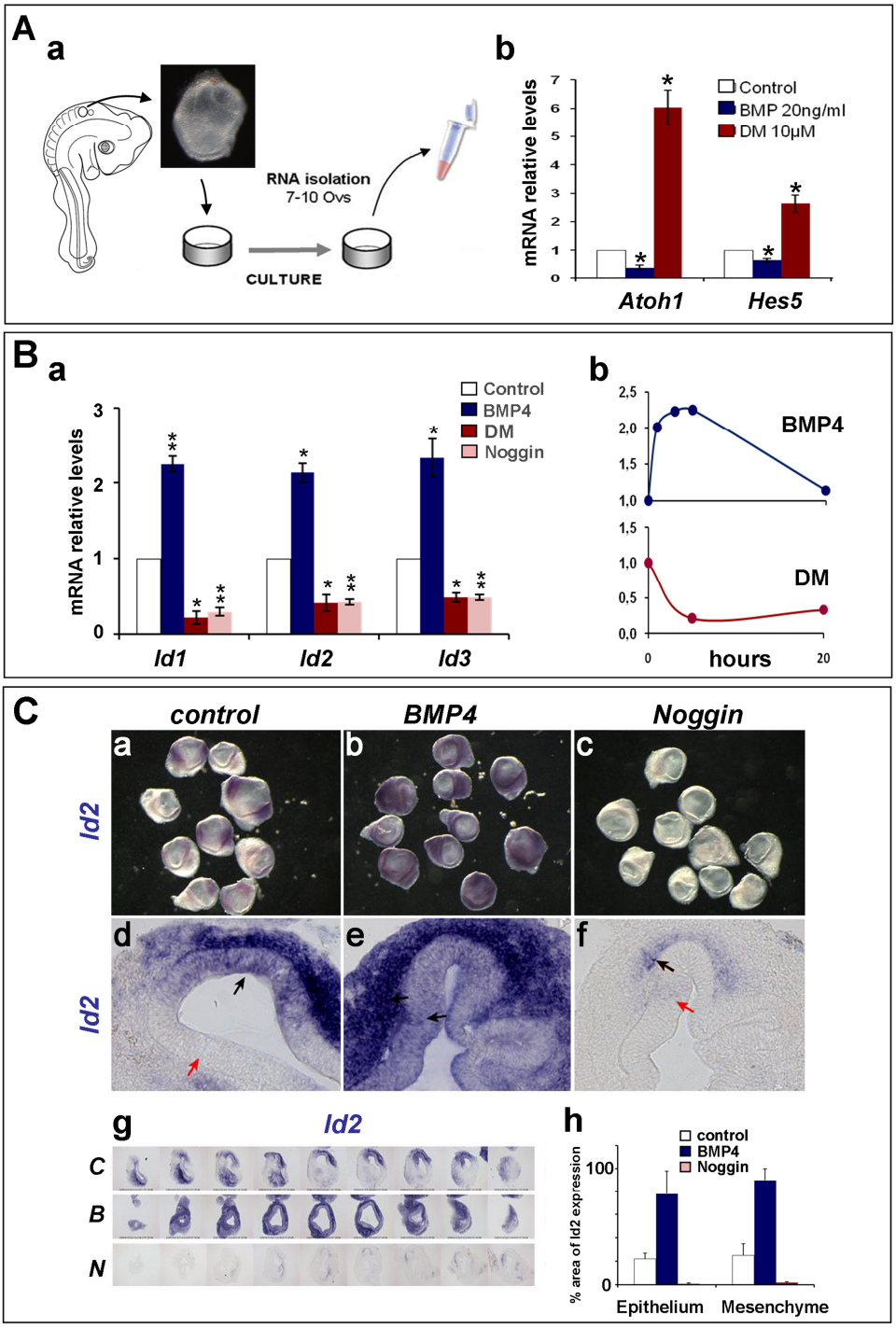
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% area of Id2 expression
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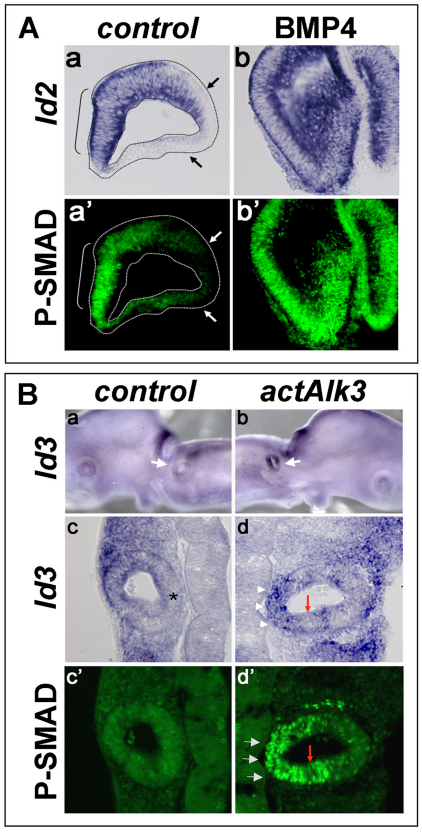

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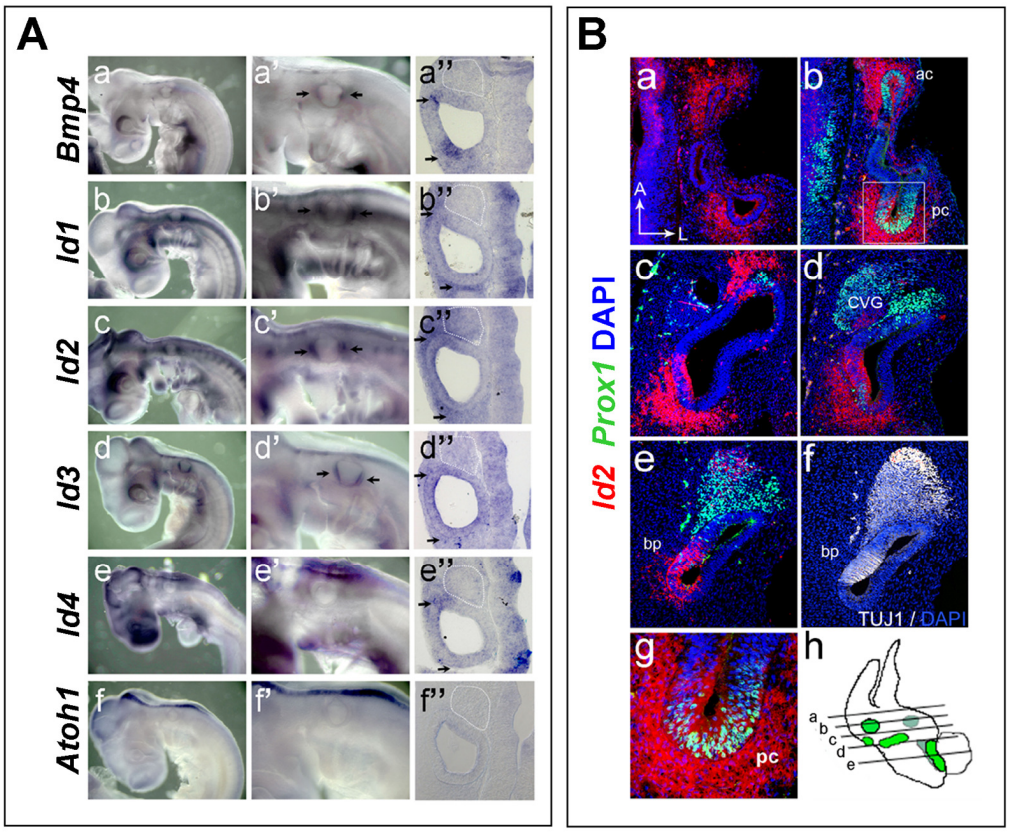

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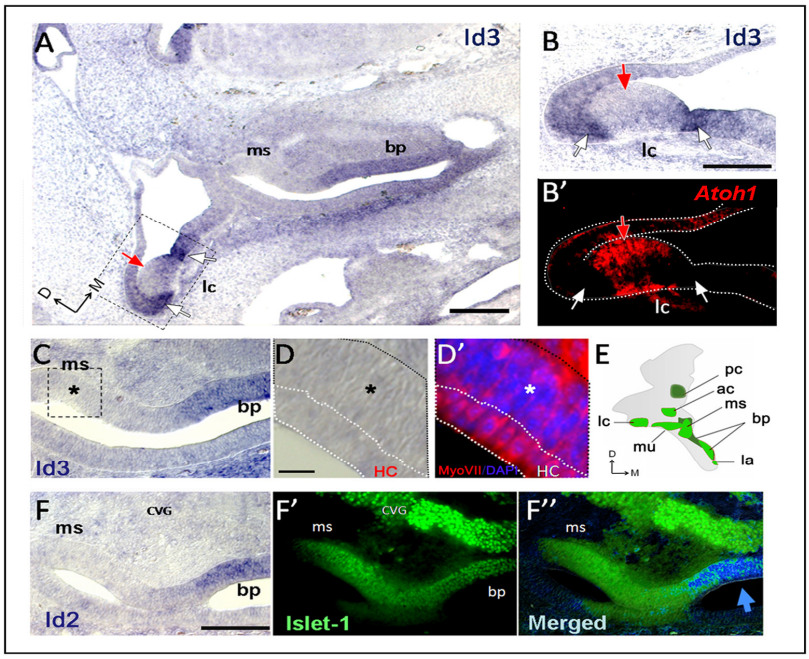

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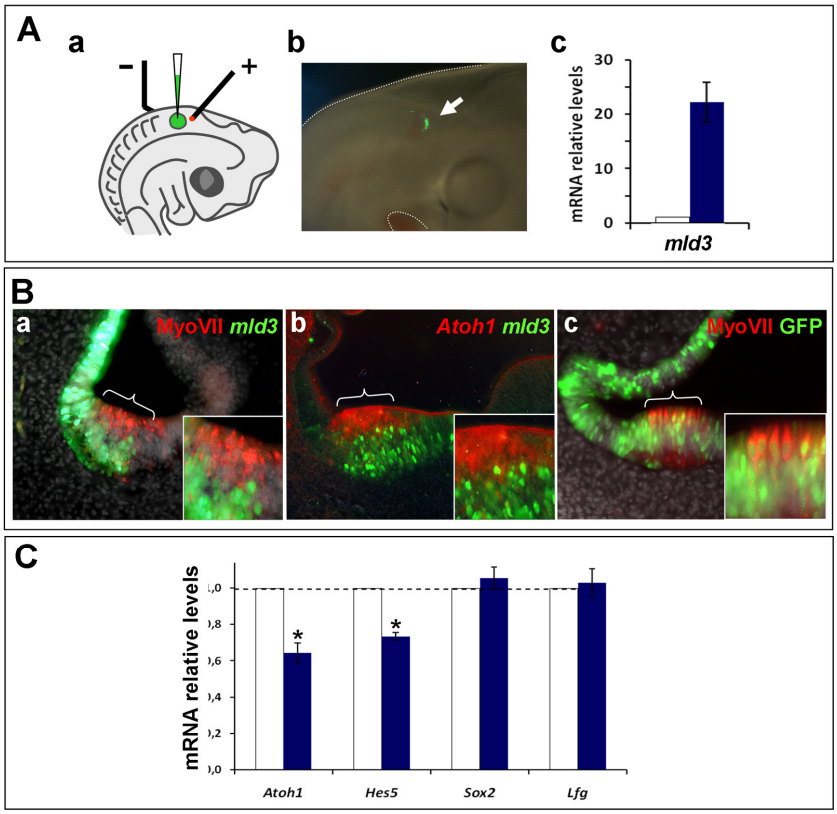

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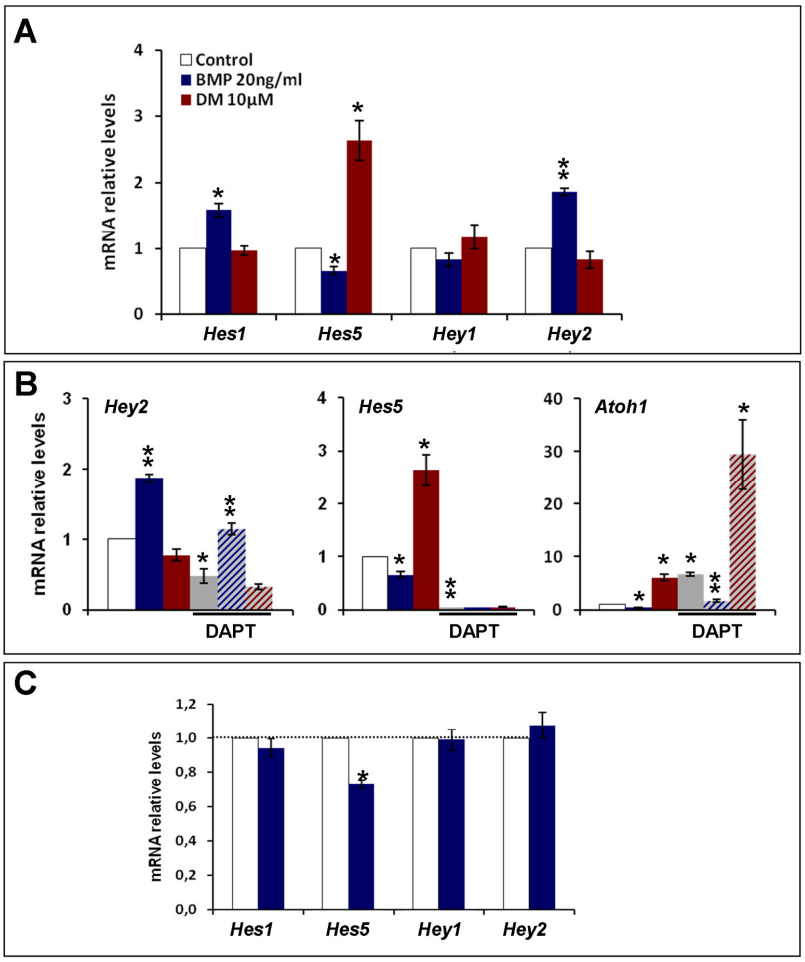

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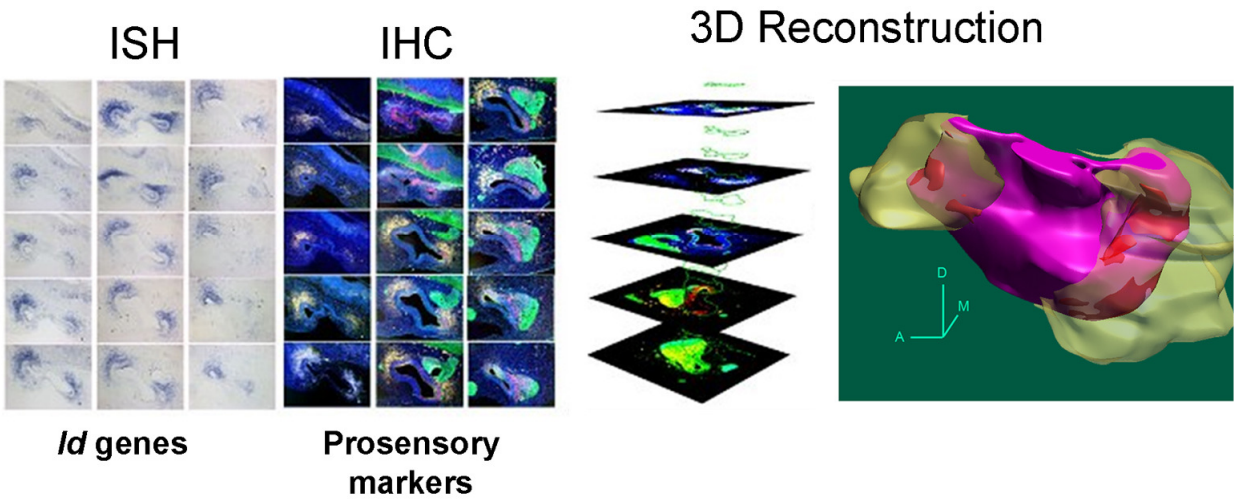
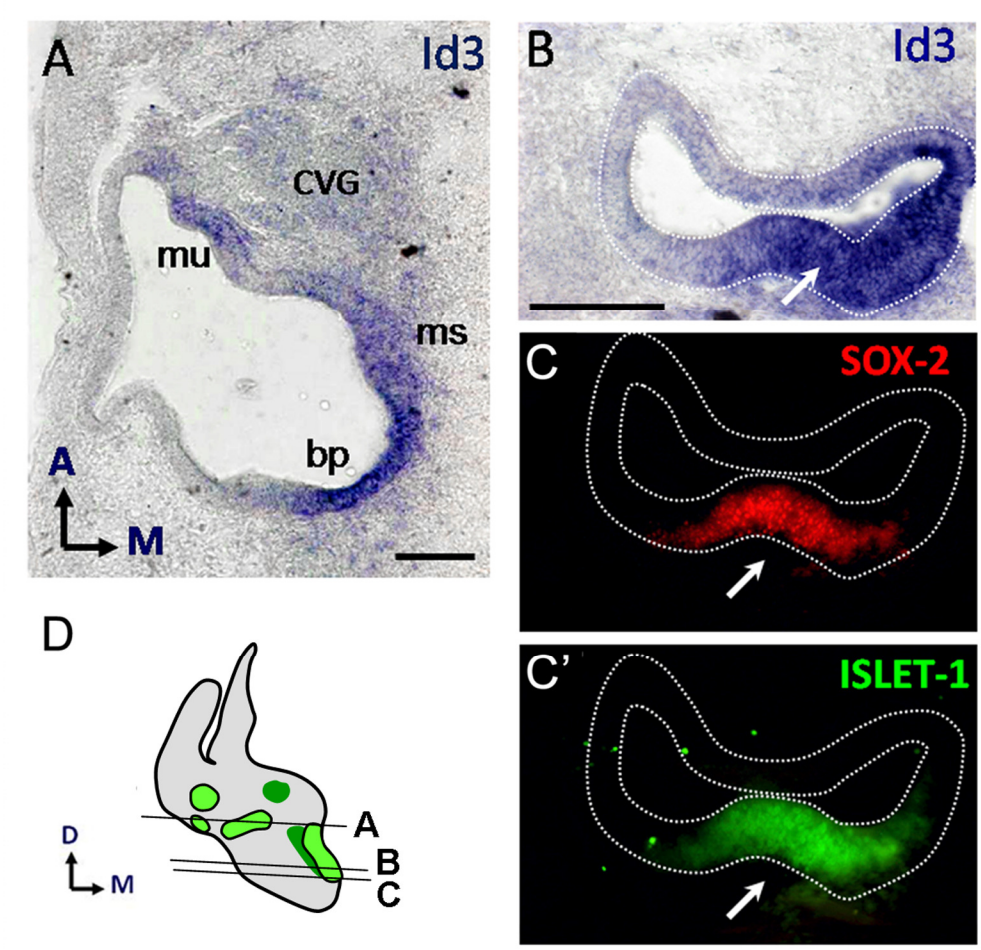
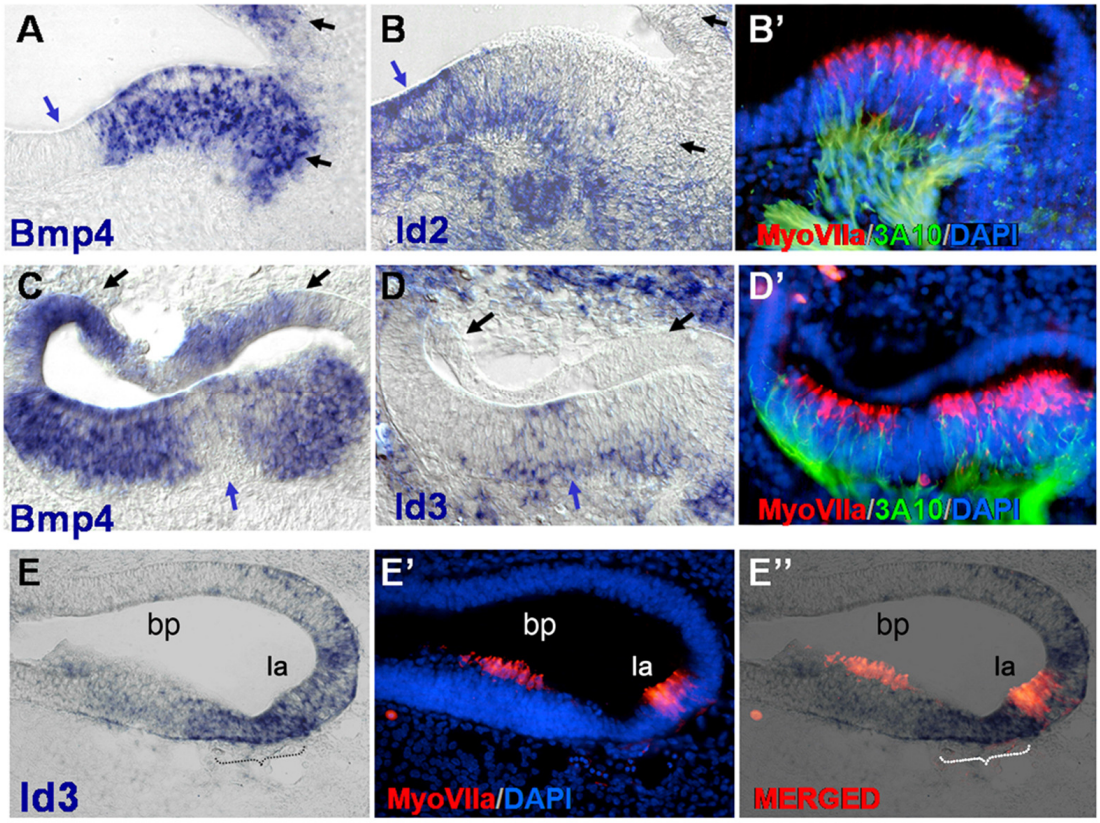


Supplemental figure 1. 3D analysis of *Id*2-3 gene expression at E3-5. A: Diagram of the procedure followed for the analysis of *Id*2-3 gene expression and 3D reconstruction of the otocyst. Left panel, coronal sections through the entire otocyst were processed for ISH, in this case *Id*2 is shown. Middle panel: after ISH, double immunofluorescence was carried out in the same sections, utilizing known markers of sensory progenitors; in this case Prox1 and TUJ1 are shown. Right panel: serial sections were analyzed using the BioVis3D® software for biological reconstruccion, which allowed precise identification of the sites of *Id* expression. To the right, one example of a 3D reconstruction of an E3-5. The external limits of the otic epithelium delineate the otocyst shape (purple). Periotic expression of *Id*2 is shown in yellow and PROX1 in dark red.



Supplemental figure 2. *Id3* expression at at E3-5. **A**: representative section throught the otocyst showing high expression in vestibular (mu and ms) and auditory (bp) prosensory regions. **B**: Section through the presumptive basilar papilla probed for *Id3* ISH. **C-C'**: adjacent section to that shown in B processed for IHC with antibodies against Sox2 (B') and Islet1 (D) to reveal the presence of sensory progenitors. White arrows (B-C'') indicate the prosensory region where *Id3*, SOX2 and Islet coincide. **D**: Schematic representation of the inner ear at indicating the locations of the different sensory organs at this stage. Abreviations: bp, basilar papila; ms, macula sacularis; mu: macula utricularis.



Supplemental figure 3. Expression of *Id*2-3 at E7. A-E'': Transversal sections of the inner ear at E7. A: transversal section at the level of the lateral crista processed for Bmp4 ISH (blue). Black arrows indicate strong Bmp4 signal in the sensory lateral crista, and in the roof of the ampula. Blue arrow indicates the non-sensory epithelium devoid of Bmp4 signal. B: Section adjacent to A, showing *Id*2 ISH signal. Arrows indicate the same relative positions than A to compare. B': the same section as B was labelled with anti-MyoVIIa (red), 3A10 (green) antibodies, and nuclei counterstained with DAPI (blue). C: transversal section at the level of the posterior crista processed for ISH with Bmp4 probe. Black arrows indicate strong Bmp4 expression in the roof of the ampula. Blue arrow indicates the non-sensory epithelium known as *cruciatum* devoid of Bmp4 signal. D: Section adjacent to C, showing *Id*3 ISH signal. Arrows indicate the same relative positions than A to compare. D': the same section as D was labelled with anti-MyoVIIa (red), 3A10 (green) antibodies, and nuclei counterstained with DAPI (blue) E-E'': Transversal sections at the most distal part of the cochlear duct stained for *Id*3 ISH (E), MyoVIIa immunofluorescence (red E') and DAPI staining (blue in E'). The merged image shows the location of the auditory hair cells in the basilar papilla (bp) and the vestibular hair cells in the macula lagena (la). The white braquet indicates the strong region of *Id*3 expression between those sensory organs.