Dual function of Notch signaling and role of *Hes/Hey* genes in the inner ear sensory development

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Litterarum radices amarae, fructus dulces

- *Cicero*

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ABSTRACT

During inner ear development, Notch exhibits two modes of operation: lateral induction, which is associated with prosensory specification, and lateral inhibition, which is involved in hair cell determination. These mechanisms depend respectively on two different ligands, Jagged1 (Jag1) and Delta1 (Dl1) and rely on a common signaling cascade initiated after Notch activation. In the chicken otocyst, expression of Jag1 and the Notch target *Hey1* correlates well with lateral induction, whereas both Jag1 and *Dl1* are expressed during lateral inhibition as are Notch targets *Hey1* and *Hes5*. Other *Hes/Hey* genes do not show restricted expression patterns in the otic epithelium. We show that Jag1 drives lower levels of Notch activity than Dl1, which results in the differential expression of *Hey1* and *Hes5*. In addition, Jag1 interferes with the ability of Dl1 to elicit high levels of Notch activity. Modeling the sensory epithelium when the two ligands are expressed together shows that ligand regulation, differential signaling strength and ligand competition are crucial for allowing the two modes of operation and for establishing the alternate pattern of hair cells and supporting cells. Jag1, while driving lateral induction on its own, facilitates patterning by lateral inhibition in the presence of Dl1. This novel behavior emerges from Jag1 acting as a competitive inhibitor of Dl1 for Notch signaling. Both modeling and experiments show that hair cell patterning is very robust. The model suggests that autoactivation of proneural factor Atoh1, upstream of Dl1, is a fundamental component for robustness. The results stress the importance of the levels of Notch signaling and ligand competition for Notch function.

Hey1 and *Hes5* are regulated by Notch, however, *Hey1* expression pattern suggests that it may be also regulated by other Notch-independent mechanisms. The results show that Bmp, Wnt and Fgf pathways modify *Hey1* and *Hes5* expression in the inner ear. Particularly, *Hey1* is regulated by Wnt through Jag1-Notch signaling and Bmps differentially regulate *Hey1* and *Hes5* expression. In addition, *Hey1* and *Hes5* show different mRNA stability that at least in part underlies differential temporal responses after Notch blockade. The gain of function of Hey1 or Hes5 shows that they cross-regulate each other in a rather complex manner. Both Hey1 and Hes5 suppress *Dl1* expression, suggesting that they cooperate during lateral inhibition. On the other hand, in spite of its association with Jag1, Hey1 is not instrumental for lateral induction, which is promoted by Hes5. We suggest that *Hey1* and *Hes5,* are subject of a rather complex regulation that includes different levels of Notch activity, the stability of their transcripts, cross regulation and other signaling pathways that may determine the different roles of Hey1 and Hes5 in inner ear.

RESUMEN

Durante el desarrollo del oído interno, Notch presenta dos modos de funcionamiento: inducción lateral, que se asocia con la especificación prosensorial, e inhibición lateral, asociada a la determinación de las células ciliadas. Estos mecanismos dependen, respectivamente, en dos ligandos diferentes, Jagged1 (Jag1) y Delta1 (Dl1) y se basan en una misma cascada de señalización iniciada con la activación de Notch. En el otocisto de pollo, la expresión de Jag1 y *Hey1* se correlacionan bien con la inducción lateral, mientras que Jag1 y *Dl1* se expresan durante la inhibición lateral junto con *Hey1* y *Hes5*. Otros *Hes/Hey* genes no muestran patrones restringidos de expresión en el epitelio ótico. Los experimentos muestran que Jag1 induce niveles más bajos de actividad de Notch que Dl1, y ello resulta en la expresión diferencial de *Hey1* y *Hes5*. Además, Jag1 interfiere con la capacidad de Dl1 para inducir niveles altos de actividad de Notch. Modelando el epitelio sensorial para los dos ligandos se demuestra que la regulación de los ligandos, la fuerza de la señalización y la competencia por la señalización son fundamentales para permitir los dos modos de funcionamiento y para establecer el patrón alterno de las células ciliadas. Jag1, opera en el modo de inducción lateral cuando está sólo, pero facilita la inhibición lateral en presencia de Dl1. Este nuevo comportamiento emerge de que Jag1 actúa como un inhibidor competitivo de Dl1 para la señalización de Notch. Los experimentos muestran que el patrón de células ciliadas es muy robusto, y el modelo sugiere que la autoactivación del factor proneural Atoh1 es un componente fundamental para la robustez del patrón. Los resultados destacan la importancia de los niveles de señalización Notch y la competencia entre los ligandos para la función de Notch.

Hey1 y *Hes5* están regulados por Notch, sin embargo, el patrón de expresión *Hey1* sugiere que puede ser también regulado por otros mecanismos. Los resultados muestran que las vías Bmp, Wnt y Ffg modifican la expresión de *Hey1* y *Hes5*. Particularmente, *Hey1* está regulado por Wnt a través de la señalización Jag1-Notch y los Bmps regulan diferencialmente a *Hey1* y *Hes5*. Además, *Hey1* y *Hes5* muestran diferentes estabilidades de mRNA, lo que al menos en parte subyace a las respuestas temporales diferentes tras el bloqueo de Notch. La ganancia de la función de Hey1 o Hes5 muestra que existe una regulación cruzada y compleja. Tanto como Hey1 y Hes5 suprimen la expresión Dl1, lo que sugiere que cooperan durante la inhibición lateral. Por otro lado, a pesar de su asociación con Jag1, Hey1 no es instrumental para la inducción lateral. Se sugiere que Hey1 y Hes5, son objeto de una regulación compleja que incluye diferentes niveles de actividad de Notch, la estabilidad de sus transcritos, la regulación cruzada y por otras vías de señalización que pueden así determinar los diferentes roles de Hey1 y Hes5 en el oído interno.

PREFACE

The vertebrate inner ear is an intricate sensory organ responsible for senses of hearing, balance and acceleration. It develops from simple epithelial thickening called otic placode that undergoes through dramatic morphogenetic and patterning events to give rise to sophisticated structure of the mature inner ear. The inner ear is lined with specialized sensory epithelium which is composed of highly ordered mosaics of hair cells and supporting cells. Hair cells are highly specialized mechanotransducers of vestibular and auditory stimuli. In mammals, hair cells have little capacity to regenerate and therefore over last decades a great effort has been put in research of hair cell regeneration and treatment options for both hearing and balance disorders. Unveiling the molecular mechanisms required for proper generation of sensory territories will give more insight and provide molecular tools to aid concerning issue on HC regeneration.

Notch signaling plays an essential role in inner ear development. As first postulated by Julian Lewis group, Notch has dual and seemingly contradictory function in otic development. Early in development, Notch is crucial for prosensory specification, whereas later on Notch drives hair cell determination. The two functions of Notch are accomplished by different Notch operational modules.

In this work we studied further the role of Notch during inner ear development, trying to understand how the singe signaling pathway operates in paradoxical manner and what determines different modes of Notch. For that purpose we made use of chick embryos that unlike other model systems provide opportunity for precise temporal and special control of *in vivo* transgenesis and *in vitro* explants.

We were able to show that different Notch signaling strength mediated by different Notch ligands results in differential expression of Notch targets and that signaling strength is crucial for patterning of sensory regions. In addition, we provided evidence on differential regulation of Notch targets in the otic epithelium that may be crucial for their different functions in the inner ear development.

In the course of this work I had also the opportunity to make a short stay in the laboratory of Dr. Doris Wu (NIDCD, NIH) and Dr. Warren Pear (University of Pennsylvania), where I acquired new technical skills and widen my knowledge in the inner ear field and Notch biology that greatly contributed to the present work.

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INTRODUCTION

THE VERTEBRATE INNER EAR

Hearing is an important ability of vertebrates to perceive information from environment and develop adequate behaviors. The hair cells of the inner ear (HCs) are the first step in audition. They transform sound waves into electrical signals processed by the brain. HCs are strikingly organized in a grid-like two-dimensional pattern in which they alternate with the so-called supporting cells (SCs), what is crucial for normal hearing. Hearing loss is one of serious disabilities that affect many people worldwide and can be caused by genetic background, noise trauma, ototoxic drugs or aging. Most defects in human audition are caused by the loss of the mechano-transducing HCs, which unlike in birds and fish show little ability to regenerate. How HCs acquire their fate and how the HC pattern is formed are major questions for developmental studies and for the improvement of regenerative therapies.

Anatomical and histological structure of the inner ear

The inner ear is a highly complex three dimensional structure. It is responsible for the senses of hearing, balance and acceleration and its structure is highly conserved throughout phyla. In all vertebrates, the inner ear originates from a simple structure called otic placode, a paired thickening of the ectoderm adjacent to the hindbrain (Fekete, 1996; Haddon and Lewis, 1996; Torres and Giraldez, 1998; Schlosser and Northcutt, 2000). From such a simple anlage, throughout development the otic placode undergoes a series of orchestrated morphogenetic movements to finally give rise to the intricate mature organ.

The inner ear is composed of an array of fluid-filled sacs and ducts that form membranous labyrinth, housed in osseous capsule (bony labyrinth). The outer wall of membranous labyrinth and bony labyrinth are separated by the perilymph, which is characterized by a high sodium concentration, whereas the membranous labyrinth baths in a fluid of high potassium concentration named endolymph. The latter is secreted by the stria vascularis (tegmentum vasculosum in birds). Differences in ionic distribution and electrical potentials allow potassium influx into sensory cells and mechanotransduction (Couloigner et al., 2006; Gillespie and Muller, 2009; Guinan et al., 2012). The membranous labyrinth is lined with specialized epithelial tissue divided into vestibular and auditory parts, each of which hosts the corresponding sensory organs. Vestibular organs are located dorsally and consist of three cristae (anterior or superior, posterior and lateral) nested in the enlarged cavities named the ampullae, at the basis of three orthogonally positioned semicircular canals, and two maculae (macula utriculi and macula sacculi). The cristae sense angular accelerations, whereas the maculae detect linear accelerations and gravity. Above each maculae lies a single (fish) or

many (birds and mammals) otholits, which are dense aggregations of proteins and calcified material that serve as inertial mass that help to stimulate macular HCs. Auditory organs differ across phyla more than vestibular organs. They are located ventrally and host straight epithelial structure called basilar papilla in the chicken inner ear (Bissonnette and Fekete, 1996). In mammals the auditory sensory organ, the cochlea, is a coiled shaped structure called organ of Corti. Ventrally to the basilar papilla, resides a small vestibular sensory organ named macula lagena (Fig. 1A). In fish, both saccule and lagena are involved in hearing (Popper and Fay, 1993; Riley and Phillips, 2003; Schneider-Maunoury and Pujades, 2007).

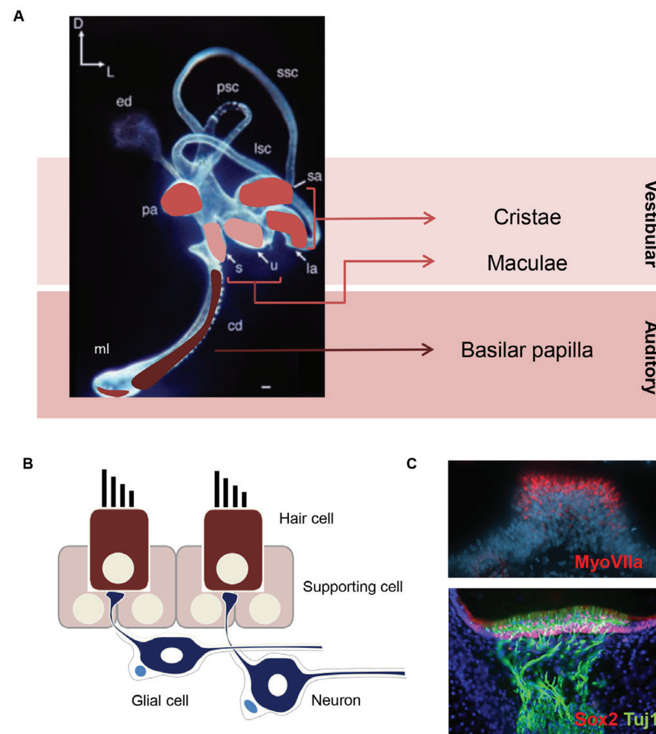

Figure 1. Structure and functional unit of the vertebrate inner ear. (A) Paint-filled inner ear of chicken otocyst at E9 with associated vestibular and auditory sensory organs. (B) Schematic drawing of functional unit of the inner ear, consisting of four cell types: hair cells, supporting cells, sensory neurons and glial cells. (C) Immunostaining of hair cells, supporting cells and sensory neurons. On the top anterior crista of an E5 chick otocyst stained against MyoVIIa (red). On the bottom macula sacculi of an E7 chick otocyst labeled with Sox2 (red), Tuj1 (green) and DAPI (blue). ssc: superior semicircular canal, psc: posterior semicircular canal, lsc: lateral semicircular canal, sa: superior ampulla, pa-posterior ampulla, la:lateral ampulla, s: macula sacularis, u: macula utricularis, cd: cochlear duct, ed: endolymphatic duct, ml: macula lagena, d: dorsal, l: lateral. Adapted from Wu et al. (1998); Giraldez and Fritzsch (2007); Kamaid et al. (2010) and Neves et al. (2013b).

The functional unit of the inner ear

Sensory organs of the inner ear accomplish diverse functions by means of a common functional unit that is composed of three main cell types: the hair cells (HCs), which are the mechano-transducing elements, the supporting cells (SCs) that play several roles in maintaining HC function, and sensory neurons, which transmit the information generated at the HCs to the brain in the form of electrical impulses (Fig. 1B).

Box 1. Homology between vertebrate sensory organs and the sensilla of a fly

Inner ear sensory organs in vertebrates largely resemble the *Drosophila* sensory bristles with which they share a similar function and developmental program (Lewis, 1991; Eddison et al., 2000) (Fig. 2). Homology is seen in several aspects: 1) each bristle is a miniature sensory organ (sensillum) that like vertebrate sensory patch has a mechanosensory function (Walker et al., 2000). 2) Each bristle is composed of four cell types: a neuron, a sheath cell, a bristle socket cell and a bristle shaft cell. There is a clear homology between bristle shaft cell and HC, bristle socket cell and SC, and between the neurons. Furthermore, the HC bundle resembles shaft of bristle shaft cell, both exhibiting a well defined planar cell polarity (Tilney et al., 1996). 3) Fly sensillum and vertebrate sensory organs share a similar developmental origin, epidermis and otic placode ectoderm, respectively. 4) Similar molecular mechanism, the lateral inhibition, underlies cell fate choices in the both sensory organs (see below).

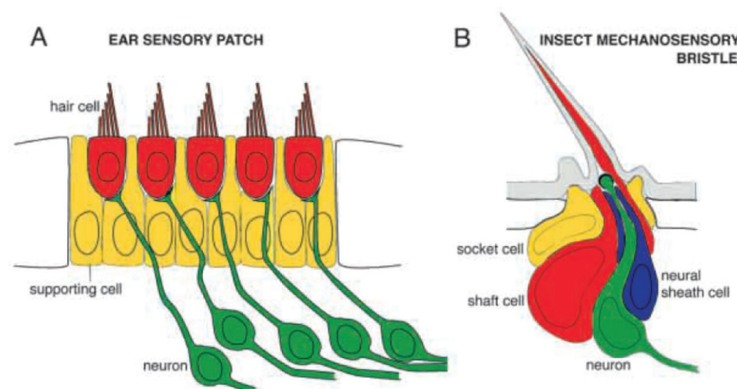

Figure 2. Homology between vertebrate sensory organs and the sensilla of a fly. Schematic representation of homology between mechanosensory patch of the inner ear (A) and mechanosensory bristles in *Drosophila melanogaster*. Adapted from Adam et al. (1998).

Hair cells

HCs are highly specialized mechano-electrical transducers of auditory and vestibular stimuli (Fig. 1C). They are characterized by the presence of stereociliary bundle on their lumenal surface (Box 2). Stimuli of auditory or vestibular origin provoke movements of stereocilia that result in the opening of transduction channels and the consequent influx of positively charged ions that depolarize the HC membrane (Eatock and Hurley, 2003). This depolarization results in a release of neurotransmitters from the base of HC into the synapse that HCs form with sensory neurons, which thereby increase their firing rate and propagate the signal to the brainstem.

Variations in HC morphology exist both between different sensory epithelia, and within the single epithelia. Vestibular sensory patches contain Type I and Type II HCs that differ in their morphology, electrophysiology and innervations (Eatock et al., 1998). Auditory epithelia in chick and mammals contain two different cell types tall and short HCs and, outer hair cells (OHCs) and inner hair cells (IHCs), respectively (Hirokawa, 1978; Lim, 1986; Nadol, 1988).

Box 2. Hair cell bundle

Each HC is characterized by the presence of stereociliary bundle that counts from 50 to 200 filamentous actin-filled microvilli (reviewed in Frolenkov et al., 2004). Asymmetric architecture of the bundle is preserved in each HC with the tallest stereocilia located in one side of the HC and gradually shorter stereocilia in adjacent rows building a staircase pattern. Bundle orientation is crucial for HC sensitivity. Additionally, each HC contains single kinocilium, the true cilium that is always located next to the tallest stereocilia. However, this kinocilium does not participate in mechano-transduction and is lost in early postnatal stages suggesting that it is not essential for bundle function (Hudspeth and Jacobs, 1979).

Stereociliary bundle is directionally sensitive as the deflection of the bundle towards the tallest stereocilia results in depolarization, whereas the deflection towards the shortest stereocilia results in hyperpolarization of the HC membrane (Hudspeth and Corey, 1977; Hudspeth and Jacobs, 1979). Tip links that stretch between the top of one stereocilium to the shaft of the next highest stereocilium provide directional sensitivity of each bundle (Assad et al., 1991).

Uniform orientation of HC bundles ensures that all HC from a given region respond uniformly to a single stimulus. The development of the bundle is a well characterized two-step process that starts with the presence of one true cilium at the center of lumenal surface of each HC that will eventually become kinocilium. First, the cilium will centrifugally

move towards the outer edge of the lumenal surface. By the time kinocilium touches the luminal edge, other stereocilia are formed. Next, the bundle exerts gradual orientation called reorientation to correct possible mistakes occurred in stereocilia orientation providing an uniform orientation of stereociliary bundles called planar cell polarity (PCP) (Cotanche and Corwin, 1991; Denman-Johnson and Forge, 1999; Dabdoub et al., 2003).

Supporting cells

SCs are non-transducing cells that in addition to the mechanical support of HCs play several important functions (Fig. 1C). In many sensory organs SCs appear to be a morphologically and molecularly homogenous cell population. However, the mammalian cochlea contains several distinct SC types: Deiters´, pillar, phalangeal, Hensen´s and Claudius cells. Available data suggest that SCs are important mediators of HC development, function, survival and phagocytosis (Jagger and Forge, 2006; Tritsch et al., 2007; Lahne and Gale, 2008; Bird et al., 2010; reviewed in Monzack and Cunningham, 2013). SCs play a role in survival and function of auditory ganglion neurons due to their ability to produce trophic factors like brain-derived neurotrophic factor (BDNF) (Zilberstein et al., 2012). SCs are also mediators of glutamine clearance at synapses. Since glutamine is an excitatory neurotransmitter, this is crucial for proper function of synapse and prevention of excitotoxicity (Pujol and Puel, 1999; Gale and Jagger, 2010). Potassium is the major cation in the endolymph, that upon given stimuli depolarizes the HC membrane. SCs play an essential role in the regulation of potassium recycling and in buffering potassium elevations (Mistrik and Ashmore, 2009; Zdebik et al., 2009). SCs have an additional role in HC death and survival (reviewed in Gale and Jagger, 2010). HC damage triggers intercellular signaling between HCs and SCs, which results in HC death (Lahne and Gale, 2008). The mechanism by which dead HCs are cleared from the epithelium is not yet well determined (Forge, 1985; Li et al., 1995; Hirose et al., 1999; Seoane and Llorens, 2005). Many of these SC functions resemble the functions of various glial cells and, therefore, SCs are thought to represent a specialized type of glia in the sensory epithelium. Finally, SCs serve as precursors for new HCs during regeneration in birds (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; reviewed in Stone and Cotanche, 2007; Monzack and Cunningham, 2013).

Sensory neurons

All sensory organs within the inner ear are innervated by bipolar sensory neurons, which reside in the cochleo-vestibular ganglion (CVG, VIIIth cranial ganglion) (Fig. 1C). They are specialized primary afferent neurons that provide transmission of electric stimuli from HCs into auditory and vestibular nuclei in the brainstem. Sensory neurons are placodal-derived elements, which are intermingled with glial Schwann cells of neural crest origin (D'Amico-Martel and Noden, 1983; Rubel and Fritzsch, 2002).

Development of the inner ear

The vertebrate inner ear originates from a very simple structure called otic placode (Alsina et al., 2009; Ladher et al., 2010). The otic placode is one of six head placodes that are transient bilateral epithelial thickenings of head ectoderm (Box 3). Until recently it has been considered that sensory and associated non-sensory elements of the mature inner ear originate exclusively from otic placode (Alsina et al., 2009). However, one recent study reveals a dual origin of the neurosensory elements of the inner ear. Genetic fate-mapping in mice shows that neuroectodermal and neural crest precursors significantly contribute to the neurosensory domain of the otic placode (Freyer et al., 2011).

In the chicken embryo, the otic placode becomes visible by HH10 and is initially located next to r4 and r5 of the developing hindbrain. By HH12, the otic placode invaginates and forms an otic cup, which is now juxtaposed to r5 and r6 (Alvarez and Navascues, 1990; Groves and Bronner-Fraser, 2000; Alsina et al., 2004). Soon after the otic cup pinches off the ectoderm and closes to form a hollow ellipsoid-shaped structure called the otic vesicle or otocyst (by HH17). By day E2, the anterior-ventral subpopulation of epithelial cells segregates from otic vesicle and populates cochleo-vestibular ganglion (CVG). Sensory neuroblasts undergo serial divisions before they differentiate into sensory neurons. Subsequently, the otic vesicle grows and changes its shape to form the mature organ with the associated sensory structures. Sensory organs emerge at specific locations and time windows (Fig. 3). At the otic vesicle stage (E3-E4), the endolymphatic duct emerges and by E6 it expands and forms endolymphatic sac. Over next few days the endolymphatic sac lengthens and bends to place dorsal to the forth ventricle over hindbrain (Bissonnette and Fekete, 1996). The mature inner ear is characterized by the presence of three orthogonally positioned semicircular canals that at the basis bulge and end into ampullae. Semicircular canals derive from vertical and horizontal pouches that are outpocketings of the dorsolateral otocyst. By E5 opposing epithelia of each pouch fuse and resorb forming a tube like structure of semicircular canals. In this way the horizontal pouch gives rise to lateral semicircular canal and the vertical

pouch forms both anterior and posterior canals joined by the common cruse (Bissonnette and Fekete, 1996; Bok et al., 2007a).

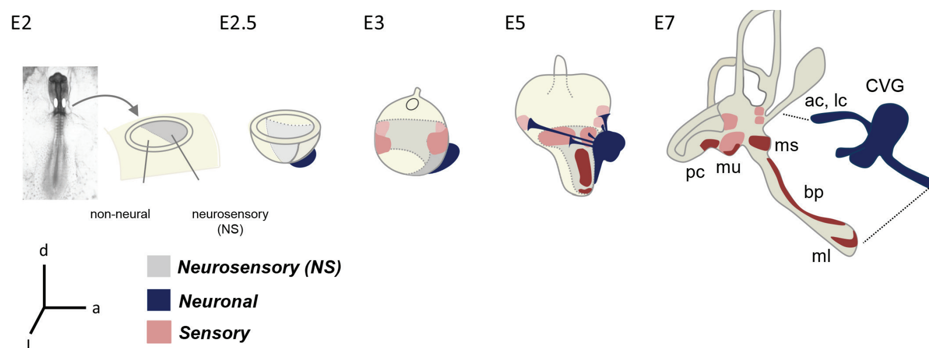

Figure 3. Inner ear development in the chick. At E2 otic placode is partitioned into neurosensory (NS) and non-neural domains. At the otic cup stage (E2.5), neurogenesis starts with delamination of neuroblasts from anteriomedial portion of the otic cup. Otic neuroblasts populate cochleo-vestibular ganglion. By E3 otic vesicle closes and undergoes morphogenesis to shape into complex structure of the mature inner ear. Sensory development is delayed with respect to neurogenesis. Between E3 and E3.5 two prosensory patches are defied in anterior and posterior poles of the otic vesicle that soon after resolve into vestibular and auditory sensory patches that can be identified by specific markers. By E5 dorsal most patches start to differentiate and by E7 all sensory patches are defined by presence of nascent hair cells innervated by sensory neurons. ac: anterior crista, pc-posterior crista, lc:lateral crista, ms: macula sacularis, mu: macula utricularis, bp: basilar papilla, ml: macula lagena, CVG: cochleo-vestibular ganglion, d: dorsal, l: lateral, a: anterior. Adapted from Neves et al. (2013b).

Box 3. Cranial placodes

Cranial placodes are transient structures that arise at precise locations during embryonic development. There are six cranial placodes found in the chicken: hypophyseal, olfactory, lens, trigeminal, otic and epibranchial (Fig. 4). With the exception of hypophyseal placode, that gives rise to the endocrine pituitary gland, they all contribute to the sensory components of the cephalic peripheral nervous system and, therefore, they are termed as sensory placodes. Cranial placodes give rise to diverse cell types including sensory receptor cells, supporting cells, neurons and endocrine cells (reviewed in Jidigam and Gunhaga, 2013). Cranial placodes originate from the neural plate border that contributes to the formation of both preplacodal region (PPR) and neural crest cells. PPR is a unique ectodermal domain distinguished by *Six/Eya* expression, which harbors the precursors for all cranial placodes (Mishima and Tomarev, 1998; Esteve and Bovolenta, 1999; Streit, 2002; Bhattacharyya et al., 2004; Ishihara et al., 2008). Establishment of PPR is a multi-step process that requires integration of different inductive and inhibitory signals from surrounding tissues (reviewed in Chen and Streit, 2013).

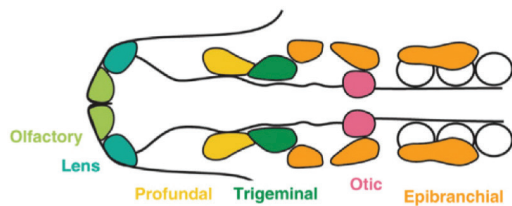

Figure 4. Vertebrate cranial placodes. Schematic representation of chick embryo at 10-somite stage. At this stage precursors of different placodes are segregated and occupy specific locations in the head ectoderm. Adapted from Patthey et al. (2014).

Otic placode induction and early otic patterning

Development of the otic placode and epibranchial placodes occur simultaneously from a common *Pax2*-positive field also known as posterior placodal area (PPA), or pre-otic field (Ladher et al., 2010). In chick, the PPA is detected by HH8 in an ectodermal domain rostral to the first somite (Groves and Bronner-Fraser, 2000), and is induced by the interplay of mesoderm- and hindbrain-derived signals (reviewed in Ladher et al., 2010; Chen and Streit, 2013). Several pieces of evidence show that Fgf family members are central for PPA induction (Ladher et al., 2000; Vendrell et al., 2000; Kil et al., 2005; Martin and Groves, 2006). Fgfs show a dynamic temporal and spatial expression pattern in tissues surrounding the developing otic placode, with some variations among species (reviewed in Schimmang, 2007). Briefly, Fgfs from the mesoderm perform a dual function acting: 1) on overlying non-neural ectoderm to induce *Pax2* (Vendrell et al., 2000; Alvarez et al., 2003; Kil et al., 2005; Ladher et al., 2005; Martin and Groves, 2006), and 2) on the surrounding hindbrain to induce Fgf and *Wnt8a* and *Wnt8c* expression in the caudal hindbrain (Ladher et al., 2000; Urness et al., 2010). Wnt signaling acts only after Fgf-mediated PPA induction (Freter et al., 2008). Its function is to instruct the medial region of PPA and direct *Pax2*-positive cells towards the otic character. In addition, while inducing the otic fate, Wnt signaling suppresses epibranchial fate laterally and thus serves as a determinant for linage choice between the otic vs. non-otic domains within PPA (Ohyama et al., 2006; Freter et al., 2008). Additional signaling refines the otic vs. non-otic field, including a positive feedback loop between Wnt and Notch (Jayasena et al., 2008), and the rapid attenuation of Fgf activity from the prospective otic territory (Freter et al., 2008). Thus, high-Wnt, high-Notch, low-Fgf promote otic identity, whereas low-Wnt, low-Notch, high-Fgf promote epidermal and epibranchial identity (Fig. 5A).

Axial patterning of the inner ear is an important step to provide positional cues for the development of the specific cell types in the correct locations. It implies the establishment of the three axes of the inner ear, anterior-posterior (AP), dorsal-ventral (DV) and medial-lateral (ML). Axial specification is driven by inductive signals from the surrounding tissues that result in and/or maintain asymmetries in gene expression (reviewed in Groves and Fekete, 2012).

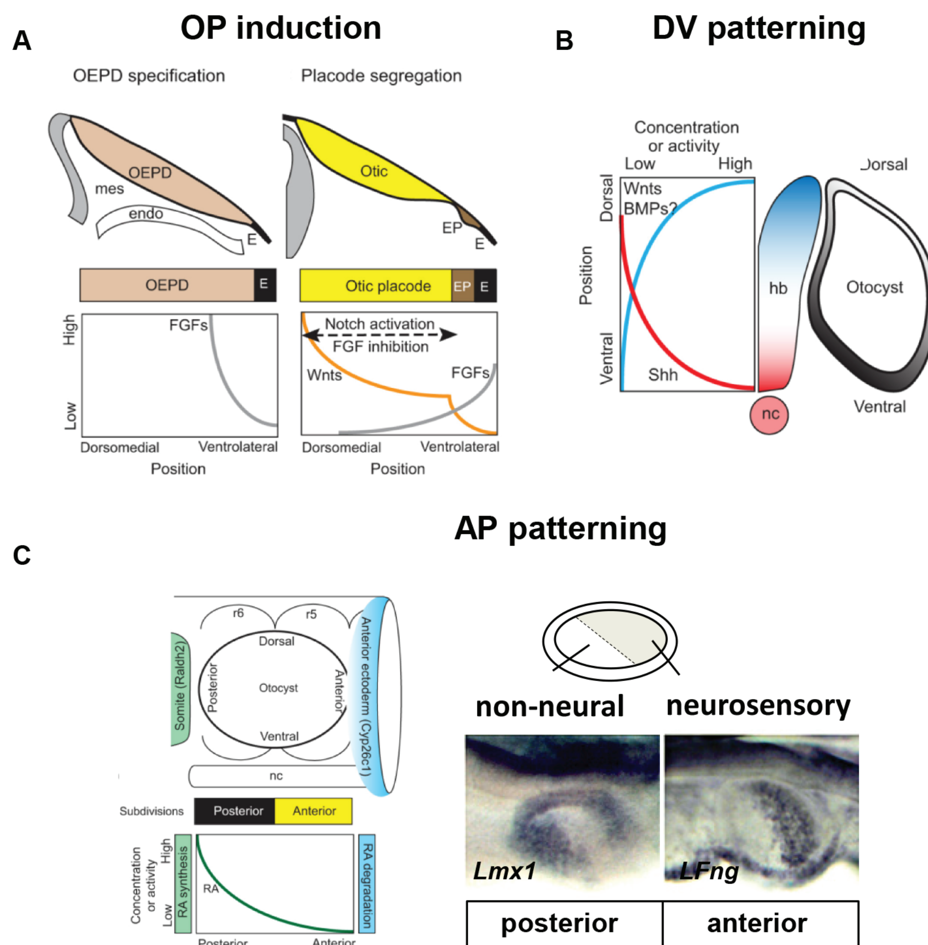

Figure 5. Otic placode induction and otic patterning by extrinsic signals. (A) Otic placode induction is two step process that requires FGFs to act on the pre-placodal domain to specify otic-epibranchial placode domain (OEPD or PPA) and separate it from ectoderm. OEPD is segregated into the otic and epibranchial placodes (EP). Wnt gradient with feedback loop involving Notch activation and FGF inhibition favors otic and represses epibranchial character. FGFs specify epibranchial fate. (B) DV patterning. Otic vesicle experiences opposing gradients of Wnt (dorsally) and Shh (ventrally) activity along DV axis. (C) AP patterning. Asymmetry of RA synthesis and degradation creates a gradient along AP axis. High RA confers posterior identity of the inner ear. Complementary *Lmx1* and *Lfng* expression shows AP patterning of the otic placode. Adapted from Groves and Fekete (2012) and Alsina et al. (2004).

The inner ear has an obvious DV polarity with the vestibular apparatus located dorsally and the auditory component located ventrally. Although asymmetric gene expression patterns in DV axis are an early event, transplantation experiments in chick indicate that DV axis is not specified until otocyst formation (Wu et al., 1998). A number of studies demonstrated that signals emanating from the hindbrain are crucial for DV axial specification of the inner ear (Giraldez, 1998; Bok et al., 2005; Liang et al., 2010). The current model suggests that DV patterning results from opposing gradients of Hedgehog and Wnt (Liu et al., 2002;

Riccomagno et al., 2002; Bok et al., 2005; Riccomagno et al., 2005; Bok et al., 2007b) (Fig. 5B). Sonic Hedgehog (Shh) is a morphogen secreted from the floor plate and notochord that acts as a ventralizing signal. A study of ear conditional Shh receptor *smoothened (Smo)* deficient mice suggests that Shh acts, respectively, in a direct and indirect manner on the ventral and dorsal regions of the otic vesicle (Brown and Epstein, 2011). Ventralizing effects of Shh are opposed by Wnts acting as dorsalizing signals from the dorsal hindbrain (Riccomagno et al., 2005). However, most probably Shh and Wnts are not the only players. Otic defects in DV patterning are seen in kr/kr mouse (Choo et al., 2006) and thought to be caused by the hindbrain deficit of Fgf signaling (McKay et al., 1996).

The first sign of asymmetry along AP axis of the otic primordium is the establishment of two complementary compartments called neurosensory (also neural) and non-neural (Fig. 5C). The term neurosensory refers to the ability of this domain to generate both sensory cells and sensory neurons (Raft et al., 2007; Neves et al., 2012). The neurosensory domain is located in the anterior part of the otic placode and it extends ventrally as the otic vesicle invaginates, whereas the non-neural domain is located in the posterior-lateral region and extends dorsally. These two domains show limited cell intermingling and unique gene expression patterns (Abello and Alsina, 2007; Abello et al., 2007). The neurosensory domain is characterized by the expression of *Sox2-3*, *Fgf10*, *LFng*, BEN, *Ngn1*, *Dl1* and *Hes5*. The non-neural domain is characterized by the expression of *Irx1*, *Lmx1b*, *Tbx1*, HNK-1, *Hairy1* and *Jag1* (Torres and Giraldez, 1998; Cole et al., 2000; Goodyear et al., 2001; Alsina et al., 2004; Raft et al., 2004; Abello et al., 2007; Neves et al., 2007; Vazquez-Echeverria et al., 2008). Experiments in chick showed that the adjacent ectoderm is critical for proper AP specification and that ectodermal RA suppresses neural fate by activating *Txb1* (Bok et al., 2011) (Fig. 5C). This has been further substantiated in zebrafish by showing that, *Hes1* acts downstream of RA and *Tbx1* (Radosevic et al., 2011). Fgfs from anterior otic ectoderm and Bmps from dorsal neural tube and/or ectoderm differentially regulate *Sox3* and *Lmx1*, and their restriction to the anterior and posterior domain, respectively (Abello et al., 2010). Analysis of *kreisler* mouse mutants suggests that Fgf signaling from the hindbrain also influences AP patterning (Vazquez-Echeverria et al., 2008). Also Notch signaling seems to be required to restrict the posterior genes like *Lmx1* and *Irx1* to the non-neural territory (Abello et al., 2007).

Medio-lateral axis specification is poorly understood. Although a defined lateral domain does not exist until otic cup closes, some aspects of ML specification occur at the time of AP and before DV axis specification (Wu et al., 1998). It is thought that the otic placode first acquires medial identity from Wnts and Fgfs emanating from the hindbrain, although the mechanisms involved is largely is unknown (Bok et al., 2007a).

Cell fate specification in the otic development

The vertebrate inner ear is a fascinating model system to study how cell fate is controlled spatially and temporally during development. As mentioned above, the first fate decision in otic development is the establishment of neurosensory and non-neural domains (Raft et al., 2004; Abello et al., 2007). The neurosensory domain is the source of both otic neurons and HCs, which are generated with different temporal profiles. The non-neural domain gives rise to the different supporting and secretory epithelia that line the wall of the mature inner ear. Neurosensory precursors generate first sensory neurons, and then give rise to prosensory patches that host HCs and SCs precursors (Fig. 6). The kinetics of neurogenesis and sensorogenesis is not identical in all species (Schneider-Maunoury and Pujades, 2007). While in amniotes, neurogenesis occurs prior to sensorogenesis, in zebrafish the two processes occur simultaneously. Both neurogenesis and sensorogenesis occur in otic epithelium cultured in the isolation, indicating that the two processes are governed by intrinsic mechanisms (Adam et al., 1998; Camarero et al., 2003).

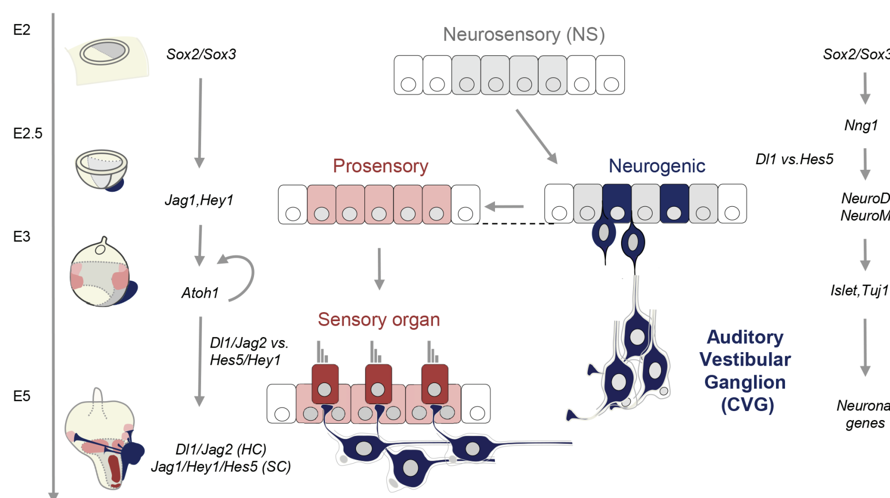

Figure 6. Cell fate specification during inner ear development. The diagram shows a model of hair cell and neuron specification during ear development in amniotes. The sequence of gene expression is indicated for sensory (left) and neuron development (right). Both hair cells (red), supporting cells (pink) and sensory neurons (blue) derive from a common domain within the otic placode, the neurosensory domain(NS) (grey) - characterized by the expression of Sox2. This domain is specified either by temporal and/or spatial cues to give rise to two main lineages: neuronal and sensory. First, the proneural gene *Neurog1* is up-regulated in neuroblasts (blue), which marks the onset of neurogenesis and via the Delta-Notch mediated lateral inhibition allows neuronal specification. After delamination of the neuroblasts from the otic epithelium prosensory specification takes place through Jag1-Notch mediated lateral induction. Expression of *Atoh1*, another proneural factor drives sensory determination via Dl1/Jag2-Notch mediated lateral inhibition. HC: hair cells; SC: supporting cells. Adapted from Alsina et al. (2009) and Neves et al. (2013a).

Commitment to neurosensory fate is given by the expression of the high-mobility HMG factors *Sox3* and *Sox2* (Fig. 6). *Sox3* is expressed only transiently until the end of the neurogenesis and is sufficient to induce *Sox2*, which labels neurosensory progenitors throughout development (Neves et al., 2007; Abello et al., 2010). Recent cell tracing experiments in chick demonstrate that both neurons and HCs derive from Sox2 positive progenitors (Neves et al., 2012). Further, evidence from both chick and mice indicate that *Sox2* is sufficient and necessary to drive sensory development (Kiernan et al., 2005b; Neves et al., 2011; Ahmed et al., 2012), and both *Sox2* and *Sox3* are able to induce neural fate (Abello et al., 2010; Puligilla et al., 2010; Neves et al., 2011).

A recent study has obtained a three-dimensional reconstruction of the mouse otocyst in which each cell can be precisely mapped into spatial expression domains by using sophisticated gene expression analysis at a single cell resolution. The work provided a dynamic transcriptional characterization of gene expression of established pathway-associated and novel otic markers (Durruthy-Durruthy et al., 2014). These tools will give crucial information on lineage relationships and molecular cell fate determinants.

Neurogenesis

Neurogenesis in the ear follows similar principles as in the central nervous system and occurs through a tightly regulated cascade of molecular events. Sox2 confers neuronal competency by directly inducing the proneural bHLH gene *Neurogenin1 (Ngn1)*, a mastergene for neuronal fate (Henrique et al., 1995; Adam et al., 1998; Ma et al., 1998; Alsina et al., 2004; Evsen et al., 2013). Like with HCs (see below), further progression from neuronal precursor state to nascent neuron requires subsequent *Sox2* downregulation by Ngn1 (Evsen et al., 2013). In the chick, by HH11 a subpopulation of cells from the neurogenic domain initiates *Ngn1* expression. Mice lacking *Ngn1* lack all sensory neurons in the inner ear (Ma et al., 1998; Ma et al., 2000). In the otic epithelium, *Ngn1* is associated with the selection of progenitors and their commitment towards neuronal fate and it is upstream of the Dl1-Notch pathway (Adam et al., 1998; Alsina et al., 2004; Abello et al., 2007; Daudet et al., 2007). *Ngn1* labels epithelial neuroblasts, but not delaminating neuroblasts. Epithelial neuroblasts also express *NeuroD* and *NeuroM*, basic helix-loop-helix (bHLH) genes associated with neuronal determination and survival (Liu et al., 2000; Kim et al., 2001). Mice lacking *NeuroD* show a near-complete loss of the cochlear ganglion and a significant loss of vestibular ganglion. The surviving vestibular ganglion displays disorganized fiber projection onto the vestibular sensory epithelium (Kim et al., 2001). Delaminated neuroblasts coalesce in the CVG (D'Amico-Martel and Noden, 1983; Hemond and Morest, 1991; Haddon and Lewis, 1996; Alsina et al., 2004) and express additional neuronal markers like the LIM homeodomain transcription factor *Islet1* and the

neuron specific βIII-tubulin, Tuj1 (Adam et al., 1998; Li et al., 2004). *NeuroD* and *NeuroM* are only transiently expressed in the sensory neuroblasts (Bell et al., 2008) (Fig. 6).

Several signaling pathways regulate otic neurogenesis. Fgf signaling promotes neuronal determination (Alsina et al., 2004). *Sox3* is one of the earliest determinants of the proneural domain, and when overexpressed is able to expand *Dl1* but does not drive *Dl1*-positive cells to full neuronal determination (Abello et al., 2007; Abello et al., 2010). As mentioned above, *Tbx1* is able to repress neural cell fate (Raft et al., 2004). Transient amplification of ganglionar precursors requires growth factors like IGF-1, for proliferation, survival and differentiation into postmitotic neurons (Camarero et al., 2003).

Sensorogenesis

After delamination of neuroblasts, the neurosensory domain gives rise to the prosensory patches (Wu and Oh, 1996; Adam et al., 1998; Daudet et al., 2007). Prosensory patches are restricted domains in the otic epithelium that anticipate the sensory organs (Fig. 6). The process by which the neurosensory domain splits into distinct prosensory patches is still not clear. It is thought that Wnt signaling may be important since several Wnt related genes are expressed concomitantly with the initiation of morphogenesis and sensory fate specification (Sienknecht and Fekete, 2008; Sienknecht and Fekete, 2009). Moreover, activated Wnt signaling transiently induces ectopic sensory patches that are of vestibular character, suggesting that the Wnt pathway may bias the choice between auditory and vestibular fates (Stevens et al., 2003). Conversely, conditional deletion of *β-catenin* results in loss of crista sensory markers and the loss of HCs in the auditory and vestibular epithelia (Rakowiecki and Epstein, 2013; Shi et al., 2014).

A number of genes have been reported to label the prosensory regions, including *Jag1* (Adam et al., 1998; Cole et al., 2000), *Sox2* (Hume et al., 2007; Neves et al., 2007), *Lfng* (Morsli et al., 1998; Cole et al., 2000), *Prox1* (Stone et al., 2003), BEN (Goodyear et al., 2001), *Bmp4* and Bmp targets *Id1-3* (Oh et al., 1996; Chang et al., 2008; Kamaid et al., 2010), *Fgf10* (Alsina et al., 2004; Chang et al., 2008), *Hey1*, *Hey2*, *HeyL* (Leimeister et al., 1999; Hayashi et al., 2008b) (Fig. 6).

Determination of HCs is associated with the bHLH gene *Atoh1*, which is a proneural transcription factor that behaves as a mastergene for HC development. *Atoh1* is highly expressed in nascent HCs (Bermingham et al., 1999; Lanford et al., 2000). The loss of *Atoh1* leads to loss of HCs and secondarily to loss of SCs (Bermingham et al., 1999; Woods et al., 2004) and, contrarily, the overexpression of *Atoh1* results in supernumerary HCs (Zheng and Gao, 2000; Jones et al., 2006). Furthermore, *in utero* gene transfer of *Atoh1* is able to produce

functional HCs that integrate well in the mouse cochlea (Gubbels et al., 2008). Taken together, these results show that *Atoh1* is both necessary and sufficient for HC development.

The regulation of *Atoh1* is, therefore, at the core of HC development and a central topic in HC development and regeneration. *Atoh1* is regulated by a conserved 1.7kb fragment located 3.5kb downstream of *Atoh1* coding region. The enhancer region contains two blocks referred as A and B that are sufficient to drive *Atoh1* expression in all *Atoh1*-positive regions, including the inner ear (Helms et al., 2000). The regulation of *Atoh1* is complex and can be modulated positively or negatively. Once initiated *Atoh1* autoactivation maintains *Atoh1* expression through binding to a "class A" E-box, located in the enhancer B (Helms et al., 2000). *Atoh1* expression is negatively regulated by Ngn1, Ids and Hes/Hey proteins (Raft et al., 2007; Kamaid et al., 2010; Tateya et al., 2011). Many of these sites are in close proximity or partially overlapping, suggesting a possibility that the final level of *Atoh1* expression depends on competition of different factors and balance between activators and repressors. Sox2 regulates *Atoh1* expression through an incoherent logic (Box 4). The expression of *Atoh1* is directly initiated by Sox2 at otic placode stage, but silenced by the parallel activation of inhibitory factors until differentiation stages, delaying HC differentiation (Neves et al., 2012). In addition, Six1 also activates *Atoh1* expression, in parallel to *Atoh1* autoactivation and Sox2 cooperates with Six/Eya complex to enhance *Atoh1* expression (Ahmed et al., 2012). In order to allow HC differentiation, *Sox2* expression has to be silenced, however, the factors involved in this process are still unknown.

Box 4. Incoherent feed forward loops

Each transcriptional network consists of sets of recurring regulatory patterns called network motifs. One of these basic building units represents a family of feed-forward loops. A forward loop is a network motif that consists of interaction among three genes. The regulator X regulates Y and Z, which is also regulated by Y, resulting in targets regulation by both X and Y (Alon, 2007). Any of these interactions may be of active or repressive nature, thus allowing generation of diverse outputs. In a coherent feed forward loop (cFFL) the outcome is ether activation or repression, whereas an incoherent feed forward loop (iFFL) results in biphasic responses of activation and repression. The outcome of iFFL depends on the strength and dynamics of individual interactions (Alon, 2007) (Fig. 7).

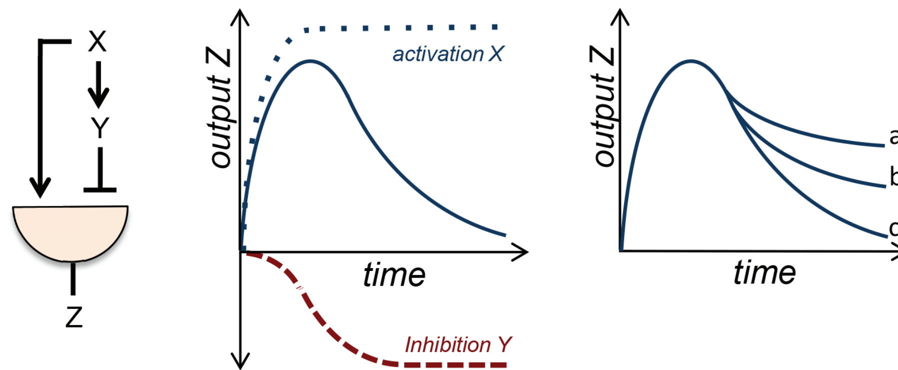

Figure 7. Schematic representation of the incoherent feed forward loop model. Left, the transcription factor X directly activates Z and at the same time it activates the repressor Y that inhibit Z: middle, the predicted output of Z is fast activation (blue dotted line) and delayed inhibition (red dotted line) that result in a transient Z output: right, the final output can vary depending on the balance in the strength of activation and inhibition at a steady state. Modified from Alon (2007) and Neves et al. (2013a).

Box 5. Common origin of neurons and sensory cells

In the sensilla of *Drosophila*, neurons, mechanoreceptors and their supporting cells arise from a single sensory organ progenitor cell (SOP). Given the homology with the functional unit of the inner ear, it is possible that neurons and HCs of inner ear share also a common lineage. Neurosensory elements of the inner ear derive from the neurosensory region of the otic placode (Adam et al., 1998; Raft et al., 2007). Expression pattern data show that sensory and neuronal lineages share *Sox2*, *Lfng*, *Dl1* and *Islet1* at least at some point during their development (Adam et al., 1998; Li et al., 2004; Radde-Gallwitz et al., 2004; Neves et al., 2007). Linage analysis in chick provides evidence of a shared neurosensory progenitor between neurons and HCs, although the study shows that bipotent progenitors are restricted to utricular macula and neurons from CVG (Satoh and Fekete, 2005). Further, fate mapping studies in chick suggest that the sensory organs and the neurons that innervate them arise from similar regions of the otic placode (Bell et al., 2008). Genetic manipulations in mouse have shown that HCs derive from *Ngn1*-positive progenitors that differentiate into neurons and HCs (Raft et al., 2007). It seems quite established that HCs and SCs derive from a common precursor (Fekete et al., 1998).

THE NOTCH SIGNALING PATHWAY

The Notch signaling pathway is highly conserved across phyla and directs multicellular development (Artavanis-Tsakonas et al., 1999; Bray, 2006; Kopan and Ilagan, 2009). It is a short-range communication pathway that requires physical interaction between membrane-bound ligands and receptors expressed in neighboring cells. The developmental outcome of Notch signaling is dependent on the cellular context. In addition, mutations in genes encoding several components of Notch signaling have been involved in diverse human diseases, including T cell acute lymphoblastic leukemia (T-ALL), Alagille syndrome, spondylocostal dysostosis, tetralogy of Fallot, Hajdu-Cheney syndrome, CADASIL syndrome and aortic valve disease (reviewed in Louvi and Artavanis-Tsakonas, 2012).

Box 6. History of Notch biology

Pioneering studies of *Notch* allele were carried out by Mohr in *Drosophila* and date from almost a century ago (Mohr, 1919). He characterized one of the first chromosomal deficiencies, which was caused by haploinsufficiency of the *Notch* locus. This *Notch* loss-of-function led to eponymous notch-like indentations of the *Drosophila* wing margin. Later on, Donald F. Poulson discovered the striking hypertrophy of the nervous system at the expense of ectoderm in the *Notch* null phenotype, naming it as *neurogenic* (Poulson, 1940). The *Notch* locus was then sequenced in both *Drosophila* (Artavanis-Tsakonas et al., 1983; Artavanis-Tsakonas, 1988) and *C. elegans* (Yochem et al., 1988), providing a solid foundation for the expansion of the field in the 90s with the discovery of the pleiotropic roles of Notch in development, tissue homeostasis and stem cell biology (reviewed in Artavanis-Tsakonas et al., 1999; Andersson et al., 2011; Hori et al., 2013).

Notch receptors

Notch receptors are multidomain type I transmembrane proteins. There are four mammalian Notch receptors (Notch1, Notch2, Notch3, and Notch4) that are orthologs of *Drosophila* Notch. Notch receptors share a common structure composed of a Notch extracellular domain (NECD), a negative regulatory region (NRR) and Notch intracellular domain (NICD). NECD consists of 29 to 36 tandem epidermal growth factor (EGF) repeats, which number varies among species and they are subject to multiple post-translational modifications. Rebay et al. (1991) showed that 11-12 EGF repeats are necessary and sufficient for receptor-ligand binding in *trans*. Interestingly, EGF-8 in *Drosophila* is involved together with EGF11-12

in Notch-Serrate, but not Notch-Dl binding, and acts independently of Fringe function (Yamamoto et al., 2012, see below). This gives an insight into a possible mechanism for Notch discrimination between Ser/Jag and Dl family ligands. NRR is composed of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD) and serves to prevent the access to S2 cleavage site in the absence of ligand (Bray, 2006; Kopan and Ilagan, 2009). The intracellular portion of Notch receptor consists of a RAM domain, an ankyrin (ANK) flanked by nuclear localization signals (NLS), a transcriptional activation domain (TAD) and a PEST domain. RAM and ANK domains are crucial for NICD interaction with CSL and Mastermind in the nucleus (Fig. 8A).

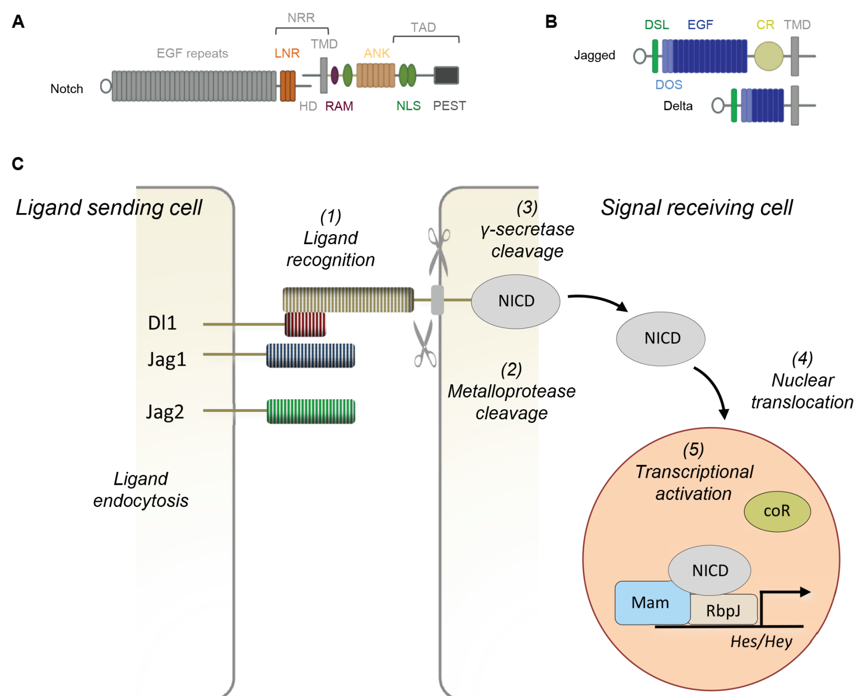

Figure 8. Structure of Notch ligands and receptors and Notch signaling pathway. The domain organization of Notch receptor (A) and DSL-family ligands (B). (C) The core of Notch signaling pathway. Interaction between Notch ligand and receptor (1) leads to series of proteolitic cleavages (S2 (2) and S3 (3) that result in release of the intracellular portion of Notch (NICD) (3). NICD translocates to the nucleus (4), where it enters into a transcriptional activation complex with CSL and Mam to activate transcription of Notch target genes (5). DSL: Delta/Serrate/LAG2; DOS: Delta and OSM-11 like proteins; EGF: epidermal growth factor motif; CR: cysteine-rich domain; TMD: trans-membrane domain; NRR: negative regulatory region; LNR: Lin12-Notch repeats; HD: heterodimerization domain; RAM: Rbpjk association module; NLS: nuclear localization sequence; ANK: ankyrin repeats; TAD: transactivation domain; PEST: proline/glutamic acid/serine/threonine rich motif; Mam: Mastermind; RBPjk: recombination signal sequence-binding protein-J kappa. Adapted from Kopan and Ilagan (2009); Neves et al. (2013b) and Gordon et al. (2008).

Notch ligands

The DSL (Delta-Serrate-Lag2) ligands are canonical Notch ligands that are responsible for the majority of Notch functions. The DSL ligands are type I transmembrane proteins characterized by multiple tandem epidermal growth factor (EGF) repeats in their extracellular domain. DSL domain together with the N-terminal region and the first two EGF repeats are required for the ligand binding to Notch receptor (Shimizu et al., 1999; Parks et al., 2006). Notch ligands are divided into two subgroups based on the homology with *Drosophila Delta* and *Serrate* genes. In mammals there are three Delta-like (Dll1, Dll3, Dll4) and two Serrate-like proteins also designated as Jagged (Jag1, Jag2) (Bray, 2006; Fiuza and Arias, 2007). However, in amniotes there are only two Delta-like (Dll1 and Dll4) and two Jag counterparts (Jag1, Jag2). Jag ligands distinguish from Dll ligands by the presence of almost twice the number of EGF repeats and additional cysteine-rich region (CR) that is not found in Delta ligands (D'Souza et al., 2008). The intracellular portion of DSL ligands is less conserved, but contains multiple lysine residues and C-terminal PDZ region, crucial for ligand signaling and interactions with cytoskeleton, respectively (Pintar et al., 2007) (Fig. 8B).

Apart from DSL ligands experimental evidences suggest the presence of non-DSL type of Notch ligands so-called non-canonical ligands capable of activating mammalian Notch receptors (reviewed in D'Souza et al., 2008). Adhesion molecule F3/Contactin and EGF-repeat factor DNER are non-DSL ligands that activate mammalian Notch during oligodendrocyte maturation and Bergmann glial cell differentiation, respectively (Hu et al., 2003; Eiraku et al., 2005). Mammalian microfibrillar proteins MAGP are also able to activate Notch receptors, but only when expressed in *cis* in the surface of the same cell (Miyamoto et al., 2006). There is no evidence of non-canonical Notch signaling in the inner ear.

Ligand-receptor binding

Notch signaling is a *juxtacrine* signaling system which activation requires the interaction between DSL ligands (Dll/Jag) expressed in one cell and Notch receptors in the surface of adjacent cell. The Notch receptor is presented to the ligand as an heteromer, resulting from cleavage by furin-like protease upon transition to the plasma membrane (Nichols et al., 2007). Atomic force microscopy revealed strong binding interaction between Delta and Notch in comparison to other ligand-receptor interactions and, likely, this helps to generate the force needed to dissociate and activate the receptor (Ahimou et al., 2004). Notch receptor interaction with DSL ligands initiates a series of proteolitic cleavages, first by a desintegrin and metalloproteases (ADAM) within the juxtamembrane domain, followed by γ-secretase

activity in transmembrane region resulting in release of intracellular portion of Notch receptor (NICD) into the cytoplasm (Fig. 8C).

The NICD transcriptional complex: the core of the pathway

Once released, and due to the presence of nuclear localization sequences (Stifani et al., 1992; Lieber et al., 1993), NICD translocates into the nucleus and forms a transcriptional complex with the CSL transcription factor (mammalian **C**-promoter binding factor 1, CBF-1 or recombination signal sequence-binding protein-J kappa, RBP-jkappa; *Drosophila* **S**uppressor of Hairless and *C. elegans* **L**ag-1) and Mastermind/MAML co-activator (Fig. 8C). Several studies revealed various conformational changes among members of the ternary complex that facilitate their mutual assembly (reviewed in Barrick and Kopan, 2006; Kovall, 2008). The RAM domain of Notch receptor allows NICD interaction with CSL, whereas the ANK domain of NICD and CSL provides an interface for Mam to bind (Choi et al., 2012). This complex further recruits other co-activators, such as histone acetyltransferases (CBP/p300) and chromatin remodeling complexes that drive the transcription of number of Notch target genes among which *Hes/Hey* genes are typical targets (Schweisguth, 2004; Bray, 2006; Fischer and Gessler, 2007; Fior and Henrique, 2009; Kopan and Ilagan, 2009; Hori et al., 2013). In the absence of NICD, CSL acts as a repressor by its association with co-repressors such as CtBP, N-CoR Hairless, Groucho, SMRT, SHRP, MINT and SPEN (reviewed in Bray, 2006; Kovall, 2008).

The direct translocation of the active Notch (NICD) into the nucleus makes Notch signaling a direct and straightforward transducer. However, equally important for precise spatio-temporal control and prevention of indefinite Notch pathway activation is NICD turnover. Disassembly of the CSL/NICD/Mam ternary complex is mediated by ubiquitination and sequential proteasomal degradation of NICD by the E3 ubiquitin ligase Fbw7/Sel10, involving phosphorylation of NICD on its TAD and C-terminal PEST domain by cyclin-dependent kinase 8 recruited by Mam (Fryer et al., 2004; Tsunematsu et al., 2004).

Notch proteolysis

Notch receptors suffer multiple modifications both before and after signaling to ligand. The Notch receptor anchored in the cell surface is in form of heterodimer processed by Furin-like convertases (S1 cleavage) occurred during trafficking through the Golgi complex (Logeat et al., 1998). The Notch heterodimer is held together by non-covalent interactions between their N- and C-terminal halves. O-linked glycosylation of NECD during Notch

receptor synthesis and secretion allows proper receptor folding and its interaction with ligand (reviewed in Rana and Haltiwanger, 2011). Receptor-ligand interaction triggers additional cleavage mediated by metalloproteinase ADAM/TACE (S2 cleavage) within the NRR, at the site that becomes exposed by ligand-induced conformational changes (Brou et al., 2000; Mumm et al., 2000). S2 cleavage is a prerequisite for subsequent S3 cleavage mediated by γ-secretase, which results in release of intracellular domain of Notch receptor (NICD). Upon γ-secretase cleavage, NECD bound to DSL ligands is *trans*-endocyted into the signal-sending cell (Gordon et al., 2008). ADAM metalloproteases also cleave DSL ligands to downregulate ligand activity (Zolkiewska, 2008).

Regulation of the Notch pathway

The Notch pathway is regulated by different means and at different levels of signal transduction. It is most commonly regulated by post-translational control, which ensures Notch pathway operation in spatio-temporal manner in a wide variety of developmental contexts.

Tuning of Notch receptor activation

Endocytosis is a process that directly regulates the pool of Notch receptors available at the plasma membrane. Different proteins have been shown to be involved in Notch endocytosis including GTPase Dynamin, Numb (cytoplasmatic protein) and Sanpodo (transmembrane protein) (Fortini, 2009; Hori et al., 2013). Numb is a membrane-associated phosphotyrosine-binding inhibitor of Notch that acts as a cell fate determinant during asymmetric cell divisions in *Drosophila* and mammalian neurogenesis. During mitosis it is unequally inherited by two daughter cells specifying the cell fate (reviewed in Schweisguth, 2004). Upon endocytosis, Notch receptor can be recycled back to plasma membrane or alternatively processed for lysosomal degradation (Yamamoto et al., 2010; Baron, 2012).

Ubiquitination is an additional important mechanism that controls membrane trafficking of Notch receptor and its regulation. Deltex (Dx) physically interacts with intracellular portion of Notch (Diederich et al., 1994; Matsuno et al., 1995; Takeyama et al., 2003) and positively regulates Notch signaling (Xu and Artavanis-Tsakonas, 1990; Busseau et al., 1994; Matsuno et al., 1995; Kishi et al., 2001; Izon et al., 2002). Dx may function by deviating Notch from the lysosomal degradation route and therefore stabilizing Notch receptor (Hori et al., 2004; Sakata et al., 2004). Dx-mediated Notch activation is likely ligand-independent. Also Dx has been reported to negatively regulate Notch pathway in the nucleus, by preventing recruitment of co-activators by NICD (Izon et al., 2002). Other E3 ubiquitin ligases like Suppressor of

Deltex/Itch and Cbl also target non-activated Notch for degradation (Hubbard et al., 1997; Jehn et al., 2002; Sakata et al., 2004; Wilkin et al., 2004).

Termination of the pathway is strictly related to NICD degradation that is mediated by Mam recruitment of CDK8, which triggers PEST-dependent degradation by the Fbw7/Sel10 ubiquitin ligase (Fryer et al., 2004; Tsunematsu et al., 2004 and see above).

GSK-3β is another kinase that phosphorylates NICD and the outcome of this phosphorylation is dependent on the cellular context and can either upregulate or downregulate Notch activity (Foltz et al., 2002; Espinosa et al., 2003).

Finally, the most recently discovered Notch modulation is acetylation and deacetylation of NICD that contributes to fine tuning NICD half-life in endothelial cells (Guarani et al., 2011).

Regulation of Notch ligand activity

Ligand ubiquitination is mediated by E3 ligases that regulate ligand expression on the cell and their availability for Notch activation (Le Borgne and Schweisguth, 2003; Chitnis, 2006; Nichols et al., 2007). Neuralized (Neur) and Mind bomb (Mib) are E3 ligases that interact with DSL ligands (both Jag and Dl) through their lysine enriched domain and add ubiquitin to enhance their endocytosis. Mib is required for initial step of DSL ligand endocytosis, whereas Neur acts downstream of Mib directing internalized ligands to lysosomal degradation (Song et al., 2006). While polyubiquitination is associated with proteasome degradation, both mono- and multi-monoubiquitination can signal for endocytosis of DSL ligands from the cell surface and further influence intracellular trafficking (D'Souza et al., 2008).

Ligand endocytosis regulates the availability of DSL ligands at the cell surface. In order to be competent to activate Notch in signal-receiving cell, the DSL ligand has to be first ubiquitinated and then internalized through endocytic pathways (Box 7). A variety of proteins regulate DSL endocytosis, although the entire mechanism is still not clear.

Box 7. Models of ligand endocytosis

Three models have been proposed to explain how ligand endocytosis promotes Notch activation (Le Borgne et al., 2005). In the "*lift and cut*" mechanism endocytosis may be largely responsible for generating a physical force needed to pull the Notch ectodomain, promoting a conformational change and exposing the metalloprotease cleavage site (Gordon et al., 2008). One possibility is that the ligand undergoes endocytosis while bound to Notch. This

internalization of Notch by signal sending cell is called *trans-endocytosis* (Parks et al., 2000). A second model predicts that newly synthesized DSL ligands have to pass through recycling endosomes and undergo unknown post-translational modification and only after trafficked back to plasma membrane they become active (Wang and Struhl, 2004). Finally, a third model postulates that DSL ligands are endocyted and trapped into multi-vesicular bodies (MVB), from where they can be degraded upon MVBs maturation into lysosomes or instructed for exocytosis by means of exosomes for subsequent delivery to the cell membrane.

Notch regulation by glycosylation at ligand-receptor interactions

EGF repeats of the Notch receptor are susceptible to various glycan modifications required for regulation of Notch receptor function and modulation of ligand binding properties (reviewed in Stanley and Okajima, 2010; Rana and Haltiwanger, 2011). Notch receptors undergo at least three types of post-translational O-linked modifications: O-glycosylation, O-fucosylation and O-GlcNAc addition.

Pofut1 is a mammalian counterpart of *Drosophila* Ofut1, localized in endoplasmic reticulum (ER) that adds O-fucose to several EGF repeats, which allow proper Notch folding required for optimal ligand binding and Notch signaling (Stahl et al., 2008).

Rumi is an ER-localized protein similar to *Drosophila O*-glycosyltransferase (Poglut) (Acar et al., 2008). *Rumi* loss-of-function in mouse cell lines and in the developing liver affects step between ligand binding and S3 cleavage (Fernandez-Valdivia et al., 2011). The Jag1 induced signaling during bile duct morphogenesis is sensitive to the gene dosage of *Rumi*, suggesting the relevance of O-glucose occupancy on Notch EGF repeats for optimal Notch signaling (Fernandez-Valdivia et al., 2011).

In mammals Rumi leaves O-linked glucose residues on Notch1 and Notch2 that are subject to further extension by addition of one or two xylose residues by xylosyltransferases (Moloney et al., 2000a; Whitworth et al., 2010; Rana et al., 2011). Functional studies in *Drosophila* revealed that xylose negatively regulates Notch signaling (Lee et al., 2013).

O-fucosylation is a prerequisite for further Notch receptor modulation by Fringe proteins. There are three mammalian Fringe prologues named Lunatic, Radical and Manic fringe. *Fringe* gene encodes for β-1,3-N-acetylglucosaminyltransferase that acts on *O*-fucosylated EGF repeats of Notch receptor. The glycosylation occurs in the Golgi apparatus, before Notch maturation and localization to the plasma membrane (Bruckner et al., 2000; Moloney

et al., 2000b; Munro and Freeman, 2000). The relevance of the Fringe-mediated modifications has been well studied, although the molecular mechanisms are still not fully understood. It is thought that Fringe/Lunatic fringe-mediated modification of Notch potentiates Notch signaling induced by Dl, while inhibiting signaling induced by Jag (Bruckner et al., 2000; Hicks et al., 2000; Shimizu et al., 2001; Lei et al., 2003; Okajima et al., 2003). Manic fringe has been reported to function similarly to Lunatic fringe (Hicks et al., 2000; Shimizu et al., 2001; Yang et al., 2005) and the role of Radical fringe is still unclear.

Sugar addition may influence Notch-ligand interactions in several ways either influencing conformational changes of Notch that favors Notch ligand-receptor interactions, and by changing ligand recognition (Haines and Irvine, 2003). Yang et al. (2005) proposed that Fringe glycosylation modulates the strength of ligand-Notch interactions affecting their ability to survive the pulling force produced by ligand endocytosis.

Cis inhibition

Contrarily to normal operation of Notch as a *trans*-interaction transducer, interaction between Notch ligand and receptor within the same cells results in Notch pathway inhibition (reviewed in del Alamo et al., 2011). It is thought that *cis*- ligand-Notch interaction sequesters Notch receptor and, thereby preventing its binding with the ligand from adjacent cell. The developmental significance of *cis*-inhibition of Notch pathway has been reported in different developmental contexts (de Celis and Bray, 1997; Yaron and Sprinzak, 2012), however, this type of interactions does not occur during inner ear development (Chrysostomou et al., 2012).

Transcriptional regulation of the Notch target genes

Notch functional diversity in a given cellular context is achieved by tightly regulated transcription of only a subset of Notch targets genes. There is growing evidence of a variety of context-dependent molecular mechanisms that provide precise spatial and temporal control of Notch-responsive gene expression.

Since upon Notch activation target gene transcription depends on CSL/RBPjk, one key question is to understand how Notch regulates transcription in a context-dependent manner. This can be achieved by different means: 1) different Notch responses may result from the combinatorial interaction between Notch transcriptional complexes with tissue-specific transcription factors; 2) cellular context may underlay several Notch paralogues which activation may result in different Notch target gene preferences (Ong et al., 2006); 3) Notch

dosage may dictate different outcomes (Mazzone et al., 2010). This hypothesis has been tested in the present work (see below); 4) transcription of conventional Notch targets may depend on Notch-independent mechanisms (Doetzlhofer et al., 2009); 5) the expression of so considered atypical Notch targets can be activated through non-canonical Notch pathways (Ross and Kadesch, 2001).

Activation of Notch targets is often insufficient to provide the required diversity of Notch-dependent gene expression. Additional mechanisms negatively control gene expression, including repression of local activators or their absence from a given context, epigenetic modifications, differences in CSL/co-repressor complexes and binding site architecture in promoter regions of target genes (reviewed in Cave, 2011 and see Box 8). In *Drosophila*, microRNAs have been reported to post-transcriptionally tune the expression of Notch targets such as E(spl) (Lai et al., 2005).

In summary, the outcome of Notch signaling is highly context dependent because of a variety of molecular mechanisms of transcriptional control. These mechanisms can act individually or in combinatorial manner to govern distinct expression patterns of individual Notch target genes.

Box 8. Selective repression of Notch target gene transcription

Chromatin remodeling enzymes associated with CSL either when part of Notch transcriptional complex or when bound to co-repressor maintain chromatin environments of primary Notch targets in active or repressive state. The type of epigenetic mark on promoter region of Notch target defines which targets remain repressed when Notch pathway is turned "on". Binding affinity and competition between NICD and co-repressors for CSL binding control transcriptional activity of Notch targets. However, upon Notch pathway activation NICD displaces Hairless which affinity to CSL is 10^3 times greater than that of the NICD (Maier et al., 2011). This is due to ability of NICD to provoke allosteric conformational change that overrides its lower binding affinity for CSL and results in destabilization of CSL/co-repressor complexes. Upon CSL/co-repressor complex dissociation new CSL transcription factors can bind DNA and, depending on Notch activation, will assemble either NICD or re-assemble co-repressors to activate or repress targets, respectively. A recent study reported the categorization of all Notch target genes into three distinct groups: genes which transcription is dependent on CSL/NICD dimmers, genes which transcription is independent on CSL/NICD dimmers and genes that utilize both monomeric and dimeric CSL/NICD complexes (Liu et al., 2010; Cave, 2011).

Notch modes of action: lateral induction vs. lateral inhibition

Notch plays many different functions during diverse developmental and physiological processes. This functional diversity results in at least two main cellular modes of tissue interactions that are called lateral inhibition and lateral induction (Fig. 9).

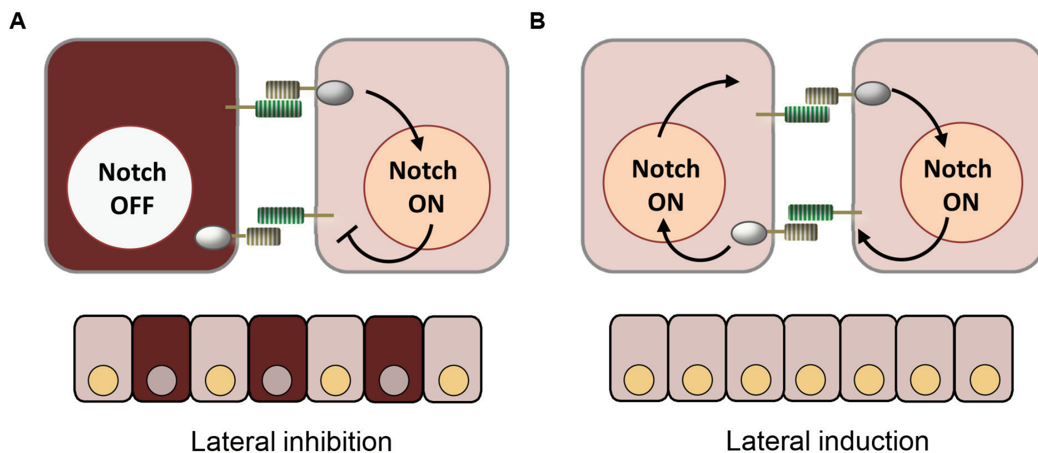

Figure 9. The two modes of operation of Notch. (A) Lateral inhibition is described as a negative feed-back loop by which Notch ligand induces Notch activity in the neighboring cell, and this causes the suppression of the expression of Notch ligand. The result is that the ligand-delivering cell shuts down Notch activity and adopts different fate from neighboring cell. Fine grained pattern of gene expression is typical hallmark of lateral inhibition. (B) Lateral induction is characterized by a positive feed-back loop between Notch and the Notch ligand. The outcome in coordinated cell behavior and uniform gene expression. Adapted from Neves et al. (2013b).

Lateral inhibition

Lateral inhibition is a mechanism by which Notch activation in one cell inhibits the expression of the Notch-activating ligand in other cell. Lateral inhibition mediates binary cell fate decisions, ensuring that the cells adopt one of two alternative fates (Fig. 9A). The first use of this term dates from 1940 by Wigglesworth in the development of bristles of the beetle, *Rhodnius prolixus*. He suggested that an inhibitory substance is produced by already formed mother bristle cells, which diffuses to inhibit neighboring epidermal cells (Wigglesworth, 1940). Ablation of neuroblasts in the grasshopper embryo showed that epidermal cells that normally acquire the epidermal fate differentiated as neuroblasts (Doe and Goodman, 1985). This suggested to the authors that, first, under normal conditions there is an inhibitory signal from neuroblasts that prevents surrounding epidermal cells from adopting neuronal fate and, secondly, that all cells from a given cluster have the potential of embarking on neurogenic pathway.

The distribution of differentiated cells as an outcome of lateral inhibition is based on the balance among three factors: distribution of activator, bias provided by an earlier patterning mechanism and the strength of the lateral inhibition (reviewed in Chitnis, 1995).

An activator is a determinant that drives a given cell fate. Stochastic variations have been proposed to create the asymmetry between initially equivalent cells. Random increase of activator´s levels in certain cells would provide them with the capacity to strongly inhibit their neighbors. One example is the adoption an anchor cell fate (AC) vs. ventral uterine precursor fate (VU) in *C. elegans*. The two cells start with initially the same potential characterized by equal levels of Notch homologue lin-12 and Delta homologue lag-2. Random variations, between neighboring cells and their amplification by feedback mechanisms contribute to one cell acquiring higher levels of lag-2 that commits to AC fate, whereas cells with lin-12 commit to VU fate.

In contrast to this stochastic model, two cells may acquire asymmetric pattern by amplifying an already pre-set intrinsic or extrinsic bias. Vulval precursor specification in *C.elegans* is an example of binary cell fate decisions biased by extrinsic cues. All six vulval precursor cells have potential to adopt any of the three cell fates named 1º, 2º and 3º, but invariant cell fate pattern arises by extrinsic cue provided by AC, previously specified by random choice (see above) (Artavanis-Tsakonas et al., 1999). During *Drosophila* sensory organ development cell fate determination is promoted by intrinsic cues in the form of unequal segregation of Numb and Neuralized into daughter cells (reviewed in Schweisguth, 2004). Similarly, in *C. elegans* P granules are cytoplasmic determinants that modulate the function of Notch homologue glp-1 (Evans et al., 1994).

The strength of lateral inhibition is also crucial to the outcome of this process. The cell with the highest amount of the activator prevails over the neighbors and is singled out from a given cluster. Thereby, lateral inhibition ensures that only one cell wins in a given cluster. This pattern has been well studied in *Drosophila* bristle development. The mechanosensory bristles, so-called macrochaetes, derive from Sensory Mother Cell (SMC) by two-step process. First, a group of cells is selected from proneural cluster in the ectoderm and specified by the expression of proneural genes of *Achaete Scute Complex* (*ac-sc*). This provides cells with competence to become SMC. Subsequently, proneural genes upregulate Notch ligand Dl1, which activates Notch in the neighboring cells and via lateral inhibition proneural gene expression is suppressed in all but in one single cell. The result is that only few cells from selected cluster become mechanosensory cells, the remaining adopting the epidermal fate (Cubas et al., 1991; Skeath and Carroll, 1991).

Several experiments in *Drosophila* exemplify bristle/epidermal cell fate choices occurring via lateral inhibition. The complete loss of proneural gene leads to all cells from a cluster developing as macrochaetes (Goriely et al., 1991). Similarly, mosaic patches of *Notch* mutant cells develop as bristles (Heitzler and Simpson, 1991). Notch is not necessary for epidermal fate, since double mutant cells for *Notch* and *ac-sc* differentiate into epidermal fate (Simpson and Carteret, 1989; Heitzler and Simpson, 1993). This suggests that Notch does not have an instructive role in determining epidermal fate, but instead in inhibiting neural fate. Work from Heitzler and Simpson (1993) suggested also that proneural blockade by Notch is mediated by Dl.

Main principles of lateral inhibition adapted from pioneering studies of *Drosophila* development are currently expanded to vertebrate neurogenesis. Overexpression of Dl1 or NICD in retina results in failure of neurogenesis and maintenance of progenitors (Austin et al., 1995; Dorsky et al., 1997; Henrique et al., 1997), whereas blockade of Notch by forced expression of dominant-negative form of Dl1 results in premature differentiation and exhaustion of progenitor pool (Henrique et al., 1997).

In summary, Dl and Notch do not determine the location or properties of proneural cluster, which depend on earlier and independent patterning mechanism. What Notch interactions serve is to compare relative potentials to adopt a certain fate among adjacent cells, and amplify minor differences so to generate binary cell fate choices and salt-and-paper patterns of gene expression.

Box 9. Oscillation model of lateral inhibition

Real-time studies of mammalian neurogenesis revealed the presence of oscillations of gene expression in neural progenitors (Shimojo et al., 2008). *Hes1* expression in neural progenitors oscillates due to a negative feedback loop in which Hes1 protein represses its own transcription. The oscillations of *Hes1* expression influence opposite oscillatory pattern of expression of proneural (*Ngn2*) and neurogenic (*Dll1*) genes (Kageyama et al., 2008; Shimojo et al., 2008). Therefore, Notch-mediated lateral inhibition continually changes gene expression in the pool of neural progenitors and it does not predict that the cell that expresses raised levels of proneural gene at a certain time point will become neuron. This suggests that lateral inhibition is not a tool for neuronal selection, but indeed keeps equipotent population of proliferating progenitors (Kageyama et al., 2008). Later in development, *Ngn2* and *Hes1* expression becomes invariable but inverse in postmitotic progenitors. *Hes1* is thought to be repressed by presence of Numb (Cayouette and Raff, 2002; Kageyama et al., 2008), and neuronal selection determined by the presence of factors

that regulate asymmetric/symmetric cell divisions. The accumulation of factors necessary for cell cycle exit above a certain threshold may instruct neural progenitors to start to divide asymmetrically or symmetrically. Whether oscillatory model of lateral inhibition underlies lateral inhibition during HC determination is completely unknown and oscillatory expression patterns of proneural or Notch genes have never been observed in the inner ear.

Lateral induction

Lateral induction is another type of Notch mode of action, which unlike lateral inhibition is less well understood. Lateral induction is an inductive process where a ligand-expressing cell stimulates their neighbors to promote the expression of the same ligand, resulting in coherent domains of Notch activity and coordinated cell behaviors (Bray, 1998; Lewis, 1998; Eddison et al., 2000) (Fig. 9B). Different ligand regulation by Notch underlies lateral induction and lateral inhibition. The two processes are characterized by positive or negative regulation of ligand by Notch signaling, respectively, and the positive feedback loop of lateral induction promotes coordinated cell specification among a group of cells that notably opposes conventional Notch mode of lateral inhibition.

A Notch inductive process was first described in boundary formation of the *Drosophila* wing. Here Notch is crucial for establishment of D-V boundary, where it is activated in the interface of dorsal and ventral compartments, keeping the two populations separated. Activation of Notch at the interface results in creation of the boundary cells that behave as organizing centers that control growth and patterning of the wing in D-V axis. Although being expressed in the entire wing disc, Notch is activated only in boundary cells by coordinated interaction between cells from dorsal and ventral territories. Cells residing in the dorsal compartment of *Drosophila* wing express the Notch ligand Serrate that activates Notch in the cells of the ventral portion of D-V boundary. Fringe protein from the dorsal side prevents Serrate-mediated Notch activation in the dorsal compartment, thus creating an asymmetry between the two cell populations. However, Delta ligand maps to the ventral portion of *Drosophila* wing and signals to activate Notch in dorsal portion of D-V boundary. Cells from a ventral compartment are prevented from signaling to each other by Delta-Notch *cis*-inhibition. As a consequence, cells that receive Delta signal in the dorsal portion of the boundary activate *Serrate* expression, which activates Notch in the ventral side of D-V boundary. The cells that are activated by Serrate in the ventral side of D-V border activate *Dl1* expression, which in turn activate Notch in the dorsal side (Bray, 1998; Irvine and Rauskolb, 2001).

There are also several examples of lateral induction in vertebrates, including the inner ear (Eddison et al., 2000, see below), eye (Onuma et al., 2002), limb (Irvine and Vogt, 1997), somites (Oates et al., 2012), lens (Le et al., 2009), hair cell follicle (Ross and Kadesch, 2004) and neural crest (Cornell and Eisen, 2005). Dl-Notch signaling has been proposed to provide a local cell coupling mechanism for oscillatory synchronization of the cells in the presomitic mesoderm (PSM) (Jiang et al., 2000). It is thought that the timing of Notch signal changes the timing of transcriptional initiation of *Hes* genes in signal receiving cell, which is communicated to neighboring cells by change in timing of *Dl* expression. Thus, by synchronizing the oscillating gene expression, Notch tunes the cells for the same behavior and attenuating the differences between neighboring cells (Oates et al., 2012). Notch signaling has been reported to play an inductive role in *Xenopus* eye development. Ectopic Notch activation results in activation of *Pax6*, which results in eye duplications and proximal eye defects that are also observed by *Pax6* missexpression (Onuma et al., 2002). In the lens, inductive Jag1 signaling is required to maintain a proliferating pool of epithelial precursor cells as well as for proper secondary fiber cell differentiation. Upon secondary fiber cell production, Jag1 positive cells that reside in transition zone, pass out and cooperatively adopt a secondary fiber cell fate (Le et al., 2009). Accordingly, a conditional lens *Jag1* mutant mice show decreased secondary fiber cell differentiation (Jia et al., 2007). This is reminiscent of Jag1 function in prosensory patches of the inner ear (see below).

NOTCH TARGET GENES: *HES* AND *HEY* GENES

Hes/Hey genes are the most extensively studied canonical Notch targets. *Hes* genes are class C basic helix-loop-helix (bHLH) factors and are the mammalian counterparts of *Drosophila Hairy* and *Enhancer of split (E(spl))* (Sasai et al., 1992). Seven *Hes* members have been identified in vertebrates so far (*Hes1-7*) (Akazawa et al., 1992; Sasai et al., 1992; Nishimura et al., 1998; Pissarra et al., 2000; Vasiliauskas and Stern, 2000; Bessho et al., 2001). *Hey* genes belong to Hes-related gene (*Hesr*) family also known as *Hrt* (Hairy-related transcription factor), *Herp* (Hes-related repressor protein), *Chf* (Cardiovascular helix-loop-helix factor) and *gridlock*. *Hey* subfamily of genes encodes for three members (*Hey1*, *Hey2* and *HeyL*) (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Iso et al., 2001a; Iso et al., 2001b).

Structure and DNA binding specificity

Hes and Hey proteins share a common structure composed of evolutionary conserved bHLH domain and Orange domain. The basic domain is crucial for DNA binding, whereas the following two α-helixes separated by the loop (HLH) provide dimerization and additional protein interactions (Massari and Murre, 2000). The Orange domain servers as an additional platform for protein interactions and for the selection of partners (Dawson et al., 1995; Taelman et al., 2004). However, Hey proteins distinguish from Hes subgroup by two striking features: first the invariant proline residue in basic domain of all Hes members is instead replaced by glycine. Secondly, the C-terminal WRPW motif that is characteristics of Hes proteins, is replaced with YRPW or YXXW (HeyL) (Fischer and Gessler, 2007; Jalali et al., 2011). The C-terminal domain provides Hes proteins with a repressive function. The YXXW motif is followed by a conserved TE(I/V)GAF peptide with unknown function (Fig. 10A). The C-terminal WRPW motif acts as polyubiquitination signal (Kang et al., 2005). Therefore, Hes factors are rapidly polyubiquitinated and degraded by proteasome with a very short half-life of approximately 20 minutes (Hirata et al., 2002).

There is an obvious difference in Hes and Hey capacity to bind DNA sequences. Most bHLH factors bind consensus sequence named E-box (CANNTG) (Kageyama et al., 2007). However, Hes factors bind with the highest affinity to different target sequences, which are class C site (CACGCG) or N-box (CACNAG) (Iso et al., 2003; Fischer and Gessler, 2007; Sun et al., 2007). However, invariant glycine in all Hey members causes their inability to bind N-box sequences, but drives their preferential binding to E-box sequences of class A (CAGGTG) or class B (CACGTG) (Iso et al., 2003; Sun et al., 2007).

Although *Hes* and *Hey* genes are thought to be conventional Notch targets, not all members respond to Notch signaling. While all *Hey* members are Notch responsive genes (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Leimeister et al., 2000a; Lin et al., 2000; Maier and Gessler, 2000; Iso et al., 2001a; Iso et al., 2002) only *Hes1*, *Hes5* and *Hes7* are induced by Notch activation (Jarriault et al., 1995; Hsieh et al., 1997; Nishimura et al., 1998; Ohtsuka et al., 1999; Bessho et al., 2001). In contrast, *Hes2*, *Hes3* and *Hes6* appear to be Notch-independent and data on *Hes4* regulation is still missing (Nishimura et al., 1998; Koyano-Nakagawa et al., 2000). *Hes/Hey* dependence on Notch signaling may vary in different cellular contexts (Doetzlhofer et al., 2009; Jalali et al., 2011).

Several additional mammalian proteins exhibit strong homologies with Hairy and E(spl), including Helt, DEC1 and DEC2. They are characterized by the lack of WRPW/YRPW motifs and there is yet no evidence for their regulation by Notch signaling (Fischer and Gessler, 2007).

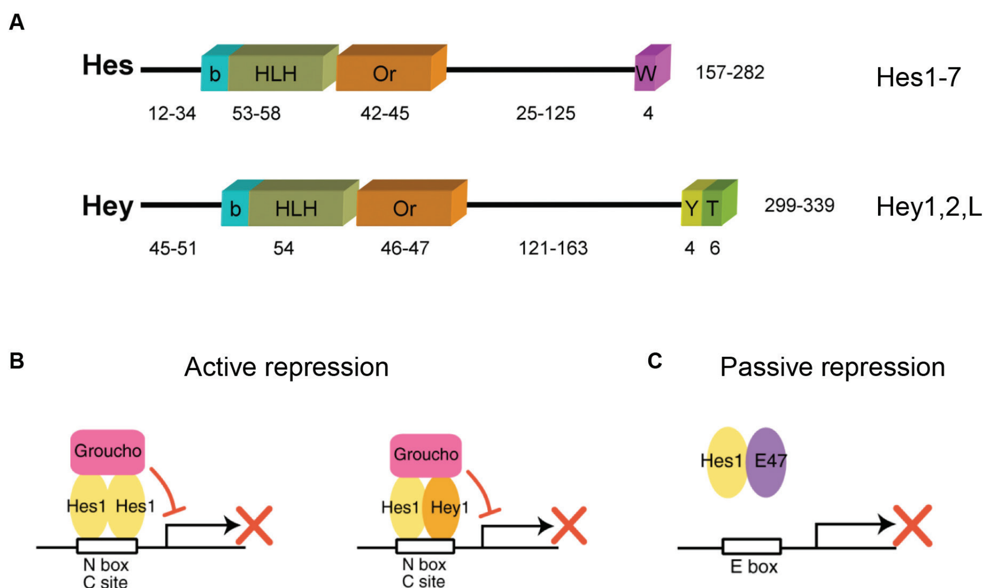

Figure 10. Structure and modes of transcriptional repression of Hes/Hey. (A) Domain organization of Hes (Hes1-7) and Hey (Hey1,2,L) proteins. Evolutionary conserved domains are labeled with distinct colors: basic-helix-loop-helix (bHLH), Orange (Or) and tetrapeptide motif (WRPW or YRPW or YXXW). Numbers indicate the amino acid content of the individual protein domains. (B) Mechanism of active repression by Hes and Hey proteins. Hes factors form homodimers or heterodimers with Hey proteins and bind N-box or class C site to actively repress transcription by interacting with co-repressors, such as Groucho through WRPW motif. (C) Dominant negative effect by passive repression. Hes factors form non-DNA binding heterodimer complex with bHLH activators such as E47 and inhibit transcription. Adapted from Fischer et al. (2007) and Kageyama et al. (2007).

Hes/Hey proteins function as homo- of heterodimers

Hes and Hey proteins may act as homo- or heterodimers via their HLH domains (Leimeister et al., 2000b; Iso et al., 2001b; Van Wayenbergh et al., 2003; Ross et al., 2006; Fischer et al., 2007) (Fig. 10B). Heterodimers bind to DNA target sequences with higher affinity than homodimers (Iso et al., 2001b). Indeed, the formation of heterodimers between Hes and Hey factors is more stable than the corresponding homodimers, this interaction being improved by the Orange domain (Leimeister et al., 2000b). The functional synergy and the co-expression of different Hes and Hey proteins in certain cell types suggest that their heterodimerization provides efficient signal amplification through their ability to recruit a more diverse set of repressors (Iso et al., 2003). However, in some case like during neural differentiation, heterodimerization can led to Hes and Hey antagonism (see below).

Hes/Hey auto- and cross-regulation

Hes/Hey genes are able to cross-regulate each other. Negative regulation of *Hes5* transcription by Hes6.2 has been shown to be a key mechanism to ensure proper modulation of Notch activity during neurogenesis (Fior and Henrique, 2005; Vilas-Boas and Henrique, 2010). Mutual exclusivity between different *Hes/Hey* members has been described in many tissues. Hes5 and Hes1 seem to negatively regulate each other's transcription in the mouse spinal cord, since *Hes1* and *Hes5* expression is complementary in wild type mice but upregulated in *Hes5*-/- and *Hes1*-/- mutant mice (Hatakeyama et al., 2004). *Hey1* and *Hey2* expression is mutually exclusive in the developing heart, where *Hey1* is expressed in the ventricles while *Hey2* is expressed in atria (Fischer and Gessler, 2003). Similarly, in the mouse inner ear *Hey2* is co-expressed with *Hey1* but never with *HeyL* (Hayashi et al., 2008b). The lack of *Hey2* and *HeyL* simultaneous expression in the ear may result from their mutual transcriptional repression, although direct demonstration of this hypothesis is missing. By contrast, *Hey1* can be co-expressed with either *Hey2* or *HeyL* in the sensory domain of otic epithelium (Hayashi et al., 2008b). Doetzlhofer et al. (2009) suggest that Hey2 represses *Hes5* expression in pillar cells, since in *Hey2* mutants *Hes5* expression is expanded into pillar cells (Doetzlhofer et al., 2009).

In addition to non-RBPjk dependent repression, a novel mechanism has been proposed for Hes and Hey repression of *Hes/Hey* promoters. In mammalian cell lines Hey2 and Hes1 negatively regulate Notch-dependent transcription of *Hes/Hey* genes through direct association with RBPjk and other co-repressors. The repression does not interfere neither with DNA-binding of nuclear Notch transcriptional complex (NICD-RBPjk) nor dissociation of

NICD from RBPjk (King et al., 2006).

Cross-regulation between Hes proteins has been exemplified in neural tube where Hes6/Hes1 interaction results in Hes6-mediated negative regulation of Hes1 activity in neural progenitors. Formation of Hes6/Hes1 prevents recruitment of TLE co-repressor, necessary for Hes1 repressive function and/or may trigger protein degradation of heterodimer (Bae et al., 2000; Gratton et al., 2003; Belanger-Jasmin et al., 2007). In the chick neural tube, Hes6.1 is proposed to bind to Hes5 and prevent Hes5 from repression transcription on its own genes (Vilas-Boas and Henrique, 2010).

Hes and Hey proteins are known to repress their own transcription. Work from several laboratories show that Hes1, Hes7, Hey1, Hey2 and HeyL factors repress their own promoters (Takebayashi et al., 1994; Maier and Gessler, 2000; Nakagawa et al., 2000; Bessho et al., 2001). However, this autonomous repression is short for Hes proteins, due to a short half-life (Hirata et al., 2002; Hirata et al., 2004). The relevance of this autoregulation is well exemplified in the segmentation clock, where synchronized oscillations of *Hes* expression result from alternate repression and de-repression.

Mechanisms for transcriptional repression by Hes/Hey proteins

With the exception of Hes6 and HeyL, all Hes and Hey proteins function as repressors of tissue specific differentiation and determination genes in a variety of systems (Iso et al., 2003; Fischer and Gessler, 2007; Kageyama et al., 2007). Despite similar structural features Hes and Hey proteins exert their repression function by different repression mechanisms.

Three mechanisms have been proposed for Hes-mediated repression. 1) Active repression requires DNA binding of the transcriptional repressor (Kageyama and Nakanishi, 1997; Kageyama et al., 2000). Here, Hes proteins either as homo- or heterodimers recruit co-repressor Groucho or its mammalian counterparts TLE1-4 via their WRPW motif (Paroush et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996). It is thought that TLE proteins can further attract additional co-repressors like histone deacetylases (HDAC) and members of Sin3 complex that result in strong transcriptional blockade (Chen et al., 1999; Choi et al., 1999) (Fig. 10B). 2) Passive repression does not require DNA-binding, but relies on protein sequestration (Sasai et al., 1992; Hirata et al., 2000). For instance, Hes1 forms a nonfunctional heterodimer with other bHLH factor like E47 that is a common partner of tissue-specific determination genes (MyoD, Mash1), thereby displaying a dominant-negative effect that prevents the formation of E47:MyoD and E47:Mash1 heterodimers (Fig. 10C). 3) Orange domain-mediated repression involves either a direct recruitment of an unknown co-

repressor and/or the stabilization or regulation of the WRPW-mediated repression through intra- or intermolecular interaction (Castella et al., 2000). Hes1 association with TLE can be dissociated by signaling pathways that convert Hes1 into a transcriptional activator (Ju et al., 2004).

Like Hes factors, Hey proteins form homo- or heterodimeric complexes both via DNA-dependent and independent mechanisms. Unlike Hes, due to the absence of WRPW motif, they cannot recruit TLE repressors, their repression activity residing primarily in the bHLH domain. Therefore, Hey proteins employ a different molecular mechanism directly interacting with other co-repressors like N-CoR, mSin3A, which then further recruit histone deacetylases and repress transcription (Iso et al., 2001b).

In addition, passive repression mechanisms have been also proposed for Hey family members. Like for Hes proteins, transcriptional regulation independent of DNA binding can be achieved by turning a transcriptional activator into a repressor, prevention of DNA binding, sequestration, degradation or interference with basal transcriptional machinery (reviewed in Fischer and Gessler, 2007).

Functional roles played by *Hes/Hey* genes

Hes/Hey genes regulate the maintenance of stem cells and progenitors

Notch signaling occupies the central place in maintenance of intestinal homeostasis. *Math1* is a mastergene for goblet, enteroendocrine and Paneth cell differentiation, whereas the activation of Notch-Hes1 pathway represses *Math1* expression and differentiation of corresponding cell types (Jensen et al., 2000; Yang et al., 2001; Fre et al., 2005; Stanger et al., 2005; Suzuki et al., 2005; van Es et al., 2005). These data suggest that the Notch-Hes pathway is essential for maintaining a pool of intestinal stem cells. The pancreatic exocrine and endocrine development is regulated by bHLH factors: Ptf1a and Ngn3, respectively (Krapp et al., 1998; Gradwohl et al., 2000; Kawaguchi et al., 2002). Notch-Hes1 signaling promotes the maintenance of pancreatic progenitors by antagonizing *Ptf1a* in exocrine cells and *Ngn3* in endocrine cells (Jensen et al., 2000; Hald et al., 2003; Esni et al., 2004; Fujikura et al., 2006).

During myogenesis, the Notch-Hey1 pathway keeps cells in an undifferentiated state antagonizing muscle differentiation. *Hey1* expression is elevated in undifferentiated C2C12 myoblast cells, but decreased as muscle differentiation proceeds, and the overexpression of Hey1 opposes the effect of MyoD-induced myogenic conversion of 10T1/2 cells. The

underlying mechanism is the formation of nonfunctional dimmers between Hey1 and MyoD that prevents formation of MyoD:E47 complexes (Sun et al., 2001).

Neurogenesis is a long lasting process that provides generation of different types of neurons and glial cells. Since, these diverse cell types are generated in different time windows and locations, neurogenesis is equipped with regulatory mechanisms that control the maintenance of progenitors and ensure a precise timing and location of cell differentiation. *Hes* genes maintain neural stem cell pool by inhibiting proneural genes like *Mash1* and *Ngn2* (Hatakeyama et al., 2004). This was documented by examining mice lacking *Hes1* and *Hes5*, which develop premature neural differentiation, exhaustion of progenitors and loss of late-born neurons. A similar function has been revealed for Hey1 and Hey2, which missexpression in mouse brains transiently maintains neural precursor cells and thereby increases late-born neurons (Sakamoto et al., 2003). Similarly, during visual development, Hes1 and Hes5 maintain retinal progenitors and prevent premature neurogenesis (Hatakeyama et al., 2004).

Hes/Hey genes regulate binary cell fate decisions

Regulation of balance between neuronal and astrocyte fate

During late neurogenesis, binary cell fate choices between neuronal and astrocyte fate take place. *Hes* genes have a well characterized role in the repression of proneural gene *Ngn1* and the promotion of astrocyte fate (Tomita et al., 2000; Nieto et al., 2001). Similarly, Hey1 and Hey2 promote astrocyte fate, likely by repressing Mash1 (Sakamoto et al., 2003). However, another Notch-independent Hes1-mediated mechanism has been proposed to promote astrocyte development. Hes1 is able to interact with Lif pathway (Leukemia inhibitory pathway) by helping Jak2-mediated phosphorylation of Stat3 that drives astrocyte differentiation (Kamakura et al., 2004). Hes/Hey are unable to promote astrocyte fate during early neurogenesis likely due to differences in epigenetic properties of astrocyte-specific promotes in early and late neural stem cells (Takizawa et al., 2001).

Regulation of binary cell fate in digestive system

In mice and zebrafish, Notch-Hes1 signaling is important for promoting enterocyte vs. non-enterocyte specification by the downregulation of Math1, which promotes non-enterocyte fates (goblet, enteroendocrine and Paneth cells) (Jensen et al., 2000; Crosnier et al., 2005; Suzuki et al., 2005; van Es et al., 2005). Similarly, in the liver, Notch-Hes1 signaling mediates specification of biliary epithelial fate vs. hepatocytes. Loss of *Hes1* results in the absence of bile ducts in the liver (Kodama et al., 2004). This scenario is phenocopied in Alagille syndrome, where mutations occur in the human *Jag1*, suggesting that Jag1 is the ligand that

activates *Hes1* expression and promotes biliary fate (Li et al., 1997; Oda et al., 1997).

Regulation of binary cell fate in endothelial development

Notch has a crucial role in controlling arterial vs. venous cell fate. The Vascular endothelial growth factor (VEGF) occupies a central place in arterial endothelial cell differentiation. Once bound to its receptor, the heterodimer Vegfr2/neurophilin1 induces Dll4, which activates the transcription of *Hey1* and *Hey2* and the arterial fate. Double *Hey1* and *Hey2* knockout mice are embryonic lethal and show loss of arterial cell fate determination (Fischer et al., 2004). Notch-mediated arterial pathway is antagonized by Coup-TFII (Nr2f2), which is a regulator of endothelial vein identity. In fact, when Dll4-Notch signaling is "on", Hey factors repress Coup-TFII and, therefore, the venous cell fate. However, ectopic expression of Coup-TFII in arteries results in their conversion into vein-like vessels (You et al., 2005). This suggests that endothelial precursors are under control of two opposing pathway, whose fine tuning determines fate choice (reviewed in Wiese et al., 2010).

Other Hes/Hey functions

Hes/Hey genes in somitogenesis

Somites are transient bilateral epithelial segments that arise by segmentation of anterior pre-somitic mesoderm (PSM) and give rise to vertebrae, ribs, skeletal muscles and dermis. Somitogenesis relies on intrinsic clock-like machinery, which first molecular evidence was the oscillatory expression of *Hes1* (Palmeirim et al., 1997). In mice *Hes1*, *Hes5* and *Hes7* share similar expression patterns in PSM, but *Hes7* seems to be the most important for somitogenesis. Loss of *Hes7* or loss of its periodicity results in fussed somites and consequently fused vertebrae and ribs (Bessho et al., 2001; Hirata et al., 2004). *Hes7* controls its own expression and cyclic expression of *Lfng* that is essential for coordinated somite segmentation (Bessho et al., 2001). Besides *Hes* genes, all three members of *Hey* family are expressed in PSM, suggesting their functional redundancy in somitogenesis (Leimeister et al., 1999; Nakagawa et al., 1999). In chick, *Hey2* expression oscillates in PSM and largely overlaps with that of *Hes1* (Leimeister et al., 2000b). Likewise, in mouse, *Hey2* expression is detected in PSM and is dramatically affected in *Dll1* and *Notch1* knockout mice (Leimeister et al., 2000b). *Hey1* expression in PSM is disrupted in Dll3 null mutants (Dunwoodie et al., 2002).

Regulation of boundary formation

The nervous system is compartmentalized and individual compartments separated by specialized boundaries. Boundaries possess unique properties and behave as organizing centers. Boundaries and compartments differ in their expression profile. This is exemplified by *Hes1* expression in the *zona limitans* intrathalamica, the isthmus and interrhombomeric boundaries and surrounding units. In these compartments *Hes1* levels are variable, suggesting that *Hes1* expression may be oscillating (Baek et al., 2006). Expectedly, since Hes1 antagonizes the *Mash1*, cells with high *Hes1* levels show low *Mash1* expression and vice versa. However, in the boundaries, *Hes1* expression is stable and high, followed by constantly low *Mash1* expression (Hirata et al., 2001; Baek et al., 2006). A similar function for *Her3* and *Her5* has been shown during zebrafish neurogenesis although through a Notch-independent mechanism. *Hes1* regulates cell cycle and cell renewal suggesting that the differential *Hes1* expression in the boundaries and compartments may underlie difference in proliferation and differentiation of the two structures (Kageyama et al., 2007).

NOTCH SIGNALING IN THE INNER EAR

The Notch signaling pathway is crucial for inner ear development. Neurosensory progenitors experience at least three waves of Notch signaling in different time windows. First, during neurogenesis Notch determines binary cell fate choices between neurons and epithelial cells. Soon after, when development of sensory organs initiates, it promotes the specification of sensory progenitors and, finally, it drives binary cell fate choices between HCs and SCs (reviewed in Kiernan, 2013; Neves et al., 2013b) (Fig. 11). In addition, Notch plays specific roles earlier, in otic placode induction (Box 10) and patterning (Box 11).

Expression of Notch components in the inner ear

Several elements of Notch signaling are expressed during the development of the inner ear with highly dynamic temporal and spatial profiles (reviewed in Neves et al., 2013b). In the chick *Notch1* and *Notch2* are the only Notch genes coded in the genome, however, *Notch1* is the only Notch receptor expressed in the chicken inner ear (Adam et al., 1998; Abello et al., 2007). *Notch1* expression is uniform and ubiquitous. It initiates at the otic placode stage (HH10-11) and continues until late stages of otocyst development (E12) in both sensory and non-sensory regions (Adam et al., 1998; Groves and Bronner-Fraser, 2000; Abello et al., 2007). During otic development *Notch1* is strongly expressed in ventral otocyst, the portion where presumptive sensory patches are formed, although later on it becomes weaker in mature sensory organs than in the surrounding regions. *Notch1* expression is excluded from dorsal regions of the developing otocyst that form the semicircular canals (Adam et al., 1998). In the mouse, ubiquitous *Notch1* and weak *Notch3* expression is detected in the otic vesicle. Upon HC determination, the expression concentrates in the SC layer and surrounding non-sensory regions (Weinmaster et al., 1991; Williams et al., 1995; Lindsell et al., 1996; Lewis et al., 1998; Lanford et al., 1999; Basch et al., 2011). Like in chicken, *Notch2* is not expressed in the mouse otic placode/vesicle (Williams et al., 1995; Lewis et al., 1998).

In contrast, the expression of Notch ligands is highly restricted throughout otic development. In the chick, the onset of *Dl1* is detected by the end of HH11 in scattered cells of anterior portion of otic placode. It labels the neurogenic domain, and is maintained during neurogenesis. As development proceeds, from E3.5 up to at least E12, *Dl1* expression is present in scattered cells of the sensory patches. However the timing of *Dl1* expression differs among the patches, according to the timing of maturation (Adam et al., 1998; Abello et al., 2007). Similar expression profile is observed in mouse inner ear, where *Dl1* transcripts stain anterio-ventral region of otic vesicle, corresponding to the neurogenic domain (Morrison

et al., 1999; Vazquez-Echeverria et al., 2008). Thereafter, staining is detected in cristae and later on in the cochlea, where it labels nascent HCs. In both animal species, *Dl1* labels nascent neuroblasts and HCs and becomes silent upon cell differentiation. *Dl1* expression is also observed in endolymphatic sac, where its function remains still obscure (Morrison et al., 1999). In mouse, in addition to *Dl1*, *Jag2* and *Dl3* are also expressed in nascent HCs (Lanford et al., 1999; Shailam et al., 1999; Hartman et al., 2007). *Jag2* and *Dl1* are reported to act synergistically in driving lateral inhibition during HC determination (Kiernan et al., 2005a). In zebrafish, *DeltaA*, *B* and *D* and *SerrateB* follow a similar pattern (Haddon et al., 1998; Riley et al., 1999).

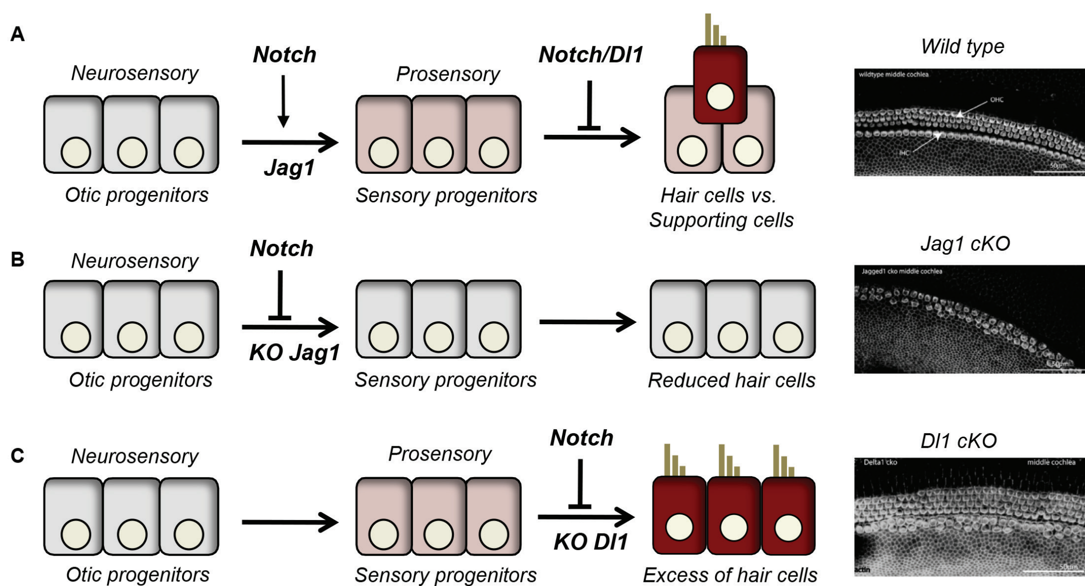

Figure 11. Dual function of Notch during inner ear sensory development. Early in development Notch is required for sensory specification. Prosensory function of Notch is mediated by Jag1. Later on, Notch prevents subsequent steps of hair cell differentiation. This function of Notch is mediated by Dl1. (B) The inhibition of Notch or the loss of function of *Jag1* prevents sensory specification and the development of hair cells. (C) However, the late inhibition of Notch, the impairment of the function of *Dl1* or the loss-of-function of some Notch downstream targets cause premature differentiation and excess of hair cells. On the right are presented confocal images of whole-mount E17.5 cochlea stained with phalloidin. Wild type cochlea contains standard pattern of one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). *Jag1* cKO cochlea contains only two disorganized rows of hair cells. By contrast *Dl1* cKO shows supernumerary inner and outer hair cells. Adapted from Neves et al. (2013b) and Brooker et al. (2006).

Jag1 expression in the chick otic placode is first detected by E2 and, in contrast to *Dl1,* it shows a uniform expression pattern. *Jag1* is initially expressed in the posterior-medial aspect of the otic placode and soon after it resolves into two poles at the anterior and posterior part

of the otocyst. These two poles are connected by a weak ventral expression domain (Myat et al., 1996; Adam et al., 1998; Cole et al., 2000; Abello et al., 2007; Daudet et al., 2007; Neves et al., 2011). By HH21 *Jag1* expression occurs in a single continuous ventral domain that, as development proceeds, resolves into presumptive sensory organs. *Jag1* expression persists at least up to E12, when it is retained by SCs (Adam et al., 1998; Cole et al., 2000). Likewise, in mouse, *Jag1* is expressed in the prosensory patches and becomes restricted to the SC layer as HCs become specified (Lewis et al., 1998; Morrison et al., 1999). Although there is evidence for prosensory function of Notch in zebrafish, the corresponding ligand is yet unknown (Millimaki et al., 2007). *Jag1* expression in the CVG is weak in the chick at HH23/24, where it is restricted to the cells close to otic epithelium and undetectable at later stages.

Lfng is also dynamically expressed during otic development. In the chick, it is first expressed by HH12 in neurogenic domain, where it overlaps with *Dl1*. At the otocyst stage, *Lfng* overlaps with *Jag1* and *Dl1* expression. Once sensory patches are restricted, *Lfng* expression labels these regions overlapping with *Jag1* and, upon HC determination *Lfng* becomes restricted to the SC layer (Cole et al., 2000; Abello et al., 2007). This pattern of expression is very similar in the mouse (Johnston et al., 1997; Morsli et al., 1998). The chicken genome codes for other *Fringe* homologues, *Radical fringe* and *Manic fringe*, but none of them is expressed in the chicken inner ear. However, both mouse and zebrafish express *Manic fringe* in the otic vesicle (Johnston et al., 1997; Qiu et al., 2004). *Lfng* is strongly expressed in the CVG where it is first detected by HH18, remaining high and scattered until late stages (Cole et al., 2000).

The *Hes/Hey* expression has been studied in some detail during mouse development, particularly in the cochlea, but at the beginning of my work, data on chicken were very scarce. *Hes5* is expressed by HH11 in the neurogenic domain and persists during neurogenesis where it is complementary to *Dl1*. Both *Hes5* and *Dl1* are expressed in a salt-and-pepper pattern and *Hes5* is dependent on Notch signaling (Abello et al., 2007; Daudet et al., 2007). Later in development, *Hes5* transcripts are detected in SCs of vestibular and auditory organs (Shailam et al., 1999; Lanford et al., 2000; Zine et al., 2001; Doetzlhofer et al., 2009; Tateya et al., 2011). In the organ of Corti its expression is widespread and extends far beyond SCs into the LER and a narrow band in the GER, to become restricted to Deiters' cells upon birth (Zheng et al., 2000; Zine et al., 2001; Doetzlhofer et al., 2009). *Hes5* expression data suggest its involvement in lateral inhibition during neurogenesis and HC determination. It is likely that similarly to Hes5 role in CNS, Hes5 negatively regulates *Ngn1* and *NeuroD* during otic neurogenesis, although this has never been directly demonstrated. Several pieces of evidence show that Hes5 represses *Atoh1* during HC determination (Zine et al., 2001; Tateya et al., 2011; Du et al., 2013).

Hes1 is first expressed at HH11 in a posterior, non-neural, aspect of otic placode, where it overlaps with *Jag1* expression and is Notch dependent (Abello et al., 2007). Spatial correspondence between *Jag1* and *Hes1* has been also reported in mouse (Jayasena et al., 2008). However, there is no direct demonstration that *Hes1* is an early readout of Jag1 mediated Notch activation in the otic placode. *Hes1* expression in non-neural domain and its repressive function suggests its possible role in antagonizing proneural gene function as recently described in the zebrafish inner ear (Radosevic et al., 2011). In the mouse, during prosensory specification of the cochlea, *Hes1* expression is detected along with activated Notch1 and it maintains the proliferation of sensory progenitors (Murata et al., 2009). Upon HC determination *Hes1* becomes restricted to SCs in vestibular and inner phalangeal cells spreading towards the LER and GER in the cochlea (Zine et al., 2001; Doetzlhofer et al., 2009; Murata et al., 2009).

Hes6 has a unique function among other *Hes* members, which is to promote neurogenesis (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Gratton et al., 2003; Fior and Henrique, 2005; Vilas-Boas and Henrique, 2010). In the mouse vestibular and auditory domains *Hes6* is expressed in HC precursors and differentiated HCs, mirroring the expression of *Atoh1*. Its expression follows the temporal base-to-apex and spatial inner-to outer gradient of cochlear HC determination. *Hes6* expression is not detected in the CVG, and there is no data on *Hes6* expression in the chicken inner ear (Qian et al., 2006; Li et al., 2008).

Hey1, *Hey2* and *HeyL* expression patterns have been carefully described in the mouse, but yet there is no information in the chick. *Hey2* is first detected in the mouse otocyst (Leimeister et al., 1999) and later on, in the medial region of the otic epithelium complementary to the neurogenic domain (Hayashi et al., 2008b; Li et al., 2008). Like *Hey2*, *Hey1* expression in also detected in the mouse otocyst (Leimeister et al., 1999). *Hey1* and *HeyL* are then expressed in the prosensory patches of vestibular epithelia and only *Hey1* in the cochlea, in a ventral broad band corresponding to the prosensory domain, to persist in Deiters' cells. *Hey2* expression in the cochlea coincides with *Hey1* but it is nested within the *Hey1* expression region. Then, *Hey2* overlaps with *Hey1* expression and becomes restricted to the apical cochlear turn. Somehow contradictory, Hayashi et al. (2008b) have shown the absence of *Hey2* at birth, while Doetzlhofer et al. (2009) reported its expression in pillar cells. *HeyL* expression is absent prior to HC determination in the cochlea, but becomes detectable at the time when *Hey2* expression starts to attenuate, to persist in Deiters' cells, inner phalangeal cells and cells in the GER, but not in pillar cells (Hayashi et al., 2008b; Li et al., 2008; Doetzlhofer et al., 2009). *Hey2* is not expressed in mouse vestibular patches (Hayashi et al., 2008b).

Box 10. Notch in otic placode induction

Notch signaling plays specific roles during the development of the otic placode (Jayasena et al., 2008). The current model suggests that Wnt signaling upregulates the expression of components of Notch pathway, such as *Jag1* in the pre-otic field, which in turn signals back through Notch1 and promotes the Wnt signaling. Wnt components from the midline generate a medial-lateral activity gradient. The Notch positive feedback mechanism acts on the lateral region that normally receives little or no Wnt activity, thus sharpening the initial medio-lateral gradient of Wnt levels into the binary pattern of high Wnt in PPA or no Wnt in adjacent epidermis (Jayasena et al., 2008). This model is supported by the following experimental evidence: 1) loss of *Notch1* does not abolish but reduces Wnt activity along with the Wnt-responsive gene *Dlx5,* 2) reduced Wnt signaling caused by *Notch1* deficiency leads to reduced otic placodes, as it does mice lacking *Pofut1* or *RBPjk* (Oka et al., 1995; de la Pompa et al., 1997; Shi and Stanley, 2003). 3) Notch deficiency in the background of constitutively active β-catenin has no effect on the size of the pre-otic field and 4) overexpression of NICD induces pre-otic field markers like *Pax8*, but not Wnt responsive gene *Dlx5* (Jayasena et al., 2008). The mechanism by which Notch augments Wnt signaling is still obscure.

Box 11. Notch in early patterning of the otic placode

Notch is also required for the early patterning of the otic placode. Notch blockade results in the expansion of non-neural genes like *Lmx1b* and *Irx1* into the anterior aspect of the otic placode, where they are not normally expressed. The expansion is not due to the cell migration, but to the lack of repression of these genes (Abello et al., 2007). However, Notch blockade does not abolish AP patterning and neurosensory domain remains restricted and Alsina et al. (2004) proposed that Fgf signaling acts upstream of Notch in neural determination. *Tbx1* has been shown to act as a selector gene that establishes proper boundary between neural and non-neural domains in the mouse otic placode (Raft et al., 2004). *Txb1* gain of function (GOF) displaces the *NeuroD* domain border anteriorly. Conversely, *Tbx1* loss-of-function (LOF) eliminates AP midline border and causes expansion of neural genes in posterior aspect of the otic placode (Raft et al., 2004). Two recent independent studies revealed that RA acts upstream of *Tbx1* in mouse and zebrafish (Bok et al., 2011; Radosevic et al., 2011). *Hes1* suppresses neural fate acting downstream of *Tbx1* in zebrafish (Radosevic et al., 2011).

Notch in sensory specification

The specification of sensory patches requires Notch signaling (Eddison et al., 2000; Daudet et al., 2007; Neves et al., 2011). The expression of the Notch ligand *Jag1* precedes cell determination and foreshadows the future sensory organs (Adam et al., 1998; Cole et al., 2000). Not only *Jag1* temporarily follows the process of sensory specification, mapping to all prosensory patches, but it also shows a uniform expression pattern within prosensory regions (Adam et al., 1998; Lewis et al., 1998; Morrison et al., 1999; Cole et al., 2000; Neves et al., 2011). This cellular distribution contrasts with the salt-and-pepper pattern of other Notch ligands like *Dl1* and *Jag2* (Adam et al., 1998; Lewis et al., 1998; Morrison et al., 1999; Shailam et al., 1999), suggesting that Jag1 does not drive lateral inhibition. Further, *Jag1* loss-of-function studies show missing prosensory patches and loss of HCs, supporting the idea that Jag1 is what drives Notch signaling during sensory specification (Kiernan et al., 2001; Tsai et al., 2001; Brooker et al., 2006; Kiernan et al., 2006; Pan et al., 2010). Sensory specification is known to require the formation of coherent domains of Notch activity associated with lateral induction (Eddison et al., 2000; Bray, 2006). These observations raise two main questions: 1) how is *Jag1* expression regulated in the inner ear? 2) What is the mechanism behind the Jag1-mediated prosensory function of Notch? It took over a decade to elucidate the mechanism of the prosensory function of Notch and the regulation of Jag1 in the ear, and I will summarize bellow the current understanding of these questions. The chicken embryo has been crucial to shed light on these problems by providing a model for precise temporal and spatial control of gene expression and *in vitro* manipulations (reviewed in Neves et al., 2013b).

Jag1 is regulated by lateral induction

Lateral induction is defined as positive feedback mechanism in which ligand-sending cell forces its neighbors to turn up its ligand production and therefore promotes ligand propagation and a coordinated cell behavior (Lewis, 1998; Bray, 2006). *Jag1* expression in the inner ear is regulated by lateral induction, implying that Notch activation upregulates Jag1 expression in a cell, which then signals to its neighbors to activate Notch and promote Jag1 expression (Fig. 12A).

The first evidence for the regulation of Jag1 by lateral induction came from pioneering studies in Julian Lewis group (Eddison et al., 2000). Notch was silenced by the electroporation of replication competent RCAS virus containing a dominant negative form of either *Dl1* or *Su(H)*. These experiments showed that upon loss of Notch signaling Jag1 expression is reduced or lost. The requirement of Notch signaling to maintain Jag1 expression was

later strengthened by blocking NICD release with γ-secretase inhibitor, which reduced Jag1 expression in the sensory domains (Daudet et al., 2007). By contrast, forced expression of activated form of Notch1 outside the sensory regions leads to ectopic Jag1 expression (Daudet and Lewis, 2005; Pan et al., 2010). Together, these experiments show that active Notch is necessary and sufficient to maintain Jag1 expression in the prosensory domains of the chick otocyst. Further insight into the entire mechanism of lateral induction was brought by two independent studies showing that Notch activation in the mouse inner ear induces Jag1 expression both cell-autonomously and non-autonomously, propagating Jag1 expression (Hartman et al., 2010) and that the ectopic expression of hJag1 in chicken otic vesicle results in Jag1 induction in a non-cell-autonomous manner (Neves et al., 2011).

These data strongly support the notion that Jag1 operates by a mechanism of lateral induction that relies on a positive-feedback loop of Notch activation and Jag1 induction.

The prosensory function of Notch depends on Jag1

Several mutant mice have been used to study Jag1 function: *slalom (Slm)*, *coloboma (Cm)* and *headturner (Htu)* (Kiernan et al., 2001; Tsai et al., 2001). These mutant mice exhibit the typical head-shaking behavior of vestibular defects and gross morphology alterations. *Htu* and *Slm* mice exhibit loss of posterior and frequently anterior ampulla, with loss of corresponding semicircular canals. The cochlear duct of the two mutants show patterning defects with one or two, instead of three rows of outer hair cells (OHCs), and slightly increased number of inner hear cells (IHCs) often with atypical OHC morphology (Kiernan et al., 2001; Tsai et al., 2001). *Coloboma (Cm)* mutant mice show a similar, but milder phenotype in both vestibular and auditory regions. However, while *Htu* and *Cm* mutants show comparable IHC phenotypes, they strikingly differ in OHC phenotypes. In the *Cm* mutant, OHC numbers vary along the length of the cochlea, with occasional presence of two OHC rows in basal and mid-basal turn similar to the other *Jag1* mutant mice, but single or two additional OHC rows in apical region (Kiernan et al., 2001). In spite of these patterning abnormalities none of mutants mentioned above is deaf (Kiernan et al., 2001; Tsai et al., 2001). The morphological defects observed in *Jag1* mutants do not depend on genetic background, which does not seem to change much the cochlear patterning defects. However, the genetic background does modify the functional phenotype (head-shaking behavior) of *Jag1* heterozygotus mice, suggesting that C3H and not B6 modifiers aggravate already existing morphological and patterning defects (Kiernan et al., 2007).

Ear conditional *Jag1* knockout mice show impaired sensory development resembling *Jag1* mutants, although more extreme (Fig. 11B). The phenotype includes the loss of the three

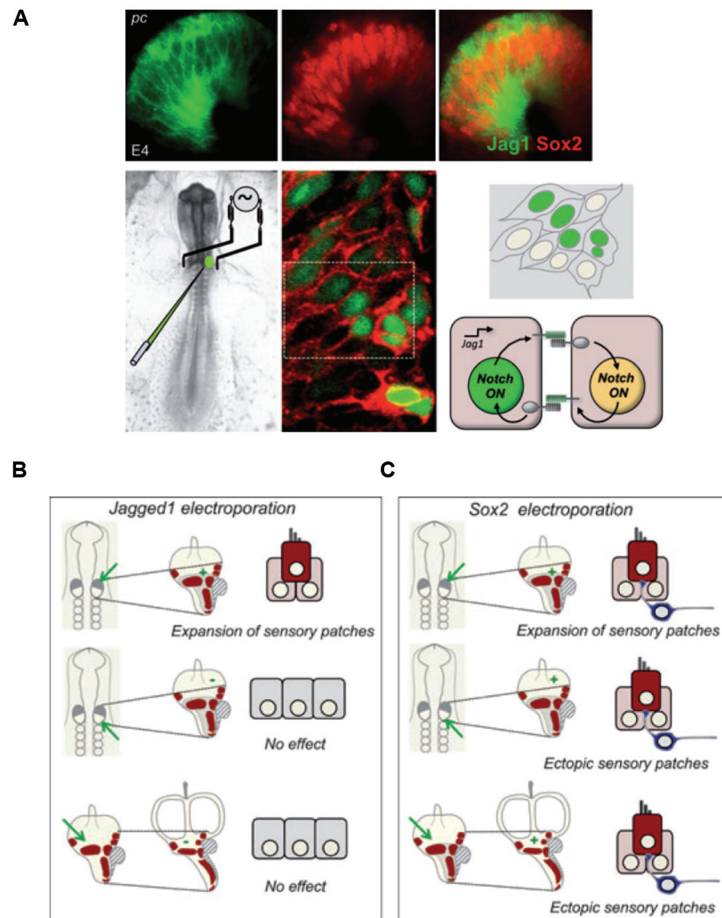

Figure 12. Jag1 drives lateral induction during prosensory specification. The prosensory function of Jag1 depends on Sox2. (A) Jag1 is expressed uniformly in the prosensory patches. The microphotographs illustrate the expression of Jag1 and Sox2 detected by immunohistochemistry. Jag1 is expressed in the cell membranes of the same cells that express Sox2 in the nucleus. Jag1 induces Jag1 in the neurosensory domains. The electroporation of hJag1 in the neurosensory domain of the otic placode (bottom left) induces the expression of Jag1 (red) in both electroporated (green) and non-electroporated cells. hJag1 was co-electroporated with a green fluorescent protein (GFP) vector and Jag1 detected by immunohistochemistry. The diagram on the bottom right illustrates an idealized view of the effects of the electroporation. (B-C) The diagrams illustrate the effects of the electroporation of Jag1 (B) and Sox2 (C) on the generation of hair cells and neurons. Embryos were electroporated in E2.5 (upper two rows) or in E3.5 (lower row), and examined after two days for neuronal and hair cell markers. The gain of function of both Jag1 (B) and Sox2 (C) in the neurosensory domain (upper rows) results in the expansion of the prosensory patches and a gain in neuronal and hair cell production. However, when electroporation is carried out in non-neurosensory domains, only Sox2 (B) is able to generate ectopic neurons and hair cells. Similarly, when electroporation is done later in development, once the prosensory patches are defined, only Sox2 is able to induce ectopic neurons and hair cells (B). Adapted from Neves et al. (2013a).

cristae, smaller utricular macula, misshapen saccular macula and undercoiled cochlea (Brooker et al., 2006; Kiernan et al., 2006). In the organ of Corti, the two studies reported different phenotypes. Kiernan et al. (2006) showed that the base of the cochlea is the most affected with no HCs and SCs, whereas in midbasal and apical region only IHC are observed, but with reduced numbers and in disorganized pattern. However, an independent study by Brooker et al. (2006) reports a milder cochlear phenotype, similar to *Jag1* mutants with reduced number of OHCs and an excess of IHCs.

Although in the absence of *Jag1* sensory specification is severally altered, it is not affected uniformly in all sensory organs. It is possible that sensory organs may have different degrees of Jag1 dependence. Possible alternatives are: 1) redundancy with *Dl1*, which partially overlaps with *Jag1* in the anteroventral aspect of the otocyst (Adam et al., 1998; Morrison et al., 1999). The loss of *Dl1* function results in defects in the macular region, the regions least affected in the conditional *Jag1* KO (Brooker et al., 2006; Kiernan et al., 2006; Pan et al., 2010). 2) A delay in Cre mediated excision of *Jag1* and the persistence of a residual Jag1 function. 3) Other signaling pathways contributing to the sensory specification.

The prosensory function of Jag1/Notch is mediated by Sox2

Recent data show that, in the chick, ectopic *Jag1* expression cannot trigger ectopic HC formation *de novo*, but only within the neurosensory domain, suggesting that Jag1 acts on a pre-existing sensory competent tissue (Neves et al., 2011) (Fig. 12B). Several genes along with *Jag1* have been reported to foreshadow the development of sensory territories like *Bmp4, Id1-3*, *Lfng, Sox2, Hey1, Hey2, HeyL* (Leimeister et al., 1999; Cole et al., 2000; Neves et al., 2007; Hayashi et al., 2008b; Kamaid et al., 2010). However, among those, only Sox2 has been shown to be required for prosensory specification (Kiernan et al., 2005b).

Light coat and circling (Lcc) and *yellow submarine (Ysb)* mice, where chromosomal rearrangements resulted in the loss or interference of specific regulatory elements that direct expression of *Sox2* within the inner ear, have been used to study *Sox2* loss-of-function phenotypes. Both mice show the absence of prosensory regions and loss of HCs and SCs that faithfully resemble *Jag1*-deficient otic phenotype, suggesting a functional relationship between *Jag1* and *Sox2* (Kiernan et al., 2005b). Several independent studies supported the idea that *Sox2* is required for prosensory specification and that Jag1 mediated Notch activity in sensory specification relies on Sox2 function. Ectopic expression of NICD in non-sensory territories of mouse otic epithelium results in the expansion of *Sox2* expression (Hartman et al., 2010; Pan et al., 2010), and these domains yield ectopic sensory patches containing HCs and SCs (Hartman et al., 2010; Pan et al., 2010; Liu et al., 2012). Notch is also able to induce *Sox2* in non-sensory

regions in inner ear stem cells (Jeon et al., 2011). Evidence for a direct regulation comes from experiments showing that CSL/RBPjk directly regulates *Sox2* transcription in the nervous system (Ehm et al., 2010). Experiments in the chick suggest that Jag1 mediated Notch activity maintains Sox2 expression rather than inducing it *de novo* (Neves et al., 2011). During normal development Sox2 expression is initially broad and contains in Jag1 patches. However, as development proceeds, Sox2 expression domains become restricted to Jag1-positive patches and therein Sox2 accompanies the prosensory domains throughout development. Ectopic Jag1 is able to maintain Sox2 expression in domains located in between the patches, where Sox2 expression is normally switched off (Fig. 12B). Accordingly, later in development when Sox2 expression is confined to prosensory domains, Jag1 is unable to expand Sox2 expression to otic non-sensory territories, while Sox2 is still sufficient to induce ectopic HCs in the otic epithelium (Neves et al., 2011; Pan et al., 2013) (Fig. 12B,C). Recent studies have attributed this function of Sox2 to its ability to directly activate *Atoh1* transcription (Ahmed et al., 2012; Neves et al., 2012).

The ectopic expression of the NICD1 in the cochlea at E13.5 is sufficient to upregulate Sox2, however, it fails to induce HCs, SCs or other prosensory markers like Hey2 or $p27^{kip1}$ (Basch et al., 2011). This suggests that Notch pathway, although being able to promote prosensory potential given by Sox2, requires other factors for establishing the prosensory fate of the cochlea. It also indicates that the cochlear sensory development may be distinct from that of the vestibular organs (Ohyama et al., 2010; Basch et al., 2011). However, early ectopic activation of Notch signaling in the mouse between E9.5 and E11.5 results in ectopic HCs and SCs including the cochlea, showing that that there is a transient competence of the cochlea to respond to Notch (Pan et al., 2010). Indeed this has been confirmed recently showing that NICD can only induce sensory progenitors before E13 in the cochlea (Liu et al., 2012; Pan et al., 2013).

In summary, Jag1 mediated Notch specification of prosensory progenitors relies on Sox2. The competence of the otic epithelium to generate HCs and SCs is transient and correlates with the restriction of Sox2 expression to the sensory regions.

Hey gene expression parallels lateral induction and sensory specification

Several members of the *Hey* family, including *Hey1*, *Hey2*, and *HeyL* are expressed during mouse sensory specification. Both *Hey1* and *Hey2* are expressed in the prosensory regions of the mouse cochlea (Hayashi et al., 2008b). *Hey2* expression corresponds well with activated Notch, which partially overlaps with Jag1. In contrast, *Hey1* expression corresponds well with Jag1, only partially overlapping with active Notch (Hayashi et al., 2008b; Murata et al.,

2009). The loss-of-function of *Jag1* results in a dramatic reduction of *Hey1* expression (Pan et al., 2010) (Fig. 13A,B). Accordingly, ectopic activation of Notch in the mouse otocyst results in the expansion of *Hey1* expression in otic epithelium (Hartman et al., 2010) (Fig. 13C,D). Cochlear cultures *in vitro* in the presence of γ-secretase inhibitors show reduced *Hey1* and *Hey2* relative mRNA levels, however, *Hey1* shows a greater change. One interesting hypothesis that came from these experiments is that *Hey1* expression may require low levels of Jag1 mediated Notch signaling that are not detected by the NICD1 antibody (Hayashi et al., 2008b; Murata et al., 2009). This important question was one of the subjects addressed in the present work.

Somehow surprisingly, *Hey1* and *Hey2* deficient mice do not to show any HC or SC phenotype in the organ of Corti (Hayashi et al., 2008b). However, combined deletions of *Hey1* with *Hes1* and *Hes5* show increased number of HCs in the cochlea, indicating that they all play a cooperative role in lateral inhibition (Tateya et al., 2011). To date, none of *Hes* members have been reported to be involved in Notch mediated lateral induction and/or prosensory specification.

Other signaling pathways have been proposed to play a role in sensory specification, which suggests the possibility that they interact with Notch signaling. I shall review the function of these other signaling pathways below.

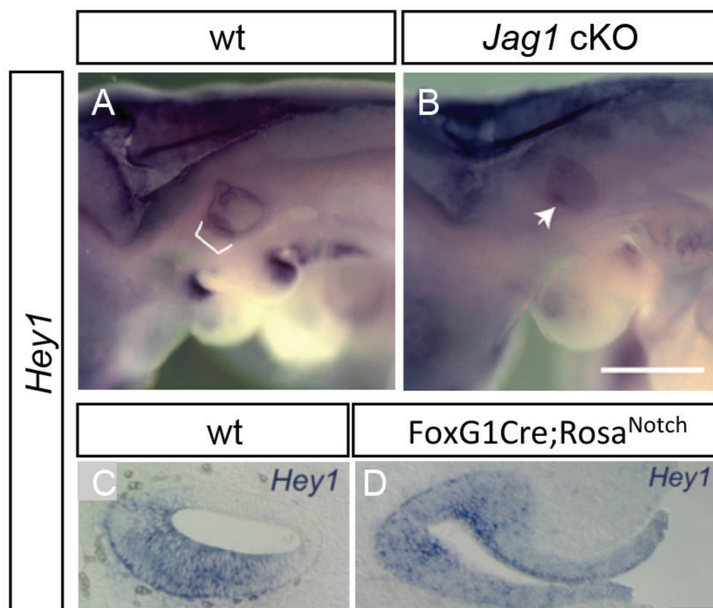

Figure 13. Hey1 expression is dependent on Notch signaling. (A-B) Whole mount in situ hybridization of *Hey1* by E10.25 in mouse embryo. *Hey1* expression is down-regulated in *Jag1* cKO inner ear. Embryos are shown in a lateral view, with anterior to the left. (C-D) In the cochlea, at E12.5 *Hey1* expression is restricted to the prosensory domain in the control (C) and expanded throughout the entire otic epithelium in double transgenic FoxG1Cre;Rosa^{Notch} embryos (D). Modified from Pan et al. (2010) and Hartman et al. (2010).

The Notch pathway in neuronal and HC determination

Neurosensory progenitors undergo two additional rounds of Notch activation prior to and after sensory specification. Notch mediated lateral inhibition controls neuronal vs. non-neuronal fate and, later in development, HC vs. SC fate. A hallmark of lateral inhibition is the negative regulation of Notch ligand by Notch signaling, which creates negative feedback and a fine grained pattern of gene expression (Lewis, 1998). In chick, mice and zebrafish *Dl1* foreshadows the determination of otic neurons and HCs through lateral inhibition (Adam et al., 1998; Haddon et al., 1998; Kiernan et al., 2005a; Brooker et al., 2006; Abello et al., 2007; Daudet et al., 2007). In mouse and zebrafish additional Notch ligand *Jag2* drives lateral inhibition during HC determination (Haddon et al., 1998; Lanford et al., 1999; Kiernan et al., 2005a).

Direct evidence of the role of *Dl* in lateral inhibition in the inner ear development came from studies on the *mindbomb (Mib)* mutant in zebrafish (Haddon et al., 1998). Mib is an ubiquitin E3 ligase required for Delta-mediated Notch activation (Itoh et al., 2003; Koo et al., 2005). The *Mib* mutant fish exhibits an increased expression of *Delta* genes and a disruption of the salt-and-pepper pattern that is accompanied by supernumerary otic neurons and HCs (Haddon et al., 1998). These phenotypes strongly suggest that Notch-mediated lateral inhibition regulates the development of neurons and HCs (Haddon et al., 1998). These observations were further confirmed in chick and mouse. In chick, the blockade of Notch signaling with DAPT increases *Dl1* expression and this is associated with the overproduction of neurons (Abello et al., 2007; Daudet et al., 2007). Ear conditional *Dl1* knockout mice show the increased size of CVG and strikingly small macula, suggesting that vestibular cells normally developing as maculae switched towards neuronal fate due to disrupted lateral inhibition (Brooker et al., 2006).

Notch pathway and hair cell determination

A second wave of Delta-Notch mediated lateral inhibition occurs during HC determination and constitutes another example of the Notch control of binary cell fate choices between HCs and SCs. The first indication for this function of Notch came from expression pattern studies of *Delta* ligands (*Dl1* in mouse and chick and *DeltaA, DeltaB* and *DeltaD* in zebrafish), showing that they are confined to the nascent HCs (Adam et al., 1998; Morrison et al., 1999 and see above). The expression of *Delta* is transient, suggesting that its expression is necessary only to initiate correct HC patterning and not for their maintenance (Adam et al., 1998; Haddon et al., 1998). Several lines of evidence provided further understanding of how lateral inhibition works on the ear. The forced expression of activated form of NICD

in the sensory patch of the chicken otocyst results in the failure of HC determination and the consequent overproduction of SCs, as expected from lateral inhibition (Daudet and Lewis, 2005). Contrarily, DAPT treatment increases the number of HCs at the expense of SCs in cochlear explant cultures (Takebayashi et al., 2007). The conditional deletion of *Dl1* results in premature and supernumerary OHCs, with occasional IHC duplications (Brooker et al., 2006) (Fig. 11C), *Dl13* mutant mice showing no discernible ear phenotype (Hartman et al., 2007). Somehow unexpectedly, *Dl1* cKO mice show excess of SCs that clashes with conventional lateral inhibition model. This has been explained by: 1) unchanged total number of SCs but their spacing in more rows within a shorter and broader cochlea. 2) Recruitment of non-sensory cells due to the excess of HCs and disruption of lateral inhibition. 3) Additional rounds of SC division due to instructive signals from supernumerary HCs.

In mouse and zebrafish, *Jag2* and *SerrateB*, respectively, are additional ligands that label nascent HCs (Haddon et al., 1998; Lanford et al., 1999; Kiernan et al., 2005a). In contrast to *Delta*, *Jag2/SerrateB* expression is more persistent in differentiated HCs (Haddon et al., 1998; Lanford et al., 1999). *Jag2* KO mice display a similar but milder phenotype to *Dl1* KO, showing a modest increase of cochlear IHCs and a slight increase of OHCs (Lanford et al., 1999).

Compound *Jag2*$^{-/-}$ homozygous and *Dl1*$^{hyp/-}$ heterozygous mice show increased cochlear HC numbers which severity depends on gene dosage (Kiernan et al., 2005a). This suggests that normal patterning of the cochlea requires a certain threshold of Notch ligand and that *Dl1* and *Jag2* are functionally redundant. In agreement with the role played in lateral inhibition, *Dl1/Jag2* compound mutants show reduced SCs, mostly affecting Dieters' cell subpopulation. However, the SC losses are modest when compared to the HC increases. One explanation came from the observation of the continuous proliferation of SCs. HCs remain non-proliferative, indicating that any overproduction of HCs should arise via Deiter's cell switch. These observations pinpoint an additional Notch role in the suppression of continuous cell proliferation in the cochlea (Kiernan et al., 2005a). Since in the nervous system Notch promotes the glial fate (Gaiano and Fishell, 2002), given the similarities between glial and supporting cells, it is tempting to suggest that Notch plays an instructive role in SC differentiation. It is thought that, in normal conditions, HCs deliver anti-proliferative signals to SCs (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Warchol et al., 1993; Matsui et al., 2002). Accordingly, zebrafish *Mib* mutant shows ten-fold increase of HCs that cannot be explained only by cell fate conversion from SCs (Haddon et al., 1998).

Although the role of Jag1 in otic development has been associated with lateral induction and prosensory specification, three studies indicate that it plays an additional role in lateral

inhibition. First, the increase of HCs in cochlear cultures with antisense-*Jag1* mRNA suggests that Jag1 keeps Notch active in SCs, thereby cooperating with lateral inhibition (Zine et al., 2000). Secondly, *Cm*/+ mice show extra rows of OHCs (Kiernan et al., 2001). Finally, conditional *Jag1* KO mice, despite showing loss of OHCs show increased number of IHCs (Kiernan et al., 2006). Since conversion of outer to inner hair cell fate is not observed, it is believed that the cochlear phenotype is a consequence of Jag1 promotion of lateral inhibition. Our work has addressed this question in some detail and related the dual function of Jag1 with the strength and competition for signaling.

In summary, the data above suggest that Dl1-mediated and Dl1/Jag2-mediated Notch lateral inhibition is crucial for generation of neurons and mosaic of HCs and SCs, respectively. Notch function in HC determination is complex. Notch prevents HC determination through lateral inhibition, but it appears to be directly or indirectly involved in SC differentiation and in the inhibition of SC proliferation.

Downstream targets of Notch during HC/SC determination

Hes/*Hey* genes are well known Notch targets during HC determination. In differentiated sensory organs they map to SC layer (see above) and their function in HC determination in the cochlea has been exhaustively studied in a set of various *Hes*/*Hey* KO mice (Zheng et al., 2000; Zine et al., 2001; Zine and de Ribaupierre, 2002; Li et al., 2008; Doetzlhofer et al., 2009; Tateya et al., 2011). For example, deletion of *Hes1* results in supernumerary IHCs, whereas loss of *Hes5* leads to supernumerary OHCs (Zheng et al., 2000; Zine et al., 2001). Double *Hes1*/*Hes5* KO mice exhibit a more robust increase in both HC populations, suggesting that *Hes1* and *Hes5* participate together in the control of HC determination (Zine et al., 2001). They also control HC determination in the macular regions, although in vestibular domains *Hes1* seems to be less important than *Hes5*. Similarly, Li et al. (2008) showed that patterning defects in the cochlea increase when *Hes1* or *Hes5* KO mice are combined with homozygous or heterozygous *Hey2* deletions. *Hey2* KO mice show patterning defect in OHCs which is reminiscent of that observed in *Hes5* KO mice (Zine et al., 2001; Zine and de Ribaupierre, 2002), thus the *Hey2* and *Hes5* compound mutant shows more severe patterning defect in OHCs in the organ of Corti. In contrast, no significant excess of IHCs is observed in *Hey2* KO, but when combined with *Hes1* KO, the compound mutant contains more IHCs than *Hes1* KO alone. This suggests that while genetic inactivation of *Hey2* and *Hes5* is additive on OHC patterning, inactivation of *Hey2* and *Hes1* is rather synergistic on IHCs (Li et al., 2008). Hes/Hey factors oppose the effect of *Atoh1,* accordingly *Atoh1* blockade is released in *Hes*/*Hey* KO mice (Zheng et al., 2000; Zine et al., 2001; Zine and de Ribaupierre, 2002; Tateya et al., 2011; Du et al., 2013).

Ear phenotypes observed in these animals are similar to those of mice deficient in *Dl1* and *Jag2* (Lanford et al., 1999; Kiernan et al., 2005a; Brooker et al., 2006) and *Notch1* (Zine et al., 2000; Kiernan et al., 2005a), suggesting that *Hes/Hey* genes are part of the cascade of lateral inhibition during HC determination (Fig. 14).

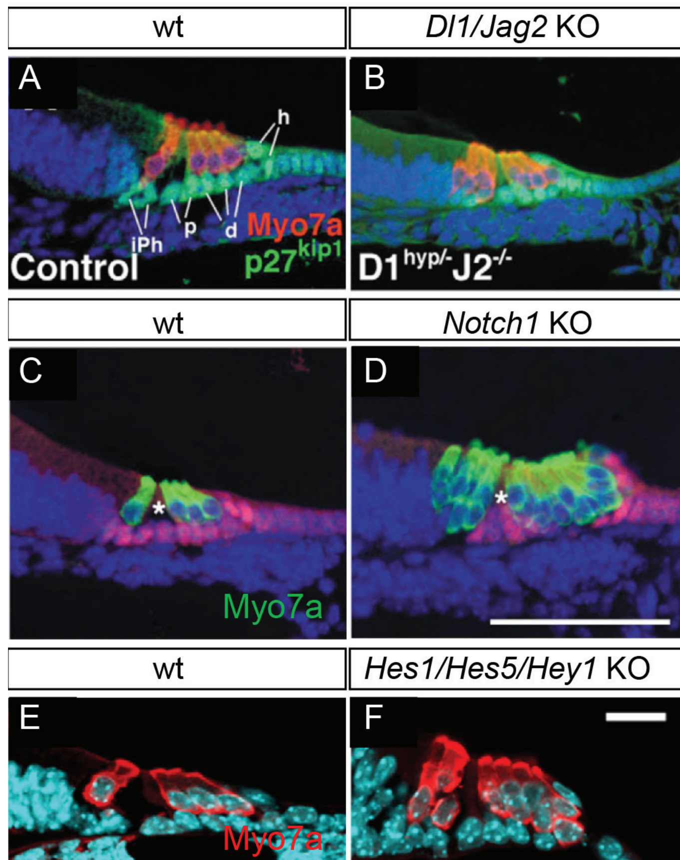

Figure 14. Disrupted lateral inhibition results in overproduction of hair cells. (A-B) Hair cells labeled against MyoVIIa (red) are dramatically increased in *Dl1/Jag2* double KO cochlea from E18.5. (C-D) Conditional *Notch1* deletion in the ear causes supernumerary hair cells in E18.5 mutant cochlea labeled with MyoVIIa (green). (E-F) Overproduction of hair cells stained for MyoVIIa (red) in triple Hes1/Hes5/Hey1 mutant cochlea at E18.5. Adapted from and Kiernan et al. (2005) and Tateya et al. (2011).

The double KOs of various *Hes* and *Hey* genes show only mild increase of HC production in comparison to *Notch1* or *Dl1* and *Jag2* mutations (Kiernan et al., 2005a; Brooker et al., 2006). Deletions of the three *Hes/Hey* genes (*Hey1*, *Hes1* and *Hes5*) result in a graded increase of HCs that corresponds to the number of *Hes/Hey* alleles inactivated (Tateya et al., 2011). In addition, supernumerary HCs are always accompanied by overproduction of SCs, if at least one allele of *Hes1*, *Hes5* or *Hey1* is present. Overproduction of HCs and SCs does not occur through expansion of prosensory domain that appears to be intact in these animals, but through prolonged cell proliferation after prosensory domain formation. In contrast, when both copies of *Hes1, Hes5* and *Hey1* are missing, SCs appeared to be decreased, and supernumerary HCs are produced at the expense of SCs, which number is balanced by their fate conversion into HC types and SC overproduction (Tateya et al., 2011). However,

even the triple mutant has a milder effect in HC patterning in the cochlea than the *Notch1* mutant (Kiernan et al., 2005a), suggesting that either there are other *Hes/Hey* factors that share functional redundancy with *Hey1*, *Hes1* and *Hes5*, or that there are other non-*Hes/Hey* related genes downstream of Notch that play an important role as effectors in lateral inhibition. The fact that SCs still form in the triple mutant suggests that fate conversion is not complete, most probably due to the compensation with other factors. *Hey2* and *HeyL* are likely candidates to perform this role. This is suggested by first, the unchanged *Hey2* and *HeyL* expression observed in the cochlea of the triple mutant (Tateya et al., 2011), secondly, the parallel functions of Hey2 with *Hes1* and *Hes5* (Li et al., 2008) and, finally, the FGF-mediated, Notch independent Hey2 function in the formation of pillar cells (Doetzlhofer et al., 2009).

In summary, perturbation of various *Hes/Hey* genes or their compound mutants suggests that *Hey1, Hey2, Hes1* and *Hes5* are good candidates to be downstream effectors of Notch-mediated lateral inhibition during generation of HCs and SCs in the sensory regions.

Conflicting results of *RBPjk* KO mice

From the above evidence, the prediction would be that the genetic deletion of *CSL/RBPjk* phenocopies the effects of *Jag1* loss of function. However, this does not turn thoroughly to be the case. Two different conditional *CSL/RBPjk* KO mice exhibit apparently contradictory phenotypes in the vestibular and auditory domains. Severe morphological abnormalities including gaps in semicircular canal formation and reduction of ampullae and both maculae are accompanied with loss of vestibular sensory territories, indicating the importance of canonical Notch signaling for vestibular sensory specification and directly or indirectly for the inner ear morphogenesis. Similar, but less affected phenotype is observed in cochlea (Yamamoto et al., 2011). However, a parallel study that used different deletion paradigm of *CSL/RBPjk* reported less severe phenotype in the cochlea. Prosensory markers, such as Sox2 and p27^{kip1} are unaffected and cochlear HCs and SCs are normally formed, but die early, due to increased cell death in sensory domains and not failure of prosensory specification, suggesting canonical Notch requirement not for formation of HC progenitors but their survival (Basch et al., 2011).

Relevant to the present work, both studies show a dramatic reduction of *Hey1* expression in the mutant cochlea (Basch et al., 2011; Yamamoto et al., 2011). Other early prosensory markers appear to be present, but reduced, supporting the original idea of Daudet et al. (2007) that Notch signaling is crucial for the maintenance rather than for the induction of prosensory domains (Yamamoto et al., 2011). Cochlear HCs still form in *CSL* deficient mice,

but they are only confined to the apical region of the mutant cochlea. Although increased cell death is observed, the loss of sensory epithelium correlates with loss of the prosensory domain in *RBPjk* mutants, since disruption of HC formation persists even when cell death is inhibited (Yamamoto et al., 2011).

Overall, phenotypic similarities between *Jag1* and *CSL/RBPjk* mutant mice strongly suggest that canonical Notch signaling is crucial for sensory specification, although it is possible that Notch signaling is not the only player in the cochlea. Other signaling pathways could also contribute to the final effect. Their potential interactions are described below.

OTHER SIGNALING PATHWAYS IN THE INNER EAR DEVELOPMENT AND THEIR INTERACTIONS WITH NOTCH SIGNALING

Fgf pathway

Fibroblast growth factor (Fgf) signaling (Fig. 15A) has multiple functions during inner ear development. Early in development Fgf signaling is required for otic placode induction (reviewed in Schimmang, 2007; Ladher et al., 2010 and see above). Later on, it is required for the determination of otic neuroblasts, acting upstream of *Ngn1* and *NeuroD* (Alsina et al., 2004). Fgf signaling is also essential for ear growth and morphogenesis (Pirvola et al., 2000; Adamska et al., 2001). In mice, the Fgf pathway has been implicated in sensory formation of the auditory epithelium. However, it still remains unclear whether Fgf signaling is important for sensory specification, differentiation or both.

The first indication of the importance of Fgf signaling in the sensory specification was provided by the conditional deletion of *Ffgr1* in the inner ear (Pirvola et al., 2002). *Fgfr1* cKO mice display reduced and isled HCs and SCs in dose dependent manner. The requirement for *Fgfr1* is specific for the cochlea, since vestibular organs appear with normal morphology. Also, *Fgfr1* mutant mice show no patterning defects in the early otocyst, probably because early functions of Fgfs are mediated by *Fgfr2* (Pirvola et al., 2000). *Fgfr1* loss-of-function mutants show reduced proliferation of sensory precursors and downregulation of *Atoh1* expression, suggesting that reduced HC and SC numbers in those animals are consequence of impaired proliferation. Similarly, inhibition of Fgf receptors with SU5402 in cochlear explants shows a reduced number of HCs and SCs (Hayashi et al., 2008a). However, the effect cannot be explained by reduction in the proliferation since the authors observed the most dramatic Fgf mediated effect when sensory progenitors are already postmitotic but not yet determined to HC or SC fate. This suggests that reduced HCs and SCs observed are due to the Fgf-mediated direct or indirect upregulation of Atoh1 (Hayashi et al., 2008a).

Although Pirvola et al. (2002) proposed that Fgf8 and Fgf10 may be the ligands driving Fgf signaling in sensory specification, subsequent studies revealed that Fgf20 is the most likely ligand for Fgfr1 (Hayashi et al., 2008a; Huh et al., 2012). Mice deficient in *Fgf10* have no HC defects in the cochlea (Pauley et al., 2003). On the contrary, *Fgf20* is expressed in presumptive sensory epithelia and its loss-of-function, either by genetic deletion in mice or with antibody against FGF20 in cochlear cultures, phenocopies the *Fgfr1* cKO. Further, HC phenotype in

the absence of *Fgf20* is rescued by addition of recombinant FGF20 protein. Together these data suggest that Fgf20-Fgfr1 pathway is required for proper sensory specification in the cochlea (Hayashi et al., 2008a).

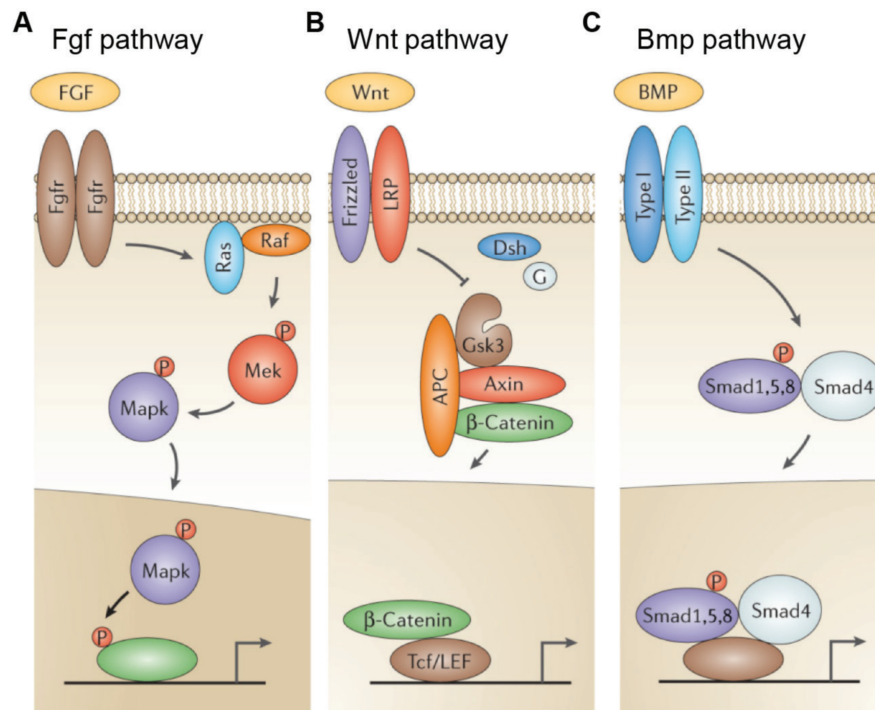

Figure 15. Fgf, Wnt and Bmp signaling pathway. (A) Fgf pathway: binding of the ligands (Fgfs) to the Fibroblast growth factor receptor (Fgfr) results in receptor dimerization and transphosphorylation. The phosphorylated receptor recruits proteins that activate the G-protein Ras, which then activates the kinase Raf. Raf phosphorylates and activates Mek, which subsequently phosphorylates and activates MAP kinase (Mapk). Mapk enters the nucleus where it phosphorylates and activates target transcription factors. (B) Canonical Wnt pathway: Wnt ligands bind to a Frizzled/LRP heterodimer which mediates the intracellular response, involving G-protein signaling, LRP phosphorylation and the activity of Dishevelled (Dsh). This results in the disruption of a large protein machine called the β-catenin destruction complex composed of Axin, APC and GSK3, which in the absence of the Wnt ligands phosphorylates β-catenin causing its degradation. When β-catenin is not degraded, it accumulates and translocates to the nucleus, where it binds members of the Tcf/LEF1 family of DNA binding factors and recruits transcriptional activators to the promoter. (C) Bmp pathway: Bmp ligands bind to Bmp receptors of type I and type II. Type II receptor phosphorilates, activating the type I receptor to phosphorilate a Smad factor. Smad1, Smad5 and Smad8 are mediators of the BMP pathway. When these Smads are phosphorylated they bind Smad4 and translocate to the nucleus where they bind to specific DNA-binding factors. The Smad proteins regulate promoter activity by interacting with transcriptional co-activators or co-repressors to positively or negatively control gene expression. Adapted from Kimelman (2006).

The phenotype of the *Fgfr1* cKO mice largely resembles *Jag1* and *Sox2* mutants (Kiernan et al., 2001; Tsai et al., 2001; Kiernan et al., 2005b; Kiernan et al., 2006), suggesting that they may act on the same gene network during prosensory specification. Both Notch inhibition and *Fgfr1* inhibition decrease *Sox2* expression (Daudet et al., 2007; Hayashi et al., 2008a; Neves et al., 2011). *Fgf20* expression in presumptive sensory region of organ of Corti is Notch dependent since it is reduced after DAPT treatment or in *Jag1* cKO mice (Munnamalai et al., 2012). Further, disruption of *Sox2* expression in the cochlea by DAPT can be partially rescued by exogenous application of FGF20, suggesting that Fgf can independently control Sox2 expression (Munnamalai et al., 2012). This indicates that in the mammalian cochlea Fgf20-Fgfr1 signaling lies downstream of Notch during prosensory specification and that maintenance of sensory progenitors is in part accomplished by Fgf-mediated control of Sox2 expression. Recently, it has been shown that this effect occurs through Fgfr1-Frs2/3 signaling and independently of Jag1 action (Ono et al., 2014).

In addition, Fgf signaling has been proposed to regulate the differentiation of OHCs and Dieters' cells from the lateral compartment of the cochlea, suggesting that OHCs and IHCs may require different signals for differentiation (Huh et al., 2012). *Fgf20* cKO mice contain undifferentiated Sox2-positive postmitotic progenitors in between HC clusters. The effect is rescued by addition of FGF9 which is similar to FGF20, suggesting that FGF20 is required for differentiation of HCs and SCs, but not for prosensory specification (Huh et al., 2012). Accordingly, Ono et al. (2014) have shown that conditional deletion of *Fgfr1* prior to HC differentiation results in OHC reduction, the effect that is independent on Sox2, as Sox2 progenitors are normally formed. At later stages of the development of the organ of Corti, Fgf8-Fgfr3 signaling likely through *Hey2* is crucial for differentiation of pillar cells (Mueller et al., 2002; Jacques et al., 2007; Doetzlhofer et al., 2009).

Most ear phenotypes of *Fgfr1* and *Fgf20* KO mice are confined to the cochlea, suggesting that Fgf signaling may be a specific requirement for the cochlear sensory development. However, there is some evidence of Fgf signaling requirement for vestibular organs, but this function is less well understood. For instance, *Fgf3* is expressed in the neurosensory domain of the mouse inner ear (Hatch et al., 2007) and *Fgf10* KO mice have missing or smaller cristae and semicircular canals.

In summary, Fgf signaling controls multiple aspects of the ear development. In the cochlea it seems to play dual role during sensory specification and OHC differentiation.

Wnt pathway

Wnt proteins belong to a large family of secreted factors coupled to at least three intracellular signaling pathways: 1) the canonical pathway, that stabilizes and translocates β-catenin into the nucleus (Dale, 1998) (Fig. 15B) 2) the release of intracellular calcium (Slusarski et al., 1997; Kohn and Moon, 2005) and 3) the activation of RhoA, linked to planar cell polarity (Mlodzik, 2002). The choice of the Wnt pathway largely depends on the cellular context.

To date mostly canonical Wnt signaling has been involved in inner ear development. Numerous Wnt ligands, Wnt receptors (Frizzleds (Frd)) and their endogenous inhibitors make puzzled expression patterns from very early to advanced stages of otic development. Typically, *Frds* are expressed in the prosensory and sensory regions, flanked by *Wnts* in non-sensory domains that transiently express also in prosensory domains. Wnt inhibitors map to both sensory and non-sensory domains, suggesting a tight temporal and spatial control of Wnt signaling in the inner ear (Sienknecht and Fekete, 2008; Sienknecht and Fekete, 2009).

At the onset of ear formation Wnt/β-catenin signaling undergoes cross-regulation with Notch signaling to regulate the size of the otic placode (Jayasena et al., 2008 and see above). Later on, Wnt signaling from the hindbrain is essential for DV axial specification of the otocyst (Riccomagno et al., 2005). During morphogenesis Wnt/β-catenin pathway is essential for correct formation of semicircular canals (Rakowiecki and Epstein, 2013). Non-canonical Wnt signaling is required for stereociliary bundle orientation (reviewed in Dabdoub and Kelley, 2005).

Retrovirus-mediated missexpression of constitutively activated β-catenin or Wnt3a in the chick otocyst gives rise to fused sensory regions, suggesting a possible role of Wnt/β-catenin in defining or maintaining sensory/non-sensory boundaries (Stevens et al., 2003). Moreover, activated Wnt signaling is able to induce ectopic sensory patches of vestibular character, indicating first, that Wnt activation is sufficient for sensory specification and secondly, that it may govern the choice between auditory and vestibular fates. This ability to instruct conversion from auditory to vestibular identity is transient and not all regions are equally competent to generate ectopic sensory patches (Stevens et al., 2003). Therefore, Wnt signaling seems to play a permissive rather than an instructive role in the sensory vs. non-sensory cell fate decisions. Since Jag1 is known to be required for prosensory specification (see above), it is likely that there is a link between these two pathways for prosensory specification. Recently, by using *TCF/Lef* reporter mice Jacques et al. (2012) showed that Wnt/β-catenin signaling surrounds the *Sox2*-positive prosensory region of the cochlea and the activation of Wnt/β-catenin causes increased proliferation of the Sox2-positive region. In contrast, Wnt/

β-catenin inactivation reduces proliferation, Sox2 expression and leads to nearly complete loss of HCs. This suggests that Wnt/β-catenin signaling regulates cell proliferation in the prosensory domain (Jacques et al., 2012). Notch overexpression induces ectopic sensory patches that express proliferation markers (Pan et al., 2013). This suggests that Notch and Wnt/β-catenin signaling may also interact in controlling proliferation of sensory progenitors. Near complete loss of HCs following Wnt inhibitor treatment after terminal mitosis suggests its additional role in HC differentiation (Jacques et al., 2012). A recent study using ear conditional *β-catenin* KO mice has shown that Wnt signaling is required for HC specification and not HC maintenance in the cochlea (Shi et al., 2014).

During postnatal stages in the mouse cochlea, ectopic activation of Wnt/β-catenin signaling induces cell proliferation and HC formation of a limited subset of SCs (Chai et al., 2012; Shi et al., 2012). The capacity of Wnt/β-catenin signaling to instruct Sox2-positive cells to re-enter the cell cycle and regenerate HCs is of importance for studies on HC regeneration (Jacques et al., 2014).

In summary, Wnt/β-catenin is necessary and sufficient for prosensory specification and it may be determinant for specification of vestibular vs. auditory fates. It remains unclear whether and how it interacts with Notch signaling. In the mouse cochlea it plays at least a dual function, in regulation of proliferation of sensory precursors and HC differentiation.

Bmp pathway

Bone morphogenetic proteins (Bmps) are diffusible molecules that belong to Transforming Growth Factor (TGFβ) superfamily (see Fig. 15C for an overview of the Bmp pathway). Several Bmp ligands are expressed in the developing inner ear where they map to sensory and non-sensory regions in both chicken and mice (Oh et al., 1996; Morsli et al., 1998). Bmp signaling performs multiple functions in the inner ear (Chang et al., 1999; Chang et al., 2002; Li et al., 2005; Pujades et al., 2006; Chang et al., 2008; Hwang et al., 2010; Kamaid et al., 2010; Ohyama et al., 2010).

Conditional deletion of *Bmp4* or Bmp type I receptors *Alk3/Alk6* in the inner ear results in the loss of the three cristae and semicircular canals (Chang et al., 2008; Ohyama et al., 2010). In the chick, downregulation of Bmp signaling by overexpression of *Smad6* or *Noggin* does not affect prosensory genes including *Sox2, Jag1* and *Fgf10*, suggesting that they function either upstream or in parallel to *Bmp4*. However, the reduction of Bmp signaling downregulates other sensory genes such as *Msx1* and *Lmo4*. In addition to regulation of some sensory markers, *Bmp4* regulates several non-sensory genes in the *septum cruciatum* including *p75Ngfr*;

Gata3 and *Lmo4*, suggesting that *Bmp4* collaborates in organizing sensory and non-sensory regions in the cristae (Chang et al., 2008).

Bmp4 expression during the stages of sensory specification suggests also a possible crossregulation with Notch. *Bmp4* expression in the crista is independent on Bmp, but maintained by Notch signaling (Daudet et al., 2007; Chang et al., 2008). *Jag1* KO mice or otic vesicle treatment with DAPT show reduced *Bmp4* expression, suggesting that Jag1/Notch signaling may act upstream of Bmp pathway during cristae sensory specification (Daudet et al., 2007; Pan et al., 2010).

Bmps repress *Atoh1* expression and maintain the undifferentiated state of sensory progenitors (Pujades et al., 2006). Exogenous BMP4 application irreversibly suppresses *Atoh1* expression by inducing apoptosis and reducing proliferation of sensory progenitors. In contrast, the Bmp antagonist Noggin upregulates *Atoh1* expression driving cell specification without requiring cell proliferation, suggesting that the balance between Bmp activity and its repression is important for deciding HC specification and the exhaustion of sensory precursors (Pujades et al., 2006). *Id1-3* genes are down-stream targets of Bmp and are expressed in sensory regions of high Bmp activity. Accordingly, the gain of function of *Id3* is able to repress *Atoh1* expression (Kamaid et al., 2010). Li et al. (2005), using low concentrations of Bmp4 reported the induction of Atoh1 expression, supporting the notion that the effect of Bmp on HC specification is concentration-dependent.

Analysis of compound *Alk3/6* KO mice shows that a gradient of Bmp signaling is necessary for patterning of the sensory and non-sensory regions of the organ of Corti (Ohyama et al., 2010). In addition, several studies propose that Bmp promotes HC formation in the mouse cochlea. Cochlear cultures incubated with BMP4 increase the number of HCs (Puligilla et al., 2007). Similarly, *Noggin-/-* mice show increased HC numbers (Hwang et al., 2010).

In summary, Bmp plays various roles during otic development. Bmps seem to be important for patterning and then maintaining sensory progenitors in undifferentiated state by repressing *Atoh1*. Further, they may promote or favor HC fate in the cochlea.

All the signaling mechanisms reviewed above seem to have potential points of interaction with the Notch pathway at different stages of development, however, there is not much information about nature of these interactions.

OBJECTIVES

OBJECTIVES

Notch signaling plays an essential role in inner ear development. Loss-of-function studies of Notch ligands *Dl1/Jag1/Jag2*, pharmacological blockade and gain of function studies of Notch revealed seemingly opposing functions. During early development Notch is required for hair cell formation, but late in development it counteracts hair cell differentiation. This behavior depends on two different modes of operation of Notch: lateral induction and lateral inhibition. Lateral induction depends on the ligand Jag1 that positively regulates its own expression in neighboring cells, forming coherent domains of Notch activity that drive progenitors towards the sensory fate. In contrast, hair cell and neuronal determination occur by lateral inhibition, where the ligand Dl1, negatively regulates its expression in neighbouring cells preventing them from adopting the same fate. The present work was aimed at studying further this problem by exploring the functional elements of the Notch pathway during inner ear development and their relationship with the different modes of operation of Notch.

The specific questions addressed are:

1. What is the expression pattern of *Hes/Hey* genes during inner ear development?

2. Is there a spatial and/or temporal correspondence between Notch ligands and Notch targets?

3. Do different Notch ligands behave differently? Are there quantitative differences in signaling? If so, does signaling strength modulate the expression of downstream targets?

4. Is there difference in Notch regulation of *Hes/Hey* genes? If so, do they have different thresholds for Notch activation?

5. Are different Notch targets instrumental for deciding between the different modes of operation of Notch? Is signaling strength?

6. Are *Hes/Hey* genes regulated by other signaling pathways?

7. Is there a mutual regulation among different Hes/Hey genes?

Part of this work has been done in collaboration with Dr. Ibañes group at the Departament d'Estructura i Constituents de la Matèria, Facultat de Física, Universitat de Barcelona and has been published in *Development* journal. The work on the differential regulation of *Hes/Hey* genes in the inner ear has been submitted for peer-review.

RESULTS

Ligand-dependent Notch signaling strength orchestrates lateral induction and lateral inhibition in the developing inner ear

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DIFFERENTIAL REGULATION OF *HES/HEY* GENES DURING INNER EAR DEVELOPMENT

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DISCUSSION

DUAL FUNCTION OF NOTCH DURING THE SENSORY DEVELOPMENT OF THE INNER EAR

One signal different outputs

In the inner ear the Notch signaling pathway is involved in at least two patterning modules, so called lateral induction and lateral inhibition (Adam et al., 1998; Eddison et al., 2000; Daudet and Lewis, 2005; Neves et al., 2011; Chrysostomou et al., 2012). Lateral induction is a process by which a ligand-signaling cell stimulates its neighbors to upregulate ligand expression and thereby it promotes a coherent cell behavior (Bray, 1998; Bray, 2006). By contrast, in lateral inhibition the ligand-signaling cell activates Notch in the neighbors and suppresses the expression of the same ligand, resulting in the adoption of a different cell fate (Heitzler and Simpson, 1991; Lewis, 1998). Both modules are present during ear neurosensory development, where the former is characteristic of the prosensory state and the latter of neuronal and HC determination (Adam et al., 1998; Eddison et al., 2000; Daudet and Lewis, 2005; Abello et al., 2007; Neves et al., 2011). Thus, neurosensory progenitors experience at least three waves of Notch activity. First, lateral inhibition drives binary cell fate choices between neuronal and epidermal fate (Adam et al., 1998; Alsina et al., 2004; Abello et al., 2007). Secondly, lateral induction ensures specification of prosensory domains that foreshadow the future sensory organs (Eddison et al., 2000; Daudet and Lewis, 2005; Hartman et al., 2010; Neves et al., 2011). Finally, a second pulse of lateral inhibition drives HC determination and provides the fine-grained mosaic of HCs and SCs (Adam et al., 1998; Lanford et al., 1999; Daudet and Lewis, 2005; Chrysostomou et al., 2012).

In otic development, different Notch ligands are associated with each module. Jag1 mediates the prosensory function (Kiernan et al., 2001; Tsai et al., 2001; Daudet and Lewis, 2005; Brooker et al., 2006; Kiernan et al., 2006; Daudet et al., 2007; Pan et al., 2010; Neves et al., 2011), whereas Dl1 mediates binary cell fate choices driving neuronal and HC determination (Adam et al., 1998; Haddon et al., 1998; Abello et al., 2007; Daudet et al., 2007; Chrysostomou et al., 2012).

Since the two modules rely on the same signaling cascade that ends with the expression of Notch downstream targets of the bHLH family of Hes and Hey repressors (Artavanis-Tsakonas et al., 1999; Bray, 2006; Fischer and Gessler, 2007; Fior and Henrique, 2009), one key question is how Notch operates in these seemingly opposing modes and what determines the different modes of action.

Notch ligands: lateral induction and lateral inhibition

In the inner ear, expression patterns and functional studies suggest that lateral induction or lateral inhibition are associated with different Notch ligands that initiate signaling, Jag1 driving lateral induction and Dl1 lateral inhibition (Adam et al., 1998; Haddon et al., 1998; Kiernan et al., 2001; Tsai et al., 2001; Daudet and Lewis, 2005; Brooker et al., 2006; Kiernan et al., 2006; Abello et al., 2007; Daudet et al., 2007; Hartman et al., 2010; Neves et al., 2011; Petrovic et al., 2014).

The association of Dl1 with lateral inhibition is a general theme in neural development (Henrique et al., 1995; Adam et al., 1998; Kageyama et al., 2010). That of Jag1 with lateral induction is seen in the lens (Le et al., 2009), developing pancreas (Golson et al., 2009), early hematopoiesis (Robert-Moreno et al., 2008) and angiogenesis (Benedito et al., 2009). However, this does not appear to be a general rule for other systems. For instance, in chick and rat, *Jag1* is expressed in complementary pattern to *Dl1* expression in the ventricular zone of developing hindbrain and in the spinal cord, before migration towards the mantle zone, suggesting their involvement in neurogenesis (Lindsell et al., 1996; Myat et al., 1996). Functional studies in *Xenopus* show that x-Serrate1 plays a role in primary neurogenesis. Overexpression of x-Serrate1 and x-Delta1 represses overproduction of primary neurons provoked by dominant negative forms of x-Delta1 and x-Serrate1, respectively, suggesting that they act in complementary manner in patterning of primary neurons (Kiyota et al., 2001). In mice, Jag1 selects ventral interneurons named V1 and dorsal interneurons named dl6 in the neural tube by lateral inhibition, the effect that can be compensated by Dll1-signalling in the absence of Jag1 (Ramos et al., 2010). On the other hand, during somitogenesis, Dl1 generates coherent patterns of expression in the presomitic mesoderm (PSM), although it actually inhibits signaling in neighboring cells. It does so by keeping the intrinsic oscillations locally synchronized through signaling delays (Oates et al., 2012).

In the inner ear, Jag1 and Dl1 are oppositely regulated by Notch signaling, which readily accounts for their association with the different circuits of lateral induction and lateral inhibition, respectively. While the inhibition of Dl1 by Notch has been associated with the repressor effect of Hes/Hey factors on bHLH proneural genes (Kageyama et al., 2010), the activation of Jag1 by Notch is poorly understood (Katoh, 2006 and see below).

The above data suggest that both ligands can generate either coherent or salt-and-pepper patterns, hallmarks of lateral induction and lateral inhibition, respectively, and that context conditions are likely candidates to determine the behavior of Dl1 and Jag1 in different tissues (see below).

Dl1 and Jag1 signal differently in the inner ear

Why Jag1 and Dl1 signal differently in the inner ear? One possibility is that Jag1 and Dl1 activate different Notch receptors. This is likely to be the case during mouse neurogenesis where Jag1 preferentially binds Notch2 expressed in the floor plate, whereas Dl1 signals through both Notch1 and Notch2 from the walls of the neural tube (le Roux et al., 2003). Similarly, in human thymocytes, while Jag2 acts through interaction with both Notch1 and Notch3, Dl4 primary binds Notch1 (Van de Walle et al., 2013). The chick genome codes for two Notch receptors Notch1 and Notch2, however only Notch1 is expressed in the inner ear (Adam et al., 1998; Abello et al., 2007 and our own data), suggesting that signaling through different Notch receptors does not occur. Since Jag1 and Dl1 signal only through Notch1 in the chick inner ear, another possibility is that the activation of Notch by different ligands results in quantitative differences in signaling strength. Our data demonstrate that this is the case for the chicken inner ear, where Dl1 induces a stronger Notch signaling than Jag1 (Petrovic et al., 2014).

Why Dl1 and Jag1 signal differently?

The interaction of different ligands with the Notch receptor can be modulated by different factors, particularly modifications of the receptor. One major modulator of Notch receptors that makes these two ligands behave differently is Fringe family glycosyltransferases (Bruckner et al., 2000; Haines and Irvine, 2003). Fringe glycosylation interferes with the efficiency of Notch cleavage triggered by the binding of Jag1 (Bruckner et al., 2000; Hicks et al., 2000; Yang et al., 2005; Benedito et al., 2009; Golson et al., 2009). This potentiates Notch signaling induced by Dl1, while inhibiting signaling induced by Jag1. Interestingly, *Lunatic Fringe* (*LFng*) is expressed in sensory regions of the mouse and chick inner ear (Morsli et al., 1998; Cole et al., 2000). Therefore, it is possible that the presence of LFng in the prosensory domains hampers Jag1 signaling, which in turn results in low levels of Notch activity. During HC production, this function is maintained, Dl1 signaling by HC precursors favored and lateral inhibition promoted.

The mechanism by which Notch activation mediated by Jag1 and Dl1 produces difference in Notch signaling strength is still obscure. "*Lift and cut*" mechanism of ligand endocytosis is thought to produce the physical force needed to pull the Notch ectodomain, promoting an exposure to metalloprotease cleavage site (S2) (Gordon et al., 2008). Fringe glycosylation may modulate the strength of ligand-Notch interactions and the ability of ligand-Notch interactions to survive the pulling force produced by ligand endocytosis (Yang et al., 2005). Alternatively, the smaller Dl1 ligand can be efficiently endocyted, creating a strong pulling

force, whereas the cell may face difficulties to endocyte the twice as bigger Jag1 ligand.

Lfng deficient mice are viable, with no obvious ear phenotype suggesting either possible redundancy with other fringes or questioning its requirement for inner ear development (Zhang et al., 2000). Both mouse and zebrafish express *Manic fringe* in the otic vesicle (Johnston et al., 1997; Qiu et al., 2004). However, in the chicken inner ear it seems that only *Lfng* is expressed. Further studies are required to determine the weight of Fringe proteins in the differences in signaling between Jag1 and Dl1 in otic development.

Jag1 and Dl1 differentially regulate Hey1 and Hes5 expression

Jag1 is uniformly expressed in the prosensory patches and later in development it becomes restricted to the basal layer of SCs in differentiated sensory organ (Adam et al., 1998; Morrison et al., 1999; Cole et al., 2000; Petrovic et al., 2014). As described above, its function is associated with prosensory specification. Jag1 maintains *Sox2* expression within the prosensory domains, and it induces its own expression through the mechanism of lateral induction (Eddison et al., 2000; Neves et al., 2011). *Dl1* is expressed in the neurogenic domain and in HCs during cell fate determination (Adam et al., 1998; Morrison et al., 1999; Alsina et al., 2004; Abello et al., 2007). We sought to assess whether there is spatial and functional correspondence between Notch ligands and targets during sensory development. Our results show that *Hey1* follows very well Jag1 expression from prosensory stages to those of HC differentiation, in agreement with mouse data (Hayashi et al., 2008b; Li et al., 2008; Tateya et al., 2011). Next, we show that *Hes5* is expressed during neuronal and HC determination and follows the temporal profile of *Dl1* expression. The absence of *Hes5* transcripts in prosensory patches suggests that it is not necessary for lateral induction and sensory specification. The speckled *Hes5* expression contrasts the uniform *Hey1* expression. The salt-and-pepper pattern of expression is a typical result of the operation of the Notch signaling during lateral inhibition mediated by Dl1, suggesting that *Hes5* may be the preferred Notch target for this mode of operation. This expression pattern data go well in line with Jag1 and Dl1 gain of function experiments, which show that Jag1 preferentially activates *Hey1,* whereas Dl1 activates both *Hey1* and *Hes5* (Petrovic et al., 2014). This further strengthens the idea that *Hey1* is associated with the activation of Notch by Jag1, whilst *Hes5* may be preferred target of Notch activation by Dl1.

Different signaling strength results in different transcriptional outcomes

Dose dependent differences in Notch response have been reported in various developmental contexts (Delaney et al., 2005; Hellstrom et al., 2007; Mazzone et al., 2010). Our results show that Dl1 and Jag1 drive Notch signaling at different strengths, eliciting differential transcriptional outcomes. This suggests that different Notch targets have different threshold for Notch activation. In the embryonic kidney Hes1 and Hes5 expression is Notch dependent and display different sensitivities to Notch levels. While Hes5 drops after short incubation with DAPT, Hes1 expression is sustained and decays only after long incubation times with same blocker (Ong et al., 2006) (but see also below the discussion on mRNA stability). The expression of *Hes5* and *Hey1/2/L* and their sensitivity to γ-secretase inhibitors has been reported also in the mouse inner ear (Hayashi et al., 2008b; Doetzlhofer et al., 2009). *Hes5* is more sensitive than *Hey1* to treatment with DAPT suggesting that it requires higher levels of intracellular Notch activity (Doetzlhofer et al., 2009). Moreover, Hayashi et al. (2008b) showed that the concentration of DAPT required to inhibit Notch signaling during lateral inhibition is lower than for the prosensory phase, suggesting that *Hes5* and lateral inhibition share a similar sensitivity to Notch. This suggests that Notch levels discriminate between different targets, and we were able to show that low Notch activation triggered by Jag1 is sufficient to induce *Hey1* but not *Hes5*, and the strong Notch signal induced by Dl1 is sufficient for transcription of both *Hey1* and *Hes5* (Petrovic et al., 2014).

What is the significance of different signaling strengths for sensory development?

Alternative cellular behaviors dependent on Notch levels have been reported in relation to the decision between cell proliferative and cell arrest states (Chapouton et al., 2010; Mazzone et al., 2010; Perdigoto et al., 2011; Ninov et al., 2012). In the adult zebrafish telencephalon, the balance between quiescence of radial glial cells in ventricular zone and neurogenesis is controlled by fluctuations of Notch activity (Chapouton et al., 2010). Similarly, in mammary epithelial cell cultures dichotomous responses to Notch are determined by the dose of pathway activation. High levels of Notch pathway result in suppression of cell proliferation, whereas low doses of Notch activation induce proliferation of epithelial cells in the acinar structures of mammary gland (Mazzone et al., 2010). Likewise, Notch signaling levels regulate decisions between cell proliferation and quiescence of zebrafish endocrine progenitors (Ninov et al., 2012). Studies in *Drosophila* reveal the importance of Notch levels in maintenance of intestine homeostasis, where Notch signaling barrier needs to be crossed

in order for intestine stem cells to exit from cell renewal program and become committed prior to terminal differentiation of specific cell fates (Perdigoto et al., 2011).

In the prosensory patches, sensory progenitors proliferate (Murata et al., 2009), whereas in the differentiating sensory organs HCs exit cell cycle and differentiate (Chen and Segil, 1999) with SCs entering a quiescent state (Oesterle and Rubel, 1993). One possibility is that gene regulation and cellular function depend on the different levels of Notch signaling elicited by the different ligands. Recently, Liu et al. (2013) showed that Notch activity is nearly undetectable during prosensory stages, but it increases during HC determination. This fits well with our results and with the notion that the prosensory state is driven by weak Jag1 signaling and HC patterning involves strong Dl1 signaling. The control exerted by Notch on both cell proliferation and differentiation resembles the dose dependent activity of Myc in epidermal cells (Watt et al., 2008). In *Drosophila* Myc is an important intermediary in Notch-induced proliferation (Krejci et al., 2009). *Myc* genes are expressed during the inner ear development and N-Myc has been shown to regulate proliferation in the mouse inner ear (Dominguez-Frutos et al., 2011). This points to the possible convergence of Myc and Notch pathways to regulate proliferation and quiescent states in the otic development.

How signal strength results in differential transcriptional outcomes?

Promoter activation of Notch target genes depends on structural properties like the arrangement and spacing of CSL binding sites or the distance from the transcriptional start site on *cis*-regulatory elements that influence the selectivity and amplitude of the response. This specific organization of the promoter regions of Notch target genes dictates the cooperative assembly of Notch transcriptional complexes, which results in different outputs (Arnett et al., 2010). Several Notch target genes harbor more than a single CSL binding site in their proximal promoter. Promoters of some *Drosophila* and mammalian *Hes* and *Hey* genes contain conserved CSL-binding sites in head-to-head orientation separated by 15-19 base pairs termed sequence paired site (SPS) (Bailey and Posakony, 1995; Nellesen et al., 1999; Cave et al., 2005; Arnett et al., 2010). Based on the CSL architecture features a recent study reported categorization of Notch targets genes which falls into at least three distinct groups: genes which transcription is dependent on CSL/NICD dimmers, genes which transcription is independent on CSL/NICD dimmers and genes that utilize both monomeric and dimeric CSL/NICD complexes (Liu et al., 2010). Transcription of mouse and human *Hes5* is CSL dimer-dependent, in contrast to human *HeyL* and mouse *Hey2* which transcription is CSL dimer-independent (Arnett et al., 2010). In the mouse T-cell lymphoma cell line, *Hey1* transcription is activated by monomeric Notch nuclear complex (Liu et al., 2010). These structural requirements underlie the effect of Notch levels on Hes1 and Hes5

expression in the embryonic kidney (Ong et al., 2006). Moreover*, in silico* analysis from our lab shows three CSL binding sites in mouse and human *Hes5* promoters and four CSL-binding sites in chick *Hes5* promoter. In contrast, the *Hey1* promoter in human, mouse and chick contains two putative CSL binding sites (unpublished data). Which CSL binding sites are functional and whether their arrangement underlies differential *Hey1* and *Hes5* response in the inner ear remains to be determined. Besides, CSL binding sites are subdivided into two groups of high and low affinity (Arnett et al., 2010). This opens the possibility that only high-affinity sites are occupied by low levels of NICD, whereas high levels of NICD occupy both low- and high-affinity CSL binding sites.

In addition, Notch activation results in the regulation of a variety of genes, sometimes with opposing functions that operate in an incoherent network logic (Krejci et al., 2009; Housden et al., 2013). Indeed, interactive loops among different Hes genes have been described in *Drosophila* muscle progenitors (Housden et al., 2013), vertebrate neurogenesis (Fior and Henrique, 2005; Vilas-Boas and Henrique, 2010) and somitogenesis (Schroter et al., 2012) and contribute to set the final steady state level of the different Hes and Hey proteins. In agreement with model proposed by Housden et al. (2013) an interesting possibility may be that there is an underlying buffer in the form of Notch dependent repressor that could prevent *Hes5* transcription from responding to low levels of Notch.

Jag1 and Dl1 compete for Notch signaling

Our results indicate that Jag1 and Dl1 drive the same type of signal through the single receptor, Notch1, but quantitatively different (Adam et al., 1998; Abello et al., 2007; Petrovic et al., 2014). In order to understand how HCs develop, we need to understand how otic progenitors cope with the coexistence of both ligands during the transition between prosensory and sensory state in the inner ear development. In other words, we analyzed the result of the combined function of Jag1 and Dl1. The results show that Jag1 and Dl1 compete for receptor signaling, the overall signal of Dl1 being reduced when both ligands are coexpressed. This suggests that Notch ligands compete for a Notch1, and that Jag1 acts as partial agonist, becoming a competitive inhibitor of Dl1/Notch signaling (Buchler et al., 2003; Petrovic et al., 2014). Similarly, during mouse angiogenesis Jag1 opposes the inhibitory effect of Dl4 mediated Notch signaling on sprouting, resulting in enhanced angiogenic growth (Benedito et al., 2009). In agreement with our observations, the combined Jag1 and Dl4 signaling results in decreased Notch activity compared to Dl4 alone. This situation resembles also the one found in *cis*-inhibition of Notch signaling, where Dl1 ligand in a cell competes with Dl1 ligand in neighboring cells to bind to Notch receptor (Formosa-Jordan and Ibanes, 2014). In the context of inner ear development, *cis*-inhibition does not occur

(Chrysostomou et al., 2012), but the competition between Dl1 and Jag1 ligands results in a similar net effect on the strength of the signal.

The results show also that the signal induced by the combined expression of Jag1 and Dl1 is closer to that of Jag1 than to Dl1. This suggests that Jag1 affinity for Notch1 is higher than Dl1 (Petrovic et al., 2014). Affinity of different Notch ligands to Notch receptors is poorly studied. One possible explanation for our observation is that sugar modifications modify the receptor in the way that it increases affinity of Jag1 over Dl1 to Notch receptor. Alternatively, the affinity may be independent on sugar status of EGF repeats of Notch, but dependent on a size of interface in Notch-ligand complex. Twice bigger Jag1 protein creates greater surface for binding and thus might exert higher affinity.

What Jag1 mediated Notch signaling is important for?

The prosensory state is characterized by the condition in which sensory progenitors are specified, but not yet determined to adopt HC or SC fate. Prosensory specification requires Jag1 mediated Notch signaling that establishes a coherent domain of low Notch activity, where Jag1/Notch signaling is expanded by lateral induction. Notch, in turn, induces Sox2 expression, which is necessary and sufficient for prosensory specification (Eddison et al., 2000; Kiernan et al., 2001; Tsai et al., 2001; Daudet and Lewis, 2005; Kiernan et al., 2005b; Kiernan et al., 2006; Hartman et al., 2010; Pan et al., 2010; Neves et al., 2011). Upon Atoh1 expression, Dl1 mediates inhibition of neighboring cells generating the HC/SC lattice, where patterning follows the rules of lateral inhibition (Haddon et al., 1998; Lanford et al., 1999; Daudet and Lewis, 2005; Chrysostomou et al., 2012). During the transition from prosensory to cell determination state it is thought that otic progenitors express both Jag1 and Dl1 ligands and thus the main question that arises here is that of how transition occurs and whether differences of Notch levels mediated by Jag1 or Dl1 observed in our experiments are important for the final patterning. Indeed, the mathematical model gives a valuable insight into answering these questions. Equal signaling strength of Jag1 and Dl1 compromises lateral inhibition and disrupts the salt-and-pepper patterning of HCs and SCs in the patch. On the contrary, the speckled pattern arises only when Jag1 signaling strength is weak. Further, our results indicate that upon Atoh1 expression, Jag1 performance switches from increasing the overall signaling and driving lateral induction, to effectively decreasing Notch signaling and facilitating HC patterning. This facilitation arises from the mutual inhibition between adjacent equivalent cells driven by Jag1 when competing with Dl1, and represents a novel role of Jag1 in lateral inhibition. We suggest a dual function of weak Jag1 signaling: it drives lateral induction and prosensory specification when acting alone, but upon Dl1 expression, it facilitates lateral inhibition and HC patterning (Petrovic et al., 2014).

What is a signature of Jag1 in lateral inhibition?

Following this idea of the function of Jag1 as a facilitator of HC patterning, we did the experiments of perturbing Jag1 levels in the sensory patch of chicken embryos. Unexpectedly, in spite of changes in cell identity and cell bias (see below) we were unable to see HC patterning defects using either numerical stimulations or experimental chick otocysts (Petrovic et al., 2014). This suggests that HC pattern formation is rather robust. Our results suggest that robustness of HC patterning to changes in Jag1 expression arises mainly from the autoactivation of Atoh1 (Helms et al., 2000). While Atoh1 autoactivation does not facilitate nor promote pattern initiation, it maintains patterning once it is formed and stabilizes the final pattern (Petrovic et al., 2014). This is in agreement with recent results showing that once sensory progenitors start to highly express Atoh1 and subsequently Dl1, cannot be prevented from becoming HCs (Driver et al., 2013). Also, Chrysostomou et al. (2012) show that HCs may develop in direct contact with several neighboring cells expressing high levels of Dl1.

Other studies had shown rather more intense phenotypes after Jag1 perturbations in the mouse cochlea. Zine et al. (2000) showed supernumerary OHCs in cochlear explants cultured with antisense-*Jag1* mRNA and, similarly, *Cm*/+ mice show extra rows of OHCs (Kiernan et al., 2001). Ear conditional *Jag1* KO mice show loss of OHCs and increased number of IHCs. These three studies indicate that Jag1 drives HC determination in the cochlea. The discrepancy between these data and our results is not completely surprising given that sensory development in mice auditory organ does not strictly parallel that of vestibular patches (Basch et al., 2011; Yamamoto et al., 2011), which were analyzed here. Still, the origin of these discrepancies remains unknown.

Given the robustness of HC patterning, what is then the signature of Jag1 in facilitating the patterning? Our experiments and numerical stimulations reveal that Jag1 does not disrupt the HC patterning, but biases towards SC fate (Petrovic et al., 2014). We believe that this occurs because of Jag1 ability to compete with Dl1. Electroporated Jag1-carring cells may reduce overall Notch signaling in unelectroporated neighboring cells, resulting in the release of Notch-mediated inhibition of *Atoh1* and, therefore, the promotion of HC fate. Contrarily, the bias of electroporated cells towards SC fate is lost when Jag1 is transiently suppressed in the sensory patch. Then, cells losing Jag1 lose also their identity as judged from the parallel loss of Sox2 (Petrovic et al., 2014). This suggests that the main function of Jag1 is to bias SC fate. The loss of SC fate as judged by loss of Sox2 expression is not enough to switch them to the HC fate. We reason that suppression of the conversion from SC to HC fate occurs because Dl1 driven Notch activation in SCs still upregulates Hes/Hey genes that keep *Atoh1* transcription suppressed.

DIFFERENTIAL HES/HEY REGULATION IN THE INNER EAR DEVELOPMENT

Hey1 and Hes5 in otic development

Hes/Hey genes are well studied in number of developmental systems, their main function being the maintenance of stem cell or progenitor state, prevention of premature differentiation and regulation of binary cell fate choices (Iso et al., 2003; Fischer and Gessler, 2007; Kageyama et al., 2007). With the exception of Hes6 and HeyL, all Hes and Hey factors function as repressors of tissue specific determination and differentiation genes in a variety of systems (Fischer and Gessler, 2007; Kageyama et al., 2007; Vilas-Boas and Henrique, 2010; Jalali et al., 2011). Similarly, in the inner ear Hes/Hey proteins oppose the effect of the HC differentiation gene Atoh1 (Zheng et al., 2000; Zine et al., 2001; Zine and de Ribaupierre, 2002; Doetzlhofer et al., 2009; Tateya et al., 2011; Du et al., 2013).

Hey1 expression maps to all prosensory regions, which is in agreement with data reported in mice (Hayashi et al., 2008b; Pan et al., 2010; Tateya et al., 2011), and suggests that Hey1 plays a functional role during prosensory specification. Given the ability of Hey1 to act as repressor of proneural factors (Sakamoto et al., 2003), it is likely that Hey1 cooperates with other bHLH factors in preventing differentiation of sensory progenitors. *Hey1* is induced by Sox2 and one good candidate to mediate the repression of Atoh1 induced by the incoherent response to Sox2 (Neves et al., 2012). Sox2 directly activates and indirectly represses *Atoh1* and thereby it maintains sensory commitment and prevents differentiation. Hey1 may be one of the factors that maintain the state of undifferentiated and proliferative state that characterizes the prosensory progenitors. Hey1 most likely cooperates with other factors for Atoh1 inhibition. In parallel to Hey1, Sox2, induces other bHLH factors that antagonize Atoh1 function (Neves et al., 2012). They include Bmp-dependent Id repressor genes (Kamaid et al., 2010) and neurogenic factors like Neurog1 and NeuroD (Ma et al., 1998; Ma et al., 2000; Kim et al., 2001). This would explain why *Hey1* deficient mice do not to show defects in the formation of HC of the organ of Corti (Hayashi et al., 2008b).

Although being a good readout of Jag1, our results show that it is unlikely that Hey1 is instrumental for Notch mediated lateral induction. This is defined as the ability of the ligand-delivering cell to induce the expression of the ligand in the neighboring, ligand-receiving cells. In principle, Hey1 could mediate lateral induction by repressing a repressor and thereby releasing the repression of Jag1. But this does not seem to be the case since the experiments show that Hey1, like Hes5, is able to repress Dl1, but has no effect on Jag1

expression (see below). Later in development, *Hey1* is expressed along with *Hes5* during HC differentiation stages in the SCs. The overlapping expression of *Hey1* and *Hes5* during hair cell differentiation suggests that these two factors cooperate in lateral inhibition between HCs and SCs. Indeed, the combined deletion of *Hey1* with *Hes1* and *Hes5* shows increased number of HCs in the cochlea (Tateya et al., 2011).

Other Hes/Hey genes

In contrast to the mice, in the chicken inner ear *Hey2* and *Hes1* do not show restricted expression in the prosensory and sensory patches. *Hey2* stains weakly the macular region and non-sensory regions of otic epithelium. In addition, *Hey2* expression occurs also in periotic mesenchyme. In the mouse, *Hey2* is expressed in the prosensory domains of the cochlea and later on it become restricted to the pillar cells (Hayashi et al., 2008b; Li et al., 2008; Doetzlhofer et al., 2009). *Hey2* expression in pillar cells is regulated by Fgf signaling and is able to antagonize *Atoh1* expression (Doetzlhofer et al., 2009). In mice, *Hey2* is not detected in vestibular organs (Hayashi et al., 2008b). Similarly to *Hey2,* in the mouse cochlea, *Hes1* is detected in the prosensory domain along with activated Notch1 receptor (Murata et al., 2009). Upon HC determination, *Hes1* becomes restricted to SCs in vestibule and inner phalangeal cells spreading towards LER and GER (Zheng et al., 2000; Zine et al., 2001; Doetzlhofer et al., 2009; Murata et al., 2009; Tateya et al., 2011). We were unable to find see this expression pattern in the chick.

Hes6 is a downstream target of Atoh1 and is expressed in HCs in mouse inner ear (Qian et al., 2006). In agreement, we observed a weak *Hes6* staining in most differentiated sensory organs. In the crista, *Hes6* expression is scattered and may map to the nascent HCs. Strong *Hes6* expression is observed in macula sacularis during prosensory stages, which fits well mice data (Qian et al., 2006). *Hes6* expression is promoted by proneural genes in the *Xenopus* neural plate (Koyano-Nakagawa et al., 2000) and it acts as a positive regulator of neurogenesis by antagonizing the repressor function of Hes1 and Hes5 (Bae et al., 2000; Gratton et al., 2003; Fior and Henrique, 2005; Vilas-Boas and Henrique, 2010). Thus, it is possible that Hes6 maintains *Atoh1* expression in the sensory patch by repressing other Hes/Hey genes.

In the chick, *Hes2* expression does not show a restricted expression pattern in the otic epithelium, however, its expression is strong in the differentiated sensory organs where it is probably restricted to the SC layer. This pattern suggests that Hes2 may repress Atoh1 expression during HC differentiation. In addition, *Hes2* is strongly expressed in non-sensory regions of otic epithelium with unknown function. *Hes2* expression has not been reported in the otocysts of other species. In mice, *Xenopus* and chick, *Hes2* expression in tissues

other than the ear is regulated by Notch-dependent and Notch-independent mechanisms (Nishimura et al., 1998; Davis et al., 2001; Cui, 2005; Solter et al., 2006; Sheeba et al., 2012). x-Hes2 is known to act as retinal gliogenic factor by antagonizing proneural gene function (Solter et al., 2006).

Cross-talk among signaling pathways and the regulation of Hey1 and Hes5

Hey1 and *Hes5* are Notch target genes in number of systems including the inner ear (Kokubo et al., 1999; Ohtsuka et al., 1999; Petrovic et al., 2014). However, the results show that during early otic development, unlike prosensory stages, *Hey1* expression does not match tightly to Jag1. Moreover, when *Hey1* becomes restricted to the future sensory regions its expression is always broader than that of Jag1. This raised the question of whether *Hey1* may be regulated by signaling mechanisms other than Notch. Several expression and functional studies suggest that Bmp, Fgf and Wnt pathways regulate diverse steps of inner ear development ranging from prosensory specification to HC differentiation (see Introduction). We explored whether Notch and these signaling pathways converge in the regulation of *Hey1* and *Hes5* in the chicken inner ear.

Hey1 and Hes5 regulation by the Bmp pathway

Various *Bmps* and their target genes (*Ids*) are expressed within and at the boundaries of sensory domains (Oh et al., 1996; Morsli et al., 1998; Kamaid et al., 2010). In addition, conditional deletion of *Bmp4* or Bmp type I receptors, *Alk3/Alk6,* in the inner ear results in loss of the three cristae and the patterning defects of the sensory and non-sensory regions of the organ of Corti (Chang et al., 2008; Ohyama et al., 2010). Our results show that, differently from *Hes5*, *Hey1* expression is not much affected by Bmp blockade. There is a small fraction of *Hey1* expression that is dependent on endogenous Bmp, and disclosed by the comparison between Notch blockade and the combined Notch and Bmp blockade. This suggests that Notch and Bmp act in a synergic manner. Jag1 induces *Bmp4* (unpublished) and the expression of *Bmp4* in the sensory patches is attenuated with DAPT or in *Jag1* cKO mice (Daudet et al., 2007; Pan et al., 2010), thus it is possible that Notch mediates the effect of Bmp on *Hey1* expression. Notch may cooperate with Bmp4 in keeping progenitors in undifferentiated state by repressing *Atoh1* transcription (Kamaid et al., 2010).

Our results show that contrarily to *Hey1*, *Hes5* transcription increases after Bmp blockade, suggesting an inhibition on *Hes5* by endogenous Bmp. This effect is likely secondary to the inhibition of *Atoh1* by Bmps (Pujades et al., 2006; Kamaid et al., 2010). The release of Atoh1

inhibition allows Atoh1 expression and lateral inhibition, with the consequent activation of *Hes5* (Pujades et al., 2006). *Hes5* regulation by Bmp, therefore, is upstream of Notch activation.

Hey1 and Hes5 regulation by the Fgf pathway

Fgf signaling components are expressed in the sensory regions and are important for auditory HC formation (Pirvola et al., 2002; Pauley et al., 2003; Hayashi et al., 2008a; Ono et al., 2014), suggesting that Fgf may affect *Hey1* expression. Our results indicate that *Hey1* and *Hes5* are inhibited by Fgf, since the blockade of Fgf signaling increases both *Hey1* and *Hes5* transcription. This effect is upstream of Notch, since it is lost when combined with Notch blockade. In parallel, Fgf blockade also increases *Jag1* expression, but the combined treatment of Fgf and Notch blockers cancels the effects, suggesting an opposed regulation of *Jag1* by Fgf and Notch. Our data indicate that Fgf represses *Hey1* expression through Notch and, in parallel, it opposes Notch effect on *Jag1*. We do not know whether these effects are direct or secondary to other factors. Interactions between Notch and Fgf pathways have been recently reported in the mouse cochlea, but in this case, Fgf seems to act downstream of Notch in regulating Sox2 expression (Munnamalai et al., 2012).

Hey1 and Hes5 regulation by the Wnt pathway

Wnt signaling elements have been characterized in detail in the developing ear, and both Wnt receptors (Frizzald proteins) and Wnt ligands show a neat compartmentalization in sensory and non-sensory domains of the developing inner ear (Sienknecht and Fekete, 2008; Sienknecht and Fekete, 2009). Moreover, the early overexpression of β-catenin results in the expansion of the sensory domains with ectopic HCs and SCs (Stevens et al., 2003; Jacques et al., 2012; Shi et al., 2014). The overexpression of NICD or Jag1 results in similar phenotype (Daudet and Lewis, 2005; Hartman et al., 2010; Pan et al., 2010; Neves et al., 2011), suggesting possible relationship between Notch and Wnt pathways during prosensory specification. In agreement with this data, our experiments show that inhibition of the Wnt pathway reduces *Jag1* expression. This explains why upon Notch blockade *Jag1* expression is only lost by 40% (Petrovic et al., 2014), and prosensory patches do not disappear completely (Daudet et al., 2007). The presence of conserved double Tcf/Lef binding sites in human and mouse *Jag1* promoters suggests that the regulation of *Jag1* by Wnt may be direct (Katoh, 2006), as reported in hair follicle formation in the adult epidermis (Estrach et al., 2006) and colonorectal and ovarian cancer (Rodilla et al., 2009; Chen et al., 2010). Similarly, the initiation of Jag1 expression in the mouse otic placode is regulated by Wnt/β-catenin (Jayasena et al., 2008). However, Wnt signaling seems to exert a net inhibitory effect on *Hey1*

since *Hey1* expression increases after Wnt blockade, and this increase is abolished by Notch blockade. Therefore, Wnt seems to operate in an incoherent manner, both activating Jag1 and inhibiting Notch, probably helping to tune the final expression levels of *Hey1*. Similarly to *Hey1*, Wnt negatively regulates *Hes5* expression, being upstream of Notch pathway.

Differential regulation of Hey1 and Hes5 by mRNA degradation

We have shown that increasing levels of active Notch result in differential target activation, low levels of NICD activating *Hey1* but not *Hes5* expression, high levels of NICD activating both (Petrovic et al., 2014). We went further to explore this question by analyzing *Hey1* and *Hes5* mRNA levels after reducing endogenous Notch levels using LY411575, probably closer to those normally encountered in the cell. Indeed, steady-state experiments suggest that *Hey1* requires lower Notch levels than *Hes5* for its activation, half-inhibition concentrations for *Hey1* being about twice the one for *Hes5* (5nM and 2.3nM, respectively). When analyzing decay experiments, we observed a rapid fall in *Hes5* and slow decay of *Hey1* expression, which at first sight suggested a confirmation of the above results. However, when analyzing the decay of *Hey1* and *Hes5* after transcriptional blockade, we found that *Hey1* mRNA was far more stable than *Hes5* and this was independent of Notch activity. The C-terminal WRPW motif in Hes members apart from a repressive function also acts as polyubiquitination signal (Kang et al., 2005). Hes factors are rapidly polyubiquitinated and degraded by proteasome with a very short half-life of approximately 20 minutes (Hirata et al., 2002). Therefore, the short Hes protein half-life may directly reflect their short mRNA stability. The lack of the WRPW motif in Hey factors may underlie their different protein interactions and thus mRNA stability. Stability of mRNA is also affected by the cell's biological state, for instance, proliferative vs. differentiated states (t Hoen et al., 2011). Prosensory patches are proliferating pools of prosensory progenitors that slow down their proliferation rate and exit cell cycle upon fate determination. The long lasting *Hey1* mRNAs in the prosensory patches accommodate to low levels of Notch activity driven by Jag1 and maintain cell proliferation until HC determination. However, when all conditions are set for differentiation, strong Notch signaling driven by Dl1 may require short-living *Hes5* mRNA for control of precise and rapid choice between HCs and SCs. Accordingly, *Jag1* mRNA is also more stable than that of *Dl1* (data not shown).

Is Hey1 instrumental for lateral induction?

A good spatial and temporal correspondence between Jag1 and *Hey1* expression during prosensory stages, dependence of *Hey1* expression on Notch signaling, Jag1 functional data that show its ability to induce *Hey1* and loss of *Hey1* expression in *Jag1* cKO mice (Hayashi et

al., 2008b; Hartman et al., 2010; Pan et al., 2010; Petrovic et al., 2014), suggest that Hey1 is a candidate gene to be a instrumental for lateral induction. However, overexpression of Hey1 shows that Hey1 is not sufficient to induce *Jag1*, which drives lateral induction (Eddison et al., 2000; Daudet and Lewis, 2005; Neves et al., 2011). This may be caused by different scenarios: 1) Underlying mechanism for lateral induction might be independent of Hey1 and Notch might directly activate other gene targets that result in *Jag1* activation. This is further supported by notion that *Hey1* KO mice display unaffected HC patterning in the cochlea, reflecting unaffected prosensory specification, the result that may be interpreted by the lack of necessity of *Hey1* expression or its redundancy with other *Hes/Hey* factors during prosensory specification (Hayashi et al., 2008b). 2) Notch activation is known to act in incoherent network logic by simultaneously inducing targets and their repressors that in this case might counteract Hey1 function in lateral induction (Krejci et al., 2009). 3) Hey1 might require other repressor proteins by which repression, Hey1 may have activator function. Possibly, these repressors may not be expressed outside the patch, and thus prevent indirect Hey1 activator function on Jag1 outside the prosensory domain. 4) Finally, Notch activation of *Jag1* in lateral induction may be direct and not accomplished through other factor(s). In fact the Jag1 promoter responds to Notch activation in mouse myoblast cell line (Castel et al., 2013), suggesting that this scenario may also be possible in the inner ear. Whether Hey1 is required for sensory specification remains undetermined. Further experiments of Hey1 effect on sensory genes like *Bmp4, Sox2, Lfng* are needed to answer this question.

Surprisingly and unlike Hey1, Hes5 appears to be able to activate *Jag1* expression, although *Hes5* is not expressed during prosensory stages along with Jag1. This positive regulation of *Jag1* may be secondary to Hes5 function in lateral inhibition. It is possible that Hes5 overexpression represses *Dl1* and thus HC determination that, in turn, would maintain prosensory state and Jag1 expression. Alternatively, Hes5 may repress an unknown repressor or replace other stronger repressor resulting in overall Jag1 activation, a situation that is not likely to occur in the sensory patch.

Both Hey1 and Hes5 are instrumental for lateral inhibition

Dl1 is Notch ligand associated with lateral inhibition during neuronal and HC determination (Abello et al., 2007; Daudet et al., 2007; Chrysostomou et al., 2012). Spatial and temporal correspondence between *Dl1* and *Hes5* and *Hey1* expression during determination stages of otic development together with Dl1 overexpression that shows induction of *Hey1* and *Hes5* suggest that both targets may be readouts of lateral inhibition (Petrovic et al., 2014). In Hey1 or Hes5 gain of function experiments we observed reduction of *Dl1* expression in either case, confirming that they behave as downstream targets of Dl1-mediated lateral inhibition.

This is in line with lateral inhibition model where Dl1 activates Notch in *trans*- to induce *Hey1* and *Hes5,* which in turn repress *Atoh1* and *Dl1* and thus the acquisition of HC fate.

Why Hey1 cannot be repressed?

Hes/Hey proteins, including Hey1 and Hes5, function as repressors either on their own promoters or in promoters of tissue specific determination and differentiation factors in a variety of systems (Iso et al., 2003; Fischer and Gessler, 2007; Kageyama et al., 2007). Gain of function of Hey1 and Hes5 reveals their complex mutual regulation in the inner ear. Both Hey1 and Hes5 are able to repress *Hes5* expression. However, surprisingly, neither of the two, when overexpressed was able to affect *Hey1* expression. Possible explanations are: 1) Hes/Hey genes might be subject of complex cross-inhibitory interactions (Hans et al., 2004; Fior and Henrique, 2005; Vilas-Boas and Henrique, 2010). One interesting possibility may be that Hey1 or Hes5 represses *Hey1* and at the same time indirectly induces *Hey1* expression by inhibiting an unknown repressor. 2) Hes/Hey proteins accomplish their repression functions acting as homo- or heterodimers, with heterodimers acting as stronger repressors due to ability to recruit a more diverse set of repressors and amplification of repression signals (Iso et al., 2003). The lack of adequate partner for heterodimerization may be crucial for the lack of repression of *Hey1*. 3) Hes/Hey factors often repress their own transcription, so Hey1 overexpression may interfere with negative autoregulatory loop that results in unchanged *Hey1* transcriptional levels. The mechanism that leaves *Hey1* expression insensitive to repressive signals remains to be determined.

In summary, *Hey1* and *Hes5* are expressed in the sensory patches with different temporal profiles, *Hey1* matching prosensory specification and both matching HC determination during inner ear development. *Hey1* and *Hes5* expression is Notch dependent, however Notch levels discriminate target transcription. In addition, *Hey1* and *Hes5* show differential regulation by other signaling pathways. Particularly, Wnt signaling appears as a good candidate to regulate *Jag1* and thereby *Hey1* expression. In addition, Bmp signaling differentially regulates *Hey1* and *Hes5* expression. Differences in mRNA stability and their cross-regulation may be coupled to the different roles played by *Hey1* and *Hes5* during inner ear development.

CONCLUSIONS

CONCLUSIONS

Ligand-dependent Notch signaling strength orchestrate lateral induction and lateral inhibition in the developing inner ear

1. There is a good correspondence between the expression pattern of Notch ligands and Notch targets during inner ear development. *Hey1* expression follows Jag1 and corresponds to lateral induction and prosensory specification. *Hes5* expression follows *Dl1*, lateral inhibition and hair cell determination.

2. Jag1 and Dl1 differentially regulate Notch targets. Jag1 preferentially induces *Hey1*, whereas Dl1 upregulates both *Hey1* and *Hes5*.

3. Different Notch ligands induce different levels of Notch activity, Jag1 signaling weaker than Dl1.

4. Different Notch levels discriminate between Notch targets. Low levels of Notch activity are sufficient to induce *Hey1*, but not *Hes5*, whereas high levels of Notch activity induce both *Hey1* and *Hes5*.

5. Jag1 and Dl1 compete for Notch signaling. The competition results in a decrease of overall signal driven by Dl1 in the presence of Jag1.

6. Differences in the Notch signaling strength driven by Jag1 and Dl1 and their competition are crucial for hair cell patterning. Jag1, while driving lateral induction on its own, facilitates lateral inhibition and pattern formation. This represents a novel function for Jag1 in inner ear development.

7. Autoactivation of *Atoh1* is a fundamental component of the robustness of hair cell patterning, which cannot be perturbed by manipulations of Jag1 levels.

8. The signature of the facilitatory function of Jag1 on hair cell patterning relies on its ability to bias but not determine supporting cell fate.

Differential regulation of Hes/Hey genes during inner ear development

1. The expression of *Hes/Hey* genes other than *Hey1* and *Hes5* such as *Hey2, Hes1, Hes2* and *Hes6* expression is not restricted in the otic epithelium.

2. *Hey1* and *Hes5* are both Notch dependent, but their regulation is also affected by other signaling pathways that include Bmp, Fgf and Wnt. These signals, in general, attenuate the activation by Notch.

3. Wnt signaling appears as a good candidate to regulate *Jag1* and thereby *Hey1* expression. Wnt and Notch pathways account for most of *Jag1* expression in the inner ear.

4. *Hey1* and *Hes5* have different mRNA stability and they cross-regulate each other in a rather complex manner. *Hes5* is repressed by Hey1 and Hes5, whereas *Hey1* is resistant to the inhibitory signals imposed by Hey1 or Hes5. It is unknown whether or not these interactions are direct.

5. Besides being a good readout of Jag1, Hey1 is not instrumental for lateral induction. Both Hey1 and Hes5 likely cooperate for lateral inhibition.

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ANNEX

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Review Article

Patterning and cell fate in the inner ear: a case for Notch in the chicken embryo

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The development of the inner ear provides a beautiful example of one basic problem in development, that is, to understand how different cell types are generated at specific times and domains throughout embryonic life. The functional unit of the inner ear consists of hair cells, supporting cells and neurons, all deriving from progenitor cells located in the neurosensory competent domain of the otic placode. Throughout development, the otic placode resolves into the complex inner ear labyrinth, which holds the auditory and vestibular sensory organs that are innervated in a highly specific manner. How does the early competent domain of the otic placode give rise to the diverse specialized cell types of the different sensory organs of the inner ear? We review here our current understanding on the role of Notch signaling in coupling patterning and cell fate determination during inner ear development, with a particular emphasis on contributions from the chicken embryo as a model organism. We discuss further the question of how these two processes rely on two modes of operation of the Notch signaling pathway named lateral induction and lateral inhibition.

Key words: delta, development, hearing, jagged, lateral induction, lateral inhibition.

Inner ear development in the chick

The inner ear is a complex three-dimensional structure that contains the auditory and vestibular sensory organs, which are the first step in the transduction of sound, balance and motion stimuli (Fig. 1A, sensory organs, see Box 1). In spite of regional differences, the functional unit of all sensory organs consists of three conserved elements: hair cells, sensory neurons and supporting cells (Purves *et al.* 2001) (Fig. 1B). Hair cells are specialized mechano-receptors that transduce the auditory and vestibular mechanical stimuli into electrical signals. Hair cells have specialized microvilli, stereocilia, that when displaced by sound or motion cause ion channel opening/closing and elicit changes in the membrane potential of hair cells (Purves *et al.* 2001). Hair cells are innervated by otic neurons, which are bipolar primary afferent neurons that are activated by neurotransmitter release in the synaptic contacts and transmit information to second order neurons in the vestibular and auditory nuclei in the brainstem (Rubel & Fritzsch 2002). Supporting cells vary greatly in morphology and functional specialization and their function goes beyond the mere mechanical scaffolding of the sensory epithelium (Kelley 2006). They maintain the correct ionic environment for the function of hair cells, they release factors that maintain the trophism and survival of the hair cells (Haddon *et al.* 1999) and, finally, they also serve as progenitors to regenerate hair cells after injury (Corwin & Cotanche 1988; Ryals & Rubel 1988), see also (Cotanche & Kaiser 2010) for review.

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Box 1

Glossary of ear development

Ear sensory organs are specialized epithelial domains containing hair cells and supporting cells in a highly specialized arrangement. Hair cells are innervated by the otic neurons. The number of sensory organs in the inner ear varies among animal species, but all have at least six differentiated sensory domains grouped into vestibular and auditory sensory organs. The former are located dorsally and they are subdivided into three *cristae* and two *maculae*. The later consists of a single domain that is located ventrally, the organ of Corti in mammals or the *basilar papilla* in birds. The *cristae* are located at the base of the semicircular canals, the *ampullae*, and detect angular accelerations. The *maculae* of the utricle and saccule detect linear accelerations in the horizontal and vertical axis, respectively, and the gravitational pull. In birds, amphibians,

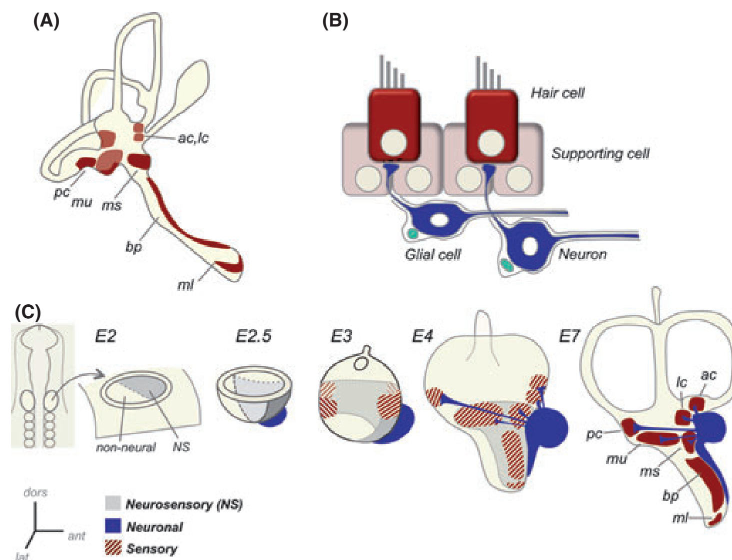

Fig. 1. The development of the inner ear in the chicken embryo. (A**)** Diagram of the post-natal inner ear. The sensory patches are indicated in red: ac, anterior crista; bp, basilar papilla; lc, lateral crista; ml, macula lagena; ms, macula sacularis; mu, macula utricularis; pc, posterior crista. (B) The functional unit of the inner ear consists of hair cells, supporting cells and neurons, all deriving from the neurosensory progenitors residing in the otic placode. Schwann cells that derive from the neural crest are also indicated. (C) Inner ear development in the chick. The day of incubation is indicated by E2–E7. The otic placode in E2 shows the first asymmetry between the Neurosensory (NS) and non-neural competent domains. At the otic cup stage (E2.5), neurogenesis starts with the delamination of neuroblasts from the anterior-medial domain. Between E3 and E3.5 the dorsal prosensory patches start to be defined and by E4 all prosensory patches can be identified by specific markers and the dorsal most start to differentiate. By E7 all sensory patches exhibit nascent hair cells and synaptic connections are established. Labels in C, like in A.

and fish there are other small *maculae* of uncertain function. In the auditory sensory epithelium, sound-wave frequency discrimination is based on the position of the hair cells along the longitudinal cochlear axis, which is correlated with the position of the sensory neurons in the cochlear ganglion. This tonotopical order is conserved in the central auditory nuclei, where sensory neurons project, reproducing in the brain the hair cell order of the cochlea. In addition to the sensory structures, the inner ear includes the endolymphatic duct (ED), which extends dorsally to communicate with the central nervous system (CNS) and is involved in the turnover of the endolymph.

Placodes, also named cranial placodes or ectodermal placodes, are transient embryonic structures that contribute to the paired sense organs and cranial sensory ganglia of the head. Placodes are discrete regions of thickened columnar epithelium that can give rise to a wide variety of cell types, including ciliated sensory receptors, sensory neurons, endocrine cells, glia, and other supporting cells (Ladher *et al.* 2010).

Neurosensory refers to neuronal and sensory cell phenotypes. The neurosensory domain in the otic placode and early otic vesicle is the one that gives rise to neurons and hair cells. It refers to the state of commitment of progenitors to neuronal and sensory fates.

Proneural genes were identified as genes involved in the early steps of neural development in *Drosophila* and, later on, found to play crucial roles in the development of the vertebrate nervous system. Proneural genes code for transcription factors that contain the basic Helix-Loop-Helix (bHLH) DNA binding domain and underlie the determination, differentiation and identity of neurons, sensory receptors and glial cells. (Bertrand *et al.* 2002; Gomez-Skarmeta *et al.* 2003). The vertebrate proneural genes discussed in this review are Neurogenin1 (Neurog1), NeuroD, NeuroM and Atoh1.

Prosensory patch/domains are restricted domains in the otocyst epithelium that are committed to develop into sensory fate, but not yet differentiated. They are defined by the expression of a characteristic set of genes that include Sox2, Jag1, LFng, BMP4, or Fgf10, which foreshadow the development of the sensory organs.

Sensory patches/domains are restricted domains in the epithelium of the otocyst that contain nascent hair cells and supporting cells. They are defined by the expression of hair cell differentiation genes. The earliest gene expressed in hair cells is the proneural gene Atoh1, followed by early differentiation genes like rare myosins MyoVI and MyoVIIa.

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Neurogenic is the property of generating neurons, and it is applied to genes or domains exhibiting such property. In the context of ear development it refers to the generation of otic (auditory and vestibular) neurons as different from the generation of hair cells. The major neurogenic genes in the ear are Neurog1, NeuroD and NeuroM, but other bHLH genes like Nhlh1 and 2 are likely important (Fritzsch *et al.* 2010).

From the otic placode to the otic vesicle

The complex structure of the inner ear derives from the otic placode that gives rise to both the sensory and non-sensory elements of the membranous labyrinth (Alsina *et al.* 2009; Ladher *et al.* 2010) (see Box 1). In the chick, the otic placode is visible as a bilateral thickening of the ectoderm adjacent to the developing hindbrain (Fig. 1C, E2). As development proceeds, the otic placode invaginates to form the *otic cup* that pinches off the ectoderm and closes to form the *otic vesicle*, an ellipsoid-shaped structure lined by a pseudo-stratified epithelium (Fig. 1C, E3). The otic vesicle undergoes an intense proliferative growth followed by differentiation that results in the otocyst and, later on, in the fully differentiated inner ear. Concomitantly, patterning and cell specification take place so that the different cell types and sensory organs develop in a precise temporal and spatial order (Fig. 1C, E4–E7). Note that during ear development the specification and differentiation of the sensory organs follow a temporal and spatial sequence by which dorsal organs develop first leading to the following sequence: cristae >maculae >basilar papilla.

The induction of the otic placode is a classic model of inductive processes in development, and the chicken embryo has greatly contributed to its understanding since the grafting experiments performed by (Waddington 1937). The current view is that the otic placode arises from a preplacodal territory, which is competent to develop into any placode but not yet specified to develop into any particular one. This has been recently substantiated by the detailed work of Andrea Streit on chicken embryos (Streit 2007). The specification of the otic identity involves at least two inductive steps: first, FGF signaling establishes an otic/epibranchial placodal domain, second, a wave of FGF and Wnt signaling refines the otic fate against epibranchial fate, by inducing otic-specific genes, see (Schimmang 2007; Ladher *et al.* 2010) for excellent reviews.

Otic patterning: The specification of the neurosensory competent domain in the otic placode

Axial patterning of the inner ear is an important step to provide the positional cues required for the development of the specific cell types in the correct places, which is crucial to the inner ear responsiveness to movements in three dimensions. 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 the otic territory (Groves & Fekete 2012).

In the chick, the specification of the AP axis precedes any other axial specification (Alsina *et al.* 2004; Bok *et al.* 2007), and establishes the first cell fate decision between neurosensory (see box 1) and non-neural domains of the otic placode (Adam *et al.* 1998; Neves *et al.* 2007). The former gives rise to otic neurons and hair cells, while the latter is the origin of the different non-sensory epithelia that line the walls of the inner ear. These two domains show limited cell intermingling (Abello *et al.* 2007). Molecular markers like Sox2 or Fgf10 allow following up of the development of the neurosensory domain as summarized in Figure 1C (Alsina *et al.* 2004; Neves *et al.* 2007).

Available evidence suggests that both neurons and hair cells derive from a common progenitor cell population (Fekete *et al.* 1998; Satoh & Fekete 2005; Raft *et al.* 2007; Bell *et al.* 2008). Commitment to neurosensory fate is dependent on the early expression of the high-mobility HMG factors Sox3 and Sox2. Sox3 is expressed only transiently until the end of neurogenesis and is sufficient to induce Sox2, which labels neurosensory progenitors throughout development (Neves *et al.* 2007; Abello *et al.* 2010). Recent tracing experiments in chick show that neurons and sensory cells derive from Sox2-positive progenitors in the otic placode (Neves *et al.* 2012), and evidence in mouse and chick indicate that Sox2 is both necessary and sufficient to drive sensory development (Kiernan *et al.* 2005b; Neves *et al.* 2011; Ahmed *et al.* 2012). Sox2 and Sox3 are also able to induce neuronal fate (Abello *et al.* 2010; Puligilla *et al.* 2010; Neves *et al.* 2011).

Development of otic neurons

As mentioned above, the neurosensory domain is specified in the anterior-medial part of the otic placode. At this stage neurogenesis starts with the specification of neuroblasts in the epithelium and their delamination to form the cochleo-vestibular ganglion

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(Hemond & Morest 1991; Adam *et al.* 1998; Alsina *et al.* 2004). Neuronal fate is specified by the expression of proneural genes like Neurogenin1, NeuroD and NeuroM, which drive neuronal delamination and differentiation (Henrique *et al.* 1995; Ma *et al.* 1998; Alsina *et al.* 2004). As it will be discussed in detail below, Notch-mediated lateral inhibition is instrumental for neuronal determination.

Development of sensory organs and hair cells

The prosensory patches/domains (see Box 1) emerge within the neurosensory domain, but are delayed with respect to the initiation of neurogenesis. The otocyst grows and undergoes profound morphogenetic changes that generate the vestibular and cochlear apparatus (Bissonnette & Fekete 1996). The prosensory patches are specified and, later on, the sensory organs differentiate and become innervated by the cochleo-vestibular neurons (Wu & Oh 1996; Adam *et al.* 1998; Rubel & Fritzsch 2002). Sox2 expression parallels sensory development from early specification until differentiation stages (Hume *et al.* 2007; Neves *et al.* 2007). In addition, other genes whose expression has been mapped to the prosensory domains have been recurrently used as prosensory markers. Those include: Bmp4 and the Bmp targets Smad1-5-7 and Id1-3 (Oh *et al.* 1996; Chang *et al.* 2008; Kamaid *et al.* 2010), the Notch signalling elements Jag1/Ser1 (Adam *et al.* 1998; Cole *et al.* 2000) and LFng (Morsli *et al.* 1998; Cole *et al.* 2000), Prox1 (Stone *et al.* 2003); BEN (Goodyear *et al.* 2001) and Fgf10 (Alsina *et al.* 2004; Chang *et al.* 2008). It is believed that they characterize a cellular state in which progenitors are committed to the sensory fate but are prevented from differentiation (Neves *et al.* 2012). Sensory differentiation is associated with Atoh1 (atonal homologue 1), a proneural gene that acts as a hair cell differentiation factor. It is expressed at the initiation of hair cell differentiation, its deletion causes hair cell loss and its overexpression is sufficient to induce hair cell fate (Bermingham *et al.* 1999; Zheng & Gao 2000; Woods *et al.* 2004). The regulation of Atoh1 is at the heart of hair cell differentiation and regeneration, and the factors that regulate its expression are beginning to be elucidated (Fritzsch *et al.* 2010; Mulvaney & Dabdoub 2012). Atoh1 expression is initiated by Sox2 in the otic placode, but it is silenced by the parallel activation of inhibitory factors. This incoherent response triggered by Sox2 results in the early specification of sensory competence but the delay of hair cell differentiation (Neves *et al.* 2012). As discussed in more detail below, Notch cooperates with Sox2 for sensory specification.

The Notch pathway

The Notch signaling pathway is a juxtacrine signaling system that regulates multiple processes throughout development. The core pathway consists of the interaction between a transmembrane Notch receptor anchored in one cell, with a transmembrane Notch

Box 2

Glossary of the notch pathway

Notch receptors: Mammals have four Notch receptors (Notch 1–4), but in birds, only Notch1 and Notch2 have been annotated. Notch is a large type-I transmembrane receptor that accumulates at the plasma membrane as a heterodimer, composed of the Notch Extracellular Domain (NECD) and a membrane bound intracellular domain. These two polypeptides are formed in the trans-Golgi as a result of proteolytic activity by a Furin protease that constitutively cleaves Notch molecules at the S1 site. Notch receptor contains a large extracellular domain with 36 tandem epidermal growth factor (EGF)-like repeats and three cysteine-rich Notch/LIN-12 repeats (Wharton *et al.* 1985; Yochem *et al.* 1988). The intracellular domain is composed of six tandem CDC10/ankyrin repeats (Breeden & Nasmyth 1987), one or two nuclear localization signals, a glutamine-rich domain (opa) and a PEST domain rich in proline, glutamate, serine and threonine (Stifani *et al.* 1992; Artavanis-Tsakonas *et al.* 1999).

Notch ligands: Notch receptors bind to type I transmembrane proteins known collectively as DSL proteins (Delta and Serrate for *Drosophila* and Lag2 for *Caenorhabditis elegans*). Mammals have five DSL ligands (*Jagged 1-2* homologous to *Serrate* and *Delta-like 1-3* homologous to *Delta*). In contrast, birds have *Jagged/Serrate1 and 2,* and *Delta-like-1 and 4*. In the extracellular domain they contain a DSL region and several EGF repeats, while the intracellular region is much smaller than in the Notch receptor and is poorly conserved among DSL family members (Fleming 1998). Ligand-receptor binding normally occurs among adjacent cells, but it can also occur in the same cell (Sprinzak *et al.* 2010); however, this seems not to be the case in the inner ear (Chrysostomou *et al.* 2012).

Intracellular signaling pathways: The binding of the ligand to the receptor occurs through the conserved DSL domain and one or more EGF-like repeats and results in a series of proteolytic cleavages. They require γ-secretase activity and lead to the release of the Notch intracellular domain (NICD) and its translocation to the nucleus. The NICD fragment is the active form of the receptor, acting in the nucleus as a transcriptional co-activator. NICD binds to the **CSL** transcription factor (mammalian **C**-promoter binding factor 1, CBF-1 or recombination signal sequence-binding protein-J kappa, **RBP-jkappa**; *Drosophila* **S**uppressor of Hairless and *C. elegans* **L**ag-1) and to the **Master mind** (**MAM** and *C. elegans*

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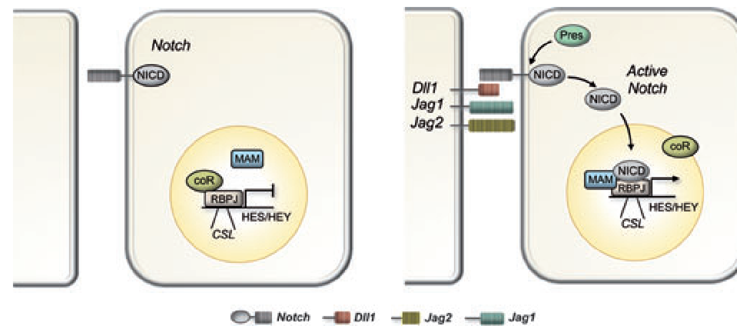

Fig. 2. The Notch pathway. The intracellular Notch pathway consists of the Notch receptor and the associated nuclear proteins that include RBPJ and Mastermind (MAM). In the absence of ligand binding (left) RBPJ is associated with co-repressors and bound to DNA CSL binding sites. The result is the repression of target genes like Hes and Hey HLH factors. Upon binding to ligands, Notch intracellular domain (NICD) is cleaved and translocated to the nucleus, where it recruits other factors like MAM and binds to CSL binding sites activating the transcription of target genes.

Lag-3) co-activator, forming a ternary complex. In the absence of NICD, the CSL transcription factor promotes the assembly of a repressor complex at the cis-regulatory regions of the CSL/NICD target genes (named Su(H) or S binding boxes), which are therefore transcriptionaly inactive. When NICD translocates to the nucleus and binds to CSL, it is able to recruit HAT (Histone Acetylase) and displace the co-repressor complexes, relieving repression. It is only when MAM binds to NICD/CSL, forming the ternary complex, that transcription is activated (Mumm & Kopan 2000). Therefore, in the absence of Notch activity, the Notch target genes are repressed by CSL. When Notch signaling is initiated, NICD makes the switch from CSL-mediated repression to NICD/CSL/MAM activation, triggering transcription of the Notch target genes (Bray 1998; Castro *et al.* 2005). In addition to this core CSL-dependent Notch pathway, in which the key signaling molecule is NICD and the ultimate output is transcription, there is also evidence for a CSL-independent Notch signaling (Martinez Arias *et al.* 2002). This CSL-independent Notch signaling seems to rely on a Deltex dependent activity and, in some cases, it relies on different ligands that do not belong to the DSL family, like Contactin and DNER (Eiraku *et al.* 2005).

Notch downstream target genes: Several genes change their expression depending on Notch activity (Krejci *et al.* 2009). However the best characterized direct targets of Notch are the **Hes** (**H**airy–**E**nhancer of **S**plit) and **Hey/Hrt** genes (**H**es **r**elated **t**ype, also known as Hesr, CHF, Herp, gridlock) (Iso *et al.* 2003). They are class C bHLH proteins that function as transcriptional repressors by binding to class C E-Box (CACGNG), N-box sequences (CACNAG) or class B E-Box sites, but not class A sites. The most striking difference between Hes and Hey factors is the lack of the WRPW tetrapeptide, which is replaced by a related YRPW peptide sequence in the members of the Hey family. This motif cannot bind TLE co-repressors, but nevertheless both factors are transcriptional repressors of tissue specific differentiation factors. They can function as homodimers and heterodimers between Hes and Hey proteins (Ohsako *et al.* 1994; Van Doren *et al.* 1994; Fisher & Caudy 1998; Iso *et al.* 2001, 2003).

Notch modulation: Interactions between Notch and either of its ligands can be differentially modulated by the **Fringe** family of glycosyltransferases (Lunatic Fringe, Manic Fringe and Radical Fringe) located in the Golgi apparatus. They glycosylate EGF repeats of Notch protein before its maturation and localization to the cell membrane (Bruckner *et al.* 2000; Moloney *et al.* 2000; Munro & Freeman 2000) Fringe proteins potentiate Notch signaling induced by Delta while inhibiting signaling induced by Serrate/Jagged1 (Bruckner *et al.* 2000; Hicks *et al.* 2000; Shimizu *et al.* 2001; Lei *et al.* 2003; Okajima *et al.* 2003; Yang *et al.* 2005). Notch ligands can inhibit signaling by co-expressed Notch in a cell-autonomous fashion, termed cis-inhibition (Glittenberg *et al.* 2006). Notch functions might be also modulated by the amount of the receptor or the ligand on the cell surface, by feedback loops that potentiate or shut off the signal, by post-transcriptional regulation mediated by microRNAs or by tissue specific co-factors. Such complex regulatory mechanisms imply that the expression of both ligands and receptors do not necessarily reflect the state of activation of the pathway (Schweisguth 2004; Bray 2006; D'souza *et al.* 2008; Borggrefe & Oswald 2009; Fortini 2009).

ligand (Delta or Serrate/Jagged) in a neighboring cell. Upon ligand–receptor binding a series of proteolytic cleavages are triggered that release the intracellular domain of Notch (NICD), allowing it to form a nuclear complex with the CSL and mastermind/MAML transcription factors. This complex then activates the transcription of target genes (Artavanis-Tsakonas *et al.* 1999; Bray 2006; Fior & Henrique 2008; Fortini 2009) (see Fig. 2 and Box 2).

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Notch signaling in inner ear development

Several elements of the Notch signaling 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 the late stages of otocyst development (Adam *et al.* 1998; Abello *et al.* 2007). In the mouse, Notch1 and Notch3 receptors are ubiquitously 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). Notch2 is neither expressed in the chick (Williams *et al.* 1995; Adam *et al.* 1998; Abello *et al.* 2007) nor in the mouse otic placode/vesicle (Williams *et al.* 1995).

In contrast to Notch receptors, the expression of the Notch ligands is restricted. Delta1 expression is first detected in scattered cells in the neurogenic domain of the otic placode, and maintained during neurogenesis. 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 (Adam *et al.* 1998). The pattern of expression of Delta1 is similar in mouse (Bettenhausen *et al.* 1995; Morrison *et al.* 1999; Vazquez-Echeverria *et al.* 2008). In both animal species, therefore, Delta1 labels nascent neuroblasts and hair cells, and becomes silent upon cell differentiation. In the mouse, in addition to Delta1, Jag2 and Delta3 are also expressed in nascent hair cells (Lanford *et al.* 1999; Shailam *et al.* 1999; Hartman *et al.* 2007). In zebrafish, DeltaA, B and D and SerrateB follow a similar pattern (Haddon *et al.* 1998; Riley *et al.* 1999).

Jag1 is first expressed in the chick otic placode by E2 and, in contrast to Delta1, it is expressed in compact domains rather than in a speckled pattern. Jag1 is initially expressed in the posterior-medial aspect of the otic placode, but it rapidly resolves into two anterior and posterior poles of expression. Then, Jag1 expression foreshadows the future sensory organs, where it remains expressed throughout development. Upon cell differentiation, hair cells downregulate Jag1 that is retained by the supporting cells. (Myat *et al.* 1996; Adam *et al.* 1998; Cole *et al.* 2000; Abello *et al.* 2007). Likewise, in mouse, Jag1 is expressed in the prosensory domains and becomes restricted to the supporting cell layer as hair cells differentiate (Lewis *et al.* 1998; Morrison *et al.* 1999). Although there is evidence for prosensory function of Notch in zebrafish (Millimaki *et al.* 2007), the corresponding ligand is yet unknown.

The Notch modulator Lunatic Fringe (LFng, see Box 2) is also dynamically expressed during otic development. In chick, LFng is first detected by E2 in the neurogenic region overlapping with Delta1. By otocyst stage LFng expression overlaps with Jag1 and Delta1 in the medial region, being stronger in the anterior ventral aspect of the otocyst. Similar to Jag1, LFng 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 expressed differentially during otic development. Hes5, Hes1/Hairy-1, Hes6, Hey1, Hey2 and HeyL expression patterns have been reported in either or both chick and mouse. The expression data in the chick is still scarce and mostly related to early stages of otic development (Abello *et al.* 2007; Daudet *et al.* 2007; Paxton *et al.* 2010). On the contrary, in the mouse, most studies on expression patterns refer solely to later stages and to cochlear development, both for Hes (Lanford *et al.* 1999; Shailam *et al.* 1999; Zheng *et al.* 2000; Zine *et al.* 2001; Qian *et al.* 2006; Jayasena *et al.* 2008; Doetzlhofer *et al.* 2009; Murata *et al.* 2009), or Hey factors (Hayashi *et al.* 2008; Li *et al.* 2008; Doetzlhofer *et al.* 2009). These studies show that in the mouse, Hes and Hey genes are differentially expressed in the various types of supporting cells of the developing cochlea. In zebrafish, the gene family is more complex and there is not yet an exhaustive description (Fischer & Gessler 2007). Hes orthologues are expressed in the otic placode (Takke *et al.* 1999; Radosevic *et al.* 2011), but there is still little information about their pattern throughout development.

The modes of operation of Notch

The complex expression pattern of receptors, ligands and modulators of the Notch signaling pathway anticipates the diverse roles that Notch plays during inner ear development. Notch is required for the induction and patterning of the otic placode (Abello & Alsina 2007; Abello *et al.* 2007; Jayasena *et al.* 2008 see Box 3). Thereafter, during neurosensory development, the Notch pathway exerts two apparently contrasting functions, which are uncovered by the opposite effects of Notch blockade in hair cell production (Fig. 3A). The blockade of Notch at late developmental stages or the loss of function of Delta1/Jag2 induce supernumerary hair cells (Haddon *et al.* 1998; Lanford *et al.* 1999; Riley *et al.* 1999; Kiernan *et al.*

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2005a; Brooker *et al.* 2006; Abello *et al.* 2007; Takebayashi *et al.* 2007; Hayashi *et al.* 2008). This is the typical "neurogenic" phenotype expected for the loss of function of Notch if working by lateral inhibition. In this case, the inhibition of Notch releases the repression on differentiation genes and allows massive cell differentiation. However, the early inhibition of Notch or the loss of function of Jag1 results in the downregulation of prosensory genes and the consequent deficit in hair cell production (Tsai *et al.* 2001; Brooker *et al.* 2006; Kiernan *et al.* 2006; Daudet *et al.* 2007; Hayashi *et al.* 2008). This suggests that besides its role in lateral inhibition, Notch is required for sensory specification through a different mechanism, which was suggested to be lateral induction (Eddison *et al.* 2000).

The classical view of Notch-mediated lateral inhibition is based on the pioneering studies of neurogenesis in *Drosophila melanogaster* (Artavanis-Tsakonas *et al.* 1999). The *Drosophila* phenotype is the overproduction of neurons after the loss of function of either Notch or its ligands, the reason why they were called neurogenic genes. In lateral inhibition, a ligand-producing cell successfully signals its neighbour to reduce ligand expression. Thus, Notch propagation is alternate and cells of an initially equipotent field either activate or silence Notch. The result is a binary cell fate decision by which adjacent signaling cells are driven to differ from one other. It is associated with salt-and-pepper patterns of gene expression (Bray 1998; Lewis 1998; Fior & Henrique 2008). Lateral inhibition operates during vertebrate neurogenesis and in the generation and the regeneration of hair cells in the inner ear sensory organs (Henrique *et al.* 1995; Adam *et al.* 1998; Lewis 1998; Lanford *et al.* 1999, see Box 3). The general model states that neurogenesis is initiated within a population of equipotent neural-competent progenitors. These cells express proneural proteins and DSL ligands (*Dll* or *Jag*), but stochastic variations of ligand levels within the cells lead some cells to express at higher levels and thus activate Notch in the neighboring cells more efficiently. Thereby, signal receiving cells express high levels of Hes/Hey genes that repress the expression of proneural genes and, consequently, the expression of Notch ligands. The final effect is that a subset of cells ends up expressing high levels of proneural genes and Notch ligands leading them to enter the differentiation pathway. In turn, they activate Notch in the neighboring cells forcing them to retain the progenitor state. Thus, the mechanism of lateral inhibition amplifies stochastic variations between neighboring cells and creates mosaic patterns of gene expression that ultimately result in the adoption of two different fates (Artavanis-Tsakonas *et al.* 1999; Schweisguth 2004; Bray 2006; Kageyama *et al.* 2008) (Fig. 3B, right).

In contrast, lateral induction was first described as a positive-feedback loop in which a ligand-expressing cell stimulates those nearby to turn up ligand expression and Notch activation, thereby promoting coherent signal activation and coordinated cell behaviour (Bray 1998) (Fig. 3B, left). Lateral induction is seen in flies in Notch-mediated induction of proneural domains in the eye (Baker & Yu 1997; Li & Baker 2001) and at the wing margin (De Celis & Bray 1997; Bray 1998). There are also several examples of lateral induction in vertebrates, including induction of proneural domains in the ear (Eddison *et al.* 2000) and in the eye (Onuma *et al.* 2002), formation of the limb bud margin (Irvine & Vogt 1997), somite boundaries (Oates *et al.* 2012), lens progenitor cell proliferation and differentiation (Le *et al.* 2009), and the establishment of the neural crest domain within the ectoderm (Cornell & Eisen 2005). Note that while in lateral inhibition Notch activation in one cell inhibits the expression of the Notch-ligand in the same cell; in lateral induction it does the opposite. We shall discuss in the next sections the different functions of the Notch signaling pathway in inner ear development highlighting the contributions of the research in the chicken embryo to shed light on the mechanisms involved in patterning and cell fate determination.

The prosensory function of Notch: Lateral induction works for patterning

The observation that the expression of Jag1 foreshadows the emergence of the sensory organs suggested the association of Notch function with prosensory specification (Fig. 4A). Loss of function studies in mice indicated a role for Jag1/Notch signaling in early ear development (Kiernan *et al.* 2001; Tsai *et al.* 2001). Moreover, contrary to Dll1 and Jag2, Jag1 expression pattern in the prosensory patches was noted to be uniform and not salt-and-pepper (Adam *et al.* 1998; Lewis *et al.* 1998; Morrison *et al.* 1999; Cole *et al.* 2000), which lead to the suggestion that Jag1 expression in the prosensory patches may be regulated by lateral induction (Eddison *et al.* 2000; Daudet & Lewis 2005; Daudet *et al.* 2007). These initial observations opened two main questions on the role of Notch and Jag1 in inner ear development: (i) how is Jag1 expression regulated by Notch? and (ii) what is the mechanism behind the prosensory function of Notch in the ear? It took over a decade to elucidate the mechanisms behind the prosensory function of Notch and the regulation of Jag1 in the ear. The chicken embryo has been crucial to shed light on these problems by

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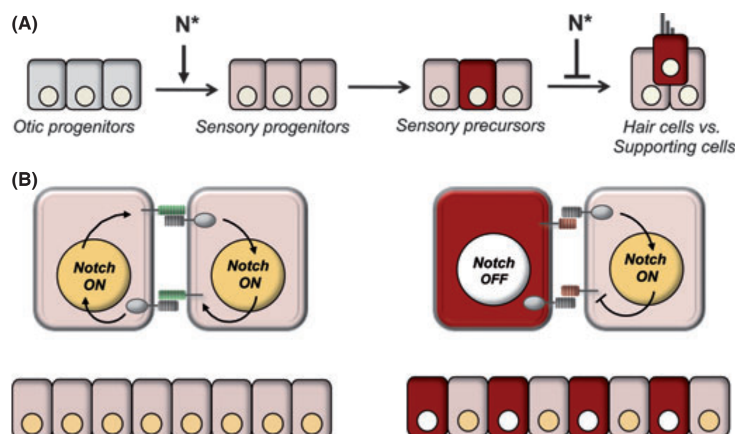

Fig. 3. The dual effects of Notch in hair cell development. (A) Notch is required for sensory specification but prevents hair cell differentiation. The inhibition of Notch or the loss of function of Jag1 prevents sensory specification and the development of hair cells. However, the late inhibition of Notch, the impairment of the function of Delta1 or the loss of function of some Notch downstream targets cause premature differentiation and excess hair cells. (B) The two modes of operation of Notch during ear development. Lateral induction (left) is characterized by a positive feed-back loop between Notch and the Notch ligand Jag1. All cells in the prosensory patch express both Jag1 and show Notch activity. They adopt the same fate and maintain Sox2 expression, which in turn, confers sensory competence to the prosensory progenitors. Lateral inhibition (right) is described as a negative feed-back loop by which Delta1 induces Notch activity in the neighboring cell, and this causes the suppression of the expression of Delta1. The result is that the ligand delivering cell shuts down Notch activity and becomes fated to differentiate, while the surrounding cells repress Delta1 expression, maintain high levels of Notch activity and adopt the supporting cell fate.

providing a model for temporal and spatial control of transgenesis and *in vitro* explants.

Jag1 is regulated by lateral induction

The notion that Jag1 is regulated by lateral induction implies that Jag1 expression is positively regulated by Notch. The first test to this hypothesis consisted of silencing Notch signaling in the otic vesicle of the chicken embryo through in ovo electroporation of a RCAS (replication competent ALV LTR with a splice acceptor) construct coding for dominant negative forms of Delta1 or Su(H). This resulted in the loss or strong reduction of Jag1 expression in the transfected prosensory regions and provided the first evidence that Jag1 expression was indeed positively regulated by Notch activity (Eddison *et al.* 2000). The requirement of Notch signaling to sustain Jag1 expression in the prosensory domains was confirmed later by experiments on isolated chicken otocysts cultured in the presence of DAPT, a γ-secretase inhibitor that prevents Notch cleavage (Daudet *et al.* 2007). Notch blockade resulted in the loss of Jag1 expression in the prosensory domains (Daudet *et al.* 2007). Previously, gain of function studies in the chick had shown that forced activation of Notch outside the prosensory domains, through in ovo electroporation of the Notch1 intracellular domain (N1ICD) in the otic vesicle, was sufficient to induce ectopic Jag1 expression (Daudet & Lewis 2005). This has been confirmed recently in the mouse (Hartman *et al.* 2010; Pan *et al.* 2010). Moreover, recent data on an inner ear conditional ablation of RBPj (see Box 2) in mice, where Notch signaling is shut down at early developmental stages, also shows the loss of Jag1 and other prosensory markers (Basch *et al.* 2011; Yamamoto *et al.* 2011). The cochlear phenotype of the RBPj mutant mice shows some resistence to Notch deletion, an unexpected feature that has suggested alternative mechanisms for prosensory induction in the mouse cochlea (Basch *et al.* 2011). But nevertheless, taken together the available data supports the idea that Notch signaling positively regulates Jag1 expression in the inner ear.

In lateral induction a positive feed-back loop is established by which Notch activation in one cell induces the expression of the Notch-activating ligand in the same cell (Bray 1998). As a result, all cells would cooperatively activate Notch and express the signaling ligand uniformly. One prediction from such a mode of action is the propagation of the signal in a cluster of cells. Recent data have indeed demonstrated that such a type of mechanism operates in the inner ear. Notch activation in the mouse inner ear not only induces Jag1 expression cell autonomously but also non-autonomously, propagating the signal to adjacent cells, up to three cell diameters (Hartman *et al.* 2010).

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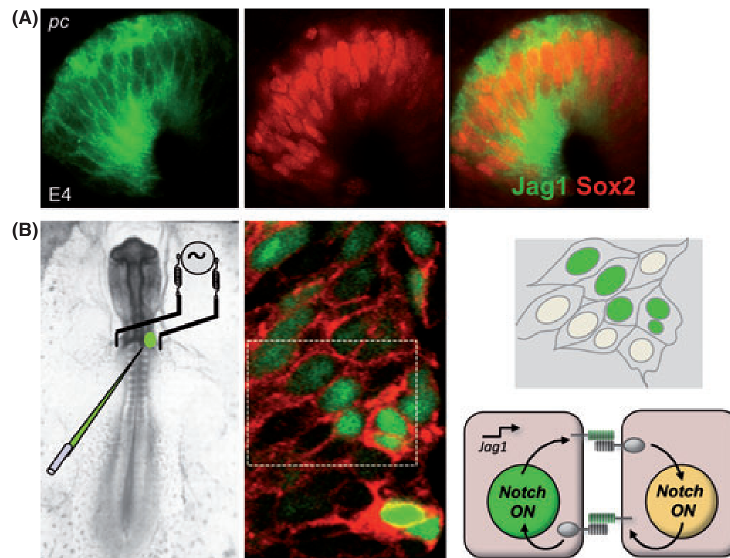

Fig. 4. Jag1 and lateral induction in the prosensory patches of the developing inner ear. (A) Jag1 is expressed uniformly in the prosensory patches. The microphotographs illustrate the expression of Jag1 and Sox2 detected by immunohistochemistry. Jag1 is expressed in the cell membranes of the same cells that express Sox2 in the nucleus. (B) Jag1 induces Jag1 in the neurosensory domains. The electroporation of hJag1 in the neurosensory domain of the otic placode (left) induce the expression of Jag1 (red) in both electroporated (green) and non-electroporated cells. hJag1 was co-electroporated with a green fluorescent protein (GFP) vector and Jag1 detected by immunohistochemistry. The diagram on the right illustrates an idealized view of the effects of the electroporation (for experimental details see Neves *et al.* 2011).

In the chick, gain of function studies showed that ectopic expression of human (hJag1) transgene in the chicken otic vesicle, outside the prosensory domains, was sufficient to induce Notch target genes and *Jag1* expression in a non-cell-autonomous manner (Fig. 4B). This strongly supports the notion that *Jag1* operates by a mechanism of lateral induction that relies on a positive-feedback loop (Neves *et al.* 2011).

The prosensory function of Notch depends on Jag1

Experiments in chick and mouse support the prosensory function of Jag1/Notch signaling during inner ear development. Several mutant mice – slalom, coloboma and headturner (Kiernan *et al.* 2001, 2006; Tsai *et al.* 2001; Brooker *et al.* 2006) – and, more recently, Pax2 and Foxg1 conditional null alleles for Jag1 in the ear have been used to analyze the effects of the loss of function of Jag1 in the inner ear (Brooker *et al.* 2006; Kiernan *et al.* 2006). Although with some phenotypic differences, Jag1 mutation/deletion leads to defects in the development of inner ear sensory epithelium. Phenotypes include truncated or missing sensory organs and loss of hair cells. The reduced number of hair cells is due neither to defects in differentiation, nor to a shift into the neuronal phenotype or cell degeneration, but to the loss of cell specification (Brooker *et al.* 2006; Kiernan *et al.* 2006; Pan *et al.* 2010). Moreover, the sustained blockade of Notch signaling with DAPT impairs hair cell production in chick otocysts cultured *in vitro* (Daudet *et al.* 2007). In contrast, the forced expression of NICD is able to trigger ectopic hair cell formation (Daudet & Lewis 2005; Hartman *et al.* 2010; Pan *et al.* 2010; Liu *et al.* 2012). However, recent data show that in the chick, ectopic *Jag1* expression cannot trigger ectopic hair cell formation *de novo*, but only within the neurosensory domain, suggesting that Jag1 acts on a pre-existing sensory competence (Neves *et al.* 2011).

The prosensory function of Jag1/Notch is mediated by Sox2

There are several genes other than Jag1 that map to the prosensory domains. They foreshadow the development of the inner ear sensory organs and include Bmp4, LFng and Sox2 (Cole *et al.* 2000; Neves *et al.* 2007). Among those, only Sox2 is required for prosensory specification, its loss of function resulting in the loss of sensory organs (Kiernan *et al.* 2005b). The question arises as to whether the prosensory function

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of Jag1/Notch is mediated by Sox2. Three independent studies have shown that ectopic expression of Jag1 in the chick otic vesicle or NICD in the mouse otocysts leads to the expansion of Sox2 expression (Hartman *et al.* 2010; Pan *et al.* 2010; Neves *et al.* 2011). Experiments in chick suggested that Jag1-mediated Notch activity maintains Sox2 expression rather than inducing it *de novo*. During normal development Sox2 expression is initially broad and contains the Jag1 patches. However, as development proceeds, Sox2 expression domains become restricted to Jag1-positive patches and therein it accompanies the prosensory domains throughout development (Fig. 4A). Jag1 is able to maintain Sox2 expression in domains located in between the patches from where it is usually switched off (Fig. 5, top left), but it is unable to induce Sox2 expression *de novo* outside the neurosensory domain (Fig. 5, middle left). Consistently, Jag1 overexpression later in development is unable to expand the expression of Sox2 (Fig. 5, bottom left). On the contrary Sox2 showed a widespread capability of inducing hair cell fate throughout the otic epithelium (Fig. 5, right), a function that has been recently associated with its ability to directly activate Atoh1 transcription (Neves *et al.* 2011, 2012; Ahmed *et al.* 2012).

Based on these observations, the suggestion is that the prosensory function of Jag1/Notch relies on its ability to maintain Sox2 expression to the prosensory domains, thus defining the regions of the otic epithelium that retain sensory potential. This provides a simple model for coupling patterning and cell fate specification: Jag1 expression specifies patches of Notch activation that maintain Sox2 expression, which, in turn, drives sensory competence. In addition, Notch induces Hes and Hey factors that are repressors of proneural gens, and Sox2 also prevents hair cell differentiation through a feed-forward incoherent loop that promotes the activation of Atoh1 inhibitors (Dabdoub *et al.* 2008; Neves *et al.* 2012). Both pathways cooperate to maintain self-renewal and the expansion of the sensory precursors before differentiation, thereby timing the birth of hair cells.

Lateral inhibition in neurogenesis and hair cell determination

In chick, mammals and zebrafish, Delta1 expression foreshadows the differentiation of otic neurons and hair cells, and Jag2 that of hair cells (Adam *et al.* 1998; Haddon *et al.* 1998; Lanford *et al.* 1999; Daudet & Lewis 2005; Brooker *et al.* 2006; Abello *et al.* 2007; Daudet *et al.* 2007). The role of Delta1 in the inner ear was first discovered by studies on the Mindbomb (Mib) mutant of zebrafish (Jiang *et al.* 1996). Mib is an ubiquitin E3 ligase required for Delta-mediated Notch activation (Itoh *et al.* 2003; Koo *et al.* 2005; Zhang *et al.* 2007). Accordingly, the Mib mutation exhibits an increased expression of *Delta1* and a disruption of the salt-and-pepper expression pattern. 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 (see Box 2) result also in neuron and 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 *within* the sensory patches prevents hair cell differentiation (Daudet & Lewis 2005) (note that this is in contrast with the ability of Notch to expand the prosensory domain when electroporated *outside* the prosensory patches, see above). Conditional deletion of Delta1 in mice induces increased CVG ganglion and macular defects, suggesting that the loss of Delta1 disrupts lateral inhibition and causes an excessive number of neurons and the exhaustion of the pool of sensory precursors (Brooker *et al.* 2006). Besides, the loss of function of Delta1 and Jag2 produce supernumerary hair cells. The effects of Jag2 predominate in the inner hair cell layer, while those of Dll1 are in the outer hair cell layers (Lanford *et al.* 1999; Kiernan *et al.* 2005a; Brooker *et al.* 2006). Dll3 mutant mice show no abnormalities in hair cell formation in the cochlea, which may be due to the redundancy between Notch ligands and not necessarily mean that it does not play a role in lateral inhibition (Hartman *et al.* 2007). Taken together, the evidence strongly suggests that the generation of neurons and the mosaic pattern of hair/supporting cells of sensory organs depend on Notch mediated lateral inhibition.

Box 3

Other functions of Notch in early inner ear development and regeneration

Notch and inner ear induction: Notch signaling is involved in the induction of the otic placode. Notch regulates the size of the otic placode, and the inactivation of Notch1 reduces the size of the otic placode. It also regulates 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 signaling does not regulate the onset of its own expression and activation in the otic placode, once that happens, it is able to enhance Wnt activity, which in turn maintains Notch activity (Jayasena *et al.* 2008). Thus, there is a positive loop between Wnt and Notch that cooperates to orchestrate otic placode specification.

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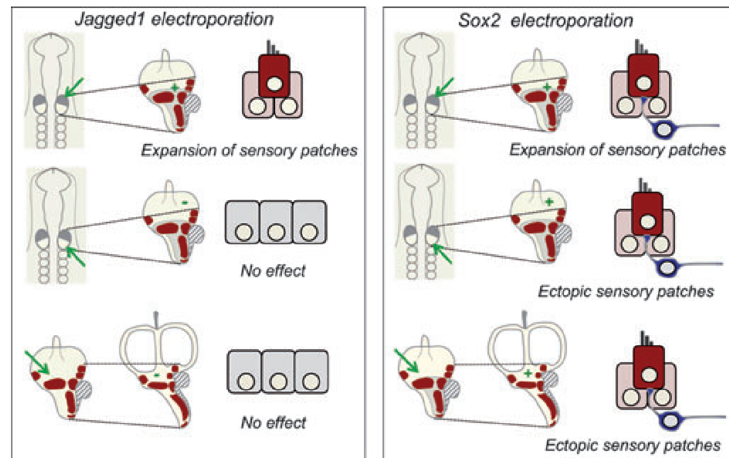

Fig. 5. The prosensory function of Jag1 depends on Sox2. The diagrams illustrate the effects of the electroporation of Jag1 (left) and Sox2 (right) on the generation of hair cells and neurons. Embryos were electroporated in E2.5 (upper two rows) or in E3.5 (lower row), and examined after two days for neuronal and hair cell markers (for details of experiments see Neves *et al.* 2011). The gain of function of both Jag1 and Sox2 in the neurosensory domain (upper rows) results in the expansion of the prosensory patches and a gain in neuronal and hair cell production. However, when electroporation is carried out in non-neurosensory domains, only Sox2 is able to generate ectopic neurons and hair cells. Similarly, when electroporation is done later in development, once the prosensory patches are defined, only Sox2 is able to induce ectopic neurons and hair cells.

Notch and early patterning of the otic placode: Notch signaling is required also for the early patterning of the otic placode that results in the specification of neurosensory and non-neural domains. Notch blockade results in the expansion of non-neural genes such as *Lmx1b* and *Irox1* into the anterior aspect of the otic placode, where they are not normally expressed. This expansion is not due to cell migration, but to the lack of repression of these genes (Abello *et al.* 2007). However, Notch blockade does not abolish AP patterning and the neurosensory domain remains restricted suggesting that other upstream mechanisms establish this domain (Bok *et al.* 2011; Radosevic *et al.* 2011).

Notch and hair cell regeneration: The avian cochlea has the ability to regenerate cochlear hair cells throughout their lifetime (Corwin & Cotanche 1988; Ryals & Rubel 1988; Stone & Cotanche 2007). During hair cell regeneration Notch drives again lateral inhibition and the selection between hair cell and supporting cell fates (Lanford *et al.* 1999; Stone & Rubel 1999; Daudet *et al.* 2009). As a consequence, the blockade of Notch signaling during regeneration results in the overproduction of hair cells at the expense of supporting cells (Daudet *et al.* 2009; Lewis *et al.* 2012). Notch1 and Jagged1 are expressed in the supporting cells of the adult basilar papilla (Stone & Rubel 1999; Daudet *et al.* 2009). During regeneration Atoh1 is rapidly induced in the supporting cells (hours) (Cafaro *et al.* 2007; Lewis *et al.* 2012), and followed by Delta1 (days). Thereafter, hair cell differentiation markers are expressed and both Atoh1 and Delta1 downregulated (Stone & Rubel 1999; Chapman *et al.* 2009).

The dual function of Notch in the ear: open questions

From the discussion above, it follows that Notch operates in two contrasting modes, in which the same intracellular machinery has to account for uniform versus, salt-and pepper expression patterns, and activation versus repression of the ligand (Fig. 6). This opens several intriguing questions on how a signaling system sharing a common cellular context can result in these two different functions. In the following, we shall speculate briefly on the possible mechanisms that allow the Notch pathway to decide between lateral inhibition and lateral induction regimes.

Does lateral induction or inhibition depend on the nature of the ligand? The case of inner ear development suggests that this is likely, because lateral induction is associated with Jag1 and lateral inhibition with Delta1. However, this is not always the case. For instance, Jag1 selects V1 neuroblasts in the neural tube by lateral inhibition (Ramos *et al.* 2010), while it is associated with lateral induction in the ear and the lens (Le *et al.* 2009; Neves *et al.* 2011). On the other hand, Delta1 generates coherent patterns of expression in the presomitic mesoderm that keeps the oscillations of the presomitic mesoderm locally synchronized by lateral induction (Jiang *et al.* 2000), but it also regulates neurogenesis in the CNS and PNS, and the generation of hair cells in the inner ear through lateral inhibition (Henrique *et al.* 1995;

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Adam *et al.* 1998). The different cellular output of the ligands may also reside in their different response to Notch, Delta being inhibited and Jagged activated. Hes and Hey genes are the canonical Notch targets and they typically act as repressors of proneural genes, which are responsible for Delta expression (Ohsako *et al.* 1994; Van Doren *et al.* 1994; Fisher & Caudy 1998; Iso *et al.* 2001, 2003). Indeed, as mentioned above Delta1 is overexpressed after Notch blockade (Abello *et al.* 2007; Daudet *et al.* 2007), whereas Jag1 behaves the opposite and decreases after Notch inhibition (Daudet *et al.* 2007), suggesting that at least in part, it is positively regulated by Notch. Matsuda and colleagues have addressed the question of the theoretical requirements for propagation of Notch signaling. Based on synthetic cell culture models, the authors suggest that the minimal network topology that is required for lateral induction requires mutual activation between adjacent cells and also signal amplification (Matsuda *et al.* 2012). The understanding of ligand regulation is critical for modeling the modes of Notch action.

Following the above discussion, one can ask what is the context leading to the different behavior of Jagged or Delta ligands resulting in different outcomes. One possibility is that the presence of the Notch modulators, like Fringe (see Box 2), makes these two ligands behave differently. Fringe proteins potentiate Notch signaling induced by Delta while inhibiting signaling induced by Jagged. Binding studies with *Drosophila* and mammalian cells have reported that Fringe alters binding of Delta or Serrate/Jagged to Notch such that Jagged signaling is inhibited in the presence of Delta (Bruckner *et al.* 2000; Shimizu *et al.* 2001; Lei *et al.* 2003; Okajima *et al.* 2003). However, in other contexts Fringe glycosylation does not prevent Jag1 binding to Notch. Instead, it interferes with the efficiency of proteolysis triggered by the binding of Jag1, effectively acting as an inhibitor of Jag1-induced Notch activation (Hicks *et al.* 2000; Yang *et al.* 2005; Benedito *et al.* 2009; Golson *et al.* 2009). It is possible that the presence of Lfng in the prosensory domains hampers Jag1 signaling, which in turn may result in low levels of active Notch. On the contrary, during hair cell production, hair cell precursors express Delta1 whose binding to glycosylated Notch1 in the neighbouring cells leads to strong Notch activation. Interestingly, it has been reported that Lfng loss of function can rescue the effects of Jag2 mutation in cochlear hair cell development (Zhang *et al.* 2000). One could speculate that in the absence of Lfng Jag1 would behave as a lateral inhibition ligand and thus compensate for Jag2 loss.

Nevertheless, besides the fact that the ligands show selectivity, the problem still remains as to how the activation of the Notch receptor results in different cellular outputs. Are the direct Notch down-stream targets such as Hes and Hey genes differentially regulated by Notch, and if so, what is the mechanism that selects the target? In some cases Hes and Hey genes are expressed simultaneously as for instance during somite formation (Leimeister *et al.* 2000). However, Hes and Hey differential expression during inner ear development (Hayashi *et al.* 2008; Tateya *et al.* 2011) suggest that the Notch may result in differential effector activation. How can active Notch result in the differential activation of Hes and Hey genes? The ability of Notch to activate a given promoter depends on structural properties like the arrangement and spacing of CSL binding sites or the distance from

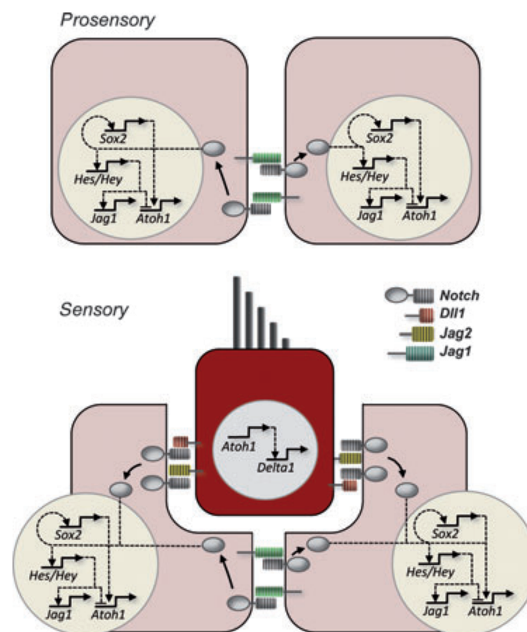

Fig. 6. Model of Notch function in sensory development. During prosensory stages (upper diagram) the activation of Notch results in the transcription of Jag1 and maintains the repression of Atoh1. Jag1-induced Notch activity maintains also the expression of Sox2, which through an incoherent loop directly activates Atoh1, but inhibits its transcription via the activation of Hes and Hey genes. This is how sensory commitment is retained by prosensory patches. The ligand Delta1 is not expressed until the repression on Atoh1 is released. It is yet unknown how Notch promotes Jag1 expression or whether Hes/Hey genes are instrumental for the regulation of Jag1. Later in development, hair cells are determined by the expression of Delta1 that is set by Atoh1. Delta1 and Jagged2 are expressed in nascent hair cells and activate Notch in the neighboring cells that adopt the supporting cell fate. Supporting cells maintain Jag1 expression and the activation of Notch by lateral induction. They also retain Sox2 expression, which is probably the basis for their ability to regenerate hair cells after injury.

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the transcriptional start site that influence the selectivity and amplitude of the response. The specific organization of the promoter regions of the target genes dictate the cooperative assembly of Notch transcriptional complexes that, in turn, result in differential outputs (Arnett *et al.* 2010). Structural requirements underlie also the fact that Notch levels are instrumental to select the quantitative differences in the activation of Hes1 and Hes5, like in the embryonic kidney (Ong *et al.* 2006). One interesting possibility that remains to be explored is that Jag1 and Delta1 expressed throughout ear development induce different levels of active Notch, which result in different gene outputs and cellular behaviors.

Finally, Notch activation results in the regulation of a variety of genes, sometimes with opposing functions. The genome-wide response to Notch analysis shows that there are several examples of pathways regulated by incoherent network logics, in which Notch activates both a gene and its repressors (Krejci *et al.* 2009). Moreover, there is an extensive cross-talk at the transcriptional level with other signaling pathways including RTK signaling. Nothing is known about Notch targets in the ear, but the above suggests that the final result of Notch activation may well vary with the state of activation of the cells even within the same general context.

In summary, we have reviewed here some aspects of Notch signaling in relation to the specification of the neurosensory territory and the development of neurons and hair cells in the inner ear. This provides an interesting example of how the same players, in this case the Notch signaling pathway, reiterate in development by performing multiple functions. Moreover, these functions rely on a core signaling pathway that is able to diversify its modes of operation. Which are the mechanisms underlying lateral inhibition versus lateral induction and those that govern their transitions are very intriguing questions still far from being resolved.

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