The regulation of Atonal 1 and the origin of hair cells in the inner ear

Héctor Gálvez García

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DIRECTORS DE LA TESI

Prof. Fernando Giráldez Orgaz

Dr. Gina Abelló i Sumpsi

DEPARTMENT OF EXPERIMENTAL AND HEATLH SCIENCES

Universitat Pompeu Fabra Barcelona

In the middle of difficulty lies opportunity – *Albert Einstein*

I strongly hold that, if an idea fits with common sense, then scientifically it is almost certain to be false – *Lewis Wolpert*

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Abstract

The vertebrate inner ear is the organ responsible for the senses of balance, acceleration and audition. Three cell types form the functional unit of the ear: hair cells, neurons and supporting cells. Atoh1 and Neurog1, two bHLH genes, drive differentiation of hair cells and neurons, respectively. Hair cell and neuronal competence is established early in development. However, hair cell differentiation is delayed with respect to neurons. In this work, I provide evidence for a molecular mechanism that underlies the repression of Atoh1 by Neurog1. Neurog1 is able to reduce the levels of Atoh1 protein and as a consequence, Atoh1 cannot activate its own expression. This provides a novel mechanism for bHLH interactions by which neurons are forced to develop before hair cells during embryonic development.

Resumen

El oído interno de los vertebrados es el órgano responsable de los sentidos del equilibrio, la aceleración y la audición. La unidad funcional del oído está formada por tres tipos celulares: células ciliadas, neuronas y células de soporte. Atoh1 y Neurog1, son genes bHLH que dirigen la diferenciación de las células ciliadas y las neuronas, respectivamente. La competencia para desarrollar células ciliadas y neuronas se establece tempranamente en el desarrollo, sin embargo, la diferenciación de las células ciliadas se retrasa con respecto a la de las neuronas. Este trabajo analiza los mecanismos moleculares que subyacen a la represión de Atoh1 por Neurog1. Los resultados muestran que Neurog1 es capaz de reducir los niveles de proteína de Atoh1 y, en consecuencia, evita que éste active su propia expresión. Se trata de un nuevo mecanismo de interacción entre factores bHLH por el que fuerza que las neuronas se desarrollen antes que el de las células ciliadas.

Preface

In this work, I have addressed the problem of understanding the formation of inner ear hair cells during embryonic development. Hair cells are specialized mechanoreceptors that transduce sound waves into electrical signals, their correct function being crucial for hearing, balance and acceleration. In amniotes, hair cells originate from a common cell population that gives raise to three cell types: neurons, supporting cells and hair cells. Atoh1 and Neurog1, two basic helix-loop-helix genes, are responsible for hair cells and neuron differentiation, respectively.

The problem of how these cells become specified and diversify throughout development is one main focus of the work. The results show that the crosstalk between proneural transcription factors, Atoh1 and Neurog1, is crucial for time and fate specification in the inner ear. I provide evidence for an unusual, non-transcriptional interaction between pro-neuronal and pro-sensory transcription factors that result in neurogenesis prevailing over sensory differentiation, thereby regulating the timing of neuron and hair cell differentiation.

In the Introduction that follows I will review briefly the structure and development of the inner ear. Then, I shall discuss in detail the role of basic helix-loop-helix factors in inner ear development, cell fate specification and sensory regeneration.

The work has been sponsored by La Marató de Catalunya and has been widely discussed in different congresses around the world. I have presented preliminary results in the following scientific meetings: Inner Ear Biology 2014, Notch Meeting 2014, Spanish Society for Developmental Biology 2014, 2015 and 2016, Societat Catalana de Biología del Desenvolupament 2015 and the 75th SDB-ISD meeting 2016.

The present work was performed by me, with the collaboration of my supervisors Gina Abelló and Fernando Giráldez. In the second year of my PhD, I performed a research stay in the laboratory of Prof. Marcelo Rivolta, at University of Sheffield thanks to the travelling fellowship granted by the Company of Biologists. During the PhD I had the opportunity to collaborate in two publications of related projects, one along with a former PhD student Jelena Petrovic, and a second one in collaboration with the group of Thomas Schimmang (IBGM-CSIC, Valladolid). The two published papers are attached in Appendix 1-2.

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INTRODUCTION

1. The inner ear

The ear is one major sensory organ of the vertebrate head that is responsible for the senses of hearing, balance and acceleration. Anatomically, it is classified in three different compartments: the outer, middle and inner ear. The **outer ear** develops from the first pharyngeal cleft and helps to collect sound waves onto the eardrum (tympanum). The **middle ear** derives from the first pharyngeal endoderm and in mammals, is composed of a cavity that contains three ossicles, the malleus, incus and stapes (a single ossicle named the columnella in birds). The function of the middle ear is to couple sound waves hitting the eardrum onto the endolymphatic fluid within the inner ear through the oval membrane, maximizing the energy transfer. The **inner ear** is responsible for mechano-electrical transduction and the afferent input to the central nervous system (CNS) (Purves et al., 2001).

1.1. Architecture of the inner ear

The inner ear is a fluid cavity named the **membranous labyrinth**, surrounded by a bone structure called **bony labyrinth**. The space between them is filled by the perilymph. **Perilymph** is similar to the extracellular fluid in its ionic composition. The fluid contained within the membranous labyrinth, the **endolymph**, is rather unique, because it contains high potassium and low sodium concentrations. This difference in ionic distribution between the endolymph and the perilymph is crucial for the mechanotransduction process (Couloigner et al., 2006; Fettiplace and Kim, 2014; see below).

The membranous labyrinth consists of an array of sacs and ducts that are subdivided by its morphology and function into **vestibular** and **auditory** parts (Fig. 1). The organization of the vestibular region is highly conserved, whereas the auditory part varies greatly across vertebrates (Fritzsch et al., 2006; Riley and Phillips, 2003; Schneider-Maunoury and Pujades, 2007; Fig. 2). The vestibular part forms the dorsal domain of the labyrinth and is responsible for the senses of motion and position. It consists of three cristae and two maculae. The former are sensory organs located at the basis of three orthogonally arranged semi-circular canals. Cristae senses angular acceleration, while macula utricularis and macula sacularis detect linear accelerations including gravity.

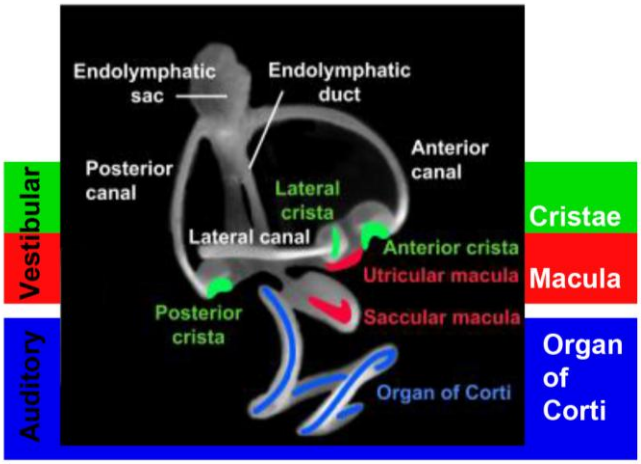


Figure 1. The vertebrate inner ear. The mouse ear is divided in the auditory and vestibular regions. Sensory patches are labelled in different colours. Organ of Corti, blue; macula, red; cristae, green (Groves and Fekete, 2012).

Te auditory organ presents more differences across phyla than their vestibular counterparts (Fig. 2). It is located ventrally to the vestibular organs. In mammals it is called the cochlea (from Greek *cochlos,* snail), a coiled structure whose sensory epithelium is the organ of Corti, or basilar papilla in birds. At the tip of the chick basilar papilla, resides a small vestibular sensory region named macula lagena. The lagena along with the saccule mediate hearing in fish (Bissonnette and Fekete, 1996; Riley and Phillips, 2003; Schneider-Maunoury and Pujades, 2007).

In addition to the sensory structures, the vertebrate inner ear includes the endolymphatic duct (ED) and endolymphatic sac that regulate the volume, pressure and pH of the endolymph (Couloigner et al., 2006).

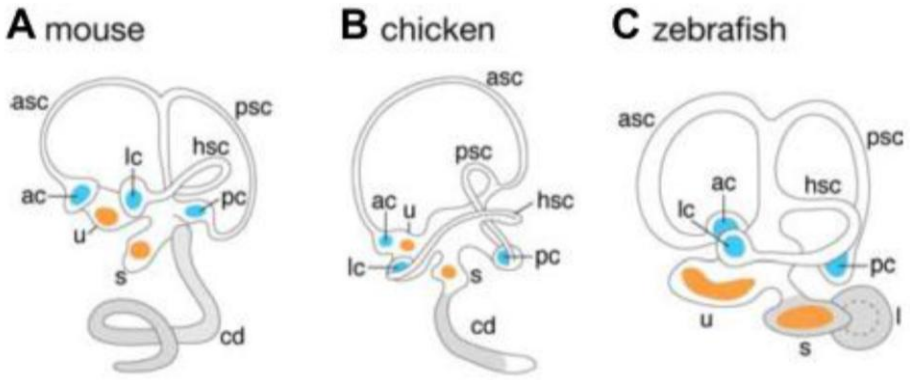


Figure 2. Evolution of the vertebrate inner ear. Structure of the adult inner ear in three different species. From right to left: **(A)** mouse, **(B)** chicken and **(C)** zebrafish. Note the high similarity of the vestibular compartments in the three animal species. Ac: anterior cristae; asc: anterior semicircular canal; cd: cochlear duct; hsc: horizontal semicircular canal; l: lagena; lc: lateral cristae; pc: posterior cristae; psc: posterior semicircular canal; s: saccule; u: utricle. Dorsal to the top, anterior to the left and medial to the reader (Fritzsch et al., 2006a; Schneider-Maunoury and Pujades, 2007).

1.2. The functional unit of the inner ear

Both vestibular and auditory organs of the inner ear share a common functional unit composed of three cell types: hair cells (HC), supporting cells (SC) and otic sensory neurons (Fig. 3), and it resembles very much the structure of the fly sensory bristles (BOX I). Sensory epithelia of the inner ear lie in between two extracellular structures, the basal lamina (or basal membrane), where sensory cells seat, and an extracellular structure associated with their luminal surface. This varies in the different sensory organs: cupula in the cristae, otolithic membrane in the macula or tectorial membrane in the auditory region.

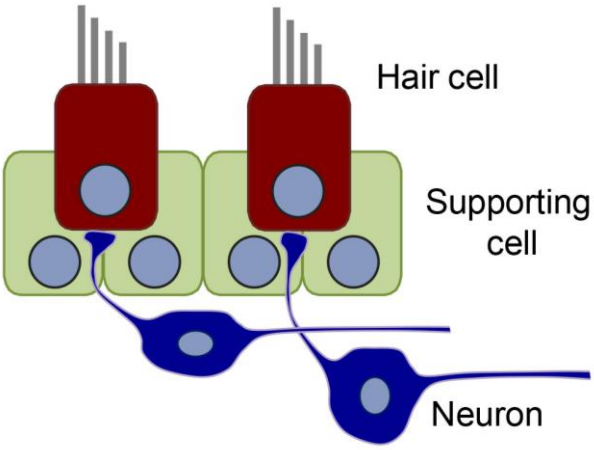
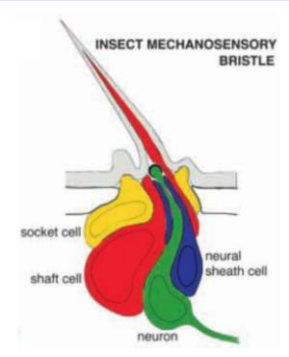


Figure 3. The functional unit of the inner ear. The three basic cell types are: hair cells in red, supporting cells in green and neurons in blue. Note the similarity with the fly mechanoreceptors shown in Box I. Adapted from: Alsina et al., 2009; Giraldez and Fritzsch, 2007; Groves and Fekete, 2012.

Otic sensory neurons are bipolar primary afferent neurons that connect hair cells with second order neurons in the vestibular and auditory nuclei of the brainstem. Their somas are intermingled with glial cells (Schwann cells) forming the auditory (cochlear) and vestibular ganglia (VIIIth cranial nerve) (Rubel and Fritzsch, 2002). Otic neurons innervate hair cells through their basal aspect, where they establish connections through highly specialised synapses (Wichmann and Moser, 2015 and Fig. 3). During ear development, the proneural factor **Neurog1** is responsible for the commitment of progenitors towards neuronal fate (see below).

BOX I. Homology between fly sensory organ and inner ear sensory organ

In Drosophila bristles, their cuticle sensory organs are formed by four cell types that derive from a common progenitor pool of cells of ectodermal origin. There is a clear homology between the bristle shaft cell and HC, the bristle socket cell and SC, and between the neurons. Fly and vertebrate organs share several common features. They are both ectoderm-derived organs and are morphologically similar. All cell types derive from a common pool of cells and the generation of cellular diversity is tightly coordinated in time and space. Finally, proneural factors and Notch-mediated lateral inhibition play a crucial role to select the different cell types (adapted from Adam et al., 1998). Figure represents a bristle structure of the fly.



Supporting cells are located in the basal side of the sensory epithelium and their lateral membrane surrounds hair cells, which project up onto the luminal side. Supporting cells are required for cell patterning, planar cell polarity, and synaptogenesis. In the mature sensory epithelia, supporting cells preserve the structural integrity of the sensory organs, modulate ion and small molecule homeostasis, and maintain the accessory extracellular matrices that enable hair cell mechanotransduction. Supporting cells clear the damaged hair cells and also are the source for their regeneration (Cotanche and Kaiser, 2010; Wan et al., 2013). They show morphological and molecular diversity in the different sensory epithelia, according to their specialized functions. This is most pronounced in the organ of Corti, which has up to five different types of supporting cells that are organized in rows along the organ's length (Fig. 4B). Several genes are expressed in the supporting cells, like Sox2 (Neves et al., 2007; Oesterle et al., 2008), Sox9 (Mak et al., 2009), Sox10 (Watanabe et al., 2000), Jagged1 (Brooker et al., 2006), and p27kip1 (White et al., 2006), which are used as supporting cell markers.

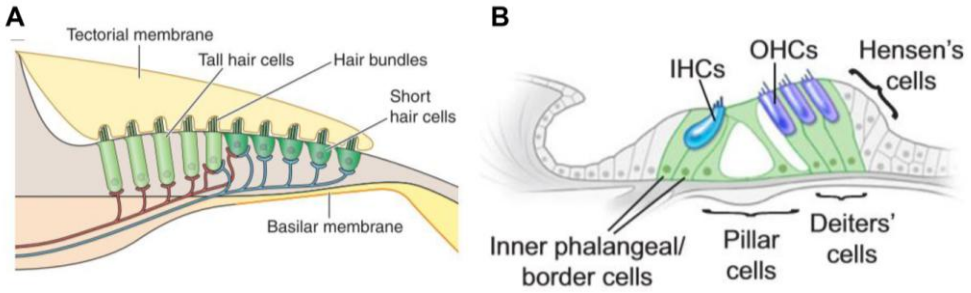


Figure 4. The auditory organ in chicken and mouse. (A) Chicken basilar papilla shows the distribution of hair cells from tall to short in contact with the tectorial membrane. **(B)** Mammalian cochlea. Hair cell distribution arranged in one row of inner hair cells and three rows of outer hair cells. (IHCs, blue), outer hair cells (OHCs, purple) and supporting cell subtypes. From outer to inner domains they are Hensen's cells, Deiters's cells, pillar cells; inner phalangeal cells; and border cells (Atkinson et al., 2015; Fettiplace and Kim, 2014).

Hair cells are highly specialized mechano-transducer sensory cells that contain a group of microvilli, referred to as stereocilia (Box II). Hair cells vary among different sensory epithelia and within sensory epithelium. They can be distinguished by differences in their morphology, electrophysiology and innervation. Vestibular epithelia contain type I and type II hair cells. The auditory epithelium also contains two types of hair cells, inner and outer hair cells in mammals, which correspond to tall and short hair cells in birds (Fig. 4; Fekete and Wu, 2002; Wichmann and Moser, 2015). **Atoh1** is a proneural factor that is crucial for the formation and specification of hair cells. Atoh1 and Neurog1 belong to the bHLH superfamily of transcription factors, and their cross-talk will be discussed extensively in this work.

BOX II. Mechano-transduction in hair cells

Stereocilia detect mechanical displacements generated by the movement of the endolymph. They are arranged in cell bundles of 30-300 specialized units. The rigid stereocilia skeleton is formed by actin filaments, which are cross-linked by fimbrin. In the auditory division, motion of the hair cell bundles is driven by their attachment to the tectorial membrane. In the vestibular part, including saccule and utricle, force is applied to the bundle via an otolithic membrane. Auditory hair cells are probably the fastest and most sensitive of all vertebrate mechanoreceptors. In the cochlea, hair cells are anchored to supporting cells and sandwiched between the tectorial membrane and the basilar membrane.

The perilymph bathing the basolateral region of the hair cell is rich in Na^+, while the endolymph is rich in K^+. The ionic gradients established between the endolymph and the intracellular compartment generates an electromotive force that depolarizes the hair cell membrane upon the opening of mechano-sensitive cationic channels. Other ionic channels like Ca^{2+} channels and Ca^{2+}-activated K^+ channels contribute to refine HC frequency tuning. **HC depolarization** increases **neurotransmitter release** at the basal membrane, where afferent synaptic transmission occurs at elaborate presynaptic active zones. Synaptic vesicles containing glutamate **activate post-synaptic afferent fibers of the VIIIth cranial nerve**, which transmit their impulses **to the cochlear and vestibular nuclear complexes on the brainstem** (Fettiplace and Kim, 2014; Hudspeth, 1997; Jaramillo, 1995; Fig. 4).

2. The development of the inner ear

The vertebrate inner ear derives from the **otic placode**, a thickening of the head ectoderm. The formation of the inner ear requires a series of cell fate decisions and morphogenetic events with a precise temporal and spatial pattern. There are three developmental steps which ensure the correct development of the mature organ: 1) the **induction of the otic placode** along with the establishment of its main **axial landmarks**, 2) the **specification of neurosensory progenitors** and 3) the **diversification of the different cell types**, i.e., neurons, hair cells and supporting cells (Fritzsch et al., 2006a; Groves and Fekete, 2012).

2.1. The induction of the otic placode

The acquisition of otic identity and competence requires both mesodermal and neuroectodermal signals (Fig. 5A). The induction of the otic placode is based on diffusible signals, which confer otic ectoderm identity to the otic placode. They are Sonic Hedgehog (Shh) from the floor plate and notochord (Riccomagno et al., 2002), FGF from mesoderm and neuroectoderm (Wright and Mansour, 2003), WNTs from the hindbrain and BMP4 from the ectoderm (Morsli et al., 1998; Ohyama et al., 2006).

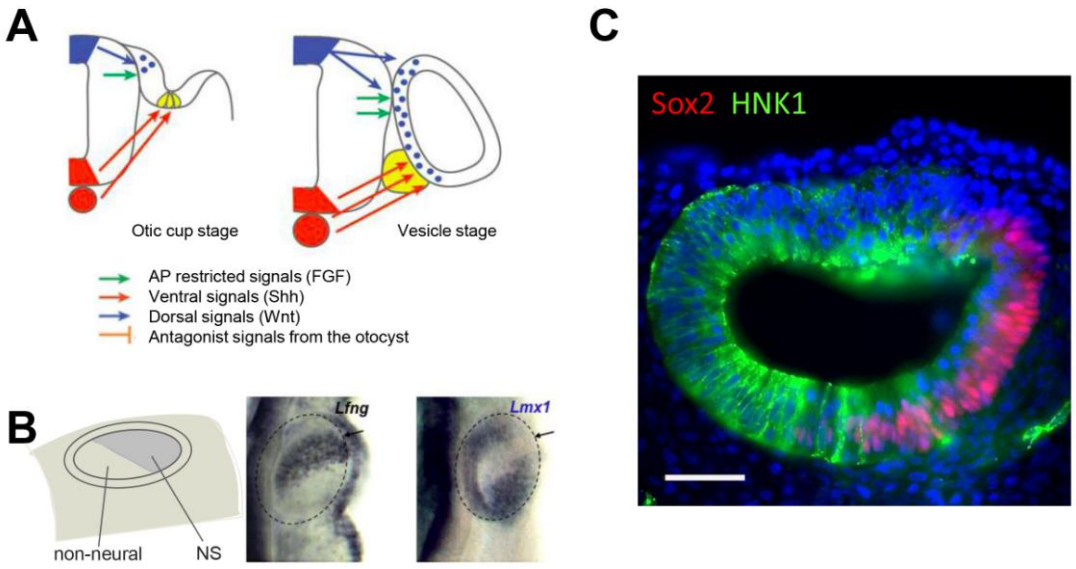


Figure 5. Otic induction and patterning. (A) DV and AP patterning of the otic vesicle are established by diffusible molecules coming from the neural tube. FGF specifies the AP axis, whereas Shh and WNT specify the DV axis (Groves and Fekete, 2012; Schneider-Maunoury and Pujades, 2007). **(B)** At otic placode stage AP markers are already expressed: Lfng is expressed in the anterior (neurosensory, NS) region, while Lmx1 is expressed in the posterior (non-neural) region of the inner ear (Abelló et al., 2007). **(C)** Sox2 expression (red) is detected in all the neurosensory anterior domain and HNK1 expression (green) at the posterior domain (parasagittal section, anterior to the right and dorsal to the top (Neves et al., 2007).

These signals are also relevant to establish the axis of the inner ear labyrinth and they result in the asymmetric expression of several markers like Sox3, Pax2 or Lmx1 (Alsina et al., 2009; Fritzsch et al., 2006a). Although the signalling pathways involved in otic placode induction are conserved among different species, its precise timing and source show considerable variations.

2.2. Otic patterning and axial specification

The inner ear has a complex morphology with a distinct polarity in all three axes. This polarity is established very early in development. Once formed, the otic placode undergoes invagination (in amniotes) or cavitation (in fish) to form a spherical otocyst. Three main axes are established during inner ear development: Dorso-Ventral (DV), Antero-Posterior (AP) and Medio-Lateral (ML).

AP patterning: The AP patterning is probably the first developmental decision in the otic epithelium and it reflects the separation of two functionally independent domains, one with neurosensory competence and another devoid of it (Abelló and Alsina, 2007). The **neurosensory domain** is located anterior and medial in the otic placode, and anterior and ventral when the otic vesicle is formed. The **non-sensory domain** extends to the posterior and lateral aspects of the otic placode and, later on, posterior, dorsal and lateral in the otic vesicle (Fig. 5B and C). The neurosensory domain gives rise to the otic sensory neurons, sensory hair cells and supporting cells in chick and mouse (Raft et al., 2007; Satoh and Fekete, 2005), and it is generally accepted that progenitors residing in the neurosensory domain are multipotent (see below).

The neurosensory domain is characterized by the expression of SoxB1 genes, such as Sox3 and Sox2, Fgf10, and members of the Notch pathway like Delta1, Hes5 and Lunatic Fringe (Abelló et al., 2007 and Fig. 5B and C). SoxB1 genes are believed to have a proneural function and to drive the expression of Neurog1 and Atoh1 (Jeon et al., 2011; Neves et al., 2012). The complementary non-neural domain shows two major patterning genes, Lmx1b and Iroquois1, and two members of the Notch pathway, Serrate1 and Hairy1 (Abelló et al., 2007).

FGF, BMP, and Notch signalling have been involved in the establishment of AP axis in mice. FGF and BMP differentially regulate the expression of Sox3 and Lmx1, and their respective restriction to the anterior and posterior domains (Abelló et al., 2007; Schneider-Maunoury and Pujades, 2007). The regionalization of the otic placode into neurosensory and non-sensory territories requires also the functional integrity of the Notch pathway for its stabilization (Abelló et al., 2007; See BOX III and IV for Notch signalling).

The non-sensory region of the otic placode receives signals that confer posterior identity (Bok et al., 2011). Retinoic acid (RA), which is known to posteriorize the

embryonic body axis, is also required to specify the posterior character of the otic placode. Expression of RA synthesizing and degrading enzymes coincides with the AP boundary of the otic placode, and experiments in chicken have disclosed a developmental window during which the otocyst receives and is sensitive to RA posteriorizing signals (Bok et al., 2011; Radosevic et al., 2011).

DV patterning is apparent in the adult ear where dorsal-vestibular and ventral-auditory regions are easily distinguished. DV cues come from adjacent tissues including the neural tube and notochord. Studies in the 30s and 40s showed that the DV pattern can be disrupted by manipulating the hindbrain, suggesting that signals from the neural tube might specify this axis of the inner ear (Groves and Fekete, 2012). This was later confirmed by showing that neural tube signals are required for the correct regional expression of the Lim homeobox transcription factor Lmx1 (Giraldez, 1998). Shh produced by the notochord and floor plate is crucial for DV patterning of the neural tube, and it also acts directly on the developing otocyst patterning (Bok et al., 2007; Riccomagno et al., 2002; Whitfield and Hammond, 2007). The perturbation of Shh signalling leads to a loss or a reduction of ventral markers with the expansion of dorsal markers and this, in turn, is translated into a loss or reduction of ventral otic structures (Riccomagno et al., 2002). Conversely, when Shh is aberrantly expressed in the dorsal otocyst, there is a loss of dorsal vestibular structures and an upregulation of ventral Shh targets in the dorsal otocyst (Riccomagno et al., 2002).

3. Cell fate commitment in the ear functional unit: neurogenesis and sensorygenesis

The adult mouse ear contains approximately 10.000 hair cells and 11.000 sensory neurons. In mice, between neurosensory specification and hair cell differentiation, ear precursors undergo approximately sixteen cell cycles of about eight and a half hours each. Assuming only symmetric divisions, just two initial cells would be needed to generate 32.000 cells in only fifteen rounds of division. This highlights the importance of selecting the right number of competent neurosensory progenitor cells during otic placode induction (Fritzsch et al., 2006a).

Once otic fate and the neurosensory competence have been established, the three cell types of the functional unit are generated in a precise and stereotyped manner (Fig. 6). This process is autonomous to the otic vesicle that can be isolated and grafted to a

heterotopic region of the embryo and still keeps its capacity to produce hair cells and neurons (Adam et al., 1998; Swanson et al., 1990).

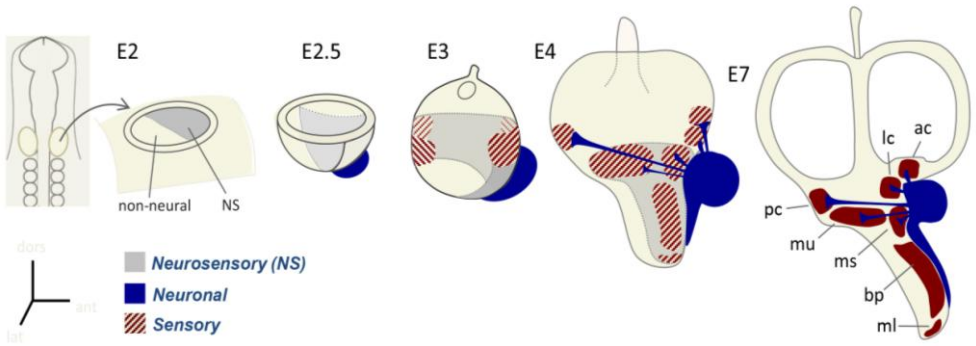


Figure 6. Chicken inner ear development. The stage of development is indicated by days of development (E2–E7). The otic placode (E2) shows the first asymmetry between the neurosensory and non-neural competent domains. At the otic cup stage (E2.5), neurogenesis starts with the delamination of neuroblasts from the antero-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 ones start to differentiate. By E7 all sensory patches exhibit nascent hair cells and synaptic connections. The sensory patches are labelled in red: ac, anterior cristae; bp, basilar papilla; lc, lateral cristae; ml, macula lagena; ms, macula sacularis; mu, macula utricularis; pc, posterior cristae. Adapted from Neves et al., 2013a. Stages E2 and E7, which are neurosensory and differentiated epithelia in chicken, correspond to E9-10 and E16 in mouse development.

The sequence of differentiation can be described as follows. First, a wave of **neurogenesis** invades the neurosensory epithelium. Neuroblasts are specified and delaminate from the otic placode to form the cochleo-vestibular ganglion. Secondly, the neurosensory epithelium losses the neurogenic potential but retains the sensory competence, thence named the **prosensory** epithelium. Later on, the prosensory epithelium gives rise to the **sensory patches** that host differentiating hair and supporting cells (Fig. 7). Lineage analyses by viral tracing in chicken embryos demonstrated that bipotential neurosensory progenitor cells are present in the otic placode (Satoh and Fekete, 2005) and dye-labelling of otic placode progenitors showed that neurons and hair cells derive from the neurosensory domain of the otic vesicle (Bell et al., 2008). Furthermore, genetic fate mapping in mouse and chick indicates that vestibular sensory hair cells derive from a neurogenic competent region of the inner ear (Neves et al., 2012; Raft et al., 2007). In zebrafish, it seems that there are three progenitor pools, one specific to neurons, another specific to hair cells and a third one that can give either neurons or hair cells until later stages (Sapede et al., 2012). It is unknown whether those pools arise from a previous neurosensory progenitor.

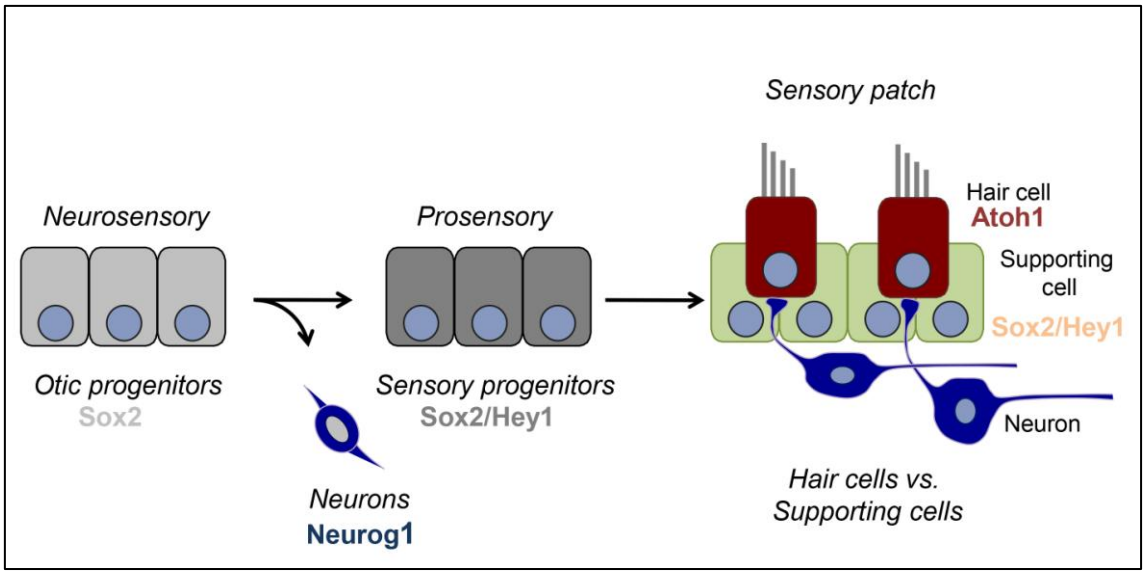


Figure 7. Cell fate specification during inner ear development. From left to right: The neurosensory epithelium is specified by Sox2. This epithelium has the potential to give rise to all three cell types. Cells that first express Neurog1 trigger lateral inhibition and single out from the epithelia. As development proceeds, the prosensory epithelia expressing Sox2 and the Notch target Hey1 remains committed but not differentiated. Finally, within the prosensory patch, cells start to express Atoh1 that by lateral inhibition differentiate into hair cells and induce the neighbouring precursors to become supporting cells, forming the sensory patch.

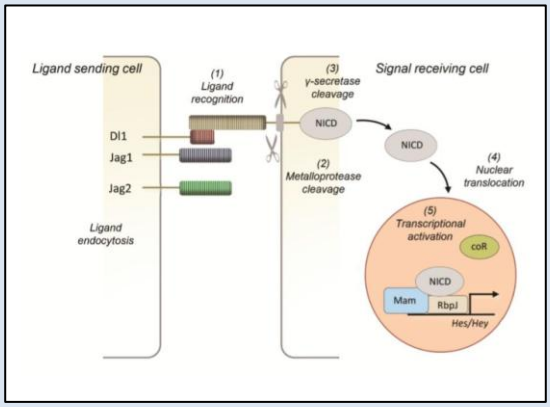
In summary, it is likely that at least in amniotes, hair cells and neurons share a common progenitor. If early in development, neurosensory cells have capacity of becoming neurons or hair cells, the question arises as to why neurons are specified immediately and before hair cells? In other words, why Neurog1 is expressed before Atoh1? In the following, I shall discuss some ideas on possible mechanisms that explain this developmental decision.

As mentioned above, the neurosensory domain is the territory of the otic placode and early otic vesicle that gives rise to neurons and hair cells. But **neurosensory** also refers to the state of competence of progenitors to become either neurons or sensory cells. The first genes with proneural function that map to this domain are the SoxB1 factors Sox3 and Sox2. Sox3 expression is transient and precedes that of Sox2, which is restricted and refined by Notch signalling in chicken and mice development (Krejcí et al., 2009; Neves et al., 2011). The loss of function of Sox3, in zebrafish and chick, has demonstrated its requirement for epibranquial placode neurogenesis (Dee et al., 2008). Loss of function of Sox2 results in impaired formation of neurons and HC in the inner ear (Basu-Roy et al., 2010; Ferri et al., 2004; Kiernan et al., 2005).

FGF signalling is important for irreversible neurosensory commitment in the inner ear, probably by the upregulation of SoxB1 factors (Alsina et al., 2009). In turn, Sox2 upregulates Neurog1 in the neurosensory domain of the otic vesicle (Neves et al., 2012; Neves et al., 2013a). However, these two factors are mutually exclusive. Once neuroblasts differentiate in the cochlear vestibular ganglion (CVG) Neurog1 downregulates Sox2 via an specific enhancer (NOP1, Nasal and Otic Placode, Kamachi and Kondoh, 2013; Evsen et al., 2013). Neurog1 upregulation precedes neuron commitment and delamination and is sufficient for neuronal commitment of otic epithelial cells, but Neurog1 expression is switched off in delaminated neuroblasts. Neurog1 activates a cascade of other bHLH factors including NeuroD, NeuroM, which specify neuronal fate and promote delamination (Abelló et al., 2007; Seo et al., 2007).

For neuroblasts selection, Neurog1 activates Delta1 (Ma et al., 1996) that leads to Notch-mediated lateral inhibition and the singling out of neuroblasts that also express the Lim-factor Islet1 (Li et al., 2004; Box IV). Once in the CVG, neuroblasts divide a few more times before entering terminal differentiation (Fritzsch et al., 2006a; Pauley et al., 2005). Successive markers of differentiated neurons are Tuj1, a neuron specific class III beta-tubulin (Li et al., 2004); Islet 1, a Lim-homeodomain factor (Li et al., 2004) and unpolimerised neurofilament proteins (Oesterle et al., 1997).

BOX III. The Notch signalling pathway

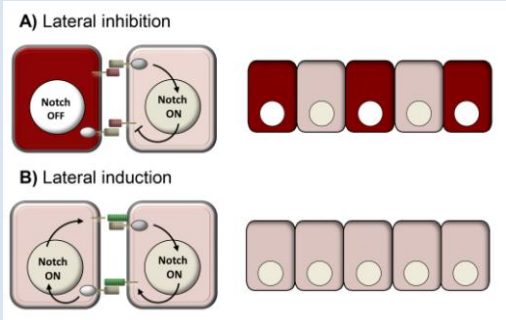


Notch signalling is an evolutionarily conserved juxtacrine signalling pathway used by metazoans. It controls a broad spectrum of developmental processes in organisms ranging from sea urchins to humans (Artavanis-Tsakonas et al., 1999; Neves et al., 2013b). The core pathway consists of the interaction between a transmembrane Notch receptor in one cell, with a transmembrane Notch ligand, a DSL protein (Delta and Serrate for Drosophila and Lag2 for Caenorhabditis elegans) expressed in a neighbouring cell. Ligand-receptor binding triggers a series of proteolytic cleavages that release the intracellular domain of Notch (NICD), allowing it to form a nuclear complex with the CSL (mammalian C-promoter binding factor 1, CBF1 or recombination signal sequence-binding protein-J kappa, rBP-jkappa; Drosophila Suppressor of Hairless and C. elegans Lag-1) and mastermind/MAML transcription factors. This complex then activates the transcription of target genes (Artavanis-Tsakonas et al., 1999; Bray, 2006). Mammals have four Notch receptors (Notch 1-4), while avian have only two (Notch1-2). Mammals have five DSL ligands (Jagged 1-2 homologous of Serrate and Delta-like 1-3 homologous to Delta) and avian have Serrate1-2 and Delta-like 1 and 4 (Abelló and Alsina, 2007).

NeuroD is activated by Neurog1 in the inner ear, and it is essential for the formation of cochlear and vestibular ganglion neurons. At an early stage, NeuroD1 is prominently expressed in the otic vesicle, delaminating neuroblasts, and the CVG. Its expression is found not only in the differentiated neurons but also in dividing neurons (Kim et al., 2001). Lack of NeuroD1 causes retention of neuroblasts precursors within the otocyst, suggesting that NeuroD1 is required in the neuroblasts precursors during the last round of cell division to promote cell cycle exit and to initiate differentiation (Huang et al., 2000; Liu et al., 2000). NeuroD null mice are deaf but hair cells do not seem to be affected (Kim et al., 2001). Interestingly, the conditional deletion of NeuroD1 in the mouse inner ear induces the expression of Atoh1 and HC markers in otic neurons (Jahan et al., 2010). This suggests that, indeed, Atoh1 is expressed in neurosensory progenitors and its repression is necessary for neuronal development.

Once neurogenesis has taken place, the neurosensory domain remains competent to develop sensory cells, but it does not yet differentiate (Fig. 7). This is called the **prosensory state**, and the corresponding domains called **prosensory patches** (Fekete et al., 1998). The territory is maintained coherent by Notch-mediated lateral induction driven by Jag1 (Daudet and Lewis, 2005; Petrovic et al., 2014, BOX IV). Prosensory cells are characterized by the expression of Notch (Adam et al., 1998), Sox2 (Neves et al., 2007), Jagged1 (Petrovic et al., 2015), BMP4 and BMP targets Id1-3 (Kamaid et al., 2010; Morsli et al., 1998), Prox1 (Stone et al., 2003) and BEN (Goodyear et al., 2001).

BOX IV. Notch modes of action



Prosensory patches are early labelled by the Notch ligand Jag1. Delta1 is detected first in differentiating neuroblasts and, later on, in differentiating hair cells. In contrast to Jag1 that shows a uniform expression pattern, the expression of Delta1 is scattered, "salt-and-pepper" (Adam et al., 1998). This corresponds to two different modes of operation: **Lateral inhibition (A)** is a mechanism by which Notch activation in one cell inhibits the expression of the Notch-activating ligand in another cell. It mediates binary cell fate decisions, ensuring that the cells adopt one of two alternative fates. **Lateral induction (B)** is an inductive process where a ligand-expressing cell stimulates their neighbours to promote the expression of the same ligand, resulting in coherent domains of Notch activity and coordinated cell behaviours (Bray, 1998; Bray, 2006; Eddison et al., 2000; Lewis, 1998). During inner ear development, Delta1-mediated lateral inhibition singles out neurons and hair cells, while Notch-mediated lateral induction contributes to establish the prosensory domains (Neves et al., 2013b).

The prosensory patches eventually initiate differentiation, giving rise to hair cells and supporting cells that along with neurons will form the **sensory patches.** The earliest gene expressed in hair cells is **Atoh1**, followed by early differentiation genes like MyoVI and MyoVIIa (Cotanche and Kaiser, 2010), Pou4f3 and Nestin (Ryan et al., 2015). As it happens during neurogenesis, Notch-Delta1 mediated lateral inhibition is responsible to single out hair cells (Pujades et al., 2006; Stone et al., 2003; Box IV).

3.1. SoxB1 factors and neurosensory competence

Sox genes are transcription factors that belong to the High Moblity Group (HMG) box domain proteins (Kamachi and Kondoh, 2013). They are present exclusively in the animal kingdom and may have evolved from canonical HMG domain proteins (Bowles et al., 2000). The SoxB group is split into two sub-groups. SoxB1 comprises Sox1, Sox2 and Sox3, being only Sox2 and Sox3 expressed in the vertebrate inner ear. SoxB2 proteins comprise Sox14 and Sox21, they are transcriptional repressors (Kamachi and Kondoh, 2013), and only Sox21 is expressed during inner ear development (Freeman and Daudet, 2012).

Sox3 is expressed in the neurosensory domain of the otic placode (Abelló et al., 2007), and it is downregulated upon neuroblast delamination (Abu-Elmagd et al., 2001). Sox3 expression is dependent on FGF signalling and its overexpression in chicken upregulates Dl1 and Sox2, and downregulates non-sensory markers like Lmx1 (Abello et al., 2010).

Besides its established function as a stem cell factor in the inner cell mass, Sox2 is involved in several developmental processes in a variety of cell types (Kiefer, 2007). It is expressed during otic development, and its function is critical in the specification of neurons and hair cells (Neves et al., 2012). Sox2 expression initially follows that of Sox3, which acts as an inductor for Sox2 (Abelló et al., 2007). Its expression is further maintained in the prosensory patches due to the Notch activity driven by Jagged1 (Neves et al., 2011). Beyond stages of sensory cell specification, Sox2 is only transiently expressed in hair cells, but it remains highly expressed in supporting cells, suggesting that these cell type retains progenitor properties (Neves et al., 2007). Fate map analysis in mice and chicken showed that Sox2-expressing cells in the early otocyst give rise to neurosensory derivatives, but also give rise to non-sensory structures (Gu et al., 2016; Neves et al., 2012). Sox2 is expressed in proliferating cells

of the otic epithelium and also in Schwann cells of the CVG, but not in otic neurons (Neves et al., 2007).

Sox2-defficient mice such as light coat and circling (Lcc) and yellow submarine (Ysb) show hearing impairment with major defects in inner ear morphogenesis (Kiernan et al., 2005). These mice lack the formation of the prosensory domains, hair cells and supporting cells. Sox2 expression however is not affected in Math1-ko mice suggesting that Sox2 acts upstream Atoh1 (Bermingham, 1999; Kiernan et al., 2005). In the inner ear, like in other areas of the nervous system, Sox2 behaves as a genuine proneural factor conferring neurosensory competence; taking over the role played by bHLH proneural factors in Drosophila (see Box V).

BOX V. bHLH in mammals: has Atoh1 lost its proneural function?

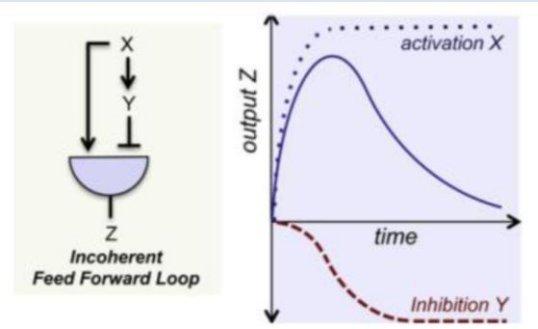
What is a proneural gene? A proneural gene must fulfill three main characteristics (Hassan and Bellen, 2000): First, its expression precedes and coincides with the selection of neuronal precursor cells. Secondly, its function is both necessary and sufficient for the specification of a given neuronal lineage in a cell autonomous fashion. Finally, its loss of function results in the deletion (and its missexpression ectopic development) of a given lineage. Proneural genes were first identified in Drosophila peripheral nervous system development. The Achaete–Scute complex (AS-C) genes were identified as proneural genes encoding bHLH factors. Later on, atonal (Atoh1 in mammals) was identified by PCR (Jarman et al., 1993). Atonal in Drosophila is the master gene for the formation of chordotonal organs, which are mechano-receptors of insect muscles. Atonal gene selects the progenitors that give rise to the mature organs. Atonal loss of function abolishes chordotonal organs and its missexpression favors their ectopic formation (Jarman et al., 1993). Are Atoh1 and Neurog1, the vertebrate homologues of atonal, also proneural genes? Atoh1 and Neurog1 overexpression drives, respectively, ectopic hair cell and neuron formation (Evsen et al., 2013; Izumikawa et al., 2005), and their loss of function results in the lack of hair cells or neurons (Bermingham, 1999; Ma et al., 1998). However, their function is far more restricted and, like in other regions of the Nervous System, they do not provide a broad neural competence, but a far more restricted lineage selection (for example, HCs and SCs in the case of Atoh1 and the inner ear). The broad neural competence is rather dependent on SoxB1 (Azuara et al., 2006; Puligilla and Kelley, 2016). This shows a proneural crisis identity in vertebrate development and the taking over by SoxB1 proteins (Hassan and Bellen, 2000).

In the otic placode, Sox2 is able to activate both Neurog1 and Atoh1. However, Atoh1 expression remains undetected until HC differentiation. Neves et al., 2012 hypothesized that this delay in Atoh1 expression could be the consequence of an incoherent feed-forward loop (I-FFL) triggered by Sox2. The model states that Sox2 activates both Atoh1 and Atoh1 repressors, which in turn prevent Atoh1 expression (Neves et al., 2012 and BOX VI). Recent experiments using a conditional gain of function system in mouse supported the I-FFL model by showing that Sox2 is required to specify prosensory competence, but it must be downregulated to allow Atoh1 expression (Puligilla and Kelley, 2016). Major candidates to mediate the repression of

Atoh1 include a variety of bHLH factors that are expressed throughout ear development.

BOX VI. Transcription regulation networks: the Incoherent Feed-Forward Loop

Transcription regulatory networks describe the interactions between transcription factor proteins and the genes that they regulate. Among conserved and prevalent networks, one is the Feed-Forward Loop (FFL). This motif consists of three genes: a regulator X, which regulates two target genes, Y, and Z, the latter being regulated by both X and Y. When the sign of the interaction of X and Y with Z is opposed, the circuit is called an Incoherent Feed Forward Loop (I-FFL). The net output in an I-FFL depends on the strength and dynamics of individual interactions (Alon, 2007). In the inner ear, the specification of sensory hair cells can be described by an I-FFL, where X (Sox2) is able to directly activate Y (Neurog1) and Z (Atoh1), but Neurog1, and probably other repressors, leads to the silencing of Atoh1 (Neves et al., 2013a).



Left, the transcription factor X directly activates Z and at the same time it activates the repressor Y that inhibits Z. Right, the predicted output of Z is a fast activation coming from the direct interaction of X (blue dotted line) and delayed inhibition, resulting from the indirect activation of the repressors (red dotted line). This results in a transient Z output, whose final steady state depends on the relative strengths of activation and repression (Alon, 2007).

4. bHLH genes, structure and classification

The basic helix-loop-helix (bHLH) proteins form a large superfamily of transcriptional regulators that are crucial for critical developmental processes in organisms ranging from yeast to humans (Jones, 2004). Over 240 HLH proteins have been identified to date (Atchley and Fitch, 1997). The **bHLH motif**, a highly conserved domain common in all bHLH proteins, was first identified in two murine transcription factors known as E12 and E47 (Murre et al., 1989). The region consisting of basic residues allows DNA binding and is called **basic domain**, while the one characterized by hydrophobic residues, the **HLH domain** allows protein-protein interactions and the formation of homo or heterodimers (Fig. 8, Murre et al., 1994).

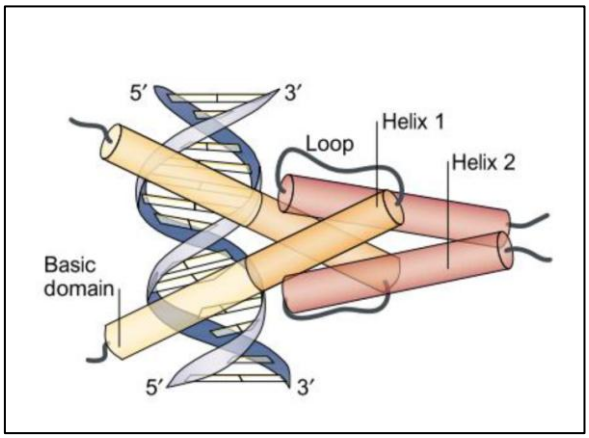


Figure 8. Schematic representation of bHLH dimer structure complexed to DNA (Bertrand et al., 2002).

Sequence comparison of the basic domains of Atonal, Daughterless, Scute, and MyoD reveal similarities in their basic domains. In addition, MyoD and Daughterless have characteristic residues that are invariant among their own family. Among the conserved residues that are not predicted to bind to DNA, some define the specific basic domain of different bHLH families (Chien et al., 1996). bHLH genes are able to bind to specific DNA consensus binding sites called E-boxes. The classification of these E-boxes is shown in **Table 1**. Neurog1 and Atoh1 are type II bHLH and they bind to class A E-boxes with more affinity than to the others.

Classification	Consensus	Examples
Class A	CANCTG	CACCTG, CAGCTG
Class B	CANGTG	CACGTG, CATGTG
Class C	CANNTG	CACGCG, CACGAG
E box	CANNTG	CACCTG, CACGTG
N box	CACNAG	CACGAG, CACAAG

Table 1. Classification of E-box DNA binding sites. Consensus sequence for each described E-box and some examples. Table adapted from Iso et al., 2003.

The classification of the different bHLH genes is based on tissue distribution, dimerization capacity, and DNA-binding specificity (Massari and Murre, 2000 , Fig. 9).

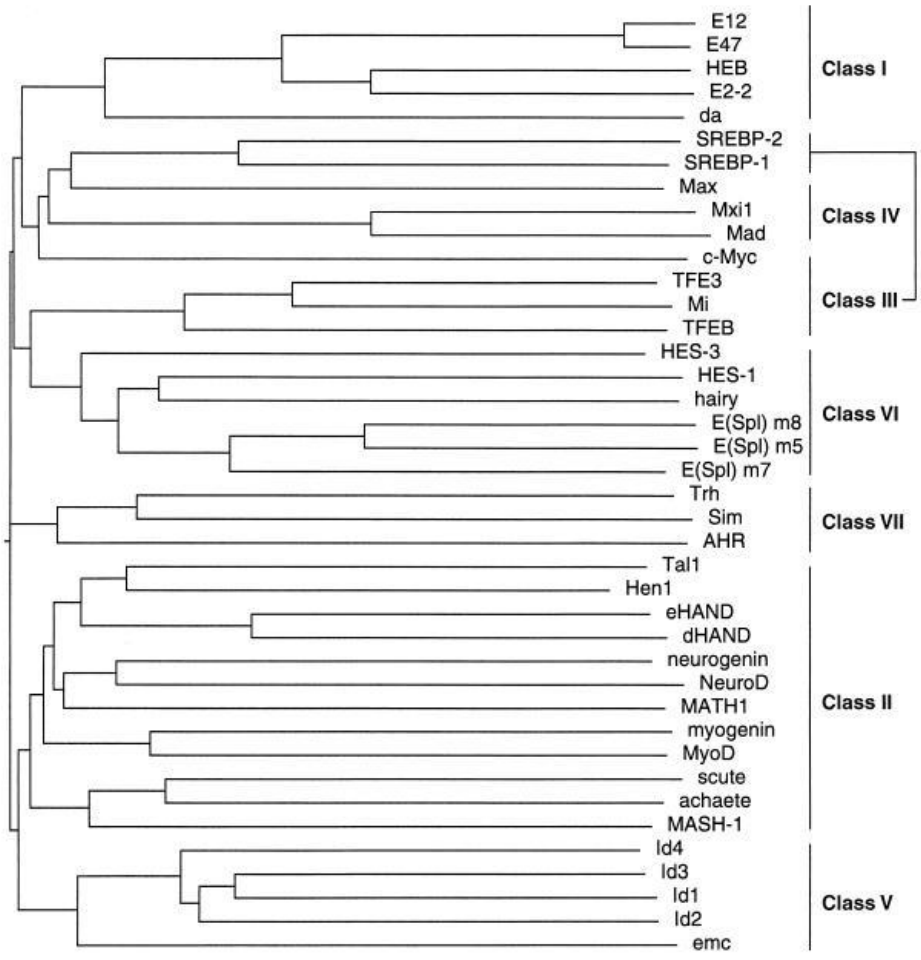


Figure 9. Classification of bHLH factors. Class II bHLH include Atoh1 and Neurog1. These two factors have to make a complex with class I bHLH to activate its downstream targets (Massari and Murre, 2000a).

Class I HLH proteins, also known as **E-proteins**, include E12, E47, HEB, and E2.2. They are widely expressed in a variety of tissues and they form homodimers or heterodimers (Murre et al., 1989). The DNA-binding specificity of class I proteins is limited to the E-box consensus sites (Table 1). **Class II** includes MyoD, Myogenin, Atoh1, Neurog1, NeuroD, and the Achaete-Scute complex, all of which show a tissue-restricted pattern of expression. With few exceptions, they are unable to form homodimers, but they heterodimerize, preferentially with E-proteins. Heterodimers of class I and class II proteins bind E-box sites, but mostly class A E-boxes (Murre et al., 1989). **Class III HLH** proteins include the Myc family of transcription factors, TFE3, SREBP-1, and the associated transcription factor, Mi. They contain a Leucine Zipper sequence adjacent to the HLH motif, which facilitate dimerization. **Class IV HLH** proteins define a family of molecules, including Mad, Max, and Mxi, which are able to dimerize with the Myc proteins or with one another. Another group of HLH proteins lack a basic region and include inhibitor of differentiation (Id) and emc, and define the **class V HLH** proteins. Class V HLH members are negative regulators of class I and class II HLH proteins. **Class VI HLH** proteins have as their defining feature a proline in their basic region. This group includes Hairy and Enhancer of split and they are typical Notch targets (see below). Finally, **class VII HLH** proteins are categorized by the presence of the bHLH-PAS domain and include members such as the aromatic hydrocarbon receptor (AHR), the AHR nuclear-translocator (Arnt), hypoxia-inducible factor 1 (Massari and Murre, 2000a). The additional domains in bHLH proteins, such as the leucine zipper, are always carboxyl-terminal to the bHLH region. However, the position of the bHLH and additional domains within the complete sequence of the protein varies widely between different families. This variability has led to the proposal that bHLH proteins have undergone modular evolution by domain shuffling, a process that involves domain insertion and rearrangement (Jones et al., 2006; Massari and Murre, 2000a).

The two major neural factors in the ear, Neurog1 and Atoh1, belong to class II bHLH proteins that heterodimerize with type I bHLH factors and bind to class A E-boxes. To follow, there is a detailed description of the molecular properties and function of Neurog1 and Atoh1, with particular reference to ear development.

4.1. Neurog1 and the development of otic neurons

Neurog1 (Neurogenin1) is a basic helix-loop-helix (bHLH) transcription factor that behaves as master regulator for neural differentiation in different vertebrates (Alsina et al., 2004; Evsen et al., 2013; Henrique et al., 1997; Ma et al., 1998). The Neurogenin family was discovered in mammals as an **Atonal related protein** (ARP) (see below). Neurog1 is the most distant relative to Atoh1, NeuroD another ARP being much closer (Hassan and Bellen, 2000) . Atoh1 and Neurog1 proteins share on average 53% amino acid identity in the bHLH domain (Sommer et al., 1996). Neurogenins differ from Atoh1 in four basic domain residues. Three neurogenins have been described in mammals. Neurog1 and Neurog2 function as neuroblast selector genes in mouse (Ma et al., 1998). They are required during the development of several neuronal lineages including proximal and epibranchial placode-derived sensory ganglia (Ma et al., 1998), dorsal root ganglia (DRG) and telencephalon (Ma et al., 1998). Although Neurog1 and Neurog2 work as neuroblast selector genes in the same tissues, they are required for distinct subsets of neuronal populations. Some examples are Neurog1 that is required for proximal cranial sensory ganglia (Ma et al., 1998) and Neurog2 that is required for distal, epibranchial placode-derived, sensory ganglia (Ma et al., 1998). Neurogenin3 (Neurog3) plays a precursor selection function in the mammalian pancreas. Neurog3 function in neural development is not well studied (Hassan and Bellen, 2000).

In the chicken inner ear, only Neurog1 is expressed during development (Evsen et al., 2013; Ma et al., 1996). Sox2 is necessary for Neurog1 up-regulation in the otic epithelium (Jeon et al., 2011; Neves et al., 2012). One feature of the neurosensory domain is that Neurog1 is upregulated while Atoh1 is repressed. The neurosensory domain has high Notch activity, and Jeon and colleagues showed that the enhancer of Neurog1 is activated by high levels of NICD transfection, while Atoh1 enhancer is not (Jeon et al., 2011). This may favour that Neurog1 expression precedes Atoh1 in the otic vesicle (Neves et al., 2011). However, later in development Notch signalling represses Neurog1 expression in the cells that remain in the epithelium. As mentioned above, this occurs by lateral inhibition and is mediated by Notch targets like Hes and Hey factors. In mice, Neurog1 is also activated by Six1 and Eya1 that synergize with Sox2 (Ahmed et al., 2012b; Zheng et al., 2003).

Neurog1 expression is controlled by different cis-elements identified 5’ and 3’ to the Neurog1 coding sequence. These enhancers drive the expression of Neurog1 in

midbrain, hindbrain, trigeminal ganglia, and ventral neural tube. For Neurog1 expression in the dorsal neural tube only a 5' enhancer has been identified (Nakada et al., 2002). Another enhancer region drives Neurog1 activity to the VIII cochlea-vestibular ganglion (Murray et al., 2000). The configuration of these enhancers is similar to the cis-elements identified for Neurog2 (Simmons et al., 2001), suggesting that there is a tight regulation of the two Neurogenins. Nakada et al., (2002) speculated that is possible that the conservation between Neurog1 and Neurog2 arise from gene duplication. The modular organisation of Neurogenins cis-regulatory regions contrasts with the single enhancer regulation described for Atoh1 (Helms et al., 2000 and see below).

Neurog1 is a transcriptional activator (Bertrand et al., 2002; Sommer et al., 1996; Sun et al., 2001). Neurog1 targets have been analyzed in xenopus and mouse, and they include genes that control regulation of transcription, signal transduction and cytoskeletal rearrangement for neural differentiation and migration. Neurog1 binds to specific enhancers that share a common core binding site CAGCTG, CAGATG, or CAAATG (Seo et al., 2007). However, Neurog1 lacking its DNA binding capacity is able to prevent the formation of glial cells in cortical precursors and stem cells cultures. This occurs by sequestering the p300/CBP-Smad1 complex from other transcription factors (Sun et al., 2001).

4.2. Notch targets: Hes and Hey

The most studied Notch canonical effectors are Hes (**H**airy and **E**nhancer of **S**plit) and Hey (Hairy and enhancer of split related). Hes and Hey genes belong to the type VI bHLH group. Seven Hes members have been identified in vertebrates (Hes1-7), while the Hey subfamily of genes encodes three members in mammals (Hey1, Hey2 and HeyL) (Iso et al., 2001; Iso et al., 2003).

The core structure of Hes and Hey proteins contains a basic and Helix-loop-Helix domain and an Orange domain at the C-terminus region (Figure 10A). The **Orange domain** serves as a region for protein-protein interactions and for partner selection (Iso et al., 2001). Hey proteins differ from the Hes subgroup by two striking features: first a glycine present in the basic domain of Hey proteins instead of a conserved proline in Hes proteins. Secondly, the C-terminal **WRPW motif** that is characteristic of Hes proteins and allow Groucho co-repressor recruitment, is replaced with YRPW or YXXW (HeyL) (Fisher et al., 1996). The YXXW motif is followed by a conserved TE(I/V)GAF

peptide with unknown function. The C-terminal WRPW motif acts as polyubiquitination signal, therefore Hes proteins are rapidly polyubiquitinated and degraded by proteasome with a very short half-life of approximately 20 minutes (Hirata et al., 2002).

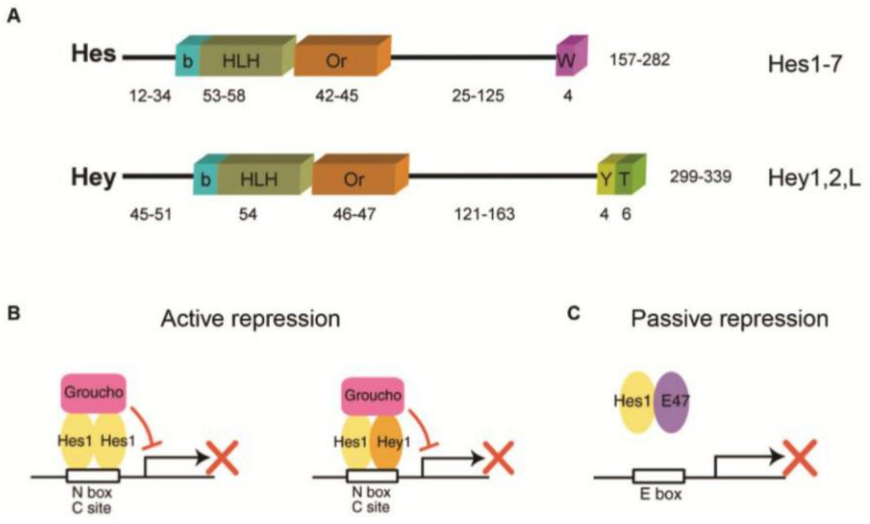


Figure 10. **(A)** Diagram representing Hes and Hey domains. Note the conserved bHLH and Orange domain. The main difference relies in the motif present at the C-terminus region. **(B)** Active repression of Hes/factors requires DNA binding of homo or hetero dimerization. **(C)** Passive repression of Hes/Hey does not require DNA binding and they sequester type I bHLH. Adapted from Fischer and Gessler, 2007; Kageyama et al., 2007.

Hes factors bind with high affinity to E-box class C or N-box. Hey1, due to the presence of a glycine residue in the basic domain has preference to class C or class B E-boxes (Iso et al., 2003). The repressive function can be either active or passive. Active repression involves DNA binding, whilst in passive repression Hey/Hes proteins sequester bHLH type I family and impair their heterodimerization with class II bHLH (Iso et al., 2003; Figure 10 B and C).

During development, several Hes and Hey genes are expressed in the inner ear. Hes5 is the major Notch target expressed during lateral inhibition. It is detected in the precursors that are not selected as neurons or hair cells. Its expression correlates well with that of Delta1 in nascent neurons and hair cells (Petrovic et al., 2014). In contrast, Hey1 is expressed in the prosensory epithelium, concomitantly with Jagged1 (Petrovic et al., 2014). Knockout mice of different Hey and Hes factors exhibit supernumerary hair cells in the cochlea, suggesting a repressor function during hair cell development. Notch inhibition of damaged sensory epithelia favours HC regeneration (Lin et al., 2011), suggesting that these factors may also regulate the ability to regenerate HCs.

4.3. Atoh1 and the development of hair cells.

In Drosophila, members of the **Achaete-Scute complex** (AS-C) are proneural genes required for the formation of external sensory organs and some multidendritic neurons (Romani et al., 1989), but not for **chordotonal organs,** which are muscle stretch-receptor organs (Dambly-Chaudière and Ghysen, 1987). Genes associated to the formation of chordotonal organs were identified by PCR amplification of Drosophila genomic DNA by using degenerate AS-C primers. This allowed the isolation of **atonal** as a chordotonal proneural factor that drives the production of chordotonal organs, one subset of olfactory sensory organs, and also some multidendritic neurons in Drosophila (Hassan and Bellen, 2000; Jarman et al., 1993).

The vertebrate atonal homolog 1 was originally named Math1 (mouse), Xath1 (Xenopus), Cath1 (chicken) and Hath1 (humans), but the current nomenclature for all is Atoh1 (Cai and Groves, 2015; Jarman et al., 1993; Mulvaney and Dabdoub, 2012). Atonal in Drosophila is described as a 1 kb open reading frame gene with predicted protein of 312 amino acids and 34 kDa, with the bHLH domain at its carboxyl terminus (Jarman et al., 1993). The mouse Atoh1 consists of an intron-less 1.053-kb coding region that produces a protein 351 amino acids and 37 kDa (Mulvaney and Dabdoub, 2012). From the two regions important for atonal function, the basic domain is crucial for the development of Atoh1-dependent cell lineages. The bHLH motif of atonal is highly similar to other bHLH proteins: 46% identity with Scute and 30% with Daughterless (homologous to mammalian E-prots). Electrophoretic mobility shift assays show that Atoh1 can form heterodimers with the ubiquitously expressed bHLH protein E47 to bind to E-boxes (Jarman et al., 1993).

In the C-terminus, Atoh1 contains highly conserved serine rich domains, which are susceptible to phosphorylation. Phosphorylation at the C-terminus region controls Atoh1 stability (Akazawa et al., 1995; Cheng et al., 2016; Forget et al., 2014a). In addition to the C-terminus, comparison of the N-terminus between mammals demonstrates that this region is well conserved. Regarding the degree of conservation between the Atoh1 sequence and the fly atonal, the bHLH is high conserved (70% sequence identity), but the C and N-terminus are not.

Vertebrate Atonal homologs were originally identified as eight members. Three of them Atoh1, Atoh7 and Atoh8 have now been designated as **Atoh family members**, the remaining being reassigned to a closely related **neurogenic differentiation factor**

family, which includes Neurog1 and NeuroD (Cai and Groves, 2015). Atoh2 and Atoh3 are now named NeuroD6 and NeuroD4, respectively, and similarly, Atoh4 and Atoh5 are Neurog2 and 3 (Cai and Groves, 2015). In mammals, Atoh1 was first shown to be required for the development of the external granular layer of the mouse cerebellum (Ben-Arie et al., 1997). During development, Atoh1 is expressed in touch receptor cells of the skin (Merkel cells), auditory and vestibular sensory epithelia (Bermingham, 1999; see BOX VII), dorsal neural tube and gut (Cai and Groves, 2015).

BOX VII. Atoh1 function in the gut, skin and central nervous system

Besides the developing inner ear, Atoh1 is also expressed in the gut, skin and in other structures of the CNS. **Gut:** Atoh1 is expressed in the mouse intestinal epithelium from embryonic to adult stages. Its function is necessary for the differentiation of the secretory lineages in the intestine, which include the goblet, Paneth, and enteroendocrine cells. In the absence of Atoh1, mice fail to form intestinal secretory cells; instead they undergo a fate switch and become absorptive cells. **Skin:** Atoh1 is required for the development of Merkel cells of touch receptors that mediate slowly adapting type I (SAI) responses. **Central Nervous System:** Atoh1 is first expressed in the dorsal neural tube adjacent to the roof plate, extending from the midbrain/hindbrain boundary along the whole length of the neural tube. As the embryo develops, Atoh1 expression in the metencephalon becomes restricted to the rhombic lip. Neurog1, whose expression is driven to the dorsal part of the neural tube, never overlaps with Atoh1 or Mash1 domains. These factors mutually exclude each other when overexpressed in ectopic domains, and they specify different neuronal subtypes in the dorsal neural tube (Gowan 2001).

The analysis of Atoh1 targets in the cerebellum led to the identification of a consensus Atoh1 binding motif based on an extended E-box variant (G/A,C/A,CA,G/T,C/A,TG,G/T,C/T) that is reminiscent of, but not identical to, that of Drosophila atonal (CAGGTG) (Klisch et al., 2011; Powell et al., 2004). The **At**oh1 **E**-box **A**ssociated **M**otif, named the **AtEAM**, is present close to the coding region of over 65% of genes bound by Atoh1 in the cerebellum. Likely, Neurog1 and NeuroD1 also have their own conserved extended E-box DNA binding motifs and subtle differences in the binding affinities have strong consequences on target selection (Seo et al., 2007). Differences in the range of targets determine the fate, either neuron or hair cell, specified by different bHLH factors (Raft and Groves, 2015). In order to bind to the AtEAM sequence, Atoh1 must dimerise with type I bHLH E-proteins (Jarman et al., 1993; Klisch et al., 2011). In Drosophila, the core sequence of the E-boxes is crucial to confer specificity to AS-C and atonal. Changes in the E-box core make genes unresponsive to the corresponding proneural bHLH. Similarly, the two nucleotides flanking the core of six nucleotides composing the E-box are essential for maintaining its function (Powell et al., 2004).

In the inner ear, Atoh1 expression foreshadows hair cell development (Fig. 11). **Loss of function** experiments in mice show that Atoh1 is required for HC development, hair cell progenitors rapidly dying in mutant mice (Bermingham., 1999; Cai and Groves, 2015; Lumpkin et al., 2003; Pan et al., 2012; Woods et al., 2004; Fig. 12A). Different effects of Atoh1 on HC development have been described using conditional mutants. Early deletions induce the loss Myo7a expression and the failure of stereocilia formation, whilst loss of Atoh1 at late stages leads to HC death (Chonko et al., 2013). In the cochlea, Atoh1 is expressed after prosensory cells withdraw from cell cycle. Prosensory cells divide until the onset of the cell cycle inhibitor p27kip, which is expressed in an apex-to-base wave (White et al., 2006). Indeed, Atoh1 is not necessary for the establishment of the post-mitotic cells in the primordium of the organ of Corti. The mutation of Atoh1 does not affect the post-mitotic sensory primordium, even though the subsequent generation of hair cells is blocked in this mutant. The cells that no proliferate but remain in the epithelia do not express the supporting cell marker Jagged1(Chen et al., 1998).

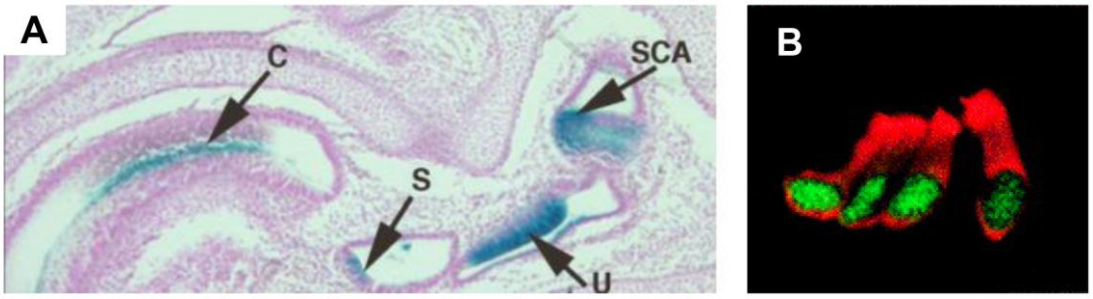


Figure 11. Atoh1 is expressed in nascent hair cells. (A) Math1$^{\beta\text{-Gal}/\beta\text{-Gal}}$ mice show Atoh1 expression throughout all the sensory primordium in E12.5 mice. **(B)** Immunohistochemistry shows Math1 protein in the nuclei of HCs in an E16.5 cochlea (Green) MyosinVIIa in the cytoplasm (Red). Cochlea (C), saccule (S), utricle (U), and semicircular canal ampullae (SCA) (Bermingham., 1999; Lumpkin et al., 2003).

The **gain of function** of Atoh1 leads to supernumerary and/or ectopic HC formation (Izumikawa et al., 2005; Kawamoto et al., 2003; Kelly et al., 2012; Liu et al., 2012; Woods et al., 2004; Zheng and Gao, 2000; Fig. 12B). In summary, Atoh1 is necessary and sufficient to drive HC commitment and differentiation and it is considered as a master gene for HC formation.

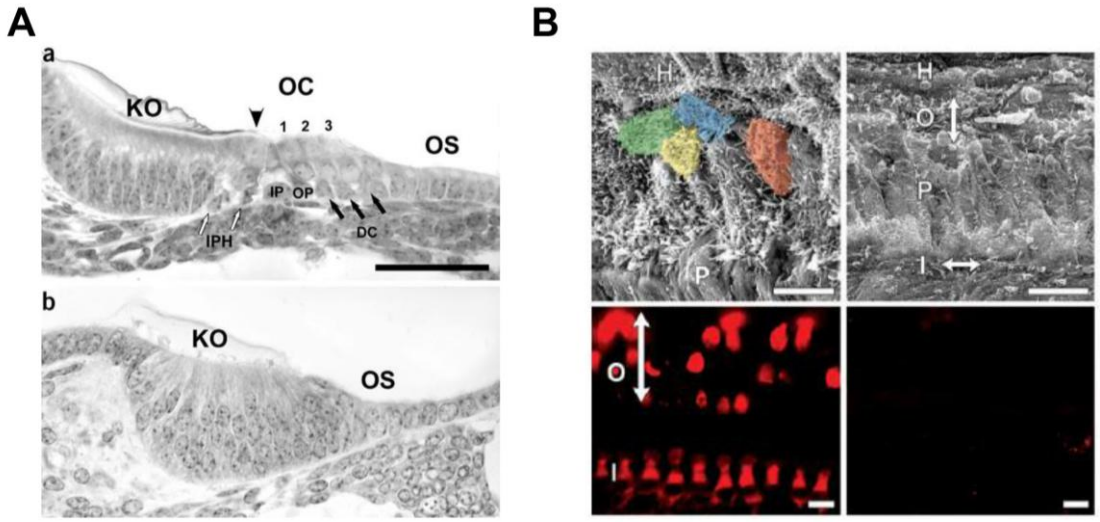


Figure 12. Atoh1 is necessary and sufficient for hair cell formation. (A) Cross-section through the cochlear duct in wild type (upper) and Math1-null mice (lower) at P0. Hair cells and four different types of supporting cells: the inner phalangeal cells (IPH; white arrows), inner pillar cells (IP), outer pillar cells (OP) and Deiter's cells (DC; black arrows). The Atoh1-null mouse shows no hair cell formation and no cells with supporting cell morphology. OC: organ of Corti. KO: Kolliker's Organ and OS: Outer Sulcus. **(B)** Deaf mice cochleae 4 weeks after Atoh1 treatment with a virus carrying the Atoh1 gene, Left, treated cochlea. Right, contralateral untreated ear. P: pillar cells; H: Hensen cells, O: Outer hair cells; I: Inner hair cells (Bermingham, 1999; Izumikawa et al., 2005; Woods et al., 2004).

5. The regulation of Atoh1 and hair cell formation.

Atoh1 is the first gene expressed in nascent hair cells, and precedes other genes like Myo7a, Pouf4f3, Gli3 that are characteristic of this cell type. A recent and comprehensive cell type-specific RNA-Seq study has documented their specific expression in HCs, along with other newly identified genes like Grxcr2 or Lmod3 (Scheffer et al., 2015). Most of these genes are expressed in inner ear sensory cells and not in other cells of the labyrinth (Ryan et al., 2015). How gene transcription is directed specifically to hair cells is unclear and this has made Atoh1 regulation a subject of intense study.

5.1. The regulation of Atoh1 transcription: the 3'Atoh1 enhancer

Pioneering work by Jane Johnson discovered a region in the Atoh1 genome landscape that recapitulated Atoh1 expression during mouse and chicken development (Helms et al., 2000). Transgene expression in mouse identified a region that directed the expression to the neural tube, external granular layer (EGL) of the cerebellum from rhombic lip, and the developing hair cells of the cochlea and semicircular canals. The region comprised a length of 21 Kb, and fragment analysis allowed the identification of a 1.7 Kb fragment located 3.4 Kb 3′ of the Atoh1 coding region that recapitulates the expression of Atoh1. This region is called the 3'Atoh1-enhancer (3'Atoh1-enh).

Two regions within the 3'Atoh1-enh show a high homology between humans and mouse, and they were named **Enhancer A** and **Enhancer B** (EnhA and EnhB). The length of A and B is highly conserved in species like chicken, mouse and human (Ebert, 2003; Fig. 13), although the distance in between EnhA and EnhB varies in these three species (Fig. 13A).

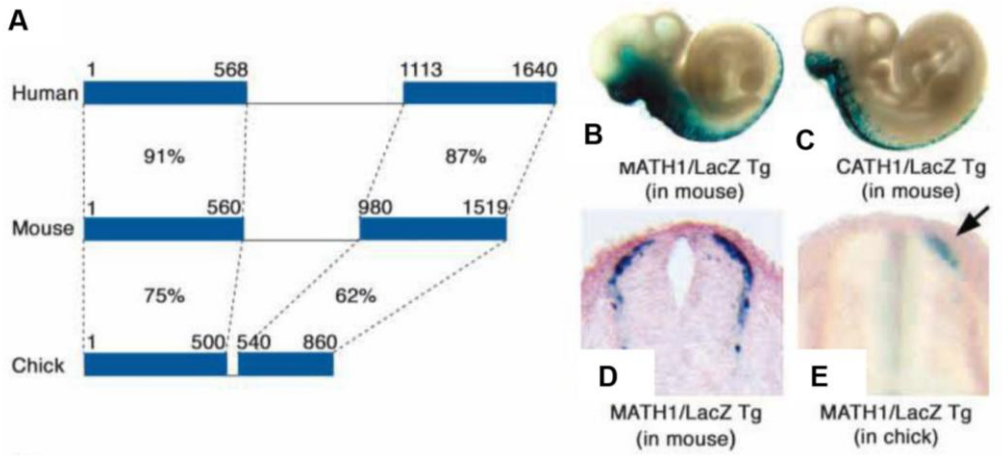


Figure 13. The 3' Atoh1 enhancer is conserved across vertebrates. **(A)** Percentage of conservation between mouse, human and chicken. **(B-C)** Mouse (Math1) and chick (Cath1) enhancers have the same activity in E10.5 mouse embryo. **(D-E)** Transverse section of the neural tube in mouse and in a chicken embryo electroporated with the Math1 enhancer (Ebert, 2003).

Interestingly, Helms et al., (2000) observed that transgenic embryos for Atoh1-enh/lacZ transgenic mice had no detectable β-gal activity in the Math1 null background, and this was shown to be also the case for the continued expression of Atoh1 in hair cells (Raft et al., 2007). This suggested that the activity of the 3'Atoh1 enhancer is dependent on

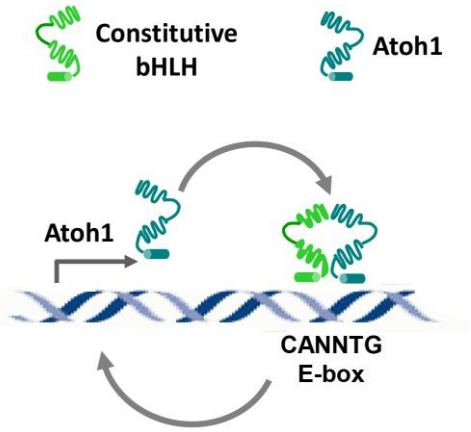


Figure 14. Atoh1 autoregulatory loop. Atoh1 is able to activate its own transcription by binding to a class A E-box located in the Enhancer B. Atoh1 requires dimerization with constitutive class I bHLH such E47 to promote transcriptional activity.

Atoh1 expression. In other words, **autoregulation** is the major mechanism for setting Atoh1 transcriptional activity (Fig. 14).

The 3'Atoh1 enhancer contains several E-boxes. One degenerated E-box in Enhancer A, and three E-boxes in Enhancer B. The E-boxes in Enhancer B are a class A, a class C and a reversed N-box. All three boxes are very close, and class A and N-box overlap (Fig.15). We have called the region that contains these three E-boxes the **CAN region**, due to the presence of **C**, **A** and **N**- boxes (see results). As mentioned above, Helms et al., (2000) already identified that Atoh1 autoactivation depends on the class A E-box located in Enhancer B.

Atoh1 autoactivation also occurs in Drosophila atonal (Sun et al., 1998), where several E-boxes are crucial for maintaining the autoregulatory loop. They are located 3' and 5' to the atonal coding region. The 3'enhancers are responsible for driving atonal expression in proneural clusters, whereas successive modular enhancers located in the 5′ region drive tissue-specific expression in chordotonal organ precursors in the embryo and larval leg, wing and antennal imaginal discs (Sun et al., 1998). In mammals only one enhancer has been identified in the 3' region and this may be related to the lack of proneural function of Atoh1 and other bHLH genes in mammals discussed above (BOX V).

Besides the CAN region, the 3'Atoh1-enh region contains candidate binding sites for a menagerie of transcriptional activators and repressors. Some of them like Sox2, Six1/Eya1, and β-catenin bind directly to the enhancer as shown by biochemical assay (Akazawa et al., 1995; Briggs et al., 2008; D'Angelo et al., 2010; Ebert, 2003; Mutoh et al., 2006; Shi et al., 2010).

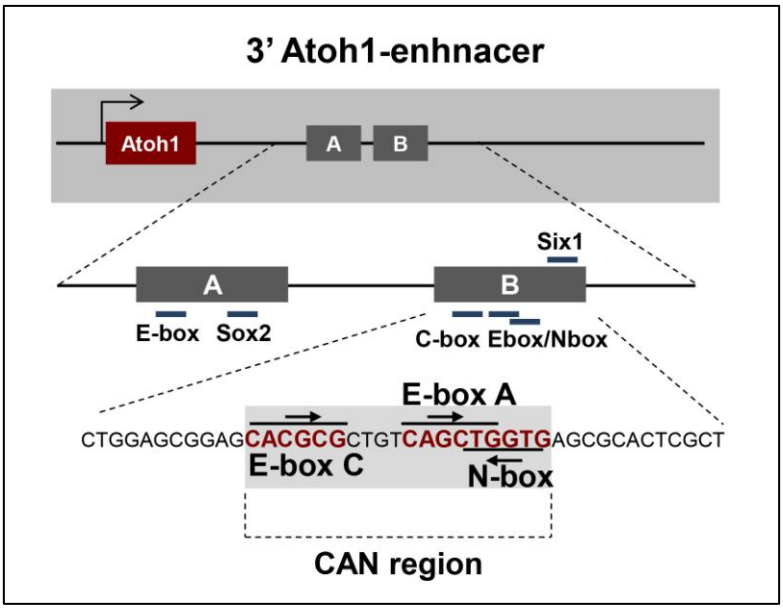


Figure 15. The 3'Atoh1 enhancer. Located 3.5 Kb downstream Atoh1 coding region, the enhancer consists of two main conserved regions named Enhancer A and Enhancer B. Within these two regions there are several putative binding sites that are predicted to be the place of bHLH repression (E-box C, E-box A and N-box: the CAN region). Other relevant consensus sequences are the binding site for Sox2 located in Enhancer A, for Six1 located in Enhancer B, and the E-box A, which is the site for Atoh1 autoregulation.

Sox2 is sufficient to activate Atoh1 and to induce ectopic hair cell formation in the chick otocyst (Neves et al., 2012). Sox2 is rapidly downregulated in hair cells as they differentiate. This downregulation is probably required for further maturation of hair cells because sustained expression of Sox2 in Atoh1 expressing cells blocks the induction of later hair cell markers such as Myosin-VIIa (Dabdoub et al., 2008). **Six1** and its transcriptional co-activator **Eya1** are expressed in the prosensory domain of the cochlea, and they bind directly to the 3'Atoh1-enh (Ahmed et al., 2012a). These two factors are sufficient to induce Atoh1 expression in competent regions and their activation is potentiated by Sox2 (Ahmed et al., 2012a). As described before, Neurog1 is also upregulated by Six/Eya with Sox2 (Ahmed et al., 2012b).

β-catenin, another activator of the 3'Atoh1 enhancer, binds directly to the enhancer (Shi et al., 2010). Manipulation of β-catenin in supporting cells of mature mouse cochlea induces de novo formation of hair cells (Shi et al., 2012), highlighting the importance of the 3'Atoh1 enhancer for hair cell regeneration (see below).

Chromatin structure and histone modifications are important for regulating gene expression. Histone H3-methylation marks different states of the chromatin. Tri-methylation of lysine 27 (H3K27m3) is a repressive mark, trimethylation of lysine K4

(H3K4m3) being a permissive one. The simultaneous modification of both methylation activities is associated with different states of gene activation, including the state in which a gene is silent but **poised** for developmentally regulated expression. This state of the chromatin is called **bivalent** (Azuara et al., 2006). Segil's group showed that such epigenetic marks occur in the Atoh1 locus. Atoh1 changes from poised in the progenitor state to an active state during hair cell development. Interestingly, the bivalent marks in Atoh1 found in the progenitor state are maintained in the perinatal supporting cells. This may be related to the latent capacity of these cells to transdifferentiate into hair cells (Stojanova et al., 2015).

5.2. Post-transcriptional regulation of bHLH protein.

Developmental studies are frequently focused on transcriptional regulation for studying gene function. However, post-transcriptional mechanisms are becoming more and more important to understand regulatory gene interactions. After transcription, several mechanisms may control a gene expression such mRNA splicing, mRNA stability, translation or post-translational events that affect protein stability and function (Day and Tuite, 1998).

Regarding mRNA regulation not only the rate of RNA synthesis is important for controlling gene expression. The half-life of individual mRNAs within a given eukaryotic cell may vary, from a few minutes to many hours, in some cases days (Day and Tuite, 1998). The **stability of mRNA** can be controlled by specific or intrinsic nucleotide sequences and is globally regulated by **RNA-binding proteins** that bind mRNAs. Furthermore, the half-life of many mRNAs can fluctuate in response to developmental or environmental stimuli such as nutrient levels, cytokines, hormones, temperature shifts and viral infection (Day and Tuite, 1998). For instance, Drosha protein regulates the amount of Neurog2 mRNA by degrading it. In this way, it prevents neuron differentiation by not permitting Neurog2 accumulation in neural progenitors (Knuckles et al., 2012). Also, E47 RNA stability changes in young versus aged B cells (Frasca et al., 2005). Therefore, specific mechanisms may regulate RNA stability. **miRNAs** (micro RNA) bind to target messenger RNA (mRNA) transcripts, and either destabilize the targets or lower their translation efficiency. Non-syndromic autosomal hearing loss has been associated with mutations in miR-96 in *Diminuendo* mice and DFNA50 human families (Lewis et al., 2009; Mencía et al., 2009), where hair cells are born but fail to fully differentiate (Kuhn et al., 2011). miRNAs are modulators of protein expression

levels rather than “off” switches, and several studies have attempted to understand their role in hair cell formation (Groves et al., 2013).

Translation initiation is an important step in both global and mRNA-specific gene regulation. Global regulation of protein synthesis is generally achieved by the modification of eukaryotic initiation factors (eIFs). Some initiation factors are phosphoproteins (Day and Tuite, 1998). Little is known about the regulation of translation during embryonic development.

Protein degradation is strictly regulated and a potential source of gene regulation. Atoh1 contains in the C-terminus a potential PEST sequence (Jarman et al., 1993). This is a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T). This sequence is associated with proteins that have a short intracellular half-life and it is hypothesized that the PEST sequence acts as a signal peptide for protein degradation. Atoh1 protein stability is very short and it is extinguished in 2 hours after protein synthesis blockade (Cheng et al., 2016; Forget et al., 2014a).

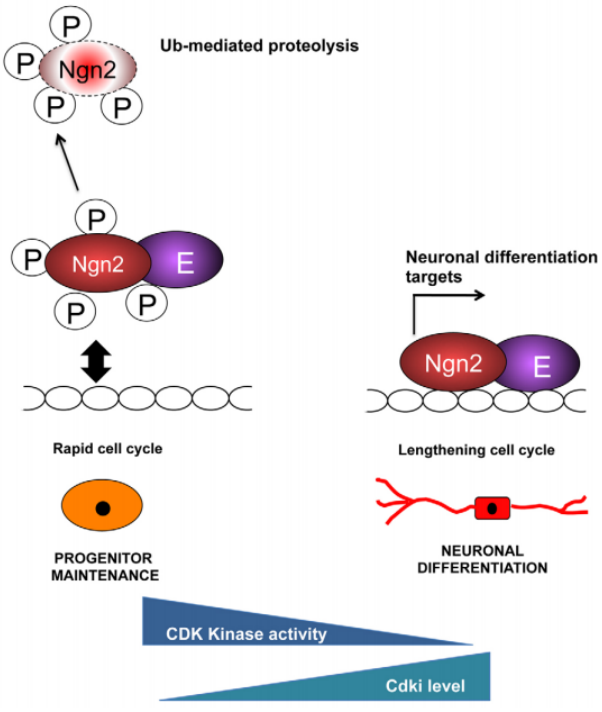


Figure 16. bHLH regulation by cdks. Model proposed by Philphott’s group were phosphorylation of Ngn2 protein occurs in rapid progenitor cell cycles. Cell cycle lengthening results in an accumulation of unphosphorylated Neurog2. This results in the activation of downstream targets and in neural differentiation. E, E-protein; Ub, ubiquitin; P, phosphorylation (Ali et al., 2011).

Aminoacid residues located at the C-terminus region of Atoh1 protein that are susceptible to phosphorylation are conserved among different species (Mulvaney and Dabdoub, 2012). In medulloblastoma cell lines, Shh regulates Atoh1 stability by preventing its phospho-dependent degradation by the E3 ubiquitin ligase Huwe1 through Serine 328 and 339 (Forget et al., 2014a). This is also the case for the phosphorylation of residue S334 in 293T cells (Cheng et al., 2016). Other bHLH proteins like Neurog2 and NeuroD4 are also less stable upon phosphorylation (Hardwick and Philpott, 2015; Hindley et al., 2012).

Cyclin-dependent kinases (Cdks) drive cell cycle progression and are known to target Serine Proline (SP) and Threonine Proline (TP) sites (Errico et al., 2010). bHLH proteins like Atoh1 and Neurog1 contain several putative ST and TP in their C and N-terminal regions. Phosphorylation in these SP/TP residues may be crucial for regulating activity and is linked to the cell cycle. For example, in Xenopus embryos and P19 cells, progenitor cells that divide rapidly show Neurog2 phosphorylation and degradation, whereas when cell cycle is lengthened, Neurog2 accumulates and activates down-stream targets (Fig. 16; Ali et al., 2011). Cell cycle exit in the cochlea is dependent on the expression of the cyclin inhibitor p27kip (White et al., 2006), and it is possible that Atoh1 is degraded in dividing prosensory progenitors until p27kip expression and cell cycle withdrawal .

Trim71 is an E3-ligase that cooperates with the microRNAs (miRNAs) machinery and promotes embryonic stem cell proliferation and maintenance. Evsen et al. (2016) recently found that over-expression of Lin-41 (TRIM71) in the chick auditory organ leads to excess progenitor cell proliferation and an inhibition of HC differentiation. This suggests that TRIM factors may regulate crucial steps in hair cell development, including Atoh1 production and stability (SDB 75th Annual Meeting-Abstract).

In summary, available information stand out that post-transcriptional modifications can affect dramatically the production and stability of bHLH factors including Atoh1 and, therefore, their function in the cell.

6. Hair cell regeneration

6.1. Deafness and Hair cell loss

Hearing loss is a major problem affecting more than 360 million people in the industrialized world (WHO). It affects speech and language and leads to severe deficits in communication. The consequence is a strong negative impact in the quality of life, including social integration. Hearing impairment is mainly caused by the failure of hair cells and/or otic neurons (sensorineural hearing loss), hair cell damage being the most frequent triggering factor. Hair cell damage arises from genetic defects, aging, noise, traumatic lesions, infections or therapeutic substances. The main problem of hair cell damage is that, unlike other animal species, humans are not able to regenerate hair cells of the auditory epithelia after damage and there is no treatment for hearing deficiencies in humans.

6.2. Hair cell regeneration and the lack of regenerative capacity of the adult mammalian cochlea

The adult mammalian cochlea shows no capacity for regenerating hair cells or neurons. In contrast to mammals, non-mammalian vertebrates like chicken, zebrafish or lizards, are able to repair and heal damaged sensory epithelia (Fig. 17). In the chick, damaged hair cells trigger supporting cells to replace lost hair cells by two different mechanisms: 1) **mitotic regeneration**, where SC divides asymmetrically and one daughter cell remains as SC and another as HC, and 2) **transdifferentiation** of SC into HC (Fig. 18). In transdifferentiation, HCs are generated at the expense of SCs, which become exhausted and hence, the epithelium is disorganized. The consequence is that, although HCs are recovered, hearing function is not (Stone and Cotanche, 2007). In birds, hair cell regeneration starts with direct transdifferentiation of SCs into HCs, followed by mitotic regeneration. This results in the correct replacement of the sensory epithelium and auditory function (Roberson et al., 2004).

Figure 17. Hair cell regeneration in the adult chicken basilar papilla after noise-induced hair cell loss. Scanning electron microscopy analysis conducted on the 1500 Hz region of chicken basilar papillae. **(A)** Control before exposure to 1500 Hz pure tone. **(B)** 24 hours after acoustic trauma shows hair cell damage. **(C)** 10 days after damage shows regeneration of basilar papilla. Arrowheads indicate new stereociliary bundles (Rubel et al., 2013).

Vestibular and auditory regions of the mammalian inner ear show different behaviours regarding their regenerative capacity. After damage, hair cells from the vestibular region retain a limited capacity for regeneration (Forge et al., 1993; Lin et al., 2011). Within the mouse utricle, there is a pool of stem cells, which are able to form spheres and differentiate into a hair cell-like in adequate culture conditions (Li et al., 2003). Regeneration in the utricle is favoured by treatment with Notch inhibitors (see below). In contrast to the utricle, the adult organ of Corti lacks this regenerative capacity (Groves, 2013; Sobkowicz et al., 1992). There is a small cell population that retains a stem cell identity after birth, but it lasts only few days (Maass et al., 2015; Shi et al.,

2012). They correspond to a population of supporting cells that express Lgfr5, a marker of adult stem cells (Shi et al., 2013).

In summary, non-mammalian vertebrate species are able to re-activate the machinery to regenerate damaged auditory and vestibular epithelia. Although mammals have some capacity to regenerate hair cells in the vestibular organs and the early post-natal cochlea, the adult auditory organ is completely devoid of this capacity.

The question arises as to what are the differences between birds and mammals that explain their different regenerative capacity. Are there signals that regulate SC quiescence and activation after HC loss in chicken? Are they similar to mammalian early post-natal regeneration? Why mammals loose the capacity of regeneration after birth?

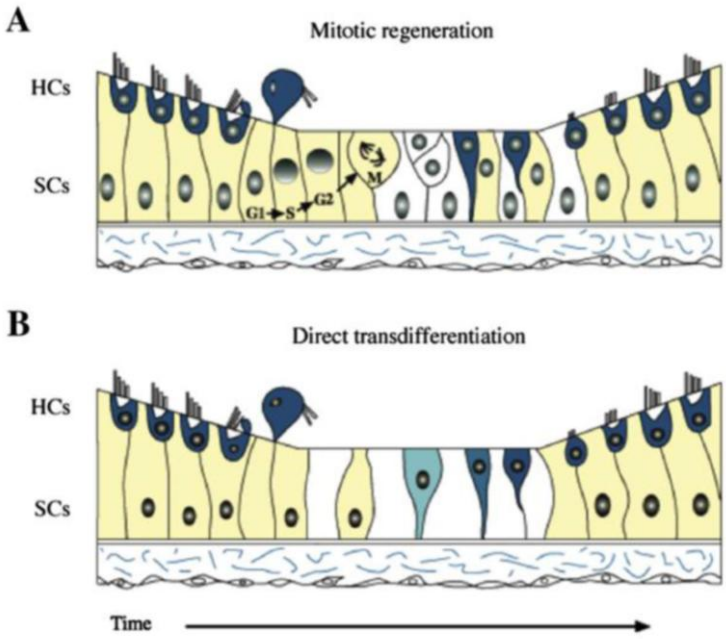


Figure 18. Hair cell regeneration from supporting cells after damage in the chick. (A) Dividing supporting cells can give rise to both hair cells and supporting cells by asymmetric cell division. **(B)** Direct conversion of supporting cells to hair cells by transdifferentiation (Rubel et al., 2013).

6.3. Development as a tool for regeneration

Studies in the chick have shown that hair cell regeneration reutilises mechanisms that operate during embryonic development. Several molecular pathways known to regulate embryonic HC progenitors are reactivated in mature chicken epithelia after HC loss. In the mature chicken basilar papilla, upon HC damage, Atoh1 becomes reactivated in

transdifferentiating and mitotically active SCs (Cafaro et al., 2007). Atoh1 reactivation is essential to form new hair cells, like it is to form hair cells during development (Bermingham, 1999). Notch signalling is down-regulated upon damage in the basilar papilla suggesting that in the mature organ it maintains a repressive sate that prevents Atoh1 expression. In agreement, different laboratories have shown that treatment with Notch inhibitors favours Atoh1 reactivation and HC regeneration in the chick basilar papilla and also in the post-natal mammalian cochlea (Cafaro et al., 2007; Mizutari et al., 2013).

A major difference between the regenerating avian cochlea and the non-regenerating mammalian counterpart is the ability of mammalian supporting cells to re-start proliferation upon damage. Supporting cells in both the neonatal and mature mammalian cochlea are mitotically quiescent (Atkinson et al., 2015). Mammalian SCs cannot down-regulate the cell cycle inhibitor p27kip after damage (Liu et al., 2012b). Therefore, damaged cells cannot re-enter the cell cycle, and remain in the SC state (White et al., 2006). Contrarily, in zebrafish p27kip is downregulated after damaged and this promotes HC regeneration (Rubbini et al., 2015). Although the effect of blocking cell cycle inhibitors in the damaged cochlea is unclear, these studies position p27Kip1 as another potential target to induce supporting cells division prior to the differentiation of regenerating hair cells.

In summary, Atoh1 upregulation, activation of the Notch pathway and cell cycle exit, which are three main steps during hair cell development, are also reactivated during hair cell regeneration. This stresses the value of developmental studies for designing rational approaches for treatments for hearing loss, some of which are briefly discussed below.

6.4. Strategies for HC regeneration

Forced Atoh1 gene expression favours de novo HC formation in mature guinea pigs and in rats by direct SC transdifferentiation into HCs (Kawamoto et al., 2003; Zheng and Gao, 2000). In vivo inoculation of adenovirus carrying Atoh1 into the endolymph results in the differentiation of immature hair cells in the organ of Corti and new hair cells in adjacent competent regions. Infected Atoh1 cells are also able to differentiate and improve hearing thresholds in damaged cochlea (Izumikawa et al., 2005). This was the first evidence supporting Atoh1 gene delivery transfer as a realistic therapy for treating hearing loos associated to hair cell damage. This possibility was further

explored by driving Atoh1 expression to the developing mouse inner ear by Atoh1 gene transfer in utero, which resulted in functional supernumerary hair cells. However, those mice showed elevated auditory thresholds, which is similar to what happens to mutant mice carrying extra rows of HCs (Brigande and Heller, 2009). Sustained Atoh1 expression in HCs also induces cell death (Liu et al., 2012b) as it does the failure to down-regulate Atoh1 in differentiating hair cells (Atkinson et al., 2014). Therefore, although uncontrolled Atoh1 expression produces new HCs, it does not necessarily restore hearing impairment.

As shown above, the 3'Atoh1 enhancer is under the control of many transcription factors such as Sox2, WNT/β-catenin or Six and Eya1, which bind directly to the 3'Atoh1 enhancer. The overexpression of β-catenin forces HC differentiation in a subpopulation of supporting cells that express Lgr5 (Shi et al., 2013) and the combined expression of Atoh1 and β-catenin in neonatal Lgr5+ cells increases by 10-fold the efficiency to generate new HCs in the cochlea (Kuo et al., 2015). The combination of Atoh1, Gfi1 and Pou4f3 has been also shown to improve HC formation in mouse embryonic stem cells *in vitro* and embryonic chick inner ear development by direct transdifferentiation (Costa et al., 2015). This highlights the importance of combining different transcription factors rather than manipulating a single gene for addressing new therapeutical approaches.

Several drug treatments seem to awaken dormant SCs by modifying **Notch signalling** or inhibiting **cyclin inhibitors**. In the mouse, Notch inhibitors favour the upregulation of Atoh1 and *de novo* formation of hair cells (Mizutari et al., 2013). As mentioned above, downregulation of p27kip in zebrafish SCs occurs before hair cell regeneration. Targeted ablation of the p27Kip1 gene leads to the production of supernumerary supporting and hair cells during development in mouse cochlea (Lowenheim et al., 1999). It is conceivable then to develop therapeutic approaches based on drugs that down-regulate Notch activity or p27kip in the inner ear. This strategy will have to deal with potential problems derived from targeting candidate molecules to the supporting cells and crossing the blood-brain barrier.

Given the lack of endogenous regeneration and the limited therapeutic range available, treatments based on the delivery of exogenous cells offer an alternative approach. The idea is to provide the damaged epithelia with hair cell-like or precursor cells that integrate in the epithelia and restore the auditory function. **Human stem cells** constitute a reasonable alternative to replace damaged hair cells. Major problems of

this approach are the difficulty to deliver treated cells to the damaged areas and their limited ability to integrate in the epithelium. Several groups have developed protocols to differentiate hair cells by mimicking the hair cell development in the embryo. This has proved successful, however the efficiency of the procedures is still very low (Chen et al., 2012; Ronaghi et al., 2014).

The first reports of otic guidance with monolayer cultured human ESCs (hESCs) revealed a propensity to differentiate along an otic neurogenic lineage rather than HC lineage (Chen et al., 2012). This problem is directly related to the question addressed in the present work, during early stages of development Neurog1 prevails over Atoh1, thereby forcing neurogenesis and delaying sensorigenesis. Since otic fate is acquired early on in inner ear development and hair cells do not differentiate until neurogenesis has taken place, it looks that the predefined fate is to become a neuron and that sensory competence is silenced. If this is so, the consequence is that hair cell production relies mainly on relieving the repression of hair cell competence rather than on the expression of activators.

AIMS

The functional unit of the ear is composed of hair cells, supporting cells and neurons. Hair cells are the transducers of sound and balance stimuli, and otic neurons convey this information to the brain. Their formation is tightly regulated during development. Both, neurons and hair cells develop from neurosensory progenitors that first generate neurons and, later on, hair cells and supporting cells. Neuronal and hair cell development is driven by proneural bHLH factors Neurog1 and Atoh1, respectively.

The competence to generate these different cell types is established early in development by the induction of both Neurog1 and Atoh1 in the otic placode. However, the onset of Atoh1 expression is delayed with respect to neurogenesis, until later stages of development. The question addressed in this work is to understand the molecular mechanism behind this delay.

When this work started, it was already known that the expression of Atoh1 is mostly recapitulated by a 3'Atoh1-enhancer located 3,5Kb downstream Atoh1 coding region. This enhancer is composed by two regulatory regions named Enhancer A and Enhancer B, and Atoh1 is able to activate its own expression by binding to a class A E-box located in the Enhancer B. This region also hosts different E-boxes that are putative binding sites for other bHLH proteins. Therefore, the work initially focused on the analysis of the interactions between the major neurogenic factor, Neurog1, and the 3'Atoh1 enhancer. Later on, it extended to other potential ways of interaction between Neurog1 and Atoh1, as well as on other bHLH factors.

Specific aims were:

a) To investigate the functional role of the 3'Atoh1 enhancer in vivo, and the contribution of regions A and B to Atoh1 regulation. This included the analysis of the Atoh1 landscape.
b) To analyse the contribution of the 3' Atoh1 enhancer to the repression of Atoh1 by Neurog1. In particular, to test whether Atoh1-autoactivation is prevented by Neurog1.
c) To correlate the effects of Neurog1 on the 3'Atoh1 enhancer with those on HC formation.
d) To explore the mechanisms by which Neurog1 interacts with the 3'Atoh1 enhancer, including the identification of the binding sites of Neurog1 and/or possible interactions between Neurog1 and other binding factors.

e) The results led to the study of mechanisms of repression other than binding to the 3'Atoh1 enhancer and to analyse the effects of Neurog1 on Atoh1 protein levels and stability.
f) Finally, the analysis extended to the effects of the prosensory gene Hey1 on the activity of the 3'Atoh1 enhancer and on hair cell formation.

The work involved the use of various techniques including ATAC-seq analysis, reporter assays, immunochemistry, immunoprecipitation and Mass-spec analysis, on different model systems like chick and mouse embryos and the P19 cell line.

The results show that the Enhancer B, besides being crucial to maintain Atoh1 autoactivation, is also the target for repression of both Neurog1 and Hey1. However, the repressor effect of Neurog1 is indirect and depends on the regulation of the levels of Atoh1 protein rather than on a direct interaction with the 3'Atoh1 enhancer. In this way, Neurog1 is able to suppress Atoh1 by decreasing the levels of the major activator of Atoh1, which is Atoh1 itself. Such a mechanism does not require DNA binding and, therefore, dissociates the repressor effect of Neurog1 on Atoh1 from the activation of the full neurogenic program. This new mode of interaction between bHLH factors may extend to other developmental contexts and have implications for hair cell regeneration.

RESULTS

CHAPTER I

NEUROG1 PREVENTS ATOH1 AUTOREGULATORY LOOP DURING INNER EAR DEVELOPMENT

These results have been submitted for publication in the journal *Development*, and the manuscript is under revision.

Table S1, S2, S3 and S4 are available in the accompanying CD.

Prevention of the Atoh1 autoregulatory loop by Neurog1

The expression of Atoh1 during inner ear development relies on an enhancer located 3.5Kb downstream its coding region, the 3'Atoh1-enhancer (Helms et al., 2000). Reporter activity of this enhancer is detected within the inner ear neurosensory domain (Neves et al., 2012). However, Atoh1 transcription is very low during neurogenesis and Atoh1 is not expressed until later stages of development when HCs start to differentiate (Lumpkin et al., 2003; Neves et al., 2013a). In the experiments that follow, we studied Atoh1 autoactivation and its regulation by Neurog1.

1. Atoh1 auto-activation depends on E-box regions located at Enhancer B.

Reporter gene analysis of the 3'Atoh1-enhancer (herein the 3'Atoh1-enh) was carried out in chicken otic vesicles and in P19 cells. After examining several cell lines, we decided to use P19 cells because they behave like the developing inner ear as to Atoh1 regulation. Further, P19 cells express considerable amounts of Sox2 and Hey1 (data not shown), mimicking the prosensory precursor state, and making them a suitable *in vitro* system for studying Atoh1 regulation. The 3'Atoh1-enh was activated by the overexpression of Atoh1, and this activation was dependent on the concentration of Atoh1 (Fig. 1A). Helms et al., showed that the E-box A (CAGCTG) present in the AtEAM motif of the Enhancer B mediates the autoactivation of Atoh1 (Helms et al., 2000). In agreement, the mutation of this E-box A abolished the ability of Atoh1 to activate the 3'Atoh1-enh reporter (Fig. 1C).

The AtEAM sequence is flanked by an E-box C (CACGNG) and by a reverse class N-box (CACNAG) that partially overlaps the E-box A (CANCTG) (Fisher et al., 1996; Iso et al., 2003 and Fig. 1B). The region containing these three E-box binding sites is referred hereafter as the CAN region. In order to test the importance of the E-boxes flanking the AtEAM motif, we mutated them and tested the resulting activity *in ovo* (Fig. 1C). Mutation of E-box C resulted in an increased activity of the reporter and, conversely, the combined mutation of E-box C, the AtEAM sequence and the N-box impaired the ability of Atoh1 to activate its own expression, mimicking the E-box A single mutation (compare E-box C mut and CAN mut in Fig. 1C). The double mutation of class C and N-boxes (without affecting the AtEAM motif) did not prevent Atoh1 autoactivation and did not overactivate the enhancer as did the E-box C mutation alone (compare E-box C mut and C-N box mut in Fig. 1C).

Together, the results suggest that the E-box C is important for 3'Atoh1-enh repression,

while the integrity of the E-box A and the overlapping N-box is required for Atoh1 autoactivation.

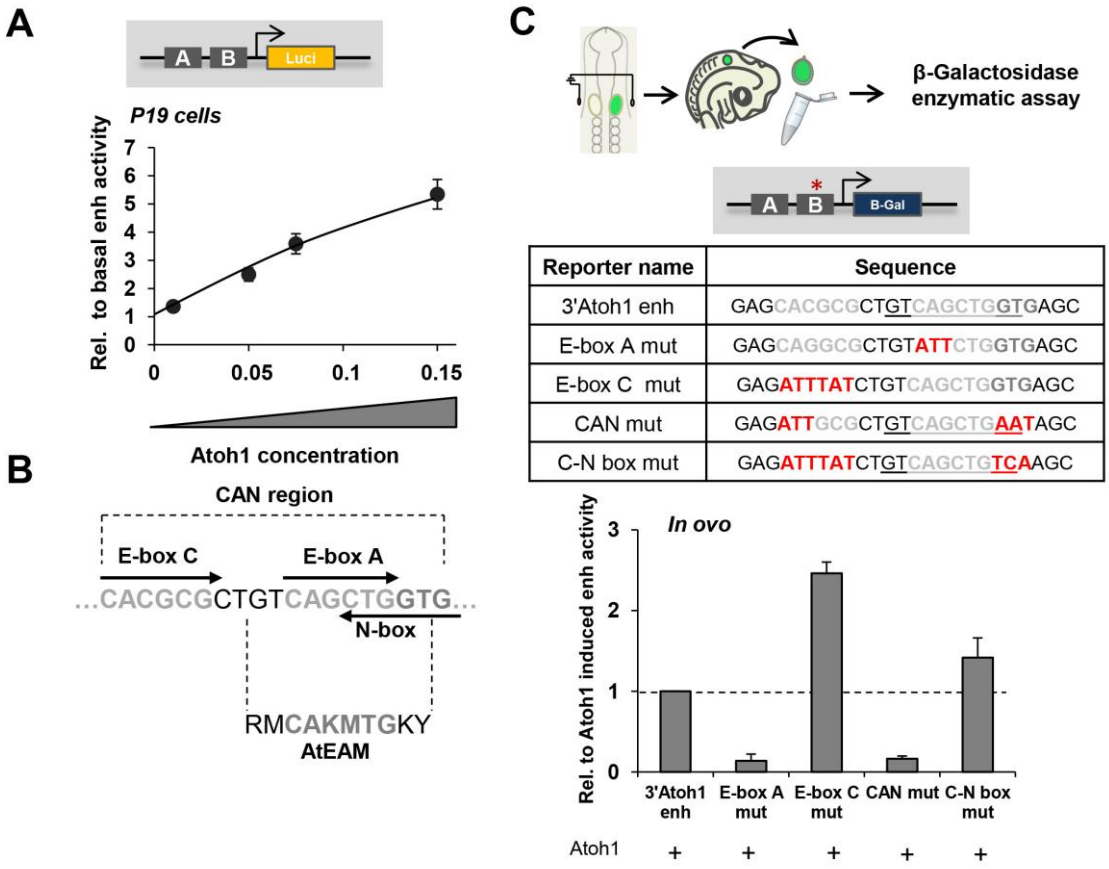


Figure 1. The 3'Atoh1-enh. (**A**) 3'Atoh1-enh activity depends on Atoh1 concentration. Quantification of the 3'Atoh1-enh reporter activity in the presence of increasing concentrations of Atoh1 in P19 cells. Values of luciferase activity (ordinates) are relative to those in the absence of Atoh1 (n=5-7). (**B**) The CAN region of the 3'Atoh1-enh is the region of EnhB that contains an E-box A, surrounded by an E-box C and a reversed and overlapping N-box. (**C**) The activity of the 3'Atoh1-enh depends on E-box A and its flanking regions. The graph shows the values of β-galactosidase activity after co-electroporation of Atoh1 and the different constructs displayed in the table in E2 chicken embryos (n=3-4). The β-galactosidase values of the experimental conditions were referred to the activated 3'Atoh1-enh by Atoh1. Mutations on E-box A and CAN abolished Atoh1-dependent activation, while mutation on E-box C caused the reverse effect. Mutation on E-box C and N-box without affecting the AtEAM showed no effect over Atoh1 activation (the two nucleotides following E-box A belonging to the AtEAM motif "GT" are replaced by "TC"). The AtEAM sequence is underlined in the table for the 3'Atoh1-enh, CAN mut and C-N box mut.

2. Neurog1 repression of Atoh1 autoregulatory loop occurs at the 3'Atoh1 enhancer

We then studied whether Neurog1 is able to repress Atoh1 expression and whether this can be accounted for by the interaction of Neurog1 with the 3'Atoh1-enh. In otic vesicles, the activity of the 3'Atoh1-enh is restricted to the neurosensory domain (Fig. 2Ab and Neves et al., 2012). Neurog1 abolished the activity of the 3'Atoh1-enh as shown by β-galactosidase staining *in ovo* and *in vitro* enzymatic quantification assays

(Figs. 2Ad, and 2B, compare values to the basal activity of the enhancer, dotted line). Neurog1 was also able to abolish the ability of Atoh1 to activate the 3'Atoh1-enh reporter *in ovo* and in P19 cells (Figs. 2B and 2C), suggesting that Neurog1 is sufficient for the repression of Atoh1 autoactivation. Cotransfection of P19 cells with Atoh1 together with decreasing amounts of Neurog1 showed that the latter was able to prevent Atoh1 autoactivation even at a concentration ratio of 1:4 (Fig. 2D). This suggests that Neurog1 repression of the 3'Atoh1-enh dominates over Atoh1 autoregulatory loop.

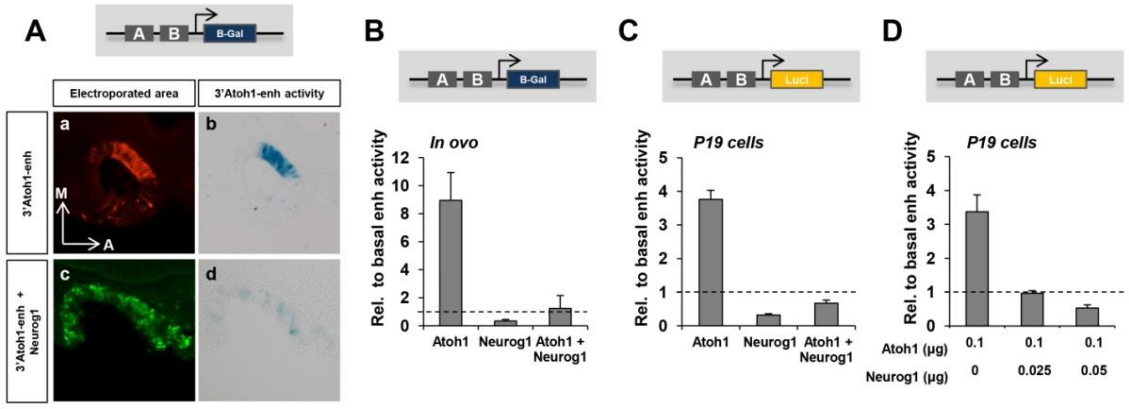


Figure 2. The repression of the 3'Atoh1-enh by Neurog1. (A) Neurog1 repressed the 3'Atoh1-enh in the otic vesicle. Otic vesicles were electroporated at E2 with the 3'Atoh1-enh with or without Neurog1. Otic vesicles were sectioned and processed for GFP immunoflurescence at E2+1 (a, c) or for β-gal staining (b, d; n=3). **(B)** Quantification of 3'Atoh1-enh activity. Otic vesicles were isolated (E2+1) and β-gal activity measured in the three conditions indicated (n=3-4). Values of β-gal activity are relative to the basal activity of the enhancer (dotted line). **(C)** Neurog1 repressed the activity of the 3'Atoh1-enh in P19 cells and prevented the activation by Atoh1. Values of luciferase activity relative to the basal activity of the 3'Atoh1-enh in the conditions indicated in abscissa (n=15). **(D)** Neurog1 is able to repress Atoh1 autoactivation at low concentrations in P19 cells. Values of luciferase activity corresponding to the 3'Atoh1-enh are represented against increasing concentrations of Neurog1 expressed as the ratio of electroporated Neurog1/Atoh1, concentrations ranging between 0 and 0.05µg (n=3-6).

3. Dissecting the 3' Atoh1 enhancer: Enhancer B at the core of repression

The 3'Atoh1-enh is divided into two different Enhancers, A and B. We used the ATAC-seq technique to analyse the accessibility of the region of the Atoh1 gene in vivo. Mouse otocysts enriched in neurosensory tissue were analysed at two different stages of development: E10.5 that corresponds to neurogenesis, when Neurog1 is expressed and Atoh1 is silent. And E14.5, when Atoh1 is already expressed in early differentiating HCs (Cotanche and Kaiser, 2010; Matei et al., 2005). A set of E14.5 otocysts were treated with LY411575, a gamma-secretase inhibitor that blocks Notch signalling (Ferjentsik et al., 2009). Under this condition bHLH Notch target genes Hes5/Hey1 are down-regulated allowing Atoh1 expression and HC overproduction (Lin et al., 2011).

Data analysis was validated by checking chromatin accessibility of otic and non-otic genes and enhancers (Fig. S1). The results show that the broad region of chromatin corresponding to the Atoh1 promoter and the 3'Atoh1-enh were both accessible to transcription factor binding in all three conditions (Fig. 3A). However, zooming into the enhancer landscape showed that the region of Enhancer A was closed at all stages explored. This observation suggests that, *in vivo*, the activity of the 3'Atoh1-enh relies mainly on Enhancer B (Fig. 3B). The ATAC signal profile of the CAN region at E10.5 showed that the E-box A, the preferred Atoh1 binding site, was not occupied by transcription factors (Fig. 3C), which is consistent with the lack of expression of Atoh1 at early stages. At hair cell differentiation stage (E14.5) the signal profile of the CAN region was different, and E-boxes A and C were both occupied. Inactivation of Notch signalling with LY411575 resulted in the release of E-box C occupancy, suggesting that at differentiation stages, Atoh1 repression relies mainly on binding to E-box C (Fig. 3C).

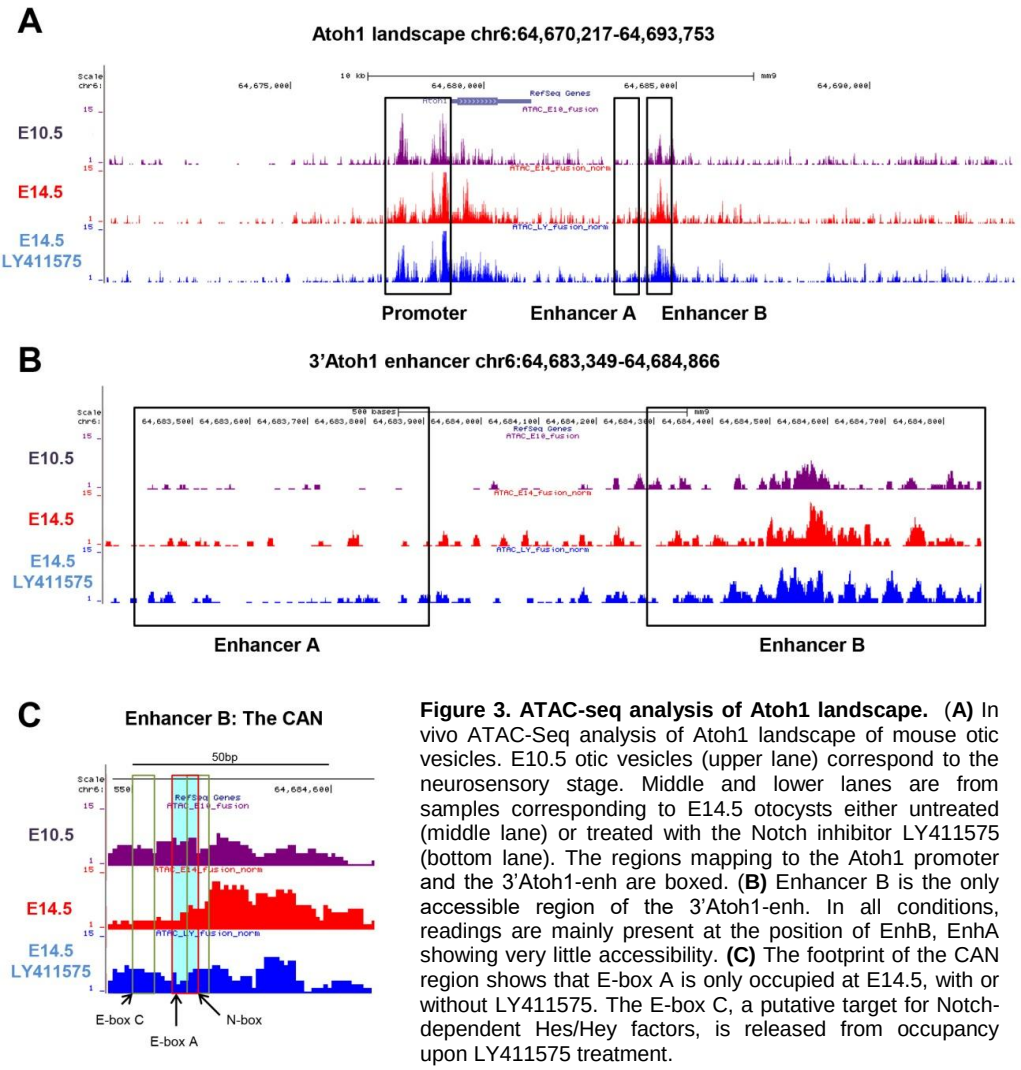


Figure 3. ATAC-seq analysis of Atoh1 landscape. (**A)** In vivo ATAC-Seq analysis of Atoh1 landscape of mouse otic vesicles. E10.5 otic vesicles (upper lane) correspond to the neurosensory stage. Middle and lower lanes are from samples corresponding to E14.5 otocysts either untreated (middle lane) or treated with the Notch inhibitor LY411575 (bottom lane). The regions mapping to the Atoh1 promoter and the 3'Atoh1-enh are boxed. (**B)** Enhancer B is the only accessible region of the 3'Atoh1-enh. In all conditions, readings are mainly present at the position of EnhB, EnhA showing very little accessibility. **(C)** The footprint of the CAN region shows that E-box A is only occupied at E14.5, with or without LY411575. The E-box C, a putative target for Notch-dependent Hes/Hey factors, is released from occupancy upon LY411575 treatment.

To further analyse the contribution of each enhancer to Atoh1 regulation, separate multimer constructs of either the Enhancer A or B (4xEnhA or 4xEnhB) were electroporated and their activity measured in otic vesicles. The spatial activity of A and B reporters was strikingly different in the chick otic vesicle. The 4xEnhA showed no activity at all (Fig. 4Af). In contrast, 4xEnhB exhibited a strong signal and expanded beyond the normotopic 3'Atoh1-enh activity domain (Fig. 4 Ae). Quantification of reporter activity showed undetectable endogenous activity of 4xEnhA, and no activation by Atoh1 or Sox2 (data not shown). This contrasted with the intense activity of 4xEnhB, even above the wild type 3'Atoh1-enh (Fig. 4B). These results correlate well with the different chromatin accessibility of Enh A and B revealed by the ATAC-seq analysis described above. Next we studied whether the EnhB was sufficient for the repression of Atoh1 by Neurog1 by analysing the effects of the co-expression of Atoh1 and Neurog1 on the activity of 4xEnhB. Indeed, 4xEnhB activity was strongly reduced by Neurog1 *in ovo* and in P19 cells, Neurog1 also preventing Atoh1 autoactivation (Figs. 4B and 4C). This indicates that the EnhB accounts for the repression by Neurog1.

4. Transcriptional repression by Neurog1 requires the CAN region

We further narrowed down the requirements of EnhB by focusing on the CAN region, the site of Atoh1 autoactivation. With this in mind, the following multimer constructs were designed and tested in P19 cells: a) E-box A multimer consisting of four tandem repeats of the E-box A without the two flanking E-boxes (tgt**CAGCTG**tcg, 4xEboxA), and b) CAN multimer consisting of four tandem repeats of E-box C, E-box A and the N-box (gag**CACGCG**CTGT**CAGCTGGT**Gagc, 4xCAN). Atoh1 and Neurog1 are both type II bHLH transcription factors that act as transcriptional activators preferentially binding to E-box A (Murre et al., 1994a). In agreement with that, the 4xEboxA was activated by either Atoh1 or Neurog1 (Fig. 4D), the mixture of both being additive. However, Neurog1 activation turned into repression when tested on the 4xCAN construct (Fig. 4E). Therefore, Neurog1 transcriptional repression needs the regions flanking the class A E-box of EnhB to function as a repressor.

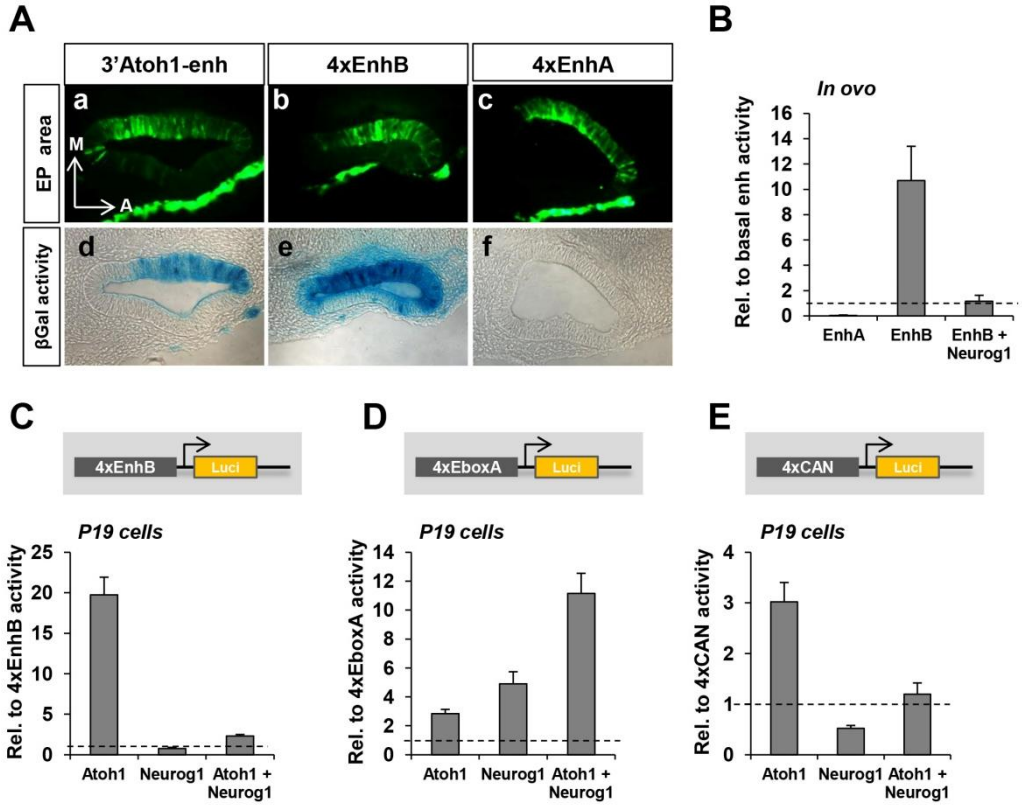


Figure 4. EnhB contains the essential elements for the repression by Neurog1. (A) EnhA shows no activity but regulates that of EnhB. Coronal sections of E2+1 otic vesicles electroporated with 3'Atoh1-enh (a, d), 4xEnhB (b, e) or 4xEnhA (c, f; n=3). EP area = Electroporated area. (B) Enhancer activity quantification electroporated E2 otic vesicles in the three conditions indicated. Values are relative to the basal β-gal activity of the 3'Atoh1-enh. Isolated EnhA showed very low activity, whereas EnhB showed an increased activity that was suppressed by Neurog1 (n=3). (C) Neurog1 counteracts Atoh1 induced activity of EnhB in P19 cells (n=13). Luciferase activity corresponding to EnhB in the three conditions indicated in abscissa. (D) The isolated E-box A is activated by both, Atoh1 and Neurog1. The activity of 4xE-box A is shown for the three conditions indicated in abscissa (n=5-7). (E) Neurog1 turned into a repressor when flanked by C and N boxes. Experiment like in D, but with the 4xCAN multimer (n=5-6).

5. Neurog1 does not require direct binding to DNA for Atoh1 repression.

Neurog1 drives neuronal commitment in the inner ear by acting as a transcriptional activator (Evsen et al., 2013) and, as shown above, Neurog1 indeed activates the Ebox-A construct. However, it turns into a repressor in the presence of the flanking E-box C and N-box, despite type II bHLH transcription factors do not bind to class C or N-boxes (Massari and Murre, 2000b; Murre et al., 1994a). This raises the question of whether Neurog1 requires actual binding to DNA for its repressor function, or else it favours the binding of other factors that result in the repression of Atoh1. We addressed this question by testing the effects of a DNA-binding deficient Neurog1 (Neurog1-AQ, Sun et al., 2001) on the 3'Atoh1-enh. Neurog1-AQ carries two mutations in the basic domain that prevents DNA-binding. Somehow unexpectedly, Neurog1-AQ was as efficient as the wild type Neurog1 in preventing the activation of the 3'Atoh1-

enh by Atoh1, both *in ovo* and in P19 cells (Figs. 5A and B). Neurog1-AQ also blocked Atoh1 autoactivation in 4xEnh B or 4xCAN constructs (Figs. 5 C and D). As expected, the Neurog1-AQ construct was not able to activate the 4xEboxA (Fig. 5E). These results suggest that Neurog1 does not prevent Atoh1 autoactivation by competing with Atoh1 for DNA binding, neither by the transcriptional activation of repressor factors.

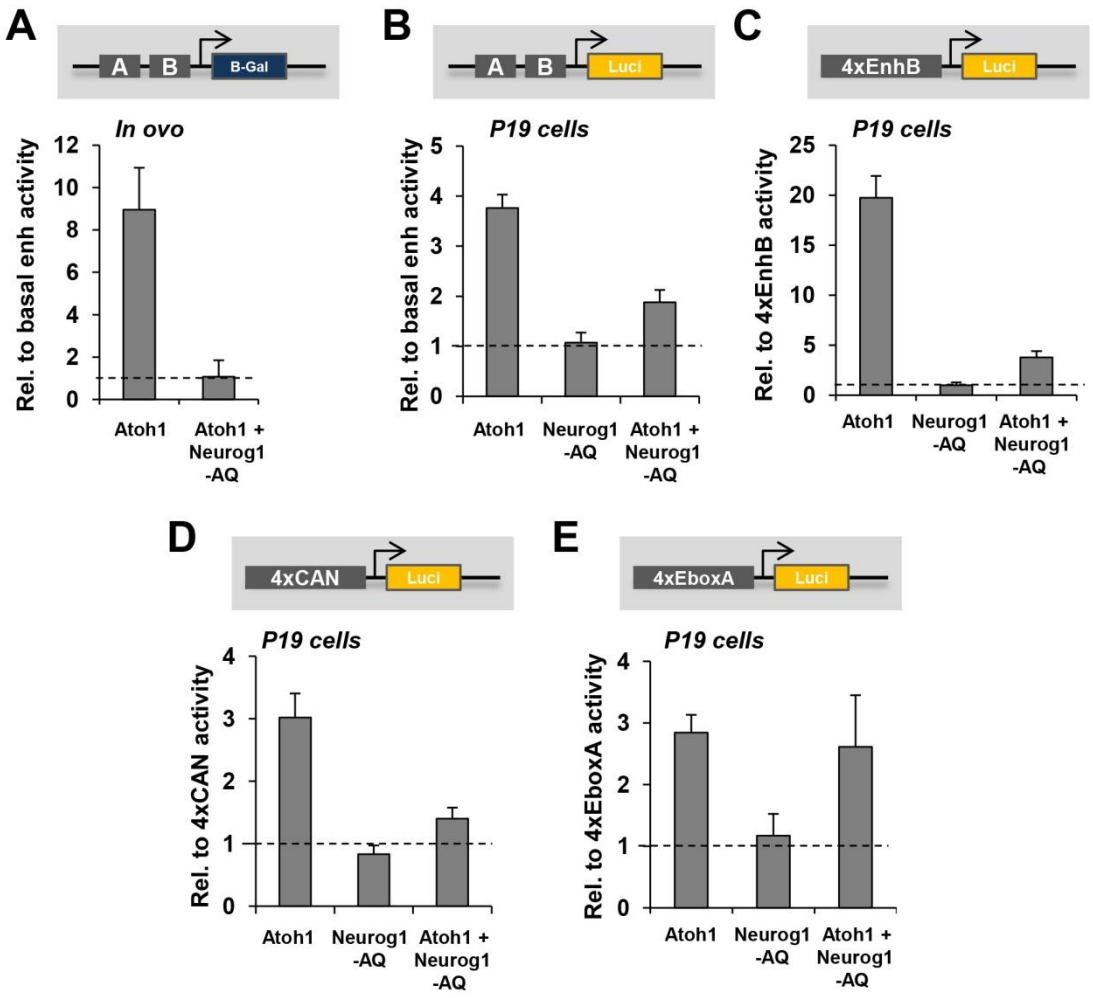


Figure 5. Neurog1 does not require DNA-binding to repress Atoh1. (**A)** Neurog1-AQ is able to repress Atoh1 dependent activation of the 3'Atoh1-enh in vivo. Quantification of β-gal activity in otic vesicles after electroporation of Atoh1 alone, or with Neurog1-AQ. Values are relative to the basal β-gal activity of the 3'Atoh1-enh (n=3). (**B)** Neurog1-AQ is able to repress Atoh1 in P19 cells. Quantification of Luciferase activity in P19 cells in the conditions indicated (n=5-15). (**C)** EnhB accounts for the repression by Neurog1-AQ (n=4-13). **(D)** Neurog1-AQ is able to repress the CAN reporter. Experiment like in C and D, but after electroporation of the 4xCAN (n=3-7). **(E)** Neurog1-AQ does not interfere with the E-box A in isolation and it does not interfere with Atoh1 activation (n=3-7).

6. Neurog1-AQ prevents Hair Cell formation *in ovo*

We further tested whether the ability of the DNA-binding deficient Neurog1 to repress Atoh1 is correlated with the impairment of hair cell formation in the chicken embryo. For this purpose we compared the effects of Neurog1 and Neurog1-AQ on HC and neuronal development. Otic vesicles were electroporated at prosensory stage (E3.5) and analysed three days later, at the stage of HC differentiation. Electroporation of Neurog1 resulted in a massive neuronal commitment as revealed by the expression of Tuj1 in electroporated cells (Fig. 6Aa, see the ganglion, CVG). Cells electroporated in either neurosensory or non-neural domains were forced to become neurons (Fig. 6Ab). No electroporated cells were observed in the sensory epithelium after three-days, and HC patterning was not substantially affected (Fig. 6Ac). Contrarily, electroporation of Neurog1-AQ caused a strong blockade of neuronal determination (Fig. 6Ad), suggesting that it acts as a Neurog1 dominant-negative during neurogenesis. In addition, the DNA-binding deficient Neurog1 caused a strong bias towards the supporting cell fate. Most electroporated cells remained in the epithelium and expressed Sox2 (Figs. 6Af and g). This is illustrated by the quantification of the fraction of cells that acquired either HC or SC fates after electroporation. For EGFP, the proportion of HCs and SCs that were electroporated was similar (33% and 28%, respectively, Fig. 6B). However, Neurog1-AQ reduced the fraction of electroporated HCs by about four-fold (from 33% to 8%, compare middle bars in Figs. 6B and C), suggesting that Neurog1-AQ biased progenitors away from HC fate. Together, the results indicate that binding to DNA is dispensable for the repressor effect of Neurog1 on Atoh1 and hair cell formation.

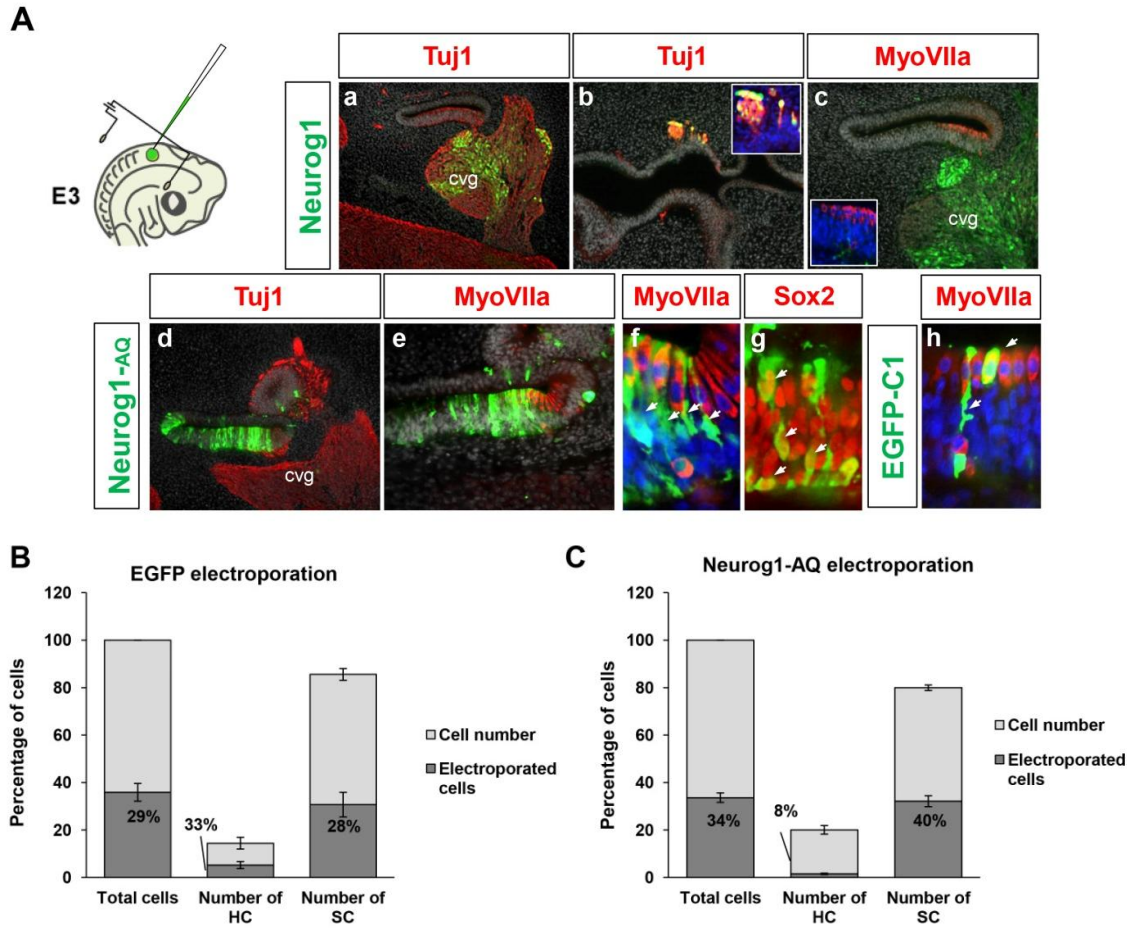


Figure 6. Neurog1 prevents HC formation *in ovo*. (A) Neurog1-AQ is able to prevent hair cell formation. Neurog1 (upper row) or Neurog1-AQ (lower row) were electroporated *in ovo* (E3.5) and otic vesicles examined for neuron (Tuj1), hair cell (MyoVIIa) or supporting cell (Sox2) markers at E6.5. Cells electroporated with Neurog1 adopted neuronal fate (a-c, cvg=cochleo-vestibular ganglion), while those with Neurog1-AQ turned into supporting cells (arrows in f-g). Control electroporation with EGFP-C1 (h). **(B, C)** Neurog1-AQ biased electroporated cells towards supporting cell fate. Bars represent the number of cells counted in two consecutive frames of electroporated macula sacularis, from three independent embryos. Cell types were identified with the markers described above. In EGFP-C1 electroporation (Ah), the fraction of electroporated cells (dark area) is similar for both hair cells and supporting cells. However, after Neurog1-AQ electroporation, very few electroporated cells became hair cells (n=3). The numbers inside the bars indicate the fraction of electroporated cells within each group.

7. Neurog1 repression is independent of Notch activity

Given that the regions flanking the E-box A are typical binding sites for Notch-dependent bHLH repressors like Hes/Hey factors (Du et al., 2013; Petrovic et al., 2014; Tateya et al., 2011) and that Neurog1 requires these binding sites for its repressor function, we explored the possibility that Neurog1 interacts with Hes/Hey factors that, in turn, bind to the flanking regions and cause Atoh1 repression. With this in mind, we tested the effects of the Notch pathway inhibitor LY411575 on the repressor effect of Neurog1-AQ. Embryos were electroporated at E2 with the 3'Atoh1-enh and Atoh1 alone or combined with Neurog1-AQ. After 6h otic vesicles were dissected, and

incubated with or without LY411575 for another 16h. As shown in Fig. 7A, Neurog1-AQ repression was similar in both cases, suggesting that the effect of Neurog1-AQ on Atoh1 autoactivation does not require the participation of Notch-dependent targets. As control for LY treatment, control ears were treated with LY411575 and levels of Hes5 and Hey1 were downregulated when measured by qPCR (data nor shown). Further, the experiments suggest that the repression of Atoh1 by Neurog1 may take place in the absence of Notch-mediated lateral inhibition.

8. Neurog1 requires the Helix-1 dimerization domain for Atoh1 repression.

To gain insight into the mechanism of action of Neurog1, we analysed which domains of Neurog1 are required for repression. We tested the functionality of different modified Neurog1 constructs carrying selective deletions of the Helix-1, Helix-2 or the C-terminal domains. The different Neurog1 constructs were cotransfected with Atoh1 and assayed for 3'Atoh1-enh activity. Helix domains 1 and 2 are used for protein dimerization, and the deletion of Helix-1 restored almost completely Atoh1-autoactivation, while Helix-2 only partially (Fig. 7B). The C-terminal part of bHLH proteins contains phosphorylation sites required for their activity and stability (Cundiff et al., 2009; Hardwick and Philpott, 2015), but its deletion did not affect the repressor effect of Neurog1 (Fig. 7B). Therefore, Helix-1 and Helix-2 dimerization domains are crucial for the repressor function of Neurog1.

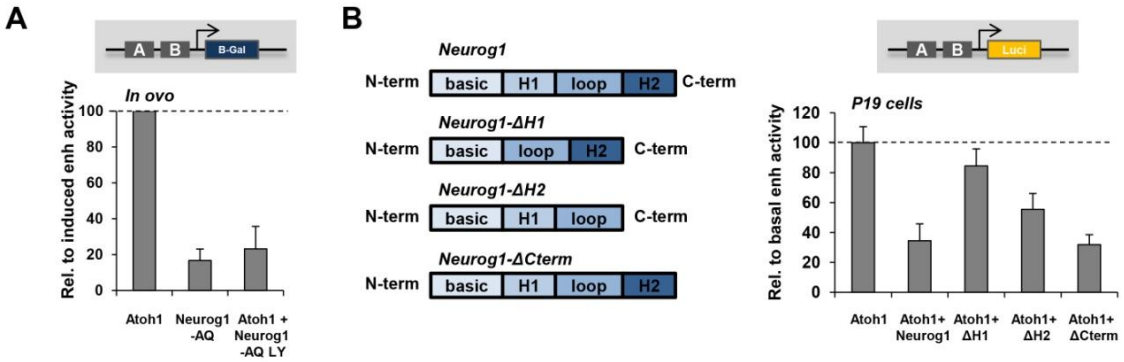


Figure 7. Helix-1 dimerization domain of Neurog1 is essential for Atoh1 repression. (A) Neurog1 does not require Notch signalling for Atoh1 repression. E2 otic vesicles were electroporated with the 3'Atoh1-enh with or without Neurog1-AQ. After 6h, they were dissected and incubated with DMSO or LY411575 o/n. The bars represent β-gal activity of the 3'Atoh1-enh relative to Atoh1 co-electroporation (n=3). **(B)** Helix-1 is required for Neurog1 repression of the 3'Atoh1-enh. Left: diagram of the different constructs tested. Right: reporter activity in P19 cells shown in the conditions indicated in abscissa. Deletion of Helix-1 and to a less extent Helix-2 hampered the repression of Atoh1 autoactivation induced by Neurog1, which was unaltered by the C-term deletion (n=4). H1: Helix-1, H2: Helix-2, N-term: N-terminal, C-term: C-terminal.

Up to now, we have shown that Neurog1 represses the activity of the 3' Atoh1-enh without sitting on it. Since Atoh1 is the major activator of its own enhancer, one

possibility for the effect of Neurog1 is that it modifies the Atoh1 protein in a way that the latter cannot activate its own enhancer. Type II bHLH activators like Atoh1 and Neurog1 bind to their specific DNA binding sites by forming heterodimers through HLH domains with type I bHLH proteins named E-proteins. Examples are E47, E2.2 and HEB, which are broadly expressed during development (Kee, 2009; Murre et al., 1994a). One possibility for the repression of Atoh1 by Neurog1 is that the later binds and sequesters shared E-proteins that are required by Atoh1 to bind to DNA. With this in mind, we tested the ability of some known E-proteins to rescue Atoh1 repression by Neurog1. However, E47, E2.2 or HEB were unable to revert the effect of Neurog1 (Fig. 8A). Neither p300/CBP and Smad1 transcriptional complex components (Sun et al., 2001) were able to prevent the repressor effects of Neurog1 (Fig. 8B). Therefore, none of the predicted partners of Atoh1 are required for the repressor effect of Neurog1.

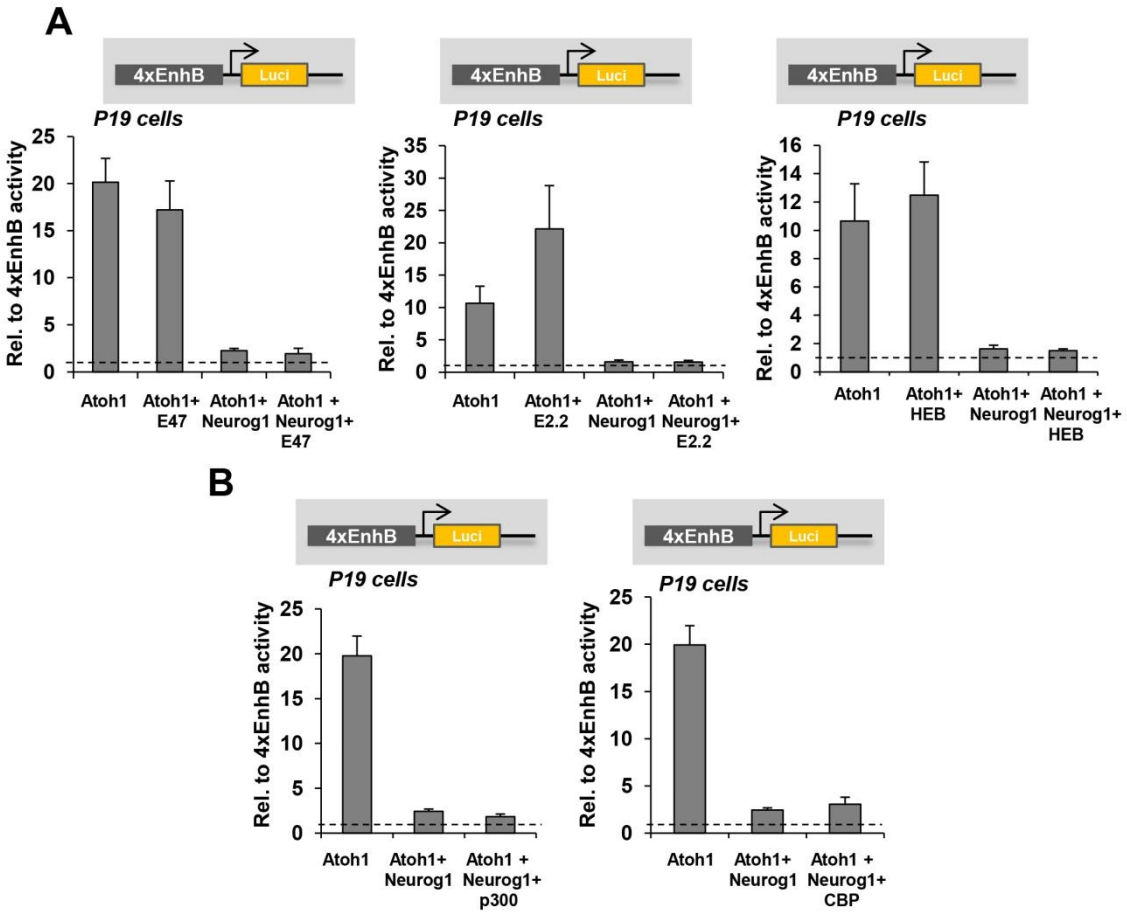


Figure 8. E-proteins and other co-activators do not rescue Neurog1 repression. (A) Overexpression of E47, E2.2 or HEB were unable to prevent Atoh1 autoactivation of 4xEnhB by Neurog1 (n=3-4). **(B)** Neither p300 nor CBP overexpression were able to prevent Neurog1 Atoh1 autoactivation of 4xEnhB reporter activity in P19 cells (n=3-14).

9. Neurog1 reduces Atoh1 protein levels

We next questioned alternative possibilities to mediate the repressor effect of Neurog1, constraints being the independence on DNA binding and the requirement of Helix-1 mediated protein interactions. The objective was to find possible protein interactions that explain the repressor effect of Neurog1.

With this in mind we analysed the Atoh1 interactome (IP Atoh1) and the Atoh1 interactome in the presence of Neurog1 (IP Atoh1-Neurog1). We also analyzed the interactome of Neurog1 in the presence of Atoh1 (IP Neurog1-Atoh1). P19 cells were transfected with Atoh1-FLAG in the presence or absence of Neurog1-myc. Atoh1 was immunoprecipitated with an anti-FLAG antibody and Neurog1 by anti-MYC antibody and the bound proteins identified by mass spectrometry. The list of bound proteins showed the presence of E2.2 and Heb in all immunoprecipitations (IP Atoh1, IP Atoh1-Neurog1, IP Neurog1-Atoh1 Table S1), indicating that E-proteins actually bind to Atoh1 regardless of the presence of Neurog1 and E-proteins are no limiting factors for Atoh1 function. Comparison of Atoh1 and Atoh1-Neurog1 interactomes, using SAINTexpress to score protein interactions, showed an 89% overlap, indicating that a large fraction of proteins bind to Atoh1 regardless of the presence of Neurog1 (Fig. 9A and Table S2). The profile of the molecular function of both interactomes was very similar as well. (Fig. S2).

When analysing the identity of the proteins, we first noted that Neurog1 was not pulled down by Atoh1 immunoprecipitation (Table S1 and S2), nor Atoh1 was recovered after Neurog1 immunoprecipitation (IP Neurog1-Atoh1, Table S1 and S3). This makes it unlikely that Neurog1 forms non-functional heterodimers with Atoh1 or that it interacts with Atoh1 directly in any way. Besides, the analysis of proteins that may interact with Atoh1 as activators, only when Neurog1 is not present, gave no potential candidates (Table S2), which suggested that no putative Atoh1 co-activators are displaced in the presence of Neurog1.

The above makes it unlikely that Neurog1 modulates the functionality or the activity of Atoh1 protein, leaving the regulation of Atoh1 protein levels as the alternative possibility to be explored. We therefore analysed whether Neurog1 promotes changes in Atoh1 protein levels by transfecting Atoh1-FLAG alone or in combination with Neurog1. With this approach we discarded effects dependent on Atoh1 autoactivation, because the atoh1-FLAG protein is constitutively expressed by the transfected plasmid.

Western Blot analysis showed that Atoh1 protein expression was reduced in the presence of Neurog1 (Fig. 9B). The reduction in optical density was of 36 % in P19 cells (Fig. 9B, bar diagram) and up to 70% in chicken otic vesicles (Fig. 10). Interestingly, the effect was abolished when Atoh1 was cotransfected with the Neurog1-ΔH1 construct (Fig. 9B, bar diagram, right), indicating that the decrease in Atoh1 protein induced by Neurog1 is strictly dependent on the integrity of the Helix-1 domain, H1. This is consistent with the loss of Neurog1 repression of the 3'Atoh1-enh when Neurog1 does not have the Helix 1 (See Fig. 7B).

Reduction in Atoh1 protein levels may be caused by at least the following and not mutually exclusive mechanisms: increased Atoh1 protein degradation or reduction of Atoh1 translation, the later being in turn related to impaired ribosomal function or to the reduction in mRNA levels. In the analysis of the IP we sorted out some proteins that only interact with Atoh1 in the presence of Neurog1. These included two proteins involved in protein degradation: the E3 ubiquitin-protein ligase TRIM56 and the S-phase kinase-associated protein 1 (SKP1), a component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex (Fig. 9A and Table S2). We confirmed that TRIM56 and SKP1 bound Atoh1 in the presence of Neurog1, but not to Neurog1 in the presence of Atoh1 (Fig. 9A and Tables S3 and S4). Since E3 ubiquitin ligases mediate polyubiquitination of proteins and target them to degradation, a plausible mechanism for the repressor effect of Neurog1 is that it favours the degradation of Atoh1.

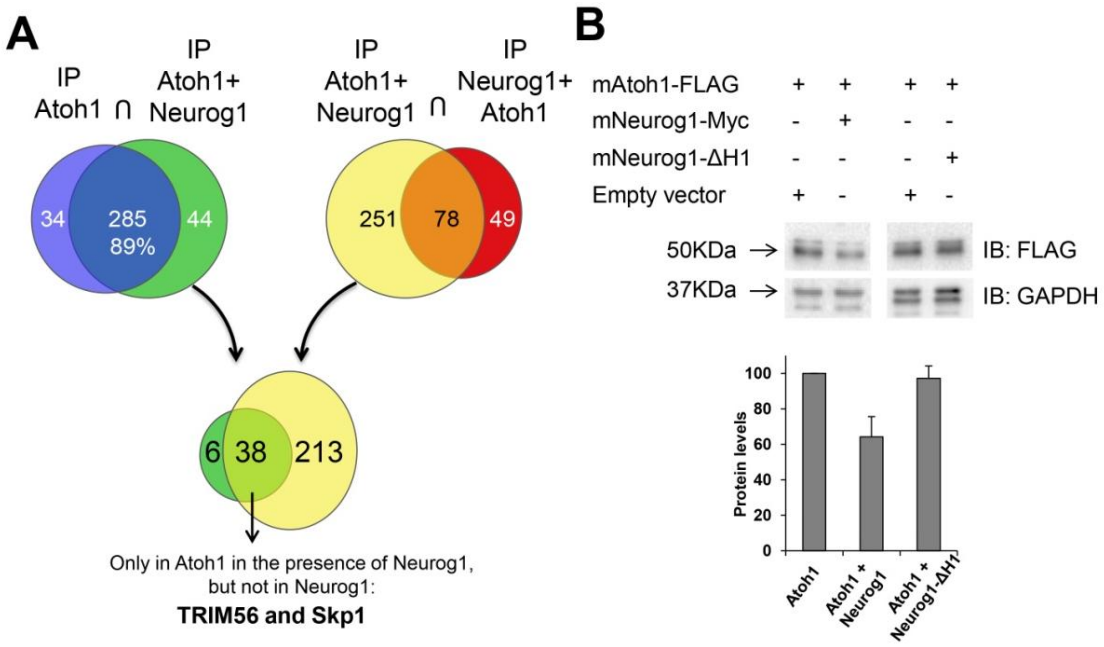


Figure 9. Neurog1 reduces Atoh1 protein levels. (A) Diagram illustrating the Atoh1 interactome of P19 cells, in the presence or absence of Neurog1 (see methods). Diagrams illustrate the number of identified proteins pulled down with an antibody against FLAG for Atoh1 IP (upper left) or with anti-Myc for Neurog1 IP (upper right). Some Atoh1 interacting proteins are lost (purple) or gained (green) in the presence of Neurog1 and some of them only interact either with Atoh1 (yellow) or with Neurog1 (red). The intersection between green and yellow (lower part) represents those proteins that interact only with Atoh1 in the presence of Neurog1. Two proteins of functional relevance are listed. **(B)** Neurog1 reduces Atoh1 protein levels. Western blot analysis of P19 cells transfected with Atoh1 in the presence or absence of Neurog1 or Neurog1-ΔH1 (n=3-7). Bar diagram showing optical density values averaged from different experiments (n=5 out of 7 for Neurog1 and n=3 for Neurog1-ΔH1) are represented relative to Atoh1-FLAG. Values were normalized by GAPDH. IP: immunoprecipitation, IB: immunoblot.

This prompted us to test the possibility that Atoh1 is degraded in the presence of Neurog1 by the proteosomal pathway. E3 Chicken otic vesicles were electroporated with Atoh1-FLAG with or without Neurog1-wt and protein levels of Atoh1-FLAG were analyzed after overnight incubation in ovo. Atoh1 protein levels in the presence of Neurog1 decreased almost 70% in the presence of Neurog1, indicating that Neurog1 dramatically reduces protein levels *in vivo*, (Fig. 10A and B). Treatment of electroporated otic vesicles with the proteosome inhibitor MG-132 for two and four hours, did not recover the Atoh1 protein levels (Fig. 10A). Indeed, note that Neurog1 continued to reduce Atoh1 levels in the presence of the inhibitor. This indicates that Neurog1 does not reduce Atoh1 protein levels by targeting Atoh1 to proteosome-dependent degradation.

In conclusion, the results suggest that the mechanism that accounts for the reduced levels of Atoh1 protein in the presence of Neurog1 is post-transcriptional, but it does not involve proteosome-dependent degradation. Potential mechanisms are the induction of mRNA de-stabilisation and/or the interference with the ribosome

machinery. Further work is required to identify such mechanisms and to understand how Neurog1 is coupled to their activation (see Discussion).

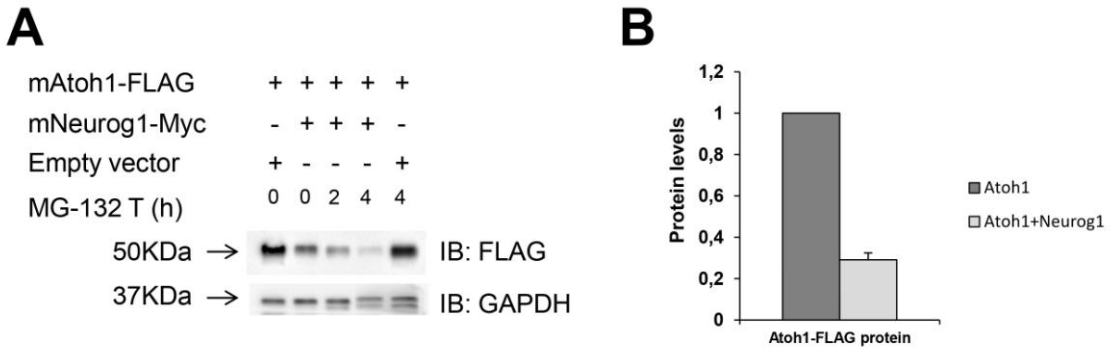


Figure 10. Atoh1 protein levels are not affected by proteosome degradation. **(A)** Western blot of protein extract from electroporated chicken otic vesicles. Three independent experiments for the Atoh1-FLAG IB without proteosome inhibitor. One observation of each MG-132 condition. **(B)** Quantification of Atoh1-FLAG protein levels corrected by GAPDH (n=3). Reduction of 70% of Atoh1-FLAG was detected in the presence of Neurog1-myc. IB: Immunoblot.

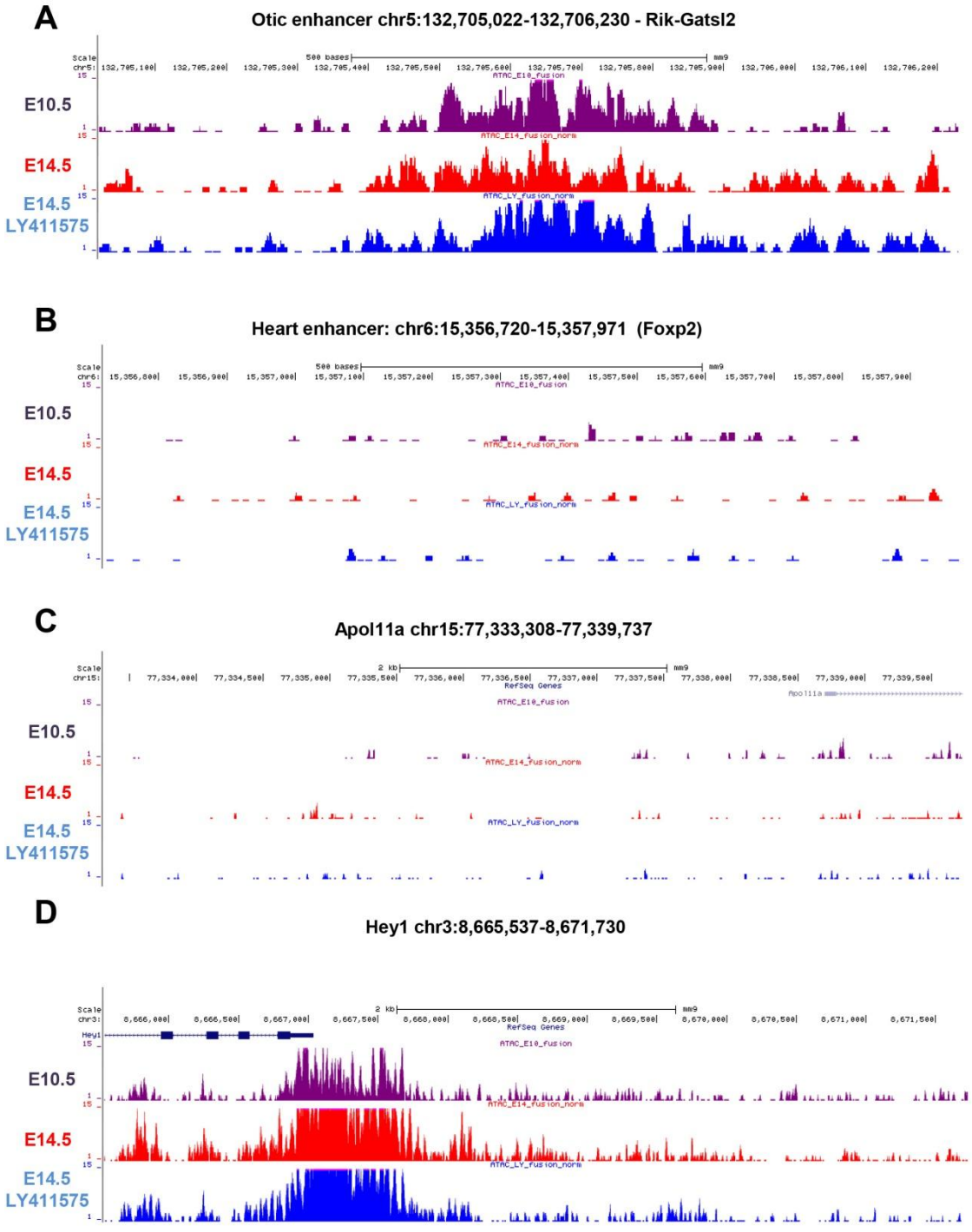


Figure S1. ATAC-Seq data validation. (A) Accessibility to the otic enhancer Rik-Gatsl2 annotated in the VISTA Enhancer Browser. Location of the region Chr5:132,705,022-132,706,230. The enhancer was accessible in all three conditions. **(B)** The region of a heart enhancer of the Foxp2 gene (chr6:15,356,720-15,357,971), which was not accessible in otic samples. **(C)** Promoter region of the Apol11a gene, which is not detected in the ear, was not accessible (chr15:77,333,308-77,339,737). **(D)** Promoter region of Hey1 gene accessible in all conditions tested (chr3:8,665,537-8,671,730).

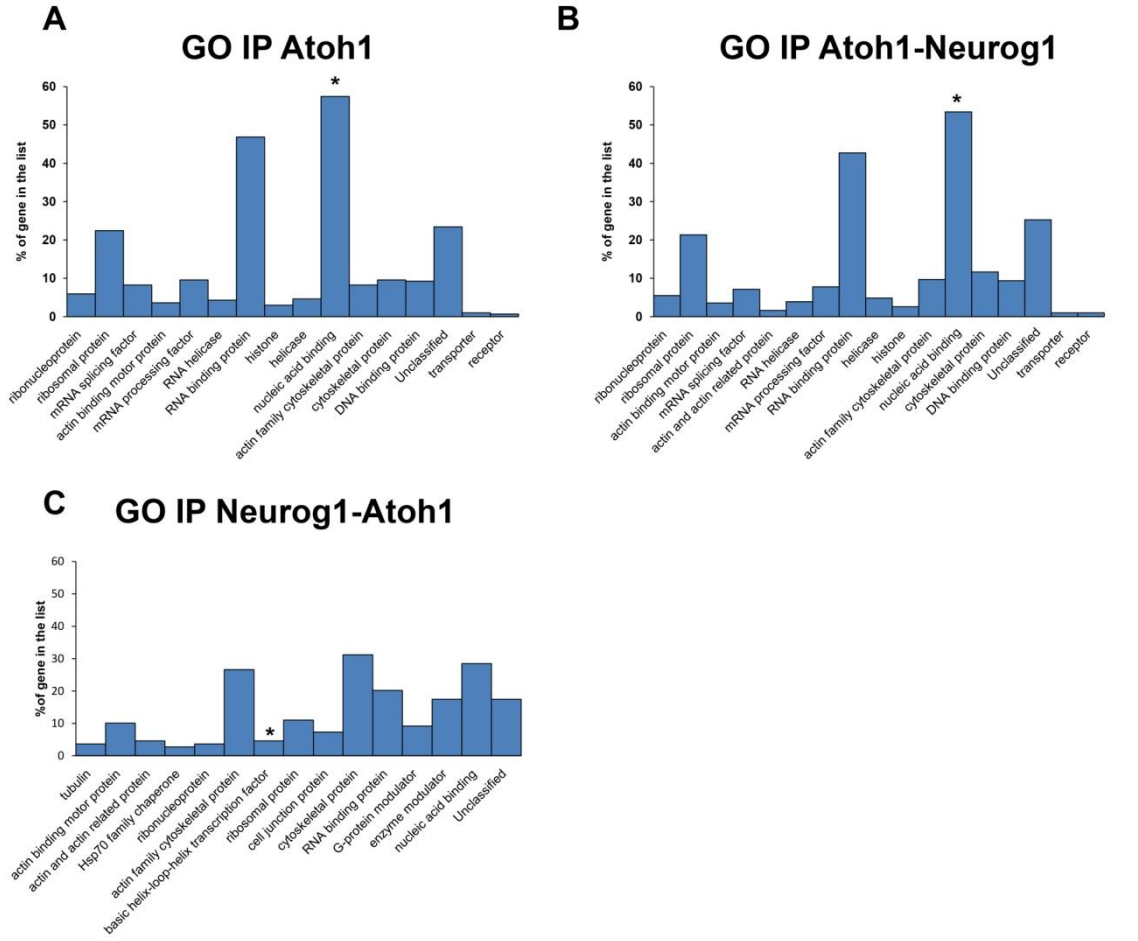


Figure S2. Analysis of the immunoprecipitated interactors. Each list of immunoprecipitated proteins were classified by protein class using gene ontology with the data set PANTER class**. (A)** Classification of the IP Atoh1 **(B)** IP Atoh1-Neurog1 and **C)** IP Neurog1-Atoh1. In A and B, the asterisk represents the most abundant group of proteins present in the interactome. In C, the asterisk indicates bHLH proteins.

CHAPTER II

HEY1 AND HES5 ARE NEGATIVE REGULATORS OF THE 3'ATOH1-ENHANCER

These results will be included in an invited review for *Frontiers in Cell and Developmental Biology*

The regulation of Atoh1 by Notch

During ear development, only Hes5 and Hey1 expression are detected within the sensory regions. Their expression correlates with the two Notch ligands expressed in the chick: Hey1 follows Jag1 expression, while Hes5 corresponds well to Dl1 expression throughout otic development (Petrovic et al., 2014). Although Hey1 and Hes5 are direct Notch downstream targets, they differ in the level of Notch required for their activation. The result is that Hes5 and Hey1 are expressed during neurogenesis, but only Hey1 during prosensory development (Petrovic et al., 2014). In both cases, this is paralleled by a very low Atoh1 expression. Upon the onset of Atoh1 and HC differentiation, Hey1 and Hes5 are expressed in the SCs. They are major suppressors of Atoh1 in SCs since the combined loss of function of Hes/Hey exhibits supernumerary HCs in the organ of Corti (Tateya et al., 2011). In this chapter, I describe experiments designed to explore the molecular mechanisms by which Hey1 and Hes5 interact with the 3'Atoh1-enh and prevent HC formation.

1. Hey1 abolishes the 3'Atoh1-enh activity in the neurosensory epithelium.

Since the developmental regulation of Atoh1 relies on the activity of its 3' enhancer, we studied whether Hey1 is able to interact with this region and repress Atoh1 transcription. The effects of Hey1 on the activity of the 3'Atoh1-enh was tested on chick E2 otic vesicles by the electroporation of the 3'Atoh1-enh with or without Hey1. As shown above, the activity of the 3'Atoh1-enh was restricted to the neurosensory domain (Fig. 1b), and Hey1 was able to prevent the basal activity of the 3'Atoh1-enh within this domain (Fig. 1d).

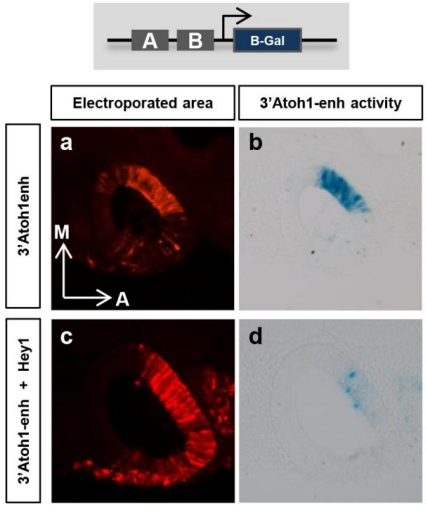


Figure 1. Hey1 missexpression abolishes 3'Atoh1-enh activity. 3'Atoh1-enh was electroporated in chicken E2 otic vesicle *in ovo* with or without Hey1. Immunohistochemistry of anti-dsRed labels the electroporated area (a-c) and β-gal staining (b-d). Electroporations were performed in E2 embryos that developed in ovo for 24h. The 3'Atoh1-enh activity was localized in the antero-medial neurosensory domain of the otic vesicle (b). Co-electroporation with Hey1 and 3'Atoh1-enh showed a strong reduction of the enhancer activity (d).

Quantification of these effects by using a β-gal reporter assay showed that Hey1 and Hes5 were able to prevent transcription of the basal activity of the 3'Atoh1-enh (Fig. 2A). Id3, another HLH which lacks the DNA binding domain and counteracts HC formation (Jones et al., 2006; Kamaid et al., 2010), also prevented the basal activity of the enhancer (Fig. 2A).

We tested also the ability of Hey1 to interfere with Atoh1 autoactivation *in ovo* (Fig. 2A) and in P19 cells (Fig. 2B and C). Both Hey1 and Hes5 were able to break the autoregulatory loop of Atoh1 and strongly reduced the activity of the 3'Atoh1-enh in the presence of Atoh1 (Fig. 2A *in ovo*; Fig. 2B and C, *in vitro*).

Taken together, the results show that Atoh1 enhancer is transcriptionally repressed by Hey1 and Hes5 and that they act as transcriptional repressors.

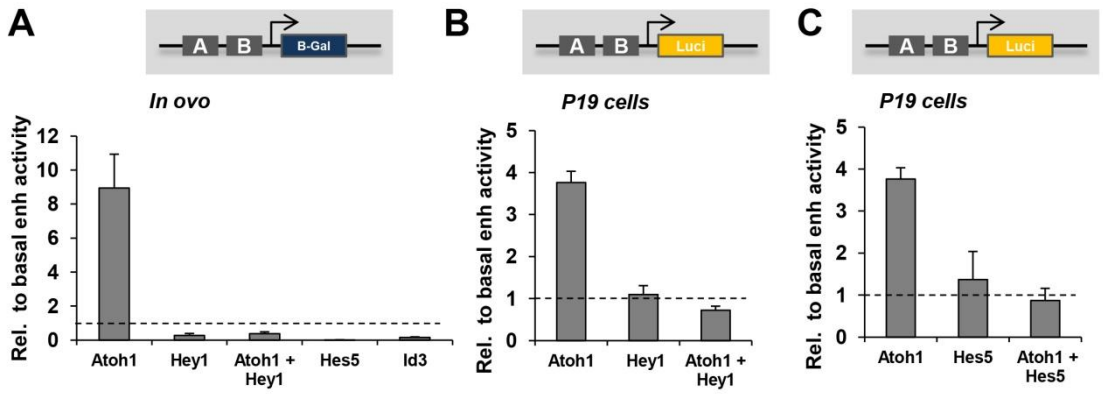


Figure 2. 3'Atoh1 regulation. **(A)** Quantification of 3'Atoh1-enh activity. Otic vesicles were isolated and developed *in ovo* for 24h (E2+1). β-gal activity measured in the five conditions indicated (n=3-4). Atoh1 activates its own enhancer. Hey1, Hes5 and Id3 repressed 3'Atoh1-enh basal activity and Hey1 prevented Atoh1 autoactivation. **(B)** Hey1 also prevented Atoh1 autoactivation in P19 cells. Values of luciferase activity relative to the basal activity of the 3'Atoh1-enh in the conditions indicated in abscissa (n=3). **(C)** Hes5 prevented Atoh1 autoactivation in P19 cells. Values of luciferase activity relative to the basal activity of the 3'Atoh1-enh in the conditions indicated in abscissa (n=3).

2. Hey1 missexpression prevents HC formation

Evidence from mice embryos indicates that Hey/Hes factors counteract HC formation and they are necessary for preventing HC overproduction. For instance, the combined knock out of Hey/Hes genes display supernumary hair cell formation (Tateya et al., 2011) as it does the blockade of Notch signalling (Lin et al., 2011; Mizutari et al., 2013). However, it is still unknown whether Hey1 is sufficient for HC fate suppression or whether the effects on the 3'Atoh1-enh have a functional correlation. The chick model is particularly suitable for gain of function studies and we used it to test the effects of Hey1 on hair cell generation. Hey1 overexpression was targeted to the prosensory

regions of E3.5 otocysts. Embryos were incubated for another three days to then analyze the production of hair and supporting cells. Hair cells were labelled with Myo7a and supporting cells with Sox2. Hair cells of macula sacularis were quantified by serial reconstructions of several embryos.

Electroporation of Hey1 prevented hair cell formation as shown by the reduction in the number of HCs at the expense of SCs (Fig. 3A). Hey1 caused a strong bias towards the supporting cell fate. This is illustrated by the quantification of the fraction of cells that acquired either HC or SC fates after electroporation. For EGFP, the fate of electroporated cells was similar and randomly distributed (33% and 28%, respectively, see Fig 3D). However, after Hey1 electroporation, the fraction of electroporated cells that acquired HC fate was reduced by about four-fold (from 33% to 7.91%, compare middle bars in Fig. 3C and Fig. 3D). This strongly suggests that Hey1 is sufficient to bias progenitors away from HC fate. Instead, Hey1-positive cells adopt a supporting cell fate.

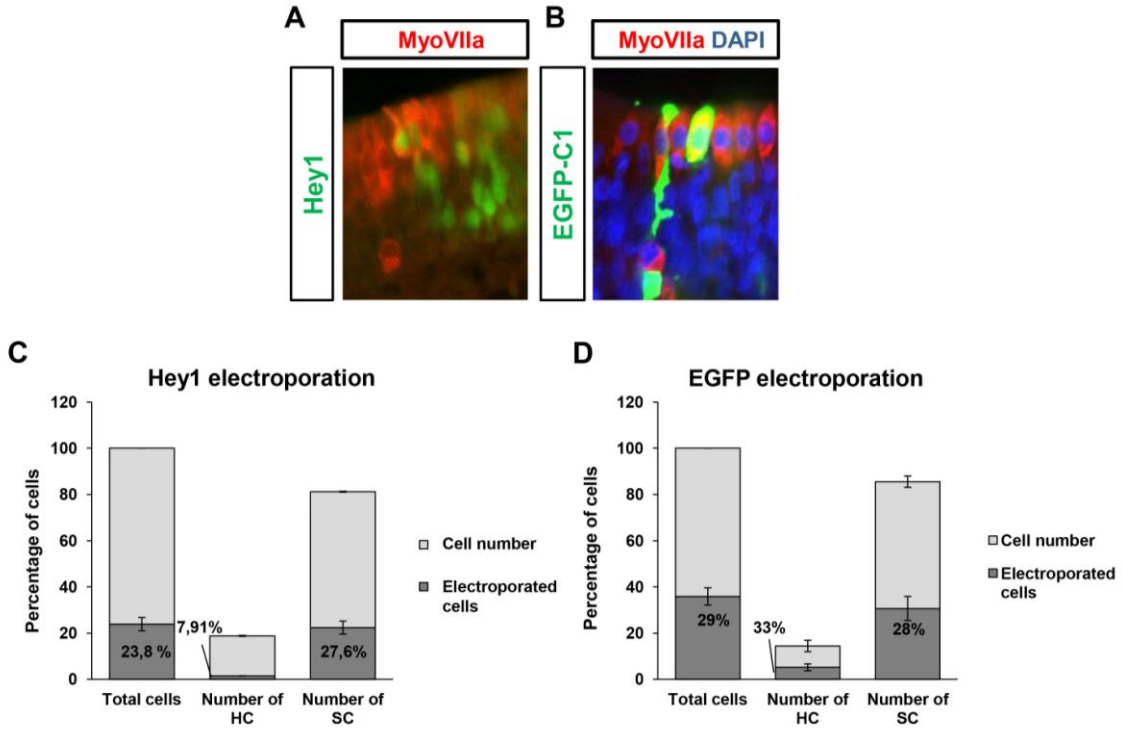


Figure 3. Hey1 prevents HC formation *in ovo*. (A) E3.5 embryos were electroporated with Hey1 and then sectioned after 3 days of incubation (E3.5+3). Analysis of the macula sacularis showed that electroporated cells were found in the SC layer, and very few developed as HC. **(B)** Control electroporation with EGFP-C1. **(C)** Hey1 electroporation biased electroporated cells towards supporting cell fate. The fraction of HCs that were electroporated was smaller than that of SCs. Bars represent the number of cells counted in two consecutive frames of electroporated macula sacularis, from three independent embryos (n=3). **(D)** Electroporation with EGFP-C1 does not show any bias for either HCs or SCs. The fraction of electroporated cells is similar for HCs and SCs (data is taken from Fig. 6 of Neurog1 chapter). The numbers inside the bars indicate the fraction of electroporated cells within each group.

3. Hey1 repression only requieres the CAN region

We next analyzed which region of the 3' Atoh1-enh may account for this effect. As shown in the previous chapter, both ATAC-seq and reporter assay data show that only the EnhB of the 3'Atoh1-enh is accessible to regulation during otic development. Accordingly, Hey1 and Hes5 were able to prevent the basal activity of EnhB in chicken otic vesicles (Fig. 4A) as they were to repress Atoh1 autoactivation (Fig. 4B). This suggests that EnhB of the 3'Atoh1-enh is critical for Hey1 and Hes5 repressor activity.

To further analyse the interaction between Hey1 with EnhB, we performed multimer reporter analysis of the CAN region, by comparing its effects on the isolated class A E-box with that on the whole CAN domain. This showed that Hey1 requires the E-boxes flanking the class A E-box to act as a repressor (Fig. 4 C and D). The CAN multimer mimicked the 3'Atoh1-enh repression promoted by Hey1. We next explored whether Hey1 requires binding to the 3'Atoh1-enh, and what is the relative weight of the E-boxes flanking class A E-box for repression.

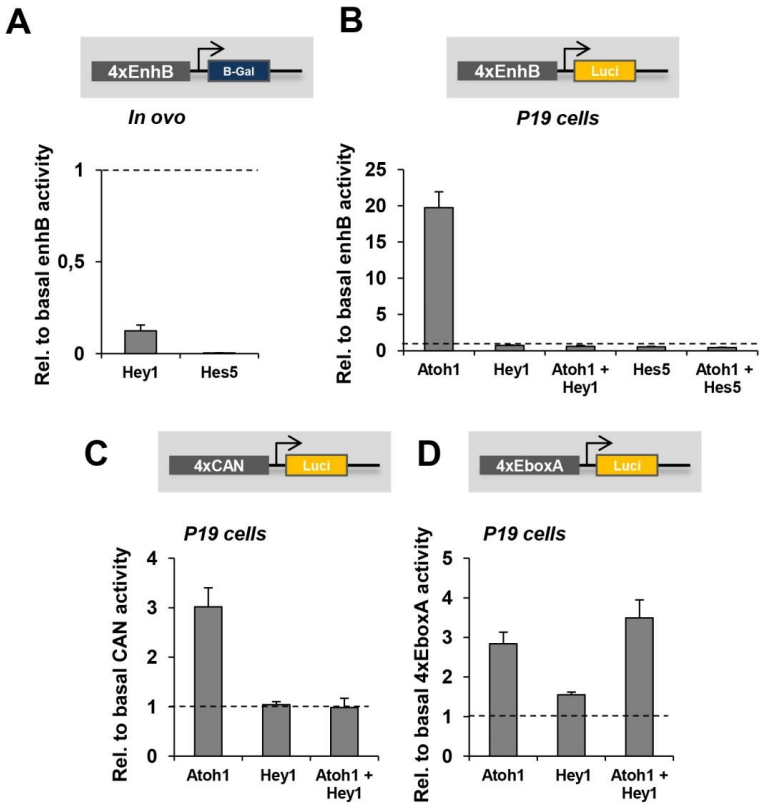


Figure 4. The regulation of the 3'Atoh1-enh is recapitulated by the CAN region (A) Quantification of EnhB activity in the presence of Hey1 or Hes5 in E2+1 otic vesicles. Hey1 and Hes5 factors were able to prevent the basal activity of EnhB (n=3). **(B)** P19 cells were transfected with EnhB and the combination of different plasmids as indicated. Luciferase activity was measured as described in Methods. Atoh1 was able to activate EnhB and the autoactivation was suppressed by either Hey1 or Hes5 (n=3). **(C)** The CAN multimer was activated by Atoh1, but Hey1 was not able to repress the basal enhancer activity. However, it prevented Atoh1 autoactivation (n=3). **(D)** Hey1 did not prevent Atoh1 activation in the E-boxA multimer (n=3).

4. Hey1 basic domain is essential for Atoh1 repression

We tested the ability of a DNA-binding deficient Hey1 construct (Hey-DN) to repress the 3'Atoh1-enh. The Hey-DN carries two mutations in the basic domain that prevents Hey1 binding to DNA (Gessler Lab). Contrary to Neurog1, Hey1-DN completely lost its repressor activity on both the 3'Atoh1-enh (Fig. 5A) and the EnhB reporter constructs (Fig. 5B). This strongly suggests that Hey1 requires binding to the CAN region to exert its repressor function. The results fit well with the change in occupancy of the E-box C in E14.5 mouse otocysts observed after LY411575 treatment in the ATAC-seq analysis (Fig. 3 Chapter I Neurog1). The class C E-box showed no occupancy after LY411575 treatment, suggesting that Notch targets mediate repression through binding this E-box. It also fits with the mutation analysis that shows that class C E-box is a site required for repressing of the enhancer (Fig. 1C, Results Chapter I).

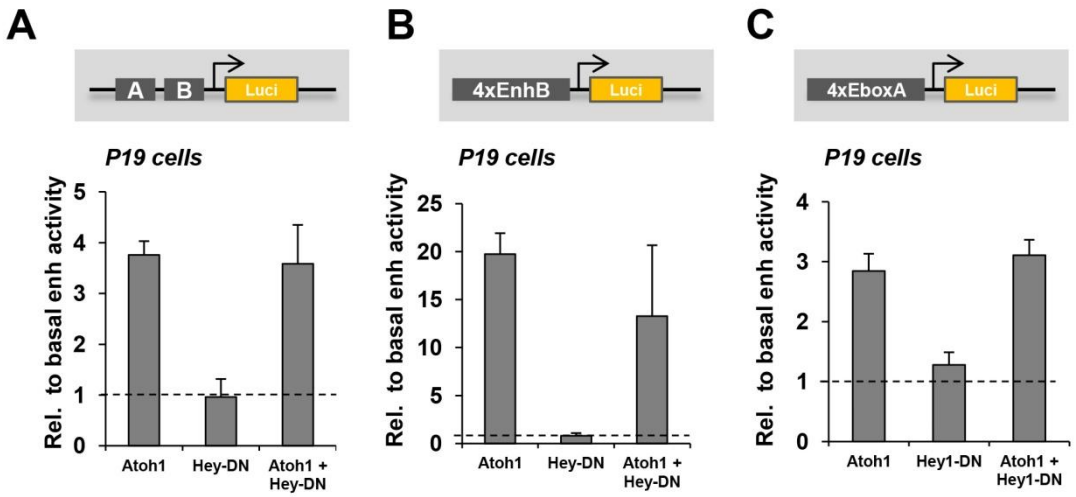


Figure 5. Hey1 requires the basic domain for Atoh1 repression. Quantification of 3'Atoh1-enh (A) or 4xEnhB (B) with Atoh1 and Hey1-DN was performed in P19 cells. Hey1-DN does not have the ability to bind to DNA. Luciferase values showed that Hey1 needed the DNA binding domain to repress the 3'Atoh1-enh **(A)** and EnhB **(B)**. **(C)** Class A E-box multimer showed that Hey1 can neither activate nor prevent Atoh1 activation.

In an attempt to identify the regions important for the repression, we performed different point mutations in the E-boxes of EnhB and tested them against Hey1 or Hes5. Mutations of the class C E-box and a double mutation with the class N-box were tested in combination with Atoh1 with or without Hey1 or Hes5. Detailed point mutations are described in Fig. 6 A. Unexpectedly, none of the mutations reduced the capacity of Hey1 or Hes5 to repress Atoh1 (Fig. 6), suggesting that Hey1 and Hes5 do not bind to their predicted putative E-boxes.

The above results are contradictory with the idea that the class C E-box of EnhB is the major site for Notch targets, as derived from ATAC-seq analysis and the effects on the basal activity. Possible explanations of this discrepancy will be discussed in the Discussion section.

A

Reporter	Sequence
Wt 3'Atoh1-enh	AG**CACGCG**CTGTC**AGCTGGTG**AG
Class C E-box mut	AG**ATT**GCGCTGTCAGCTGGTGAG
Class C-N box mut	AG**ATT**GCGCTGTCAGCTG**AAT**AG

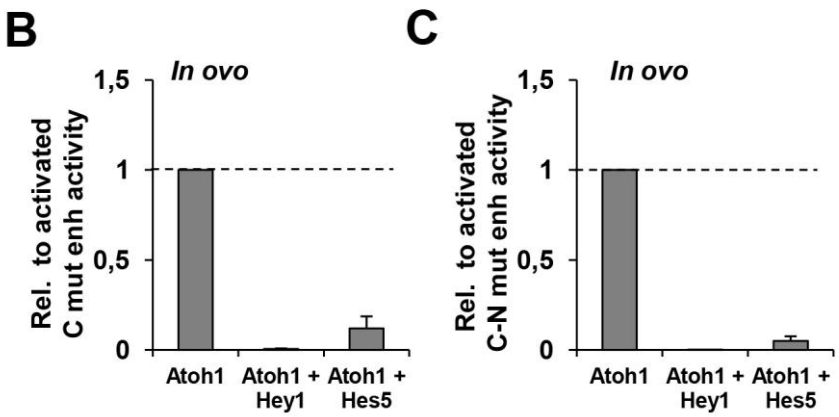


Figure 6. Hey1 and Hes5 predicted binding sites were not enough to prevent the repression by Hey1 or Hes5. **(A)** Table showing the mutations generated in the E-boxes. The three E-boxes are depicted in black and bold, in red the mutated nucleotides **(B)** Hey1 and Hes5 were able to block Atoh1 autoactivation when class C E-box was mutated (n=3). **(C)** The same results were obtained when the E-box C and N-box were mutated together. Two replicates for Hey1 and three replicates for Hes5.

DISCUSSION

One central question in development is to understand how different cell types are generated at specific times and locations throughout embryonic life. The vertebrate inner ear is a simple example in which the three major cell types of its functional unit, hair cells, supporting cells and neurons, arise with a precise timing and a specific spatial arrangement during development. The aim of the present work was to understand how molecular interactions result in cell fate decisions during inner ear development. In particular, I focused my work on the decision between neurogenesis and sensory development by analysing the molecular mechanisms underlying the repression of Atoh1 by Neurog1 and other bHLH factors. The experiments reported here show that Neurog1 prevents the autoregulatory loop that drives Atoh1 expression and HC formation. Further, the repression by Neurog1 is not due to its interaction with the enhancer regions of Atoh1, as expected from a transcription factor, but by protein-protein interactions that result in the modification of Atoh1 protein levels. In other words, Neurog1 prevents Atoh1 transcription by lowering the levels of the major Atoh1 activator, which is Atoh1 itself. This explains why Neurog1 requires the integrity of the 3' Atoh1-enh -the site of Atoh1 autoregulation- to exert its function, but not to bind DNA. The work provides a molecular explanation for the dominant effect of neurogenesis against sensorigenesis, and a novel mechanism for the interaction among proneural factors.

1. Neurons vs. hair cells

The three cell types of the sensory unit of the inner ear arise from the neurosensory domain that, early in development, is specified in the otic placode. Multipotent progenitors express the proneural factor Sox2 which gives the proneural (neurosensory) identity to the neurosensory region (Neves et al., 2012). Contrary to what happens in the development of central and peripheral nervous system in Drosophila, bHLH proteins, are not the selectors of the precursors cells. In vertebrates, this selection is done by the SoxB1 factors, which set up the expression of neuronal and hair cell master genes, Neurog1 and Atoh1, respectively (Ahmed et al., 2012a; Ahmed et al., 2012c; Jeon et al., 2011; Neves et al., 2012). Neurog1 drives neuronal generation and delamination at early stages of otic development (Evsen et al., 2013; Ma et al., 1996), but the onset of Atoh1 is delayed with respect to Neurog1, as it is hair cell formation. The result of this stereotyped timing is that hair cells differentiate at the time that the peripheral projections of otic neurons invade sensory epithelia. The early expression of Neurog1 and the latter of Atoh1, suggest that their expression is

regulated by positional cues set by other factors. When Neurog1 is expressed instead of Atoh1 in mice inner ear, the formation of hair cells is severely affected (Jahan et al., 2015), suggesting that the correct timing of Atoh1 and Neurog1 expression is crucial for the formation of neurons or hair cells.

This sequence of events poses one major question: why Atoh1 is not expressed along with Neurog1 when induced by Sox2 in the neurosensory domain? In other words, what prevents the precocious differentiation of hair cells? Inhibitory interactions between Neurog1 and Atoh1 have been documented in mouse but the underlying molecular mechanisms are still unknown (Matei et al., 2005; Raft et al., 2007).

2. The Enhancer B is at the core of Atoh1 regulation

Pioneering work in Jane Johnson's laboratory identified two Enhancers, A and B, within 21Kb sequence flanking the Atoh1 coding region that are able to drive Atoh1 expression. Together, they are called the 3'Atoh1 enhancer (3'Atoh1-enh). It has a length of 1.7Kb, it is highly conserved among vertebrates and is sufficient to recapitulate Atoh1 expression in mouse and chicken (Ebert, 2003; Helms et al., 2000). Atoh1 expression relies mainly on its own expression, being an autoregulatory loop the major mechanism that regulates Atoh1 transcription (Helms et al., 2000). Given that Atoh1 is activated by Sox2 but its expression is detected only after neurogenesis (Neves et al., 2012; Pujades et al., 2006; Raft et al., 2007), the implication is that it must be repressed during neurogenesis. Along this line of thought, our experiments focused on the mechanism by which Neurog1, the master gene for neuronal commitment, interferes with the Atoh1 autoregulatory loop.

The experiments show that Neurog1 is able to block Atoh1 auto-activation by acting on the Enhancer B. This repression requires the integrity of not only the class A E-box of EnhB but also the whole CAN region (E-box C, E-box A and N-box located at EnhB) and (see below). Indeed, EnhB holds most of the activity and accessibility to transcription factors as indicated by ATAC-seq and reporter experiments. ATAC-seq analysis revealed that only EnhB of the 3'Atoh1-enh is in the form of accessible chromatin, EnhA remaining closed. This corresponds well with the behaviour of the separate enhancer regions in vivo, as shown by reporter analysis of the two regions in the chick otic vesicle. EnhA shows no activity on its own, although it modified that of EnhB, suggesting that the EnhA is important for the spatial restriction and the level of

activation of EnhB. The latter, contains all the information required for the regulation by Neurog1.

3. The mechanism of Atoh1 repression by Neurog1

Neurog1 is a class II bHLH that binds to class A E-boxes (CANNTG) (Bertrand et al., 2002) and activates the expression of target genes. Among them, NeuroD is one major effector of Neurog1 in the ear, being essential for neuroblasts delamination (Huang et al., 2000; Kim et al., 2001; Ma et al., 1996) and for shutting down Sox2 expression in the neurons (Evsen et al., 2013). *In vivo*, Neurog1 transcriptionally activates target genes that cooperate for Atoh1 suppression. Conditional NeuroD deficient mice show that NeuroD suppresses Atoh1 expression in auditory-vestibular neurons as indicated by the ectopic expression of Atoh1 after NeuroD deletion (Jahan et al., 2010). However, during early stages of neurosensory development, Neurog1 is expressed homogeneously in the neurosensory epithelium, including hair cell precursors (Raft et al., 2007), and only those cells that express high levels of Neurog1 trigger lateral inhibition and delaminate from the epithelium. Therefore, alternative mechanisms are required to prevent Atoh1 without necessarily driving neuronal differentiation. Prevention of HC formation dissociated from neurogenesis may reinforce/enhance the progenitor neurosensory state (Fritzsch et al., 2006b; Sun et al., 2001). The fact that Neurog1 is able to repress Atoh1 and hair cell formation by a DNA-binding independent mechanism may be particularly adapted to this situation.

Reporter experiments discard the possibility that Neurog1 makes an unproductive binding to the same class A E-box used by Atoh1, in a competitive inhibition or a partial agonist manner. Indeed, Neurog1 is able to activate the isolated class A E-box and Neurog1-AQ still able to repress the whole enhancer. However, the repressor effect of Neurog1 still requires the integrity of the CAN region of the 3'Atoh1-enh. Neurog1 does not require binding to DNA to repress Atoh1. However it requires the helix1 dimerization domain. This suggests that repression by Neurog1 relies on an indirect mechanism that results in the loss of activity of the 3' Atoh1-enh. Whatever the mechanism, it is cell autonomous and does not depend on Notch signalling, since the blockade of the Notch pathway does not interfere with Neurog1 repression.

Since, on one hand Neurog1 represses the activity of the 3' Atoh1-enh without binding to the enhancer and, on another hand, Atoh1 is the major activator of the enhancer, one likely possibility is that Neurog1 modifies the activation capacity of Atoh1 to

activate its own enhancer. In principle, this may happen by reducing the activity of Atoh1 protein, reducing its levels, or both, and those possibilities were explored here.

The H1 (helix1) domain is crucial for heterodimerization of bHLH proteins with other bHLH (Longo et al., 2008). This led us to test the hypothesis that Neurog1 sequesters the E-proteins required for Atoh1 function. Several E-proteins such as E47, E2.2 and HEB are likely candidates for this function (Flora et al., 2007; Zhang et al., 2006). However none of them are able to prevent the repression by Neurog1 *in vitro*. Similarly, other factors that act as co-activators of transcription such as p300 or CBP, which are sequestered by Neurog1 in neural stem cells (Sun et al., 2001), are also unable to prevent the repression of Atoh1.

Considering that the CAN region is required for the repressor effect of Neurog1, factors that inhibit differentiation may bind to the regions flanking the class A E-box, preventing in this way Atoh1 interaction with the enhancer. One candidate for such function is Sal1, which was present in the Mass-spec analysis among the proteins bound to Atoh1 in the presence of Neurog1. Sal1 protein is targets for repression and inhibitors of differentiation in other tissues, like in mice kidney (Basta et al., 2014). Unfortunately, there is no information on its expression and function during ear development.

Given that none of the known E-proteins partners of Atoh1 accounted for the loss of Atoh1 activity, we next explored whether Neurog1 directly binds to Atoh1, the complex resulting inefficient. This possibility is unlikely, since immunoprecipitation combined with Mass-spec analysis showed that Neurog1 is not present in the Atoh1 pull-down and vice-versa. This discards the possibility that the direct association of Neurog1 with Atoh1 is responsible for preventing Atoh1 function.

One other general mechanism for the regulation of long-term protein interactions is the control of protein levels. We therefore tested whether Atoh1 protein levels were reduced by Neurog1 and, indeed, this was the case. This decrease was abolished when Neurog1 lacked its Helix1 domain, suggesting that both the loss of Atoh1 protein and the repressor effect on the 3'Atoh1-enh have a common link. The reduction in Atoh1 protein levels, in turn, may be caused by at least these three mechanisms, which are not mutually exclusive: first, the loss of Atoh1 mRNA, secondly, the reduction of Atoh1 translation rate and finally, the increase of Atoh1 degradation (See Discussion Fig 1).

Degradation of bHLH proteins has been extensively documented and it is accounted by phosphorylations in their C-terminal region in different model systems (Forget et al., 2014b; Hardwick and Philpott, 2015; Quan et al., 2016). Atoh1 post-transcriptional downregulation has been reported during cerebellar granule neuron differentiation, where BMP2 and BMP4 inhibit proliferation and induce differentiation through proteosome mediated degradation of Atoh1 (Zhao et al., 2008). BMPs induce the expression of Id1 and Id2 that upon dimerization with Atoh1 target the complex for degradation. In cerebellar granule neuron progenitors, Shh prevents Atoh1 degradation by preventing the recruitment of Atoh1 by Huwe3, an E3 ligase (Forget et al., 2014b). Atoh1 is degraded by the proteosome pathway when dimerizing with Huwe1 in HEK cells, and the conditional deletion of Huwe1 generates supernumerary hair cells in the mouse cochlea (Cheng et al., 2016).

Immunoprecipitation of Atoh1 in the presence of Neurog1 disclosed two proteins bound to Atoh1 that are involved in the proteosomal degradation pathway, like skp1 and TRIM56. The former is an E3-ubiquitin ligase that degrades target proteins that contain phosphorylation modifications (Sardiello et al., 2008). TRIM proteins are also involved in blocking translation (see below). The role of TRIM proteins in the developing inner ear has been recently investigated by Evsen et al., (2016) who found that TRIM 71 prevents HC formation in chicken basilar papilla (Abstract SDB). The other protein, Skp1, is a component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription (Uniprot code: Q9WTX5). Skp1 expression in the inner ear is restricted to the supporting cells layer and this suggests that prevents HC fate (Andy Groves, personal communication). These observations suggested us that Neurog1 possibly enhances Atoh1 degradation through the proteosome pathway. However, in our hands, the effects of Neurog1 over Atoh1 protein levels were not altered after inhibition of protein degradation, discarding this mechanism for the reduction of Atoh1.

Leaving aside degradation, other alternatives for the loss of Atoh1 protein in the presence of Neurog1 are that Atoh1 mRNA is degraded, or that Atoh1 translation is hampered. The blockage of protein synthesis has been shown to be important in Drosophila development, where the TRIM32/Argonaut1 protein complex interferes with Atoh1 protein synthesis and neural differentiation (Stegmüller and Bonni, 2010). Interestingly, Argonaut 2 and TRIM56 were present in the Atoh1 pull-down in the

presence of Neurog1. De-stabilisation of mRNA is a mechanism to avoid mRNA accumulation in the cell and avoid cell differentiation in neural stem cells (Knuckles et al., 2012). This mechanism is accomplished by RNAse III protein called Drosha, that destabilises Neurog2-mRNA (Knuckles et al., 2012). The stability of mRNA of other bHLH like E47 changes upon age (Frasca et al., 2005). These observations encourage us to think that Atoh1 regulation by Neurog1 is mediated by mRNA de-stabilization or by inhibiting its translation.

The stability of mRNA depend on RNA-binding proteins that bind mRNAs, and the half-life of many mRNAs can fluctuate in different developmental contexts (Day and Tuite, 1998; Knuckles et al., 2012). Also, micro-RNAs (miRNAs) are known regulators of mRNA stability or translation efficiency and modify protein expression levels. Some miRNAs like the miR-183 family (miR-96, miR-182 and miR-183) are expressed in young hair cells and ganglion neurons (Li et al., 2010; Weston et al., 2006) and the manipulation of miR-183 levels modify the number of hair cells (Groves et al., 2013; Li et al., 2010). Regulation of translation and protein synthesis depends on initiation factors (eIFs), some of which are phosphoproteins susceptible of regulation (Day and Tuite, 1998), but little is known about their behaviour during embryonic development.

In summary, the experiments point to the notion that the reduction of Atoh1 protein levels induced by Neurog1 is due to a post-transcriptional regulation of Atoh1 that does not involve, proteosome-dependent degradation. Potential mechanisms are the induction of mRNA de-stabilisation and/or inhibition of protein translation. However, the identity of the proteins that bind to the helix1 and the nature of the mechanisms triggered by Neurog1 to induce the loss of Atoh1 protein are still unknown.

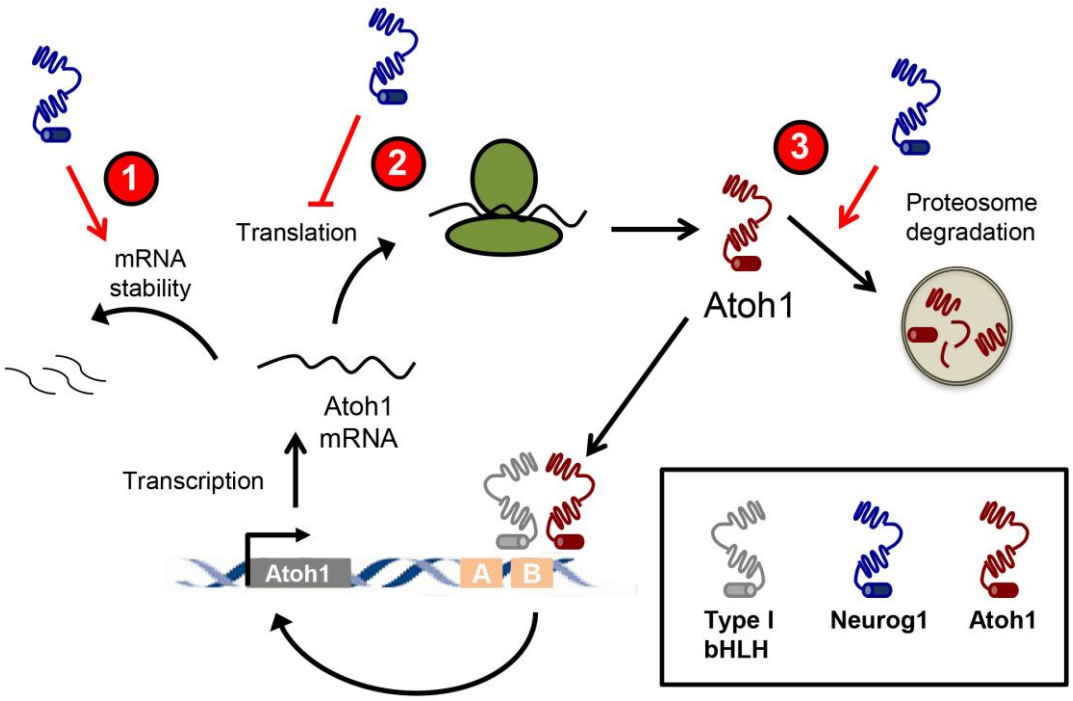


Figure 1. Proposed model of Neurog1 mechanism. Neurog1 can affect Atoh1 production by promoting 1- mRNA degradation; 2- Inhibition of protein synthesis and; 3- protein destabilization by promoting Atoh1 protein to proteosome degradation.

4. The flanking region of E-box A selects the binding affinity of Atoh1

Although several factors like Sox2, Six/Eya and β-catenin are known to activate the 3'Atoh1-enh (Ahmed et al., 2012a; Neves et al., 2012; Shi et al., 2010), Atoh1 regulation can be mainly accounted by its own activation (Helms et al., 2000). This occurs through the binding of Atoh1 to the class A E-box in the CAN region of Enhancer B (Helms et al., 2000; Lai et al., 2011; and present work). In the absence of ChIP-seq data in the inner ear, *in silico* analysis indicates potential regions for binding of transcription factors and, in particular, bHLH factors. Overlapping the class A E-box in the 3' Atoh1-enh there is a reverse N-box, while few nucleotides upstream there is a class C E-box consensus site (We refer to this region as the CAN region). The 3' Atoh1-enh is highly conserved among human, mouse and chicken (Ebert, 2003) and, interestingly, only the CAN region is conserved also in zebrafish (Fig. 2). The arrangement of the CAN region invited us and others to think that bHLH repressor factors may occupy those sites and prevent the binding of Atoh1 (Groves et al., 2013; Jarman and Groves, 2013; Mulvaney and Dabdoub, 2012).

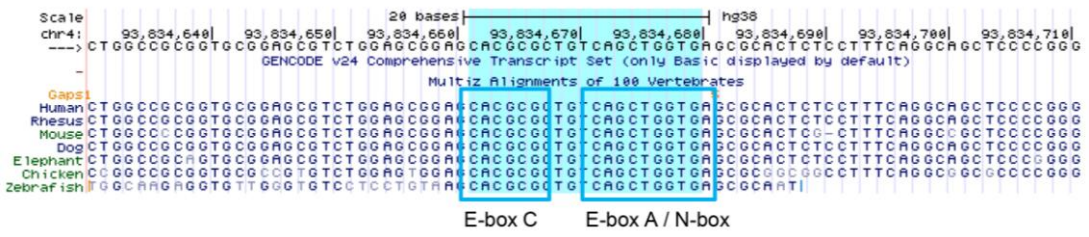


Fig 2. Enhancer B region of Atoh1 3' enhancer. Reference sequence is the human region downstream Atoh1 coding region. The only conserved region among the species shown is the CAN region.

The class C E-box acts as a repressor site, since mutation of the E-box C results in the increase of the 3'Atoh1-enh activity. In agreement, ATAC-seq analysis shows the occupation of E-box C during Atoh1 repression, and its vacancy after Notch inhibition, which promotes the occupation of class A E-box. Class C E-boxes are preferred binding sites for Notch downstream targets Hey/Hes (Iso et al., 2003; Murre et al., 1994a), which are known repressors of Atoh1 (Kiernan, 2013). This provides a direct mechanism for the repression of Atoh1 by Hes and Hey factors. However, the repression by Hes and Hey factors is likely to be more complex because they have different modes of repression (Fischer and Gessler, 2007; Kageyama et al., 2007). A recent work has shown that Hes5 and Hey2 have the ability to prevent Atoh1 expression by binding to the promoter region in several in vitro cell lines (Abdolazimi et al., 2016). This repression seems to be crucial for the quiescence of the supporting cell state, which, contrarily to what occurs in development, it depends very little on the activity of the 3' Atoh1-enh. ATAC-seq data did not show occupancy of the E-boxes present in the Atoh1 promoter, neither in the suggested E-boxes bound by Hes5 (Abdolazimi et al., 2016), indicating that he promoter region is not instrumental for repression during development. The picture is that the promoter region and the 3'Atoh1 enhancer show a reciprocal and complementary behaviour with respect to Atoh1 repression during development and post-natal life.

A general feature of Atoh1 regulation is that its transcriptional activity is severely impaired by the mutation of the last nucleotides of the AtEAM motif (Klisch et al., 2011; Powell et al., 2004). Accordingly, Atoh1 transcription is impaired after mutation of the N-box, but only when the AtEAM motif is affected. This indicates that the last two nucleotides of the AtEAM motif are crucial for proper Atoh1 function and that, in fact, the overlapping N-box is required for Atoh1 autoactivation. The activation of the Drosophila atonal enhancer is also reduced after changing two nucleotides flanking the

core of class A E-box (Powell et al., 2004). Suboptimal binding motifs and spacing in enhancer also attenuate enhancer activity (Farley et al., 2015). Thus, the specificity of Atoh1 to activate class A E-box during hair cell formation is likely the consequence of the surrounding E-box A region.

The results show that Neurog1 is also able to activate the class A E-box of the 3' Atoh1 enhancer, but this interaction is not competitive with Atoh1. In fact, Neurog1 is not able to prevent the activation of the monomers of class A E-box by Atoh1. We have no explanation for this observation besides the possibility that the isolated class A E-box may have a high affinity and be activated at a very low Atoh1 concentration. Since the activation of the class A E-box by Neurog1 turns into repression in the presence of the flanking regions, it is possible that these flanking regions are required to set the affinity of the class A E-box for the type II bHLH factor. Should they differentially lower the affinity for Atoh1 and Neurog1, the net result would be the selection of Atoh1 over Neurog1.

5. Prosensory and SC cell fate: just Notch for the repression of Atoh1?

Hey1 is the major Notch effector expressed during the expansion and proliferation of the prosensory patches, and the preferred target of Jag1 activity. Hes5 is expressed during lateral inhibition as the major target of Delta1 activity (Petrovic et al., 2014). The combined loss of function of Hes5, Hey1 and Hes1 results in supernumerary hair cells (Tateya et al., 2011) and Atoh1 is upregulated after interference of Hey1/Hes5 expression with siRNAs (Du et al., 2013). Therefore, Hey1 and Hes5 repress Atoh1 and our experiments show that they are able to silence the 3'Atoh1-enh. In addition, both factors are able to block Atoh1 autoactivation, suggesting that, under these conditions, the repression of Atoh1 prevails upon activation. To further test the functional consequences of Hey1 repressor activity, we analysed the effects of the gain of function of Hey1 on hair cell development. The results show that in parallel to 3'Atoh1- enh repression, Hey1 is sufficient to prevent HC generation in chick sensory epithelia. Taken together, these observations suggest that during otic development, Notch targets Hey1 and Hes5 act on the 3'Atoh1-enh repressing Atoh1 expression in prosensory precursors and supporting cells.

Hey1 needs to bind DNA in order to repress Atoh1, since the mutation of Hey1 DNA binding domain abolishes repression. However, the identification of the region bound

by Hey1 and Hes5 has been difficult to demonstrate and still remains elusive. On one hand, mutations of either the class C E-box or the N-box of the CAN region were unable to prevent Hey1 or Hes5 Atoh1 repression. This is in agreement with the results of ChiP-seq analysis performed on HEK 293, which shows that Hey1 does not bind to the 3'Atoh1-enh (Heisig et al., 2012). This is somehow surprising and suggests alternative binding sites and/or mechanisms of repression. On the other hand, ATAC-seq data shows that E14.5 cochlea treated with Notch inhibitor releases the occupancy of E-box C, suggesting that Hey1 and Hes5 interact with this E-box. We have no explanation for this apparent contradiction, whose elucidation requires further study. Besides, it is possible that Hey1 blocks the transcription of Atoh1 by interfering with the class A E-box. This possibility is difficult to explore since the mutation of the E-box A silences the 3'Atoh1-enh. It is worth noting here that being Atoh1 autoactivation the major mechanism for Atoh1 regulation, any molecular change that impairs Atoh1 binding to the class A E-box results in Atoh1 inactivation.

In summary, Hey1 requires binding to the 3'Atoh1-enh to suppress its activity. However, evidence available does not allow us to clarify at the moment whether Hey1 binds to the class C E-box or to other sites that indirectly results in the inactivation of the 3' Atoh1 enhancer.

6. Building up Atoh1 expression: The priming of the 3'Atoh1-enhancer

Sensory competence of the inner epithelia is established by the activation of Atoh1 and this requires Sox2. Atoh1 is activated by the direct interaction of Sox2 with the Enhancer A of the 3'Atoh1-enh (Neves et al., 2012). However, the mechanism of action of Sox2 is intriguing in two respects. First, Sox2 is able to activate the 3'Atoh1- enh, but not EnhA alone (not shown). This suggests that the interaction of Sox2 with the 3'Atoh1-enh requires DNA folding and the interaction between Enhancers A and B. Indeed, Sox2 co-partners Six1/Eya1 have binding sites close to the CAN domain in EnhB (Ahmed et al., 2012a). Secondly, ATAC-Seq experiments showed that EnhA was not accessible at the stages analyzed. One explanation for this seemingly paradox is that like Ascl, Sox2 is able to bind regions of closed chromatin (Raposo et al., 2015). This view is supported by the observation that Sox2 has been identified recently as a pioneering transcription factor able to bind nucleosomal DNA (Soufi et al., 2015).

However, priming of Atoh1 in the developing ear may be also promoted by other mechanisms. Changes in histone modifications can change the transcriptional hierarchy that controls cell differentiation (Azuara et al., 2006). The dynamic changes in the histone modifications H3K4me3/H3K27me3, H3K9ac and H3K9me3 indicate that there is a progression from poised to active, and finally to repressive marks in the Atoh1 locus. This sequence correlates with the onset of Atoh1 expression and its subsequent silencing during the perinatal period (Stojanova et al., 2015). The inhibition of acetylation blocks Atoh1 mRNA expression in nascent hair cells, as well as the ongoing hair cell differentiation during embryonic organ of Corti development (Stojanova et al., 2015). Contrarily, histone deacetylase inhibition favours the expression of hair cell markers in mouse utricle progenitor cells (Hu and Wang, 2014). Cochlear explants treated with histone deacetylase inhibitor increase the levels of Atoh1 mRNA in early post-natal mice (Stojanova et al., 2015). This suggests that Atoh1 is poised during developmental stages and thereby ready to be activated. However, after birth the Atoh1 locus becomes methylated and cannot be transcribed when hair cells are damaged. The epigenetic status of Atoh1 locus during organ of Corti development shows a bivalent mark of the Atoh1 locus by H3K27me3and H3K4me3, prior to the upregulation of Atoh1 (Stojanova et al., 2015). This is consistent with the idea that Sox2 poises/primes the Atoh1 locus until Atoh1 itself is able to bind to the E-box A and trigger Atoh1 expression.

7. Implications for HC regeneration

The results of the work presented here address the mechanisms of neuronal and hair cell fate specification during development. But beyond, this knowledge may have implications for designing regenerative therapies as applied to hearing loss. First, Atoh1 is a master gene for making HCs, and an exhaustive understanding of its regulation is crucial for deriving hair cells in vitro and for awakens the non-regenerative mammals supporting cells. We provide here valuable information on the properties of the 3'Atoh1-enh and its regulation by bHLH factors expressed during development, and describe a novel mechanism by which Neurog1 interferes with Atoh1 expression and prevents hair cell formation. Neurog1 induces the lowering of Atoh1 protein levels, which in turn suppresses Atoh1 auto-activation. The interaction between Neurog1 and Atoh1 shows a dominant role for the former, the result being that neurons develop before hair cells. This is interesting, because it resembles very much what happens in the culture dish when trying to differentiate HCs from stem cells (Chen et al., 2012;

Ronaghi et al., 2014). Different attempts to generate HCs in culture commonly face the problem that most cells derive to the neuronal cell fate, making it very difficult to enrich them in HCs (Chen et al., 2012; Ronaghi et al., 2014). This is reminiscent of what happens in the embryo, where neurogenesis prevails over sensory development. The cellular context of conditionally derived stem cells may be similar to that in the embryo and interference with heterodimerizing properties of Neurog1 may open a way to improve the efficiency of HC production.

CONCLUSIONS

Conclusions

1. The 3'Atoh1 enhancer is composed of two regions, Enhancer A and Enhancer B, which show different accessibility and activity. Enhancer A is silent but determines the spatial restriction of the 3'Atoh1 enhancer. Enhancer B accounts for the activity and regulation of Atoh1 in the neurosensory domain.
2. Atoh1 activates its own enhancer through the class A E-box of the CAN region located at Enhancer B, the flanking E-boxes being crucial for Atoh1 activation.
3. Neurog1 is able to repress the activity of the 3'Atoh1 enhancer and to block Atoh1 autoactivation. This repression is independent of Notch activity and requires the integrity of the CAN region of Enhancer B.
4. Neurog1 does not require DNA binding for 3'Atoh1 repression and prevention of HC formation. Instead, it requires the Helix1 dimerization domain.
5. Neurog1 represses the 3'Atoh1 enhancer indirectly by protein-protein interactions that results in the reduction of Atoh1 protein levels. The mechanism probably involves the inhibition of protein translation or mRNA de-stabilisation but not protein degradation by the proteosome pathway.
6. Hey1 is sufficient to block the 3'Atoh1 enhancer and to prevent hair cell differentiation. This requires binding to DNA, yet the identity of the binding sites is still elusive.
7. Together, the results deep into the molecular mechanisms of Atoh1 repression, a crucial step for hair cell development and regeneration.

MATERIALS AND METHODS

1. Chicken embryos

Fertilized hens' eggs were obtained from Granja Gibert located in Tarragona (Spain). Eggs were stored at 15ºC and incubated at 38ºC for the desired times. Embryos were staged according to Hamburger and Hamilton (V. Hamburger, 1951).

2. In ovo electroporation

2.1. In ovo electroporation of HH12-14 (E2)

Electroporation uses electrical fields to create transient pores in the cell membrane that allow the entry of normally impermeable macromolecules. The electroporation technique has been mostly used to transfer DNA to bacteria, yeast, and mammalian cells in culture, but now applied to living animals. In this work we used the in ovo electroporation technique as developed by Nakamura.

HH12-14 (E2) chicken embryos were electroporated into the otic cup by using the following procedure: a window was opened in the shell with curve scissors after removal of 5ml of albumin with a regular 5ml syringe, and the vitelline membrane was gently opened to get access direct to the embryo. The cathode platinum electrode was placed next to the right otic cup and the anode electrode parallel to it, on the other side of the embryo. It was very important not to touch the embryo with the electrodes to avoid embryos death.

DNAs used for electroporation were: EGFP-C1 (0.5 µg/µl, Promega), pDsRed (0.5µg/µl Clontech), CMV-Luciferase (0.2µg/µl, R.Perona, Madrid), β-galactosidase reporter (1µg/µl) and expression vector (1-2µg/µl). Fast green (0.04 µg/µl) was added to the DNA mix to visualize the injection. The delivery was performed by delivering air pressure through a glass micropipette. Information about the expression vectors and reporters used is summarized in Table1 and Table2.

Square pulses (8 pulses of 9V, 50Hz, 250ms) were generated by a Square CUY-21 (BEX Co., Ltd, Japan) electroporator. Medium-199 (22350018-Invitrogen) with 1x Antibiotics (Pen/strep, 15140-122, GIBCO) was added to the top of the embryo while electrodes were removed. Eggs were sealed and re-incubate for designated time.

Table 1. Plasmid constructs table

Vector	Source
pCiG-mHey1^{+}*	Petrovic et al., 2014
pCAGGs-cHes5^{+}*	Fior and Henrique, 2005
pMiW-mNgn1^{+}*	Gowan et al., 2001
pMiW-mNgn1-AQ^{+}*	Gowan et al., 2001
pCIG-mId3^{+}	Elisa Marti's Lab
pIRES2-hAtoh1^{+}*	Thomas Schimmang's Lab
pCDNA3.1-hHey1^{+}	De la Pompa's Lab
pMiW-mNeurog1-ΔH1 *	Fernando Giraldez's Lab
pMiW-mNeurog1-ΔH2*	Fernando Giraldez's Lab
pMiW-mNeurog1-ΔC-term*	Fernando Giraldez's Lab
CMVβ-hp300 *	Eckner et al., 1994
pRc/RSV-mCBP *	Richard Godman's lab
pCIG-hSmad1 *	Elisa Marti's Lab

Table 2. Reporter table

Reporter	Source
3'Atoh1-enhancer-BgZA^{+}	Helms et al., 2000
4xEnhancer A-BgZA^{+}	(Tg14, Helms et al., 2000)
4xEnhancer B-BgZA $^{+}$	(Tg17, Helms et al., 2000)
E-box A mut-BgZA^{+}	Fernando Giraldez's Lab
E-box C mut-BgZA^{+}	Fernando Giraldez's Lab
C-N box mut-BgZA^{+}	Fernando Giraldez's Lab
CAN mut-BgZA^{+}	Fernando Giraldez's Lab
4xEboxA-pGL3-basic vector*	Fernando Giraldez's Lab
4xCAN-pGL3-basic vector*	Fernando Giraldez's Lab
pGL3b-3'Atoh1-enh*	Fernando Giraldez's Lab
4xEnhB-pGL3b*	Fernando Giraldez's Lab

Concentration of expression plasmid for in ovo electroporation was 1-2 μg/μl**.** Expression plasmids used for transfection in cells were used at 0.075 μg/μl. Reporters used at 1 μg/μl in ovo and 0.075 μg/μl in P19 cells. + For vectors/reporters used in ovo and * for P19 cells.

2.2. In ovo electroporation HH20-21 (E3.5)

Electroporations of HH20-21 (E3.5) otic vesicles were performed as described in Kamaid et al., (2010). The cathode consisted of a 0.3 mm diameter Pt tip attached to a handle and the anode was a 0.5 mm diameter Pt electrode that was placed underneath the embryo. DNA was injected into the otic vesicle at a concentration of 6–8 µg/µl and electroporation conditions were 8 pulses of 10–12 V, 250 ms, 50 Hz. A second train of pulses was performed by changing the polarity of the electrodes when aiming at massive electroporations. Like before, the embryo was covered with medium and eggs were sealed and incubated for the designated stage.

Plasmids used in HH20-21 electroporations were pCiG-mHey1, pMiW-mNgn1 and pMiW-mNgn1-AQ. EGFP-C1 was added to trace the electroporation. Fast green (0.04 µg/µl) was added to the DNA mix to visualize the injection. The high concentration of the DNA mix sample is crucial for the efficiency of the electroporation. Concentrations lower than 4 µg/µl of DNA were not successful.

3. β-gal staining

For *whole mount* β-galactosidase staining, embryos were fixed with 4% Para-Formaldehyde for 1h at 4ºC, washed in PBS and incubated at room temperature for 30-180 minutes with β-gal solution (1mg/ml X-gal, 5mM Ferrocyanide, 5mM Ferricyanide and 2mM $MgCl_2$). Embryos were then washed with PBS, fixed again with 4% Para-formaldehyde for 30 minutes and processed for cryosection.

4. Immunostaining

4.1. Whole mount immunostaining

Staged embryos were dissected in cold PBS. Two washes with PBS were done before O/N fixation with 4%PFA at 4ºC. After fixation embryos were washed three times with PBS-1%Tween for 30'. After rinse, proceed directly to block for 1h at RT with 10% goat or horse serum in PBS-1%Tween. Incubate with primary antibody O/N at 4ºC in a sacker. Incubate with primary antibody diluted in blocking solution O/N at 4ºC in a shaker. Wash 4-8 times during 10' the following day with PBS-1%Tween with shaking. Incubate with the secondary antibody O/N at 4ºC in a shaker. Wash 4-8 times for 10' the following day with PBS-1%Tween with shaking. Incubate DAPI (100mg/ml) staining 10-20' at room temperature. Additionally, you can rinse twice after DAPI staining with

PBS-1%Tween. Finally, mount embryos with Glycerol.

4.2. Standard protocol for slide immunofluorescence.

Embryos were fixed O/N with 4%PFA at 4ºC and washed with 1xPBS and cryoprotected with 15% sucrose at 4ºC. Samples were embedded in pre-warmed (37ºC) 7.5% gelatine and 15%sucrose at 37ºC. Blocks were frozen cold 2-methylbutane and stored at -80ºC until sectioned (20µm) with a cryostat (LeycaCM1950). Take slides out from freezer and let at RT for 15-30min. Circle your tissue with hydrophobic pen and wait until it dries. Rinse the slides for 30 min in PBT (PBS+0.1% Triton) to remove agar/embedding medium. Incubate for 30 min at RT in block solution (PBS + 0.1%Tween + 10% Goat serum or any serum matching the secondary antibody species). Incubate 2h at RT or overnight at 4ºC with 100µl of primary antibody(ies) solution in PBT- *If overnight incubation, use coverslips made of parafilm.* Rinse in PBT for 3x 10 min- *if coverslips were used; remove them during the first rinse.* Incubate 2h at RT with secondary goat anti-IgG antibody(ies) in the dark. *The secondary has to be directed against Ig of the primary antibody.* Rinse samples with PBT for 3 x 10 min. If necessary, stain nuclei with DAPI (100ng/ml) (optional, some mounting media contains DAPI, **and do not use DAPI if an A405-conjugated secondary was used**). Mount in glycerol or Mowiol mounting medium and seal with nail varnish and store at 4ºC.

Table 3. Primary antibodies for Immunofluorescence

Antibody	Species	Source	Dilution
class III β-tubulin	rabbit polyclonal	Covance PRB435P100	1:500
GFP	rabbit polyclonal	Torrey Pines 401	1:400
GFP	mouse monoclonal	Invitrogen A11120	1:400
Myo7a	mouse monoclonal	Hybridoma Bank	1:100
Sox2	goat polyclonal	Y-17 Santa Cruz	1:400
Ds-Red	rabbit polyclonal	Takara 632496	1:400

Secondary antibodies were Alexa Fluor 488-, 555-, 546-, and 594-conjugated anti-mouse, anti-goat and anti- rabbit (Molecular Probes Invitrogen, 1:500). Fluorescence was analyzed by conventional fluorescence microscopy (Leica DMRB Fluorescence Microscope with Leica CCD camera DC300F). Images were processed with Adobe Photoshop.

5. In vitro culture of electroporated otic vesicles

Electroporated and control chick otic vesicles or mouse inner otocysts were dissected with PBS 4ºC, transferred into a four-well chamber and incubated with DMEM (BE-12614-F, Lonza Iberia) supplemented with 1% FBS (S06910S1900, Attendbio Research) as described in Neves et al., 2011.

6. β-galactosidase and Luciferase reporter assays

The otic vesicles from electroporated embryos and selected for positive electroporation by using GFP fluorescence. Protein extracts were prepared by using Reporter Lysis buffer (E397A, Promega, (Petrovic et al., 2014). β-galactosidase and Luciferase measurements were done as in Neves et al. (2012). β-galactosidase activity was normalized for the level of electroporation using a Luciferase reporter.

7. Site directed mutagenesis

Point mutations in the 3'Atoh1-enhancer reporter BgZA vector were introduced by using the Quick Change XL site-directed mutagenesis kit (200517, Agilent technologies) following the manufacturer's instructions. E-Box C mut-BgZa was generated with the 3'Atoh1-enhancer-BgZA as a template and the primers described in the Table 4. C-N box mut-BgZa and E-box A mut-BgZa were already generated in Fernando Giraldez's Lab. CAN mut was generated with the primers described in Table 4 and E-box C mut-BgZa as a template.

Reporter	Forward	Reverse
E-box C mut-BgZA	GCGGAGCGTCTGGAGCGGA GATTTATCTGTCAGCTGGTG AGCGCAC	GTGCGCTCACCAGCTGACA GATAAATCTCCGCTCCAGAC GCTCCGC
CAN mut-BgZA	GGAGATTTATCTGTCAGCTG TCAAGCGCACTCGCTTTCAG GCC	GGCCTGAAAGCGAGTGCGC TTGACAGCTGACAGATAAAT CTCC

8. Neurog1 deletions

Neurog1-ΔH1 and Neurog1-ΔH2 deletion expression plasmids were generated by inserting designed sequences (Ultramers 4nmols, IDT) into an NcoI/XbaI pMiw digested plasmid. Neurog1-ΔCterm was generated by PCR using pMiw-mNeurog1 as a template. PCR product was digested with SalI/XbaI and inserted into SalI/XbaI digested

pMiw. mNGN1 deltaC-term was generated by PCR using the following primers: FWD 5-CCAAGCTTGTCGACGGTAT-3 and REV 5- CTAGATTAATCTGCCAGGCGCAGTGT-3

9. E-box reporter systems

4xEboxA and 4xCAN reporter constructs were generated by inserting annealed oligonucleotides (Ultramers 4nmols, IDT) into a pGL3-basic vector (Promega) digested with NheI/BglII. The forward oligonucleotide had a NheI site, while the reverse oligonucleotide had a BglII site. 4xEboxA contains 4 copies of E-box A (4xTGT**CAGCTG**TCG**)**, while 4xCAN contained 4 copies of the class C E-box (E-box C), E-box A and N-box (4xGAG**CACGCG**CTGT**CAGCTGGTG**AGC**).** The 4xEboxA multimer not affecting the AtEAM motif (Klisch et al., 2011) had the two nucleotides following the E-box A "GT" replaced by "TC".

10. P19 transfection and Luciferase Reporter Assay

P19 cells (embryonic carcinoma cells derived from teratocarcinoma mice) were grown in DMEM with L-glutamine and antibiotics and 10% FBS. Ninety-six well dishes were seeded with 10^4 cells and transfected with Fugene HD (E2311, Promega) with 0.075ng expression plasmids, 0.075ng luciferase reporter plasmid, and 0.03ng pRenilla-TK vector (Promega) as internal control. Cells were lysed 48 hr after transfection. Cell lysis and Luciferase/Renilla measurements were performed using Dual-Glo Luciferase Assay System (E2940, Promega). Luciferase reporter plasmids and expression plasmids described in Table 1 and 2.

11. ATAC-seq analysis

ATAC-Seq experiments were performed as described in (Buenrostro et al., 2015). Experiments on mice (C57BL/6, wild-type) were approved by the ethics committee of the Barcelona Biomedical Research Park (PRBB) and by the Catalan Government. E10.5 mouse otocysts or E14.5 mouse cochleae were dissected, the tissue was digested with collagenase (C0130, Sigma, 0.3mg/ml, 45 minutes at 37ºC) and cells were suspended and counted in a Neubauer chamber ($7{,}5x10^4$ cells for transposition). LY411575 treatment (University of Dundee, UK, 100nM) was performed overnight on a set of E14.5 cochleas previous to ATAC-Seq procedure in a DMEM media with 1% FBS bovine serum with L-glutamine and antibiotics. The resulting library was sequenced in Illumina Hiseq 2000 pair-end lane. Reads were aligned with Bowtie2

software (Langmead and Salzberg, 2012), using mouse July 2007 (mm9) as reference genome. Duplicated pairs or those ones separated by more than 2Kb were removed. The enzyme cleavage site was determined as the position -4 (minus strand) or +5 (plus strand) from each read start, and this position was extended 5 bp in both directions.

12. Immunoprecipitation and Mass spectrometry

$2x10^6$ P19 cells were seeded and transfected with 4µl PEI/µg DNA and 4µg of pcDNA3.1-mAtoh1-FLAG (Quan et al., 2016) $\pm$ pMiw-mNgn1. Cells were lysed with buffer containing 1mM EDTA, 100µM Na_3VO_4, 0.5% Triton-x 100, 20mM β-Glycerolphosphate, 0.2mM PMSF in PBS with 1 complete EDTA free tablet (11873580001, Roche). Starting material for immunoprecipitation was 5-10mg. The lysate was pre-cleared with Dynabeads protG beads (10003D, Thermo Fischer) during 2h at 4ºC, and incubated with 3-5µg of 9E10, monoclonal mouse c-Myc (9E10, Santa CruzBiotech) or rabbit FLAG (F7425, Sigma) overnight at 4ºC. Dynabeads protG beads previously blocked (0,5% BSA) were added to the lysate and incubated for 2h at 4ºC.

Beads were washed three times with 500 µl of 0.2M NH4HCO3, resuspended in 60 µl of 6M urea 0.2M NH4HCO3, reduced with dithiotrhreitol (10µl DTT 10mM, 37ºC, 60 min), alkylated with iodoacetamide (10µl IAM 20mM, 25ºC, 30min), diluted up to 1M urea with 0.2M NH4HCO3 and digested overnight with trypsin (1 µg, 37ºC).Samples are desalted using C18 stape tips (UltraMicroSpin Column, SUM SS18V, The Nest group,Inc., MA)

45% of the peptide mixture was analyzed using a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a nano-LC (Proxeon, Odense, Denmark) equipped with a reversed-phase chromatography 2 cm C18 pre-column (Acclaim PepMap-100, Thermo; 100 µm i.d., 5 µm), and a reversed-phase chromatography 25 cm column with an inner diameter of 75 µm, packed with 1.9 µm C18 particles (Nikkyo Technos, Japan). Chromatographic gradients started at 7% buffer B with a flow rate of 300 nL/min and gradually increased to 35% buffer B in 60 min. After each analysis, the column was washed for 15 min with 90% buffer B (Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile). The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.2 kV and source temperature at 300 °C. Ultramark 1621 for the FT mass analyzer was used for external calibration prior the analyses. The background polysiloxane ion signal at m/z 445.1200 was used as lock mass. The instrument was operated in data-

dependent acquisition (DDA) mode, and full MS scans with 1 microscan at resolution of 60 000 were used over a mass range of m/z 350−2000 with detection in the Orbitrap. Auto gain control (AGC) was set to 1e6, dynamic exclusion was set at 60s, and the charge-state filter disqualifying singly charged peptides for fragmentation was activated. Following each survey scan, the 20 (CID) most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation at normalized collision energy of 35%. Fragment ion spectra produced via CID were acquired in the linear ion trap, AGC was set to 1e4 and isolation window of 2.0 m/z, activation time of 10 ms and maximum injection time of 100 ms were used. All data were acquired with Xcalibur software v2.2. Data Analysis Acquired data were analyzed using the Proteome Discoverer software suite (v1.4.1.14, Thermo Fisher Scientific), and the Mascot search engine (v2.5.1, Matrix Science; Perkins et al., 1999) was used for peptide identification. Data were searched against a Mus musculus protein database derived from SwissProt plus the most common contaminants (total of 17335 sequences). A precursor ion mass tolerance of 7 ppm at the MS1 level was used, and up to three missed cleavages for trypsin were allowed. The fragment ion mass tolerance was set to 0.5 Da. Oxidation of Methionine and N-terminal protein acetylation was defined as variable modification and carbamidomethylation of Cysteines was set as fixed modification. The identified peptides were filtered 5%FDR. SAINTexpress (Teo et al., 2014) was used to score protein interactions.

13. Immunoblot analysis

Western blot was performed as described in Neves et al. (2012). For chicken western blot protein extraction was performed with 10%Glycerol, 2%SDS and 50mM Tris (pH7.5). Three otic vesicles per condition were incubated with the buffer 10’ shacking at RT, 20’ at -80ºC and 10’RT. Protein samples were separated in 12% polyacrylamide gel, membranes incubated overnight with anti-FLAG (F7425, Sigma, 1:5000) or anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, 1:15000). Secondary, goat anti rabbit (P0488, Dako, 1:2000) and donkey anti-mouse (715-036-150, Jackson/Affinipure, 1:2000) coupled to horseradish incubated for 1h at room temperature. Optical density of immunoreactive bands was quantified on a ChemiDoc XRS System and Quantity One software v4.6.3 (Bio-Rad). Values were normalized to the GAPDH levels.

14. Statistics

Data are displayed as Mean+S.E.M from at least three different experiments. p-values (t-Student's test) were calculated and are at least below 0.05 for comparisons shown and discussed in the paper. However, given the small size of the samples, we followed Halsey et al., (2015) and the directions of the journal Development and avoided the use of statistics as inappropriate for such sample sizes. Instead, we show average values, S.E.M. and the number of experiments, which give an estimate of effect size and precision.

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APPENDIX I

Petrovic J, Gálvez H, Neves J, Abelló G, Giraldez F. Differential regulation of Hes/Hey genes during inner ear development. Dev Neurobiol. 2015 Jul;75(7):703–20. DOI: 10.1002/dneu.22243

APPENDIX II

Vendrell V, López-Hernández I, Durán Alonso MB, Feijoo-Redondo A, Abello G, Gálvez H, et al. Otx2 is a target of N-myc and acts as a suppressor of sensory development in the mammalian cochlea. Development. 2015 Aug 15;142(16):2792–800. DOI: 10.1242/dev.122465