Id4: an inhibitory function in the control of hair cell formation?

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I, Sara Weber confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Mechanosensitive hair cells in the sensory epithelia of the vertebrate inner ear are essential for hearing and the sense of balance. Initially formed during embryological development they are constantly replaced in the adult avian inner ear after hair cell damage and loss, while practically no spontaneous regeneration occurs in mammals. The detailed molecular mechanisms that regulate hair cell formation remain elusive despite the identification of a number of signalling pathways and transcription factors involved in this process.

In this study I investigated the role of Inhibitor of differentiation 4 (Id4), a member of the inhibitory class V of bHLH transcription factors, in hair cell formation. I found that Id4 is expressed in both hair cells and supporting cells of the chicken and the mouse inner ear at stages that are crucial for hair cell formation. In addition, forced overexpression of Id4 appeared to inhibit hair cell formation in the developing chicken inner ear while deletion of the *Id4* gene did not result in an obvious phenotype in mice. When looking at *Id4* gene regulation, luciferase reporter experiments revealed that *Id4* expression is driven by Notch activity; however, blockade of this pathway did not reduce the levels of Id4 and it seems likely that *Id4* expression is regulated by different pathways and mechanisms at a time.

Together, these results indicate that Id4 could regulate hair cell formation during embryological development; however its exact function remains unclear. The fact that *Id4* expression was also found in differentiated hair cells is inconsistent with the hypothesis of Id4 merely blocking hair cell formation. It seems likely that the function of Id4 is rather determined by expression levels and the presence or absence of interacting bHLH partners. Thus, a detailed analysis of levels of expression in a tissue- and developmental stage-specific manner as well as consideration of other expressed bHLH transcription factors are required for a deeper understanding of the complex transcriptional network that regulates hair cell formation.

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1. Introduction

The vertebrate inner ear is a highly elaborate sensory organ that subserves both the perception of sound and the sense of balance. Even though the basic structure and shape as well as the organisation of the sensory epithelia within the inner ear are widely conserved across species, there are differences especially in terms of initial cell specification and the capacity to regenerate after cell damage. The formation of hair cells in the auditory and vestibular sensory epithelia is a crucial step in inner ear embryological development. Hair cells are specialized neuroepithelial cells that are required for the conversion of mechanical sound stimuli into electrical impulses in the auditory and vestibular nerve through the process of mechanosensory transduction. Damage and death of hair cells can lead to a permanent loss and is one of the foremost underlying causes of hearing and balance impairments in humans. In contrast to the mammalian auditory epithelium, where hair cell loss results in irreversible damage, hair cells in the sensory epithelia of birds and most lower vertebrates undergo constant renewal and are able to regenerate after damage.

A greater understanding of the mechanisms underlying hair cell formation and regeneration could contribute significantly to the development of therapies that will aid those suffering from hearing or balance impairment.

1.1 Embryological development of the inner ear

The vast majority of the cells of the inner ear originate from a specific region of the head ectoderm, the otic primordial epithelium, which is flanking the embryonic neural tube. A schematic illustration of the major steps in inner ear development and formation is depicted in figure 1-1. The first morphological sign of otic differentiation is the formation of the otic placode, a thickening of the embryo's ectoderm that is located on both sides of the hindbrain (Torres and Giraldez 1998). In the course of embryological development, the otic placode

sinks into the underlying mesenchyme by a process referred to as invagination to form the otic cup. During this process, neuroblasts start to delaminate ventrally from the otic cup into the mesoderm. These cells will later on give rise to cochleovestibular neurons that innervate the sensory organs (Fekete and Wu 2002). Closure of the otic cup denotes formation of the auditory vesicle, also referred to as the otocyst. Cells of the otocyst will later on give rise to the complex membranous labyrinth consisting of the three semicircular canals of the vestibular system and their associated cristae, the saccule, the utricle, the cochlea (Cotanche and Kaiser 2010), and the endolymphatic sac and duct (Fekete 1999). The otocyst is a spherical structure that can be divided into different regions according to the three main axial orientations of the future inner ear (anterior-posterior (AP), dorsal-ventral (DV), and medial-lateral (ML)). The early patterning of the otocyst is to a large extent established by diffusible signals produced by surrounding tissues. According to the combination of genes that are expressed, cell fates and tissue identity of the respective regions are determined. Many of the signals involved are highly conserved in avian, fish, and mammalian species. Among the most important signalling pathways regulating early cell fate decisions are for example the Wnt signalling and the Sonic hedgehog (Shh) signalling pathway which both play a role in the establishment of the DV axis of the future inner ear (Liu, Li et al. 2002, Riccomagno, Martinu et al. 2002, Bok, Bronner-Fraser et al. 2005, Riccomagno, Takada et al. 2005). Wnt signalling is exerted from the most superior part of the neural tube and it defines the future dorsal part of the inner ear structures such as the endolymphatic duct. Accordingly, interruption of the pathway has been reported to lead to a failure of development of any vestibular structures in the inner ears of mutant mice (Riccomagno, Takada et al. 2005). Sonic hedgehog-producing cells, in contrast, are located in the notochord below the otocyst. The ventral region of the otocyst will give rise to the ventral auditory component of the inner ear, i.e. the cochlea (Bok, Chang et al. 2007). Blockade of Shh hedgehog signalling by either gene deletion or blocking protein activity has

led to a complete absence of ventral inner ear structures in both mice and chicken (Riccomagno, Martinu et al. 2002, Bok, Bronner-Fraser et al. 2005). A major candidate gene for the specification of AP identity is the T-box transcription factor *Tbx1*, which is exclusively expressed in the posterior half of the otic cup. Deletion of *Tbx1* has been shown to alter the expression patterns of anterior-specific genes (such as *Ngn1*, *NeuroD1*, *Lfng*, and *Fgf3*) and to abolish the expression of posterior-specific genes (*Oxt1/2*, *Goosecoid*) within the developing otocyst (Vitelli, Viola et al. 2003, Raft, Nowotschin et al. 2004, Arnold, Braunstein et al. 2006). Besides Tbx1, retinoic acid (RA) has been reported to be involved in AP patterning of the inner ear in chicken and mouse (Bok, Raft et al. 2011, Wu and Kelley 2012). Application of RA on the anterior half of the developing otic cup led to the development of an inner ear with two posterior domains in chicken, and it has been suggested that RA is involved in the regulation of Tbx1 (Bok, Raft et al. 2011).

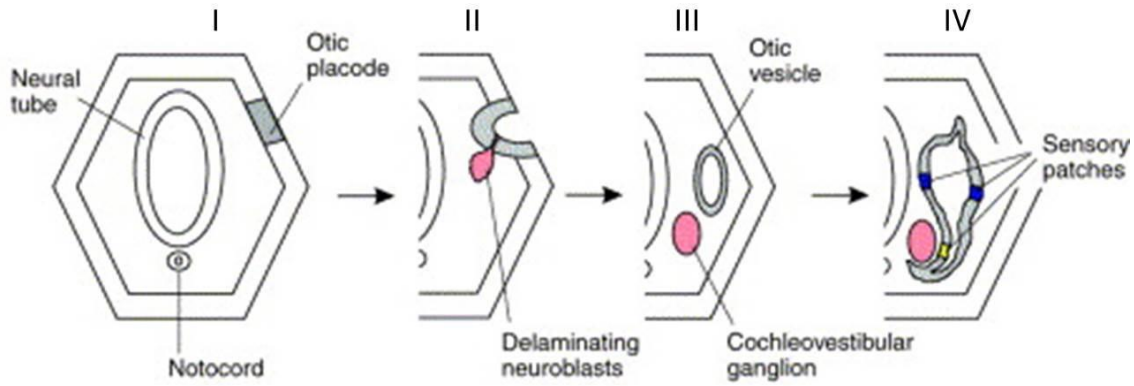


Figure 1-1: Formation of the inner during embryological development. (I) Cells from the otic primordial epithelium, which is flanking both sides of the embryonic neural tube, assemble to form an ectodermal thickening referred to as the otic placode. (II) The otic placode then invaginates into the underlying mesenchyme to form the otic cup. At the same time, neuroblasts start to delaminate ventrally from the otic cup into the mesoderm. (III) The otic cup closes and forms the auditory vesicle, also referred to as the otocyst or otic vesicle. Delaminated neuroblasts will give rise to cochleovestibular neurons that will innervate the sensory organs. (IV) Cells of the otocyst start forming the different sensory patches that will later on give rise to the complex membranous labyrinth of both the auditory and the vestibular system. Figure adapted from (Muller and Littlewood-Evans 2001).

The presumptive sensory epithelia of the developing inner ear are defined as prosensory patches, and they contain the precursors of hair cells and supporting cells. In the mature sensory epithelia hair cells are surrounded by non-sensory supporting cells in a highly accurate, mosaic-like pattern. This pattern is established primarily through a process known as lateral inhibition, a common mechanism in many developmental processes driven primarily by the Notch signalling pathway (see also chapter 1.2.3). Once a precursor differentiates into a hair cell, it expresses the Notch ligands Delta1 and Jagged2. Binding of these Notch ligands to Notch receptors on the surface of neighbouring undifferentiated cells triggers a signalling cascade that represses hair cell formation (Slowik and Bermingham-McDonogh 2013). According to the “either-or” principle, the neighbouring cells will subsequently differentiate into supporting cells. This mosaic-like pattern of hair cells and supporting cells is found in all inner ear epithelia, and the basic mechanisms controlling its formation appear highly conserved throughout a variety of vertebrate classes. However, there are important differences in the timing of hair cell formation during embryological development and the potential for regeneration of hair cells in different tissues and vertebrate classes.

The following sections will focus on the development of the inner ear in chicken and mouse, two model organisms for mammalian and avian species that have been used in this study.

An overview of the mouse inner ear anatomy as well as an illustration of the cochlea’s sensory epithelium, the organ of Corti, is shown in figure 1-2 A and B. In the cochlea, the onset of hair cell differentiation is around embryonic day 12 (E12). Terminal mitosis is first observed at the cochlear apex and subsequently extends as a wave of differentiation along the longitudinal axis towards the base. Hair cell production reaches a peak between E13 and E14 with more than 80% of the progenitor cells undergoing their terminal mitosis at this stage (Ruben 1967, Katayama and Corwin 1989, Chen and Segil 1999). Cell cycle withdrawal is generally regulated by cyclin-dependent kinases (CDK). During embryological development,

the CDK inhibitor $p27^{KIP1}$ is expressed in the prosensory precursors of the cochlea, and it is thought to play a crucial role in triggering cell cycle exit and thus inducing terminal mitosis (Chen and Segil 1999). By E17-18, hair cell production in the mouse cochlea is virtually complete (Ruben 1967, Sher 1971, Lim and Anniko 1985). By then, approximately 3,300 hair cells will have formed in an accurate pattern of three rows of outer hair cells and one row of inner hair cells (Ehret and Frankenreiter 1977). All hair cells are surrounded by various types of non-sensory supporting cells sitting on the basilar membrane. At their bottom end, hair cells are innervated by the axons of auditory sensory neurons which assemble and send their projections via the auditory nerve to the cochlear nuclei of the auditory brainstem. At their top end, the hair cells' mechanosensing stereocilia are embedded into the tectorial membrane (Fig. 1-2 B). Once hair cells have formed their total number is definite - no production of new auditory hair cells will occur in the adult tissue. The cells remain postmitotic throughout adult life (Ruben 1967), and will not be replaced if damaged. However, in contrast to the mammalian cochlea, there seems to be a limited capacity for hair cell regeneration in the sensory epithelia of the mammalian adult vestibular system (Forge, Li et al. 1993, Forge, Li et al. 1998, Kawamoto, Izumikawa et al. 2009, Warchol 2011). As mentioned above, the formation of hair cells in the cochlea initiates at the apex and proceeds towards the base. Interestingly, the final differentiation of newly born hair cells proceeds in the opposite direction, starting at the base and proceeding towards the apex of the cochlea (Jahan, Pan et al. 2010, Jahan, Pan et al. 2013). Moreover, the morphology of hair cells in the mammalian organ of Corti varies according to their location along the epithelium. While hair cells in the more basal region exhibit shorter stereocilia at their top end, the length of stereocilia increases towards locations at the apical end of the cochlea (Furness, Richardson et al. 1989, Kaltenbach, Falzarano et al. 1994). Given the tonotopic organisation of the cochlea, higher tone frequencies are detected by the hair cells with short stereocilia in the

more basal part and lower tone frequencies are detected by hair cells with long stereocilia in the more apical part.

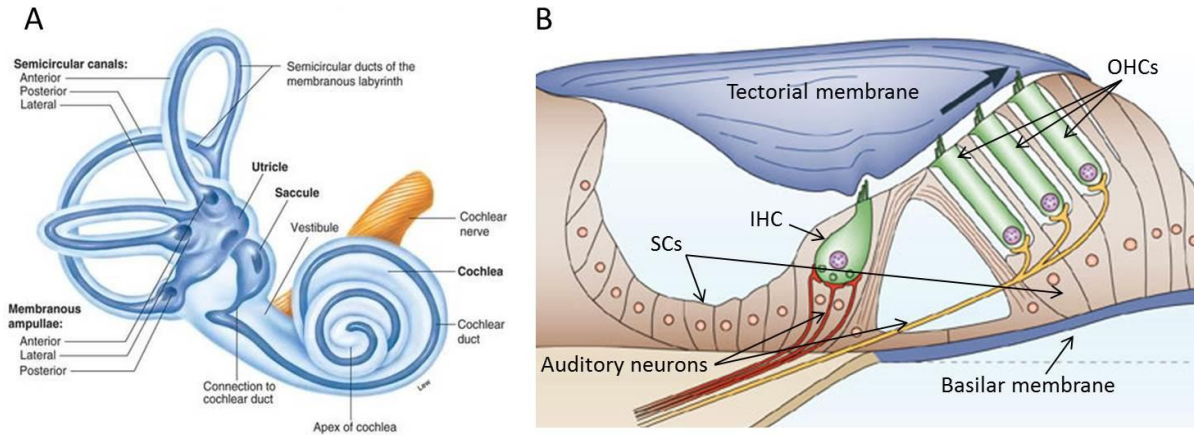


Figure 1-2: Anatomy of the mammalian inner ear. (A) The vertebrate inner ear can be divided into two parts: the vestibular system which is in charge of the sense of balance, and the auditory system which is required for hearing. The vestibular system consists of three semicircular canals and their associated ampullae, as well as the utricle and the saccule. The auditory system comprises the cochlea, a snail shell-like coiled structure containing the auditory sensory epithelium referred to as the organ of Corti. (B) Within the organ of Corti, three rows of outer hair cells (OHC) and one row of inner hair cells (IHC) are surrounded by various types of non-sensory supporting cells (SC) sitting on the basilar membrane. Hair cells are connected to the axons of auditory neurons at their bottom ends and covered by the tectorial membrane at their top ends. Mechanosensing stereocilia placed on the top ends of hair cells are embedded into the tectorial membrane and are displaced when sounds waves hit the inner ear and start moving the membranes. Displacement of the stereocilia leads to the opening of integrated ion channels, a depolarization of the cells, and finally to the emergence of action potentials that are sent to the auditory cortex of the brain via the auditory nerve. Figures adapted and modified from (A) http://humanphysiology2011.wikispaces.com and (B) (Fettiplace and Hackney 2006).

The overall basic structure of the inner ear is much conserved between mammals and birds. However, unlike the mammalian cochlear duct the auditory sensory epithelium of birds, referred to as the basilar papilla (BP), is not coiled but forms a slightly crescent-shaped structure. It does not exhibit separate rows of inner and outer hair cells, but a high number of two types of hair cells, short hair cells and long hair cells, that are evenly spread over the length of the epithelium and that show the same characteristic gradient within the length of their stereocilia as seen in the hair cells of the mammalian organ of Corti (Tilney and

Saunders 1983, Corwin and Cotanche 1989). A schematic illustration and a picture of the embryonic avian inner ear are shown in figure 1-3. As in mammals, a mosaic-like pattern of hair cells and supporting cells is achieved through lateral inhibition processes mediated by Notch signalling (Adam, Myat et al. 1998, Stone and Rubel 1999, Chrysostomou, Gale et al. 2012). The first hair cells in the chick BP are produced between E5 and E6 in a longitudinal band within the superior region of the basal end. Terminal mitosis events then proceed progressively towards the apex in a “center-to-periphery” manner (Katayama and Corwin 1989). Like in the mammalian organ of Corti, differentiation of newly produced hair cells proceeds in the opposite direction compared to terminal mitosis, starting around E6 in a circular patch at the distal (apical) end of the BP and proceeding towards the base. Hair cell production peaks around E11 and by E12 approximately 12,000 hair cells have formed and differentiated along the length of the sensory epithelium (Tilney and DeRosier 1986, Goodyear and Richardson 1997). Even though no post-mitotic cell division or hair cell production occurs in the BP under normal conditions quiescent supporting cells are able to re-enter the cycle and give rise to new and fully functional hair cells throughout adulthood when hair cell death occurs (Corwin and Cotanche 1988, Ryals and Rubel 1988, Stone and Cotanche 2007).

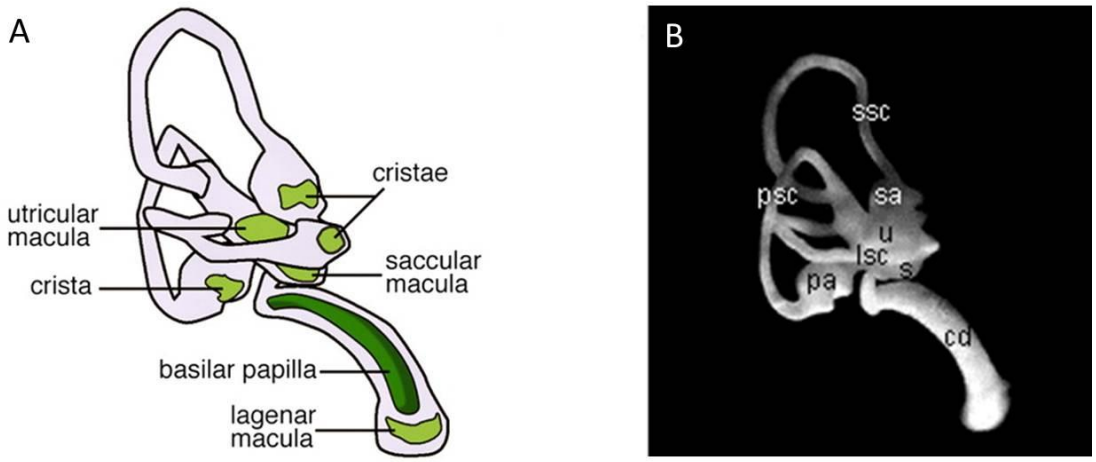


Figure 1-3: Anatomy of the chicken inner ear at (A) E10 and (B) E16. Like the mammalian inner ear, the chicken inner ear is composed of the vestibular and the auditory apparatus. The organisation of the vestibular system is similar to that in mammals and it consists of the three semicircular canals with associated cristae, as well as the saccule and the utricle. Other than the mammalian cochlea, however, the chicken cochlear duct is not coiled but displays an elongated and slightly crescent-shaped structure. Hair cells of the chicken auditory sensory epithelium, referred to as basilar papilla, are not organized in rows of outer and inner hair cells but are evenly spread along the length of the epithelium. As in the mammalian organ of Corti, hair cells are surrounded by non-sensory supporting cells in a highly organized, mosaic-like pattern. Abbreviations: cd, cochlear duct; lsc, lateral semicircular canal; pa, posterior ampulla (houses the posterior crista); psc, posterior semicircular canal; s, saccule (houses the saccular macula); sa, superior ampulla (houses the superior crista); ssc, superior semicircular canal; u, utricle (houses the utricular macula). Figures adapted from (A) (Daudet and Lewis 2005) and (B) (Brigande, Kiernan et al. 2000).

1.2 Molecular mechanisms of inner ear patterning and hair cell formation

Sensory hair cells and non-sensory supporting cells derive from the same pool of progenitors within the developing otocyst (Fekete, Muthukumar et al. 1998). A number of different molecular players and signalling pathways is involved in cell fate decisions and the patterning of auditory and vestibular sensory epithelia. Some of them are intrinsic and others can be exerted from neighbouring cells and surrounding tissues. Many of these factors are interacting, thus creating a complex regulatory network. In the following paragraphs, the most important pathways and transcription factors will be outlined and their role in inner ear development will be described.

1.2.1 First things first: Prosensory specification

The establishment of defined prosensory domains within the developing otocyst can be considered the earliest event in terms of cell specification and patterning of the inner ear sensory domains. A key factor for prosensory specification is the transcription factor Sox2, which has been found to be expressed in the putative prosensory domains of the otocyst along with the Notch ligand Jagged1/Serrate1 (Jagged1 in mouse and humans, Serrate1 in chicken), and the Sox2 downstream target Prox1 (Adam, Myat et al. 1998, Stone, Shang et al. 2003, Neves, Kamaid et al. 2007, Dabdoub, Puligilla et al. 2008). Downregulation of *Sox2* expression in the developing mouse otocyst has been reported to lead to a complete failure of prosensory domain formation as well as an absence of both hair cells and supporting cells (Kiernan, Xu et al. 2006). There is evidence that Sox2 is acting downstream of Jagged1, which has been shown to be involved in prosensory specification via a Notch-driven mechanism referred to as lateral induction (Eddison, Le Roux et al. 2000, Kiernan, Ahituv et al. 2001, Daudet and Lewis 2005). When interacting with Notch receptors on the surface of surrounding cells, Jagged1 ligands induce their own expression in the signal-receiving cells as well as the expression of prosensory marker genes such as *Sox2* (Eddison, Le Roux et al. 2000, Neves, Kamaid et al. 2007, Neves, Parada et al. 2011). Similar to *Sox2*, reduced expression or deletion of *Jagged1* leads to a disrupted establishment of prosensory regions and to a reduction in the total number of progenitor cells in the mammalian inner ear (Kiernan, Ahituv et al. 2001, Brooker, Hozumi et al. 2006, Kiernan, Xu et al. 2006). These findings are consistent with data reporting that increasing Notch activity by overexpression of the Notch intracellular domain (NICD) leads to the expression of prosensory markers in the mammalian cochlea, the formation of ectopic sensory patches in the chick inner ear (Daudet and Lewis 2005, Dabdoub, Puligilla et al. 2008), and induction of ectopic hair cells in the mouse inner ear (Hartman, Reh et al. 2010, Pan, Jin et al. 2010).

Besides Notch signalling, another important pathway to mention in the context of prosensory patch specification is the bone morphogenetic protein (BMP) signalling pathway. BMPs are multifunctional growth factors that act via binding to specific membrane-bound receptor molecules, thus triggering diverse intracellular signalling cascades. They play important roles in a number of developmental events, including cardiovascular development, the induction of bone and cartilage formation, and the regulation of neural development (Hogan 1996, Chen, Zhao et al. 2004). Within the developing chick inner ear, BMP4 and BMP7 have been shown to be expressed in regions mapping the prosensory patches of the otocyst (Oh, Johnson et al. 1996), thus suggesting a role in prosensory specification. It has also been reported that BMP signalling is required for the specification of supporting cells and other non-sensory cells arising from the prosensory domains (Chang, Lin et al. 2008). A possible link between BMP signalling and hair cell formation at later embryonic stages has been suggested by Kamaid and colleagues. In a study focusing on a group of transcription factors, namely the family of inhibitors of differentiation (Id), they found evidence that several members of the Id family (Id1, 2, and 3) inhibit *Atoh1* expression and thus hair cell formation downstream of BMP signalling (Kamaid, Neves et al. 2010). Taken together, there is evidence that BMP signalling is not only involved in the early determination of prosensory domains, but also in the subsequent specification of supporting cells and hair cells. This is consistent with published data suggesting a sequential role for BMP4 in prosensory development and the specification of non-sensory cells and supporting cells (Chang, Lin et al. 2008).

1.2.2 Becoming a hair cell: Atoh1

The basic helix-loop-helix (bHLH) transcription factor Atoh1 (atonal homolog 1) is the vertebrate homologue of the *Drosophila* proneural gene atonal (ato) (Jarman, Grau et al. 1993). In mammals, Atoh1 was first described as MATH-1 (mammalian atonal homolog 1) (Akazawa, Ishibashi et al. 1995). While being essential for sensory organ precursor (SOP)

cells in *Drosophila* (Jarman, Sun et al. 1995), *Atoh1* is often referred to as the master gene for hair cell differentiation in both the mammalian and the avian inner ear. It is the earliest hair cell-specific gene and it has been shown in several studies that it is both required and sufficient for hair cell formation (Bermingham, Hassan et al. 1999, Zheng and Gao 2000). Deletion of this gene leads to a complete absence of hair cells even though precedent specification of prosensory domains is not affected (Bermingham, Hassan et al. 1999, Chen, Johnson et al. 2002). In the mouse inner ear, *Atoh1* is first expressed around E12 in the sensory region of the developing vestibular system (Bermingham, Hassan et al. 1999). Within the cochlea, first *Atoh1* transcripts are found in the very basal region between E13 – E13.5 (Lanford, Shailam et al. 2000, Woods, Montcouquiol et al. 2004). It is to note that expression of *Atoh1* has been reported to be exclusive to cells that have exited the cell cycle and that appear to subsequently differentiate into hair cells (Chen, Johnson et al. 2002). At later developmental stages, around E17, *Atoh1* expression becomes restricted to the single row of inner hair cells and the three rows of outer hair cells (Jones, Montcouquiol et al. 2006, Cotanche and Kaiser 2010). Expression in these cells starts to be downregulated in the basal region of the cochlea by postnatal day three (P3) (Lanford, Shailam et al. 2000, Mulvaney and Dabdoub 2012). In the developing chicken inner ear, *Atoh1* expression is first detected at E4 within two of the prosensory patches of the otocyst that correspond to the putative anterior and posterior cristae (Stone, Shang et al. 2003, Pujades, Kamaid et al. 2006).

While deletion of *Atoh1* leads to a complete failure of hair cell formation, forced overexpression has been reported to be sufficient for the generation of supernumerary hair cells in the mammalian cochlea both *in vivo* and *in vitro* (Zheng and Gao 2000, Kawamoto, Ishimoto et al. 2003, Zhao, Zou et al. 2011). In addition to its function during initial hair cell formation, Atoh1 has been reported to be expressed in differentiating hair cells in the regenerating avian basilar papilla (Cafaro, Lee et al. 2007) as well as in supporting cells after cell damage (Lewis, Hume et al. 2012). Forced overexpression of Atoh1 by viral gene

transfer has further been reported to lead to hair cell regeneration in the damaged cochlea of adult guinea pigs (Yang, Chen et al. 2012). Taken together, these data strongly imply a fundamental role of Atoh1 in both hair cell formation and regeneration processes. However, the capacity to regenerate remains extremely limited in the mammalian sensory epithelia, and regenerative therapies targeting other factors such as cell cycle regulators in addition to *Atoh1* gene delivery seem to be a promising approach (Collado, Burns et al. 2008).

Despite the extensive work on Atoh1 and its known role in inner ear development and maintenance, the precise signalling factors and mechanisms regulating its activity still remain unclear. It is known that the *Atoh1* gene is able to drive its own expression in an autoregulatory manner via a positive feedback loop. This feedback loop is exerted by binding of the Atoh1 protein to an E-box consensus site in its own enhancer region (Helms, Abney et al. 2000). It has further been reported that the *Atoh1* enhancer is active in hair cells during development (Woods, Montcouquiol et al. 2004), suggesting that autoregulation plays an important role in elevating *Atoh1* expression levels during cell fate decisions in the inner ear. Apart from the mechanism of autoregulation, several other molecules have been considered as candidate regulators of *Atoh1* activity. The transcription factor Sox2, which is suggested to act downstream of Notch signalling (Kiernan, Xu et al. 2006, Puligilla and Kelley 2009), and Atoh1 have been reported to regulate each other in a negative manner (Dabdoub, Puligilla et al. 2008, Wu and Kelley 2012). Sox2 has been reported to directly antagonize the pro-hair cell effect of Atoh1, a finding consistent with data showing that Sox2 overexpression inhibits hair cell formation. Conversely, overexpression of Atoh1 in cell lines was able to downregulate *Sox2* expression levels (Dabdoub, Puligilla et al. 2008). However, based on a recent study by Neves and colleagues there is also evidence that Sox2 directly induces *Atoh1* expression at the time of prosensory specification by binding to its enhancer region (Neves, Uchikawa et al. 2012). One possible explanation advanced by the authors for this contrary effect is that Sox2 first drives progenitor cells towards the hair cell fate by activating

Atoh1 expression, but then keeps them in an undifferentiated intermediate state until prosensory specification has finished (Neves, Uchikawa et al. 2012). In addition to its role in regulating Atoh1 on its own, Sox2 has further been reported to be implicated in driving *Atoh1* expression in cooperation with the phosphatase and transcriptional coactivator Eya1 and its cofactor Six1 (Ahmed, Wong et al. 2012). Eya1 and Six1 have been shown to be involved in sensory organ formation in previous studies, and genetic inactivation led to a failure of otic development at an early embryonic stage in mice (Xu, Adams et al. 1999, Zheng, Huang et al. 2003). In their study, Ahmed and colleagues provided evidence that Eya1/Six1 and Sox2 act synergistically to activate *Atoh1* transcription and thus hair cell formation in the non-sensory epithelium of the murine cochlea. They could show that a direct interaction between Eya1/Six1 and Sox2 on the protein level significantly increased binding to responsive elements within the *Atoh1* enhancer when compared to the Eya1/Six1 complex alone. Even though ectopic expression of Eya1/Six1 alone was able to induce hair cell formation, the rate of transfected cells expressing hair cell markers was nearly one hundred percent only when Eya1/Six1 and Sox2 were overexpressed simultaneously (Ahmed, Wong et al. 2012).

As will be explained in more detail in the following sections there are also studies suggesting a role for two members of the Hairy and enhancer of split (HES) family of transcription factors, HES1 and HES5, in the regulation of *Atoh1* expression. The transcription factors HES1/5 are well-known effectors of the Notch signalling pathway and their role in regulating Atoh1 levels during Notch-driven lateral inhibition processes in the development of the inner ear has been well characterized (see chapter 1.2.3). In addition, some members of the Id protein family have been found to be expressed in the inner ear and have been reported to inhibit *Atoh1* activity downstream of the BMP signalling pathway (see chapter 1.2.4).

1.2.3 Notch signalling and lateral inhibition

The Notch signalling pathway is a common and well understood cell-to-cell signalling pathway which mediates its action via the principle of receptor-ligand binding. It is evolutionary highly conserved and occurs in a variety of different organisms and species. During development, Notch signalling plays a crucial role in cell differentiation, and it is involved in processes such as angiogenesis, somitogenesis, or the development of the heart and the central nervous system (Bolos, Grego-Bessa et al. 2007). In vertebrates, four Notch receptors (NOTCH1-4) and a variety of Notch ligands, including members of the Delta/Serrate/LAG-2 (DSL) family of proteins, have been identified so far (Artavanis-Tsakonas, Rand et al. 1999). Both Notch ligands and Notch receptors are transmembrane proteins and are thus present on the cell surface. Binding of Notch ligands to their receptors on neighbouring cells leads to a γ-secretase-mediated cleavage of the Notch receptor and to the release of the Notch intracellular domain (NICD) into the cytoplasm. The NICD translocates into the nucleus and regulates transcription of various Notch target genes (Baron 2003, Andersson, Sandberg et al. 2011). Crucial for the transcriptional regulatory activity of Notch is an interaction between the released NICD and the transcriptional regulator RBPjκ, also known as CSL (CBF-1 in mammals, Suppressor of hairless (SuH) in *Drosophila*, Lag-2 in *C.elegans*). The protein RBPjκ is a highly conserved DNA-binding protein that is associated with the mediation of canonical Notch signalling (Kopan and Ilagan 2009). In the absence of NICD, RBPjκ is reported to mainly act as a transcriptional repressor and is found in complexes with other co-repressor proteins (Zhou and Hayward 2001). However, as soon as Notch becomes activated, RBPjκ binds to the released NICD and initiates the recruitment of a protein co-activator complex. This complex involves proteins of the Mastermind-like (MAML) protein family and drives transcriptional regulation of various Notch target genes by DNA-binding to specific CSL binding sites (CBS) (Wu, Aster et al. 2000, Kitagawa, Oyama et al. 2001, Lubman, Korolev et al. 2004).

In the given context, a mechanism referred to as lateral inhibition is of particular relevance. Originally discovered in *Drosophila*, Notch-mediated lateral inhibition has been well-studied in the context of sensory organ precursor (SOP) selection (Hartenstein and Posakony 1990, Parks and Muskavitch 1993, Pitsouli and Delidakis 2005), and many analogies and comparisons have been drawn between SOP formation and the development of the inner ear (Adam, Myat et al. 1998, Eddison, Le Roux et al. 2000).

In the auditory and vestibular system, Notch signalling has been reported to play various roles during development and in tissue maintenance. As for SOPs in *Drosophila*, the characteristic mosaic-like pattern of hair cells and supporting cells is established by Notch-mediated lateral inhibition processes. Newly formed hair cells express the Notch ligands Delta1 and Jagged2 (Adam, Myat et al. 1998, Lanford, Lan et al. 1999, Morrison, Hodgetts et al. 1999, Shailam, Lanford et al. 1999). Binding of Delta1 to Notch receptors on the surface of undifferentiated neighbouring cells activates the Notch signalling pathway and thus specific Notch effectors in these cells (Woods, Montcouquiol et al. 2004, Kiernan, Cordes et al. 2005, Daudet, Gibson et al. 2009, Cotanche and Kaiser 2010). Crucial Notch effectors in the context of lateral inhibition within the inner ear are members of the HES family of transcription factors (Zheng and Gao 2000, Zine, Aubert et al. 2001). HES proteins belong to the bHLH superfamily of transcription factors. They form homo- and hetero-dimers and regulate the expression of their target genes by binding to specific hexanucleotide DNA sequences (Ferre-D'Amare, Prendergast et al. 1993, Murre, Bain et al. 1994, Ledent and Vervoort 2001). Two members of this gene family, HES1 and HES5, have been reported to prevent hair cell formation by directly inhibiting the expression of the required pro-hair cell transcription factor Atoh1 (Zheng and Gao 2000, Zine, Aubert et al. 2001). Consistent with the model of Notch negatively regulating hair cell formation, it has been reported that inhibition of Notch activity by either treatment with γ-secretase inhibitors, such as N-[N-(3,5-Difluorophenylacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), or downregulation/

deletion of Notch1 and Jagged2 can increase the total number of hair cells generated both *in vitro* and *in vivo* (Lanford, Lan et al. 1999, Zine, Van De Water et al. 2000, Kiernan, Cordes et al. 2005, Brooker, Hozumi et al. 2006, Slowik and Bermingham-McDonogh 2013). A schematic illustration of Notch-mediated lateral inhibition during inner development is shown in figure 1-4.

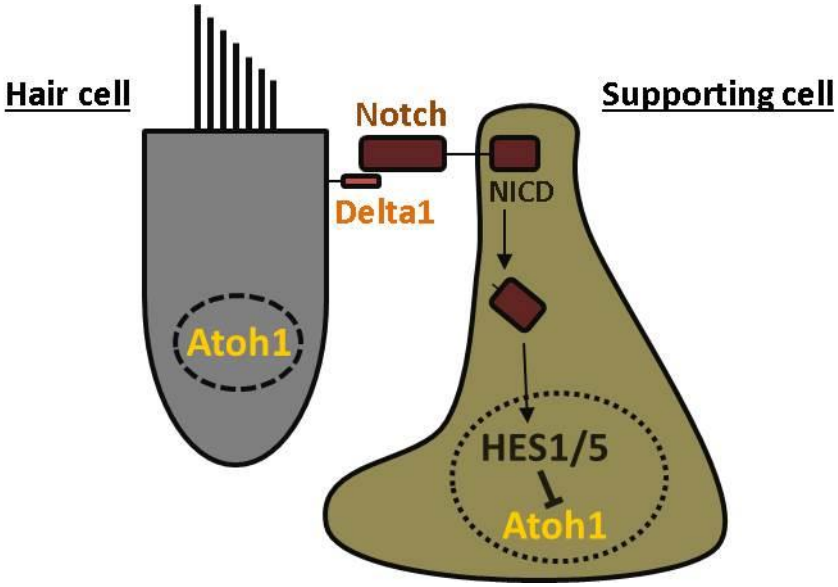


Figure 1-4: Notch-mediated lateral inhibition in the context of hair cell formation. Atoh1-positive differentiating hair cells express the Notch ligand Delta1. Binding of Delta1 to Notch receptors on the surface of surrounding cells leads to a γ-secretase-mediated cleavage of the Notch receptor and to the release of NICD into the cytoplasm. The NICD translocates into to nucleus and induces the expression of two members of the HES family of bHLH transcription factors, HES1 and HES5, which directly inhibit *Atoh1* expression and thus prevent hair cell formation.

1.2.4 Id proteins

Id proteins are key regulators of development that belong to the superfamily of bHLH transcription factors. They have been associated with a number of developmental processes, such as angiogenesis and neurogenesis (Lanford, Shailam et al. 2000), and are generally involved in cell fate determination and regulating the timing of cell differentiation. They mostly

act by controlling a cell's cell cycle state and gene expression, thus maintaining for example progenitor cells in an undifferentiated state and preventing premature or excessive cell proliferation (Benezra, Davis et al. 1990, Norton, Deed et al. 1998, Norton 2000). Expression of the different members of the Id protein family has been reported to be deregulated in a range of human cancers, and Id1 and 3 have been suggested to be required for tumour angiogenesis (Lasorella, Uo et al. 2001, Hasskarl and Munger 2002, Ruzinova and Benezra 2003, Lasorella, Benezra et al. 2014).

Id proteins are classified as class V helix-loop-helix (HLH) proteins (Murre, Bain et al. 1994). HLH proteins are transcription factors that in most cases consist of a basic region (b) and a characteristic region of 2 α-helices connected by a variable loop (HLH) (illustrated in Fig. 1-5). In general, bHLH transcription factors are activated and able to regulate gene expression when assembled as protein dimers. The dimers bind to the DNA and inhibit or enhance transcription of specific target genes. DNA-binding usually occurs to a specific hexanucleotide DNA sequence (CANNTG) referred to as enhancer box (E-box). The binding is mediated through the connected basic regions of two dimerized bHLH proteins, and binding is not possible in their monomeric state. Most bHLH proteins that are tissue-specific form heterodimers with ubiquitously expressed, non-tissue-specific bHLH proteins referred to as E-proteins (Benezra, Davis et al. 1990, Jen, Weintraub et al. 1992, Norton and Atherton 1998). A common feature of all class V HLH transcription factors, however, is the lack of the basic DNA binding site (Fig. 1-5). When such proteins dimerize with other tissue-specific bHLH proteins transcriptional activity is inhibited by preventing the binding of the complex to the DNA. Class V HLH proteins have also been reported to bind and sequester E-proteins with even higher affinity. Given these characteristic features, Id proteins are potent inhibitors of other bHLH transcription factors, and they function either on the level of transcriptional regulation or on the protein level in the context of heterodimer formation (Ledent and Vervoort 2001). In addition to that, there is also evidence that Id proteins trigger degradation

of other bHLH transcription factors (Vinals, Reiriz et al. 2004, Vinals and Ventura 2004), and the theory exists that this particular mechanism might be involved in Id-induced downregulation of Atoh1 in medulloblastoma cells (Zhao, Ayrault et al. 2008).

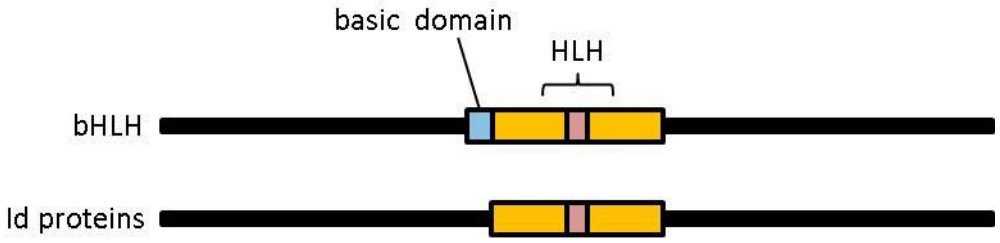


Figure 1-5: Schematic structure of Id proteins and common bHLH transcription factors. Id proteins are classified as class V helix-loop-helix (HLH) transcription factors. A common feature of class V HLH transcription factors is the lack of the basic domain (b, blue) which is required for DNA binding and the regulation of gene expression of specific target genes. By forming heterodimers with other bHLH transcription factors Id proteins inhibit their binding to the DNA. Figure adapted and modified from (Norton 2000).

In vertebrates, including humans, mammals and birds, four members of the Id protein family have been characterized so far: Id1, Id2, Id3, and Id4. The overall expression pattern of Id1, 2, and 3 appears to be overlapping to a large extent in various tissues and organs throughout embryogenesis, such as lung, kidney, spinal cord, and brain (Jen, Manova et al. 1996, Jen, Manova et al. 1997). Notwithstanding, Id4 has been reported to be expressed in a differing manner, and it seems to be restricted to the developing nervous system (Riechmann and Sablitzky 1995, Jen, Manova et al. 1996, Jen, Manova et al. 1997, Yokota 2001, Bedford, Walker et al. 2005). To characterize Id protein function in more detail, knockout mouse models have been generated for all Id family members in previous studies. These studies revealed that single deletions of either *Id1* or *Id3* were not sufficient to cause an obvious phenotype, and animals showed an overall normal development. Combined deletion of *Id1/3*, however, has been reported to be lethal at embryonic stage E13.5 with striking defects in angiogenesis as well as premature terminal mitosis and aberrant differentiation of neural

precursor cells (Lyden, Young et al. 1999). This result, along with the reported overlapping expression of *Id1* and *Id3*, suggests some functional redundancy between these two Id protein family members. On the other hand, single deletion of *Id2* led to a mild but mostly viable phenotype showing defects in the proliferation and differentiation of various immune cells, such as T cells, and a lack of lymph nodes and Peyer's patches (Perk, Iavarone et al. 2005). In contrast to all other *Id* genes, absence of *Id4* causes a significant neurogenic phenotype (Bedford, Walker et al. 2005). The brains of *Id4* $^{-/-}$ mice are reported to be severely malformed, with a reduction in the overall size and enlarged 3rd, 4th, and lateral ventricles. In addition, *in vitro* studies with neurospheres generated from adult and embryonic brain tissue of *Id4* $^{-/-}$ mice revealed that the proliferation of neural precursor cells (NPCs) is impaired by an *Id4* deletion. The morphological alterations in the brains of *Id4* $^{-/-}$ mice are believed to be caused by both a premature neuronal differentiation and the seen impairment of NPC proliferation (Yokota 2001, Yun, Mantani et al. 2004, Bedford, Walker et al. 2005). Altogether, these studies have highlighted the crucial role that Id4 plays in the regulation of the proliferation and differentiation of neural precursor cells.

Within the inner ear, Id1, 2, and 3 have been reported to be expressed in the developing mouse otic vesicle as early as E11.5 as well as in the cochlear duct by E13 (Riechmann, van Cruchten et al. 1994, Jen, Manova et al. 1997, Lin, Ozeki et al. 2003, Jones, Montcouquiol et al. 2006). Previous studies have suggested that Id1 and Id3 could antagonize the pro-hair cell transcription factor Atoh1, and overexpression inhibited hair cell formation in organotypic cultures of the mouse organ of Corti (Jones, Montcouquiol et al. 2006). The exact regulation of Id proteins in this particular context remained unclear at that time; however, there are several studies reporting a link between *Id* gene expression and the BMP signalling pathway in other systems such as embryonic stem cells (Hollnagel, Oehlmann et al. 1999) or during neuro- and myogenesis (Nakashima, Takitawa et al. 2001, Yanagisawa, Takizawa et al. 2001, Katagiri, Imada et al. 2002, Ruzinova and Benezra 2003). In the chicken inner ear,

expression of Id proteins has been detected in the otic placode (*Id3*) as well as in the otic vesicle at later stages (*Id1, Id2*) (Kee and Bronner-Fraser 2001, Kamaid, Neves et al. 2010).

Surprisingly, Id4 could not be detected within the mammalian or chicken inner ear sensory epithelia using PCR methods or *in situ* hybridization so far (Riechmann, van Cruchten et al. 1994, Jones, Montcouquiol et al. 2006, Kamaid, Neves et al. 2010). One possible explanation for that is the fact that previous studies only looked at stages E3 and below, while *Id4* transcripts weren't found until stage E3.5 of chick embryos during this study. A previously conducted study by Jen and colleagues, however, reported traces of expression of *Id4* in the otic epithelium of E11.5 – E16.5 mouse embryos, but no further details about the spatial localization or the exact time point of detected expression are available from this publication (Jen, Manova et al. 1996). However, unpublished data from a genome-wide microarray screening in the embryonic chicken inner ear suggested that *Id4* might be a Notch target gene, opening the possibility that it could act as a regulator of *Atoh1* activity in the inner ear sensory epithelia (N. Daudet and M. Gomez, unpublished data).

1.3 Research aims

This study was aimed at further understanding the role of the bHLH transcription factor Id4 in hair cell formation during inner ear development. As described in the foregoing chapters, hair cell formation is to a large extent regulated via lateral inhibition events mediated by the Notch signalling pathway. A recent genome-wide microarray screening for targets of Notch activity revealed that levels of Id4 are significantly decreased after artificial blockade of Notch activity in the developing chicken utricle *in vitro*, suggesting an involvement in this process. The exact mechanisms however by which Id4 might exert its actions are currently unknown. A working hypothesis showing a model for possible interactions between Notch signalling, Id4, and hair cell formation is shown in figure 1-6. In a first step, I analysed the expression pattern

of *Id4* during development of the chicken inner ear by the means of *in situ* hybridization. In addition, I studied the effects of Id4 overexpression in early stage chick embryos using *in ovo* electroporation. The data collected thus far show specific expression of Id4 in the chicken inner ear prosensory domains at the time of hair cell formation, and that Id4 overexpression inhibits hair cell formation. Preliminary data from these experiments further suggest that levels of Atoh1 protein are reduced in cells overexpressing Id4.

To follow on from these studies, I investigated the expression pattern of Id4 in the developing and mature mouse inner ear using an *Id4-lacZ* knock-in mouse model as well as immunohistochemistry. I found that Id4 is expressed in all inner ear sensory epithelia at embryonic and adult stages, and preliminary data from looking at perinatal stages of homozygous *Id4-lacZ* animals did not reveal any effect on hair cell formation caused by the absence of Id4.

To investigate the signals that drive and regulate *Id4* gene expression in the inner ear, I performed an *in silico* analysis on the *Id4* promoter. I found regions that are highly conserved across mammalian species and that these regions contain regulatory elements that are responsive to the Notch and the BMP signalling pathway, respectively. BMP signalling has previously been reported to upregulate expression of other members of the Id protein family (Id1/2/3) and was thus considered another pathway to be potentially involved in the regulation of Id4. I tested the responsiveness of a portion of the *Id4* promoter towards Notch and BMP signalling *in vitro* by performing luciferase reporter assays using an otic cell line and found that Notch signalling, but not the BMP pathway is able to upregulate luciferase activity driven by the *Id4* promoter. In contrast, neither blocking of the Notch or the BMP signalling pathway did seem to have any effect on endogenous *Id4* activity.

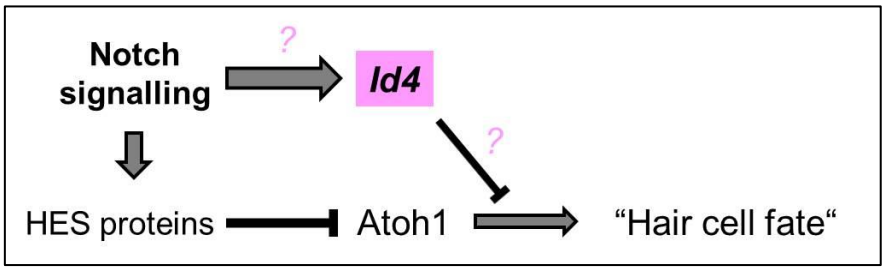


Figure 1-6: Working hypothesis for the action of Id4. In a genome-wide microarray screening for potential Notch targets in the developing chicken vestibular system levels of Id4 mRNA were found to be significantly decreased after treatment with the Notch inhibitor DAPT (N. Daudet and M. Gomez, unpublished data). Based on findings from previous studies which provided evidence that other Id protein family members are able to inhibit hair cell formation by inhibiting Atoh1 in a manner downstream of the BMP signalling pathway, our working hypothesis suggests that Id4 might function in a similar manner downstream of Notch.

2. Materials and Methods

2.1 Animals

2.1.1 Chicken

Embryos from fertilized white Leghorn chicken eggs (Henry Stewart & Co. Ltd) were used for *in ovo* electroporation, *in situ* hybridization and immunohistochemical experiments. Eggs were kept at 12°C for a maximum of 12-14 days and incubated at 37.5°C in an air damped rotating incubator if needed for experiments. 24 hours of incubation correspond to one day of embryological development (E).

2.1.2 Mice

Dissected and fixed P0 embryos (WT, heterozygous, homozygous) from an *Id4-lacZ* knock-in line and two heterozygous stud males for in-house breeding were kindly provided by Dr Li Yi, Baylor College of Medicine, Houston/ United States of America. The line was originally generated on a CD1 background by aggregating targeted and genetically modified ES cells with CD1-derived morulae (Bedford, Walker et al. 2005). Rederivation of the line was carried out by crossing heterozygous *Id4-lacZ* mice with CD1 WT mice. Any further breeding was carried out by crossing heterozygous males and females to generate hetero- and homozygous offspring.

2.1.2.1 DNA extraction and genotyping of Id4-lacZ mice

To genotype mice from the *Id4-lacZ* line DNA was extracted from either brain tissue or tail snips using the DirectPCR® Lysis Reagent Ear Kit (ViagenBiotech, cat #402-E). Tissue samples of ~0.2 cm were collected in 1.5 ml Eppendorf tubes and provided with 150 µl/tube

of DirectPCR® Lysis solution. 0.04 mg/ml of Proteinase K was added to the tubes and samples were incubated at 55°C on a shaker overnight. The next day, crude lysates were incubated at 85°C on a heating block for 45 min and precipitated by centrifuging at 1.000 g for 10 seconds. For immediate use in genotyping 3 µl of DNA lysate was used per PCR reaction. PCR reactions were prepared using the Phusion® High-Fidelity DNA Polymerase and the appropriate 5x Phusion HF Buffer. PCR was carried out in an Eppendorf Mastercycler Gradient PCR machine. PCR settings and primers sequences of all used primers are depicted in table 2-1.

Id4		• 98 °C, 30 seconds	
sense antisense	5'-GCGATATGAACGACTGCTAC-3' 3'-TCACCCTGCTTGTTGACGGC-5'	• 98 °C, 10 seconds 60 °C, 30 seconds 72 °C, 60 seconds	(35x)
Band size = ~580 bp		• 72 °C, 10 min	
LacZ		• 98 °C, 30 seconds	
sense antisense	5'-CGTCGTGACTGGGAAAACCC-3' 3'-CGCGTAAAAATGCGCTCAGG-5'	• 98 °C, 10 seconds 69 °C, 30 seconds 72 °C, 60 seconds	(35x)
Band size = ~500 bp		• 72 °C, 10 min	

Table 2-1: Primer sequences and PCR settings for the *Id4-lacZ* genotyping PCR.

2.2 Buffers and solutions

All buffers and solutions were used in the following composition and concentrations. They and their ingredients were either purchased ready for use or prepared according to common laboratory protocols.

Alkaline phosphatase (AP) buffer, 10ml	1 ml Tris (1M pH 9.5) 500 µl $MgCl_2$ (1M) 200 µl NaCl (5M) 100 µl Tween20 (10%) 8.2 ml H2O
Beta galactosidase substrate solution	5 mM $K_3Fe(CN)_6$ 5 mM $K_4Fe(CN)_6$ 0.5 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) Dimethylformamide
Blocking solution	1xPBS 10% goat serum 0.3% Triton-X
DMEM complete	1x DMEM (Dulbecco's Modified Eagle Medium, Gibco) 10% fetal bovine serum
10x HBS (pH 7.12)	8.18% NaCl (w/v) 5.94% HEPES (w/v) 0.2% Na_2HPO_4 (w/v) H_2O
HBSS complete	1x HBSS (Hank's Balanced Salt Solution, Gibco) 1 M HEPES
Hybridization buffer, 100ml	50 ml Formamide (99%) 25 ml 20xSSC 2 g Boehringer blocking powder 1 ml Triton-X (10%) 1 ml Chaps buffer (10%) 50 µg/ml heparin

	50 µg/ml yeast tRna 1 ml EDTA (0.5M) 22 ml DEPC H_2O
MEM complete	1x MEM + Glutamine (Minimal Essential Medium, Gibco) 10% fetal bovine serum Interferon-γ (50 U/ml)
0.1% Phosphate-buffered saline plus Triton (PBT)	50 ml 1xPBS 500 µl Triton-X (10%)
10x Tris-buffered saline plus Triton (TBST), 800ml	240 ml NaCl (5M) 80 ml Tris-HCl (1M pH 8.0) 8 ml Triton-X 472 ml H_2O
X-gal permeabilisation buffer	2 mM $MgCl_2$ 0.02% NP_4O 0.01% Sodium deoxycholate 1xPBS

2.3 Plasmids and antibodies

All plasmids used for *in ovo* electroporation, *in situ* hybridization, *in vitro* transfection, and molecular cloning experiments as well as their origins are listed in table 2-2.

Name	Description	Insert	Plasmid type	Origin
chEST135d4	Gallus gallus cDNA clone	Id4 (chicken)	pBluescript II KS+	Simon Hubbard, University of Manchester
pT2K-CAG-rTA-M2	Tetracycline transactivator protein	rTA-M2	pT2K	Yoshiko Takahashi, NAIST, Japan
pCAGGS-TP	Transposase expression construct	Transposase	pCAGGS	Yoshiko Takahashi, NAIST, Japan
pT2K-B1-EGFP	GFP control plasmid (Tol2 inducible)	B1-TRE-EGFP	pT2K	Yoshiko Takahashi, NAIST, Japan
pT2K-B1-TRE-Id4-EGFP	Id4 overexpression construct (Tol2 inducible)	Id4 mouse	pT2K	Nicolas Daudet, University College London
pCAB-IRES-GFP	GFP expression construct	IRES-GFP	pCAB	Jonathan Gilthorpe, King's College London
pCAB-HA-NICD-IRES-GFP	NICD-GFP expression construct	Notch1 ICD + HA-tag chicken	pCAB-IRES-GFP	Nicolas Daudet, University College London
pCAB-Smad6-IRES-GFP	Smad6 expression construct	Chicken Smad6	pCAB	Claudio Stern, University College London
pGL3-Basic	Luciferase reporter vector	Firefly luciferase	pGL3	Promega (# TM033)
pGL3-Id4-luc	Id4-luciferase reporter construct	Id4 promoter + firefly luciferase	pGL3	Haibo Wang, Cincinnati Children's Hospital Medical Center

Table 2-2: Details and origin of plasmids used for *in ovo* electroporation, *in situ* hybridization, *in vitro* transfection, and molecular cloning experiments.

Primary antibodies used for immunohistochemical experiments are listed in table 2-3. Secondary antibodies were purchased from Invitrogen™/ Thermo Fisher Scientific and used at 1:1,000 unless indicated differently.

Name	Antigen	Host (Isotype)	Conjugate	Working dilution	Origin
Anti-Atoh1	Atoh1 (mouse)	Chicken (IgY)	-	1:5,000 – 10,000	Matthew W Kelley, NIDCD/NIH
Anti-HCA	Hair cell antigen (chicken)	Mouse (IgG1)	-	1:500	Guy Richardson, University of Sussex
Anti-Id4	Id4 L-20 (mouse)	Rabbit (IgG)	-	1:200	Santa Cruz (sc-491)
Anti-Jagged1	Jagged1 (human)	Rabbit (IgG)	-	1:100	Santa Cruz (sc-8303)
Anti-Myo7A	MyosinVIIA (human)	Mouse (IgG)	-	1:250	DSHB (138-1)
Anti-Sox2	Sox2 (human)	Mouse (IgG1)	-	1:300	BD Pharmingen (561469)
Anti-Sox2	Sox2 (human)	Rabbit (IgG)	-	1:500	Abcam (ab97959)

Table 2-3: Details of used primary antibodies and their origin.

2.4 In ovo electroporation

DNA working solution

Plasmid DNA solution for electroporation experiments was prepared immediately before use or stored at -20°C and thawed 1 – 2 x as a maximum. DNA working solution was prepared in a total volume of 10 µl and supplied with 2 µl of fast green/sucrose solution. The total amount of DNA differed between the used plasmids but was usually held between 3-4 µg/µl in total. Plasmids were used in the following concentrations: Tol2 transposon 0.6 µg/µl, transposase 0.8 µg/µl, Id4-EGFP 1 µg/µl, EGFP 1 µg/µl.

Experimental procedure

In ovo electroporation of early stage chicken embryos was carried out according to the laboratory's protocol (Freeman and Daudet 2012). To perform overexpression experiments embryos were electroporated on day 2 of embryological development (E2). Three to four ml of albumen were removed carefully using a 20 ml syringe with a 1.2 mm x 40 mm BD Microlance needle before opening the eggshell. The eggs were placed under a stereomicroscope equipped with a cold-light source. Two electrodes connected to a BTX ECM 830 Electro Square Porator were positioned one on each side of the embryo's right otic cup. An OD 1.2 mm x ID 0.94 mm glass needle filled with the prepared DNA working solution was positioned in between the two electrodes right above the otic cup. DNA was injected into the otic cup by air-blowing and a 9 V electric pulse (3 x 100 ms, 500 ms intervals) was given immediately. Air bubbles were removed by adding a drop of 1xPBS. The eggs were sealed with transparent adhesive tape (Sellotape®) and incubated at 37.5°C until the desired stage.

Doxycycline induction

Doxycycline treatment of embryos was performed 3 days after electroporation (E5) in order to induce protein expression of EGFP or Id4-EGFP, respectively. The total amount of administered Doxycycline per embryo was 20 µg in 200 µl of sterile 1xPBS. The Doxycycline solution was injected into the embryo's surrounding liquid by carefully piercing the vitelline membrane with a 0.45 mm x 10 mm BD Microlance needle attached to a 5 ml syringe. After Doxycycline treatment the eggs were re-sealed and incubated at 37.5°C until the desired stage.

2.5 Embryo fixation

Early stage whole mount chicken embryos and embryonic inner ear tissue were fixed in 4% PFA for 1-2 hours at room temperature (RT) or overnight at 4°C, respectively. For stages E3 and below, chicken embryos were removed from the yolk sac using a fine pair of scissors and transferred into a 15 ml falcon tube containing 4% PFA. For stages E4 and above embryos were decapitated *in ovo* using a fine pair of tweezers and heads were transferred into a 15 ml or 50 ml falcon tube containing 4% PFA. After fixation, the inner ear was dissected under a standard stereoscope and processed for further experiments.

2.6 Cryosectioning

Cryostat sections were prepared from fixed whole mount chicken embryos (E3+4) and dissected inner ears (E5, 7, 9) as well as dissected heads (E13+14) and inner ears (P0+32) from mouse embryos in order to perform *in situ* hybridization and immunohistochemistry experiments. To prepare the tissue for freezing, samples were incubated in a 30% sucrose in 1xPBS solution overnight at 4°C. Samples were then embedded in 1% agarose + 18% sucrose in 1xPBS and let to set at RT or 4°C. To freeze the samples, portions were cut out of

the agarose and transferred onto a filter paper containing O.C.T. mounting medium (VWR). Samples were frozen in liquid nitrogen-cooled isopentane at -60°C to -90°C and stored at -80°C. Cryosectioning of frozen samples was performed on a Leica CM1850 cryostat. Samples were mounted on the object holder using O.C.T. medium and cut at a thickness of 10 - 20 µm. Sections were collected on Superfrost® Plus Micro Slides (VWR) and kept at -20°C until further use. In case of adult tissue, samples were decalcified by incubation in 10% EDTA (pH 7.3) overnight at 4°C on a rotator prior to cryosectioning.

2.7 In situ hybridization

Synthesis of DIG-riboprobe

A DIG-labelled RNA probe against chicken Id4 was synthesized using the EST clone chEST135d4 as a template (*Gallus gallus* cDNA, see table 2-2). Synthesis of the probe was carried out according to the following protocol. To linearize the template 5 – 10 µg of plasmid DNA was digested with Not1 in a total volume of 50 µl. Restriction digest was confirmed by gel electrophoresis using 1 µl of the digested plasmid. The reaction was stopped by heat inactivation at 65°C for 15 min and DNA was precipitated with 1/10 volume of 3 M NaOAc and 2.5 x volume of 100 % EtOH for at least 1 hour at -20°C. DNA was washed in 70% EtOH by centrifugation and resuspended in 20 µl of nuclease-free H_2O. The concentration was checked using a NanoDrop spectrophotometer and the digested template plasmid was kept at -20°C until further use. To transcribe antisense RNA T3 transcription enzyme was chosen according to the Id4 plasmid vector card. The transcription mix was composed in a total volume of 40 µl in the following composition:

1 µg template DNA
2 µl 10x DIG-nucleotide mix (Roche)

4 µl 10x transcription buffer (Roche)
1 µl RNasin® Plus RNase Inhibitor (Promega)
3 µl T3 RNA Polymerase (Promega)
H_2O nuclease-free up to 40 µl

The mix was vortexed and transcribed at 37°C for 2 hours. The mix was transferred to ice to stop the reaction and transcription products were verified by gel electrophoresis. To precipitate the probe 4 µl of 0.5 M EDTA, 30 µl of 8 M LiCl and 26 µl of H_2O were added to the transcription mix for 30 min at -20°C. The probe was then washed with 70% EtOH by centrifugation and finally resuspended in 50 µl nuclease-free H_2O. If required the probe was again checked by gel electrophoresis and kept at -20°C until further use.

Experimental procedure

In situ hybridization on cryostat sections was carried out using microscope slide boxes for incubation steps and 50 ml glass coplin jars for washing steps. The bottom of the microscope slide boxes was covered with a damped whatman paper to avoid drying out of the samples. *In situ* hybridization was performed according to the following protocol. Slides were air-dried for 30 min at RT. Then, 100 – 200 µl of DIG-probe diluted in hybridization buffer was applied on each slide and the slides were incubated at 70°C overnight to hybridize. On the next *day* slides were rinsed in the following buffers at 70°C for 2 x 30 min each: 1xSSC + 50% formamide + 0.1%Tween, 2xSSC + 0.1%Triton, 0.2xSSC + 0.1%Triton. A final rinsing step was carried out at RT for 2 x 30 min in 1xTBST. Slides were blocked with 1xTBST + 10% sheep serum and incubated with 200 µl of anti-DIG-AP in blocking solution at 4°C overnight. On day 3, slides were rinsed in 1xTBST at RT for at least 4 x 20 min. To reveal the signal slides were pre-incubated with freshly prepared AP buffer for >10 min at RT before adding the substrate solution. The NBT/BCIP substrate solution was prepared according to the

supplier's manual (1 ml AP buffer + 4.5 µl NBT + 3.5 µl BCIP) and applied onto the slides in the dark. To stop the reaction slides were rinsed in 1xTBST and covered with a glass cover slip for microscopic analysis and imaging.

2.8 Immunohistochemistry

Whole mount

Immunohistochemistry on whole mount inner ears was performed after electroporation experiments in order to visualize sensory patches and to label different cell types. After PFA fixation the tissue was processed in a 3 cm petri dish filled with 1xPBS. Dissected inner ears were transferred into a 1.5 ml Eppendorf tube and rinsed in 0.1%PBT (1xPBS + 0.1%Triton) for 2 x 10 min each. Samples were incubated in blocking solution (1xPBS + 0.3%Triton + 10% goat serum) for >30 min at RT and then incubated with the primary antibody diluted in blocking solution for 2 hours at RT or overnight at 4°C, respectively. After the primary antibody incubation samples were rinsed in 0.1%PBT for at least 3 x 15 min at RT. Samples were incubated with the secondary antibody diluted in 0.1%PBT for 2 hours at RT and again rinsed in 0.1%PBT for at least 3 x 15 min. If required, samples were counterstained with DAPI (1:500 of 5mg/ml stock solution). Samples were mounted on microscope slides using FluorSave® mounting medium, covered with a glass cover slip and sealed with nail varnish before used for microscopic analysis.

Cryostat sections

Immunostaining of mounted cryostat sections was performed after chicken ISH experiments and on LacZ-stained and unstained sections from the mouse inner ear. Steps were carried out in the dark if required by the presence of any fluorescent component. Slides with the cryostat sections were rinsed in 0.1%PBT for >10 min in a glass coplin jar at RT while

shaking (an additional rinsing step in 0.1%PBT + 0.05% H_2O_2 was required if used for later detection with a peroxidase-conjugated secondary antibody). Slides where blocked with 250 µl/slide of 0.1%PBT + 10% goat serum for ~20 min in a covered microscope box and incubated with ~200 µl/slide of primary antibody diluted in 0.1%PBT for 2 hours at RT or overnight at 4°C. Slides were rinsed in 0.1%PBT for at least 3 x 15 min and then incubated with the secondary antibody diluted in 0.1%PBT for 1-2 hours at RT. After incubation slides were rinsed for at least 3 x 10 min in 0.1%PBT. Samples were covered with a glass cover slip after applying FluorMount® mounting medium and sealed with nail varnish before used for microscopic analysis.

Atoh1 immunostaining

An aliquot of the chicken-anti-Atoh1 primary antibody as well as a detailed immunostaining protocol was kindly provided by the Matthew W. Kelley lab, National Institute on Deafness and Other Communication Disorders (NIDCD)/ National Institute of Health (NIH), United States. To increase antibody affinity, immunostaining was exclusively performed on short-fixed inner ear tissue (30 min or less). After fixation, samples were processed for cryosectioning and staining was performed on microscope slides. Sections were rinsed in 1xPBS for 20 – 30 min and incubated in 0.5%PBT for further 30 min to permeabilize. Sections were then blocked with 0.5%PBT + 10% goat serum + 10% Henblocker blocking agent (BlokHen®, AvesLab BH-1001) for 1 hour at RT. Primary antibody solution was prepared in 0.5%PBT at different concentrations (1:5,000 – 1:20,000). After blocking, sections were incubated with the primary antibody for 3 hrs at RT and then rinsed in 0.1%PBT for at least 4 x 30 min at RT. In the meantime, the secondary antibody solution was prepared in 0.5%PBT. Here, goat-anti-chicken-IgY was used at 1:2,000. Note that the secondary antibody was centrifuged for 30 min at 4°C and maximum speed before use.

Sections were incubated with the secondary antibody solution overnight at 4°C in the dark. Sections were then rinsed in 0.1%PBT for at least 4 x 30 min and mounted for microscopy.

2.9. LacZ (X-gal) staining

Beta galactosidase (lacZ) staining was performed on whole mount inner ears dissected from heterozygous animals of the *Id4-lacZ* line. After fixation in 4% PFA samples were rinsed in 1xPBS for a minimum of 15 min at 4°C on a rolling platform. Samples were then incubated with X-gal permeabilisation buffer in a 5 ml glass vial for 30 min at RT. Permeabilisation buffer was removed and replaced with 1 ml/vial of beta galactosidase substrate solution. To develop the lacZ signal samples were incubated for a minimum of 6 hours at 4°C on a rolling platform in the dark. When fully stained samples were imaged as whole mounts and then further processed for cryosectioning and immunohistochemistry.

2.10 Cell culture methods

2.10.1 Culturing of cells

Conditionally immortalized auditory hair cell precursor cells (OC-2 cells, Rivolta, Grix et al. 1998) were kindly provided by Dr Sally Dawson, Ear Institute, UCL London. Cells were cultured in a total volume of 10 ml MEM complete in T-75 flasks (Nunc^{TM} Thermo Scientific) at 33°C and 5% CO_2. Cells were usually split at approximately 80% of confluency to maintain the cultures. To split the cells, medium was removed from the flasks with a serological pipette and replaced by 3ml/flask of trypsin to detach the cells. Flasks were incubated at 33°C for 5-10 min and gently shaken if required. 3 ml of MEM complete was added to the flasks and cells were collected in a 50 ml falcon tube. Cells were centrifuged at 1000 rpm for 3 min and cell pellets were resuspended in 10 ml of fresh MEM complete. For further

culturing 1 ml of the cell suspension was added to 9 ml of MEM complete in a fresh T75 flask and incubated at 33°C and 5% CO_2.

2.10.2 Calcium phosphate transfection

Cells were seeded in 6-well-plates (Falcon™ Sterile R) at 2 x 10^5 cells/well in a total volume of 2 ml of MEM complete and incubated at 33°C and 5% CO_2 overnight. On the day of transfection the medium was removed from the wells and replaced by 2 ml of prewarmed DMEM complete. Plates were incubated at 37°C and 5% CO_2 for at least one hour to prepare the cells. In the meantime DNA solutions for transfection were prepared under sterile conditions. The total amount of DNA used for transfection was 1200 ng/well for the various expression constructs, 200 ng/well for the luciferase reporter constructs and 10 ng/well for the *Renilla* luciferase. Expression constructs and luciferase reporters were used at a stock solution of 100 ng/µl prepared from EtOH-precipitated DNA. *Renilla* luciferase was used at 10 ng/µl. For each well, the DNA stock solution was mixed with 72.6 µl of H_2O and 12.4 µl of 2 M $CaCl_2$ and incubated for 20 – 30 min at RT to allow the DNA precipitate to form. The DNA precipitate was then carefully mixed with an equal amount of 2xHBS buffer (pH 7.12) just before adding the transfection mix to the cells. 200 µl of transfection mix were added to each well of the 6-well-plates and cells were then incubated at 37°C and 5% CO_2 for 24 hours.

Glycerol shock

To enhance transfection efficiency a glycerol shock was carried out 24 hours after adding the transfection mix to the cells. Medium was removed from the wells and replaced by 2 ml DMEM complete + 15% glycerol. The solution was left for 2:30 min at RT, removed, and wells were 2x washed with HBSS complete. After the washing steps all medium was

removed from the wells and 2 ml of MEM complete were added. Cells were incubated at 33°C and 5% CO_2 overnight to recover and grow and used for further experiments on the following day.

2.10.3 Notch inhibition (LY-411575 treatment)

The γ-secretase inhibitor LY-411575 (Cayman Chemical, CAS 209984-57-6) was used for *in vitro* inhibition of the Notch signalling pathway. The crystalline powder was dissolved in DMSO and kept as a stock 5 mM stock solution at -20°C. To inhibit the pathway, LY-411575 was added to the cell culture medium at a final concentration of 1 µM or 5 µM, respectively. Differences in the amount of added 5 mM stock solution were equalized by adding the appropriate amount of DMSO. The total amount of DMSO in cell culture media was 0.1% under all conditions. For luciferase experiments, cells were cultured in the absence of LY-411575 until transfection. Cell culture medium was replaced by LY-411575-containing medium immediately before the transfection mix was added and further kept in LY-411575-containing medium after the glycerol shock and all washing steps.

2.10.4 BMP treatment

To increase the level of BMP activity *in vitro*, cells were treated with human BMP4 protein (hBMP4, Invitrogen, Cat.# PHC9534) at different concentrations. Aliquots of hBMP4 were kindly provided by Dr Jonathan Gale and were kept as a 100 ng/µl stock solution at -20°C. For luciferase experiments, cells were cultured under normal conditions until the glycerol shock. After the washing steps medium was replaced by 1 ml/well of serum-free MEM complete medium supplied with 50 ng/µl or 250 ng/µl of hBMP4, and incubated for 18 hours before proceeding to further experiments.

2.10.5 Luciferase reporter assay

Luciferase reporter assays were performed using the Dual-Luciferase® Reporter (DLR™) Assay System (Promega). All steps were carried out according to the manufacturer's protocol. Cells were harvested 48 hours after transfection and 24 hours after the glycerol shock. To harvest the cells, wells were rinsed 2x with 1xPBS. All liquid was removed using a serological pipette and 100 µl of Passive Lysis Buffer (PLB, Promega) were added to each well. Cells were detached manually by scratching the surface with the inner part of a clean 1 ml syringe (BD). The cell lysate suspension was collected in labelled 1.5 ml Eppendorf tubes and samples were incubated for 10 min at -20°C and 10 min at RT. Samples were centrifuged for 1 min at 14.000 rpm and the cleared lysate was transferred into a fresh 1.5 ml Eppendorf tube. 20 µl of the lysate were mixed with an equal amount of Luciferase Assay Reagent II (LAR II, Promega) predispensed in a separate 1.5 ml Eppendorf tube. The tube was placed into the tube holder of a Turner TD-20e luminometer immediately and Firefly luciferase activity was measured using a delay time of 5 seconds and a reading time of 30 seconds. To measure *Renilla* luciferase activity, 50 µl of Stop&Glo® Reagent (Promega) were added to the same tube, mixed by pipetting and the measurement was taken using the same settings. Steps were repeated for each sample of one experimental set and reaction tubes were discarded.

2.11 Sequencing

Sanger sequencing to confirm the identity of expression and reporter constructs was carried out using the SourceBioScience Sanger Sequencing Service (Source Bioscience UK Limited).

2.12 Microscopy

Light microscopy

Mounted cryostat sections from chicken ISH experiments and mouse lacZ staining experiments were analysed using a Zeiss Axioplan2 light microscope connected to a Zeiss HBO100 microscope illuminating system. Pictures were taken with a Zeiss AxioCam HRc 12V camera using the Axiovision Rel 4.3 imaging software. All pictures were processed with the Fiji software and assembled in Adobe Photoshop CS2.

Confocal microscopy

Fluorescent whole mount chicken inner ears from Id4 gain-of-function experiments and immunostained mouse cryostat sections were analysed under a Zeiss LSM 510 Meta inverted confocal microscope connected to a Zeiss LSM 510 Laser module. All confocal images were taken as 12_bit files at different magnifications and further processed and analysed using the Fiji software. Final figures were assembled using Adobe Photoshop CS2.

2.13 Quantification and statistics

Quantification of transfected cells and differentiated hair cells or supporting cells was carried out using the Cell Counter plugin for the Fiji software (Vos 2014). 12_bit confocal images were taken after immunostaining to visualize hair cells and supporting cells, respectively, and either single confocal slices or confocal stack projections were used for quantification depending on the orientation and the thickness of the mounted tissue. Brightness and contrast levels were adjusted during analysis of the confocal images in order to not leave out any transfected cell with low levels of fluorescence. All transfected cells were included that were found within the sensory region as indicated by the expression of hair cell or supporting

markers. Using the Cell Counter plugin, transfected cells were tagged and quantified with counter type a in the first place, and examined for fluorescent signal from used cell type markers to determine cell identity in a second step using counter type b. Statistical analysis of the quantitative data was performed by applying a two-tailed Student’s t-test based on an unequal variance (Microsoft Excel).

3. Results

3.1 Expression and function of Id4 in the avian inner ear

3.1.1 Id4 is expressed in the developing chicken inner ear at stages crucial for hair cell formation

A genome-wide microarray screening for transcriptional targets of Notch activity was carried out in our laboratory prior to this study and provided first evidence that Id4 is expressed in the developing chicken inner ear at a time point relevant for hair cell formation (N. Daudet and M. Gomez, unpublished data). However, the screening was carried out using tissue from stage E9 embryos, which in respect to hair cell formation is a rather late stage, and only tissue from the utricle was used. In order to determine the expression pattern of *Id4* at earlier stages and over a longer period of time, as well as to take into account all sensory regions of the inner ear, I performed *in situ* hybridization experiments using a DIG-labelled RNA hybridization probe against the chicken Id4 mRNA.

Hair cell formation in chicken embryos starts approximately at E5 in the vestibular organ, E6 in the auditory epithelium, and terminates around E12 in the BP (Tilney and DeRosier 1986, Goodyear and Richardson 1997). Stages E5, E7 and E9 were chosen as they represent early to late time points of hair cell formation. Stages E3 and E4 were further included in order to examine *Id4* expression prior to the onset of hair cell formation. ISH experiments were performed on 20 µm cryostat sections that had been prepared from either fixed whole mount embryos (E3, E4) or separated heads (E5, E7, E9). All results are depicted in figure 3-1, 3-2, and 3-3.

Figure 3-1 A and B show a longitudinal (coronal) section of an E3 embryo. At this stage, Id4 was found to be broadly expressed in the hindbrain (Fig. 3-1 A) and was not detected within

the developing otocyst (Fig. 3-1 A, B arrows). Figure 3-1 C and D show an example of a transverse section of an embryo 24 hours later at stage E4. *Id4* transcripts were still detected in the hindbrain where they appeared in a defined and striped pattern in the luminal region (Fig. 3-1 C, brackets). At this stage, Id4 mRNA was also detected in the ventro-medial wall of the developing otic vesicles on both sides of the body axis (Fig. 3-1 C, D arrow). These data suggest that *Id4* expression might set in between E3 and E4 and that expression precedes the onset of hair cell formation.

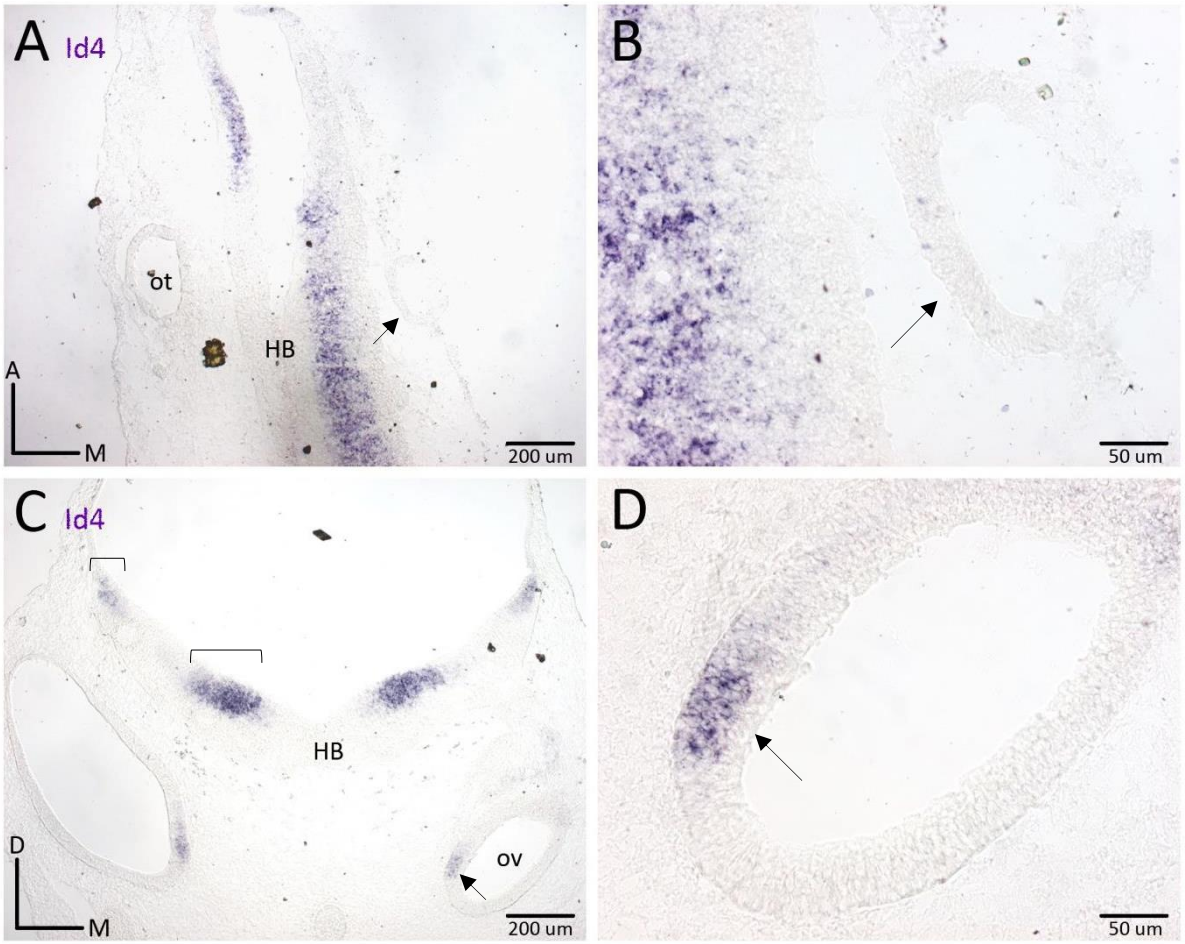


Figure 3-1: *Id4* expression in the developing chicken inner ear at stages E3 (A, B; longitudinal (coronal) section) and E4 (C, D; transverse section). (A, B) Id4 is broadly expressed in the hindbrain at stage E3 and is absent from the otocyst (arrow). (C, D) At E4, *Id4* expression becomes restricted to a defined striped pattern in the hindbrain (brackets) and is also found in the ventro-medial wall of the developing otocyst at this stage (arrow). Abbreviations: A, anterior; D, dorsal; HB, hindbrain; ot, otocyst; ov, otic vesicle.

The next analysed stages were stages at which hair cell formation had already started in all (E7, E9) or some (E5) of the sensory patches. Figure 3-2 shows transverse sections of the vestibular system of E5, E7, and E9 embryos, whereas figure 3-3 shows the same stages with respect to the auditory apparatus. Since I was particularly interested in *Id4* expression within the sensory patches, those regions were visualized by immunostaining against the characterized progenitor and supporting cell marker Jagged1.

At E5, *Id4* transcripts were found in the hindbrain in a pattern that resembled the one observed at an earlier stage (Fig. 3-2 A brackets, Fig. 3-3 A). In some of the sections *Id4* expression seemed to have broadened along the luminal region and appeared in three rather than two stripes on each side, reaching up all the way to the rhombic lip (Fig. 3-3 A brackets). Within the inner ear, expression of Id4 was detected in the macula utriculi of the vestibular apparatus (Fig. 3-2 A, B arrows) as well as in the ventro-medial wall of the developing basilar papilla (Fig. 3-3 A, B arrows). Both regions showing *Id4* expression were also positive for Jagged1, thus confirming their sensory identity. It is important to point out that at E5 hair cell formation has most likely not yet started in the apical end of the basilar papilla. Hence, the onset of *Id4* expression appears to precede auditory hair cell formation. At stage E7, *Id4* transcripts were detected along the ventricular zone of the hindbrain and the defined striped pattern was no longer seen (Fig. 3-2 C, Fig. 3-3 C). In addition to the ventricular zone, *Id4* transcripts were also detected in the underlying tissue of the hindbrain in a rather widespread pattern. Within the inner ear, *Id4* expression was detected in both the vestibular system (cristae; Fig. 3-2 C, D arrows) and the basilar papilla (Fig. 3-3 C, D arrows) overlapping with *Jagged1* expression. In addition to the hindbrain and the inner ear, Id4 was also found to be expressed by auditory neurons at this stage (Fig. 3-3 C, D arrowheads, F). At E9, *Id4* expression became even more widespread within the hindbrain compared to earlier stages and was further still found within the auditory nerve (Fig. 3-2 E, Fig. 3-3 E, F). In the inner ear, *Id4* expression was detected in all sensory regions of both the vestibular and

the auditory apparatus (Fig. 3-2 E, F, Fig. 3-3 E, F). Within the lateral crista and the macula sacculi, *Id4* transcripts were found in close colocalization with Jagged1 (Fig. 3-2 E, F arrows). In the basilar papilla, Id4 was found to be expressed along the length of the sensory epithelium, reaching from the basal end to the apical end, as well as in the non-sensory regions of the tegmentum and auditory neurons (Fig. 3-3 E, F). Jagged1-positive cells were found within the basilar papilla where they colocalized with *Id4* expression (Fig. 3-3 E, F arrowheads).

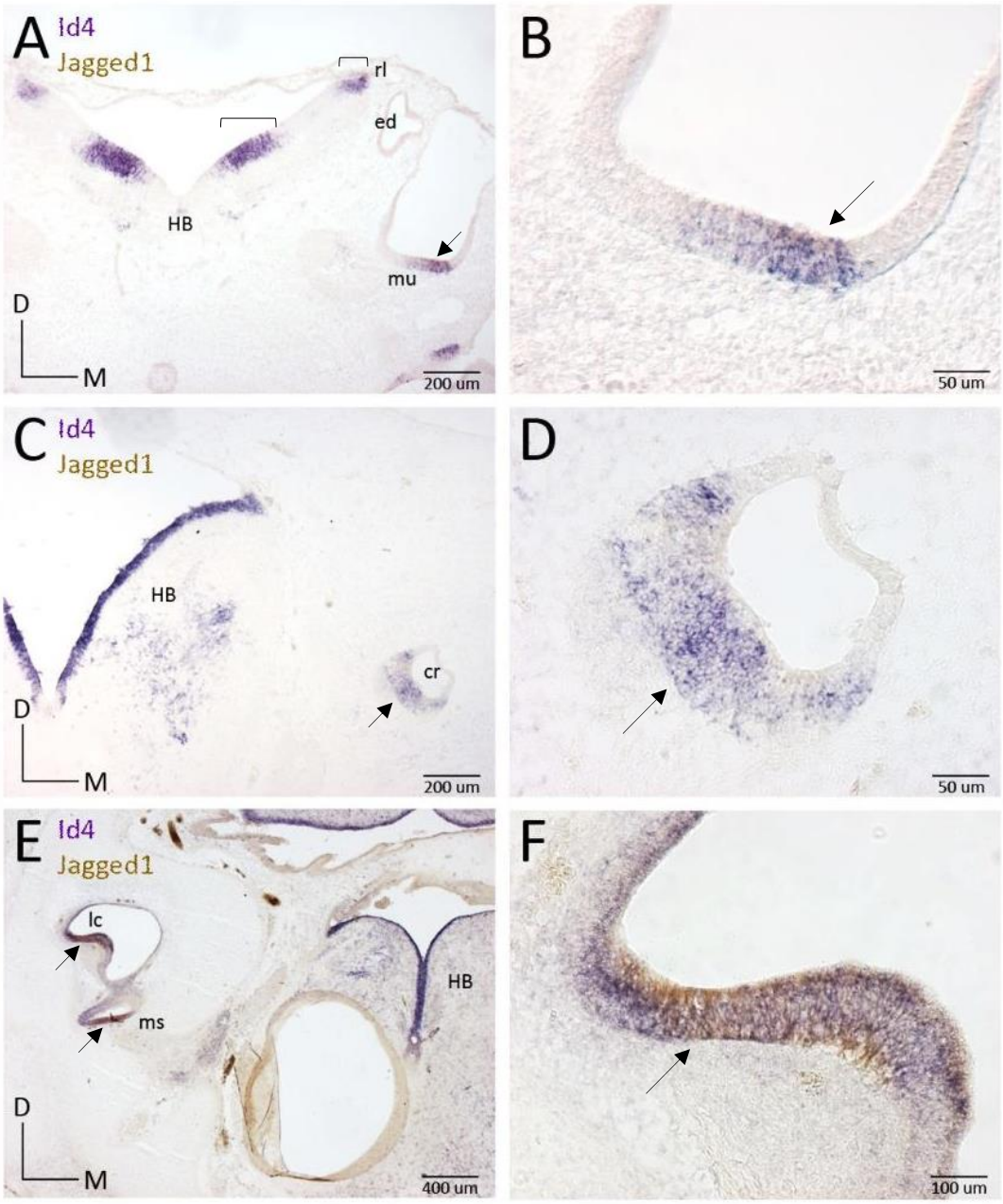


Figure 3-2: *Id4* expression in the vestibular system and the hindbrain of stage E5 (A, B), E7 (C, D), and E9 (E, F) chicken embryos (transverse sections). Sensory regions were verified by immunostaining against the supporting cell marker Jagged1. (A, B) At E5, *Id4* transcripts are found in distinct regions of the hindbrain in a stripy pattern (brackets) as well as in the macula utriculi of the vestibular organ (arrows). (C, D) At E7, *Id4* expression is becoming more widespread in the hindbrain, stretching ventrally. *Id4* expression is also found in the vestibular organ at this stage (arrows). (E, F) At E9, Id4 is expressed in large parts of the hindbrain, as well as in the lateral crista and the macula sacculi of the vestibular apparatus (arrows). At all stages, *Id4* expression colocalizes with Jagged1, confirming its localization in the sensory domains of the developing inner ear. Abbreviations: cr, cristae; D, dorsal; ed, endolymphatic duct; HB, hindbrain; lc, lateral crista; ms, macula sacculi; mu, macula utriculi; rl, rhombic lip.

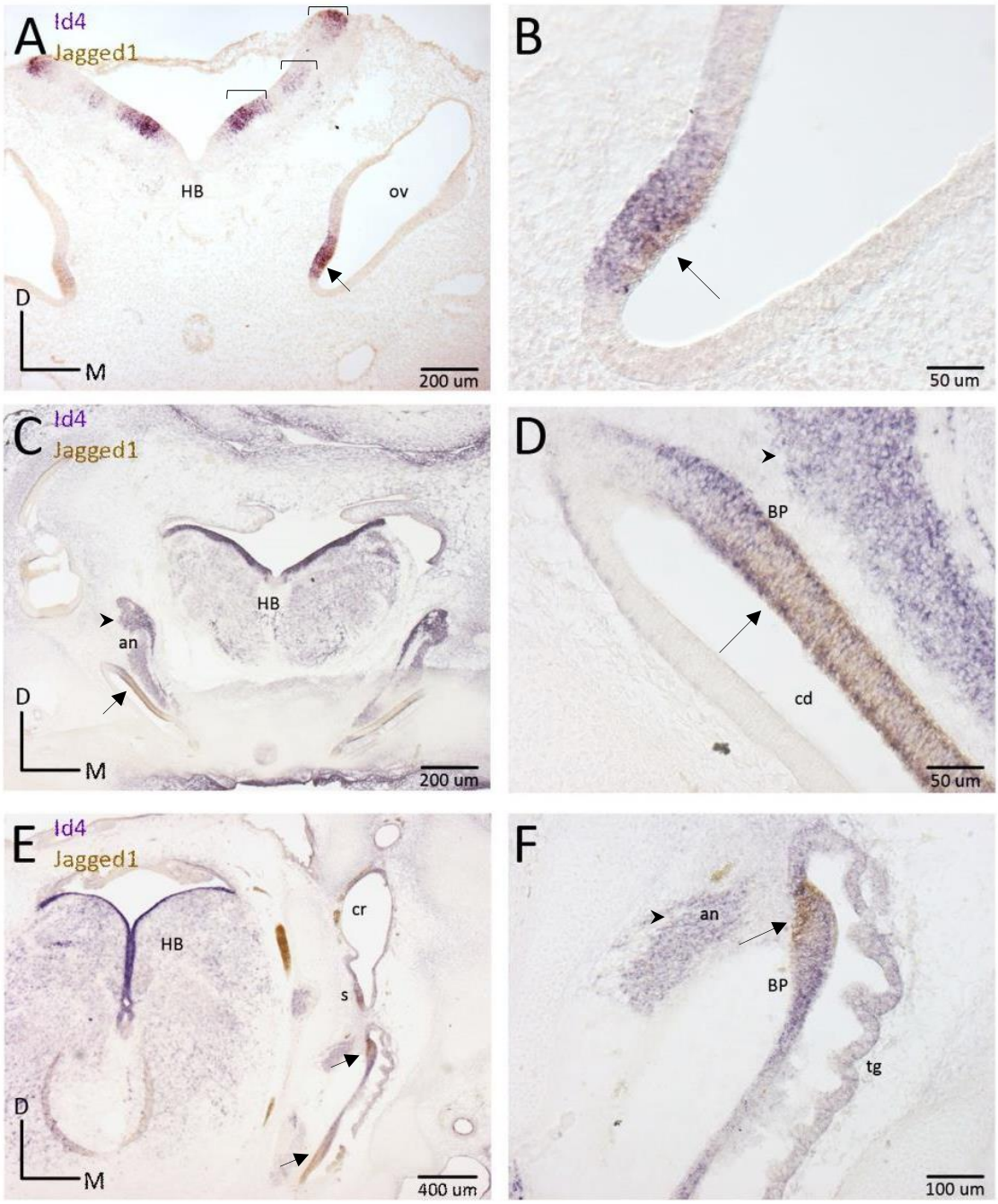


Figure 3-3: *Id4* expression in the basilar papilla and the hindbrain of stage E5 (A, B), E7 (C, D), and E9 (E, F) chicken embryos. Sensory regions were verified by immunostaining against the prosensory and supporting cell marker Jagged1 (A, B) At E5, *Id4* expression is found in the dorsal-medial part of the developing basilar papilla (arrows). (C-F) At E7 and E9, Id4 is expressed along the length of the auditory sensory epithelium. At both stages, Id4 is found within regions of *Jagged1* expression (arrows). *Id4* expression is further detected in the auditory nerve at E7 and E9 (C-F arrowheads) as well as in a changing pattern within the hindbrain at all depicted stages (A brackets, C, E). Abbreviations: an, auditory nerve; BP, basilar papilla; cr, cristae; cd, cochlear duct; D, dorsal; HB, hindbrain; M, medial; ov, otic vesicle; s, saccule; tg, tegmentum (vasculosum).

The data collected in the ISH experiments using different developmental stages provided evidence that Id4 is expressed in the prosensory and sensory domains of the chicken inner ear at time points that are relevant for hair cell formation. So far, this observation supports the initial hypothesis of Id4 playing a role in the regulation of hair cell formation.

3.1.2 Id4 gain-of-function: Id4 overexpression inhibits hair cell formation in the developing chicken inner ear

After having confirmed the expression of Id4 in tissues and stages relevant for hair cell formation, the next step was to test directly the function of Id4 during inner ear development. As previously described in the working hypothesis Id4 might be acting in a negative regulatory manner to control the formation and differentiation of hair cells. To test a putative inhibitory effect of Id4 on hair cell formation, I performed gain-of-function experiments using a Doxycycline-inducible (Tet-ON) Id4-GFP expression construct enabling a temporal control of gene expression in transfected cells (Kawakami 2007, Freeman, Chrysostomou et al. 2012). The experiment involved co-electroporation of the following constructs: i) The inducible construct for protein overexpression (pT2K-B1-TRE-Id4-EGFP, in the following referred to as Id4-eGFP), ii) a plasmid encoding a transposase, and iii) a plasmid encoding a transactivator protein. A schematic illustration is shown in figure 3-4 A. The inducible expression construct contains Tol2 transposon elements, which after co-electroporation with the transposase-encoding plasmid enable stable integration of the transgene into the genome of transfected cells. In absence of Doxycycline (Dox), however, protein expression is not active. When applied at a desired stage, Dox binds and activates a tetracycline transactivator protein (rtTA) which is encoded by the additional co-electroporated plasmid construct. The activated rtTA protein then binds to a tetracycline-responsive element within the expression construct and activates protein expression. Here, a bidirectional promoter was used in order to drive simultaneous expression of *Id4* and an enhanced *GFP* (*eGFP*). Thus, presence of green

fluorescent signal after Dox treatment was indicative of successfully transfected and induced cells.

In ovo electroporation experiments were carried out as previously described. The timeline of overexpression experiments is indicated in figure 3-4 B. Embryos were electroporated within the right-hand otic cup at stage E2 for reason of accessibility. In order to minimize the risk of any possible side effect of Id4 overexpression at stages prior to hair cell formation E5 was chosen as the stage for induction of protein expression. Doxycycline was administered for 48 hours and embryos were fixed and examined at stage E7. Choosing these early stages I was able to examine the direct effect of Id4 overexpression on hair cell formation. Concomitant control experiments were carried out by using an inducible GFP expression construct.

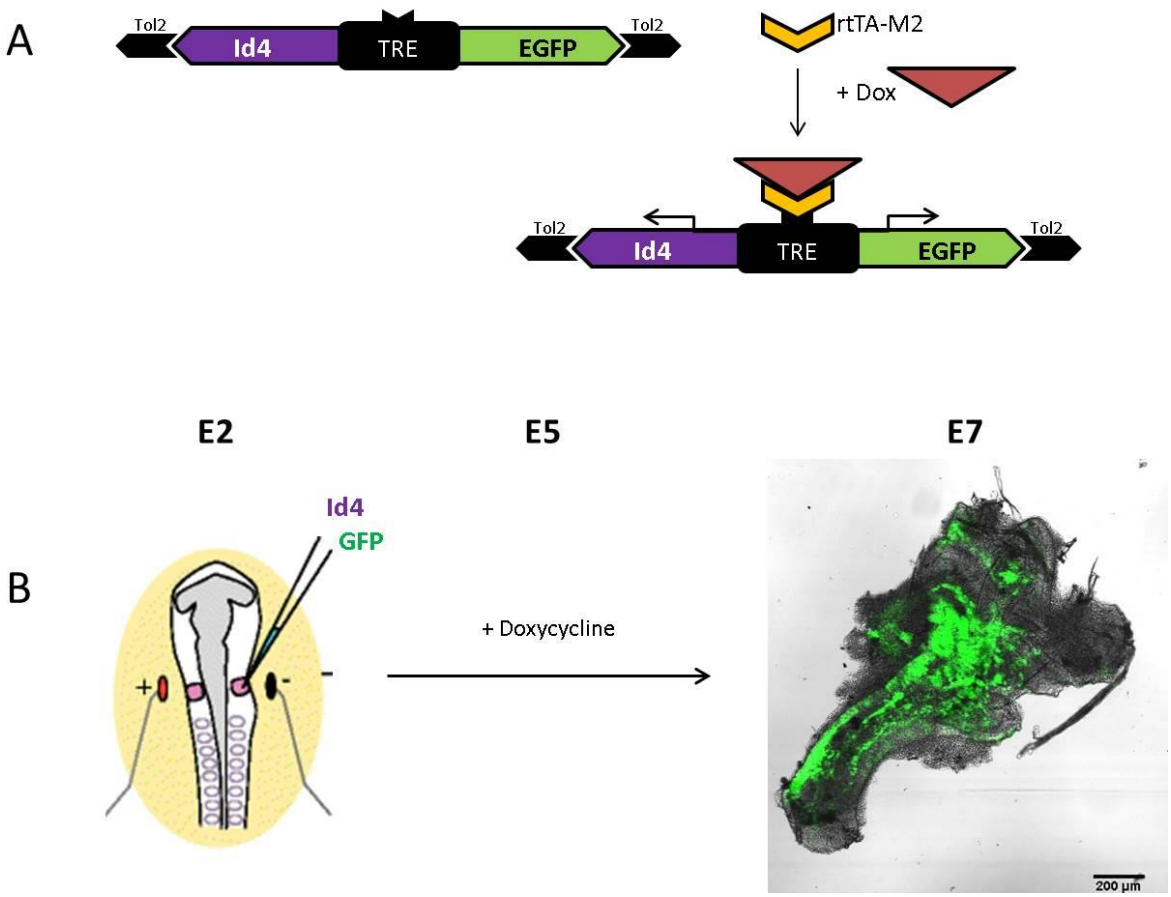


Figure 3-4: Id4-eGFP overexpression construct and experimental set-up for *in ovo* electroporation. (A) An inducible expression construct based on the Tet-ON system was used for overexpression experiments. The presence of Tol2 transposons and co-electroporation with a transposase-encoding plasmid enable stable integration of the expression construct into the cells' genome. Protein expression is inactive in the absence of the tetracycline analogue Doxycycline (Dox). Appliance of Dox leads to the activation of a co-electroporated tetracycline transactivator protein (rtTA-M2), binding of the complex to a tetracycline-responsive element (TRE) located within the expression construct, and finally to the initiation of protein expression. In case of the Id4-eGFP overexpression construct protein expression is driven by a bidirectional promoter. This allows simultaneous expression of Id4 and an enhanced GFP (eGFP), and thus a straightforward identification of successfully transfected and induced cells. Electroporation of control embryos was carried out using a corresponding Dox-inducible GFP expression construct (not shown). (B) Stage E2 embryos were electroporated into the right-hand otic cup with the Id4-eGFP or the GFP overexpression construct, respectively. Protein expression was induced at stage E5 by Dox treatment. Embryos were fixed at stage E7 and dissected whole-mount inner ears were examined using confocal microscopy. Successfully transfected and induced cells were identified by the presence of GFP fluorescence.

Fixed and dissected whole mount inner ears were counterstained with antibodies against the hair cell antigen (HCA), a polypeptide specific to the apical surface of sensory hair cells

(Richardson, Bartolami et al. 1990), and Myosin VIIA (Myo7A) in order to label both hair cells' cell bodies and stereocilia. The HCA, also referred to as Ptprq, has been identified as a member of the receptor-like protein tyrosine phosphatase family (Goodyear, Legan et al. 2003) and is a component of the shaft connectors between neighbouring stereocilia (Goodyear and Richardson 1992). Qualitative and quantitative analysis was carried out with focus on the vestibular system, as the number of formed hair cells at the examined stages is much higher in these regions compared to the basilar papilla. Confocal microscopy images of Id4-electroporated embryos and GFP-electroporated control embryos are shown in figure 3-5 and 3-6.

In Id4-overexpressing embryos, two main phenotypes were observed. In some of the embryos a clear reduction in the density of hair cells was observed in those regions that overexpressed Id4 (Fig. 3-5 A-C dotted line). Other samples showed a rather mixed situation in which a large-scale reduction in the total number of hair cells was not as obvious at lower magnifications (Fig. 3-5 D-F dotted line). However, looking at these samples at higher magnifications revealed an inhibitory effect on hair cell formation. The majority of cells that had been transfected with the Id4 overexpression construct had not differentiated into hair cells, and conversely, most of the cells that had committed to a hair cell fate did not express the Id4-eGFP construct (Fig. 3-5 D-I, G-I asterisks). However, some few cells were detected that were both Id4-overexpressing and positive for hair cell markers (Fig. 3-5 G-I arrows). In contrast to Id4, overexpression of GFP alone had no effect on hair cell formation (Fig. 3-6). The overall density of hair cells within vestibular sensory patches was not altered (Fig. 3-6 A-C), and GFP-overexpressing cells did differentiate into hair cells as indicated by the presence of hair cell markers (Fig. 3-6 D-F asterisks, G-I arrows). To check whether overexpression of Id4 does not only affect hair cell but also supporting cell formation samples of Id4-electroporated embryos were additionally immunostained against the supporting cell markers Jagged1 and Sox2. As shown in figure 3-7, no obvious change in the levels or the

pattern of *Jagged1* and *Sox2* expression could be observed within Id4-overexpressing cells (Fig. 3-7 A-L).

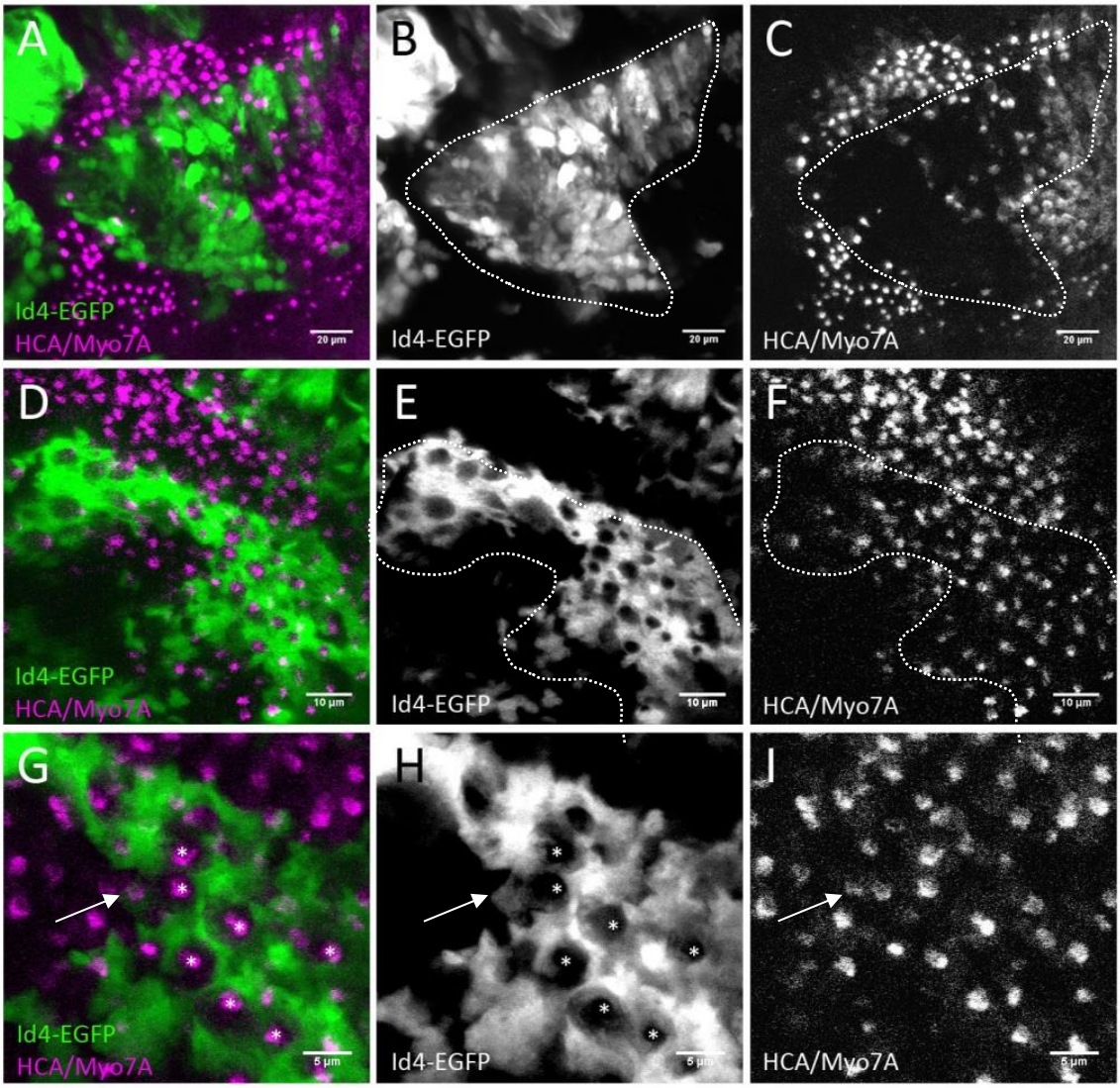


Figure 3-5: Overexpression of Id4 affects hair cell formation in the vestibular system of E7 chicken embryos. To examine the effect of an Id4 overexpression on hair cell formation, differentiated hair cells were labelled by immunostaining against the hair cell marker HCA/Myo7A. Two main phenotypes were observed in embryos electroporated with the Id4 overexpression construct. In a portion of the examined embryos, overexpression of Id4 led to a broad reduction of hair cell density within the sensory patches (A-C dotted line), while other samples did not show such an obvious phenotype at lower magnifications (D-F). However, when analysed at higher magnifications, it was observed that cells overexpressing Id4 had not differentiated into hair cells. Similarly, cells expressing hair cells markers did not show any sign of *GFP* expression.

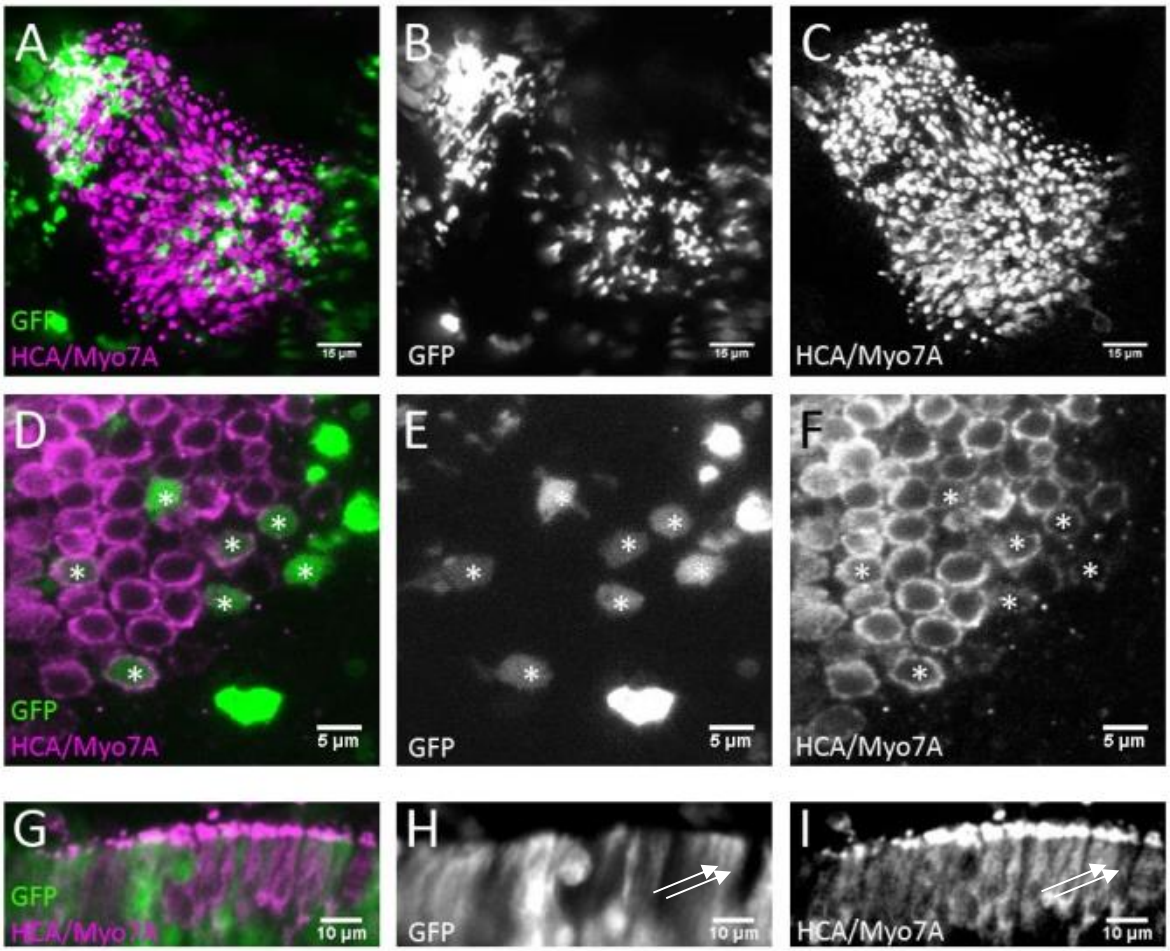


Figure 3-6: Overexpression of GFP has no obvious effect on hair cell formation in the developing chicken vestibular system. In control embryos electroporated with the GFP expression construct no significant effect on hair cell formation was seen. The overall distribution of hair cells within vestibular patches did not seem to be affected (A-C). Cells overexpressing GFP were further found to have differentiated into hair cells as indicated by the expression of hair cells markers (D-F asterisks, G-I arrows).

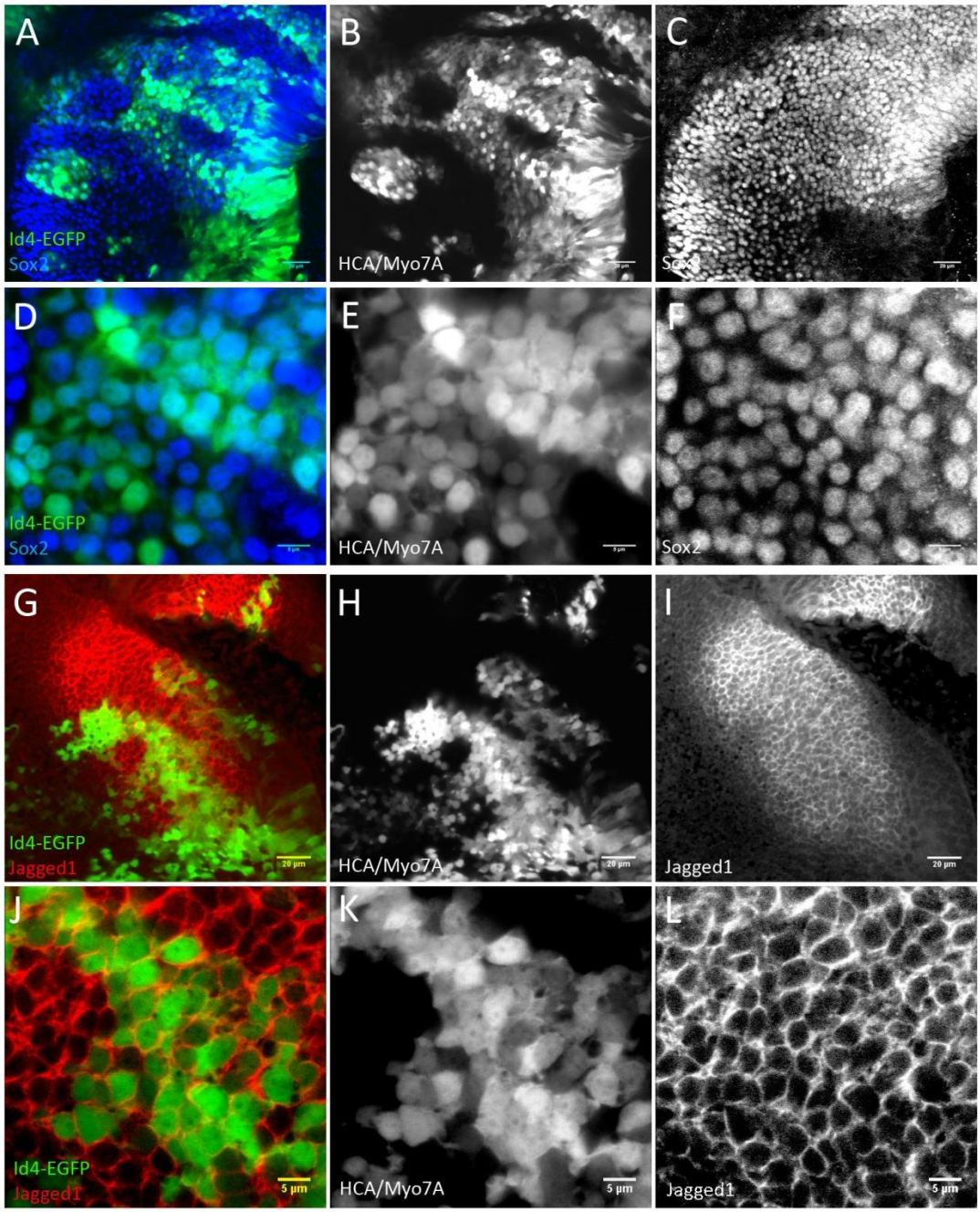


Figure 3-7: Overexpression of Id4 has no effect on supporting cell formation. To check for a potential effect of Id4 on supporting cell formation, Id4-electroporated inner ears were immunostained against the supporting cell markers Sox2 (A-F) and Jagged1 (G-L). No obvious change in the pattern of expression of these markers within supporting cells was found in any of the examined samples.

To get an idea about the efficiency of inhibition of hair cell formation by Id4 overexpression I performed quantitative analysis based on the number of differentiated hair cells in Id4-

electroporated versus GFP-electroporated embryos. The total numbers of counted and analysed cells are presented in figure 3-8. A region within the vestibular sensory patches was chosen and the total number of transfected cells was counted. Cells were then examined for the expression of hair cell markers individually to decide about their hair cell identity. The percentage of transfected cells that showed expression of hair cell markers was first calculated for each sample individually and then averaged across each of the two phenotypes (Id4/GFP). In case of GFP-electroporated control embryos a total number of 387 induced cells was analysed across 9 individual samples. The percentage of induced cells expressing hair cell markers ranged between 0 – 77.8%, thus resulting in an average of 33.96% of GFP-overexpressing cells that had differentiated into hair cells (Fig. 3-8 B, Fig. 3-9). In contrast, the average number of Id4-overexpressing cells that had differentiated into hair cells was much lower. As shown in figure 3-8 A, from a total of 567 induced cells analysed across 7 individual samples only an average of 4.26% had committed to a hair cell fate (Fig. 3-8 A, Fig. 3-9).

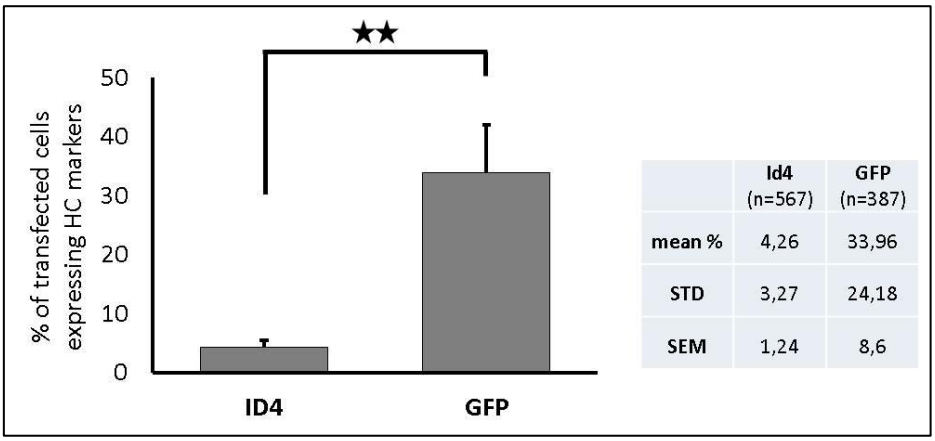
A

	Id4-1	Id4-2	Id4-3	Id4-4	Id4-5	Id4-6	Id4-7
# transfected cells	101	105	44	25	55	175	62
hair cells	2	1	3	2	4	0	3
%	1.98	0.95	6.8	8	7.3	0	4.8

B

	GFP-1	GFP-2	GFP-3	GFP-4	GFP-5	GFP-6	GFP-7	GFP-8	GFP-9
# transfected cells	9	8	12	23	62	51	71	45	106
hair cells	7	4	6	10	21	7	8	0	27
%	77.8	50	50	43.5	33.87	13.7	11.3	0	25.5

Figure 3-8: Quantitative analysis of hair cell formation in stage E7 vestibular sensory patches of Id4- (A) and GFP- (B) electroporated chicken embryos. The total number of electroporated and induced cells was determined by the presence or the absence of GFP fluorescence. Successfully induced cells were examined for the expression of hair cell markers (HCA/Myo7A) to decide about their hair cell identity. The percentage of induced cells that had differentiated into hair cells was calculated for each sample individually and then averaged in a next step (Fig. 3-9). In case of Id4-electroporated embryos a total of 567 transfected cells have been counted across 7 samples. The percentage of cells showing expression of hair cell markers ranged between 0 – 8 % (A). As for control embryos, 387 cells were scored across 9 samples, and the percentage of differentiated hair cells within these cells ranged from 0 – 77.8% (B).



	Id4 (n=567)	GFP (n=387)
mean %	4,26	33,96
STD	3,27	24,18
SEM	1,24	8,6

Figure 3-9: Id4 overexpression inhibits hair cell formation. In average, 4.26% of cells electroporated with the Id4 overexpression construct had differentiated into hair cells as indicated by the expression of hair cell markers. The number of differentiated hair cells in the GFP control embryos in contrast was found to be significantly higher with a mean of 33.96%. N = total number of cells analysed per condition. STD = Standard deviation. SEM = Standard error of the mean. Statistical significance was confirmed by two-tailed Student's t-test (unequal variance, p=0.00606986).

Taken together, both qualitative and quantitative analysis of the data provides strong evidence that Id4 is sufficient to inhibit the formation of vestibular hair cells. Control experiments using overexpression of GFP show that the effect is specific to Id4 and is not generally caused by the introduction of foreign DNA via electroporation or by Doxycycline treatment. The observation that Id4 overexpression is affecting transfected cells but not any of the surrounding cells suggests that Id4 interferes with hair cell differentiation in a cell-autonomous manner. This observation is consistent with the presented working model in which Id4 could prevent hair cell formation by counteracting the activity or expression of the pro-hair cell transcription factor Atoh1.

3.1.3 Id4 and Atoh1: expression of Atoh1 seems to be downregulated in cells overexpressing Id4 in the developing chicken inner ear

In previous experiments, induced overexpression of Id4 has been shown to inhibit hair cell formation in the developing chicken inner ear. As suggested by the working hypothesis, this inhibition could be caused by a direct or indirect inhibition of the pro-hair cell factor Atoh1 through Id4. To examine a putative effect of Id4 overexpression on endogenous Atoh1 expression, cryostat sections prepared from an Id4-electroporated chicken inner ear were immunostained against Atoh1 protein. The number and distribution of Atoh1-positive cells was then analysed with respect to cells overexpressing Id4. Results are shown in figure 3-10. Overexpression of Id4 is indicated by the presence of GFP. Cells positive for Atoh1 are depicted in the red channel. Three different regions within the successfully electroporated vestibular system were analysed, including the saccule and the utricle. In all three analysed areas cells with the strongest signal intensity for Atoh1 seemed to be cells in which no Id4 overexpression was induced (Fig. 3-10 A–I arrows). Some cells were found that were both positive for Atoh1 and overexpressing Id4, however the antibody signal intensity and thus the levels of Atoh1 protein seemed to be reduced in these cells when compared to

unelectroporated cells. Moreover, Atoh1-positive cells were not found in regions where Id4-overexpression was more widespread.

Taken together, this data might suggest that Id4 is indeed able to inhibit *Atoh1* expression, a finding that would be consistent with the hypothesis of Id4 inhibiting hair cell formation by inhibiting Atoh1. However, it is to note that the presented data is preliminary and based on a single experiment. More data will be required in order to draw conclusions about a putative interaction between Id4 and Atoh1.

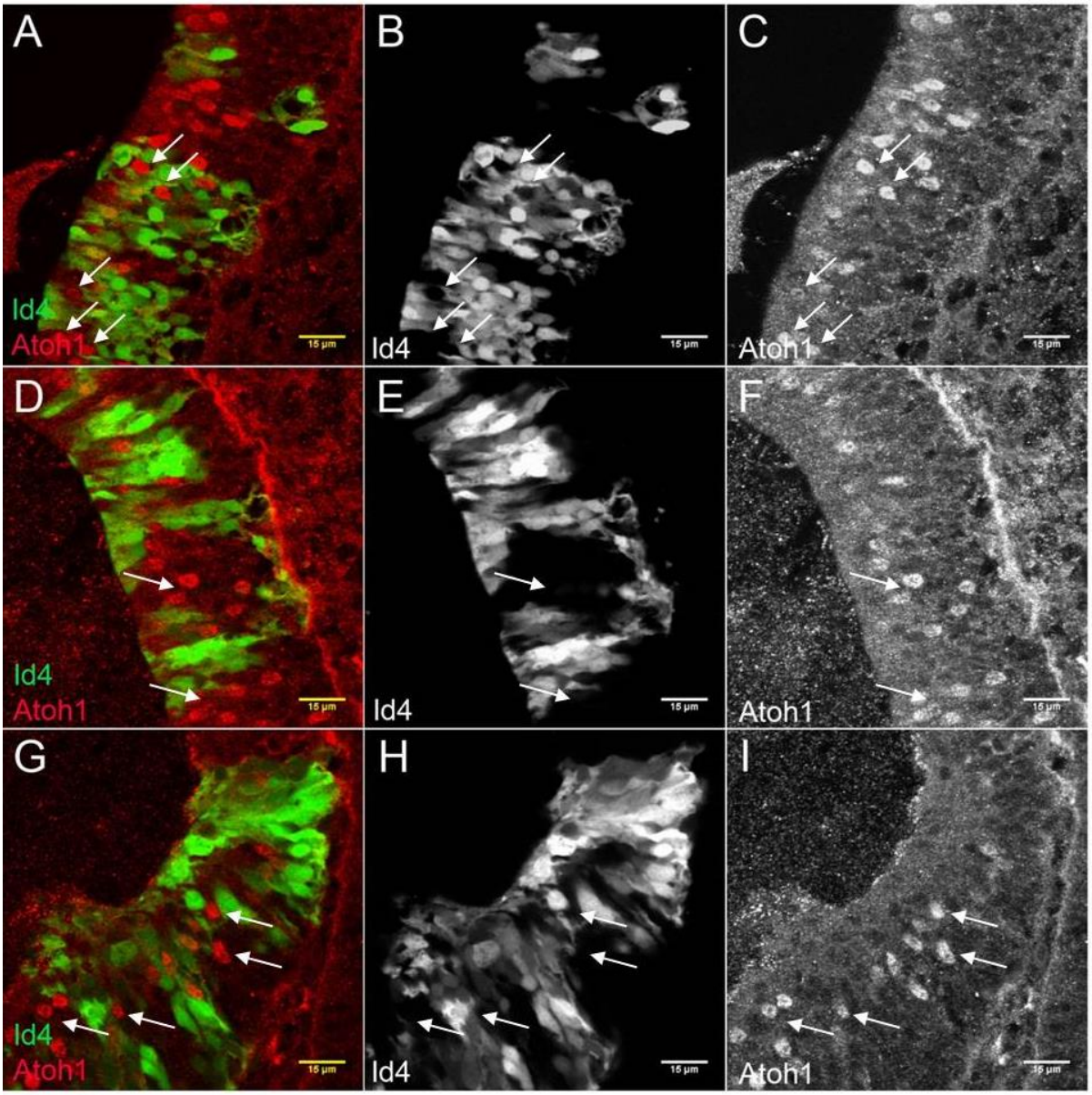


Figure 3-10: Id4 overexpression seems to lower levels and distribution of Atoh1 protein. Cryostat sections prepared from an Id4-electroporated chicken inner ear were immunostained against Atoh1 protein in order to examine a putative effect of Id4 overexpression on Atoh1 levels. Three different regions of the vestibular system were analysed (A–C, D–F, G–I). In all analysed regions, levels of Atoh1 seemed to be highest in cells not overexpressing Id4 (A–I arrows). Some cells were found to be both Atoh1-positive and overexpressing Id4, but antibody signal intensity seemed to be lower in these cells.

3.2 Expression and function of Id4 in the mammalian inner ear

3.2.1 Id4 is expressed in the mouse inner ear at embryonic and postnatal stages

To date, no data has been published concerning the spatial expression and putative function of Id4 within the mammalian inner ear at any developmental stage. In a study in 1996, Jen and colleagues reported traces of *Id4* expression in the epithelium of the otic vesicle of E11.5 – 16.5 mouse embryos, but no further details or images are available from this publication (Jen, Manova et al. 1996). However, in addition to that the importance of Id4 e.g. in neuronal differentiation during development has been studied intensively in the murine brain (Yun, Mantani et al. 2004, Bedford, Walker et al. 2005), and given the fact that expression has been found within the sensory epithelia of the developing chicken inner ear it seems conceivable that Id4 is also playing a role in the development of the mammalian inner ear. To determine whether Id4 is expressed in the mouse inner ear I used two different approaches. To study endogenous sites of expression, I used an *Id4-lacZ* knock-in mouse model which was originally generated by Fred Sablitzky and colleagues. In this particular model the 3' end of the *Id4* gene, including the region encoding the HLH domain required for protein dimerization, has been replaced with a *LacZ/neomycin* cassette (Bedford, Walker et al. 2005). This leads to the expression of a fusion protein consisting of the N-terminal 65 amino acids of Id4 and beta galactosidase, and endogenous expression of Id4 can be visualized by lacZ staining. To additionally label the Id4 protein I performed immunohistochemical experiments using an anti-Id4 antibody. Due to a lack of transgenic animals at early embryonic stages, *Id4* expression at E13 and E14 was analysed by the means of immunohistochemistry only. Perinatal (P0) and adult (P32) stages were examined by both immunohistochemistry and lacZ staining. Results are depicted in figure 3-11 – 3-16.

To get an initial idea about the specificity and signal-to-noise ratio of the Id4 antibody test stainings were performed on E13 brain tissue (Fig. 3-11). *Id4* expression within the murine

brain has been reported in previous studies (Riechmann, van Cruchten et al. 1994, Riechmann and Sablitzky 1995) and did thus serve as a positive control. In addition, a no primary antibody control was included to factor out any unspecific binding of the used secondary antibodies. Different concentrations between 1:100 and 1:1,600 were tested for the anti-Id4 primary antibody. Secondary antibodies were used at 1:1,000 in all cases. The neural tube was labelled with the sensory and neural marker Sox2 as a reference. As seen in figure 3-11 C, a strong signal for Id4 was obtained in the developing brain in close colocalization with Sox2 (Fig. 3-11 A-C), and no specific staining was seen in the no primary antibody control (Fig. 3-11 D-F). Based on the staining results from the different anti-Id4 primary antibody concentrations a final working dilution of 1:200 was chosen for subsequent experiments.

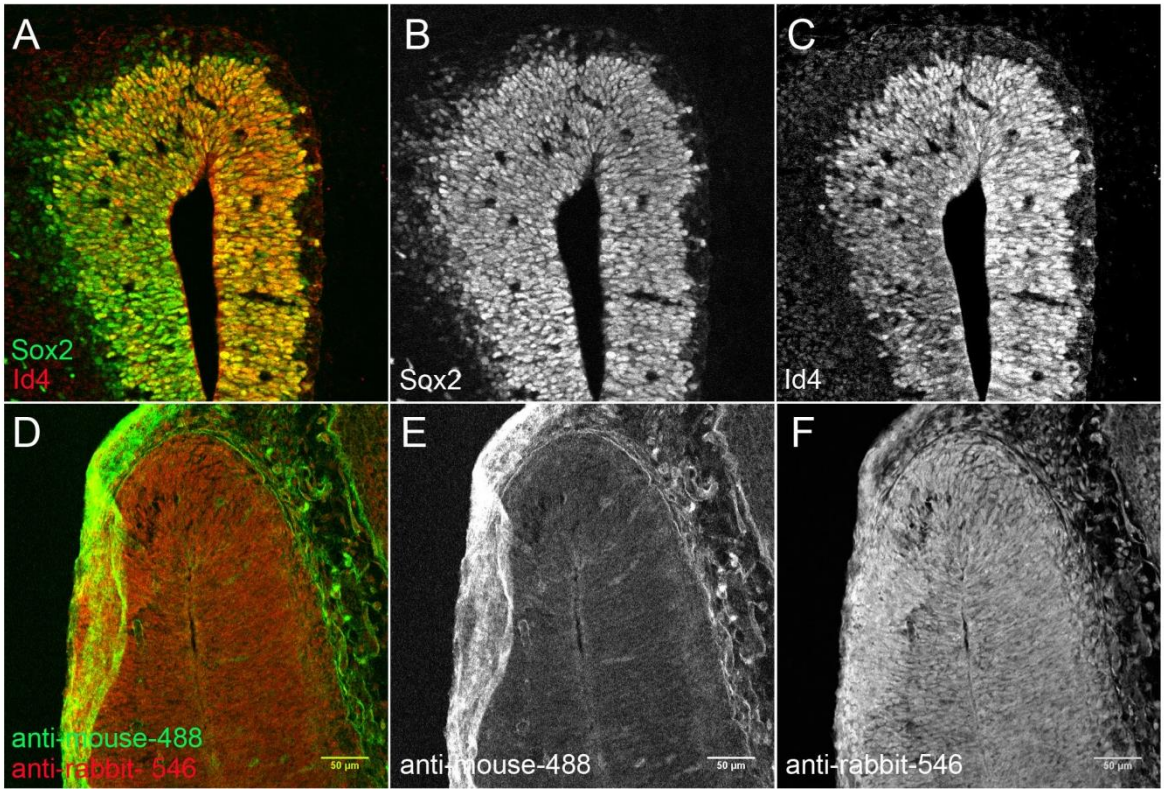


Figure 3-11: Id4 antibody test and no primary control staining on E13 mouse brain tissue. (A–C) The anti-Id4 antibody was tested on brain tissue from stage E13 mice in order to get an idea about antibody specificity and signal intensity as well as to determine the optimal antibody working dilution for subsequent experiments. The neural tube was visualized by staining against the neural marker Sox2. A strong and specific signal for Id4 (1:200) was obtained in close colocalization with Sox2. (D–F) No primary antibody control. Secondary antibodies were tested in the absence of any primary antibody. Some background signal was detected for both secondary antibodies used, but the staining was negligible and unspecific when compared to the positive control.

Figure 3-12 and 3-13 show cryostat sections through the embryonic mouse inner ear at stage E13 and E14, respectively. These stages have been chosen as the earliest stages as they represent the time point around the onset of hair cell formation. For both stages, the collected cryostat sections contained several turns of the cochlea as well as various regions of the vestibular system.

At E13, no signal from the Id4 antibody was detected in the sensory epithelium of the cochlea (Fig. 3-12 C-E). However, as shown in figure 3-12 F and H, expression seemed to be coming up within at least the cristae of the vestibular apparatus (Fig. 3-12 F, H arrows).

The sensory identity of the domain of expression was confirmed by the presence of Sox2 in this region (Fig. 3-12 F, G). The fact that Id4 protein was found in the vestibular system, but not in in the cochlea in colocalization with sensory markers might indicate that expression begins in the inner ear sensory epithelia at this particular stage. Surprisingly, Id4 protein was also detected in a region of the cochlea outside of the sensory domain (Fig. 3-12 I–K arrowheads). 24 hours later, at stage E14, the observed results were similar. As shown in figure 3-13 A–D, no Id4 protein was detected in the sensory epithelium of the cochlea, and low expression was detected in cells within the cristae of the vestibular system (Fig. 3-13 E-H arrows). Sensory regions are again marked by the presence of Sox2. As already seen at stage E13, Id4 protein was further detected within the cochlea but outside of the sensory domain at this stage (Fig. 3-13 A-D arrows).

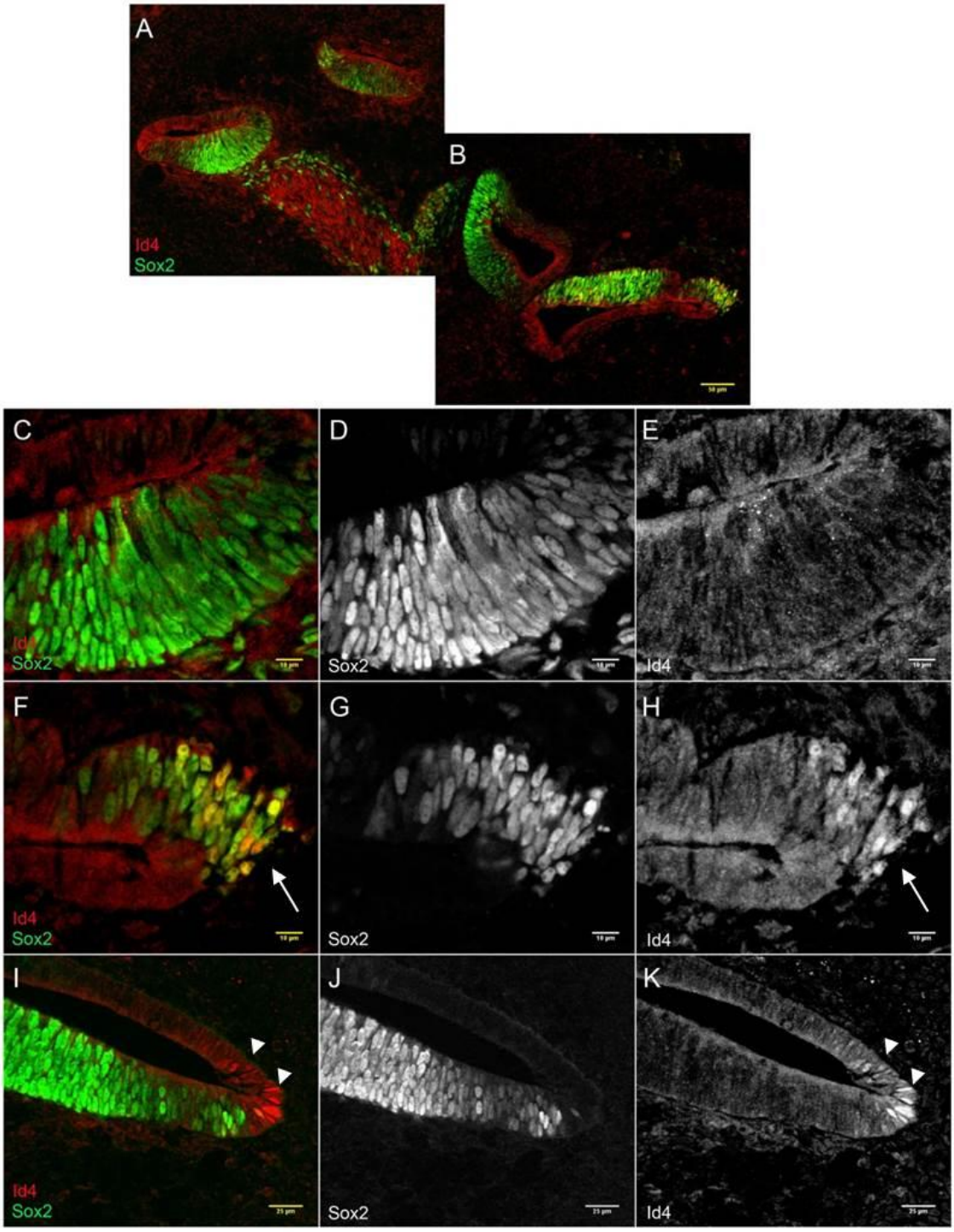


Figure 3-12: Expression of Id4 in the auditory and vestibular system of a stage E13 mouse inner ear. *Id4* expression was analysed by immunohistochemistry against the Id4 protein. Sensory domains were visualized by immunostaining against the sensory marker protein Sox2. (C–E) No expression of Id4 was detected within the sensory epithelium of the cochlea. However, low levels of expression were found in some regions of the vestibular system in colocalization with Sox2 (F–H arrows) suggesting that Id4 might start to be expressed in these epithelia in the first place. Unexpectedly, expression of Id4 was also detected in regions of the cochlea that were outside of the sensory domain (I–K arrowheads).

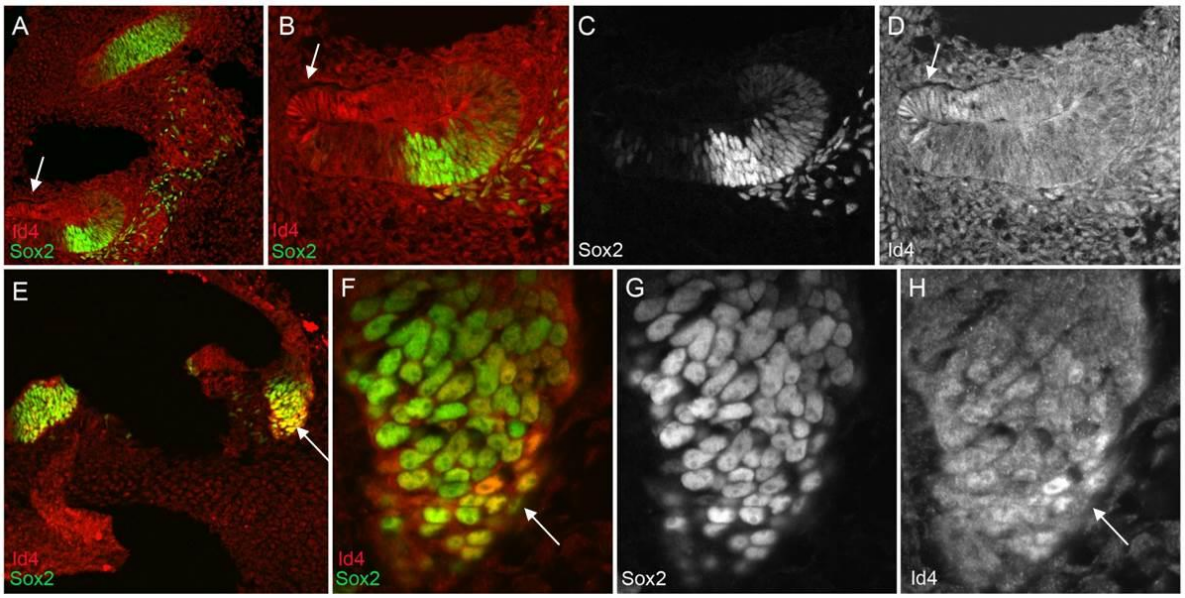


Figure 3-13: Analysis of *Id4* expression in the stage E14 mouse inner ear by immunohistochemistry. Id4 protein was labelled using an anti-Id4 antibody. Sensory regions were visualized by immunostaining against the sensory marker protein Sox2. At this stage, the expression pattern of Id4 was similar compared to earlier stage cryostat sections. (A–D) No expression of Id4 was detected within the sensory domain of the cochlea. However, Id4 protein was found outside of the sensory epithelium in a region similar to that seen at E13 (A, B, D arrows). Within the vestibular system, low expression of Id4 was detected in the sensory epithelia as confirmed by spatial colocalization with Sox2 (E–H arrows).

Taken together, looking at early stages of mouse inner ear development confirmed the presence of Id4 protein in the vestibular system at a time point relevant for the onset of hair cell formation. Expression was found in colocalization with Sox2, confirming sensory identity of the tissue and suggesting a possible involvement of Id4 in cell fate choices in this region. However, no sign of Id4 protein was detected in colocalization with Sox2 within the cochlea at this stage. In contrast, it was found outside of the sensory domain. This finding was surprising and not consistent with the model of Id4 controlling hair cell formation. Interestingly, this finding is also contrary to the data obtained from gene expression studies in chicken embryos where Id4 is not only expressed but even proceeds hair cell formation in the sensory epithelia during early developmental stages. Altogether, these data raise new questions about the actual role of Id4 during early stages of hair cell formation. It might

indicate that there are differences between its timing or role within the auditory and the vestibular system, and there seem to be differences between mammals and avian species as well.

To take into consideration *Id4* expression at stages when hair cell production is completed, I analysed the expression of Id4 at perinatal and adult stages by the means of immunohistochemistry and lacZ staining. Fixed and dissected P0 inner ears of heterozygous *Id4-lacZ* mice were processed for lacZ staining and imaged as whole mounts before being cut and imaged as cryostat sections. Corresponding sections from WT littermates were used for immunostaining to label different cell types and thus to identify putative sensory regions in the domains of *Id4* expression. Summarized results from lacZ staining and Id4 immunostaining experiments are presented in figure 3-14 and 3-15. As indicated by the blue lacZ stain in the whole mount preparation in figure 3-14 A-C, *Id4* expression in the inner ear seems to be specific and to some extent exclusive to the sensory epithelia of the cochlea, the saccule, the utricle, and the cristae of the vestibule (Fig. 3-14 A-C arrows). In addition, lacZ signal was detected in both the auditory and the vestibular nerve (Fig. 3-14 B, C, F, I arrowheads). Cryostat sections from lacZ stained ears were further examined and compared with WT sections that were immunostained for different cell type markers. Sox2 and Myosin7A were used to label sensory domains and supporting cells or hair cells, respectively. As shown in figure 3-14 D-H, Id4 is clearly expressed within the sensory domain of the cochlea. Consistent with the observations made at early embryonic stages, Id4 was further found within the cochlea but outside of the sensory domain (Fig. 3-14 G arrow). Figure 3-14 I–O shows the results for the lacZ staining within the vestibular system. A strong and highly specific signal for *Id4* expression was found in the cristae (Fig. 3-14 I–K) as well as in the saccule and the utricle (Fig. 3-14 L arrows). Immunostaining on corresponding WT cryostat sections confirmed the sensory identity of the domains of *Id4* expression in the crista (Fig. 3-14 M) and the saccule (Fig. 3-14 N, O). Other than in the cochlea, Id4 seemed to be

enriched in the hair cell layer within these regions (Fig. 3-14 J, M arrows). In addition to the lacZ staining, *Id4* expression at P0 was further examined by immunohistochemistry on WT cryostat sections. Results are shown in figure 3-15. In the cochlea, a weak signal from the Id4 antibody was detected in the nucleus of both hair cells and supporting cells (Fig. 3-15 A–C arrowheads, D - F). Consistent with previous findings, Id4 protein was further found in a region outside of the sensory epithelium (Fig. 3-15 D–F arrows). Within the vestibular system, presence of Id4 protein was indicated by antibody staining within the sensory regions (as shown for saccule, Fig. 3-15 G–J, K–M arrows). Based on the detected antibody staining, Id4 seems to be enriched in the supporting cell layer (Fig. 3-15 I, J, L, M arrowheads), a finding that is contrary and inconsistent with the corresponding results from the lacZ staining.

Taken together, data from the lacZ staining experiments and Id4 immunohistochemistry provided evidence that Id4 is expressed in both the auditory and vestibular epithelia of the P0 mouse inner ear. However, contrary results were obtained regarding the distribution of Id4 within hair cells and supporting cells. Assuming the lacZ signal represents the actual site of endogenous *Id4* expression one might thus question the specificity and reliability of the used anti-Id4 antibody. To rule out the presence of a false or unspecific signal, a control staining was performed on tissue from a homozygous P0 *Id4-lacZ* mouse. Results are shown in figure 3-16. No specific nuclear signal was detected in the cochlea and the vestibular system. A few bright speckles were detected next to the hair cells in the cochlea (Fig. 3-16 C, arrows) and in a region close to the hair cells' stereocilia within the vestibular system (Fig. 3-16 F, arrows). However, the exact cause for this punctuate staining is not clear at the moment. It might be artefactual or indicating the presence and binding of the Id4 antibody to a non-functional Id4-lacZ fusion protein.

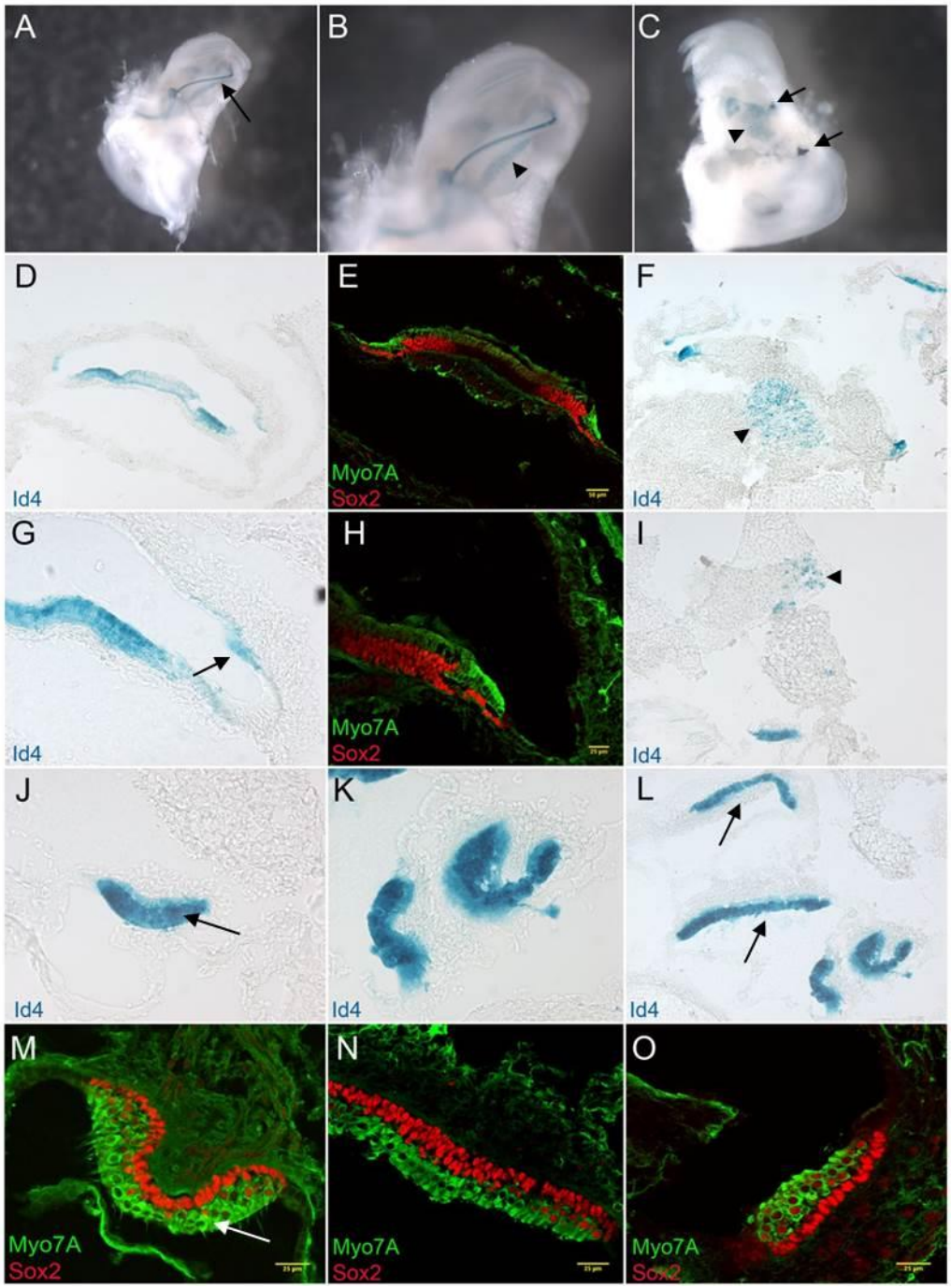


Figure 3-14: Analysis of *Id4* expression by lacZ staining on P0 whole mount inner ears and cryostat sections. Inner ears of heterozygous *Id4-lacZ* mice were dissected, fixed and processed for whole mount lacZ staining and cryosectioning. To bring the sites of *Id4* expression into context with inner ear sensory regions, corresponding sections from WT littermates were used for immunohistochemistry against Myosin7A and Sox2. (A–C) Examination of a whole mount inner ear after lacZ staining revealed specific expression of Id4 in the cochlea and the vestibular system, as well as in the auditory and vestibular nerve (B, C, F, I arrows, arrowheads). Expression in these epithelia was confirmed when looking at cryostat sections from the same sample. Comparing cryostat sections from the lacZ stained ear with corresponding WT sections immunolabelled for Myosin7A and Sox2 further confirmed that Id4 is expressed within the sensory epithelia. Surprisingly, within the cochlea the

detected lacZ signal was strongest in supporting cells (D–H), while *Id4* expression seemed to be enriched in the hair cell layer within the vestibular system (J–O, J, M arrows). In addition to the sensory epithelia, a lacZ signal indicating *Id4* expression was also detected outside the sensory region of the cochlea (G arrow).

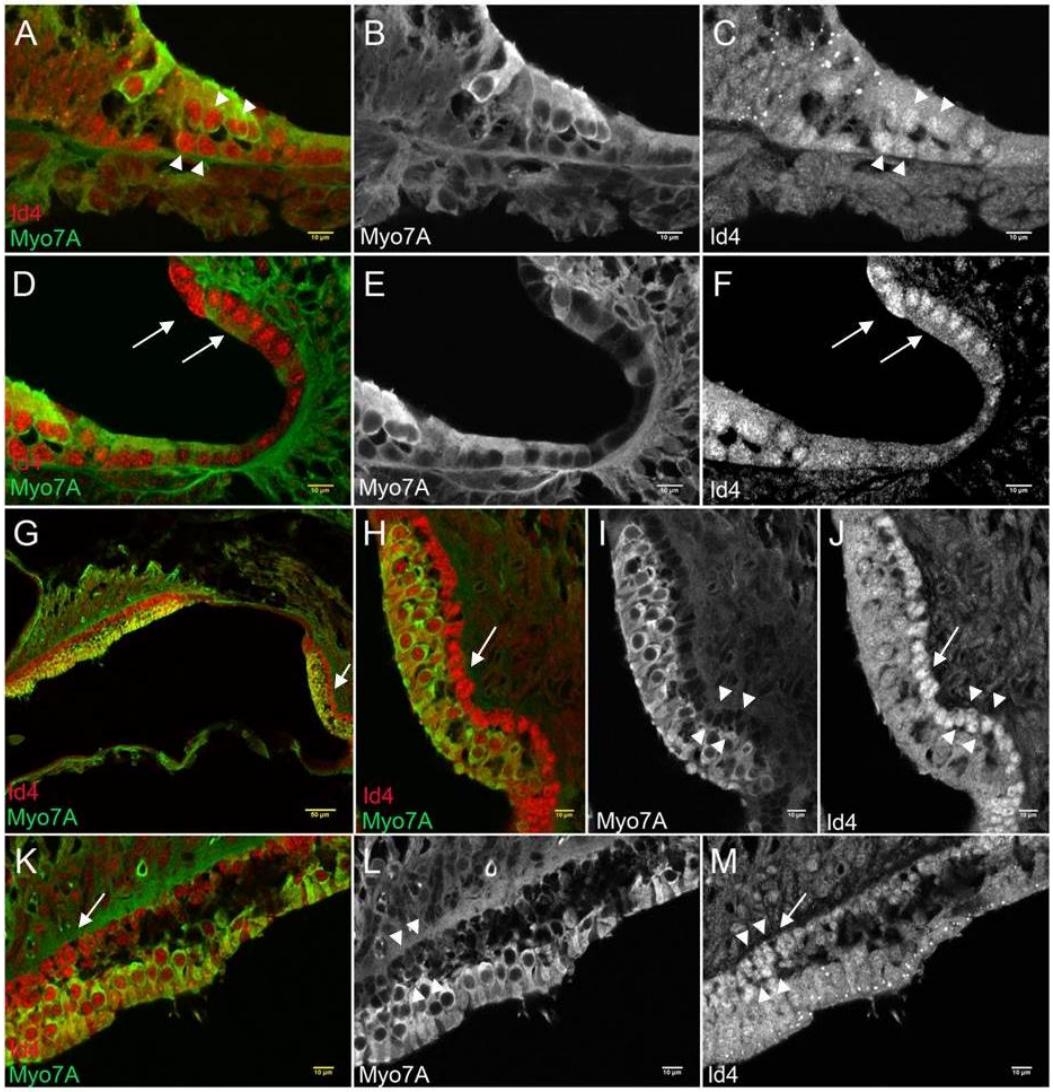


Figure 3-15: Id4 immunostaining on P0 cryostat sections. Immunohistochemistry was performed on cryostat sections from WT littermates of the *Id4-lacZ* line. Id4 protein was detected using the tested anti-Id4 antibody. Sensory epithelia and hair cells were visualized by immunostaining against Myosin7A. Id4 protein was detected within the cochlea in both the hair cell layer and the supporting cell layer (A–C arrowheads). Consistent with the results from the lacZ staining, Id4 was further found outside the sensory region of the cochlea (D–F arrows). In the vestibular system, Id4 protein was found within the sensory epithelium. However, contrary to the site of *Id4* expression revealed by lacZ staining, Id4 protein seemed to be accumulated in the supporting cell layer rather than the hair cell layer in these epithelia (H–M arrowheads).

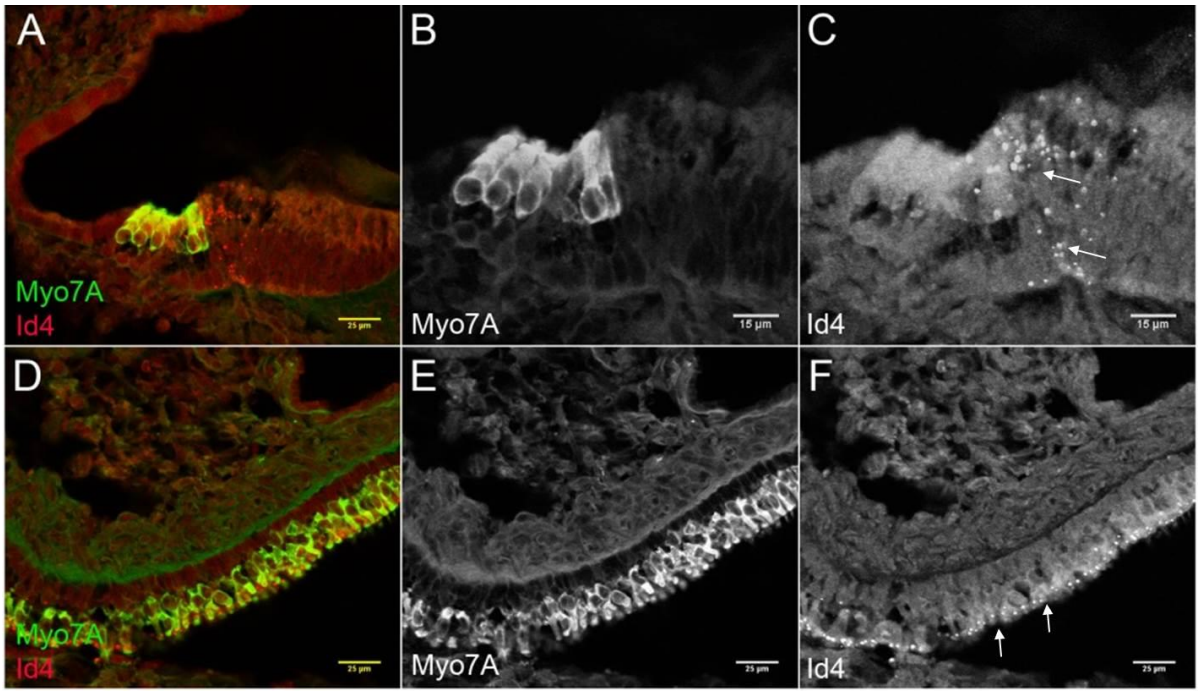


Figure 3-16: Id4 control immunostaining using tissue of a homozygous P0 *Id4-lacZ* mouse. No nuclear signal from the Id4 antibody was found in the cochlea (A–C) and the vestibular system (D–E). However, a punctuate staining was detected in both sensory epithelia which might be artefactual or caused by an Id4-lacZ fusion protein (C, F arrows).

To get an idea about a putative role played by Id4 e.g. in the maintenance of the mature inner ear sensory epithelia, *Id4* expression was examined at stage P32 by the means of both lacZ staining and Id4 immunohistochemistry. Tissue from a heterozygous mouse of the *Id4-lacZ* in-house colony was used for whole mount lacZ staining and was then processed for cryosectioning and immunohistochemistry. Results are depicted in figure 3-17. As seen from the whole mount lacZ stain, Id4 is exclusively expressed in the sensory epithelium along the cochlea, as well as in the sensory epithelia of the saccule, the utricle, and the three cristae of the vestibular system (Fig. 3-17 A arrows). Figure 3-17 B and C show a picture from a cryostat section of the lacZ stained cochlea in combination with immunostaining against the hair cell marker Myosin7A. Figure 3-17 D–F show roughly the same region on a cryostat section from an ear not used for lacZ staining but immunostained against Id4 and the hair cell marker Myosin7A. Looking at the lacZ signal, only very low *Id4* expression was detected

within the hair cells in this region (Fig 3-17 B arrow). However, a fairly strong signal was detected in a structure underneath which most likely refers to the auditory nerve (Fig. 3-17 B, C arrowheads). So far, this is consistent with observations made at earlier stages. The presence of Id4 within the auditory nerve was confirmed by Id4 immunostaining on separate sections (Fig. 3-17 D–F arrowheads). Furthermore, immunostaining for Id4 suggested a relatively strong expression in at least some of the hair cells within the cochlea (Fig. 3-17 D–F arrows). In the vestibular system, a strong signal for Id4 was obtained by lacZ staining in perfect colocalization with the hair cell marker Myosin7A (Fig. 3-17 G–J). Expression of Id4 within both the hair cell and the supporting cell layer of the vestibular system was confirmed by Id4 immunostaining (Fig. 3-17 K–M).

Taken together, combined analysis by immunohistochemistry and lacZ staining revealed that Id4 is expressed in all sensory epithelia of the adult mouse inner ear. While the obtained data were consistent for both methods when looking at the vestibular system, the same was not the case within the auditory apparatus. However, it seems likely that Id4 is expressed at least to a certain amount in the hair cells of the cochlea at this stage.

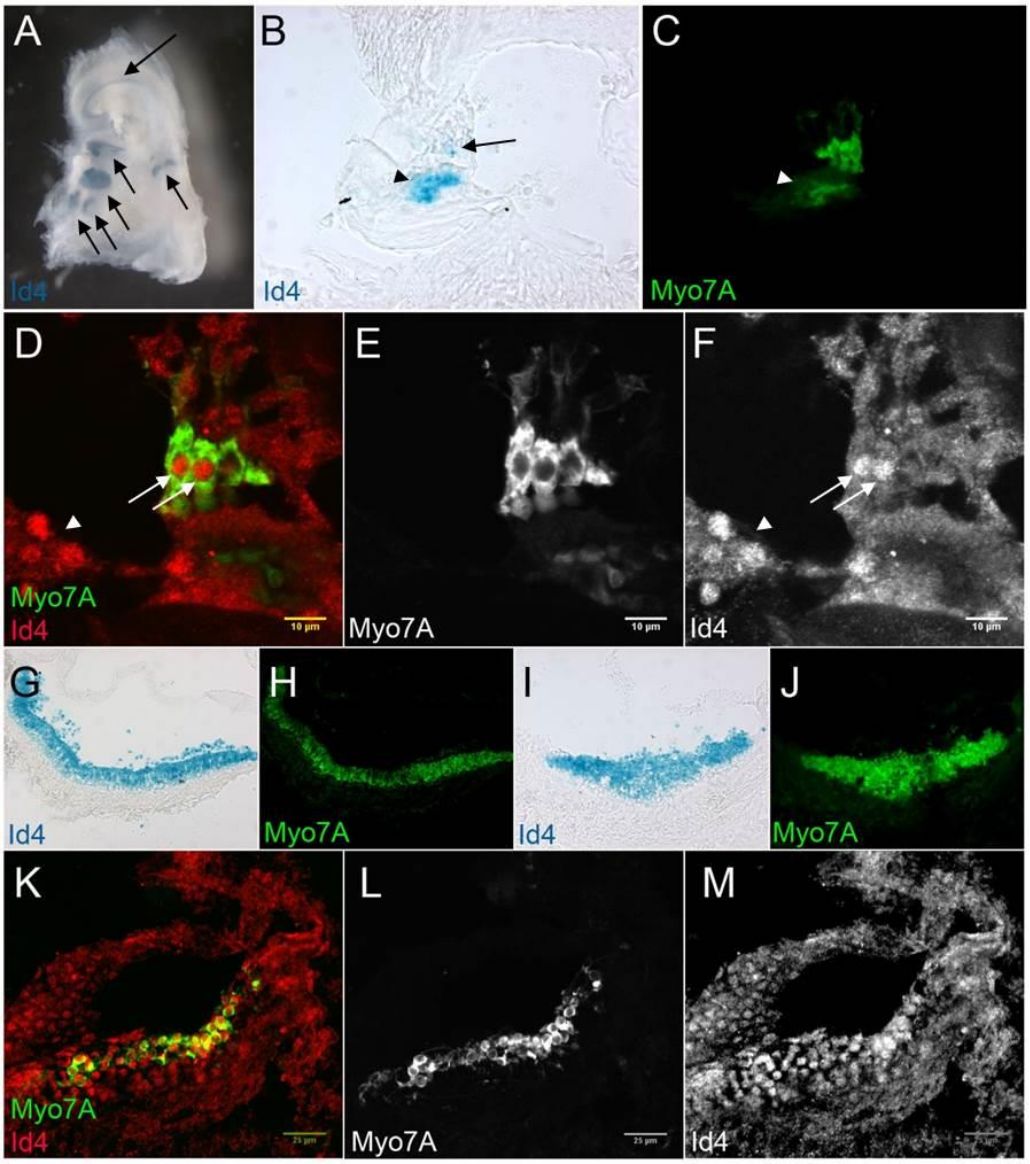


Figure 3-17: *Id4* expression in the adult mouse inner ear (P32). *Id4* expression was examined by lacZ staining and immunohistochemistry using an anti-Id4 antibody on tissue of heterozygous mice of the *Id4-lacZ* line. Sensory regions and hair cells were visualized by immunostaining against the hair cell marker Myosin7A in all cases. (A) Examination of a whole mount lacZ stained inner ear revealed highly specific and exclusive expression of Id4 in all sensory epithelia of the auditory and the vestibular system (arrowheads). Looking at prepared cryostat sections from the same sample further revealed expression in a region underneath the sensory epithelium probably referring to the auditory nerve (B, C, D, F arrowhead). A very low signal was detected within the hair cells (B arrow, C). LacZ staining in the vestibular system revealed strong expression of Id4 within the sensory epithelia. Colocalization with the hair cell marker Myosin7A further revealed that Id4 is expressed within the hair cell layer in these epithelia (G–J). Results from Id4 immunohistochemistry were similar in the vestibular system, with a signal for Id4 colocalizing with Myosin7A (K–M). Within the cochlea, a fairly strong signal from the Id4 antibody was detected within at least some of the hair cells in the epithelium.

3.2.2 Id4 loss-of-function: deletion of Id4 has no effect on hair cell formation at peri- and postnatal natal stages in the murine cochlea

Even though no sign of *Id4* expression was detected within the sensory epithelia of the cochlea at early embryonic stages of development it was found in both hair cells and supporting cells of perinatal and adult mice. To get an idea about whether deletion of the *Id4* gene has any lasting effect on hair cell formation within the organ of Corti, I looked at the overall pattern of hair cells in the inner ear of homozygous Id4-lacZ ($Id4^{lacZ/lacZ}$) mice at stages P0 and P14. Scanning electron microscope (SEM) images are shown in figure 3-18. At both stages, no obvious difference was found in the hair cell pattern of the *Id4*-depleted mice when compared to WT animals ($Id4^{+/+}$) at similar stages. One row of outer hair cells and three rows of inner hair cells had formed in all cases (Fig. 3-18 A – D arrows) and the overall look of hair cells appeared normal. Note that the differential length of stereocilia in $Id4^{+/+}$ and $Id4^{lacZ/lacZ}$ mice at P14 is due to different locations on the cochlea regarding the base (Fig. 3-18 C) and apex (Fig. 3-18 D).

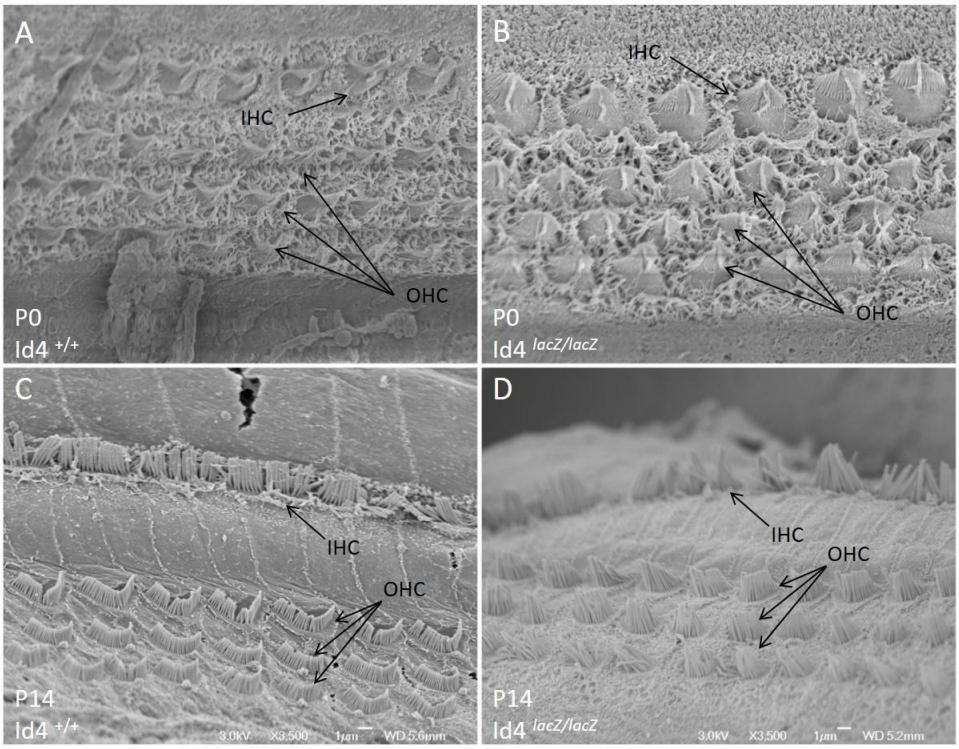


Figure 3-18: Hair cell pattern in P0 and P14 *Id4*-depleted (Id4$^{lacZ/lacZ}$) and WT (Id4$^{+/+}$) mice. No obvious difference in the pattern of hair cell formation was found in Id4$^{lacZ/lacZ}$ mice when compared to WT animals at similar stages. One row of outer hair cells and three rows of inner hair cells had formed in all cases (arrows).

3.3 In silico analysis of the Id4 promoter region

So far, I have shown that Id4 is expressed within the chicken and the mouse inner ear sensory patches at stages crucial for hair cell formation, and that overexpression of Id4 is able to inhibit hair cell differentiation in a cell-autonomous manner. As described in the working hypothesis, Id4 might exert its inhibitory action downstream of the Notch signalling pathway. In this context, the *Id4* promoter region is of particular interest as it represents the site of putative Notch-responsive element. An *in silico* analysis of the *Id4* promoter region of different vertebrate species was performed to learn more about the conservation and possible regulation of Id4.

I first looked at the coding region of the human, mouse, rat, zebra finch, and *Xenopus Id4* gene to check the level of evolutionary conservation across this region in different vertebrate

species. To identify conserved regions, a multispecies alignment was performed using the MLAGAN alignment program of the mVISTA Comparative Genomics software (Dubchak, Brudno et al. 2000, Frazer, Pachter et al. 2004). All gene sequences were extracted from the Ensembl genome database using the FASTA format. Coding region sequences included all exons and interjacent introns of the corresponding species. Results are depicted in figure 3-19. The human *Id4* coding region of 3,299 bp was chosen as a reference sequences and is represented on the x-axis. Regions with sequence alignment of 70% and more were considered conserved and are indicated by the salmon-coloured areas. Evolutionary conservation was found in several areas which mostly refer to the locations of exon 1, 2, and 3 of the human *Id4* gene (Fig. 3-19 black brackets). However, some conservation was also found within intronic regions. Overall, the levels of conservation seemed to be highest within the mammalian group of human, mouse, and rat (Fig. 3-19 red brackets, red arrows).

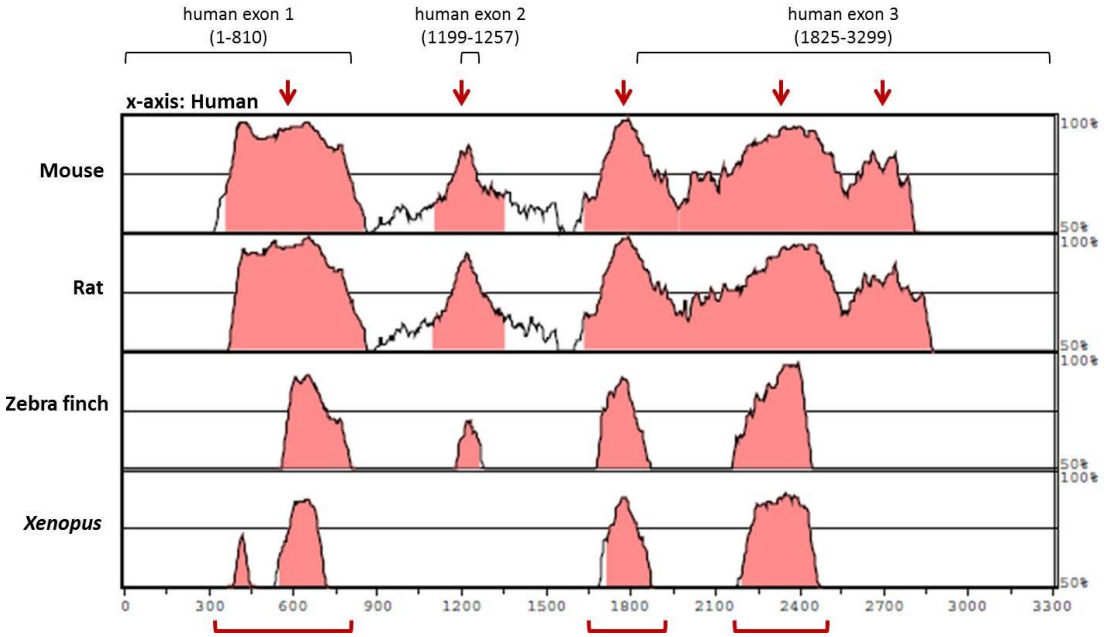


Figure 3-19: Multispecies alignment of the *Id4* coding region. The coding region of the mouse, rat, zebra finch and *Xenopus Id4* gene including all exons and interjacent introns were aligned against a 3,299 bp reference sequence representing the human *Id4* coding region. Evolutionary conservation was found within the exonic and intronic region of the reference sequence (red brackets lower end). Particular high levels of conservation were found within the mammalian group of human, mouse, and rat (red arrows upper end).

In a next step, the conservation of the *Id4* promoter sequence was compared between the human, mouse, rat, and zebra finch *Id4* gene. Due to a currently incomplete data set regarding the chicken *Id4* promoter region in the existing databases the chicken sequence was not included in this analysis. Figure 3-20 shows the alignment of a region located 1 kb upstream of the transcription start site (TSS) of the human, mouse, rat, and zebra finch *Id4* coding region using human as a reference sequence (x-axis). Evolutionary conserved regions were found across a long stretch of the examined region between human, rat, and mouse as indicated by the salmon-coloured areas. However, plotted conservation levels varied significantly between the mammalian group of human, mouse, and rat and human and zebra finch (Fig. 3-20 red brackets). Altogether, the existence of conservation of different sections of the analysed 1 kb portion of the *Id4* promoter region across mammalian species, but also between mammals and birds suggests that this region might be of particular relevance for the regulation of *Id4* gene expression.

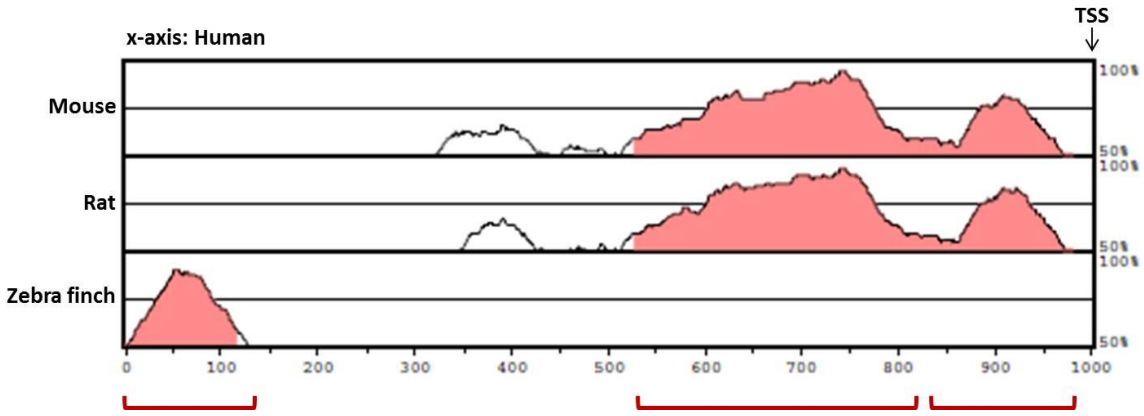


Figure 3-20: Different regions of the *Id4* promoter region are evolutionary conserved between human, mouse, and rat and human and zebra finch. Sequences of the region 1kb upstream of the transcription start site (TSS) from mouse, rat, and zebra finch were aligned against human using the mVISTA Comparative genomics software. Peaks indicating evolutionary conservation between the sequences were found along a wide stretch of the analysed region between human, mouse and rat. Levels of conservation were significantly lower and located to a more upstream region when comparing human and zebra finch, and conserved regions did not align with those plotted for the mammalian group (brackets).

To investigate the location of potential RBPjκ binding sites within the conserved region of the *Id4* promoter, a search for consensus transcription factor binding sites using different online tools was performed. An automated search was carried out by the Genomatix Gene2Promoter software (Genomatix Software GmbH) using the V$RBPF (vertebrate RBPjκ) matrix family. Two RBPjκ binding sites were predicted for the human *Id4* promoter within a region of 0.6kb upstream of the TSS while none was found in the mouse (data not shown). To double check this result, a second automated search was carried out with the mVISTA software in combination with the rVISTA tool (regulatory Vista (Loots, Ovcharenko et al. 2002)) using the TRANSFAC matrix RBPjκ (TRANSFAC Professional, Biobase). The search was conducted for the human, rat and mouse *Id4* promoter region. Sequences of the mouse and rat promoter were aligned against human (represented on the x-axis). Results are depicted in figure 3-21. For a region of 1kb upstream of the mouse *Id4* TSS 10 RBPjκ binding sites were predicted in total, three of which were considered conserved (Fig. 3-21 upper graph, blue and green marks). Likewise, of the 10 binding sites predicted for the rat *Id4* promoter the corresponding three binding sites were marked as conserved (Fig. 3-21 lower graph, blue and green marks).

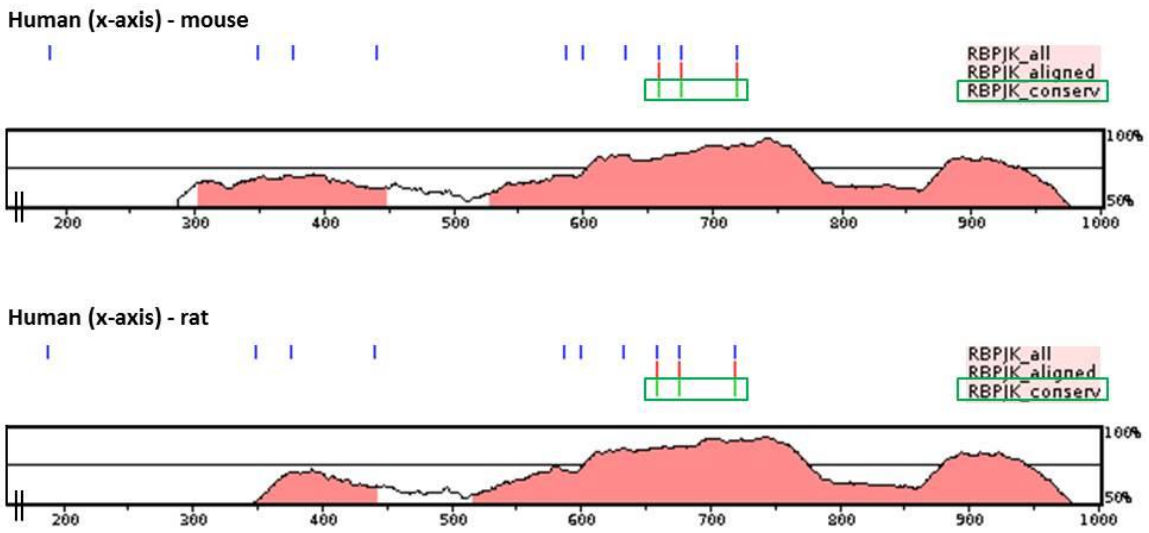


Figure 3-21: RBPjκ binding sites in the *Id4* promoter region. An automated search for consensus transcription factor binding sites within the *Id4* promoter region of human, mouse, and rat was carried out using the rVISTA (regulatory Vista) tool of the mVISTA software. The search was conducted for the TRANSFAC matrix RBPjκ, which corresponds to the CSL binding sites that are required for gene regulation exerted by the Notch signalling pathway. Within a region of 1kb upstream of the transcription site three RBPjκ binding sites were identified to be conserved between mouse and human (A and B green marks).

Considering the different results obtained by the different automated online search tools, an additional manual search for RBPjκ binding sites was performed using the ApE (A plasmid Editor) software (M. Wayne Davis). The search was conducted using two commonly known CBS binding motifs, YGTGRGAA (Y denotes T on the upstream site or C on the downstream site, R denotes A or G) for high affinity binding sites and RTGRGAR (R denotes A or G) for low affinity binding sites (Nellesen, Lai et al. 1999, Ong, Cheng et al. 2006). Within the 1kb region upstream of the TSS four low affinity binding sites were found in the mouse and the rat *Id4* promoter while only two were present in human (data not shown). When the search was expanded to a region of 5kb upstream of the TSS, four additional low affinity binding sites were found in rat and seven in both human and mouse. No high affinity binding site was predicted for the examined conserved region.

In view of the fact that members of the Id protein family have been reported to be regulated by the BMP signalling pathway, I additionally screened the chosen 1 kb *Id4* promoter region for BMP-responsive elements using the mVISTA software. As previously described for the RBPjκ binding sites, mouse and rat were aligned against human and the search was conducted using the TRANSFAC matrices for Smad1 and Smad4. Results are depicted in figure 3-22. For the mouse *Id4* promoter region, ten binding sites for Smad1 and six binding sites for Smad4 were predicted within the conserved region (Fig. 3-22 upper graph, green marks). For the rat *Id4* promoter, eight binding sites within the conserved region were predicted for both Smad1 and Smad4 (Fig. 3-22 lower graph, green marks).

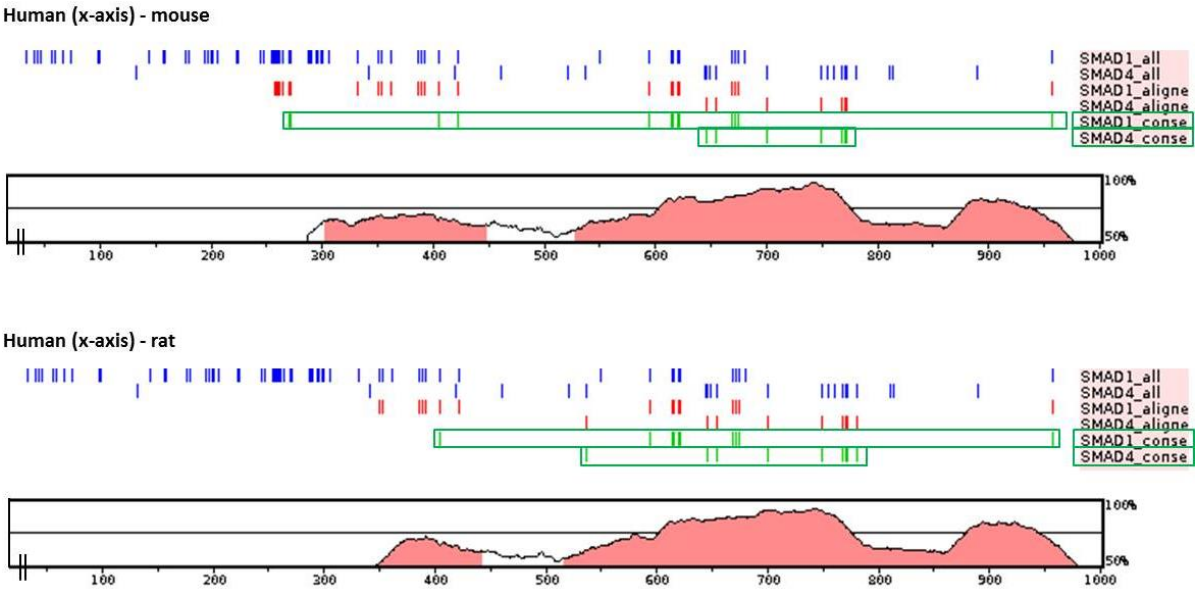


Figure 3-22: BMP-responsive binding sites in the *Id4* promoter region. A portion of 1 kb of the *Id4* promoter region was analysed for putative Smad1 and Smad4 binding sites. Sequences of mouse and rat were aligned against human, and predicted binding sites were analysed in the conserved region.

3.4 Id4 expression levels are affected by Notch signalling but not by the BMP pathway in vitro

The observed conservation of different portions of the *Id4* promoter region across different mammalian species as well as between human and zebra finch suggested a particular role of this region probably in terms of gene expression regulation. The presence of several CBS domains within this conserved region further supports the hypothesis that regulation of the *Id4* gene might at least partially be regulated by the Notch signalling pathway. To test this hypothesis, a set of luciferase assay experiments was performed using an Id4-luciferase reporter construct (pGL3-Id4-Luc). The construct used was kindly provided by Dr Qing Richard Lu (UC Department of Pediatrics, Cincinnati) and consisted of a ~3.8 kb portion of the rat *Id4* promoter cloned upstream of the Firefly luciferase gene in a basic pGL3 luciferase reporter vector (Promega, Cat.# E1751) (Chen, Wang et al. 2011). Orientation and identity of the rat *Id4* promoter insert was verified by sequencing, and the presence of eight CBS domains within this region was confirmed by manual analysis using the ApE software (data not shown). A vector map generated with the Graphic Map tool of the ApE software is depicted in figure 3-23.

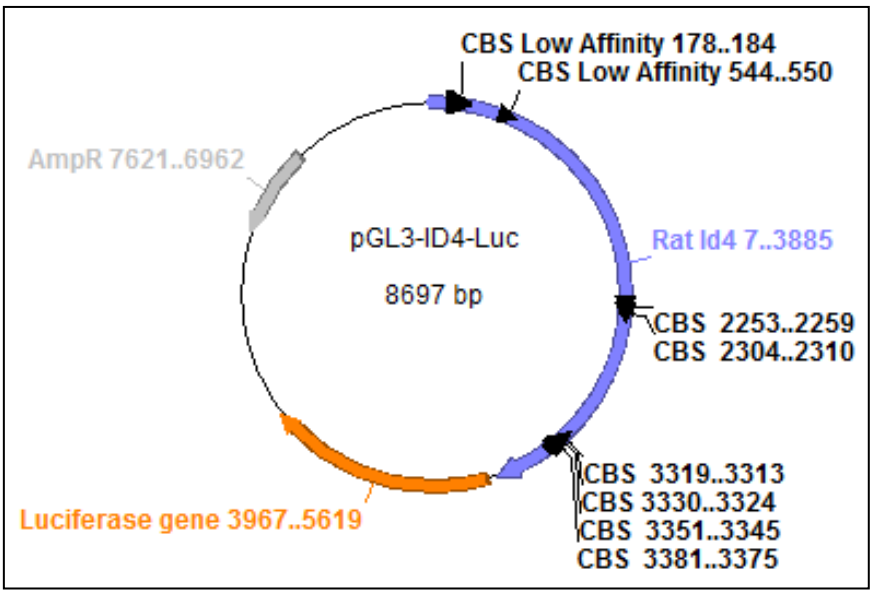


Figure 3-23: Vector map of pGL3-Id4-Luc. A ~3.8 kb portion of the rat *Id4* promoter containing eight CBS domains was cloned upstream of the Firefly luciferase gene in a basic pGL3 luciferase reporter vector (Promega, Cat.# E1751). Orientation and identity of the insert was verified by sequencing.

All luciferase experiments were carried out in an otic cell line (OC-2 cells, Rivolta, Grix et al. 1998). To test the responsiveness of the *Id4* promoter to the Notch signalling pathway, OC-2 cells transfected with the pGL3-Id4-Luc reporter construct were co-transfected with different amounts of a NICD expression construct (pCAB-HA-NICD-IRES-GFP) to mimic different levels of Notch activity. Cells were transfected with 400 ng, 800 ng, or 1200 ng of NICD and the total amount of DNA per transfection was equalized by adding appropriate amounts of the empty expression vector (pCAB-IRES-GFP). In addition to the luciferase reporter and the expression construct, cells were co-transfected with *Renilla* luciferase to monitor transfection efficiency and normalize levels of measured Firefly luciferase activity accordingly. Each experiment was carried out as a triplicate using the same DNA solution, and three independent sets of experiments were conducted using DNA from different plasmid preparations. An empty pGL3 luciferase reporter vector was used as an additional control to confirm that any effect of NICD on luciferase activity was due to the presence of the *Id4* promoter elements. Luciferase activity was measured in arbitrary luciferase units and is

displayed as Average Relative Luciferase (ARL). Summarized results are shown in figure 3-24 A. All values of measured luciferase activity from NICD overexpressing cells were normalized to baseline levels from cells transfected with the Id4-luciferase reporter, but not the NICD expression construct (Fig. 3-24 A NICD 0ng). The mean value of luciferase activity in these cells was normalized to 100 ARL units. The relatively high levels of baseline luciferase activity suggested a certain amount of endogenous *Id4* expression in OC-2 cells under normal conditions. Indeed, expression was confirmed by immunohistochemistry in independent experiments (data not shown). Luciferase activity increased when levels of Notch activity were increased by overexpression of NICD (Fig. 3-24 A Id4-luc). Cells transfected with 400 ng of NICD showed an average luciferase activity of 133.7 ARL, corresponding to a fold change of 1.34 compared to the untreated control. At 800 ng of NICD luciferase activity increased by a fold change of 1.57, and was highest in cells transfected with the maximum of 1200 ng of NICD with a fold change of 2.45 compared to the control. In contrast, cells transfected with the empty luciferase reporter construct did not show any increase in the amount of luciferase activity when levels of Notch were augmented (Fig. 3-24 A pGL3 basic). To examine the effect of a Notch downregulation on *Id4* expression levels in a next step, a similar set of experiments was conducted while treating the cells with different concentrations of the γ-secretase inhibitor LY-411575. However, transient blocking of the Notch signalling pathway did not result in a significant change in the levels of measured luciferase activity (Fig. 3-24 B). As in previous experiments, the mean value of luciferase activity from control cells treated with DMSO was normalized to 100 ARL units. Measured levels of luciferase activity were slightly lower in cells treated with 1 µM of the inhibitor with a fold change of 0.94, and similar results were seen when the concentration of the inhibitor was increased to 5 µM.

Taken together, these data suggest that the Notch signalling pathway is able to upregulate the expression of Id4 in a dose-dependent manner when artificially enhanced. Associated

control experiments have shown that this effect is specific to the *Id4* promoter. However, the fact that *Id4* expression levels are not altered when Notch signalling is transiently blocked might indicate an involvement of other regulative mechanisms in the control of *Id4* expression.

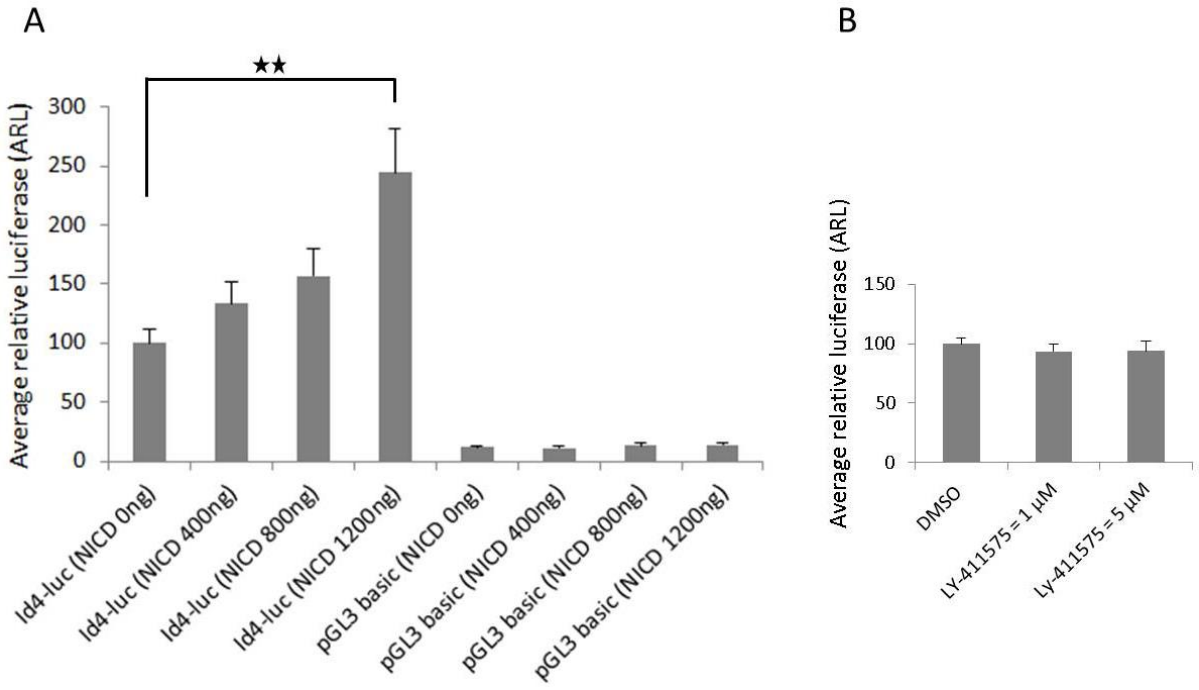


Figure 3-24: Luciferase activity driven by the *Id4* promoter in response to varying levels of Notch activity. (A) OC-2 cells transfected with an Id4-luciferase reporter (pGL3-Id4-Luc) were co-transfected with a NICD expression construct. Measured mean values are displayed in ARL units: NICD 0 ng=100 ARL, NICD 400 ng=133.7 ARL, NICD 800 ng=156.7 ARL, NICD 1200 ng=244.5 ARL. Statistical significance: p=0.0038806. (B) OC-2 cells transfected with the luciferase reporter construct were treated with different amounts of the Notch inhibitor LY-411575. Measured mean values: DMSO control=100 ARL, LY-411575 1µM=93.6 ARL, LY-411575 5µM=93.8 ARL.

In order to test whether *Id4* expression is also influenced by other cell signalling pathways such as BMP, luciferase experiments were repeated with different levels of BMP activity. BMP signalling is known to be able to directly regulate members of the Id protein family in a number of systems, and *in silico* analysis of the *Id4* promoter region confirmed the presence

of BMP-responsive elements. To alter levels of BMP activity *in vitro*, OC-2 cells transfected with the Id4-luciferase reporter construct were either co-transfected with a Smad6 expression construct (pCAB-Smad6-IRES-GFP) to inhibit potential endogenous BMP signalling, or treated with varying amounts of BMP4 protein to increase activity. All experiments were performed as triplicates, and two independent sets of experiments were conducted using different DNA preparations. Readouts of Firefly luciferase activity were performed as described. Summarized results are shown in figure 3-25 A and B. In all cases, luciferase activity was normalized to baseline levels from control cells in which the BMP pathway had not been manipulated (Fig. 3-25 A and B, first bar). With a fold change of 1.06 the measured luciferase activity was slightly higher in cells transfected with 400 ng of the Smad6 expression construct when compared to the empty control vector (Fig. 3-25 A, second bar). The levels of luciferase activity further increased in a dose-dependent manner with increasing levels of Smad6. Cells transfected with 800 ng of Smad6 showed a fold change in luciferase activity of 1.23, and levels were highest in cells transfected with 1200 ng of Smad6 with a fold change of 1.42 when compared to the control (Fig. 3-25 A, third and fourth bar).

These data suggest that *Id4* expression is upregulated under inhibition of the BMP signalling pathway in a dose-dependent manner. However, when BMP signalling was increased by treatment with the BMP4 protein no significant effect on luciferase activity was measured (Fig. 3-25 B, mean values BMP4 50 ng = 89.7 ARL and 250 ng = 98.2 ARL).

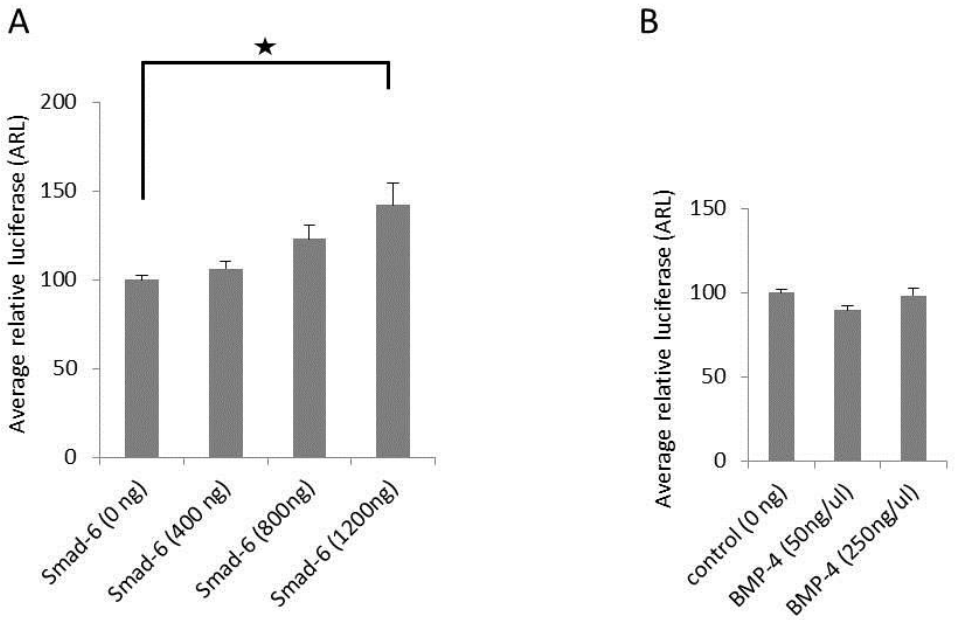


Figure 3-25: Luciferase activity at different levels of BMP signalling. OC-2 cells transfected with the Id4-luciferase reporter construct were either (A) co-transfected with a Smad6 expression construct to block BMP signalling, or (B) treated with BMP4 protein to enhance BMP activity. Measured mean values of luciferase are displayed in ARL units: Smad6 0 ng=100 ARL, Smad6 400 ng=106.2 ARL, Smad6 800 ng=122.8 ARL, Smad6 1200 ng=142.1 ARL, statistical significance: p=0.0187463; BMP4 50 ng/µl=89.7 ARL, BMP4 250 ng/µl=98.2 ARL.

4. Discussion

The aim of this study was to find out more about the mechanisms that regulate the patterning of inner ear sensory epithelia, and especially the possible involvement of the dominant inhibitory helix-loop-helix transcription factor Id4 in the process of hair cell versus supporting cell fate decisions. It is well known that the mosaic-like pattern of hair cells and supporting cells is to a large extent regulated by Notch-mediated lateral inhibition, and a recent genome-wide microarray screening has identified Id4 as a potential Notch target and effector in the developing chicken inner ear. In the context of this thesis I conducted experiments aiming to study the expression pattern of Id4 in the vertebrate inner ear during embryological development, as well as its regulation *in vitro* and the effects of a gain-of-function and a loss-of-function on the overall extent of hair cell production.

The results indicate that Id4 is expressed in both the sensory epithelia of the chicken and the mouse inner ear at stages that are relevant for hair cell formation. Overexpression experiments have shown that Id4 is able to directly inhibit hair cell formation in the chicken inner ear, but no obvious phenotype was seen when the *Id4* gene was deleted in mice. *In vitro* experiments using an otic cell line confirmed a positive regulation of *Id4* expression by the Notch signalling pathway, whilst BMP signalling did not seem to be involved in Id4 regulation in this context.

Taken together, these data suggest that Id4 is involved in cell fate choices in the developing inner ear, and there is clear evidence that Id4 is able to directly block hair cell formation in a cell-autonomous manner when expressed at high levels. However, whether this is also the function of Id4 *in vivo* under normal conditions remains unclear, and there are subtle differences in the timing and the cell-type specific expression between the auditory and the vestibular system that need to be further explored. In the following sections I will shine a

more detailed light onto the complex network of transcription factors regulating hair cell differentiation, and I will discuss the possible roles of Id4 in this particular context.

4.1 Id4 – more than an inhibitor of hair cell formation?

Id4 expression was found in the sensory region of both the auditory and the vestibular system at different stages of the developing chicken and mouse inner ear. Surprisingly, expression was not restricted to the layer of supporting cells in these epithelia but was also found within differentiated hair cells. This finding was unexpected; based on the working hypothesis outlined earlier, *Id4* expression was predicted to be found in supporting cells but not hair cells as it is thought to inhibit hair cell formation and favour a supporting cell fate instead. Consistent with this hypothesis, forced expression of Id4 in the chicken inner ear led to a reduction in the overall number of hair cells while supporting cells did not seem to be affected. However, at this point it is important to mention that a forced overexpression does not reflect normal expression levels. This consideration might be relevant as it is well conceivable that the effects of Id4 could vary depending on its level of expression in particular tissues and cell types, and it is possible that regulation of expression might be the factor that determines its function (Patel, Morton et al. 2015). Since all HLH proteins function as heterodimers (Murre, Bain et al. 1994), sequestration of E-proteins by Id4 or competition with the formation of functional E-protein/Atoh1 heterodimers could well explain the results seen in the Id4 overexpression experiments. Another intriguing finding made was that of Id4 being expressed differentially in the murine cochlea and vestibular system during early stages of development. Even though hair cell formation had started in all sensory epithelia at the examined stages, no sign of *Id4* expression was found within the organ of Corti at E13 and E14. Moreover, expression was found outside of the sensory region in a domain that is not considered relevant for hair cell or supporting cell formation. Based on these results it

seems that, compared to the vestibular system, *Id4* expression sets in at a later stage in the organ of Corti of mice. Notably, this appears to be after the onset of hair cell formation, as it was also the case in both the chicken vestibule and basilar papilla. The obvious differences in the timing of *Id4* expression in the mammalian vestibular and auditory system suggests that Id4 might be playing differential and probably contrary roles in the context of hair cell formation within these particular epithelia. In the light of these new findings and considerations, the initial working model of Id4 strictly counteracting hair cell formation clearly needs to be revised. However, some of the results obtained during this study came up to expectations. Consistent with the finding that forced Id4 overexpression blocks hair cell formation, preliminary data was obtained that Atoh1 protein levels are also reduced in cells that are overexpressing Id4. The same was not seen in cells overexpressing GFP (data not shown), and the apparent downregulation of Atoh1 levels thus seemed to be specific to Id4. How exactly this downregulation may be accomplished in this particular context however is yet to be elucidated. With Atoh1 and Id4 both belonging to the family of HLH transcription factors, regulation could either occur on the transcriptional level, the protein level, or on both. It is conceivable that Id4 protein directly interacts with Atoh1 protein to form inactive protein dimers and thus inhibits any transcriptional activity of Atoh1 on its target genes. Moreover, forming inactive dimers with Atoh1 could also inhibit the Atoh1 auto-regulatory feedback loop (Helms, Abney et al. 2000) and thus limit expression levels of Atoh1. The use of an Atoh1 reporter in co-expression with the Id4 overexpression construct would be helpful to find out more about the nature of interaction and regulation between Id4 and Atoh1 in the inner ear.

In general, the possible modes of action of any bHLH protein are manifold and can vary depending on the system and other molecular factors and interaction partners that are expressed. As a member of this protein superfamily the same applies to Id4. Indeed Id4 has been the subject of a number of studies and its reported functions range from inhibition of differentiation (Kondo and Raff 2000, Samanta and Kessler 2004, Junankar, Baker et al.

2014) to the induction of apoptosis (Andres-Barquin, Hernandez et al. 1999, Carey, Knowell et al. 2013) and promotion of cell differentiation (Bedford, Walker et al. 2005, Tokuzawa, Yagi et al. 2010, Srikanth, Kim et al. 2014) depending on the particular tissue investigated. Studies of human cancers have also highlighted the versatility of this transcription factor – while Id4 acts as a tumour promoter in cancer types affecting the brain and the central nervous system it functions in a contrasting way in many other cancers by acting as a tumour suppressor (Oncomine Database, Rhodes, Yu et al. 2004, Patel, Morton et al. 2015). In line with and on top of that, the overall data I obtained from the *Id4* expression studies suggest that even within a single organ such as the inner ear Id4 might participate in several differential aspects of its development. These clearly include cell fate decisions within the epithelium, such as hair cell versus supporting cell fate, and probably regulation of cell proliferation. Previous studies have revealed a function for Id4 in the regulation of cell proliferation by keeping progenitor cells in an undifferentiated state (Kondo and Raff 2000, Bedford, Walker et al. 2005), and Id4 could exert a similar function in the inner ear at stages prior to hair cell formation. This would be consistent with the fact that *Id4* expression was found to precede hair cell formation in the chicken inner ear and the mouse vestibular system. Additionally, the fact that *Id4* expression was detected in both hair cells and supporting cells in the adult organ of Corti suggests that it not only plays a role during embryological development and regulation of initial hair cell formation but also seems to be involved in tissue maintenance in the mature inner ear.

Besides the implication that Id4 might play differential roles depending on the developmental context, these latest data also increasingly promote the idea that not only Id4 and its putative target Atoh1, but the entirety of all expressed HLH transcription factors in the inner ear might regulate hair cell formation. Along with Id4, all other members of the Id protein family, namely Id1, Id2, and Id3, are expressed in the relevant sensory epithelia of the developing chicken and mouse inner ear at stages prior to and during hair cell formation (Riechmann, van

Cruchten et al. 1994, Jen, Manova et al. 1997, Kee and Bronner-Fraser 2001, Lin, Ozeki et al. 2003, Jones, Montcouquiol et al. 2006, Kamaid, Neves et al. 2010). As assumed for Id4, Id1 and Id3 have previously been reported to directly antagonize Atoh1 function and to be able to inhibit hair cell formation when overexpressed in organotypic cultures of the mouse organ of Corti (Jones, Montcouquiol et al. 2006). Given their presence in the inner ear as well as the fact that they seem to function similarly at least in their capacity to counteract Atoh1 and thus hair cell formation, the obvious question arises whether Id4 and some of the other Id protein family members may act redundantly. Such redundancy might explain why the single *Id4* gene knockout did not exhibit a clear phenotype in terms of hair cell production. However, even though all Ids are expressed in the inner ear, the pattern of expression varies quite significantly. While Id4 is mostly specific to the auditory and vestibular sensory epithelia and, apart from the described patch in the cochlea, not expressed in other inner ear tissue, the expression of Id1, 2, and 3 is much broader and includes not only the otic epithelium but also surrounding mesenchymal regions (Jen, Manova et al. 1997, Jones, Montcouquiol et al. 2006, Kamaid, Neves et al. 2010). This suggests that Id4 function and regulation differ from that of the other Id family members in the context of inner ear development.

The actual roles of Id proteins in the inner ear might also depend on their relative expression levels. In fact, an interesting interaction between Id4 and Id1/2/3 has been described in a recent study by Sharma and colleagues (Sharma, Chinaranagari et al. 2015). Looking at human cancer cell lines, they found that Id4 is able to promote general DNA-binding of bHLH factors by directly interacting with Id1/2/3 and thus neutralizing their inhibitory effect on other bHLH factors. More precisely, they reported that inhibition of the E-protein E47 by Id1 and Id2 was fully alleviated by expression of Id4, an effect that could not be achieved by any of the other Ids (Sharma, Chinaranagari et al. 2015). By analogy, it is conceivable that similar processes could take place during inner ear development and hair cell formation. Low levels of Id4 could thus favour hair cell formation by counteracting Id1/2/3, and high levels of Id4

would inhibit hair cell formation probably in cooperation with the other Ids. Indeed, this consideration could explain away the fact that *Id4* expression was found in differentiated hair cells while artificial overexpression inhibited hair cell formation.

4.2 Making sense of the HLH factors network during hair cell fate decisions: lessons from Drosophila?

Besides Id proteins and Atoh1, there is a number of additional HLH transcription factors expressed in the developing inner ear that most likely all account to the complex regulatory network directing inner ear patterning and hair cell formation to some extent. These include for instance HES proteins that act downstream of Notch signalling to counteract Atoh1 function, HEY1 protein which is involved in both Notch-mediated lateral induction during prosensory specification and Notch-mediated lateral inhibition during hair cell formation (Petrovic, Formosa-Jordan et al. 2014), ubiquitously expressed E-proteins such as E47, or Neurog1, Neurod1 and Nhlh1 that are involved in sensory neuron formation (Fritzsch, Eberl et al. 2010). Understanding the interplay of all these factors is essential, especially when it comes to considering potential therapeutic approaches targeting one of them. In the following section I will explain how insights into the development of the *Drosophila* sense organs have greatly contributed to our current understanding of hair cell formation and could be particularly relevant to elucidate Id4 functions in the inner ear.

There are striking similarities between the *Drosophila* bristle sensory organs and vertebrate inner ear hair cells. Bristle sensilla are small mechanoreceptors that belong to the peripheral nervous system and that cover the body surface of the *Drosophila* imago. Depending on their size they are also referred to as macrochaetae or microchaetae, respectively (Fig. 4-1). Not only their anatomy and mode of action show great resemblance with inner ear hair cells.

Many of the mechanisms and genes regulating bristle sensory organ formation are well conserved and have homologues in vertebrates.

In the given context, the longer macrochaetae are of particular interest. Their number is more restricted than that of the microchaetae, and their position is predetermined and follows a strict and invariant pattern (Smith and Sondhi 1961). As for sensory hair cells and non-sensory supporting cells, all cells of the macrochaetae arise from a common pool of progenitors in so-called proneural clusters (Huang, Dambly-Chaudiere et al. 1991, Furman and Bukharina 2011). These clusters can be considered equivalent to the prosensory patches of the early otocyst, and they are defined through the expression of two tissue-specific class II bHLH transcription factors (Murre, Bain et al. 1994), *achaete* (ac) and *scute* (sc). These members of the *Drosophila achaete-scute* complex are characterized proneural genes that convey cells into a proneural state, and combined deletion of *ac* and *sc* has been reported to result in a virtually complete loss of bristles and other sensilla (Garcia-Bellido 1979). To become transcriptionally active, ac and sc proteins dimerize with the ubiquitously expressed class I bHLH protein Daughterless (Da), the only *Drosophila* homologue of mammalian E-proteins (Dambly-Chaudiere and Leyns 1992). However, the activity of *ac*, *sc* and *Da* is also regulated by *extramacrochaetae* (*emc*), a member of the inhibitory class V HLH transcription factors that is homologous to the vertebrate *Id* genes. The *emc* locus was first discovered by Craymer in 1980 and was named after the observation that supernumerary macrochaetae were formed in flies carrying mutations in this gene (Craymer 1980, Report of new mutants – Drosophila melanogaster). To inhibit ac, sc and Da function, emc forms inactive dimers with these factors and prevents their action on downstream targets (Van Doren, Ellis et al. 1991, Van Doren, Powell et al. 1992, Alifragis, Poortinga et al. 1997). Once the proneural clusters have formed, a single cell is selected within each cluster that will give rise to four specialized cells to form the bristle sensillum of the *Drosophila* imago. This single selected cell is referred to as sensory organ precursor (SOP) cell, and its

selection within the proneural clusters marks one of the key events of sensory organ morphogenesis (Furman and Bukharina 2011).

The current model for SOP selection is based on Notch-mediated lateral inhibition processes exerted by Delta as the main Notch ligand (Pitsouli and Delidakis 2005), and members of the Enhancers of split complex (E(spl)-C) as the main Notch targets that antagonize proneural genes and thus suppress SOP formation in addition to the inhibitory effect of emc. Consistent with this model, loss of either Notch or Delta has been reported to allow all cells of a proneural cluster to become a SOP (Hartenstein and Posakony 1990, Parks and Muskavitch 1993).

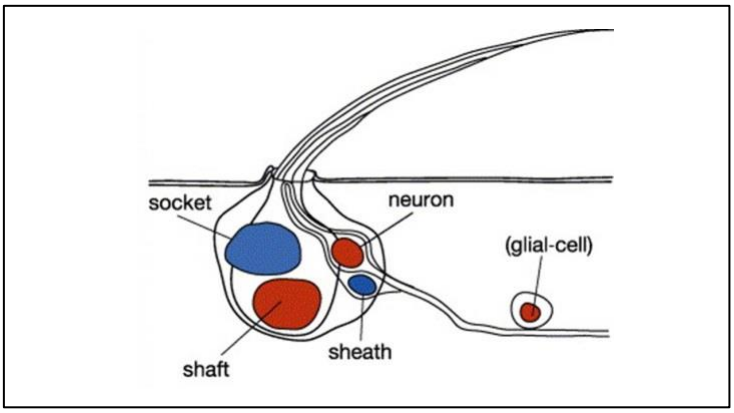


Figure 4-1: *Drosophila* bristle sensory organ. Each large bristle (macrochaete) consists of four differential, specialized cells. The shaft and the socket cell represent the external part which is visible on the imago's surface, while the neuron and the sheath cell are considered the neural elements. All four cells of the mature bristle sensillum arise from a single sensory organ precursor (SOP) cell. Figure adapted from (Lai and Orgogozo 2004).

However, a recent study by Troost and colleagues (Troost, Schneider et al. 2015) proposes a new kind of interactions between the various HLH transcription factors involved in SOP formation. According to this model (Fig. 4-2 A), not Notch-mediated lateral inhibition processes but the existence of a proneural, band-like region is required for the initial selection of proneural clusters. They suggest this proneural band to be defined by two

essential factors; a baseline activity of Notch, which prevents all cells located in between the clusters from adopting a SOP fate by suppressing proneural activity, and, more importantly, a differential expression of emc. Thus, low levels of emc are suggested in the presumptive proneural regions and high levels of emc are suggested in the regions in between. An interesting finding that led to the development of this new model was the fact that macrochaetae were able to form even in the absence of *ac* and *sc* expression when *emc* expression was abolished at the same time. Consistent with that, the authors found that levels of emc protein were low in regions where *ac* and *sc* expression was elevated (Fig. 4-2 A). They then hypothesized that ac and sc neutralize emc protein by directly binding and that this interaction leads to the release of Da protein which is otherwise bound and inhibited by emc monomers. Released Da could then form transcriptionally active homodimers or heterodimers with ac and sc proteins and thus create a high proneural activity in this particular region where ac and sc are expressed at high levels (Troost, Schneider et al. 2015).

If a similar model was applied in the context of the inner ear, it could mean that the primary function of Atoh1 is to relieve an imposed blockade from Id4 on Atoh1 itself or on other bHLH transcription factors, e.g. ubiquitously expressed E-proteins or other yet unidentified pro-hair cell factors (Fig. 4-2 B). Thus, the simple presence of Id4 in a cell would not necessarily mean that hair cell formation is inhibited; the adopted cell fate would rather depend on the actual level of Id4 as well as on the level of available Atoh1 protein to sequester Id4 monomers. Interestingly, this model would be consistent with the fact that Id4 was found in both supporting cells and hair cells in the inner ear. Furthermore it would be consistent with data reporting the ability of cells to differentiate into hair cells even in the absence of Atoh1 in the mouse inner ear (Jahan, Pan et al. 2015). These recent findings underline the need of a more detailed analysis of the levels of expression and interactions of the HLH transcription factors that regulate hair cell formation.

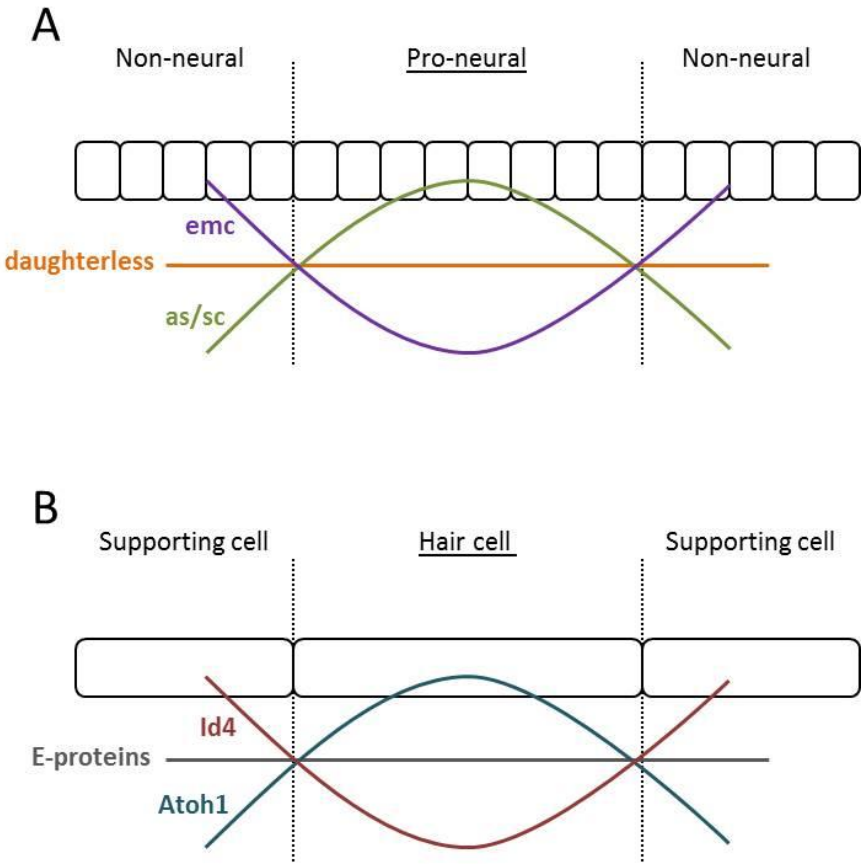


Figure 4-2: Model for potential interactions between different HLH proteins during bristle development in flies and hair cell formation. (A) Troost and colleagues suggested a new model for SOP selection during *Drosophila* sensory organ development. In this model, high levels of the bHLH proteins ac and sc neutralize the inhibitory impact from emc on other bHLH proteins such as Daughterless (Da). (B) In the context of the inner ear this model could imply a role for Atoh1 in neutralizing Id4 function and allow cells to differentiate into hair cells. Figure adapted and modified from (Troost, Schneider et al. 2015).

4.3 How to keep the temper: signalling pathways and the regulation of Id4 expression

Keeping in mind the complexity of the network of transcription factors controlling hair cell fate decisions it seems obvious that the timing and levels of expression of these factors must be tightly regulated and would have a profound influence on the pace and extent of hair cell formation. The regulation of expression and activity by cell-cell signalling pathways is

therefore of particular relevance and interest. As for the mode of action of Id4, interesting hypotheses about the regulation of its expression can be drawn from other systems, including *Drosophila*. In fact, previous studies have shown that *emc* expression is dependent on Notch signalling during *Drosophila* wing development and vein differentiation, and evidence exists that *emc* acts synergistically with other Notch effectors such as members of the (E(spl)-C) group of transcription factors (Baonza, de Celis et al. 2000). Dependence of *emc* expression on Notch has further been reported in *Drosophila* eye development and follicle cell differentiation (Baonza and Freeman 2001, Adam and Montell 2004, Spratford and Kumar 2015). Compared to *emc*, much less data is available about the regulation of *Id4* expression by the Notch signalling pathway. A study in *Xenopus* by Liu and Harland has revealed opposing results indicating a negative regulation of Id4 by Notch (Liu and Harland 2003). However, experiments conducted in the course of this thesis showed that increasing levels of Notch activity are able to upregulate *Id4* expression in an otic cell line derived from mouse. Notch inhibition, in contrast, did not have any effect on *Id4* expression levels. However, it is difficult to draw conclusions about the impact of a loss of Notch activity on *Id4* expression based on data obtained from the use of Notch inhibitors *in vitro*. It is possible that endogenous Notch signalling is not present in the particular cell line used; that would well explain the lack of effect of inhibition of the pathway. Overall, considering the fact that a basal endogenous *Id4* expression was detected in OC-2 cells these data might imply that expression of Id4 is regulated by several mechanisms and pathways at a time. These mechanisms and pathways might also be redundant or act in a cooperating manner. Moreover, since the results implicating an upregulation of *Id4* expression by Notch signalling are not consistent with the *Xenopus* data, it could imply that the regulation is cell type-specific and could result in opposing outcomes depending on the system. Another relevant signalling pathway in terms of *Id4* expression is the BMP signalling pathway. A positive regulation of Id4 by BMP4 has been reported in several studies (Liu and Harland 2003,

Samanta and Kessler 2004, Srikanth, Kim et al. 2014), and other members of the Id protein family are also known to act downstream of BMP signalling (Hollnagel, Oehlmann et al. 1999, Ruzinova and Benezra 2003, Samanta and Kessler 2004, Kamaid, Neves et al. 2010). Here, upregulation of BMP4 activity *in vitro* did not have any effect on Id4 levels, while a blockade of the pathway resulted in a slight upregulation of *Id4* expression. How these data fit into the published literature remains unclear at the moment; one possible explanation could be that OC-2 cells do not express a sufficient number of BMP receptors to be able to respond to such high levels of BMP4 activity. It is also conceivable that BMP4 signalling is not active in these cells. However, that still would not explain the upregulation of *Id4* expression in the presence of BMP inhibitors, and more data will clearly be needed to further elucidate the impact of BMP4 signalling on *Id4* expression in an otic environment. In conclusion, the use of a luciferase reporter is a reasonable method to get a fair impression about regulation and dependence of expression levels on different factors in a system; however, one must bear in mind that its capacity is limited and that only a small portion of the *Id4* promoter region can be tested using this experimental approach. Even though *in silico* analyses of the appropriate region of the *Id4* promoter revealed the presence of both Notch- and BMP-responsive elements the possibility remains that the actual critical regulatory elements that determine *Id4* expression *in vivo* are not included in this particular domain.

Furthermore, *Id4* expression could also be regulated by various alternative regulatory mechanisms such as post-transcriptional modifications, epigenetics, and hormonal regulation (Patel, Morton et al. 2015). Whether and to what extent any of these regulatory mechanisms apply to the inner ear during hair cell formation is a question that has not been addressed in the scope of this thesis. Further studies and investigations in this particular system would clearly be helpful in order to learn more about how the interactive network of HLH factors might be affected and probably orchestrated by differential levels of *Id4* expression in dependence of different stages and cell types. Here, using the *Id4-lacZ* knock-in mouse

model seems like an appropriate approach to investigate regulation of Id4 in an ear-specific context. Organotypic inner ear tissue cultures derived from transgenic animals could be used to directly study *Id4* expression in response to modifications of relevant signalling pathways or other regulatory mechanisms as mentioned above.

4.4 Outlook – Id4 as a potential therapeutic target in regenerative medicine?

Id4 is expressed in hair cells and supporting cells in the developing and the mature inner ear, and it is most likely involved in the regulation of hair cell formation. The exact nature of the role of Id4 in this process remains unclear at the moment, but these findings clearly raise the question whether Id4 could also be involved in hair cell regeneration. By the current state of scientific knowledge there is no evidence for naturally occurring hair cell regeneration in the adult mammalian cochlea and only limited regenerative potential has been reported in the vestibular system (Forge, Li et al. 1993, Forge, Li et al. 1998, Kawamoto, Izumikawa et al. 2009, Yang, Chen et al. 2012). The precise causes for the inability of the mammalian system to regenerate naturally are not currently known, but the possibility that Id4 might be involved to some extent is not to be excluded. As opposed to mammals, the avian inner ear holds great capacity to regenerate hair cells in both the mature auditory and vestibular sensory epithelium (Corwin and Cotanche 1988, Ryals and Rubel 1988, Stone and Cotanche 2007), and looking at this system might give indication about a possible involvement of Id4. In this context, most interesting data has been published by Ku and colleagues who characterized the transcriptome of regenerating hair cells in the chicken utricle (Ku, Renaud et al. 2014). According to their data, the *Id1*, *Id2*, and *Id4* genes were among the most highly expressed genes in the regenerating epithelium, and they were assumed to be present in vast excess compared to their target genes. Further, they found that Id1/2/4 were expressed in strong positive correlation with Atoh1, suggesting some kind of interaction between these HLH

factors (Ku, Renaud et al. 2014). Given the fact that Atoh1 activity is required for hair cell formation and regeneration it seems unlikely that the role of Id4 in the regenerating epithelium is inhibitory to Atoh1. To the contrary, Id4 might enhance Atoh1 activity perhaps by binding other bHLH that otherwise inhibit Atoh1. If that is the case, considering a combined gene delivery of both *Atoh1* and *Id4* seems like a conceivable approach to trigger hair cell formation in the damaged mammalian sensory epithelium. The ability of Atoh1 to induce hair cell regeneration in the mature mammalian cochlea and vestibular organs after hair cell damage has been reported in a fair number of studies (Kawamoto, Ishimoto et al. 2003, Izumikawa, Minoda et al. 2005, Staecker, Praetorius et al. 2007, Yang, Chen et al. 2012, Atkinson, Wise et al. 2014); however, the capacity to regenerate is still limited and providing high levels of *Id4* expression at the same time might further support and enhance the regenerative potential initiated by Atoh1.

For all that, since expression of Id4 was also found in the mouse inner ear where no regeneration occurs, its mere presence does not seem to be the critical factor in the context of hair cell regeneration. Thus, quantitative analysis and comparison of the actual levels of Id4 in both damaged and undamaged chicken and mouse inner ear tissue are needed and will help to clarify the possible roles of Id4 in the regeneration of the inner ear auditory and vestibular sensory epithelia.

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