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The Function of Hedgehog and Wnt Signaling Pathways in Otic Development

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Abstract
The inner ear is a complex sensory organ essential for hearing and balance. During embryonic development, the inner ear depends on signaling information originating from the embryonic hindbrain to establish dorsoventral and anteroposterior identity. The Hedgehog (Hh) and Wnt signaling pathways are active in the hindbrain and implicated in otic development, but their exact mechanisms of action remained unclear. We investigated the function of Hh in ear development using a mouse model where we conditionally inactivated Hh signaling in the otic vesicle, a transient embryonic structure that gives rise to the inner ear, while leaving nearby Hh dependent tissues unaffected. We found Hh signaling within the otic vesicle functions to establish ventral otic identity and drive the proliferation of cochlear-vestibular ganglion (cvg) neuroblasts that will innervate the ear. We identified presumptive Hh target genes in the developing inner ear using microarrays. Several of these presumptive Hh targets are known to function in ear development or hearing. We also identified many novel targets that have not been characterized in the ear. Many of these novel presumptive Hh target genes are expressed in the ventral otic vesicle, a region that will give rise to the cochlear duct. To interrogate the function of Wnt signaling in ear development, we used a Wnt responsive inducible Cre recombinase (TopCreERT2) to genetically label cells at different stages of ear development. We found cells that make up dorsal, vestibular, structures and cvg neurons are Wnt responsive for prolonged periods of ear development. In the cochlear duct, we found both sensory and support cells originate from a Wnt responsive population. Surprisingly, we found the Wnt responsive population of cochlear progenitors was also labeled using a cre recombinase expressed from the Gbx2 locus. TopCreERT2 and Gbx2 expression overlap in the dorsomedial wall of the otic vesicle, suggesting this region is a likely source for auditory cells.

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THE FUNCTION OF HEDGEHOG AND WNT SIGNALING PATHWAYS IN OTIC DEVELOPMENT

Dedication

I would like to dedicate this thesis to my parents, who have always supported me, even when they didn't understand what I was doing or why it seemed so difficult.

Acknowledgements

This this would not exist without the help of many people. I am deeply indebted to my wife Christine, who fed me when I was too distracted to eat, who patiently listened to my terrible ideas for experiments, and who tolerated the nights I worked so late I didn't come home. I'm also grateful to my advisors, Mike Klymkowsky who convinced me to apply to graduate school, and Doug Epstein who shepherded me through grad school and oversaw the work that makes up this thesis.

ABSTRACT

THE FUNCTION OF HEDGEHOG AND WNT SIGNALING PATHWAYS IN OTIC DEVELOPMENT

Alexander S. Brown

Douglas J. Epstein

The inner ear is a complex sensory organ essential for hearing and balance. During embryonic development, the inner ear depends on signaling information originating from the embryonic hindbrain to establish dorsoventral and anteroposterior identity. The Hedgehog (Hh) and Wnt signaling pathways are active in the hindbrain and implicated in otic development, but their exact mechanisms of action remained unclear. We investigated the function of Hh in ear development using a mouse model where we conditionally inactivated Hh signaling in the otic vesicle, a transient embryonic structure that gives rise to the inner ear, while leaving nearby Hh dependent tissues unaffected. We found Hh signaling within the otic vesicle functions to establish ventral otic identity and drive the proliferation of cochlear-vestibular ganglion (cvg) neuroblasts that will innervate the ear. We identified presumptive Hh target genes in the developing inner ear using microarrays. Several of these presumptive Hh targets are known to function in ear development or hearing. We also identified many novel targets that have not been characterized in the ear. Many of these novel presumptive Hh target genes are expressed in the ventral otic vesicle, a region that will give rise to the cochlear duct. To interrogate the function of Wnt signaling in ear

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Attributions

The work described in chapter two was previously published in:
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The work described in chapter three was performed in collaboration with Yao Yao and Alex Rohacek with technical assistance from Diane Dolson.

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Chapter 1: Introduction

Function of the inner ear

The ability to detect gravity, and its counterpart acceleration, is present throughout the animal kingdom. To this end, different detection schemes have been employed throughout evolution ranging from simple structures like Johnston's organ in insects[1, 2] to the complex, multipart vertebrate inner ear.

Mechanosensory cells

The basic information gathering unit of the inner ear is the mechanosensory hair cell (Figure 1.1). These highly specialized cells are polarized with actin based protrusions lining the apical surface. These actin based microvilli, termed stereocilia, are the site of mechanosensation[3, 4]. The distribution of stereocilia on the apical surface of the cell is not random. Instead, they form in a cluster or chevron, as a result the majority of sterocilia have a common orientation[5]. The common orientation of steociliary bundles makes hair

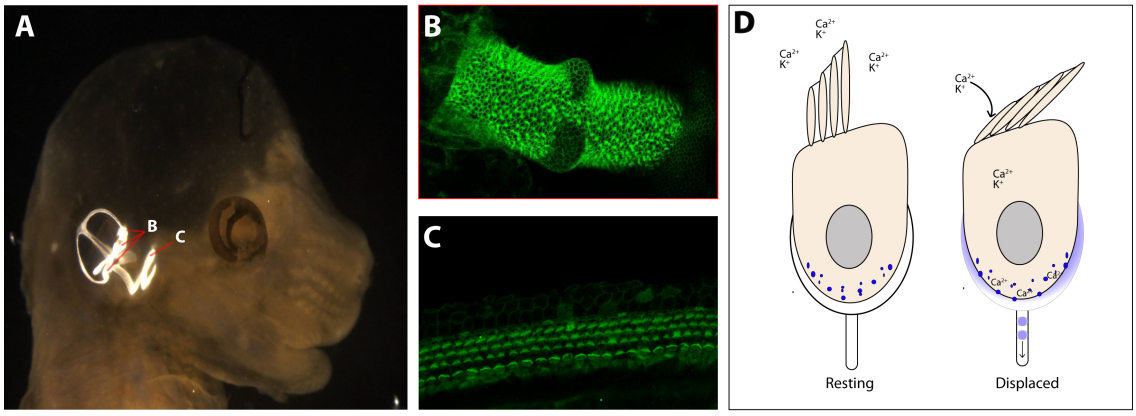

Figure 1.1: Mechanosensory hair cells
In the mouse, mechanosensory hair cells are located within the inner ear **(A).** Displacement is detected by stereocilia, which are arranged in different patterns for vestibular **(B)** and auditory **(C)** hair cells. Movement of the stereocilia leads to an influx of calcium and potassium ions, which depolarizes the hair cell **(D)**. Depolarization causes the release of glutamate (blue), exciting neurons which signal to higher processing centers.

cells most sensitive to particular vectors of displacement[6, 7]. The tips of stereocilia are physically linked together, and this linkage is essential for hearing[8]. The tip links do not force stereocilia to move as a group, a property due to their physical structure independent of their tip links[9]. Instead, displacing the stereocilliary bundle pulls on the tip links causing ion channels of uncertain identity to open. The newly opened channels allow calcium and potassium ions to enter the cell[10]. This influx of cations depolarizes the cell leading to a local increase of Ca^{2+} at the base of the hair cell adjacent to the synapse. The local increase of calcium causes the release of glutamate containing vesicles into the synapse[11, 12], completing the transduction of mechanical energy to neural impulse. The stereociliary bundle is an exquisitely sensitive motion detector, displacing it as little as 600 pm leads to detectible changes in membrane voltage potential[13].

Hair cells located in the inner ear synapse with neurons in the VIIIth cranial nerve, which carries information to the auditory or vestibular nucleus in the central nervous system. Although an individual sensory hair cell is capable of transducing motion into neural impulses, the functions of hearing and balance depend on a variety of additional cell types and the physical structure of the ear itself.

The vestibular system

In mammals, the inner ear contains six groups of sensory hair cells, five that detect acceleration and one that detects sound (Figure 1.2). The structures that detect acceleration are located in the dorsal half of the ear termed the

vestibulum. Vestibular structures can be further divided into the three cristae, each housed in an ampulla located at the base of a semicircular canal, that detect angular acceleration (Fig. 1.2 blue shading), and the utricule and saccule that detect linear acceleration and gravity (Fig. 1.2 green shading). The hair cells of the utriclular and saccular maculae are covered with otoconia, a mixture of protein and $CaCO_3$ crystals[14] whose mass imparts inertia. The inertia of the otoconia makes the utricle and saccule sensitive to gravity and linear acceleration[15]. Alternatively, the cells in each crista are covered by a cupula, a protein matrix that helps distribute the force imparted by circulating endolymph within the semicircular canal. Angular acceleration, for example a turn of the head, displaces endolymph within the canals which stimulates ampullar hair cells[7]. The inner ear is the organ that perceives balance, and to do so each vestibular structure must function correctly. Blocking only the formation of the lateral semicircular canal severely disrupts balance in mice[16], and improper stimulation of the lateral ampulla in humans results in benign paroxysmal

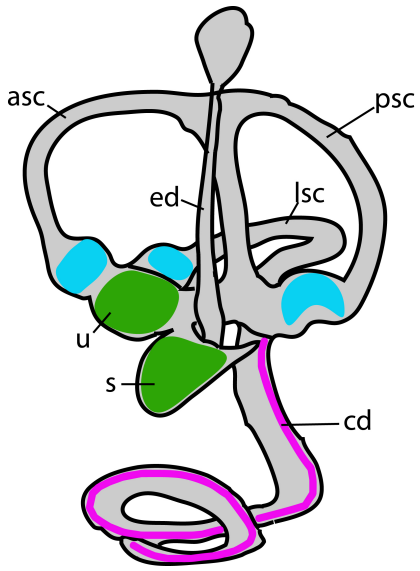

Figure 1.2: Sensory patches of the mammalian inner ear
The vestibulum contains structures to detect gravity (green), and angular acceleration (blue). The auditory apparatus (magenta) detects sound. Anterior semicircular canal (asc), lateral semicircular canal (lsc), posterior semicircular canal (psc), utricle (u), saccule (s), cochlear duct (cd), endolymphatic duct (ed).

positional vertigo (bppv). Fortunately, bppv is readily treated by a specific series of head movements designed to reposition rouge otoconia particles that may have drifted into the lateral canal[17]. The use of semicircular canals in the inner ear is evolutionarily ancient with examples spread across at least 500 million years of evolution ranging from lamprey to human[18, 19].

The auditory system

In mammals the auditory organ, the cochlear duct, contains a stripe of mechanosensory hair cells along its length that respond to different frequencies of sound (Fig 1.2 magenta shading). The structure of the cochlear duct, and within it the organ of Corti that houses the hair cells (Figure 1.3), plays an essential role in hearing. Amphibians and birds hear using an analogous structure, the basal papilla, which also houses a collection of sensory cells but in a different arrangement than the cochlea.

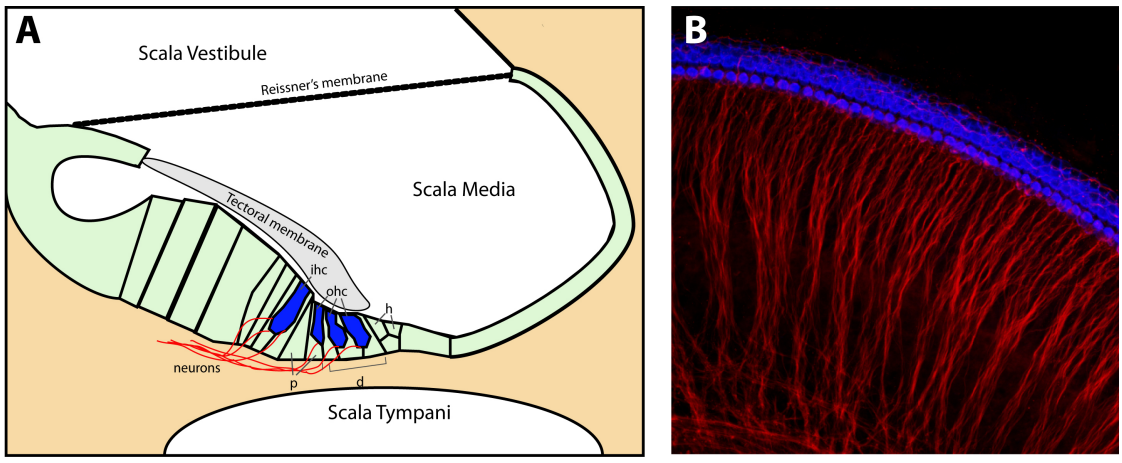

Figure 1.3: Organ of Corti
The organ of Corti is housed within the cochlear duct and contains hair cells. **(A)** Schematic cross section through an embryonic organ of Corti. At this stage of development mature cells are present, but tissue wide remodeling still needs to occur for optimal hearing. **(B)** a confocal micrograph showing hair cells (blue) and neurons (red). inner hair cell (ihc), pillar cell (p), outer hair cell (ohc), deiter's cells (d), henson's cells (h).

The incredible sensitivity of the mammalian cochlea is due to three characteristics: the ability of hair cells to detect tiny displacements, the physical structure of the cochlea to dissect complex sounds into pure tones, and the movement of outer hair cells to physically amplify sounds.

The cochlear duct houses three fluid filled channels, the scala vestibule, scala media, scala tympani (Fig. 1.3A). Vibrations that makeup sound are transduced by the middle ear to generate waves of pressure in the endolymph of the scala vestibule and scala media, which displace the basilar membrane housing the organ of Corti. Each frequency of sound creates a different pressure wave along the length of the cochlear duct. These different pressure waves, maximally displace a unique region of the basilar membrane[20], allowing a limited section of cochlear to respond a unique frequency of sound. Frequency selectivity creates a tonotopic map, where basal regions of the cochlear duct respond to high frequency sound, while more apical regions respond to increasingly lower frequencies. This tonotopic map is reflected in the innervation pattern of the cochlear nucleus in the brainstem. Each frequency of sound detected by different hair cells leads to a spatially distinct innervation pattern[21].

Although changing the mechanical properties of the basilar and tectoral membranes alters resonant properties of the ear, the physical structure of the cochlea is not the sole cause of hair cell stimulation. An additional active amplification step[22] increases sensitivity 100 fold using force generated by the outer hair cells. For this amplification step, changes in transmembrane voltages causes outer hair cells change their length and move their stereocilia at a

frequency that matches a sound stimulus[23, 24]. This increases the amplitude of stimulation on the inner hair cell. These differences in hair cell properties, where inner hair cells detect vibration and outer hair cells amplify vibration, are reflected in their innervation patterns. Inner hair cells are synapsed by multiple afferent spiral ganglion neurons, while multiple outer hair cells can be innervated by a single efferent neuron (Fig. 1.3B).

Of all the sensory systems, the inner ear has the finest temporal resolution where hair cells respond on the order of microseconds[25], and exquisite sensitivity with the ability to detect acceleration as small as 10^{-6}g[26]. Despite all the complexities of the inner ear, its embryonic origin and development is controlled by a limited number of cell signaling pathways. These pathways are often used repeatedly during development to create remarkably different cells and tissues depending on the time and context of signal activity. A variety of genetic studies and embryo extirpation experiments support roles for the Hedgehog and Wnt signaling pathways in establishing dorsoventral polarity in the ear[27, 28], which in turn, guides the formation of the vestibulum and cochlear duct[29].

The Hedgehog signaling pathway

Since its discovery in the fruit fly *Drosophila Melanogaster*[30], the Hedgehog signaling pathway has been found to function in the patterning, proliferation and differentiation of many organs and tissues. Damage to the Hedgehog pathway

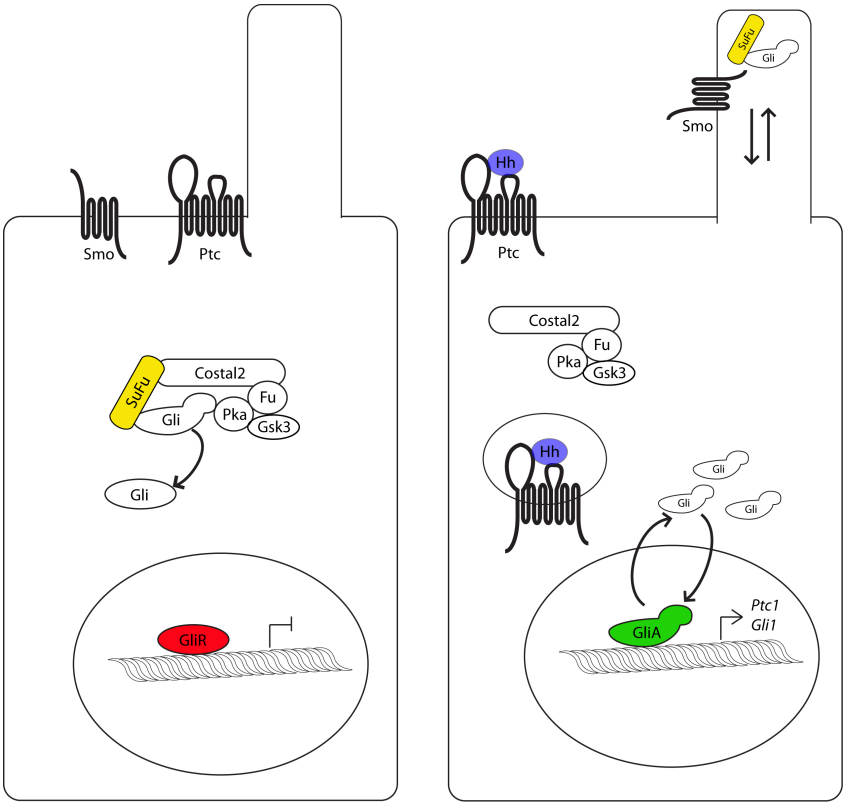

Figure 1.4: The Hedgehog signaling pathway
In the cell on the left, the Hh pathway is inactive because it has not received any ligand. Gli is phophorylated and proteolyitically cleaved into a transcriptional repressor. The cell on the right is exposed to ligand, allowing Ptc to clear from the cilia and be internalized. This allows Smo to move onto the cilia, and recruit Sufu and Gli to the cilia tip. Full length Gli protein accumulates, and a labile fraction is able to enter the nucleus and serve as a transcriptional activator. Two direct targets of Hh signaling are *Ptc1* and *Gli1.*

frequently results in birth defects and a variety of cancers, making the pathway medically important. In the context of the developing ear, the ligand *Sonic Hedgehog (Shh)*[29, 27], the transducing protein *Smoothened (Smo)*[31], and the transcriptional effectors *Gli2* and *Gli3*[32] have been studied experimentally.

Although core components of the Hedgehog pathway are conserved from fly to human, important differences have evolved among species. In all cases, the first step of a cell's response to hedgehog signaling begins at the cell membrane when the a ligand, Hedgehog in flies, or any of the three ligand Sonic

Hedgehog (Shh), Indian Hedgehog (Ihh), Desert Hedgehog (Dhh) proteins in mammals[33-35], binds a complex of receptor proteins. This receptor complex must contain the twelve pass transmembrane protein Patched (Ptc), which antagonizes Hh signal transduction in the absence of ligand[36]. In the absence of Ptc, unrestrained Hh signaling occurs which can lead to lethal embryonic defects. Less severe cases of *Ptc* disruption result in Gorlin's syndrome which is characterized by increased frequencies of basal cell carcinoma[37, 38] and medulloblastoma[39]. Ptc was originally characterized as the primary Hh receptor[40, 41]. However, an increasing number of co-receptors have been found to be necessary for signal transduction, including Ihog and Boi[42] in fly and their mammalian homologs Cdo and Boc[43], as well as vertebrate specific co-receptors Gas1[44, 45] and LRP2[46].

Structurally Ptc is similar to cholesterol transport proteins like NPC-1[47], and bacterial RND permeases[48]. Despite the apparent similarity to membrane transport proteins the exact mechanism of Ptc activity is unclear. However, Ptc has been observed to function in a catalytic manner[49] that may involve lipid or sterol intermediates[50]. Further support for the idea that Ptc functions to modulate the levels of small molecules comes from the structure of antagonists to the Ptc target Smoothened (Smo). The first discovered Smo antagonist, cyclopamine[51], a steroidal alkaloid isolated from *veratum californicum* was identified for causing cyclopia in offspring of pregnant livestock that consumed the plant[52 53]. Additional small molecule Smo antagonists have been discovered[54], but their divergent structures give little insight to the identity of a potential endogenous regulator.

Hh binding to the Ptc complex relieves antagonism of the eight pass transmembrane protein Smoothened (Smo). Smo activity is essential for Hh signal transduction[55]. At this point, the hedgehog pathway begins to diverge between flies and vertebrates. In flies, active Smo antagonizes an intracellular complex containing the kinesin Costal2[56, 57], the kinases PKA[58] and Fused (Fu)[59], and the novel protein Suppressor of Fused (Sufu)[60]. When this protein complex functions in the absence of Hh, it sequesters and phosphorylates the zinc finger transcription factor Cubitus Interruptus (Ci)[61, 62]. Ci contains an N-terminal transcriptional repression domain and a C-terminal activation domain, allowing it to function as a transcriptional activator or repressor in a signal dependent manner[63, 64]. The phosphorylation of Ci marks the C-terminus for degradation[65] creating a truncated repressor isoform consisting of the N-terminal repressor domain and DNA binding zinc fingers.

In vertebrates, the primary cilium is an essential site of hedgehog signaling. The cilium is a microtubule based organelle that protrudes from the cell surface and functions as a signaling center and as a sensor for the local environment. The requirement for cilia in Hedgehog signal transduction was initially discovered in mouse embryos mutant for members of the IFT family of ciliary transport proteins[66, 67]. These mutants had poorly formed or missing cilia and phenotypically resembled embryos mutant for Gli transcription factors, the three vertebrate homologs of Ci[68]. These observations lead to a model where: In response to ligand, Ptc is displaced from the cilium[69]. Clearance of Ptc is followed by an accumulation of Smo the cilium[70], ciliary Smo then activates

through an unknown mechanism[71] leading to an accumulation of effector proteins such as Sufu and full length Gli proteins, at the cilia tip[72]. Sufu directly interacts with Gli proteins to promote the formation of truncated Gli repressor isoforms and assists in forming labile Gli activator isoforms[73]. Divergently from flies, Sufu serves as a prominent vertebrate inhibitor of hedgehog signaling[74]. However, Sufu successfully antagonizes signaling in the absence of cilia[75], calling into question whether the accumulation of effector proteins at the cilia tip plays a functional role in signal transduction. Other aspects of hedgehog signal transduction appear well conserved. The Costal2 homolog Kif7[76-78] functions in a complex with PKA, CK1 and GSK3 to mark Gli for degradation[65], or to establish labile full length transcriptional activator.

The endpoint of the hedgehog signaling pathway is the differential expression of target genes in response to relative levels of Gli-activator and Gli-repressor proteins. Using differential activator/repressor activity generates many possible responses to ligand and allows Hedgehog to function as a morphogen, specifying different cell fates in a time and concentration dependent manner[79]. Shh emanating from the floor plate and notochord specifies different classes of neurons along the dorsoventral axis of the neural tube. In this case, different concentrations of Shh are reflected in different amounts of Gli-activator or Gli-repressor activity[80-82]. A similar logic is seen in the developing limb bud, where Shh originating from the zone of polarizing activity (ZPA)[35] establishes a gradient of Gli3 repressor activity to specify individual digits[83]. The hedgehog receptor and negative pathway regulator Ptc is a direct transcriptional target of hedgehog

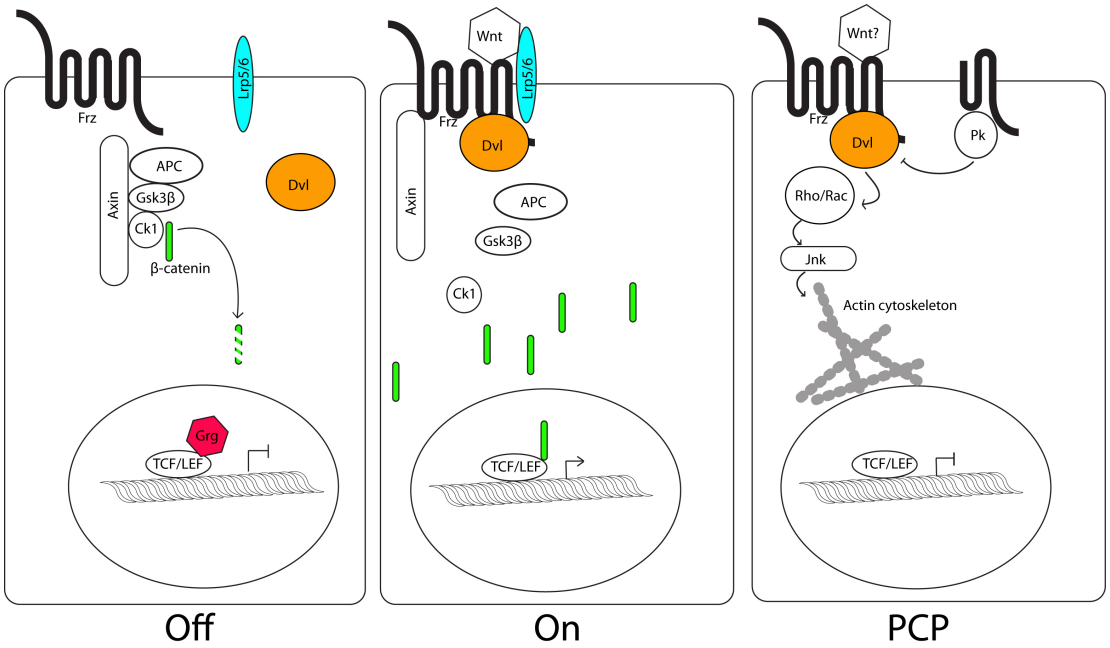

Figure 1.5: The Wnt signaling pathway
In the canonical Wnt/β-catenin pathway the cadherin subunit β-catenin is phosphorylated and degraded by a cytoplasmic destruction complex consisting of the scaffold protein Axin2, APC and Ck1, Gsk3β kinases, while TCF/LEF family members bind DNA in complex with Groucho transcriptional repressors. In the presence of Wnt ligand, Dvl recruits Axin to the cell membrane disrupting the β-catenin destruction complex. β-catenin accumulates and enters the nucleus where it displaces Groucho and activates transcription. Within Planar Cell Polarity (PCP) pathway, a Wnt ligand binds Frz, which recruits Dvl to modulate the function of Rho/Rac kinases, ultimately resulting in remodeling of hte actin cytoskeleton. The localization of active Dvl is in part restricted by Vangl, and Prickled (Pk) activity.

signaling, establishing a negative feedback loop[84]. This regulatory loop prevents runaway signaling, and may function to help a cell interpret different levels hedgehog ligand[85].

The Wnt Signaling Pathway

The Wnt signaling pathway has many more ligands and receptors than the hedgehog pathway, as well as multiple extracellular signaling inhibitors[86-88]. This plethora of ligand, receptor, and inhibitor combinations feed into a pathway that can have multiple readouts including changes in transcription, cytoskeletal

remodeling, and activation of heterotrimeric G proteins to modulate intracellular calcium levels [89-92].

Wnt ligands are lipid modified proteins[93] that interact with one or more of the 10 *Frizzled* (*Frz*) receptors[94] and the obligate co-receptor *LRP5/6*[95-97]. Mammals have 19 Wnt ligands, which are expressed in partially overlapping patterns, and there seems to be some variability as to how a given ligand or receptor activates the Wnt pathway[98]. Once ligand is bound, Frz recruits disheveled (Dlv)[99], which in turn recruits the tumor suppressor *adenomatous polyposis coli (APC)*[100]and the scaffold protein *Axin2*[101]. This recruitment of APC and Axin to the cell membrane disrupts the β-catenin destruction complex. The β-catenin destruction complex consisting of APC, Axin[102], Glucose Synthase Kinase 3β (Gsk3β)[103], Casein kinase I (CKI)[104-106] phosphorylates the cytoskeletal protein β-catenin, leading to its ubiquitylation and degradation[107]. The disruption of the destruction complex in the presence of Wnt signal leads to an accumulation of β-catenin, allowing it to enter the nucleus and interact with *TCF/LEF* transcription factors[108-110]. The recruitment of β-catenin to TCF/LEF displaces *Groucho* corepressors[111-113], activating transcription. This transcriptional activation can be detected by increased expression of *Axin*[114] or the use of reporter constructs driven by synthetic promoters consisting of multimerized TCF/LEF consensus binding sites[115, 116].

In addition to the canonical β-catenin mediated pathway, some Wnt ligands function through the planar cell polarity (PCP) pathway during ear development[117, 118]. The PCP pathway shares several key mediators of Wnt

signaling with the β-catenin pathway including Frz[119] and Dlv[120-122], but can signal through a Rho[123], Rac[124], Jun-kinase[125], cascade to control the cytoskeleton. Ultimately, a cell displays polarized distribution of PCP effector proteins, where Frz,Dlv are enriched in a domain distinct from the effector proteins Prk, and Vangl[126-128]. This molecular polarity is then reflected in the shape and organization of the cell giving rise to tissue wide properties.

In vertebrates, the most striking examples of tissue wide organization due to PCP signaling are convergent extension movements necessary for neural tube closure, cochlear duct outgrowth, and the the orientation of hair cells within the cochlear duct.

Development of the inner ear

Morphogenesis

A variety of fate mapping experiments in chick and mouse reveal that an overwhelming majority of the cells that make up the inner ear come from a

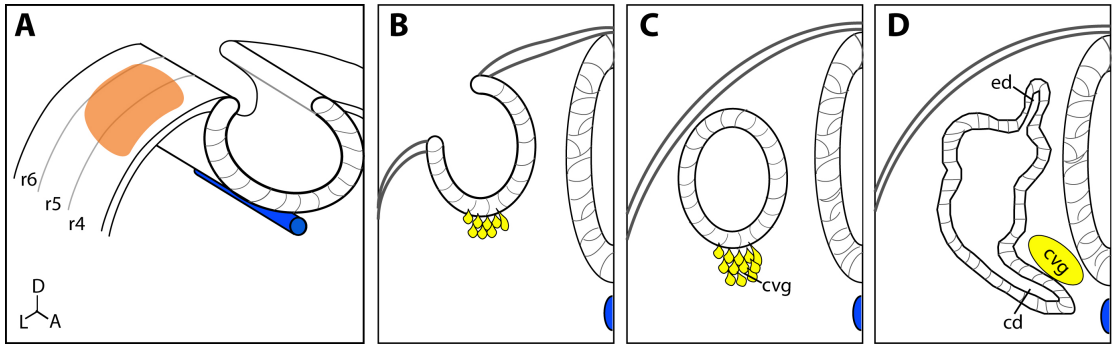

FIgure 1.6 Early otic Development
Transverse sections through the developing hindbrain show ear development starts as the otic placode (**A**, orange). As development progresses the placode invaginates to form the otic cup (**B**) and neuroblasts begin to delaminate (yellow). The otic cup closes to form the otic vesicle (**C**), and delaminating neuroblasts begin to aggregate to form the cochlear vestibular ganglion (cvg). (**D**)The otic vesicle begins to elongate along the dorsoventral axis, generating the endolymphatic duct (ed) and cochlear duct (cd).

common embryonic origin, the ectoderm adjacent to the developing hindbrain. Series of heterotopic grafting experiments, where different regions of ectoderm are replaced within age matched embryos, revealed that amphibians and fish have large regions of ectoderm competent to contribute to the ear. This competency is largely due to the activity of several Wnt and Fgf signals, whose identity varies between organisms. In zebrafish *Fgf8* and *Fgf3* are necessary for otic induction[129], a role filled in the mouse by *Fgf3* and *Fgf10*[130, 131], and *Fgf19* and *Wnt8c* in chick[132]. In mouse, the region of otic competency is gradually restricted and a portion of the ectoderm thickens creating the otic placode by 10 somites of age. The choice between otic placode and cranial ectoderm seems to be governed by Wnt/β-catenin signaling, as ectopic activation of the pathway leads to expanded placodes at the expense of ectoderm and inhibited Wnt signaling results in microvesicles that arrest early in development[133].

As otic development progresses roughly to the 15 somite stage, the placode beings to invaginate forming the otic cup. Fate mapping experiments in chick show that cells are already organized into presumptive dorsal, ventral, anterior and posterior regions at this time point[134]. This presumptive regionalization is reinforced by extirpation experiments in salamander and chick, where rotating the otic placode resulted in defects in structures along the anteroposterior axis, but not along the dorsoventral axis. Rotating the otic vesicle a few hours later resulted in defects along the dorsoventral axis[135, 136]. These results suggest that the anteroposterior axis becomes fixed in development before the dorsoventral axis. The ability for both axes to be

reprogrammed from an initial pattern to that of its host suggests the developing ear receives positional information from nearby tissues. Although the invaginating otic placode displays regionalized gene expression and restricted cell fates, there remains a possibility for additional cell movement. Cells have been observed migrating into the otic vesicle[137, 138], while fish forgo otic cup formation all together, instead relying on cavitation to hollow a mass of cells into the otic vesicle[139].

The factors responsible for axis specification and the development of otic structures may vary between species. In mouse and chick, Wnt signals emanating from the dorsal hindbrain[28] and Hedgehog emanating from the ventral neural tube and notochord[29, 27] establish the dorsoventral axis in the otic vesicle, while a wave of retinoic acid signaling imparts anteroposterior polarity[140]. Conversely, in frogs and fish, hedgehog activity largely establishes posterior identity[141-143], and promotes ventromedial identity[144] while Fgf signaling establishes anterior identity[145]. This is use of different signaling pathways to create an evolutionarily conserved organ is somewhat puzzling. A role for Hh signaling in auditory development is common among each of these examples, yet significant differences remain. One commonality is that Hh is required for the cochlear duct in mammals, and the fish auditory organ, the posterior macula. Additionally, in both mice and fish Hh promotes the proliferation of cvg progenitors. Yet Shh antagonizes hair cell formation in mice[146], while Hh promotes late forming saccular hair cells in fish through the regulation of *atoha1* expression[142]. These differences should not be brushed off as simply an example

of convergent evolution. These molecular differences likely help explain the dramatic morphological differences in auditory structures between tetrapods and teleost fish, but more study is required for a full understanding of the molecular mechanisms that pattern the otic vesicle.

The ventral outgrowth that gives rise to the cochlear duct is governed by at least two overlapping signaling pathways. Hedgehog signaling within the otic vesicle establishes ventral otic identity establishing gene expression patterns to support cochlear duct outgrowth[31, 27]. As the cochlear duct develops, Gli-activator activity is required for full elongation[32]. Cochlear duct outgrowth also depends on convergent extension governed by Wnt5a[118], Wnt7[117], and multiple PCP effector proteins[147-149]. Although Wnt and Hh both coordinate cochlear duct outgrowth, there are distinct differences in their mutant phenotypes. Altering Hh activity truncates the cochlear duct and induces ectopic patches of sensory hair cells, but does not cause hair cell orientation defects within the organ of Corti[146]. Perturbing PCP signaling also truncates the cochlear duct, but differs from Hh by randomizing hair cell orientation within the organ of Corti.

The dorsal outgrowths that give rise to the semicircular canals will undergo an even more dramatic series of morphogenetic changes. The initial domain of the canals is defined by the expression of the homeobox transcription factor *Dlx5*[150]*.* The cells that makeup a region within the growing out pouches, termed the fusion plate, will ultimately die or be resorbed into the canal proper. The action of the fusion plate depends on the expression of *Netrin1 (Ntn1)*[151], but the mechanism that restricts *Ntn1* to the fusion plate is not fully understood[16].

Nonetheless, *Ntn1* expression is necessary for the wave of apoptosis[152] and possibly cell movement that create the canals. Surprisingly, canal formation does not seem to be affected by mutations in PCP components, indicating that the dramatic morphogenesis that occurs in the developing canals functions independently of PCP signaling.

Neurogenesis

Like the majority of inner ear cells, the neurons that innervate the ear trace their origin to the otic placode. The presumptive neurogenic domain that contains the cells that create in the anteroventral region of the otic placode and vesicle. This region is initially defined by the expression of the neurogenic master regulator *Neurogenin 1 (Ngn1)*[153]*.* Cells within this neurogenic domain proliferate, then express *NeuroD*[154] and begin to delaminate from the otic vesicle. After delaminating, neuroblasts begin to express *Islet-1 (Isl1)*[155] and cease to proliferate. The newly delaminated neuroblasts aggregate to form the cochlear-vestibular ganglion (cvg), which will ultimately split, giving rise to the spiral ganglion innervating the cochlea and Scarpa's ganglion innervating the vestibulum. The exact process that selects a neuroblast for auditory of vestibular fate is poorly understood. Fate mapping studies showed auditory neurons are generated slightly later in development than vestibular neurons[156], and birth dating studies reached similar conclusions[157].

During development an overabundance of inner ear neurons are generated. These excess neurons compete for survival factors expressed by their target tissues. Only a handful of survival factors have been implicated in cvg

maturation. These include *brain derived neurotrophic factor (bdnf)* and *neurotrophin 3(Nt3).* Mutants lacking both factors have an almost complete loss of inner ear neurons[158]. The amount of pruning following this overproduction of neuroblasts is remarkable. In the cvg up to half of all neurons born will fail to receive sufficient trophic support die during development[159, 160].

The prosensory domain

The medial wall of the developing cochlear duct contains a region fated to give rise to auditory hair cells, termed the prosensory domain. This region consists of an equivalence group defined by the expression *Sox2*[161] and Notch signaling components. The presumptive hair cells express high levels of notch ligands *Dll1*[162]*, Jagged2, Jagged1*[163]*,* which signal through *Notch1* in adjacent support cells[164]. Differential notch activity in the prosensory domain limits the number of sensory hair cells through a classic lateral inhibition mechanism. As presumptive hair cells begin to differentiate, they begin to express *Atoh1*, a factor necessary and sufficient for hair cell fate[165, 166], while cells with high levels of Notch activity become support cells, and maintain high levels of the cell cycle inhibitor P27^{kip1}[167, 168]. Several factors in addition to *Sox2* and Notch function within the prosensory domain. *Shh* has been shown to antagonize Notch activity in the prosensory domain and limit the number of sensory hair cells[146], and Wnt/ β-catenin signaling is sufficient to transform auditory hair cells to a vestibular fate in chick[169]. Regardless of the identity of input signals, the entire prosensory domain undergoes terminal mitoses by e15.5[157], and further growth growth is largely a function of tissue remodeling.

Chapter 2: Requirements for Hedgehog signaling in otic development

Introduction

The mammalian inner ear is a sensory organ with dual roles in sound and motion detection. The partitioning of these functions within the inner ear to auditory and vestibular components occurs early in embryonic development, allowing each of these senses to operate independently[170]. The auditory portion of the inner ear, the cochlea, derives from the ventral outgrowth of the otic vesicle, which progressively extends and coils as it matures. Mechanosensory hair cells lining the cochlear duct from base to apex respond to sound waves in a tonotopic manner, and transmit information along auditory (spiral) neurons to sound processing centers in the brain[21, 171]. Vestibular structures, on the other hand, mostly derive from dorsal out-pockets of the otic vesicle and through incompletely understood mechanisms are sculpted into the three semicircular canals, utricle and saccule[170, 172]. Sensory patches associated with each of these structures detect angular movements of the head (semicircular canals) and linear acceleration along the horizontal (utricle) and vertical (saccule) planes. Vestibular neurons innervating each of these sensory patches transmit sensory information to visual, vestibular and proprioceptive centers to coordinate balance[173].

The hindbrain is a critical source of signals necessary for dorsoventral patterning of the otic vesicle and subsequent morphogenesis into auditory and

vestibular components[29, 174-176, 28, 177]. Members of the Wnt and Hedgehog (Hh) families play prominent roles in establishing dorsoventral identity within the otic epithelium. Wnt1 and Wnt3a secreted from the dorsal hindbrain regulate the expression of dorsal otic determinants, such as the homeodomain transcription factors *Dlx5* and *Dlx6* [28, 178]. Consequently, vestibular morphogenesis is completely impaired in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutants[28]. Sonic hedgehog (Shh), secreted from the floor plate of the hindbrain and notochord, opposes the dorsalizing effects of Wnts by repressing *Dlx5*, and activating ventral otic genes, including the transcriptional regulators *Otx2* and *Pax2*[27, 28]. The failure to regulate the ventral otic program in *Shh*$^{-/-}$ embryos results in cochlear agenesis[179-181, 27, 182]. Interestingly, *Shh*$^{-/-}$ embryos also display profound deficits in vestibular development including, malformations of the semicircular canals, utricle, saccule and endolymphatic duct. Each of these morphological defects can be traced back to alterations in otic vesicle patterning genes[27]. For example, the misexpression of *Otx1* and *Gbx2* in the *Shh* mutant otocyst likely explains the absence of the lateral semicircular canal and endolymphatic duct, respectively[183, 184].

Shh also functions in inner ear neurogenesis. The cochlear and vestibular neurons that make up the VIIIth cranial nerve originate from progenitors in the anteroventral region of the otic vesicle that express *Ngn1*, a neural determinant required for their specification[153]. The establishment of the neurogenic domain is one of the earliest signs of asymmetry along the anteroposterior axis of the otic vesicle. The T-box containing transcription factor *Tbx1*, is expressed in a complementary pattern to *Ngn1* and is required to restrict the neurogenic domain

to the anterior portion of the otocyst[185]. *Shh*$^{-/-}$ embryos show a significant reduction in *Ngn1* expression, suggesting a possible involvement in the regulation of anteroposterior identity within the otic vesicle, although the underlying mechanism has not been elucidated[27].

What remains uncertain from these previous studies is the extent to which the inner ear phenotype in *Shh*$^{-/-}$ embryos can be attributed to a direct loss of Shh signaling within the otic epithelium versus an indirect consequence that the absence of Shh has on tissues surrounding the inner ear. The hindbrain and periotic mesenchyme are sources of other signals essential for inner ear development that are also disrupted in *Shh*$^{-/-}$ embryos[29, 186, 176, 187, 27, 28, 188, 189]. Thus, their misregulation could also explain the inner ear defects observed in *Shh*$^{-/-}$ mutants.

The best evidence in support of Shh acting directly on the otic epithelium comes from the observation that *Gli1*, a transcriptional target of the Shh pathway, is expressed in a graded manner along the dorsoventral axis of the otocyst, with higher levels detected ventrally, closer to the source of Shh, and lower levels tapering off dorsally[32]. While suggestive, this result does not resolve the functional significance of this signaling gradient. The analysis of single and compound mutants in *Gli2* and *Gli3*, the transcriptional mediators of Shh signaling, support a model whereby reciprocal gradients of Gli activator and Gli repressor function are required to shape inner ear morphology along the entire dorsoventral axis in response to Shh[32]. Of particular interest was the finding that vestibular, but not auditory, defects could be prevented in *Shh*$^{-/-}$ mutants by

removing a wild type allele of *Gli3* (*Shh*$^{-/-}$;*Gli3*$^{+/-}$). This result suggests that Shh promotes vestibular morphogenesis by reducing Gli3 repressor function[32]. However, it does not address the tissue specificity of this action. Recovery from the vestibular defects in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos could equally be explained by the reduction of Gli3 repression in the inner ear as it could the neural tube, which also shows improvements in patterning and morphology compared to *Shh*$^{-/-}$ embryos[32, 81].

In order to distinguish between the primary requirements for Shh in inner ear development from its secondary roles in surrounding tissues, we generated conditional mutants in which Smoothened (Smo), an essential Hh signal transduction component, was selectively inactivated in the otic epithelium (*Smo*^{ecko}). Our results demonstrate that Shh acts directly on the otic epithelium to regulate ventral target genes that are necessary for the outgrowth of the cochlear duct and saccule. On the other hand, the development of dorsal otic derivatives is indirectly dependent on Shh, as these vestibular structures were absent or malformed in *Shh*$^{-/-}$ mutants but maintained in the ears of *Smo*^{ecko} embryos. The role of Hh signaling in cochlear-vestibular ganglion (cvg) formation is more complex, as it is dependent on both direct and indirect signaling mechanisms. Our data suggest that the loss of cvg neurons in *Shh*$^{-/-}$ animals is partly due to an increase in Wnt responsiveness in the otic vesicle (indirect signaling), resulting in the ectopic expression of *Tbx1* in the neurogenic domain and subsequent repression of *Ngn1* transcription. An unanticipated role for Shh as a mitogen for cvg progenitors was also revealed in our analysis of *Smo*^{ecko} embryos (direct

signaling). These data contribute to a better understanding of the intrinsic and extrinsic signaling properties of Shh during inner ear development.

Materials and Methods

Animals

Foxg1$^{Cre/+}$ and *Smo*$^{loxp/loxp}$ mouse lines were described elsewhere[190, 191]. *Smo*$^{loxp/loxp}$ mice were maintained on a mixed Swiss-Webster, C57BL6/J background. *Shh*$^{+/-}$ [192] and *Rosa*$^{Gfp/Gfp}$ [193] mice were obtained from Jackson Labs (Bar Harbor, ME). *Tbx1*$^{+/-}$ mice were provided by J. Epstein[194]. Topgal mice were provided by E. Fuchs[115].

Immunohistochemistry

For immunohistochemistry, embryos were fixed in 4% paraformaldehyde for 1 hour, cryoprotected in 30% sucrose overnight, mounted in OCT embedding media (Sakura Finetek Torrence, CA) and snap frozen. Embryos were sectioned at 14 µm and stained with DAPI and the following antibodies: Mouse anti-Islet 1 (DSHB) 1:100, Rabbit anti-Phospho Histone H3 (Cell Signaling Technology, Danvers, MA) 1:1000, Rabbit anti-cleaved caspase 3 (Cell Signaling Technology) 1:1000, Rabbit anti-MyosinVIIa (Proteus Biosciences Ramona, CA) 1:300, Mouse anti-Neurofilament (DSHB) 1:200, Chicken anti-GFP (Aves Labs, Tigard, OR) 1:1000, Mouse anti-Gata3 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:50. Primary antibodies were detected with one of the following secondary antibodies: Donkey anti-mouse IGG conjugated to Cy3 (Jackson ImmunoResearch West Grove, PA) or Alexa488 (Molecular Probes Eugene, OR);

Donkey anti-Rabbit IGG conjugated to Cy3 or Alexa488; or Goat anti-Chicken IGG conjugated to Alexa488.

In situ hybridization

For section in situ hybridization, embryos were processed in the same manner as for immunohistochemistry. Sections were rehydrated in PBS containing 0.1% Tween-20, and hybridization was performed as in [195]. For antibody detection after in situ hybridization the following modifications were made: Proteinase K treatment was omitted. After completion of the BM purple reaction, slides were washed three times in PBS-Tween, fixed for 10 minutes with 4% paraformaldehyde then washed three times in PBS-Tween. Slides were then incubated in primary antibody and the immunohistochemistry protocol was followed. Whole mount in situ hybridization was carried out as in [196] using digoxigenin –UTP labeled riboprobes.

Embryo culture

Embryo roller culture was performed as described in [197]. Briefly, E9.5 embryos were collected in ice-cold L-15 media without damaging the yolk sac. Embryos were grown under 95% O_2: 5% CO_2 at 37°C in 100% rat serum (Gemini Bio-Products, West Sacramento CA) supplemented with 0.175 mg/ml glucose, 2 mM glutamine, 1x Penn-Strep. Embryos were re-gassed every 12 hours. LiCl treatment: embryos carrying a *TopGal* transgene were cultured with increasing amounts of LiCl to determine an optimal concentration (50 mM LiCl) that maximized Wnt reporter activity without excessive toxicity (data not shown). Fgf

inhibitor: 3mg/ml EMD341603 dissolved in DMSO was added to culture media to a final concentration of 25μM.

Inner ear paint fill

Inner ear paint fills were performed essentially as described in [172], with the exception that White-out Plus (Bic Corp. Milford CT) was used to fill the inner ears instead of latex paint.

Cell counts

The total number of cells in the cvg was determined by counting Isl1^{+}, cRet^{-} cells in each sequential section through the entire otic vesicle. Bright field images of section in situ hybridizations were inverted, assigned a color and merged with DAPI and antibody channels in Image J. Cells were hand counted using the cell counter plug-in in Image J.

Area measurements

To determine the percent of otic vesicle expressing *Tbx1*, the area of positive staining in lateral whole-mount views was traced in ImageJ and measured, and then divided by the total area of the otic vesicle.

Results

Inactivation of Hedgehog signaling in the otic epithelium

To determine the specific requirements of Hedgehog (Hh) signaling in the inner ear, we generated embryos in which a floxed allele of Smo (*Smo^{loxp})*, an essential mediator of Hh signaling, was selectively inactivated in the otic epithelium using the *Foxg1$^{cre/+}$* mouse line[190, 191]. The *Foxg1$^{cre/+}$* line was

particularly advantageous for our studies because it is active in all otic progenitors well in advance of when Shh signaling is known to be required in the otic vesicle[190, 27]. Moreover, *cre* showed negligible expression in tissues surrounding the otic vesicle including the neural tube and periotic mesenchyme (**Fig. 2.1**). In all experiments described below, at least three to five *Foxg1$^{cre/+}$; Smo$^{loxp/-}$* embryos (herein referred to as *Smo^{ecko}* for ear conditional knockout of *Smo*) were compared to an equal number of control littermates (*Foxg1$^{cre/+}$; Smo$^{loxp/+}$* and *Smo$^{loxp/-}$*). No differences were seen in ear morphology or vesicle patterning between *Foxg1$^{cre/+}$* and *Foxg1$^{+/+}$* genotypes.

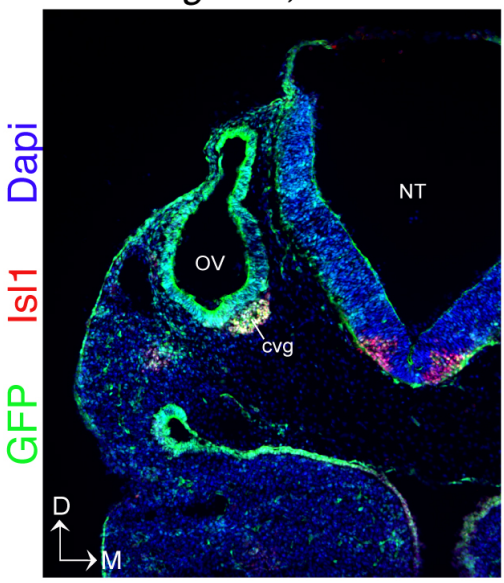

Figure 2.1: *Foxg1^{Cre}* activity
A transverse section through an E10.5 *Foxg1$^{Cre/+}$;Rosa$^{Gfp/+}$* embryo stained for GFP (green), Isl1 (red) and Dapi (blue). Gfp expression indicates robust cre activity in the otic vesicle (OV) while Isl1 labels the presumptive cvg and motor neurons in the neural tube (NT). Little cre activity can be detected in the periotic mesenchyme and neural tube.

We first assessed the effect of deleting *Smo* in the inner ear by examining the expression of *Gli1* and *Ptc1*, two transcriptional targets of Hh signaling. In control embryos, *Gli1* expression initiated weakly at E9.5 in the ventral most region of the otic vesicle (Fig. 2.2A, n=4). At this stage, *Ptc1* was not yet detected in the otic epithelium despite its strong expression in other Shh responsive cell types, including the ventral neural tube and periotic mesenchyme (Fig. 2.2C, n=3). By E10.5, Shh signaling intensified resulting in robust *Gli1* and *Ptc1*

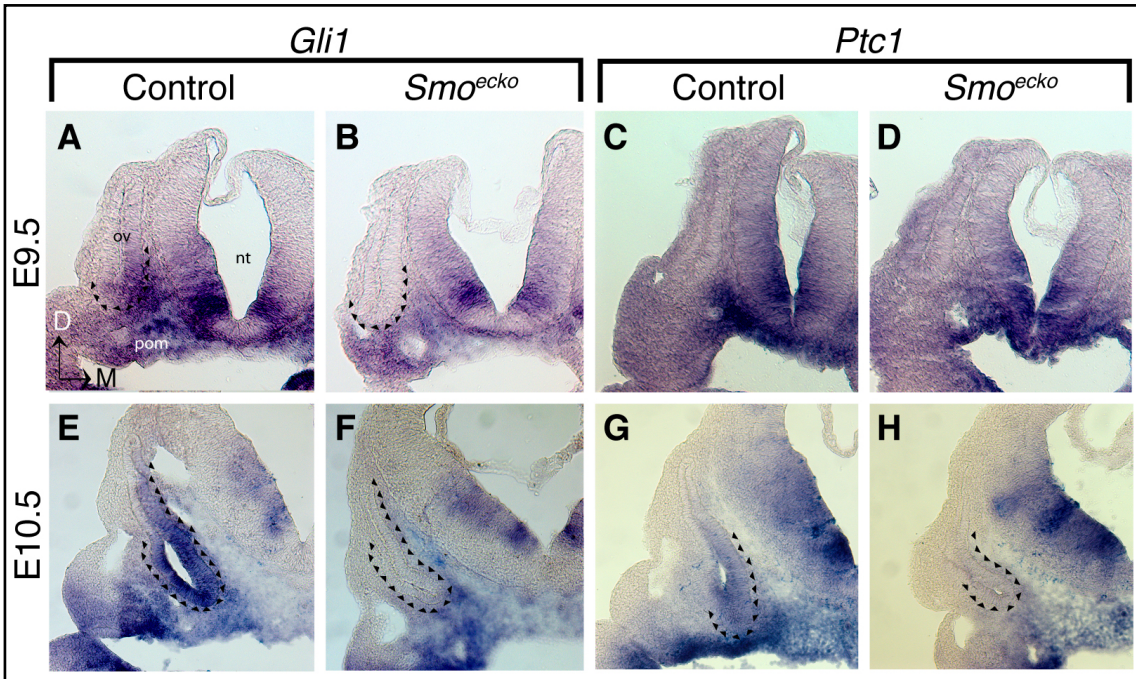

Figure 2.2: Inactivation of Hedgehog signaling in *Smo*^{ecko} otic vesicles.
In situ hybridization for *Gli1* (A,B,E,F) and *Ptc1* (C,D,G,H) on transverse sections through the otic vesicle of control and *Smo*^{ecko} embryos. Arrowheads indicate staining within the otic epithelium. At E9.5, *Gli1* was detected in the otic epithelium of control (A), but not *Smo*^{ecko} (B**)** embryos. *Ptc1* was not detected in the otocyst of either control (C), or *Smo*^{ecko} (D) embryos at this stage. By E10.5, both *Ptc1* and *Gli1* were detected in the otic epithelium of control (E,G), but not *Smo*^{ecko} (F,H) embryos. *Ptc1* and *Gli1* were also detected in the neural tube and periotic mesenchyme of control and *Smo*^{ecko} embryos. Abbreviations: D, dorsal; L, lateral; nt, neural tube; ov, otic vesicle; pom, peri-otic mesenchyme.

staining in ventral regions of the otic vesicle and along the medial wall in a ventral (high) to dorsal (low) gradient (Fig. 2.2E,G,n=3 and 3, respectively) and [32]. *Smo*^{ecko} embryos consistently failed to express *Gli1* and *Ptc1* in the otic epithelium at both stages analyzed, yet robust expression of these markers was observed in the neural tube and periotic mesenchyme (Fig. 2.2B,D,F,H,n=4,3,3,3). Therefore, the disruption to Hh signaling was both specific and complete in the inner ears of *Smo*^{ecko} embryos.

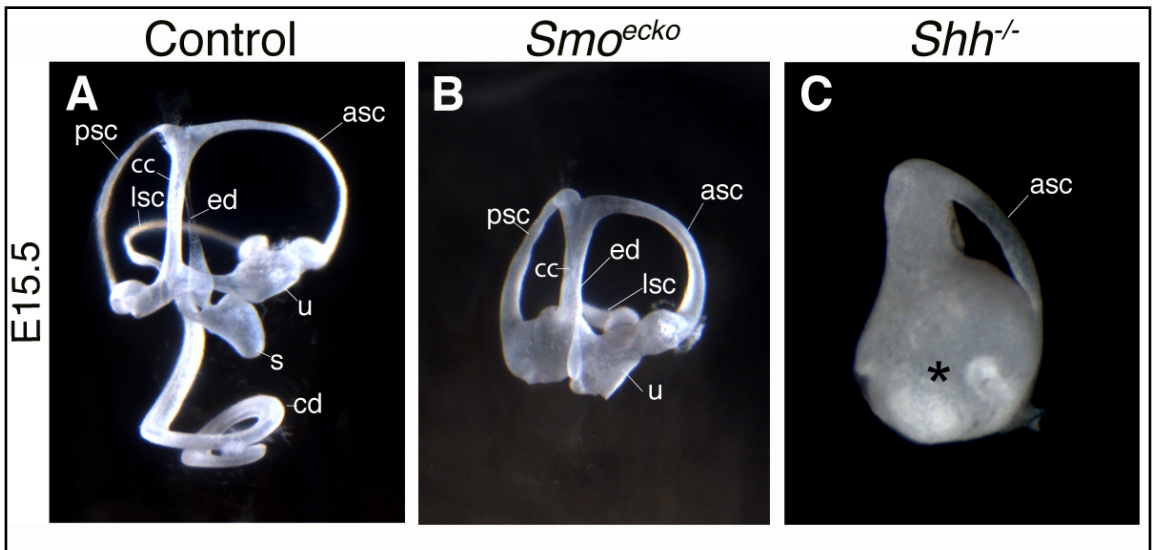

Figure 2.3: Cochlear, but not vestibular, morphogenesis is directly dependent on Hh signaling. Medial view of inner ear paint fills at E15.5. (A) Control inner ears reveal the morphology of the anterior, posterior and lateral semicircular canals (asc,psc,lsc), endolymphatic duct (ed), common crus (cc) utricle (u) saccule (s), and cochlear duct (cd). (B) *Smo*^{ecko} inner ears lacked a cochlear duct and saccule, but all other structures were present. (C) *Shh*$^{-/-}$ inner ears possessed an anterior semicircular canal, but all other structures were missing. The asterisk marks a large cystic structure.

Cochlear, but not vestibular, morphogenesis is dependent on direct Hh signaling within the otic epithelium

Shh$^{-/-}$ embryos show profound vestibular and auditory defects including cochlear agenesis, missing or malformed semicircular canals, as well as absence of the utricle, saccule and endolymphatic duct (Fig. 2.3A,C). If these defects are wholly attributed to the loss of Shh signaling in the otic epithelium, then they should be recapitulated in *Smo*^{ecko} embryos. On the other hand, if some, or all, of these phenotypes result from secondary consequences of perturbing Shh signaling in tissues surrounding the inner ear, then they should be milder in *Smo*^{ecko} embryos.

We visualized the gross anatomy of *Smo*^{ecko} and control inner ears by paint-fill at E15.5 (Fig. 2.3A,B). At this stage, the morphology of the inner ear has reached near full maturity in wild type embryos. The vestibulum, comprising the

three semicircular canals, utricle, saccule, endolymphatic duct and common crus were readily discerned, and the cochlear duct had elongated and coiled 1.5 turns (Fig. 2.3A). Ventral ear structures, namely the cochlear duct and saccule, were entirely absent in *Smo*^{ecko} embryos (n=14 ears), a phenotype similar to that observed in *Shh*$^{-/-}$ mutants (Fig. 2.3B,C). Remarkably, all dorsal otic derivatives, including the semicircular canals, utricle and endolymphatic duct, were present in *Smo*^{ecko} embryos (Fig. 2.3B). The appearance of dorsal vestibular structures in *Smo*^{ecko} embryos contrasts with the pronounced vestibular dysmorphology observed in *Shh*$^{-/-}$ mutants and suggests that dorsal otic derivatives are not directly dependent on Shh for their development. Conversely, the consistent loss of ventral inner ear structures in *Smo*^{ecko} and *Shh*$^{-/-}$ embryos suggests that Shh signaling, acting directly on the otic epithelium, is required for cochlear duct outgrowth and saccule formation.

Direct Hh signaling within the otic epithelium establishes ventral otic identity

At E10.5, the otic vesicle displays regionalized patterns of gene expression that mark competency domains for subsequent development into distinct adult structures[170, 198]. Several of these otic patterning genes are misexpressed in *Shh*$^{-/-}$ embryos[27]. In order to distinguish the genes that are dependent on Hh signaling within the otic epithelium from those that are misregulated due to the secondary effects of disrupting Shh in neighboring tissues, we surveyed their expression by in situ hybridization in *Smo*^{ecko} embryos.

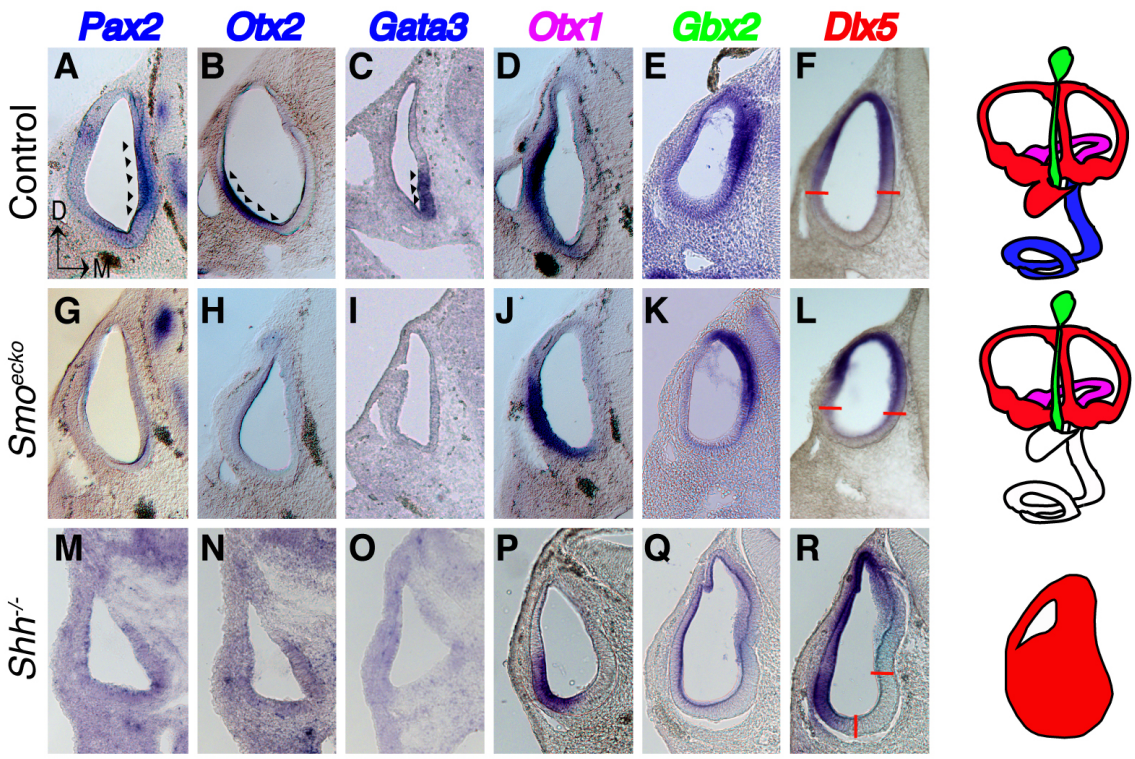

Figure 2.4: Loss of ventral otic markers in *Smo^{ecko}* embryos.
In situ hybridization on transverse sections through the otic vesicle of control (A,B,C,D,E,F), *Smo^{ecko}* (G,H,I,J,K,L) and *Shh$^{-/-}$*(M,N,O,P,Q,R) embryos at E10.5. Arrowheads point to ventral otic expression of (A)*Pax2,* (B)*Otx2*, and (C)*Gata3* in control embryos, which was absent in *Smo^{ecko}* (G,H,I) and *Shh$^{-/-}$ (M,N,O)* embryos. *Otx1* expression in the lateral wall of the otocyst of control embryos (D) was not altered in *Smo^{ecko}* mutants (J), but shifted ventrally in *Shh$^{-/-}$(P)*. The expression of dorsal otic markers *Gbx2* (E,K) and *Dlx5* (F,L) was similar between control and *Smo^{ecko}* embryos. The ventral extent of *Dlx5* expression is marked by red lines (F,L,R). The inner ear schematic is color coded to reflect the requirement of genes for particular structures.

Pax2, Otx2 and *Gata3* are three transcription factors expressed in partially overlapping domains in the ventral otocyst, which are necessary for cochlear duct development[179, 180, 199, 200, 181, 182]. *Pax2* is broadly expressed throughout the otic placode before becoming restricted to the ventromedial wall of the otic vesicle (Fig. 2.4A and data not shown). *Otx2* is also expressed in the ventral region of the otocyst (Fig. 2.4B). The pattern of *Gata3* expression at early stages of otic development is dynamic, but is then localized to the elongating cochlear duct and spiral ganglion (Fig. 2.4C) and [199]. Each of these genes was previously

shown to be downregulated in *Shh*$^{-/-}$ embryos (Fig. 2.4 M-O) and [27]. A comparable reduction in the expression of *Pax2*, *Otx2* and *Gata3* was observed in *Smo*^{ecko} embryos (Fig. 2.4G-I, n=3), suggesting that Shh signaling within the otic vesicle is required for ventral otic identity and subsequent cochlear duct morphogenesis.

Otx1 is expressed in the lateral wall of the otic vesicle at E10.5 and is required for lateral semicircular canal formation (Fig. 2.4D)[183, 201, 202]. A significant ventral shift in the expression of *Otx1* was observed in the otic vesicle of *Shh*$^{-/-}$ embryos (Fig. 2.4P), which likely explains the absence of the lateral semicircular canal in these mutants[27]. In *Smo*^{ecko} embryos, *Otx1* was properly localized to the lateral wall of the otocyst, indicating that Hh signaling within the otic epithelium is not required for lateral otic identity (Fig. 2.4J, n=3). This result also suggests that the lateral semicircular canal defect in *Shh*$^{-/-}$ embryos is an indirect consequence of perturbing Shh signaling in tissues adjacent to the inner ear.

The additional vestibular dysmorphologies observed in the ears of *Shh*$^{-/-}$ mutants can also be explained by patterning changes in the otic vesicle. For instance, the expression of *Gbx2*, a homeodomain containing transcription factor required for endolymphatic duct formation[184], is not maintained in the dorsomedial otocyst of *Shh*$^{-/-}$ mutants (Fig. 2.4Q) and [27]. Moreover, the dorsal otic expression of *Dlx5*, a homeodomain containing transcription factor required for semicircular canal development[203, 150, 204] is expanded ventrally in *Shh*$^{-/-}$ embryos in a Wnt dependent manner (Fig. 2.4R)[28]. These observations

suggested that Shh was necessary for the expression of certain dorsal otic genes (*Gbx2*), while antagonizing the expression of others (*Dlx5,* Topgal). However, the regulation of these dorsal otic genes by Shh appears to be indirect as neither is misexpressed in *Smo*^{ecko} embryos (n=3) (Fig. 2.4E,F, K,L). These data also indicate that the antagonistic interaction between Shh and Wnt signaling pathways responsible for setting up the dorsoventral axis of the otocyst does not stem from a cell intrinsic mechanism within the otic epithelium, but must reside from interplay between these pathways outside of the ear.

Ngn1 expression is not directly dependent on Hh signaling

The neurons that make up the VIIIth cranial nerve and innervate the sensory patches within the inner ear originate from a common progenitor pool in the anteroventral region of the otic vesicle. The bHLH transcription factor, *Ngn1*,

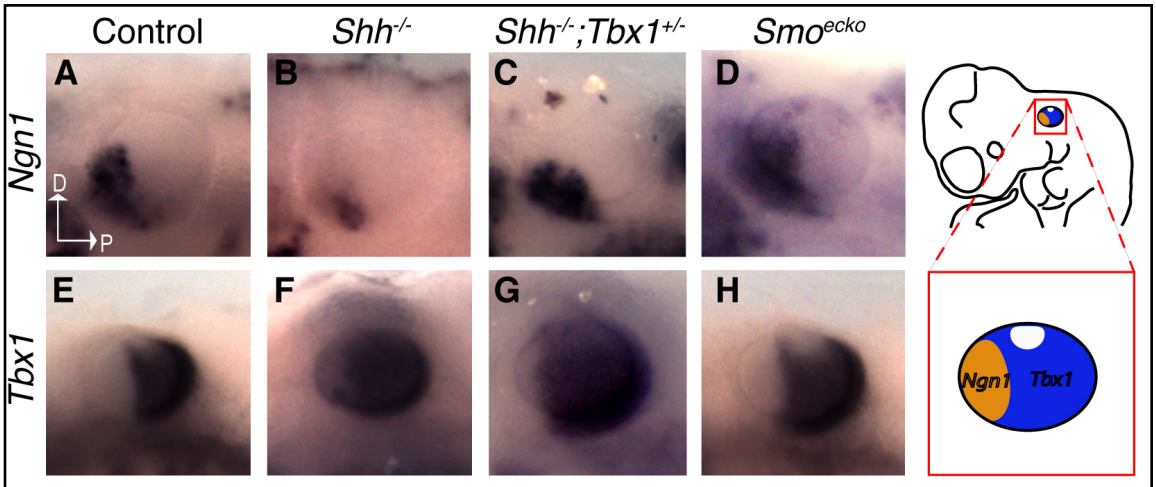

Figure 2.5: Neurogenic patterning is indirectly regulated by Shh.
Lateral surface views of embryos stained by whole mount in situ hybridization for *Ngn1* (A-D), and *Tbx1* (E-H) at E9.5 arranged by genotype. In control embryos, *Ngn1* (A) marks the neurogenic domain, whereas, Tbx1 (E) shows a complementary pattern of expression. In *Shh*$^{-/-}$ embryos, *Ngn1* expression was reduced (B), while *Tbx1* was expanded into the anterior otocyst (F). *Shh*$^{-/-}$*;Tbx1*$^{+/-}$ embryos, showed restored expression of *Ngn1* (C), despite the partial expansion of *Tbx1* into the presumptive neurogenic domain (G). *Smo*^{ecko} embryos revealed a similar pattern of expression for *Ngn1* (D), and *Tbx1* (H) compared to controls. Schematic of embryo shows orientation of otic vesicle in panels (A-H).

is expressed in these neuroblast progenitors (Fig. 2.5A), and is required for their specification[153]. The spatial restriction of *Ngn1* to the anteroventral otic domain is mediated, in part, by the repressive action of *Tbx1*, a T-box containing transcription factor expressed in a complementary pattern to *Ngn1* (Fig. 2.5E) and [185]. In *Tbx1*$^{-/-}$ embryos, *Ngn1* is ectopically expressed resulting in the posterior expansion of neuroblast progenitors[185].

Previous studies demonstrated that *Ngn1* expression is greatly reduced in *Shh*$^{-/-}$ embryos, causing a significant reduction to the size of the cochlear-vestibular ganglia (cvg) (Fig. 2.5B, n=3) and [27]. However, the mechanism underlying the regulation of *Ngn1* transcription by Shh was unclear. Novel insight to this problem came from our observation that *Tbx1* expression had expanded into the neurogenic domain of *Shh*$^{-/-}$ mutant otic vesicles (Fig. 2.5E-G, n=5). This raised the possibility that the failure to repress Tbx1 is an effect of the loss of Shh, and not Ngn1 function. Alternatively, the downregulation in Ngn1 may have prompted the expansion of Tbx1. Since *Tbx1* was not expanded in the otic vesicle of *Ngn1*$^{-/-}$ mutants, the latter prospect was ruled out (data not shown). To address the former possibility, we generated embryos lacking a wild type allele of *Tbx1* on a *Shh*$^{-/-}$ mutant background. We reasoned that if *Tbx1* was responsible for the repression of *Ngn1* in *Shh*$^{-/-}$ embryos, then reducing its dosage should restore *Ngn1* transcription. Notably, the pattern of *Ngn1* expression in *Shh*$^{-/-}$*;Tbx1*$^{+/-}$ embryos was greatly enhanced compared to *Shh*$^{-/-}$ mutants and closely resembled that of controls (Fig. 2.5C, n=3). Thus, Shh indirectly regulates *Ngn1* by restricting *Tbx1* from the neurogenic domain.

We next determined whether the repression of *Tbx1* from the neurogenic domain was a direct or indirect action of Shh on the otic epithelium. Both *Tbx1* and *Ngn1* were properly localized to their respective otic territories in Smo^{ecko} embryos (Fig. 2.5D,H, n=3 and 6, respectively), arguing that their misregulation in $Shh^{-/-}$ mutants was a secondary consequence of disrupting Shh signaling in tissues extrinsic to the inner ear.

Opposing roles for Wnt and Fgf signaling pathways in cvg neurogenesis

If Shh is not acting directly on the otic epithelium to regulate the anteroposterior positioning of the neurogenic lineage, then what is the responsible signal(s)? Select members of the Wnt and Fgf families appeared to be excellent candidates based on prior studies. For instance, Wnts secreted from the dorsal hindbrain were shown to partially suppress the neurogenic lineage[28]. Whereas, Fgfs were shown to both repress and activate neuronal determinants in the otic epithelium[205-207]. In $Fgf3^{-/-}; Fgf10^{-/-}$ mouse embryos, neuroblast progenitors were ectopically expressed in the posterior otocyst[207]. Conversely, the pharmacological inhibition of Fgf signaling in the chick otic vesicle caused a dramatic reduction in the expression of *Ngn1* and *NeuroD* and a corresponding loss of cvg neurons[206]. These seemingly contradictory results may be attributed to species-specific differences in Fgf signaling activity and/or temporal differences in Fgf ligand utilization.

To investigate whether modulation of Wnt or Fgf signaling pathways could mimic aspects of the $Shh^{-/-}$ neurogenic phenotype, we cultured wild type mouse

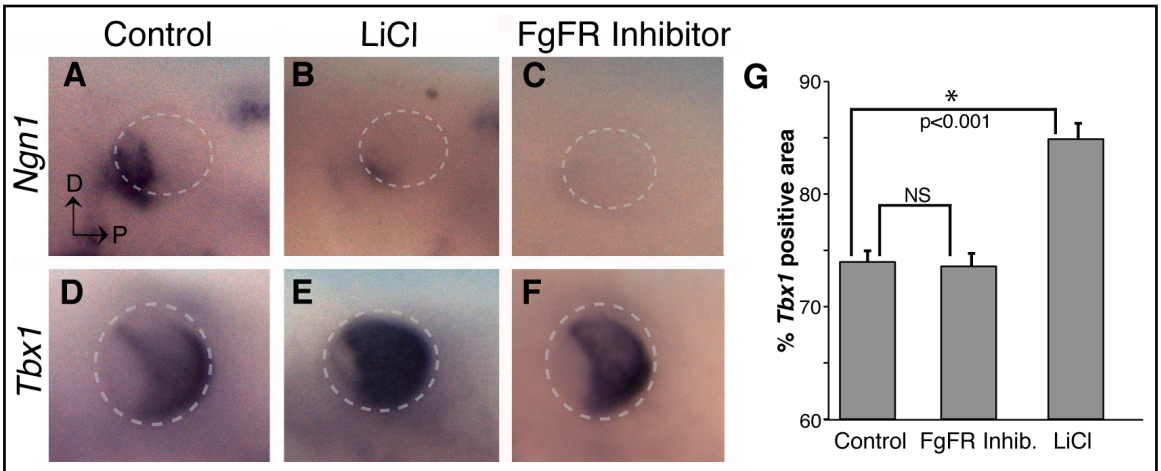

Figure 2.6: Extrinsic signals regulate *Tbx1* expression.
Lateral surface views of embryos stained by whole mount in situ hybridization for *Ngn1* (A-C), and *Tbx1* (D-F), after being cultured for 18 hours in the presence of control media (A,D), 50 mM LiCl (B,E), or 25 μM of the FgfR inhibitor EMD 341603 (C,F). Control embryos showed a normal pattern of *Ngn1* (A) and *Tbx1* (D,G). Embryos cultured in LiCl showed reduced *Ngn1* expression (B), concomitant with an anterior expansion of *Tbx1* (E,G). In contrast, embryos cultured in the FgfR inhibitor showed a severe reduction in *Ngn1* (C), with no change in the domain of *Tbx1* expression (F,G). (G) Quantification of the area of the otic vesicle expressing *Tbx1* in terms of percentage. Error bars represent S.E.M., NS not statistically significant by unpaired t-test.

embryos in the presence or absence of the canonical Wnt signaling agonist, LiCl [208], or Fgf signaling antagonist, EMD 341608 [209], and assayed the expression of *Ngn1* and *Tbx1*. Wild type embryos harvested at E9.5 and cultured in control media for 18 hours showed proper anterior and posterior expression of *Ngn1* and *Tbx1*, respectively (Fig. 2.6A,D, n=8/9 and 17/20, respectively). However, when embryos were cultured in either LiCl (n=5/5) or EMD 341608 (n=6/8), they showed a consistent and profound downregulation of *Ngn1* in the anterior otocyst (Fig. 2.6B,C). These results confirm that Wnt signaling antagonizes, while Fgf signaling is necessary for, *Ngn1* expression in the mouse otocyst. Interestingly, the anterior otic expansion of *Tbx1* was only observed in embryos cultured in LiCl (n=6/8), and not EMD 341608 (n=0/15) (Fig. 2.6E-G, P<0.001, unpaired t-test). Thus, heightened Wnt signaling better recapitulated the a/p polarity defects observed in *Shh*$^{-/-}$ embryos than did Fgf inhibition. The upregulation of canonical

Wnt signaling in the otic vesicles of *Shh*$^{-/-}$ embryos is a fitting explanation for the anterior expansion of *Tbx1* and consequent reduction in *Ngn1* transcription [28].

Shh is a mitogen for cvg progenitors

To determine if Shh has other functions in cvg formation we quantified the number of Isl1^{+} neurons in *Smo*^{ecko} embryos at E10.5 (36-38 somites), corresponding to the midway point of cvg neurogenesis (Fig. 2.7). Double labeling studies were performed with *c-Ret*, to distinguish the cvg (Isl1^{+}, cRet^{-})

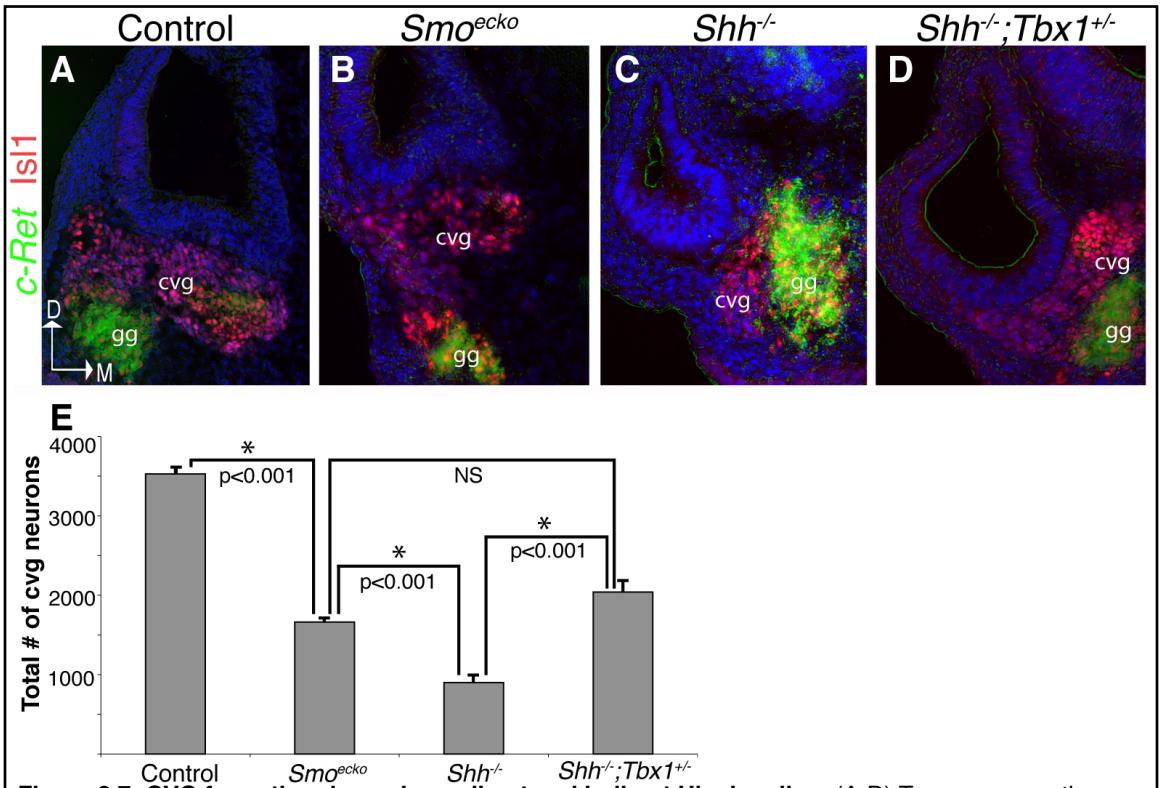

Figure 2.7: CVG formation depends on direct and indirect Hh signaling. (A-D) Transverse sections through the anterior region of the otic vesicle of E10.5 embryos arranged by genotype were stained for Isl1 (red) and *cRet* (green). Neurons of the cochlear-vestibular ganglia (cvg) (Isl1^{+}, cRet^{-}) were distinguished from the adjacent geniculate ganglia (gg) (Isl1^{+}, cRet^{+}) by the absence of cRet staining. Control embryos (A), showed a greater number of cvg neurons compared to *Smo*^{ecko} mutants (B). An even greater reduction of cvg neurons was observed in *Shh*$^{-/-}$ embryos (C). The number of cvg neurons in *Shh*$^{-/-}$;*Tbx1*$^{+/-}$ embryos (D), was increased compared to *Shh*$^{-/-}$ embryos, and comparable to that observed in *Smo*^{ecko} mutants. (E) Quantification of the total number of cvg neurons in embryos from each genotype is shown. Error bars represent S.E.M., NS not statistically significant using Tukey-Kramer multiple comparisons test.

from the nearby neural-crest derived geniculate ganglia (Isl1^{+}, cRet^{+}) [210, 155]. *Smo^{ecko}* embryos displayed a 47% reduction in the number of Isl1^{+} cvg neurons (1661+/- 51, n=3) compared to control littermates (3529 +/- 86, n=4), indicating that Hh signaling has an additional function within the otic epithelium to regulate an aspect of cvg neurogenesis that is distinct from its role in controlling *Ngn1* expression (Fig. 2.7A,B,E). Interestingly, the number of cvg neurons was not significantly different between *Shh$^{-/-}$;Tbx1$^{+/-}$* (2039+/- 146, n=3) and *Smo^{ecko}* embryos (Tukey-Kramer multiple comparisons test) (Fig. 2.7B,D,E), implying that the further reduction in cvg neurons in *Shh$^{-/-}$* (899+/- 95, n=3) compared to *Smo^{ecko}* embryos, was indeed due to the decrease in *Ngn1* expression (Fig. 2.7B,C,E).

Shh signaling is essential for the proliferation and survival of several populations of neurogenic progenitors[211-213]. To determine whether Shh functions as a mitogen or survival factor for cvg neuroblasts, we compared the number of mitotically active (phospho-Histone H3^{+}) and apoptotic (activated Caspase-3^{+}) cells in distinct otic regions between *Smo^{ecko}* and control embryos at E9.5 (23

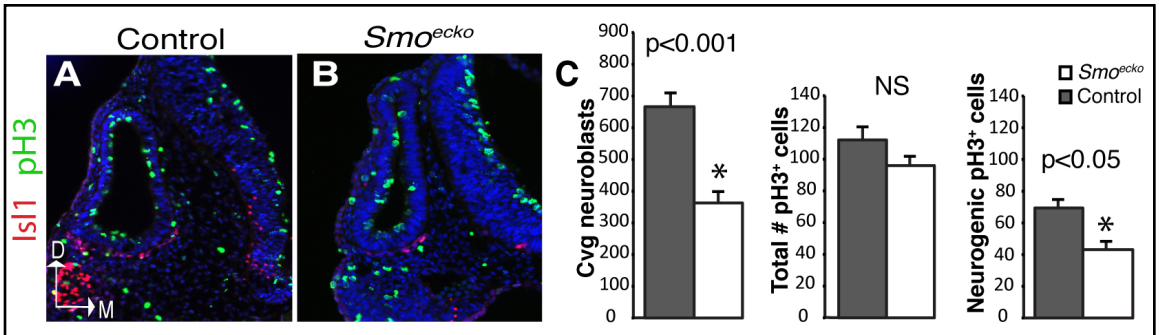

Figure 2.8: A mitogenic role for Hh on cvg progenitors. Transverse sections through control (A), and *Smo^{ecko}* (B) embryos at the 23 somite-stage stained for Isl1 (red) and phospho-histone H3 (green). (C) The number of Isl^{+} cvg neurons and proliferating neuroblasts was significantly reduced in *Smo^{ecko}* embryos compared to control littermates, whereas the total number of phospho-histone H3^{+} cells throughout the otic vesicle was unchanged. Error bars are S.E.M., NS not statistically significant by unpaired t-test.

somite stage), shortly after otic vesicle closure. While the total number of mitotically active cells throughout the otic epithelium not statistically different between *Smo*^{ecko} (96+/-7 cells) and control embryos (112+/-7 cells), a significant reduction in the number of phospho-Histone H3^{+} cells was observed in *Smo*^{ecko} embryos when only the anteroventral domain was considered (43+/-4 vs. 69+/-5 cells, $p<0.05$ unpaired t test) (Fig. 2.8A-C, n=4). The reduction in proliferating otic neuroblasts in *Smo*^{ecko} embryos correlated with a deficit in cvg neurons, which was readily apparent at E9.5 (Fig. 2.8A-C). The number of apoptotic cells did not differ between *Smo*^{ecko} and control embryos (data not shown). Taken together, these results indicate that Shh is necessary for the proliferation of neurogenic progenitors in the inner ear.

Auditory and vestibular neurons are specified in *Smo*^{ecko} embryos

The cvg comprises a heterogeneous population of presumptive auditory and vestibular neurons. Lineage tracing studies suggest that the fate of these inner ear neurons is decided early, possibly prior to their delamination from the otic vesicle[156]. Shh plays a prominent role in assigning identity to neuronal progenitors in the ventral neural tube[214]. To address whether Shh functions in a similar capacity to promote the fate of vestibular and auditory neurons, we evaluated cell-type specific properties of the remaining neurons in *Smo*^{ecko} embryos. Unfortunately, no molecular markers have been described that distinguish auditory from vestibular neurons at progenitor stages of their development. We therefore examined unique aspects of their identity after their

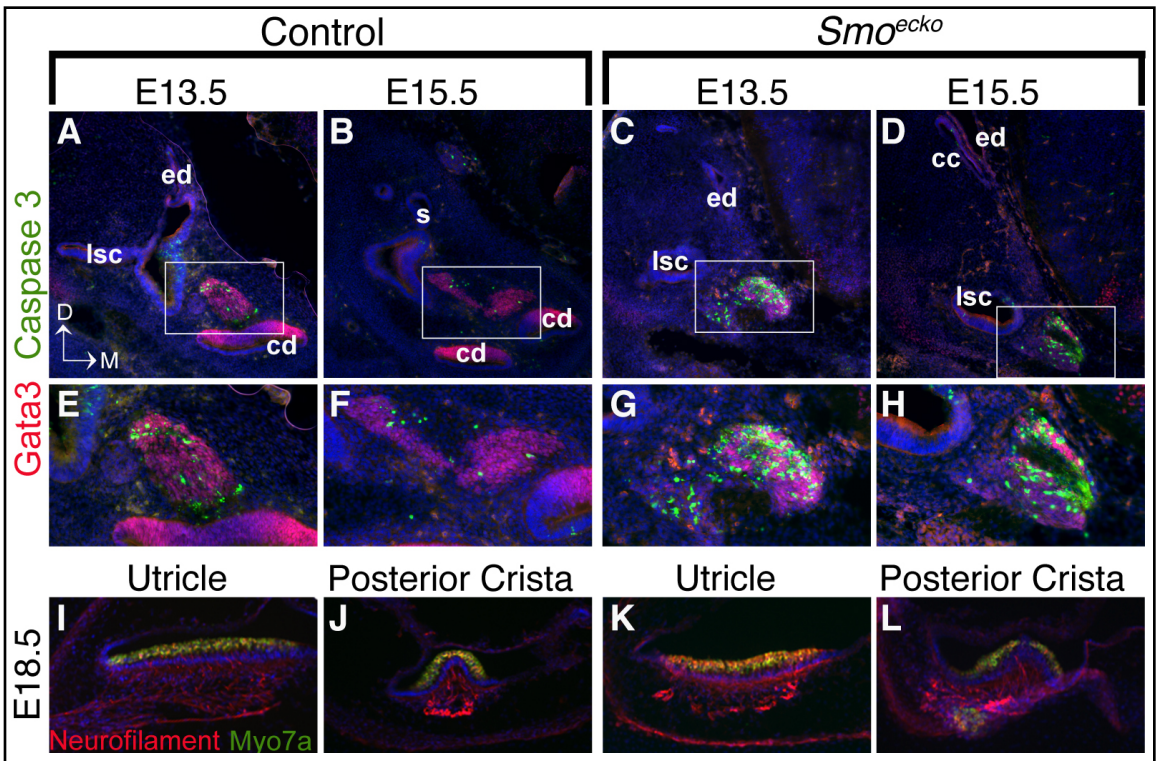

Figure 2.9: Specification of both spiral and vestibular neurons in *Smo*^{ecko} embryos. Transverse sections through embryos at E13.5 (A,C,E,G) and E15.5 (B,D,F,H) stained for active Caspase 3 (green) and Gata3 (red). (E,F,G,H) are higher magnification views of the regions outlined by white boxes in (A,B,C,D). (A,E) In control embryos at E13.5, Gata3 staining was observed in the spiral ganglia (sg) and prosensory domain of the cochlear duct (cd), which showed negligible amounts of apoptosis. By E15.5, the cochlear duct had completed an additional coil (B) and there was limited apoptosis in the spiral ganglia (F). In E13.5 *Smo*^{ecko} embryos, Gata3 staining was observed in the vicinity of the spiral ganglia (C,G) despite the absence of a cochlear duct. These neurons were apoptotic and stained strongly for active caspase 3. By E15.5, only a few remaining Gata3^{+} spiral ganglion neurons were detected in *Smo*^{ecko} embryos (D,H), which were Caspase 3^{+}. (I-L) Transverse sections through the utricle (I,K) and posterior ampula (J,L) at E18.5. Both control (I,J) and *Smo*^{ecko} (K,L) embryos displayed MyoVIIa^{+} hair cells (green) innervated by Neurofilament^{+} neurons (red). Abbreviations: lsc, lateral semicircular canal; s, saccule.

physical separation into spiral (auditory) and vestibular ganglia. At E13.5, spiral neurons can be identified by their expression of the *Gata3* transcription factor, which also marks the prosensory domain of the cochlear duct (Fig. 2.9A,E)[199, 215]. A population of cells expressing Gata3 was observed in *Smo*^{ecko} embryos, despite the absence of a cochlear duct (Fig. 2.9C,G). Unlike control embryos, however, the Gata3^{+} cells from *Smo*^{ecko} mutants also stained positively for activated Caspase-3 (Fig. 2.9C,G), suggesting that spiral neurons were specified

in the absence of Hh signaling but subsequently underwent apoptosis. By E15.5, the Gata3^{+}, Caspase-3^{+} cell population was dramatically reduced compared to control littermates (Fig. 2.9B,F,D,H), and by E18.5, Gata3^{+} cells could no longer be detected in *Smo^{ecko}* embryos (data not shown). The timing of the death of spiral neurons in *Smo^{ecko}* ears is consistent with when they normally become dependent on Bdnf and NT-3, two neurotrophins secreted from cochlear hair cells, which are required for their survival[158].

The innervation of vestibular hair cells by vestibular neurons was evaluated by immunostaining with antibodies against Myosin VIIa and Neurofilament, respectively, at E18.5. Vestibular hair cells in all three cristae and the utricular macula showed proper patterns of innervation in *Smo^{ecko}* embryos compared to control littermates (Fig. 2.9I-L). These data indicate that Hh signaling is not required for the specification of vestibular hair cells or the neurons that innervate them.

Discussion

Ventral, but not dorsal, otic identity is dependent on direct Hh signaling in the otic epithelium

Previous work described an essential role for Shh, secreted from the notochord and floor plate of the hindbrain, in shaping inner ear development along its dorsoventral axis[29, 27]. However, given the pleiotropic nature of Shh function, it was unclear from these studies whether Shh dependent phenotypes were directly attributed to a blockade in Shh signaling within the otic epithelium,

or instead, were due to the loss of secondary signals from Shh responsive tissues surrounding the inner ear.

To determine which aspects of otic development are dependent on direct Hh signaling we generated *Smo^{ecko}* embryos, in which only the otic epithelium was prevented from responding to Hh, and compared them to *Shh$^{-/-}$* mutants. The occurrence of cochlear and saccular agenesis in both *Smo^{ecko}* and *Shh$^{-/-}$* embryos indicated that these otic phenotypes were directly attributed to impaired Shh signaling within the ventral otocyst. Our data further showed that Shh promotes ventral otic identity by regulating the expression of *Pax2, Otx2,* and *Gata3,* which are downregulated in both *Shh$^{-/-}$* and *Smo^{ecko}* mutants (see model in Fig 2.10A-C).

Our unanticipated finding that *Smo^{ecko}* embryos did not exhibit any of the other vestibular defects observed in *Shh$^{-/-}$* mutants (absence or malformation of semicircular canals, utricle and endolymphatic duct) suggests that Shh signaling in the dorsal otocyst is dispensable for vestibular morphogenesis. In keeping with these findings was our observation that a select number of dorsal otic genes (e.g. *Dlx5*, *Gbx2),* which were misregulated in *Shh$^{-/-}$* embryos, were appropriately expressed in *Smo^{ecko}* mutants (Fig. 2.10). Given these results, the validity of the prevailing model that a graded distribution of Shh signaling activity patterns the extent of the dorsoventral axis of the otic vesicle is drawn into question (see below).

Dorsal otic patterning does not require discrete levels of Gli-R

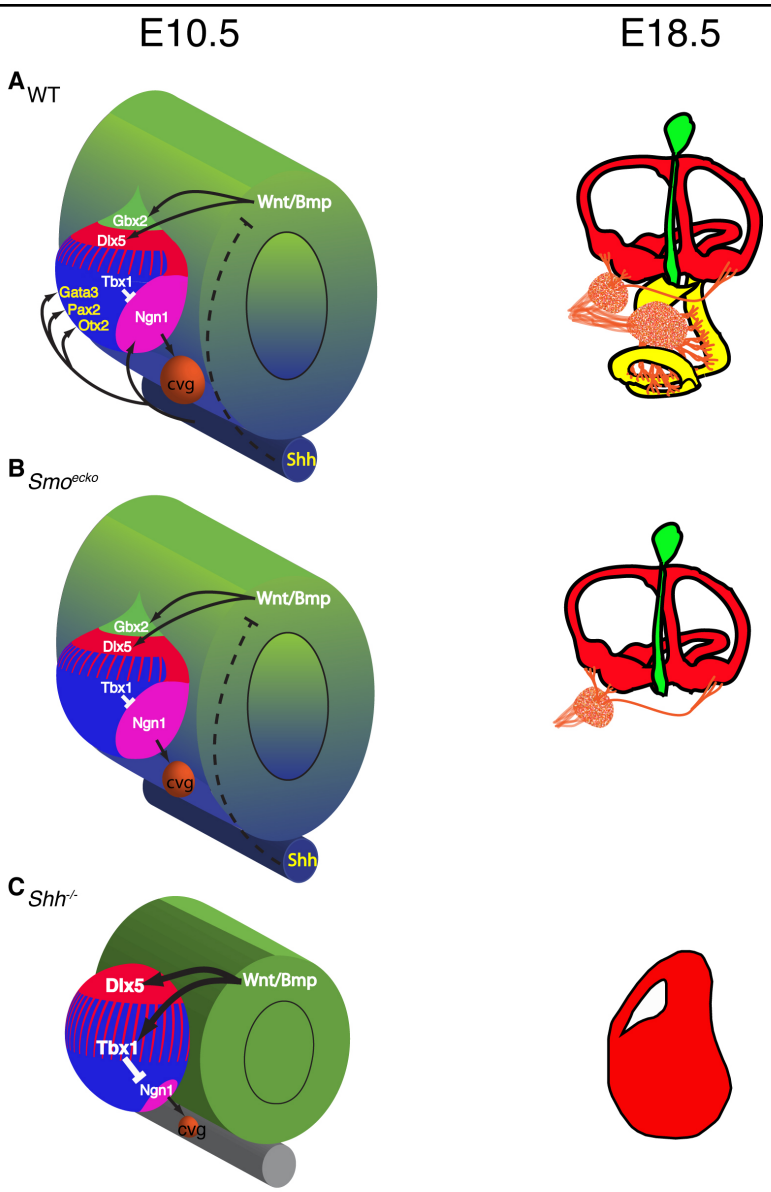

Figure 2.10: A model depicting the direct and indirect roles of Shh during otic development. (A) In wild type embryos at E10.5, Shh secreted from the floor plate and notochord signals directly to the otic epithelium to regulate ventral otic identity (*Gata3, Pax2, Otx2)* and proliferation of Ngn1^{+} neuroblasts. Shh also acts outside of the ear (dashed line) to restrict canonical Wnt signaling activity to the dorsal otocyst (red). This indirect role of Shh is also important for preventing Tbx1 (blue) from being expressed in the neurogenic domain (magenta). The schematic of a morphologically mature inner ear (E18.5) is shown to the right. Color-coding of vestibular (red) auditory (yellow) and neuronal cell types matches their developmental origin in the otic vesicle. (B) In *Smo^{ecko}* embryos, the otic epithelium is no longer responsive to Shh, resulting in the loss of ventral otic identity (*Gata3, Pax2, Otx2)* and a reduction in the number of proliferating Ngn1^{+} neuroblasts, reflected by the smaller cvg. However, Shh dependent functions outside the ear (indirect signaling) are maintained. The loss of ventral otic identity results in cochlear and saccular agenesis at E18.5, but the remaining vestibular structures are intact, including their pattern of innervation. (C) In *Shh$^{-/-}$* embryos, all Shh signaling activity is lost (both direct and indirect). Consequently, ventral otic identity is compromised and Wnt signaling is no longer restricted to the dorsal otocyst. The heightened Wnt responsiveness in the ear causes expanded domains of *Dlx5* ventrally, and *Tbx1* anteriorly, which alters vestibular development and cvg neurogenesis at E18.5.

The observation that *Gli1* is expressed in a graded manner along the dorsoventral axis of the ear supported the hypothesis that a gradient of Hh activity patterns the otocyst[32]. According to this model, the loss of Shh signaling causes levels of Gli-Repressor (Gli-R) to increase at the expense of Gli-Activator (Gli-A), resulting in otic patterning defects and consequent inner ear dysmorphologies[32]. The failure of cochlear duct outgrowth in $Shh^{-/-}$ and Smo^{ecko} embryos, which are predicted to have high ratios of Gli3R:GliA in ventral otic regions, is in agreement with this model. Moreover, the restoration of vestibular development in $Shh^{-/-};Gli3^{+/-}$ embryos was taken as evidence in favor of a lower ratio of Gli3R:GliA being required for the patterning of dorsal, compared to ventral, otic structures. However, the absence of vestibular defects in Smo^{ecko} embryos does not fit the hypothesis that graded Hh signaling is responsible for patterning the entirety of the dorsoventral otocyst. The presence of a well-formed vestibulum in Smo^{ecko} embryos indicates that higher levels of Gli3-R in the dorsal otocyst do not influence vestibular development. Instead, we attribute the cause of the vestibular dysmorphologies displayed by $Shh^{-/-}$ embryos to secondary consequences of disrupting Shh signaling in periotic tissues. The rescue of dorsal otic structures in $Shh^{-/-};Gli3^{+/-}$ embryos can be viewed in a similar manner, where removing one allele of *Gli3* partially rescues Shh dependent cell types and secondary signals present in the hindbrain, which influence dorsal vestibular morphogenesis. Therefore, if graded Hh signaling is acting on the otic epithelium, it is only necessary for patterning ventral otic structures.

Cvg neuroblasts are directly dependent on Shh for their proliferation

Our study unmasked a previously unappreciated role for Shh in regulating the expansion of cvg progenitors. This mitogenic role for Shh is similar to that demonstrated for other neuronal cell types in the cerebellum and ventral neural tube[211, 216, 212, 213, 217]. Proliferation was reduced in the neurogenic domain of *Smo*^{ecko} embryos by 47%. The resulting deficit in cvg progenitors did not selectively eliminate one class of neurons as both vestibular and spiral neurons were present in *Smo*^{ecko} embryos. Interestingly, the vestibular neurons in *Smo*^{ecko} embryos innervated their sensory targets in the cristae and maculae, whereas the auditory neurons failed to survive, likely due to a lack of trophic support normally provided by cochlear hair cells[158].

It is intriguing to speculate why a normal pattern of vestibular innervation was achieved in *Smo*^{ecko} embryos, despite the significant reduction in cvg neurons. As with many neuronal cell types, progenitors are usually generated in excess, which over time undergo apoptosis after failing to compete for a limited number of synaptic targets. The process of eliminating surplus neurons by cell death is highlighted in *Bax*$^{-/-}$ mice, which lack a key apoptotic regulator, and have a 83% increase in vestibular ganglia[160]. In chick, 24% of vestibular neurons undergo apoptosis during synapse formation, suggesting that this mechanism is not limited to mammals[159]. Given the large fraction of vestibular neurons that are normally lost to cell death, the reduction in cvg neurons in *Smo*^{ecko} embryos is not likely to profoundly affect the elaboration of vestibular neural circuitry.

Tbx1 expression is indirectly regulated by Shh

An additional role for Shh in regulating cvg neurogenesis was indicated by the reduced expression of *Ngn1* in the anteroventral otic region of *Shh*$^{-/-}$ embryos [27]. We now show that Shh signaling does not regulate *Ngn1* transcription directly, but rather is required to restrict Tbx1, a known repressor of *Ngn1*, from the neurogenic domain[185]. This conclusion is supported by the observation that *Ngn1* expression and many of the cvg neuroblasts are restored in *Shh*$^{-/-}$*;Tbx1*$^{+/-}$ compound mutants (Figs. 2.5,2.7). Nevertheless, the anterior otic expansion of *Tbx1* appears to be a secondary consequence of losing Shh, since *Tbx1* and *Ngn1* were unaffected in *Smo*^{ecko} embryos. We determined that heightened Wnt signaling is likely responsible for this neurogenic phenotype, given that it correlates with the anterior otic expansion of *Tbx1* in both *Shh*$^{-/-}$ mutants and embryos treated with LiCl (Fig. 2.6; and [28]).

The antagonism of Wnt signaling by Shh is not mediated in a cell autonomous manner within the otocyst. Therefore, the negative interaction between these two pathways must take place in tissues extrinsic to the ear (Fig. 2.10B,C). One way this might occur is if Shh limits the range of Wnt ligands secreted from the neural tube. Wnt1 and Wnt3a in the dorsal hindbrain are known to regulate *Dlx5* expression in the dorsal otocyst[28]. The range of these Wnts appeared to expand in *Shh*$^{-/-}$ embryos as evidenced by the ventralized expression of *Dlx5*, as well as the Wnt responsive Topgal reporter[28]. A similar mechanism may also explain the anterior expansion of *Tbx1* in the otic vesicles of *Shh*$^{-/-}$ embryos (Fig. 2.10C).

While the upregulation of Wnt signaling activity may explain the anteroposterior polarity defects observed in the otic vesicle of *Shh*$^{-/-}$ embryos, it remains unclear what role Wnts normally play in the regulation of *Tbx1* expression. The conditional inactivation of β-catenin, a transcriptional mediator of canonical Wnt signaling, at early stages of otic development caused a profound reduction in the size of the otic vesicle, yet *Tbx1* expression remained properly localized[218]. Interestingly, retinoic acid was recently shown to positively regulate *Tbx1* expression in the posterior domain of the otic vesicle[140]. Thus, Wnt signaling may only impact on *Tbx1* expression in the absence of Shh, whereas retinoic acid normally activates *Tbx1* in the otic epithelium.

The regulation of *Tbx1* by Shh is context dependent. As described above, Shh negatively regulates *Tbx1* in the neurogenic domain of the otic epithelium. However, within the pharyngeal and periotic mesoderm Shh promotes *Tbx1* expression[219, 27, 189]. Mice lacking *Tbx1* in the periotic mesoderm show reduced expression of Cyp26 family members, which regulate the catabolism of retinoic acid[186]. Consequently, retinoic acid signaling is upregulated in the otic epithelium of these mice, resulting in cochlear outgrowth defects. Therefore, cochlear development is dependent on Shh signaling in both the otic epithelium and periotic mesenchyme.

In summary, we found that Shh signaling acting directly on the otic epithelium is necessary for the establishment of ventral otic identity and the proliferation of cvg neural progenitors. We found that Shh acting in periotic tissues regulates the anterior-posterior expression of *Tbx1* in the

otic vesicle, possibly through a canonical Wnt intermediate. Overall these studies contribute to the understanding of Shh signaling in the developing ear and refute the idea that discrete amounts of Gli-Repressor activity is required for vestibular development.

Chapter 3: Hedgehog target genes in otic development

Introduction

Hedgehog signaling within the mammalian inner ear is necessary for the establishment of ventral otic identity and the formation of auditory structures[31, 220, 27]. However, the targets of Hh in the ear, and by extension the mechanism of Hh action within the ear, remain unknown. To address the mechanism of Hh action in the ear we have investigated gene expression levels in *Smo*^{ecko} mutant otic vesicles. Hedgehog signaling is disrupted specifically in the *Smo*^{ecko} otic vesicle leaving adjacent Hedgehog responsive tissues unperturbed. *Smo*^{ecko} mutants lack ventral otic identity and auditory structures, while retaining a properly formed vestibulum[31], suggesting the dorsal and ventral domains of the inner ear can form independently. Since *Smo*^{ecko} mutants specifically lack ventral, but not dorsal otic tissue, we reasoned that genes with reduced expression in *Smo*^{ecko} mutants when compared to control litter-mates likely function during cochlear duct formation. We used microarrays to interrogate gene expression levels in otic vesicles isolated from the surrounding mesenchyme shortly after the start of

cochlear duct outgrowth at E11.5 (Fig. 3.1). We identified many genes known to have reduced expression in *Smo*^{ecko} mutants including: *Smo, Gli1, Ptc1, Pax2, Otx2*[31]*.* We also found many genes essential for auditory function had reduced expression in *Smo*^{ecko} mutants. Based on the positive identification of genes known either to be altered in *Smo*^{ecko} mutants or function in the ear, we are optimistic that novel genes identified by this approach likely function during ear development. We failed to detect a significant difference in levels of dorsally expressed genes including *Gbx2* or *Dlx5*, consistent with our previous study of *Smo*^{ecko} mutants. Genes with increased expression in *Smo*^{ekco} mutants included several with mesenchymal function such as *Foxc2*[221].

Materials and Methods

Animals:

Foxg1$^{Cre/+}$, *Smo*$^{loxp/loxp}$ and *ShhP1* mouse lines were described elsewhere[190, 191, 27]. *Smo*$^{loxp/loxp}$ mice were maintained on a mixed Swiss-Webster, C57BL6/J background. *Shh*$^{+/-}$ [192] mice were obtained from Jackson Labs (Bar Harbor, ME). Cag-GFP mice were described in [222], and obtained from the Campbell lab, University of Cincinnati.

Tissue isolation:

Otic vesicles were microdissected at E11.5 from *Foxg1*$^{Cre/+}$;*Smo*$^{loxp/-}$*;Cag-GFP*$^{+/-}$ and *Foxg1*$^{Cre/+}$;*Smo*$^{loxp/+}$*;Cag-GFP*$^{+/-}$ litter-mates in ice cold PBS. Isolated vesicles were treated for 10 min with 2 mg/ml collagenase P (Roche, Indianapolis, IN) to disrupt the periotic mesenchyme, then further removed from

mesenchyme using forceps. Otic vesicles were then submerged in RNAlater (Qiagen Valencia, CA) at 4°C.

RNA isolation and Microarray analysis

Total RNA was extracted using RNAeasy kit (Qiagen Valencia, CA). Quality was assessed using a Nanodrop2000 (Thermo Scientific Wilmington, DE) to confirm the $A_{260/280}$ was greater than 1.9, The RNA Integrity Number, a measure of sample quality, was determined to be above 9 using a Pico RNA chip (Agilent Bioanalyzer Pico assay). cDNA was generated using WT-Ovation RNA amplification system (NuGEN San Carlos, CA) and hybridized to to GeneChip Mouse Gene 1.0ST array (Affymetrix Santa Clara, CA). Signal intensity was determined using an Axon GenePix 4000B scanner (Molecular Devices Sunnyvale, CA) in the University of Pennsylvania MicroArray Core facility.

qPCR

Otic vesicles were dissected from E9.5 embryos using tungsten needles. E18.5 whole brain and whole ears were dissected with forceps. Total RNA was extracted using Trizol (Invitrogen Carlsbad, CA) according to manufacturers instructions. cDNA was synthesized using an Applied Biosystems High capacity cDNA kit (Applied Biosystems Carlsbad, CA) according to manufacturers instructions. Two step real time quantitative PCR was performed on an Applied Biosystems 7900 thermocycler using Applied Biosystems SYBR green master mix according to manufacturers instructions. Primers used are listed in Table 3.1. Relative expression was determined by comparing to *GapDH* using the ΔΔCT method established in [223].

Section In situ hybridization

For in situ hybridization, embryos were fixed in 4% paraformaldehyde 1 hour, cryoprotected in 30% sucrose overnight and embedded in OCT embedding media (Sakura Finetek Torrence, CA) and snap frozen. Embryos were sectioned

Table 3.1 qPCR primers

Gene Name	Forward Primer	Reverse Primer	Size
GapDH	CCTCGTCCCGTAGACAAAATG	TGTAGTTGAGGTCAATGAAGGG	
Sox2	ACATGATGGAGACGGAGCTGAA GCC	TCATGGGCCTCTTGACGCGGT	
Irx3	CGCAGCCGCCTATGCTCGG	ACCAGAGCAGCGTCCAGATGGT	
Irx5	CTCGCCCGGCTACAACTCGC	GCCCAGAGGTGCTGCATAAGG G	
RorB	CCAGGCACCAGCAGCTAGGAC	GTCGTGGCCACAGGGTGACG	
Emx2	CCCGTCCACCTTCTACCCCTGG	GGACGGCGAGAAGGCGGTT	
Otx2	GGCACAGCTCGACGTTCTGGAA G	TGGCGGCACTTAGCTCTTCGAT TC	
ThrB	TGCAGTCGCCACCGCACTC	ACCCTGTGGCTTTGTCCCCACA	
Eya4	ATCCCTCCCCACCTCCGGACA	TCCGGAGCCCGAGAGTCACAG	
Brip1	CGCCCGTGCTGTCATAACCGT	TGACGGCCAGGCAGAAGACCT	
Isl1	TCTGCCGTGCAGACCACGATG	TGGCTGCCTAGCCGAGATGGG	
Pax2	GGCTGCTAGCCGAGGGCATC	GAACGATGGTGTGGCCGGGG	
Tceal1	TGGCCCGTATCCGCCCTCAA	GCGGCTCCGTTTTGCCTTCC	
Zfp691	AACTTGGGCTTCAGTTTTGCCAC CT	GGGACGCTGCTCCTTCTCGC	
Six1	ACGCCAGCCACTCGGAGTCTA	GCGCGGCTGCTCCTAACCC	
Nr0B1	TGCGGTCCAGGCCATCAAGAGT	ATGTATTTCACGCACTGCAGGC CA	
Nr2f1	GCTGCCTCAAAGCCATCGTGC	TGCCAAAGCGGCTGGGCTG	
Chd7	ACGGACTGTCCTGAGCTGCGT	GGGTCCCGGTCAGCAGGACTT	
Tox3	GGCCGAGGCAAACAACGCCT	GGAGGAGGCGTGATCGGTGGA	
Elp4	TCCTGTGGCTACATGAGGCTGCT	TCCCCATAACGGTGAGCCAAGG T	

at 14 µm. Sections were rehydrated in PBS containing 0.1% Tween-20, and hybridization was performed as in [195].

Results

Identification of genes with reduced expression in *Smo^{ecko}* mutants

To confidently identify differentially expressed genes, we limited our analysis to a 5% false discovery rate (Appendix 1). This threshold yielded 138 genes with reduced expression in *Smo^{ecko}* mutants, and 173 genes with increased expression. Searching for gene ontology (GO) terms describing the molecular function of these genes using the GOTERM_MF_FAT database in the DAVID bioinformatic resource[224, 225] revealed that genes with reduced expression in *Smo^{ecko}* mutants, and genes with increased expression in *Smo^{ecko}* mutants are enriched for slightly different molecular functions (Figure 3.2).

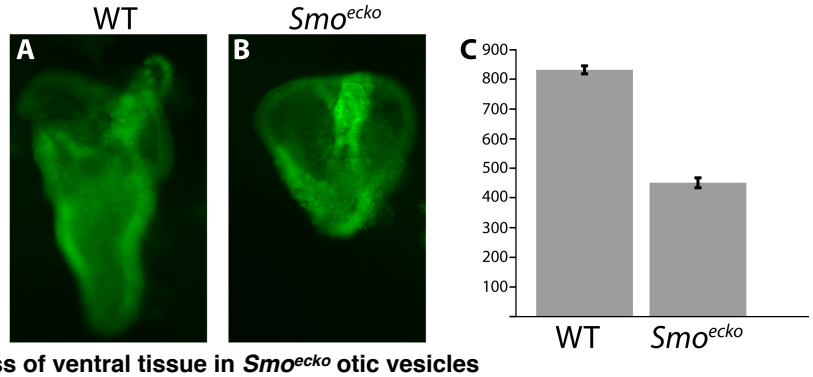

Figure 3.1 Loss of ventral tissue in *Smo^{ecko}* otic vesicles
Microdissected E11.5 *Foxg1$^{cre/+}$;Smo$^{loxp/+}$,cag-GFP$^{+/-}$* otic vesicles (**A**) display an elongating cochlear duct. *Foxg1$^{cre/+}$;Smo$^{loxp/-}$,cag-GFP$^{+/-}$* otic vesicles completely lack cochlear duct (**B**). This complete lack of ventral tissue reduces the overall length of the otic vesicle, quantified in (**C**).

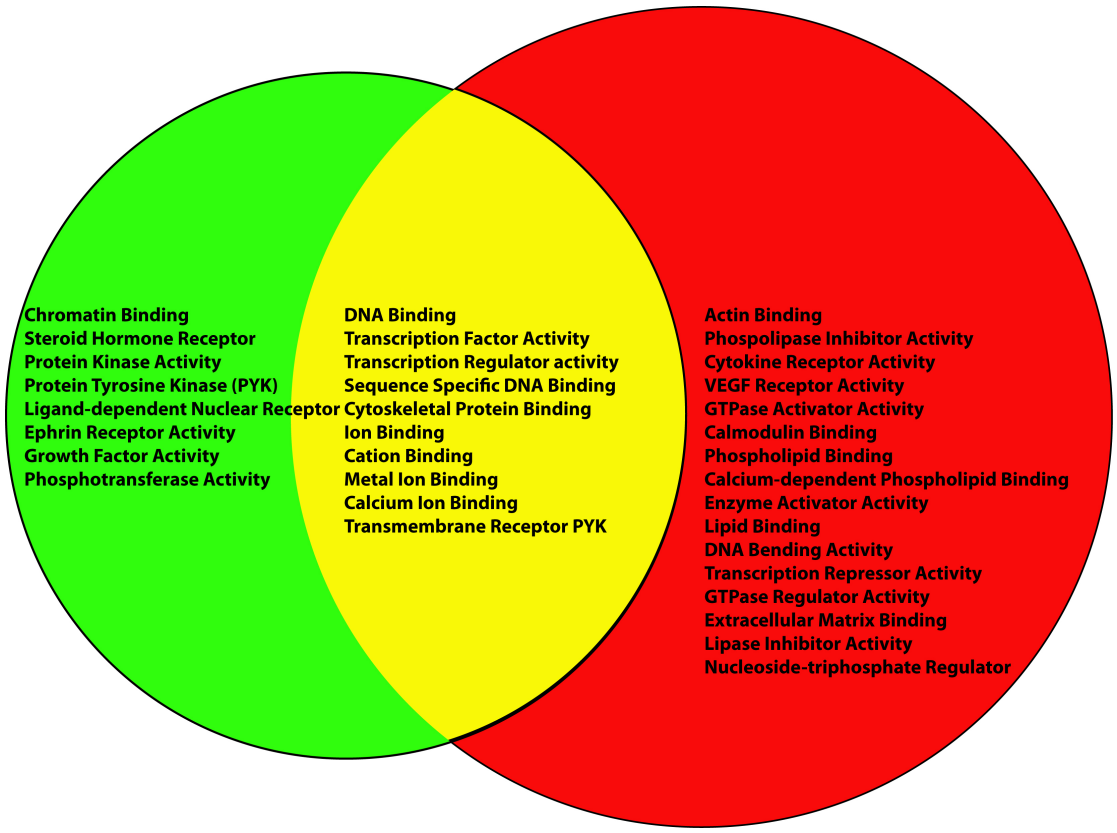

Figure 3.2 GO Terms enriched in *Smo^{ekco}* dataset
Molecular function gene ontology (GO) terms identified using the GOTERM_MF_FAT database are shown for genes with reduced expression (green) or increased expression (red) in *Smo^{ecko}* mutants. Yellow represents terms shared by both classes of genes.

Identification of putative Shh dependent transcription factors

The development of the inner ear depends on gene regulatory cassettes, which can function in parallel, sequentially or iteratively. At otic vesicle stages, at least two different regulatory cassettes function in parallel to promote cochlear duct formation. One regulatory cassette is defined by the transcription factor *Eya1*, and its target *Six1*[226]. *Six1*$^{-/-}$ mutants lack a cochlear duct and have dramatic vestibular defects[227]. The morphological defects present in *Six1*$^{-/-}$ mutants are preceded by reduced *Fgf3, Fgf10* and *Gata3* expression. However, loss of *Six1* function does not impact the expression of the essential cochlear

duct factor *Pax2*[227, 228]. Another regulatory cassette implicated in cochlear duct formation depends on *Shh.* The *Shh* dependent cassette also modulates the expression of *Fgf3, Fgf10* and *Gata3,* but includes *Pax2*[31, 27]. After initial cochlear duct outgrowth, the *Eya1/Six1* cassette is employed in the prosensory domain of the cochlear duct to specify hair cells[229], demonstrating that these regulatory networks can be used iteratively during organogenesis.

We identified 19 genes with reduced expression in *Smo*^{ekco} otic vesicles reported to function in transcription. The complete lack of ventral tissue in the E11.5 *Smo*^{ecko} otic vesicle (Fig 3.1), likely perturbs both *Shh* dependent regulatory cassettes, as well as regulatory cassettes that function in parallel. To determine whether any of these genes are putative targets of Hh signaling, we employed co-expression analysis. The first defects detectible when Hh signaling is ablated in the inner ear are a failure to maintain *Pax2* expression[27] and reduced proliferation in the neurogenic domain that produces the neurons that make up the cvg[31]. These deficits begin to manifest shortly after otic vesicle closure, at 23 somites of development. We reasoned that genes that display low expression at this early time point, and robust expression at later points in development, may be regulated by Hh. Conversely, genes with robust expression at this early point may function in parallel to Hh signaling, or depend on an earlier, previously unappreciated, Hh signal.

To determine which transcriptional regulators with reduced expression in *Smo*^{ecko} mutants were likely Hh targets we evaluated their relative expression levels by qPCR in otic vesicles isolated at 19-22 somite stages (E9.5) shortly

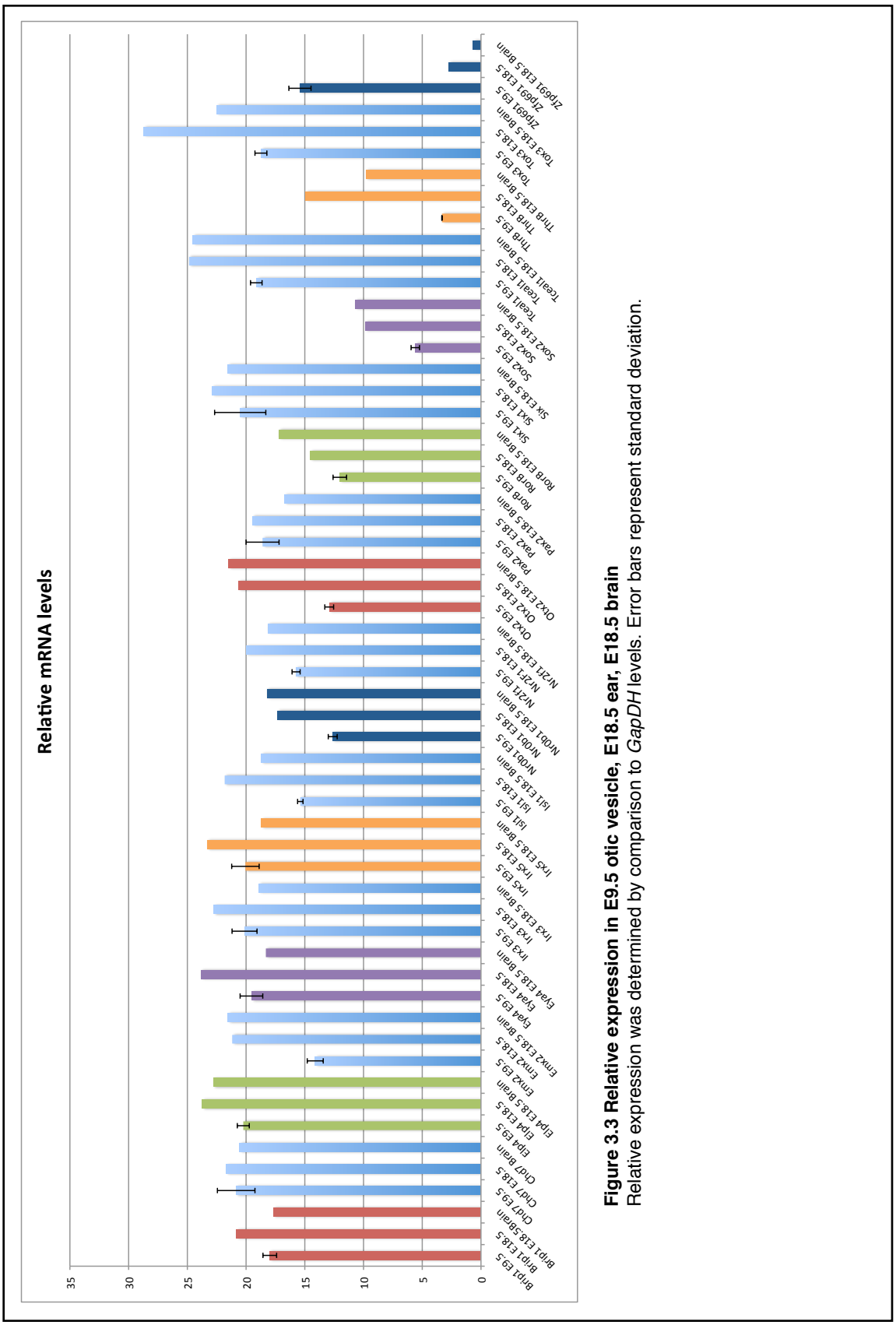

Figure 3.3 Relative expression in E9.5 otic vesicle, E18.5 ear, E18.5 brain
Relative expression was determined by comparison to *GapDH* levels. Error bars represent standard deviation.

before otic vesicle closure, as well as whole ear and whole brain cDNA libraries prepared from e18.5 tissue, a stage when all cell types present in the mature ear have differentiated (Fig 3.3).

Of the genes evaluated, *Sox2* and *Thyroid Hormone Receptor β (Thrβ)* stood out for having low expression at E9.5. *Sox2,* functions within the developing cochlear duct to establish the prosensory domain and generate sensory hair cells[161]. *Thrβ* is essential for the proper formation of the tectoral membrane and the maturation of cochlear hair cells[230, 231]. Unsurprisingly, these two genes clustered together (Fig. 3.4). The low expression level of these genes shortly after otic vesicle closure, and their known requirement in hair cell development, suggest they are putative Hh targets.

Categorizing genes by co-expression

Eight of the nineteen genes evaluated by qPCR display robust expression at early otic vesicle stages function during ear development, or are previously known to be expressed in the developing ear. Their pattern of clustering may help elucidate the role of the seven remaining genes with no previously reported otic function. Several clusters seem to group based on expression patterns. *Isl1* and *Nr2f1* group together. *Isl1* is expressed in the cvg and prosensory wall of the cochlear duct[155], and is important for the development of multiple types of neurons[232], while *Nr2f1* is also expressed in the prosensory wall of the cochlear duct[233], and functions in cochlear duct elongation and sensory cell specification[234]. Both *Isl1* and *Nr2f1* are expressed in the prosensory wall of the cochlear duct, but not the otic vesicle, and cluster together accordingly (Fig 3.4).

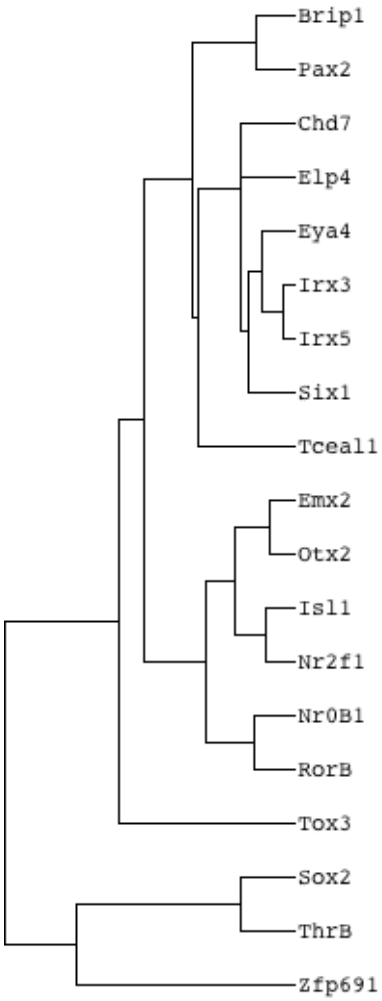

Figure 3.4 Clustering of transcription effectors based on expression pattern Factors were clustered based on average linkage using E9.5, E18.5 and whole brain expression levels.

Several more intriguing comparisons are established between genes with known otic function. *Emx2* and *Otx2* are expressed in the ventral otic vesicle, are essential in ear development[235, 236, 181], and cluster together. *Irx3* and *Irx5* are IrxB group genes, that are expressed in the ventral otic vesicle[237, 238], but have no previously reported otic function. The seeming success of grouping genes with known otic activity, suggests this approach may help characterize novel otic genes. One particularly promising result is the grouping of the known gene *Pax2* and the novel gene *Brip1*. Followup experiments show *Brip1* is expressed in the ventromedial otic vesicle (Fig. 3.5) in a pattern overlapping *Pax2.*

Grouping genes based on co-expression is one method to impose order on a chaotic list of genes, and in doing so, generate testable hypotheses. The clusters displayed in Figure 3.4 are fascinating, but will require considerable validation. The grouping of *Pax2* and *Brip1*, combined with their overlapping expression pattern, suggests they may be subject to similar regulatory controls. *Brip1* expression will need to be evaluated in Smo^{ecko} or $Shh^{-/-}$ mutants to

determine whether, like *Pax2,* it requires *Shh* to maintain expression. Similarly, the grouping of *Eya4, Irx3, Irx5* with *Six1* is intriguing, but they too will have to be evaluated in Hh mutants to determine whether *Eya4, Irx3, Irx5* function in parallel to Shh like *Six1.*

Ventral expression of down regulated genes

To complement our qPCR studies, we evaluated the spatial distribution of transcripts in wild-type embryos shortly after the initiation of cochlear duct outgrowth at E11.5. We selected genes for evaluation by the fold change between mutant and control tissue with the assumption that genes with the most dramatic fold change would be best detected by in situ hybridization.

We validated our list of genes by examining the expression of several genes known to function in the developing cochlea. *Six1, Eya1,* and *Jag1* had reduced expression in Smo^{ecko} otic vesicles, and are known to function in the prosensory domain of the cochlear duct[239, 163] during hair cell specification. We confirmed *Six1, Eya1, Jag1* are expressed in the ventromedial wall of the otic vesicle (Fig. 3.5). We found novel expression of several genes within the prosensory domain including: *Carbonic anhydrase 13 (Car13), Family member 107a (Fam107a), Growth arrest specific 2 (Gas2), Brac1 interacting protein C-terminal helicase 1(Brip1), Anoctamin 1 (Ano1),* and *Protocadherin 11 x-linked (pcdh11x).* These genes represent a variety of cellular processes. Carbonic anydrases catalyze the reaction of CO_2 and water and are best know for catalyzing the release of CO_2 from red blood cells, and can also regulate pH. *Car13* is a cytoplasmic protein, whose exact function remains unknown, is

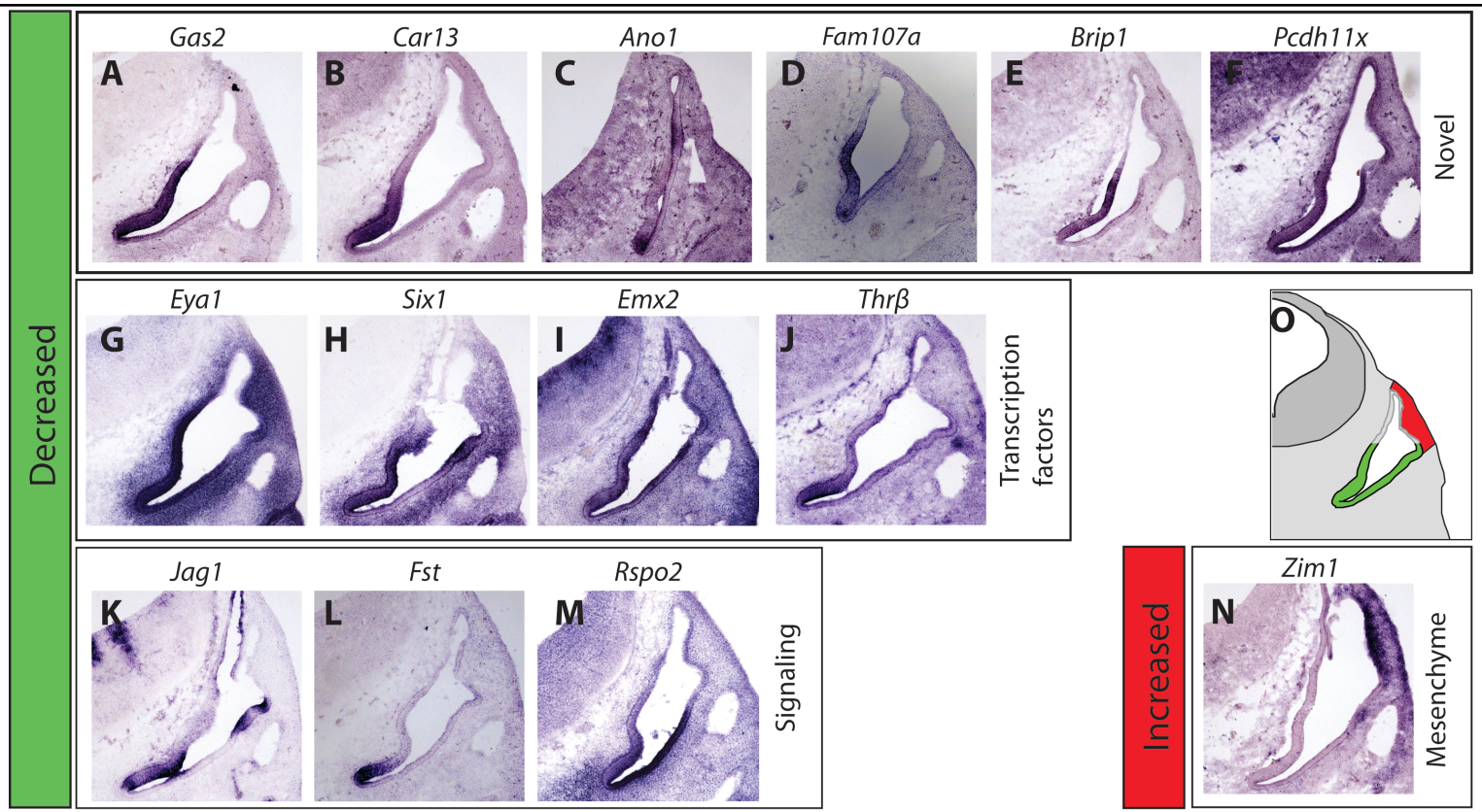

Figure 3.5 Ventral expression of genes with reduced expression in *Smo*^{ecko} mutants
Expression was evaluated at E11.5, shortly after the initiation of cochlear duct outgrowth. Several genes with no reported function in otic development were showed expression in the ventral otic vesicle. These novel genes include *Gas2*(A), *Car13*(B), *Ano1*(C), *Fam107a*(D), *Brip1*(E), *Pcdh11x*(F). *Eya1* (G), *Six1* (H), *Emx2* (I), *Thrβ* (J) are transcription factors known to function in ear development and have reduced expression in *Smo*^{ecko} mutants. Their expression was restricted to the ventral part of the otic vesicle. *Jag1*(K), *Fst*(L), *Rspo2*(M), function in the Notch, BMP and Wnt pathways respectively. Each of these pathways functions during otic development. *Zim1* (N) is a transcription factor expressed in the mesenchyme adjacent to the forming semicircular canal, and is an example of a gene with increased expression in *Smo*^{ecko} mutants. Diagram summarizing different expression patterns (O). Genes with reduced expression in *Smo*^{ecko} are generally expressed in the ventral (green) region while genes with increased expression (red) may be expressed in mesenchyme adjacent to the otic vesicle.

expressed in a variety of cell types including oligodendrocytes[240]. *Fam107a* was initially identified as a tumor suppressor[241, 242], but has also been shown to function as an actin bundling protein that modulates synapse formation in response to stress[243]. *Gas2* seems to function as a microtubule binding protein that antagonizes cell division[244]. *Brip1* functions to help repair double stranded DNA breaks[245], and has been implicated in Fanconi Anemia[246]. *Ano1* is a chloride channel, a family that regulates cell size and osmotic pressure. However *Ano1*$^{-/-}$ mice fail to thrive and die by 1 month of age, but their inner ears have not been evaluated[247]. *Pchd11x* is a member of the protocadherin superfamily of cell adhesion proteins, and is expressed at high levels in the brain[248]. Interestingly, *Pcdh11x* has been implicated in non-syndromic language delay[249], but it remains unclear whether *Pcdh11x* functions in auditory processing or another aspect of language.

Although the exact functions of *Car13, Fam107a, Gas2, Ano1, Pcdh11x* in the ear remains unknown, they serve as a starting point for a better understanding of the cellular processes occurring during sensory formation.

Conclusions

We have used microarrays to identify genes with reduced expression in *Smo*^{ecko} otic vesicles, and in doing so generated a list of genes enriched for expression in the developing cochlear duct. Many of these genes have been shown to be essential for inner ear development and hearing, and many more are expressed in the ventral otic vesicle but have not been functionally evaluated.

The presence of multiple genes implicated in otic development and human disease make us confident that our list of genes with reduced expression in *Smo*^{ecko} mutants likely includes many novel factors essential for otic development.

We have started to investigate the Hh dependent gene regulatory network necessary for cochlear duct formation. Based on co-expression data we have identified candidate Hh targets (*Rorβ, Nr2f1, Nr0b1)* as well as candidate genes that depend on Hh for continued expression (*Brip1)*. However, a full evaluation of these candidates in *Smo*^{ecko} and *ShhP1* mutant embryos is required. To truly generate a list of direct Hh targets a different approach is necessary. One possibility would be to identify direct Hh targets using ChIP-seq to determine the genome wide occupancy of Gli1[250, 251], a constitutive Gli activator.

The inaccessibility of the mammalian inner ear has been a barrier to fully understanding the cellular processes that give rise to the organ of Corti. So far, we have evaluated only 20% of gene identified by our microarray studies, and found at least six genes with unappreciated expression in the ventral inner ear. These genes are distributed across a broad range of cellular processes including ion transport, pH regulation, and control of the cytoskeleton. These genes represent exciting targets for further study, which should ultimately increase our understanding of how the inner ear develops.

Chapter 4: A Fate Map of Wnt responsive cells

Introduction

Fate mapping experiments using *TopCreERT2* to indelibly label Wnt/β-catenin responsive cells early in development and visualize what tissues they populate later revealed an unexpected contribution of Wnt responsive cells to the cochlear duct[28]. Although Wnts function within the otic placode[132, 133] and are necessary for the formation of dorsal otic structures[28], their role in the cochlea is mysterious. Wnt signaling has been implicated in cochlear duct formation. *Wnt5a* and *Wnt7* function through the PCP pathway to modulate the convergent extension movements that drive later cochlear duct outgrowth[117, 118]. Wnt/β-catenin signaling may also function earlier in cochlear duct formation, as shown by the truncated cochlea in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutant mice[28]. However, it remains unclear whether truncated cochlea present in *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutants is due to a requirement for Wnt/β-catenin signaling in early cochlear duct formation, or is a secondary consequence of defects elsewhere in the embryo. Wnt signaling may also function in hair cell specification. Forced activation of the Wnt/β-catenin pathway in chick, using degradation resistant β-catenin, was sufficient to induce ectopic hair cells in the cochlea and to transform auditory hair cells to a vestibular morphology[169]. These disparate lines of evidence suggest Wnts may be active in the cochlear duct, but this later cochlear function has been masked by the requirements for Wnt early in placode formation and otic vesicle patterning. To

better understand the role of Wnt/β-catenin signaling in the cochlear duct we used *TopCreERT2* to construct a fate map of Wnt responsive cells in the ear.

Materials and Methods

Animals

Rosa$^{Gfp/Gfp}$ [193] and *Rosa*$^{LacZ/LacZ}$ [252] mice were obtained from Jackson Labs (Bar Harbor, ME). *TopCreERT2*, *Gbx2*$^{CreER/+}$ and *TopGal* mice were described elsewhere [115, 184, 28].

Cre activation

Pregnant dams were fed 150µg/g body weight tamoxifen (Sigma Aldrich St. Louis ,MO) dissolved in corn oil by oral gavage.

Immunohistochemistry

The heads of E18.5 embryos were bisected and the brain removed, fixed in 4% paraformaldehyde for 3 hours then the otic capsul was dissected out, cryoprotected in 30% sucrose overnight, mounted in OCT embedding media (Sakura Finetek Torrence, CA) and snap frozen. Embryos were sectioned at 14 µm and stained with DAPI and the following antibodies: Rabbit anti-MyosinVIIa (Proteus Biosciences Ramona, CA) 1:300, Chicken anti-GFP (Aves Labs Tigard, OR) 1:1000, Rabbit anti-Prox1 1:500 (Chemicon Billerica, MA). Primary antibodies were detected with one of the following secondary antibodies: Donkey anti-mouse IGG conjugated to Cy3 (Jackson ImmunoResearch West Grove, PA) or Alexa488 (Molecular Probes Eugene, OR); Donkey anti-Rabbit IGG

conjugated to Cy3 or Alexa488; or Goat anti-Chicken IGG conjugated to Alexa488.

Wholemount cochlear preparations

The heads of E18.5 embryos were bisected and the brain removed, then fixed in 4% paraformaldehyde for 3 hours. The membranous labyrinth was dissected, and sensory patches were exposed by opening opening the ampulae, maculae, and cochlear duct with forceps. Dissected tissue was then treated using the same antibodies as sections, mounted on slides and imaged using a Leica SP2 confocal microscope.

Xgal staining

The heads of E18.5 embryos were bisected, the brain removed, and fixed in 4% paraformaldehyde for 1 hour. Bisected heads were then incubated in at 37°C overnight, and post fixed 3 hours in 4% paraformaldehyde. The otic capsul was dissected, dehydrated in a graded methanol series, and cleared using a 1:2 solution of benzyl alcohol:benzyl benzoate. Cleared ears were imaged on a Leica dissecting microscope.

Fluorescent in situ hybridization

Section in situs were performed as in[31] with the following modifications: Slides were incubated in PBS containing 0.3%H_2O_2 0.1% Tween-20 for 30 minutes to quench endogenous peroxidase activity. Digoxigenin-UTP and flourescein-UTP labeled probes were hybridized to tissue. Tissue was then incubated with HRP conjugated anti-Fluorescein (1:100), and detected with fluorescein tyramide signal amplification (Perkin-Elmer Waltham, MA). After detection, slides were

fixed in 4% paraforaldehyde, washed 3 times in PBS-Tween, incubated in PBS-Tween-H_2O_2, washed three times in PBS-tween then incubated with HRP conjugated anti-digoxigenin. Signal was detected with tetramethylrhodamine tyramide signal amplification. Embryos were cleared using ScaleU2[253] and imaged on a Leica SP2 laser scanning confocal microscope.

Multiphoton imaging and embryo culture

E9.5 TopCre;Rosa$^{Gfp/+}$ embryos were collected in ice-cold L-15 media without damaging the yolk sac. Yolk sacs were opened with forceps without damaging major blood vessels. Embryos were grown in 35mm Mat-tek glass bottom dishes in a Cell Biosystems stage top environmental chamber under 95% O_2: 5% CO_2 at 37°C in 100% rat serum (Gemini Bio-Products, West Sacramento CA) supplemented with 0.175 mg/ml glucose, 2 mM glutamine, 1x Penn-Strep. Culture media was circulated using a peristaltic pump.

Embryos were imaged using a chameleon femtosecond pulsed IR laser tuned to 880nm excitation fitted to a Zeiss LSM510 microscope using a 20x objective in the non-descanned detection mode. Excitation laser power was adjusted for the depth of the sample to minimize exposure and phototoxicity.

Results

Wnt responsive cells contribute to the cochlear duct

During otic development Wnt ligands are expressed in nearby tissues including the neural tube , and in the otic vesicle itself[254]. To determine whether the observed contribution of Wnt responsive cells to the cochlear duct reflects a

temporally restricted response, possibly to a single Wnt ligand, or a more general response to the variety of ligands present over time we labeled Wnt responsive cells at different points during development and evaluated their fate. We found that labeling at E8.5, during early placode formation, revealed a broad contribution of Wnt responsive cells to many tissues within the ear (Fig. 4.1A). This contribution included both auditory and vestibular hair cells, support cells, neurons, and the epithelia that makes up the semicircular canals. Labeling at later time points gradually restricted the tissues and cell types containing Wnt responsive cells. Inducing recombination at E9.5 reduced the contribution of labeled cells to the cochlear duct (Fig. 4.1B), while inducing recombination at E10.5 almost completely abolished labeling of cochlear cells while having little impact on the contribution of labeled cells to the vestibulum or spiral ganglion (Fig. 4.1C). The only ventral cells labeled by inducing recombination at E11.5 or later in development are spiral ganglion neurons (Fig. 4.1 D,E,F).

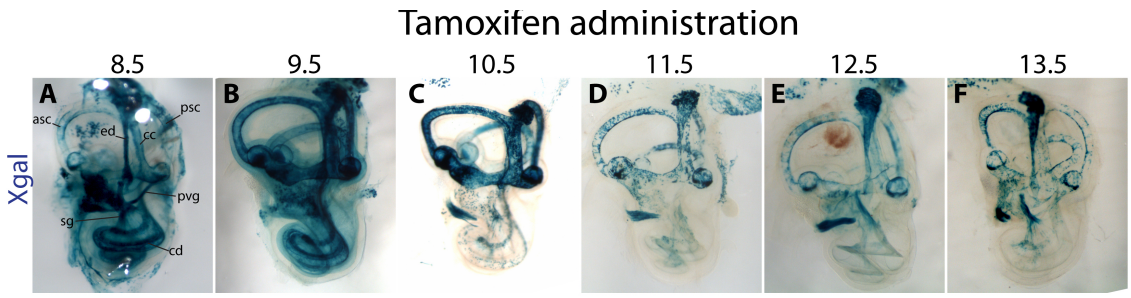

Figure 4.1: Temporal dependent contribution of Wnt responsive cells to the cochlear duct
Whole mount views of Xgal stained TopCre;Rosa$^{lacZ/+}$ inner ears where tamoxifen was administered at the stated time to induce recombination. Administering tamoxifen at E8.5 labeled the semicircular canals, endolymphatic duct, cochlear duct and inner ear neurons (**A**). Administering tamoxifen at E9.5 (**B**) produced similar results. Inducing recombination at E10.5 caused robust labeling in vestibular structures, with little labeling in the cochlear duct (**C**). Ventral labeling seen at E10.5 is largely the spiral ganglion. Administrations at E11.5 (**D**), E12.5 (**E**), E13.5 (**F**) caused labeling in the endolymphatic duct and vestibulum. The only ventral labeling is present in the spiral ganglion. anterior semicircular canal **asc,** posterior semicircular canal **psc,** endolymphatic duct **ed**, common cruz **cc,** spiral ganglion **sg**, cochlear duct **cd**, posterior vestibular ganglion **pvg**.

Contrasting the temporal sensitivity of labeling in the cochlear duct, the anterior and posterior semicircular canals and both auditory and vestibular neurons could be labeled at each time point evaluated, however we found minimal contribution of labeled cells to the lateral crista.

TopCre activity is present in multiple cochlear cell types

The prosensory domain of the cochlear duct consists of an equivalence group of *Sox2*$^{+}$ cells[161] that will give rise to both hair cells and support cells in response to differential levels of Notch signaling activity[164]. To determine whether an early exposure to Wnt activity biased cells towards low levels of Notch activity resulting in a sensory fate, or high levels of Notch activity resulting in a support cell fate we determined the contribution of *TopCreERT2* labeled cells to sensory or support populations.

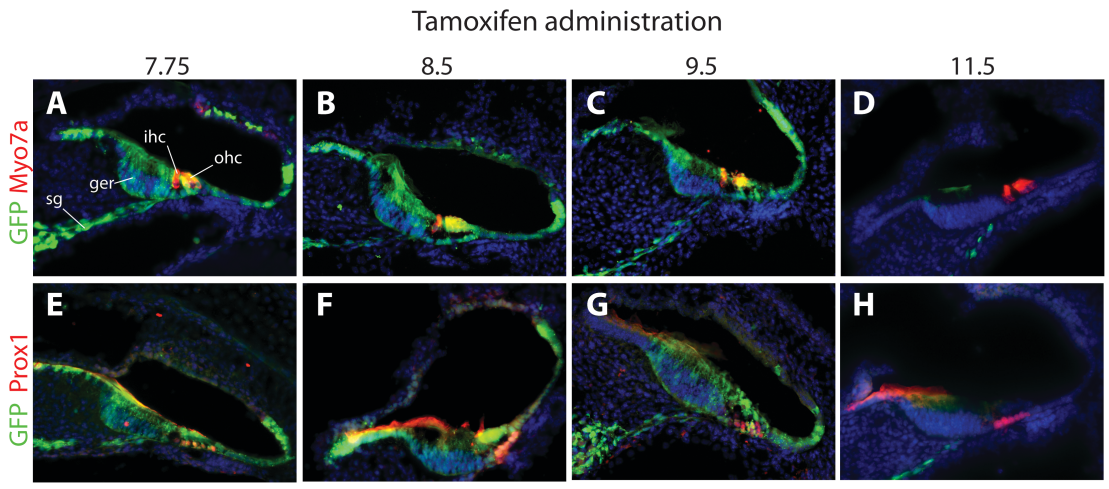

Figure 4.2: Sensory and support cells originate from a Wnt responsive progenitor
Labeling in the cochlear duct appeared stochastic, never achieving 100% efficiency. Inducing recombination at E7.75 robustly labeled sensory hair cells (**A**) and support cells (**E**), as well as spiral ganglion neurons and cells throughout the greater epithelial ridge. Labeling remained robust when recombination was induced at E8.5 (**B,F**). The amount of labeled cells in the cochlear duct started to decrease at E9.5 (**C,G**), and labeled cells no longer contributed to the cochlea by E11.5 (**D,H**). spiral ganglion **sg**, greater epithelia ridge **ger**, inner hair cell **ihc**, outer hair cells **ohc**.

Sensory hair cells can readily be identified by the expression of atypical motor protein Myosin VIIa[255], while support cells are identifiable by expression of the transcription factor Prox1[256]. We identified cells in *TopCreERT2;Rosa*$^{Gfp/+}$ embryos based on the colocalization of GFP and MyoVIIa or Prox1, and quantified their relative abundance. We found that inducing recombination at E7.75 labeled inner hair cells, outer hair cells and support cells within the cochlear duct with a 60% efficiency. This efficiency is reduced to labeling 5% of hair cells by E10.5 with no bias between inner and outer hair cells (data not shown).

The cochlear duct is organized in a tonotopic manner, where cells near the apex detect low frequency sound and cells near the base detect high frequency sound. This sensitivity gradient is mirrored by a temporal gradient of hair cell maturation. Birth dating studies suggest that hair cells leave the cell cycle in a wave that moves from the apex to the base of the cochlea[157] while morphological observations find that hair cells mature first in the base of the cochlea followed by the apex[257]. To determine whether prior Wnt responsiveness correlated with a given maturation time or cell cycle exit we evaluated the distribution of labeled hair cells along the length of the cochlear duct. We found no bias in the distribution of labeled cells (data not shown).

Wnt responsive cells likely do not migrate ventrally in the otic epithelium

One hypothesis to explain the ventral contribution of *TopCreERT2* labeled cells is that they move ventrally within the otic epithelium, possibly in response to

a chemoattractant or chemorepulsant cue. To test this hypothesis we imaged cells within *TopCreERT2;Rosa*$^{Gfp/+}$ otic vesicles using multiphoton microscopy. We were unable to detect ventral cell movement within the otic epithelium (Fig. 4.2A,B). However, we did observe ventral cell migration in nearby neural crest cells (Fig 4.2B, white arrowheads).

The absence of detectible cell migration ventrally could be attributed to problems with our embryo culture system. As a complementary approach, we evaluated the distribution of GFP^{+} cells marked by *TopCreERT2*, and the

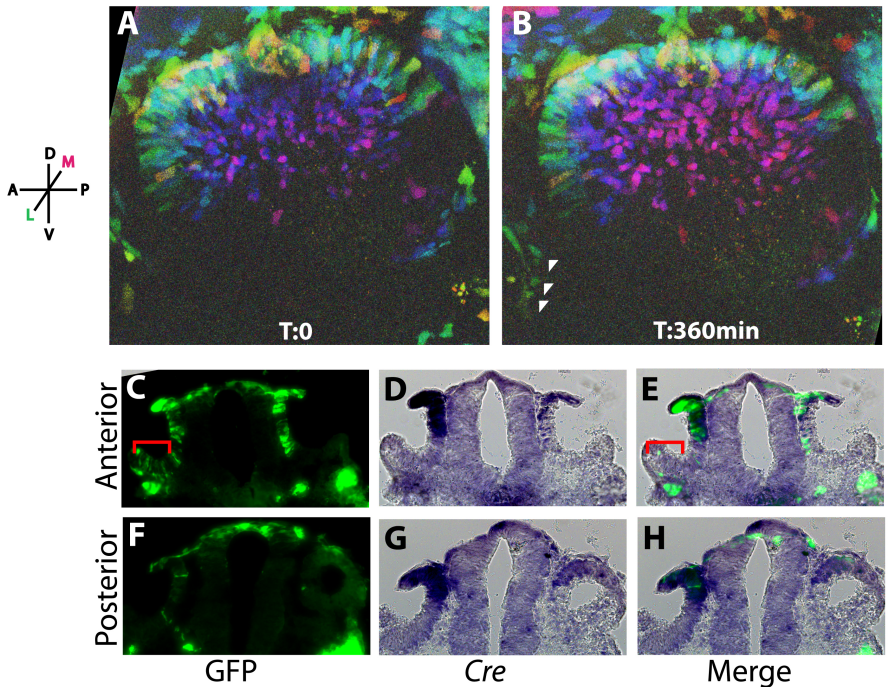

Figure 4.3 Presumptive ventral movement is restricted to the anterior otic vesicle
A depth coded lateral view shows Topcre activity is not uniformly distributed throughout the otic vesicle, instead it is restricted to the medial wall and extends further ventrally in the anterior otic vesicle (**A,B**). Multiphoton live imaging failed to detect cell movement within the medial wall of the otic vesicle. Cells outside the otic vesicle were observed to migrate ventrally during this time (**B,** white arrowheads). In transverse sections, cre activity visualized by GFP expression extends further ventrally in anterior parts of the otic vesicle (**C**). The localization of Topcre activity (GFP) closely correlates with *Cre* transcript in the posterior vesicle (**F,G,H**). In the anterior vesicle, GFP extends further ventrally than *Cre* transcript (**C,D,E,** red bracket).

distribution of *Cre* transcript at otic cup stages. We reasoned, a migratory behavior would be reflected by a ventral expanse of GFP^{+},*Cre*$^{-}$ cells.

We found that inducing recombination at E7.75 or E8.5 was sufficient to label the otic vesicle. At 17 somites of development (E9.0), we found a close correlation between GFP and *Cre* expression in the posterior and middle sections of the otic cup (Fig. 4.2F,G,H). In the most anterior sections of the otic cup GFP expression extended slightly further than *Cre* expression (Fig. 4.2C,D,E).

It remains unclear whether the extension of GFP beyond the domain of *Cre* in the most anterior parts of the otic vesicle is due to a role for cell movement within the otic placode, or a result of the transition from otic placode to otic cup.

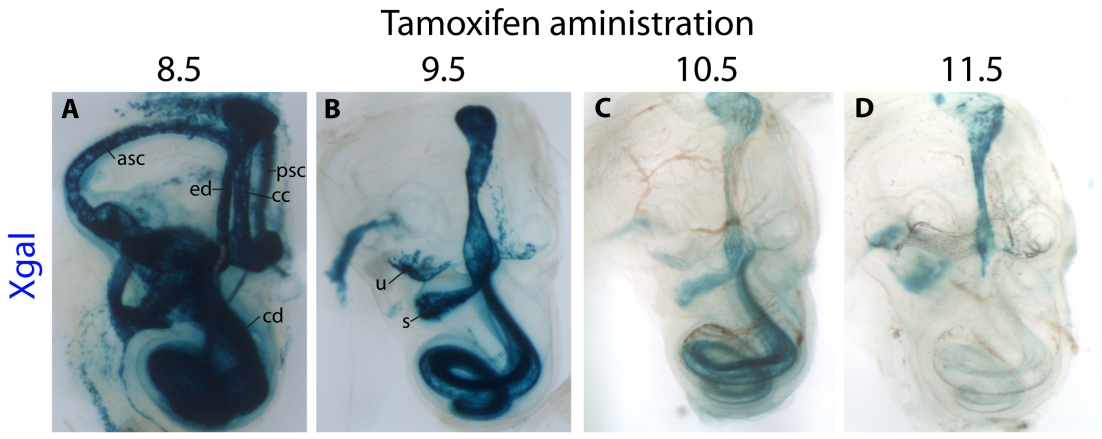

Figure 4.4: Contribution of *Gbx2*$^{Cre/+}$ cells to the cochlear duct
Inducing recombination at E8.5, otic placode stage, (**A**) results in labeled cells contributing to all otic structures at E18.5. Inducing recombination at late otic cup stages, E9.5, (**B**) marks cells that selectively contribute to the endolymphatic duct, cochlear duct, utricle and saccule. Labeling at E10.5 (**C**), marks the endolypmatic duct, cochlear duct and saccule. By E11.5 the cochlear duct starts to outgrow from the otic vesicle, and recombination induced at this time only contributes to the endolymphatic duct (**D**). anterior semicircular canal **asc**, posterior semicircular canal **psc**, endolymphatic duct **ed**, common cruz **cc**, cochlear duc **cd**, utricle **u**, saccule **s**.

Contribution of Gbx2 fate mapped cells to the cochlear duct

The transcription factor *Gbx2* is expressed in the dorsomedial wall of the otic vesicle and is essential for the formation of the endolymphatic duct[184]. We found that cells labeled with $Gbx2^{Cre}$ contribute to the endolyphatic duct as expected based on the mutant phenotype. Surprisingly, we found cells labeled using $Gbx2^{Cre}$ between E8.5 and E10.5 also contributed to the sensory wall of the cochlear duct (Fig. 4.4A,B,C). This contribution of labeled cells to the cochlear duct is temporally responsive, with cochlear labeling being lost by E11.5 while endolymphatic duct labeling is maintained (Fig. 4.4D). The unexpected contribution of $Gbx2^{cre/+}$ cells to the cochlear duct suggests the spatial origin of auditory hair cells resides in the *Gbx2* expression domain in the dorsomedial wall of the otic vesicle.

A population of TopCre^{+}, Gbx2^{+}, Ngn1^{-} cells are cochlear sensory progenitors

Our fate mapping data suggest that *TopCreERT2* and $Gbx2^{Cre}$ label a population of cells that will later populate the prosensory domain in the cochlear duct. *Gbx2* are broadly expressed in the otic placode before being restricted to the dorsal otic vesicle[258], which likely explains the contribution of early labeled cells to many otic structures and a gradual restriction of labeled tissue over time. To better define the region of the otic vesicle that gives rise to sensory cells within the cochlear duct, we examined the distribution of *TopCreERT2* and *Gbx2* transcripts at E9.5, the last time point where both Cres contribute to the cochlear duct. We also compared the expression of *TopCreERT2* and *Gbx2* transcripts to

Ngn1. Fate mapping studies using a *Ngn1Cre* that is expressed in the anteroventral wall of the otic vesicle were unable to robustly label cochlear duct progenitors[156, 259]. We reasoned that the region of otic vesicle that expresses *Gbx2* and *TopCreERT2* is likely enriched for cochlear duct progenitors, while the region that expresses *TopCreERT2* and *Ngn1* is likely enriched for cvg progenitors.

We compared the expression of *TopCreERT2, Gbx2, Ngn1* transcripts using fluorescent in situ hybridization. Surprisingly, *Ngn1* expression did not appear to overlap with *TopCreERT2* despite the strong *TopCreERT2* activity in the cvg. Instead, *Ngn1* and *TopCreERT2* seem to be expressed in adjacent domains within the otic vesicle, and *TopCreERT2* is robustly transcribed in neuroblasts that have delaminated from the otic vesicle and no longer express *Ngn1* (Fig. 4.5A). We also found that *Gbx2* and *Ngn1* expression also do not

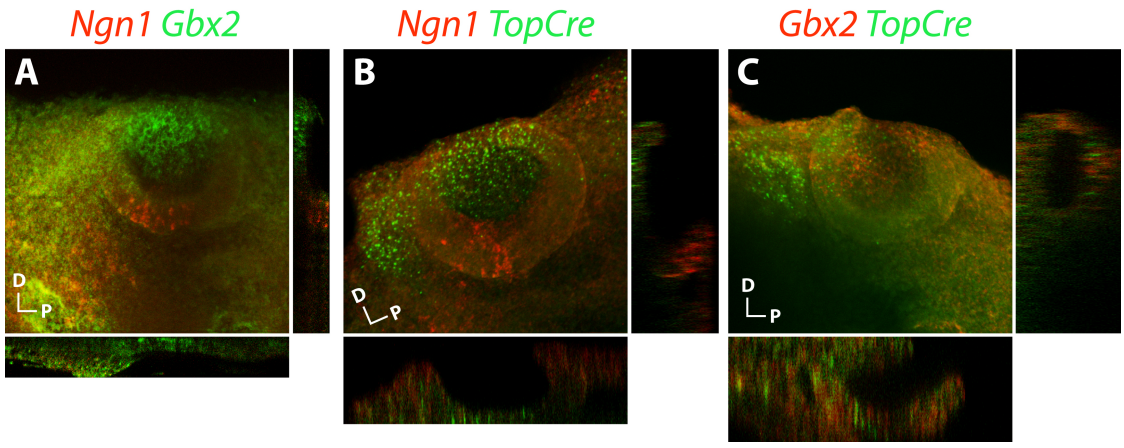

Figure 4.5 *TopCre, Gbx2* expression do not overlap with *Ngn1*
Z projections and orthogonal views of two color fluorescent whole mount in situ hybridizations demonstrate that *Gbx2* and *Ngn1* transcripts are expressed in spatially distinct regions of the otic cup (**A**). *Ngn1* and *TopCre* expression follow a similar distributions, where *Cre* is restricted to the dorsomedial wall of the otic cup, and to the cvg neuroblasts that have already delaminated, while *Ngn1* is largely restricted to the anteroventralateral section of the otic cup (**B**). *Gbx2* and *TopCre* expression colocalizes in the medial wall of the otic vesicle (**C**), marking the domain of cochlear sensory progenitors.

overlap at E9.5 (Fig 4.5B), while *TopCreERT2* and *Gbx2* expression do overlap in the dorsomedial wall of the otic vesicle (Fig 4.5C). These results validate the idea that cochlear progenitors originate from the medial wall of the otic vesicle, but lack the resolution to determine their origin along the anteroposterior axis.

The transition from otic cup to otic vesicle has been best described in chick where cells from the rim of the closing otic cup are excluded from the ventromedial wall of the otic vesicle, a region that ultimately gives rise to auditory hair cells[134]. Further studies in chick found cells in the posterolateral quadrant of the otic placode were most likely to contribute to the chick analog of the cochlea, the basal papilla[260]. Together these two studies provide a rough fate map of the closing otic cup where auditory sensory cells likely originate from a posterior section of the ventromedial wall of the otic cup. Our studies suggest a similar regionalization occurs in mouse. We find *TopCreERT2* and *Gbx2^{cre}*, activity are the earliest markers for a population of cells that contain auditory hair cell progenitors.

Conclusions

Fate mapping studies and embryo extirpation experiments in chick support a regionalization of the otic placode. However, the extent of these domains has not been defined at high resolution, and few comparable studies have been conducted in mouse. Using inducible Cre recombinases expressed in different domains of the otic placode and otic vesicle to indelibly label cells, we have identified the medial wall of the otic vesicle as a region that develops into the

sensory wall of the cochlear duct. These results suggest the regionalization seen in the chick otic vesicle also applies to mammals.

We have demonstrated *TopCreERT2* is active in cells that contribute to the sensory wall of the cochlear duct and the spiral ganglion. Since labeling appears stochastic, *TopCreERT2* will likely serve as a useful tool to study cell autonomous processes within the cochlear duct and spiral ganglion.

Chapter 5: Summary and Future Directions

The developing inner ear employs a host of cell signaling pathways and morphogenetic processes making it an ideal organ to study basic biology, while the near universal penetrance of age related hearing loss makes the ear a medically relevant organ system.

Amphibians and birds are capable of regenerating auditory hair cells lost to insult[261-263], while mammals are not. This difference in regenerative capacity suggests the inability to regenerate hair cells evolved sometime after mammals and birds diverged roughly 200 million years ago[18]. The ability for non-mammals to regrow hair cells also suggests that changing the activity of a select number of genes might return mammalian cells to their ancestral state and allow hair cell regeneration. One tissue that may provide insight into reactivating quiescent cells is the mammalian musculature. In striated muscle, mature cells are terminally differentiated and regeneration occurs through the activation of an endogenous stem cell pool. The major barrier to muscle cells re-entering the cell cycle and regenerating are the tumor suppressor genes *Rb* and *Ink4a*.

Genetically removing both genes allows myocytes to proliferate and integrate into mature muscle fiber[264]. Perhaps a similar mechanism occurs in the ear, which expresses multiple cell cycle inhibitors during development. Deleting *Rb* alone, or deleting *Ink4d* in combination with *p27*, is sufficient to induce proliferation in cochlear cells, but they undergo apoptosis[265, 257]. All three factors have not been inhibited in the cochlea, so it may be possible that the apoptosis seen in response to mutating a single gene is a direct result of the activity of other tumor suppressors. In the event that reactivating proliferation in cochlear cells proves impractical, an alternative approach to treating hair cell loss may be through transplanting progenitor cells. There is hope for this approach since embryonic stem cells, and induced pluripotent stem cells (iPS), can be differentiated into functional hair cells[266]. However, cell transplantation will have to overcome several major hurdles to be a viable therapy including: the requirement that hair cells be placed in the correct position and orientation within the cochlea, as well as innervated correctly in order to function. Regardless of the approach, replacing sensory hair cells will require a better understanding of the mechanisms of inner ear development and a deeper understanding of terminal differentiation and cell cycle arrest.

Although the majority of molecular pathways that govern inner ear development have been identified, much remains to be learned about how they function and interact to control the growth of the ear. The Wnt and Hedgehog pathways are both used several times during ear development. They initially were thought to have both synergistic and antagonistic interactions in the

establishment of dorsoventral polarity[28]. We have been able to show the antagonistic interaction between Wnt and *Shh* during otic vesicle patterning does not occur in the ear itself. Instead, *Shh* likely promotes the expression of a Wnt antagonist in peri-otic tissue. We were unable to definitively identify this Wnt inhibitor, but the Wnt antagonist *Sfrp2* is a strong candidate. *Sfrp2* has been shown to be a target of *Shh* in the somite[267], and we did observe altered *Sfrp2* expression in the neural tube of *Shh*$^{-/-}$ mutants.

The Hedgehog and Wnt pathways also interact to mediate cochlear duct outgrowth. Hh signaling within the otic vesicle is necessary for the initiation of cochlear duct outgrowth[31], and Gli-Activator function mediates full extension of the cochlear duct[32]. Concurrently, Wnt/βcatenin signaling is active in cells that contribute to the prosensory wall of the cochlear duct ([28] and Chapter 4), and *Wnt5a, Wnt7* dependent convergent extension completes the elongation of the cochlear duct[117, 118]. Exactly how these pathways interact during cochlear duct outgrowth remains unclear, and several confusing examples exist within the literature. Cilia function in both Hh and PCP pathways[268, 269]. Yet perturbing ciliary function in the otic vesicle results in a PCP phenotype characterized by hair cell orientation defects and a truncated cochlear duct[270] rather than a Hh phenotype of shortened cochlear duct and altered hair cell numbers[32, 146]. Perhaps the apparent lack of Hh phenotype in conditional ciliary mutants is an effect of the timing of recombination and kinetics of ciliary protein turnover. In this case cilia would be fully disrupted after Hh is required, but not before PCP requirements have passed. Alternatively, the lack of Hh phenotypes in conditional ciliary

mutants may reveal the limitations and possible pleiotropic effects of using small molecule Hh inhibitors in organ culture to study sensory cell formation and global gene knockouts to study the requirements for Gli-activator function in cochlear duct outgrowth. Nonetheless, PCP effector genes have been shown to influence ciliary structure and Hedgehog signal transduction[271, 272], suggesting there are more interactions between these pathways to be understood.

A role for Hh signaling in auditory development is not limited to amniotes, but also extends to lower vertebrates. In zebrafish and frogs, Hh signaling is required for the development of the posterior macula, a sensory organ with auditory function, as well as its associated innervation[141-143] . While the reliance on Hh signaling for auditory development appears to be conserved across phyla, there are more differences than similarities in how the Hh pathway is used to fulfill this function. Firstly, in mice and chicken, Shh patterns the otic vesicle along its dorsoventral axis to promote cochlear duct morphogenesis[29, 27] . This contrasts with the role of Hh signaling in zebrafish, which patterns the otocyst along its anteroposterior axis to promote the development of the posterior saccular and lagenar maculae[141, 142] . Not surprisingly, these species-specific requirements of Hh do not depend on the same complement of target genes. Secondly, significant differences exist in the manner that Hh signaling regulates sensory epithelial development. In the mouse cochlea, Hh functions to repress hair cell formation[146] . Whereas, in zebrafish, Hh specifies late forming saccular hair cells through the regulation of *atoh1a* expression in the posterior otic region[142] . Finally, the Hh dependent regulation of cvg progenitor proliferation is a

common feature shared between mice and fish[31, 142] . However, additional neurogenic roles for Hh, including the spatial segregation of utricular and saccular otic neurons along the anteroposterior axis, and the conferring of specificity to their patterns of innervation, have solely been adapted for zebrafish[142] . Given the distinct morphologies of the auditory structures in mice and zebrafish, it is not unexpected that signaling pathways such as Hh will elicit different developmental outcomes. The comparative analyses of the auditory organs in these and other species should be helpful in identifying additional signals that cooperate with Shh to promote cochlear duct outgrowth in amniotes.

Several lines of evidence support a requirement for Wnt signaling in vestibular morphogenesis[273, 28], but the pleiotropic nature of many Wnt mutants makes determining the function of Wnt in auditory development more challenging. Conditionally ablating β-catenin in the otic placode[133]or otic vesicle[218] arrests development before cochlear duct outgrowth, preventing an analysis of the requirement for Wnt signaling in sensory cell specification. Modulating the activity of Wnt ligands emanating from the hindbrain generates a less severe mutant phenotype that highlights the requirements for Wnt signaling in establishing dorsal otic identity. *Wnt1*$^{-/-}$*;Wnt3a*$^{-/-}$ mutant mice lack all vestibular structures and have a severely truncated cochlear duct[28], while performing a comparable experiment in chick by removing the embryonic hindbrain abolishes the vestibulum and has minimal effect on auditory organ,the basal papilla[29]. These subtle differences in results may be due to the differing approaches taken to abolish hindbrain Wnt signals.

To truly assay the role for Wnt/β-catenin in auditory development, experimental manipulations have to avoid perturbing the early roles for Wnt in placode formation and establishing dorsal otic identity. A few lines of evidence point to requirements for Wnt signaling later in otic development. Increasing Wnt activity in chick after dorsoventral polarity has been established using a degradation resistant β-catenin resulted both in ectopic hair cells in the basal papilla and a transformation of auditory hair cells to a vestibular morphology[169]. Two additional studies tie β-catenin to regulating hair cell fate. The master hair cell regulator *Atoh1*, is in part regulated by a 3‘ enhancer containing *Tcf/Lef* responsive elements[274]. The greater epithelia ridge, a transient structure adjacent to the inner hair cell, is competent to give rise to ectopic hair cells[275] and contains a transient population of cells that express the Wnt target *Lgr5* and differentiate into hair cells in culture[229]. Together these three studies suggest a role for Wnt/β-catenin signaling in auditory hair cell specification, but elucidating the function of Wnt signaling in vivo and mechanism of action require further studies.

We have been able to unmask previously unappreciated roles for Hedgehog signaling in driving the proliferation of cvg neuroblasts and establishing ventral otic identity independent of dorsal or lateral identity. We have identified dozens of presumptive Hedgehog target genes that are expressed in the developing cochlear duct. In doing so we have laid the groundwork for a better understanding of the gene regulatory networks and cell biological processes that control the formation of auditory structures. Finally, we have used

fate mapping studies to define the domain in the otic vesicle that gives rise to auditory sensory cells.

Appendix 1: *Smo*^{ecko} microarray results

Gene Symbol	RefSeq	Fold-Change
Otx2	NM_144841	-7.61759
Clu	NM_013492	-5.50419
Rorb	NM_001043354	-5.35633
Fst	NM_008046	-4.76437
Slc27a2	NM_011978	-4.19096
Muc15	NM_172979	-3.95275
Slc39a8	NM_001135149	-3.46418
Car13	NM_024495	-3.32742
Pcdh11x	NM_001081385	-3.30586
Sall1	NM_021390	-3.03052
1600029D21Rik	NM_029639	-2.89138
Moxd1	NM_021509	-2.76587
Gas2	NM_008087	-2.72027
Gm414	NM_001018031	-2.69983
Tacstd2	NM_020047	-2.68471
Cdkn1a	NM_007669	-2.61984
Emx2	NM_010132	-2.60864
E330013P04Rik	NR_026942	-2.56787
Fgf18	NM_008005	-2.53177
Fat3	NM_001080814	-2.46979
Calml4	NM_138304	-2.34371
Rspo2	NM_172815	-2.3196
Vmo1	NM_001013607	-2.29987
Pls1	NM_001033210	-2.29572
9930013L23Rik	NM_030728	-2.22254
Thrb	NM_009380	-2.19971
Smo	NM_176996	-2.15013
Ano1	NM_178642	-2.12321
Fam107a	NM_183187	-2.10726
Eya1	NM_010164	-2.09774
Ptch1	NM_008957	-2.09006
Eya4	NM_010167	-2.06935
Crym	NM_016669	-2.05948
Capn6	NM_007603	-2.05097
Brip1	NM_178309	-2.0403
Isl1	NM_021459	-2.0009
AK220484	NM_001083628	-1.99688
Frem1	NM_177863	-1.98346
Irgm1	NM_008326	-1.95945
Gipc2	NM_016867	-1.95169
IAP element encoding integrase	X16670	-1.92717
Gm4638	XM_001480931	-1.92717
Fam102b	ENSMUST00000046924	-1.91413
4933436C20Rik	ENSMUST00000034183	-1.90247
Pgf	NM_008827	-1.90108
Itih5	NM_172471	-1.81822
Pax2	NM_011037	-1.81296
AK220484	NM_001083628	-1.78556

Gene Symbol	RefSeq	Fold-Change
Gpr98	NM_054053	-1.73681
Mpzl2	NM_007962	-1.73328
Dsp	NM_023842	-1.71659
Ephb1	NM_173447	-1.70214
Trdn	NM_029726	-1.70112
Lect1	NM_010701	-1.70004
Sox2	NM_011443	-1.64665
Atp1b1	NM_009721	-1.64236
Nr0b1	NM_007430	-1.63853
C330024D21Rik	NR_015582.1	-1.63533
Emb	NM_010330	-1.61383
Rhpn2	NM_027897	-1.59696
Trdn	NM_029726	-1.5944
BC048679	ENSMUST00000073406	-1.58141
Epha7	NM_010141	-1.57672
Fgf20	NM_030610	-1.56852
Gli1	NM_010296	-1.55559
Lad1	NM_133664	-1.55358
Kcnh8	NM_001031811	-1.54047
Irx5	NM_018826	-1.53744
Cldn10	NM_021386	-1.53615
Gramd1b	NM_172768	-1.53604
Gpld1	NM_008156	-1.52982
Ckmt1	NM_009897	-1.52977
Nr2f1	NM_010151	-1.52779
2610018G03Rik	NM_133729	-1.52342
Elp4	NM_023876	-1.51896
B930095G15Rik	BC096543	-1.51484
Dsel	NM_001081316	-1.51143
Wfdc2	NM_026323	-1.49639
Cubn	NM_001081084	-1.48416
Sfrp1	NM_013834	-1.48144
Myo7a	NM_008663	-1.47525
Cldn10	NM_023878	-1.47171
Erbb3	NM_010153	-1.47162
Kif5c	NM_008449	-1.47054
Tmem30b	NM_178715	-1.46492
Cdca7l	NM_146040	-1.46464
Matn1	NM_010769	-1.46421
Gldc	NM_138595	-1.46318
Chd7	NM_001081417	-1.46093
Dennd4a	NM_001162917	-1.45503
Socs2	NM_007706	-1.45094
Trpc4	NM_016984	-1.44682
Sulf1	NM_172294	-1.44531
Cuedc2	NM_024192	-1.44114
Capg	NM_007599	-1.43697
Plch1	NM_183191	-1.43655

Gene Symbol	RefSeq	Fold-Change
Tox3	NM_172913	-1.43517
Tceal1	NM_146236	-1.42433
Ninl	NM_207204	-1.42199
Bmpr1b	NM_007560	-1.41843
Jag1	NM_013822	-1.41265
Itgb8	NM_177290	-1.40677
Irx3	NM_008393	-1.40231
Dtna	NM_207650	-1.36429
Acer2	BC051923	-1.36231
Ccnjl	NM_001045530	-1.36106
Ramp3	NM_019511	-1.35873
Gm8584	XR_033495	-1.35465
Prr15	NM_030024	-1.35352
Grtp1	NM_025768	-1.35273
Rragb	NM_001004154	-1.35129
Uevld	NM_001040695	-1.34868
Cpd	NM_007754	-1.3485
Mylk	NM_139300	-1.34495
Hook1	NM_030014	-1.34097
Nek1	NM_175089	-1.32747
Tmem144	NM_027495	-1.32559
Pdgfa	NM_008808	-1.30931
Got1	NM_010324	-1.30446
Rspo3	NM_028351	-1.30068
Obfc2a	NM_028696	-1.3005
Krt18	NM_010664	-1.29666
Glrx	NM_053108	-1.28438
Krtcap3	NM_027221	-1.28346
4933415A04Rik	ENSMUST00000056256	-1.28131
Fzd7	NM_008057	-1.27146
Ppp1r9a	NM_181595	-1.26946
1110012J17Rik	NM_001114098	-1.26854
Kit	NM_001122733	-1.26826
Sms	NM_009214	-1.2592
Nnat	NM_010923	-1.24473
Zfp691	NM_183140	-1.2436
Chd7	NM_001081417	-1.24055
Six1	NM_009189	-1.23963
Cdh2	NM_007664	-1.2316
Slc12a6	NM_133649	-1.21004
Rfesd	NM_178916	-1.18822
Fam60a	NM_019643	-1.14921
Gvin1	NM_029000	1.18735
Map3k3	NM_011947	1.18969
Lats2	NM_015771	1.19871
Tpm1	NM_024427	1.20196
Spnb2	NM_175836	1.20331
Acap2	NM_030138	1.20598

Gene Symbol	RefSeq	Fold-Change
Lgals3bp	NM_011150	1.22855
Cbln1	NM_019626	1.24815
Add1	NM_001102444	1.24819
Pcdh1	NM_029357	1.27764
Apbb2	NM_009686	1.28488
Gm13305	NM_001099348	1.28789
Il11ra1	NM_010549	1.28812
Axin2	NM_015732	1.28856
Junb	NM_008416	1.29419
Tmem35	NM_026239	1.30184
Gng2	NM_010315	1.30294
Epb4.1l2	NM_013511	1.30779
C130074G19Rik	NM_178692	1.31867
Bag3	NM_013863	1.32158
Unc5c	NM_009472	1.33203
B2m	NM_009735	1.33289
Dixdc1	NM_178118	1.33848
Gm12164	XR_031806	1.33863
Igfbp4	NM_010517	1.33962
Zfp521	NM_145492	1.34308
Srpx	NM_016911	1.34619
Prkg2	NM_008926	1.34741
1200009O22Rik	BC043099	1.34823
Zfp36l2	NM_001001806	1.35892
Rbp4	NM_001159487	1.36587
St5	NM_001001326	1.36683
Glt8d4	NM_198612	1.37219
Cachd1	NM_198037	1.37697
Synpo	NM_001109975	1.38297
Crtap	NM_019922	1.38416
Fkbp14	NM_153573	1.38444
Pdzrn3	NM_018884	1.40346
Cadm2	NM_178721	1.4053
Gm5098	ENSMUST00000104904	1.41528
Ccnd2	NM_009829	1.41567
St6galnac6	NM_016973	1.41994
Dock5	NM_177780	1.42836
Peg3	NM_008817	1.42905
Notum	NM_175263	1.43013
Cald1	NM_145575	1.43952
C630028N24Rik	NM_177351	1.44169
Cdh11	NM_009866	1.44285
Ifitm3	NM_025378	1.46089
Wnt5a	NM_009524	1.46492
Arhgap28	NM_172964	1.46567
Gamt	NM_010255	1.46778
Itpripl2	NM_001033380	1.47258
Phactr2	NM_001033257	1.47795

Gene Symbol	RefSeq	Fold-Change
Twist2	NM_007855	1.47968
Ebf3	NM_001113415	1.48304
Gng12	NM_025278	1.48544
Grm8	NM_008174	1.48607
Elk3	NM_013508	1.48955
Thra	NM_178060	1.48981
Nab1	NM_008667	1.48989
Cited1	NM_007709	1.49555
Cgnl1	NM_026599	1.49691
Aff2	NM_008032	1.49737
Fbn2	NM_010181	1.4976
Slc40a1	NM_016917	1.4984
Prex2	NM_029525	1.50506
Maged2	NM_030700	1.50813
Reck	NM_016678	1.50819
Erg	NM_133659	1.51337
Zic4	NM_009576	1.51422
Msn	NM_010833	1.52026
Fkbp7	NM_010222	1.53517
Sema6d	NM_199241	1.53625
Plagl1	NM_009538	1.54153
Plxnd1	NM_026376	1.55206
Emp1	NM_010128	1.56248
Gm15498	ENSMUST00000110990	1.57463
Ttc28	ENSMUST00000100894	1.57557
Tubb6	NM_026473	1.58189
Pcdh18	NM_130448	1.5882
Tanc2	NM_181071	1.59032
Trim9	NM_053167	1.6026
Tbc1d2b	NM_194334	1.60315
Fmod	NM_021355	1.60383
Tmem119	NM_146162	1.61182
Blvra	NM_026678	1.61449
Lef1	NM_010703	1.61685
Ebf2	NM_010095	1.62012
Lama2	NM_008481	1.6217
Ednrb	NM_007904	1.62225
Mmp2	NM_008610	1.62261
6330442E10Rik	BC079613	1.62945
Cpxm2	NM_018867	1.64293
Mecom	NM_021442	1.64562
Dab1	NM_177259	1.66426
Atp6v0d2	NM_175406	1.69078
Cdgap	NM_020260	1.70624
Inpp4b	NM_001024617	1.70866
4933409K07Rik	BC059060	1.70955
Tmsb15a	NM_030106	1.72141
Corin	NM_016869	1.7244

Gene Symbol	RefSeq	Fold-Change
Zcchc24	NM_001101433	1.72709
Rftn2	NM_028713	1.72961
4933409K07Rik	BC059060	1.73211
Pou3f4	NM_008901	1.73706
Gap43	NM_008083	1.74198
Scube1	NM_022723	1.74331
Slc38a4	NM_027052	1.74794
Ets1	NM_011808	1.75828
Arhgap29	NM_172525	1.76559
Dab2	NM_023118	1.77935
Foxp2	NM_053242	1.79091
Crispld1	NM_031402	1.79181
4933409K07Rik	BC059060	1.79896
Col12a1	NM_007730	1.80282
Anxa6	NM_013472	1.80389
4933409K07Rik	BC059060	1.81173
Timp3	NM_011595	1.81275
Islr	NM_012043	1.82877
Ctsc	NM_009982	1.84875
Plk2	NM_152804	1.85285
Anxa2	NM_007585	1.85977
Kank4	NM_172872	1.86288
Heg1	NM_175256	1.87375
Lgals1	NM_008495	1.89683
Zic2	NM_009574	1.89837
4933409K07Rik	BC072647	1.91697
Cd93	NM_010740	1.93647
Egfl6	NM_019397	1.94426
Pcolce	NM_008788	1.98985
Zim1	NM_011769	2.00726
Aard	NM_175503	2.01453
Fbn1	NM_007993	2.06432
Zeb2	NM_015753	2.07184
Zic1	NM_009573	2.0774
Gng8	NM_010320	2.10779
Sepp1	NM_009155	2.11273
Pdgfrb	NM_001146268	2.11583
Tmem45a	NM_019631	2.13286
6230427J02Rik	BC115538	2.1435
Nrp1	NM_008737	2.14489
Leprel1	NM_173379	2.15066
Nid1	NM_010917	2.15527
Abca9	NM_147220	2.15866
Bgn	NM_007542	2.19578
Arhgdib	NM_007486	2.33324
Twist1	NM_011658	2.34709
Foxd1	NM_008242	2.36302
Lix1	NM_025681	2.38287

Gene Symbol	RefSeq	Fold-Change
Aplnr	NM_011784	2.39194
Bmp5	NM_007555	2.42594
9030425E11Rik	NM_133733	2.42609
Cdh5	NM_009868	2.45582
Apod	NM_007470	2.51276
Anxa3	NM_013470	2.59438
Pdgfra	NM_011058	2.60061
Epha3	NM_010140	2.62098
Rhoj	NM_023275	2.64151
Ebf2	NM_010095	2.70925
Tgfbi	NM_009369	2.71426
Prrx1	NM_175686	2.80316
Ppbp	NM_023785	2.82878
Lix1l	ENSMUST00000062058	2.83123
A430107O13Rik	BC151018	2.87507
Hoxa2	NM_010451	2.90103
Mecom	NM_007963	3.04467
4930466F19Rik	ENSMUST00000098116	3.04883
Lrrc17	NM_028977	3.07445
Fli1	NM_008026	3.11411
Foxc2	NM_013519	3.56397
Lepr	NM_001122899	3.60172
Pf4	NM_019932	3.67285

Confirmed in SmoEcko
Ventral (this study)
Mesenchyme (this study)

Bibliography

[1]Johnston, C., *Auditory Apparatus of the Culex mosquito.* Quarterly Journal of Microscopical Sciences, 1855(3): p. 97-102.

[2]Kamikouchi, A., H.K. Inagaki, T. Effertz, O. Hendrich, A. Fiala, M.C. Gopfert, and K. Ito, *The neural basis of Drosophila gravity-sensing and hearing.* Nature, 2009. **458**(7235): p. 165-71.

[3]Beurg, M., R. Fettiplace, J.H. Nam, and A.J. Ricci, *Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging.* Nat Neurosci, 2009. **12**(5): p. 553-8.

[4]Hudspeth, A.J. and R. Jacobs, *Stereocilia mediate transduction in vertebrate hair cells (auditory system/cilium/vestibular system).* Proc Natl Acad Sci U S A, 1979. **76**(3): p. 1506-9.

[5]Lowenstein, O., *A functional interpretation of the electron-microscopic structure of the sensory hairs in the cristae of the elasmobranch Raja clavata in terms of directional sensitivity.* Nature, 1959. **184**: p. 1807-1808.

[6]Hudspeth, A. and D. Corey, *Sensitivity, polarity, and conductance change in the response of vertebrate hair cells to controlled mechanical stimuli.* Proceedings of the National Academy of Sciences, 1977. **74**(6): p. 2407.

[7]Lowenstein, O. and A. Sand, *The mechanism of the semicircular canal. A study of the responses of single-fibre preparations to angular accelerations and to rotation at constant speed.* Proceedings of the Royal Society of London. Series B-Biological Sciences, 1940. **129**(855): p. 256-275.

[8]Kazmierczak, P., H. Sakaguchi, J. Tokita, E.M. Wilson-Kubalek, R.A. Milligan, U. Muller, and B. Kachar, *Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells.* Nature, 2007. **449**(7158): p. 87-91.

[9]Kozlov, A.S., T. Risler, and A.J. Hudspeth, *Coherent motion of stereocilia assures the concerted gating of hair-cell transduction channels.* Nat Neurosci, 2007. **10**(1): p. 87-92.

[10]Corey, D.P. and A.J. Hudspeth, *Kinetics of the receptor current in bullfrog saccular hair cells.* J Neurosci, 1983. **3**(5): p. 962-76.

[11]Parsons, T.D., D. Lenzi, W. Almers, and W.M. Roberts, *Calcium-triggered exocytosis and endocytosis in an isolated presynaptic cell: capacitance measurements in saccular hair cells.* Neuron, 1994. **13**(4): p. 875-83.

[12.]Seal, R.P., O. Akil, E. Yi, C.M. Weber, L. Grant, J. Yoo, A. Clause, K. Kandler, J.L. Noebels, E. Glowatzki, L.R. Lustig, and R.H. Edwards, *Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3.* Neuron, 2008. **57**(2): p. 263-75.

[13.]Denk, W. and W.W. Webb, *Forward and reverse transduction at the limit of sensitivity studied by correlating electrical and mechanical fluctuations in frog saccular hair cells.* Hear Res, 1992. **60**(1): p. 89-102.

[14.]Anniko, M., *Development of otoconia.* Am J Otolaryngol, 1980. **1**(5): p. 400-10.

[15.]Lowenstein, O. and T. Roberts, *The equilibrium function of the otolith organs of the thornback ray (Raja clavata).* The Journal of physiology, 1949. **110**(3-4): p. 392-415.

[16.]Abraira, V.E., T. Del Rio, A.F. Tucker, J. Slonimsky, H.L. Keirnes, and L.V. Goodrich, *Cross-repressive interactions between Lrig3 and netrin 1 shape the architecture of the inner ear.* Development, 2008. **135**(24): p. 4091-9.

[17.]Epley, J., *The canalith repositioning procedure: for treatment of benign paroxysmal positional vertigo.* Otolaryngology--head and neck surgery: official journal of American Academy of Otolaryngology-Head and Neck Surgery, 1992. **107**(3): p. 399.

[18.]Kumar, S. and S.B. Hedges, *A molecular timescale for vertebrate evolution.* Nature, 1998. **392**(6679): p. 917-920.

[19.]Wray, G.A., J.S. Levinton, and L.H. Shapiro, *Molecular evidence for deep Precambrian divergences among metazoan phyla.* Science, 1996. **274**(5287): p. 568-573.

[20.]Von Bekesy, G., *Experiments in hearing.* 1960.

[21.]Rubel, E.W. and B. Fritzsch, *Auditory system development: primary auditory neurons and their targets.* Annu Rev Neurosci, 2002. **25**: p. 51-101.

[22.]Kemp, D.T., *Stimulated acoustic emissions from within the human auditory system.* The Journal of the Acoustical Society of America, 1978. **64**(5): p. 1386-1391.

[23.]Kennedy, H.J., A.C. Crawford, and R. Fettiplace, *Force generation by mammalian hair bundles supports a role in cochlear amplification.* Nature, 2005. **433**(7028): p. 880-3.

[24]Liberman, M.C., J. Gao, D.Z. He, X. Wu, S. Jia, and J. Zuo, *Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier.* Nature, 2002. **419**(6904): p. 300-4.

[25]Corey, D. and A. Hudspeth, *Response latency of vertebrate hair cells.* Biophysical journal, 1979. **26**(3): p. 499-506.

[26]Narins, P.M. and E.R. Lewis, *The vertebrate ear as an exquisite seismic sensor.* The Journal of the Acoustical Society of America, 1984. **76**: p. 1384.

[27]Riccomagno, M.M., L. Martinu, M. Mulheisen, D.K. Wu, and D.J. Epstein, *Specification of the mammalian cochlea is dependent on Sonic hedgehog.* Genes Dev, 2002. **16**(18): p. 2365-78.

[28]Riccomagno, M.M., S. Takada, and D.J. Epstein, *Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh.* Genes Dev, 2005. **19**(13): p. 1612-23.

[29]Bok, J., M. Bronner-Fraser, and D.K. Wu, *Role of the hindbrain in dorsoventral but not anteroposterior axial specification of the inner ear.* Development, 2005. **132**(9): p. 2115-24.

[30]Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila.* Nature, 1980. **287**(5785): p. 795-801.

[31]Brown, A.S. and D.J. Epstein, *Otic ablation of smoothened reveals direct and indirect requirements for Hedgehog signaling in inner ear development.* Development, 2011.

[32]Bok, J., D.K. Dolson, P. Hill, U. Ruther, D.J. Epstein, and D.K. Wu, *Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear.* Development, 2007. **134**(9): p. 1713-22.

[33]Echelard, Y., D.J. Epstein, B. St-Jacques, L. Shen, J. Mohler, J.A. McMahon, and A.P. McMahon, *Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity.* Cell, 1993. **75**(7): p. 1417-30.

[34]Krauss, S., J.P. Concordet, and P.W. Ingham, *A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos.* Cell, 1993. **75**(7): p. 1431-44.

[35]Riddle, R.D., R.L. Johnson, E. Laufer, and C. Tabin, *Sonic hedgehog mediates the polarizing activity of the ZPA.* Cell, 1993. **75**(7): p. 1401-16.

[36]Ingham, P.W., A.M. Taylor, and Y. Nakano, *Role of the Drosophila patched gene in positional signalling.* Nature, 1991. **353**(6340): p. 184-7.

[37]Hahn, H., C. Wicking, P.G. Zaphiropoulous, M.R. Gailani, S. Shanley, A. Chidambaram, I. Vorechovsky, E. Holmberg, A.B. Unden, S. Gillies, K. Negus, I. Smyth, C. Pressman, D.J. Leffell, B. Gerrard, A.M. Goldstein, M. Dean, R. Toftgard, G. Chenevix-Trench, B. Wainwright, and A.E. Bale, *Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome.* Cell, 1996. **85**(6): p. 841-51.

[38]Johnson, R.L., A.L. Rothman, J. Xie, L.V. Goodrich, J.W. Bare, J.M. Bonifas, A.G. Quinn, R.M. Myers, D.R. Cox, E.H. Epstein, Jr., and M.P. Scott, *Human homolog of patched, a candidate gene for the basal cell nevus syndrome.* Science, 1996. **272**(5268): p. 1668-71.

[39]Goodrich, L.V., L. Milenkovic, K.M. Higgins, and M.P. Scott, *Altered neural cell fates and medulloblastoma in mouse patched mutants.* Science, 1997. **277**(5329): p. 1109-13.

[40]Fuse, N., T. Maiti, B. Wang, J.A. Porter, T.M. Hall, D.J. Leahy, and P.A. Beachy, *Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 10992-9.

[41]Marigo, V., R.A. Davey, Y. Zuo, J.M. Cunningham, and C.J. Tabin, *Biochemical evidence that patched is the Hedgehog receptor.* Nature, 1996. **384**(6605): p. 176-9.

[42]Zheng, X., R.K. Mann, N. Sever, and P.A. Beachy, *Genetic and biochemical definition of the Hedgehog receptor.* Genes Dev, 2010. **24**(1): p. 57-71.

[43]Yao, S., L. Lum, and P. Beachy, *The ihog cell-surface proteins bind Hedgehog and mediate pathway activation.* Cell, 2006. **125**(2): p. 343-57.

[44]Allen, B.L., T. Tenzen, and A.P. McMahon, *The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development.* Genes Dev, 2007. **21**(10): p. 1244-57.

[45]Martinelli, D.C. and C.M. Fan, *Gas1 extends the range of Hedgehog action by facilitating its signaling.* Genes Dev, 2007. **21**(10): p. 1231-43.

[46]Christ, A., A. Christa, E. Kur, O. Lioubinski, S. Bachmann, T.E. Willnow, and A. Hammes, *LRP2 is an auxiliary SHH receptor required to condition the forebrain ventral midline for inductive signals.* Dev Cell, 2012. **22**(2): p. 268-78.

[47]Carstea, E.D., J.A. Morris, K.G. Coleman, S.K. Loftus, D. Zhang, C. Cummings, J. Gu, M.A. Rosenfeld, W.J. Pavan, D.B. Krizman, J. Nagle, M.H.

Polymeropoulos, S.L. Sturley, Y.A. Ioannou, M.E. Higgins, M. Comly, A. Cooney, A. Brown, C.R. Kaneski, E.J. Blanchette-Mackie, N.K. Dwyer, E.B. Neufeld, T.Y. Chang, L. Liscum, J.F. Strauss, 3rd, K. Ohno, M. Zeigler, R. Carmi, J. Sokol, D. Markie, R.R. O'Neill, O.P. van Diggelen, M. Elleder, M.C. Patterson, R.O. Brady, M.T. Vanier, P.G. Pentchev, and D.A. Tagle, *Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis.* Science, 1997. **277**(5323): p. 228-31.

[48.] Ioannou, Y.A., *Multidrug permeases and subcellular cholesterol transport.* Nat Rev Mol Cell Biol, 2001. **2**(9): p. 657-68.

[49.] Taipale, J., M.K. Cooper, T. Maiti, and P.A. Beachy, *Patched acts catalytically to suppress the activity of Smoothened.* Nature, 2002. **418**(6900): p. 892-7.

[50.] Khaliullina, H., D. Panakova, C. Eugster, F. Riedel, M. Carvalho, and S. Eaton, *Patched regulates Smoothened trafficking using lipoprotein-derived lipids.* Development, 2009.

[51.] Chen, J.K., J. Taipale, M.K. Cooper, and P.A. Beachy, *Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened.* Genes Dev, 2002. **16**(21): p. 2743-8.

[52.] Binns, W., L.F. James, J.L. Shupe, and E.J. Thacker, *Cyclopian-type malformation in lambs.* Arch Environ Health, 1962. **5**: p. 106-8.

[53.] Keeler, R.F. and W. Binns, *Teratogenic compounds of Veratrum californicum (Durand). I. Preparation and characterization of fractions and alkaloids for biologic testing.* Can J Biochem, 1966. **44**(6): p. 819-28.

[54.] Chen, J.K., J. Taipale, K.E. Young, T. Maiti, and P.A. Beachy, *Small molecule modulation of Smoothened activity.* Proc Natl Acad Sci U S A, 2002. **99**(22): p. 14071-6.

[55.] van den Heuvel, M. and P.W. Ingham, *smoothened encodes a receptor-like serpentine protein required for hedgehog signalling.* Nature, 1996. **382**(6591): p. 547-51.

[56.] Robbins, D.J., K.E. Nybakken, R. Kobayashi, J.C. Sisson, J.M. Bishop, and P.P. Therond, *Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2.* Cell, 1997. **90**(2): p. 225-34.

[57.] Sisson, J.C., K.S. Ho, K. Suyama, and M.P. Scott, *Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway.* Cell, 1997. **90**(2): p. 235-45.

[58]Chen, Y., N. Gallaher, R.H. Goodman, and S.M. Smolik, *Protein kinase A directly regulates the activity and proteolysis of cubitus interruptus.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2349-54.

[59]Preat, T., P. Therond, C. Lamour-Isnard, B. Limbourg-Bouchon, H. Tricoire, I. Erk, M.C. Mariol, and D. Busson, *A putative serine/threonine protein kinase encoded by the segment-polarity fused gene of Drosophila.* Nature, 1990. **347**(6288): p. 87-9.

[60]Preat, T., *Characterization of Suppressor of fused, a complete suppressor of the fused segment polarity gene of Drosophila melanogaster.* Genetics, 1992. **132**(3): p. 725-36.

[61]Wang, G., K. Amanai, B. Wang, and J. Jiang, *Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus.* Genes Dev, 2000. **14**(22): p. 2893-905.

[62]Zhang, W., Y. Zhao, C. Tong, G. Wang, B. Wang, J. Jia, and J. Jiang, *Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of Cubitus interruptus.* Dev Cell, 2005. **8**(2): p. 267-78.

[63]Alexandre, C., A. Jacinto, and P.W. Ingham, *Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins.* Genes Dev, 1996. **10**(16): p. 2003-13.

[64]Aza-Blanc, P., F.A. Ramirez-Weber, M.P. Laget, C. Schwartz, and T.B. Kornberg, *Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor.* Cell, 1997. **89**(7): p. 1043-53.

[65]Tempe, D., M. Casas, S. Karaz, M.F. Blanchet-Tournier, and J.P. Concordet, *Multisite protein kinase A and glycogen synthase kinase 3beta phosphorylation leads to Gli3 ubiquitination by SCFbetaTrCP.* Mol Cell Biol, 2006. **26**(11): p. 4316-26.

[66]Huangfu, D., A. Liu, A.S. Rakeman, N.S. Murcia, L. Niswander, and K.V. Anderson, *Hedgehog signalling in the mouse requires intraflagellar transport proteins.* Nature, 2003. **426**(6962): p. 83-7.

[67]Liu, A., B. Wang, and L.A. Niswander, *Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors.* Development, 2005. **132**(13): p. 3103-11.

[68]Aza-Blanc, P., H.Y. Lin, A. Ruiz i Altaba, and T.B. Kornberg, *Expression of the vertebrate Gli proteins in Drosophila reveals a distribution of activator and repressor activities.* Development, 2000. **127**(19): p. 4293-301.

[69]Rohatgi, R., L. Milenkovic, and M.P. Scott, *Patched1 regulates hedgehog signaling at the primary cilium.* Science, 2007. **317**(5836): p. 372-6.

[70]Corbit, K.C., P. Aanstad, V. Singla, A.R. Norman, D.Y. Stainier, and J.F. Reiter, *Vertebrate Smoothened functions at the primary cilium.* Nature, 2005. **437**(7061): p. 1018-21.

[71]Rohatgi, R., L. Milenkovic, R.B. Corcoran, and M.P. Scott, *Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3196-201.

[72]Haycraft, C.J., B. Banizs, Y. Aydin-Son, Q. Zhang, E.J. Michaud, and B.K. Yoder, *Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function.* PLoS Genet, 2005. **1**(4): p. e53.

[73]Humke, E.W., K.V. Dorn, L. Milenkovic, M.P. Scott, and R. Rohatgi, *The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins.* Genes Dev, 2010. **24**(7): p. 670-82.

[74]Svard, J., K. Heby-Henricson, M. Persson-Lek, B. Rozell, M. Lauth, A. Bergstrom, J. Ericson, R. Toftgard, and S. Teglund, *Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway.* Dev Cell, 2006. **10**(2): p. 187-97.

[75]Jia, J., A. Kolterud, H. Zeng, A. Hoover, S. Teglund, R. Toftgard, and A. Liu, *Suppressor of Fused inhibits mammalian Hedgehog signaling in the absence of cilia.* Dev Biol, 2009. **330**(2): p. 452-60.

[76]Cheung, H.O., X. Zhang, A. Ribeiro, R. Mo, S. Makino, V. Puviindran, K.K. Law, J. Briscoe, and C.C. Hui, *The kinesin protein Kif7 is a critical regulator of Gli transcription factors in mammalian hedgehog signaling.* Sci Signal, 2009. **2**(76): p. ra29.

[77]Endoh-Yamagami, S., M. Evangelista, D. Wilson, X. Wen, J.W. Theunissen, K. Phamluong, M. Davis, S.J. Scales, M.J. Solloway, F.J. de Sauvage, and A.S. Peterson, *The mammalian Cos2 homolog Kif7 plays an essential role in modulating Hh signal transduction during development.* Curr Biol, 2009. **19**(15): p. 1320-6.

[78]Liem, K.F., Jr., M. He, P.J. Ocbina, and K.V. Anderson, *Mouse Kif7/Costal2 is a cilia-associated protein that regulates Sonic hedgehog signaling.* Proc Natl Acad Sci U S A, 2009. **106**(32): p. 13377-82.

[79]Dessaud, E., L.L. Yang, K. Hill, B. Cox, F. Ulloa, A. Ribeiro, A. Mynett, B.G. Novitch, and J. Briscoe, *Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism.* Nature, 2007. **450**(7170): p. 717-20.

[80]Bai, C.B., D. Stephen, and A.L. Joyner, *All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3.* Dev Cell, 2004. **6**(1): p. 103-15.

[81]Litingtung, Y. and C. Chiang, *Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3.* Nat Neurosci, 2000. **3**(10): p. 979-85.

[82]Persson, M., D. Stamataki, P. te Welscher, E. Andersson, J. Bose, U. Ruther, J. Ericson, and J. Briscoe, *Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity.* Genes Dev, 2002. **16**(22): p. 2865-78.

[83]Hill, P., K. Gotz, and U. Ruther, *A SHH-independent regulation of Gli3 is a significant determinant of anteroposterior patterning of the limb bud.* Dev Biol, 2009. **328**(2): p. 506-16.

[84]Jeong, J. and A.P. McMahon, *Growth and pattern of the mammalian neural tube are governed by partially overlapping feedback activities of the hedgehog antagonists patched 1 and Hhip1.* Development, 2005. **132**(1): p. 143-54.

[85]Balaskas, N., A. Ribeiro, J. Panovska, E. Dessaud, N. Sasai, K.M. Page, J. Briscoe, and V. Ribes, *Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube.* Cell, 2012. **148**(1-2): p. 273-84.

[86]Glinka, A., W. Wu, H. Delius, A.P. Monaghan, C. Blumenstock, and C. Niehrs, *Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction.* Nature, 1998. **391**(6665): p. 357-62.

[87]Hsieh, J.C., L. Kodjabachian, M.L. Rebbert, A. Rattner, P.M. Smallwood, C.H. Samos, R. Nusse, I.B. Dawid, and J. Nathans, *A new secreted protein that binds to Wnt proteins and inhibits their activities.* Nature, 1999. **398**(6726): p. 431-6.

[88]Rattner, A., J.C. Hsieh, P.M. Smallwood, D.J. Gilbert, N.G. Copeland, N.A. Jenkins, and J. Nathans, *A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2859-63.

[89]Katanaev, V.L., R. Ponzielli, M. Semeriva, and A. Tomlinson, *Trimeric G protein-dependent frizzled signaling in Drosophila.* Cell, 2005. **120**(1): p. 111-22.

[90].Sheldahl, L.C., M. Park, C.C. Malbon, and R.T. Moon, *Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner.* Curr Biol, 1999. **9**(13): p. 695-8.

[91].Slusarski, D.C., V.G. Corces, and R.T. Moon, *Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling.* Nature, 1997. **390**(6658): p. 410-3.

[92].Slusarski, D.C., J. Yang-Snyder, W.B. Busa, and R.T. Moon, *Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A.* Dev Biol, 1997. **182**(1): p. 114-20.

[93].Willert, K., J.D. Brown, E. Danenberg, A.W. Duncan, I.L. Weissman, T. Reya, J.R. Yates, 3rd, and R. Nusse, *Wnt proteins are lipid-modified and can act as stem cell growth factors.* Nature, 2003. **423**(6938): p. 448-52.

[94].Bhanot, P., M. Brink, C.H. Samos, J.C. Hsieh, Y. Wang, J.P. Macke, D. Andrew, J. Nathans, and R. Nusse, *A new member of the frizzled family from Drosophila functions as a Wingless receptor.* Nature, 1996. **382**(6588): p. 225-30.

[95].Pinson, K.I., J. Brennan, S. Monkley, B.J. Avery, and W.C. Skarnes, *An LDL-receptor-related protein mediates Wnt signalling in mice.* Nature, 2000. **407**(6803): p. 535-8.

[96].Tamai, K., M. Semenov, Y. Kato, R. Spokony, C. Liu, Y. Katsuyama, F. Hess, J.P. Saint-Jeannet, and X. He, *LDL-receptor-related proteins in Wnt signal transduction.* Nature, 2000. **407**(6803): p. 530-5.

[97].Wehrli, M., S.T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz, D. Vaizel-Ohayon, E. Schejter, A. Tomlinson, and S. DiNardo, *arrow encodes an LDL-receptor-related protein essential for Wingless signalling.* Nature, 2000. **407**(6803): p. 527-30.

[98].Boutros, M., J. Mihaly, T. Bouwmeester, and M. Mlodzik, *Signaling specificity by Frizzled receptors in Drosophila.* Science, 2000. **288**(5472): p. 1825-8.

[99].Klingensmith, J., R. Nusse, and N. Perrimon, *The Drosophila segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal.* Genes Dev, 1994. **8**(1): p. 118-30.

[100].Rubinfeld, B., B. Souza, I. Albert, O. Muller, S.H. Chamberlain, F.R. Masiarz, S. Munemitsu, and P. Polakis, *Association of the APC gene product with beta-catenin.* Science, 1993. **262**(5140): p. 1731-4.

[101].Zeng, L., F. Fagotto, T. Zhang, W. Hsu, T.J. Vasicek, W.L. Perry, 3rd, J.J. Lee, S.M. Tilghman, B.M. Gumbiner, and F. Costantini, *The mouse Fused locus*

encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell, 1997. **90**(1): p. 181-92.

[102.]Behrens, J., B.A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich, and W. Birchmeier, *Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta.* Science, 1998. **280**(5363): p. 596-9.

[103.]Yost, C., M. Torres, J.R. Miller, E. Huang, D. Kimelman, and R.T. Moon, *The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3.* Genes Dev, 1996. **10**(12): p. 1443-54.

[104.]Amit, S., A. Hatzubai, Y. Birman, J.S. Andersen, E. Ben-Shushan, M. Mann, Y. Ben-Neriah, and I. Alkalay, *Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway.* Genes Dev, 2002. **16**(9): p. 1066-76.

[105.]Liu, C., Y. Li, M. Semenov, C. Han, G.H. Baeg, Y. Tan, Z. Zhang, X. Lin, and X. He, *Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism.* Cell, 2002. **108**(6): p. 837-47.

[106.]Yanagawa, S., Y. Matsuda, J.S. Lee, H. Matsubayashi, S. Sese, T. Kadowaki, and A. Ishimoto, *Casein kinase I phosphorylates the Armadillo protein and induces its degradation in Drosophila.* EMBO J, 2002. **21**(7): p. 1733-42.

[107.]Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler, *beta-catenin is a target for the ubiquitin-proteasome pathway.* EMBO J, 1997. **16**(13): p. 3797-804.

[108.]Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier, *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. **382**(6592): p. 638-42.

[109.]Molenaar, M., M. van de Wetering, M. Oosterwegel, J. Peterson-Maduro, S. Godsave, V. Korinek, J. Roose, O. Destree, and H. Clevers, *XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos.* Cell, 1996. **86**(3): p. 391-9.

[110.]van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin, and H. Clevers, *Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF.* Cell, 1997. **88**(6): p. 789-99.

[111.]Brannon, M., M. Gomperts, L. Sumoy, R.T. Moon, and D. Kimelman, *A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus.* Genes Dev, 1997. **11**(18): p. 2359-70.

[112]Cavallo, R.A., R.T. Cox, M.M. Moline, J. Roose, G.A. Polevoy, H. Clevers, M. Peifer, and A. Bejsovec, *Drosophila Tcf and Groucho interact to repress Wingless signalling activity.* Nature, 1998. **395**(6702): p. 604-8.

[113]Roose, J., M. Molenaar, J. Peterson, J. Hurenkamp, H. Brantjes, P. Moerer, M. van de Wetering, O. Destree, and H. Clevers, *The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors.* Nature, 1998. **395**(6702): p. 608-12.

[114]Jho, E.H., T. Zhang, C. Domon, C.K. Joo, J.N. Freund, and F. Costantini, *Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway.* Mol Cell Biol, 2002. **22**(4): p. 1172-83.

[115]DasGupta, R. and E. Fuchs, *Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation.* Development, 1999. **126**(20): p. 4557-68.

[116]Maretto, S., M. Cordenonsi, S. Dupont, P. Braghetta, V. Broccoli, A.B. Hassan, D. Volpin, G.M. Bressan, and S. Piccolo, *Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3299-304.

[117]Dabdoub, A., M.J. Donohue, A. Brennan, V. Wolf, M. Montcouquiol, D.A. Sassoon, J.C. Hseih, J.S. Rubin, P.C. Salinas, and M.W. Kelley, *Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea.* Development, 2003. **130**(11): p. 2375-84.

[118]Qian, D., C. Jones, A. Rzadzinska, S. Mark, X. Zhang, K.P. Steel, X. Dai, and P. Chen, *Wnt5a functions in planar cell polarity regulation in mice.* Dev Biol, 2007. **306**(1): p. 121-33.

[119]Vinson, C.R. and P.N. Adler, *Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of Drosophila.* Nature, 1987. **329**(6139): p. 549-51.

[120]Heisenberg, C.P., M. Tada, G.J. Rauch, L. Saude, M.L. Concha, R. Geisler, D.L. Stemple, J.C. Smith, and S.W. Wilson, *Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation.* Nature, 2000. **405**(6782): p. 76-81.

[121]Theisen, H., J. Purcell, M. Bennett, D. Kansagara, A. Syed, and J.L. Marsh, *dishevelled is required during wingless signaling to establish both cell polarity and cell identity.* Development, 1994. **120**(2): p. 347-60.

[122.]Wallingford, J.B., B.A. Rowning, K.M. Vogeli, U. Rothbacher, S.E. Fraser, and R.M. Harland, *Dishevelled controls cell polarity during Xenopus gastrulation.* Nature, 2000. **405**(6782): p. 81-5.

[123.]Strutt, D.I., U. Weber, and M. Mlodzik, *The role of RhoA in tissue polarity and Frizzled signalling.* Nature, 1997. **387**(6630): p. 292-5.

[124.]Eaton, S., R. Wepf, and K. Simons, *Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of Drosophila.* J Cell Biol, 1996. **135**(5): p. 1277-89.

[125.]Boutros, M., N. Paricio, D.I. Strutt, and M. Mlodzik, *Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling.* Cell, 1998. **94**(1): p. 109-18.

[126.]Bastock, R., H. Strutt, and D. Strutt, *Strabismus is asymmetrically localised and binds to Prickle and Dishevelled during Drosophila planar polarity patterning.* Development, 2003. **130**(13): p. 3007-14.

[127.]Das, G., A. Jenny, T.J. Klein, S. Eaton, and M. Mlodzik, *Diego interacts with Prickle and Strabismus/Van Gogh to localize planar cell polarity complexes.* Development, 2004. **131**(18): p. 4467-76.

[128.]Jenny, A., R.S. Darken, P.A. Wilson, and M. Mlodzik, *Prickle and Strabismus form a functional complex to generate a correct axis during planar cell polarity signaling.* EMBO J, 2003. **22**(17): p. 4409-20.

[129.]Liu, D., H. Chu, L. Maves, Y.L. Yan, P.A. Morcos, J.H. Postlethwait, and M. Westerfield, *Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification.* Development, 2003. **130**(10): p. 2213-24.

[130.]Alvarez, Y., M.T. Alonso, V. Vendrell, L.C. Zelarayan, P. Chamero, T. Theil, M.R. Bosl, S. Kato, M. Maconochie, D. Riethmacher, and T. Schimmang, *Requirements for FGF3 and FGF10 during inner ear formation.* Development, 2003. **130**(25): p. 6329-38.

[131.]Wright, T.J. and S.L. Mansour, *Fgf3 and Fgf10 are required for mouse otic placode induction.* Development, 2003. **130**(15): p. 3379-90.

[132.]Ladher, R.K., K.U. Anakwe, A.L. Gurney, G.C. Schoenwolf, and P.H. Francis-West, *Identification of synergistic signals initiating inner ear development.* Science, 2000. **290**(5498): p. 1965-7.

[133.]Ohyama, T., O.A. Mohamed, M.M. Taketo, D. Dufort, and A.K. Groves, *Wnt signals mediate a fate decision between otic placode and epidermis.* Development, 2006. **133**(5): p. 865-75.

[134]Brigande, J.V., L.E. Iten, and D.M. Fekete, *A fate map of chick otic cup closure reveals lineage boundaries in the dorsal otocyst.* Dev Biol, 2000. **227**(2): p. 256-70.

[135]Harrison, R.G., *Relations of Symmetry in the Developing Ear of Amblystoma Punctatum.* Proc Natl Acad Sci U S A, 1936. **22**(4): p. 238-47.

[136]Wu, D.K., F.D. Nunes, and D. Choo, *Axial specification for sensory organs versus non-sensory structures of the chicken inner ear.* Development, 1998. **125**(1): p. 11-20.

[137]Ali, M.M., S. Jayabalan, M. Machnicki, and G.S. Sohal, *Ventrally emigrating neural tube cells migrate into the developing vestibulocochlear nerve and otic vesicle.* Int J Dev Neurosci, 2003. **21**(4): p. 199-208.

[138]Freyer, L., V. Aggarwal, and B.E. Morrow, *Dual embryonic origin of the mammalian otic vesicle forming the inner ear.* Development, 2011. **138**(24): p. 5403-14.

[139]Haddon, C. and J. Lewis, *Early ear development in the embryo of the zebrafish, Danio rerio.* J Comp Neurol, 1996. **365**(1): p. 113-28.

[140]Bok, J., S. Raft, K.A. Kong, S.K. Koo, U.C. Drager, and D.K. Wu, *Transient retinoic acid signaling confers anterior-posterior polarity to the inner ear.* Proc Natl Acad Sci U S A, 2011. **108**(1): p. 161-6.

[141]Hammond, K.L., H.E. Loynes, A.A. Folarin, J. Smith, and T.T. Whitfield, *Hedgehog signalling is required for correct anteroposterior patterning of the zebrafish otic vesicle.* Development, 2003. **130**(7): p. 1403-17.

[142]Sapede, D. and C. Pujades, *Hedgehog signaling governs the development of otic sensory epithelium and its associated innervation in zebrafish.* J Neurosci, 2010. **30**(10): p. 3612-23.

[143]Waldman, E.H., A. Castillo, and A. Collazo, *Ablation studies on the developing inner ear reveal a propensity for mirror duplications.* Dev Dyn, 2007. **236**(5): p. 1237-48.

[144]Hammond, K.L., F.J. van Eeden, and T.T. Whitfield, *Repression of Hedgehog signalling is required for the acquisition of dorsolateral cell fates in the zebrafish otic vesicle.* Development, 2010. **137**(8): p. 1361-71.

[145]Hammond, K.L. and T.T. Whitfield, *Fgf and Hh signalling act on a symmetrical pre-pattern to specify anterior and posterior identity in the zebrafish otic placode and vesicle.* Development, 2011. **138**(18): p. 3977-87.

[146]Driver, E.C., S.P. Pryor, P. Hill, J. Turner, U. Ruther, L.G. Biesecker, A.J. Griffith, and M.W. Kelley, *Hedgehog signaling regulates sensory cell formation and auditory function in mice and humans.* J Neurosci, 2008. **28**(29): p. 7350-8.

[147]Curtin, J.A., E. Quint, V. Tsipouri, R.M. Arkell, B. Cattanach, A.J. Copp, D.J. Henderson, N. Spurr, P. Stanier, E.M. Fisher, P.M. Nolan, K.P. Steel, S.D. Brown, I.C. Gray, and J.N. Murdoch, *Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse.* Curr Biol, 2003. **13**(13): p. 1129-33.

[148]Mao, Y., J. Mulvaney, S. Zakaria, T. Yu, K.M. Morgan, S. Allen, M.A. Basson, P. Francis-West, and K.D. Irvine, *Characterization of a Dchs1 mutant mouse reveals requirements for Dchs1-Fat4 signaling during mammalian development.* Development, 2011. **138**(5): p. 947-57.

[149]Montcouquiol, M., R.A. Rachel, P.J. Lanford, N.G. Copeland, N.A. Jenkins, and M.W. Kelley, *Identification of Vangl2 and Scrb1 as planar polarity genes in mammals.* Nature, 2003. **423**(6936): p. 173-7.

[150]Depew, M.J., J.K. Liu, J.E. Long, R. Presley, J.J. Meneses, R.A. Pedersen, and J.L. Rubenstein, *Dlx5 regulates regional development of the branchial arches and sensory capsules.* Development, 1999. **126**(17): p. 3831-46.

[151]Salminen, M., B.I. Meyer, E. Bober, and P. Gruss, *Netrin 1 is required for semicircular canal formation in the mouse inner ear.* Development, 2000. **127**(1): p. 13-22.

[152]Cecconi, F., K.A. Roth, O. Dolgov, E. Munarriz, K. Anokhin, P. Gruss, and M. Salminen, *Apaf1-dependent programmed cell death is required for inner ear morphogenesis and growth.* Development, 2004. **131**(9): p. 2125-35.

[153]Ma, Q., Z. Chen, I. del Barco Barrantes, J.L. de la Pompa, and D.J. Anderson, *neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia.* Neuron, 1998. **20**(3): p. 469-82.

[154]Kim, W.Y., B. Fritzsch, A. Serls, L.A. Bakel, E.J. Huang, L.F. Reichardt, D.S. Barth, and J.E. Lee, *NeuroD-null mice are deaf due to a severe loss of the inner ear sensory neurons during development.* Development, 2001. **128**(3): p. 417-26.

[155]Radde-Gallwitz, K., L. Pan, L. Gan, X. Lin, N. Segil, and P. Chen, *Expression of Islet1 marks the sensory and neuronal lineages in the mammalian inner ear.* J Comp Neurol, 2004. **477**(4): p. 412-21.

[156.]Koundakjian, E.J., J.L. Appler, and L.V. Goodrich, *Auditory neurons make stereotyped wiring decisions before maturation of their targets.* J Neurosci, 2007. **27**(51): p. 14078-88.

[157.]Ruben, R.J., *Development of the inner ear of the mouse: a radioautographic study of terminal mitoses.* Acta Otolaryngol, 1967: p. Suppl 220: 1.

[158.]Ernfors, P., T. Van De Water, J. Loring, and R. Jaenisch, *Complementary roles of BDNF and NT-3 in vestibular and auditory development.* Neuron, 1995. **14**(6): p. 1153-64.

[159.]Ard, M.D. and D.K. Morest, *Cell-Death during Development of the Cochlear and Vestibular Ganglia of the Chick.* International Journal of Developmental Neuroscience, 1984. **2**(6): p. 535-547.

[160.]Hellard, D., T. Brosenitsch, B. Fritzsch, and D.M. Katz, *Cranial sensory neuron development in the absence of brain-derived neurotrophic factor in BDNF/Bax double null mice.* Dev Biol, 2004. **275**(1): p. 34-43.

[161.]Kiernan, A.E., A.L. Pelling, K.K. Leung, A.S. Tang, D.M. Bell, C. Tease, R. Lovell-Badge, K.P. Steel, and K.S. Cheah, *Sox2 is required for sensory organ development in the mammalian inner ear.* Nature, 2005. **434**(7036): p. 1031-5.

[162.]Morrison, A., C. Hodgetts, A. Gossler, M. Hrabe de Angelis, and J. Lewis, *Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear.* Mech Dev, 1999. **84**(1-2): p. 169-72.

[163.]Kiernan, A.E., J. Xu, and T. Gridley, *The Notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear.* PLoS Genet, 2006. **2**(1): p. e4.

[164.]Lanford, P.J., Y. Lan, R. Jiang, C. Lindsell, G. Weinmaster, T. Gridley, and M.W. Kelley, *Notch signalling pathway mediates hair cell development in mammalian cochlea.* Nat Genet, 1999. **21**(3): p. 289-92.

[165.]Bermingham, N.A., B.A. Hassan, S.D. Price, M.A. Vollrath, N. Ben-Arie, R.A. Eatock, H.J. Bellen, A. Lysakowski, and H.Y. Zoghbi, *Math1: an essential gene for the generation of inner ear hair cells.* Science, 1999. **284**(5421): p. 1837-41.

[166.]Zheng, J.L. and W.Q. Gao, *Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears.* Nat Neurosci, 2000. **3**(6): p. 580-6.

[167.]Chen, P. and N. Segil, *p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti.* Development, 1999. **126**(8): p. 1581-90.

[168.]Lowenheim, H., D.N. Furness, J. Kil, C. Zinn, K. Gultig, M.L. Fero, D. Frost, A.W. Gummer, J.M. Roberts, E.W. Rubel, C.M. Hackney, and H.P. Zenner, *Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 4084-8.

[169.]Stevens, C.B., A.L. Davies, S. Battista, J.H. Lewis, and D.M. Fekete, *Forced activation of Wnt signaling alters morphogenesis and sensory organ identity in the chicken inner ear.* Dev Biol, 2003. **261**(1): p. 149-64.

[170.]Bok, J., W. Chang, and D.K. Wu, *Patterning and morphogenesis of the vertebrate inner ear.* Int J Dev Biol, 2007. **51**(6-7): p. 521-33.

[171.]Schwander, M., B. Kachar, and U. Muller, *Review series: The cell biology of hearing.* J Cell Biol, 2010. **190**(1): p. 9-20.

[172.]Martin, P. and G.J. Swanson, *Descriptive and experimental analysis of the epithelial remodellings that control semicircular canal formation in the developing mouse inner ear.* Dev Biol, 1993. **159**(2): p. 549-58.

[173.]Straka, H., N. Vibert, P.P. Vidal, L.E. Moore, and M.B. Dutia, *Intrinsic membrane properties of vertebrate vestibular neurons: function, development and plasticity.* Prog Neurobiol, 2005. **76**(6): p. 349-92.

[174.]Giraldez, F., *Regionalized organizing activity of the neural tube revealed by the regulation of lmx1 in the otic vesicle.* Dev Biol, 1998. **203**(1): p. 189-200.

[175.]Kil, S.H., A. Streit, S.T. Brown, N. Agrawal, A. Collazo, M.H. Zile, and A.K. Groves, *Distinct roles for hindbrain and paraxial mesoderm in the induction and patterning of the inner ear revealed by a study of vitamin-A-deficient quail.* Dev Biol, 2005. **285**(1): p. 252-71.

[176.]Liang, J.K., J. Bok, and D.K. Wu, *Distinct contributions from the hindbrain and mesenchyme to inner ear morphogenesis.* Dev Biol, 2010. **337**(2): p. 324-34.

[177.]Schneider-Maunoury, S. and C. Pujades, *Hindbrain signals in otic regionalization: walk on the wild side.* Int J Dev Biol, 2007. **51**(6-7): p. 495-506.

[178.]Robledo, R.F. and T. Lufkin, *Dlx5 and Dlx6 homeobox genes are required for specification of the mammalian vestibular apparatus.* Genesis, 2006. **44**(9): p. 425-37.

[179.]Burton, Q., L.K. Cole, M. Mulheisen, W. Chang, and D.K. Wu, *The role of Pax2 in mouse inner ear development.* Dev Biol, 2004. **272**(1): p. 161-75.

[180.]Favor, J., R. Sandulache, A. Neuhauser-Klaus, W. Pretsch, B. Chatterjee, E. Senft, W. Wurst, V. Blanquet, P. Grimes, R. Sporle, and K. Schughart, *The mouse*

Pax2(1Neu) mutation is identical to a human PAX2 mutation in a family with renal-coloboma syndrome and results in developmental defects of the brain, ear, eye, and kidney. Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13870-5.

[181.]Morsli, H., F. Tuorto, D. Choo, M.P. Postiglione, A. Simeone, and D.K. Wu, *Otx1 and Otx2 activities are required for the normal development of the mouse inner ear.* Development, 1999. **126**(11): p. 2335-43.

[182.]Torres, M., E. Gomez-Pardo, and P. Gruss, *Pax2 contributes to inner ear patterning and optic nerve trajectory.* Development, 1996. **122**(11): p. 3381-91.

[183.]Acampora, D., S. Mazan, V. Avantaggiato, P. Barone, F. Tuorto, Y. Lallemand, P. Brulet, and A. Simeone, *Epilepsy and brain abnormalities in mice lacking the Otx1 gene.* Nat Genet, 1996. **14**(2): p. 218-22.

[184.]Lin, Z., R. Cantos, M. Patente, and D.K. Wu, *Gbx2 is required for the morphogenesis of the mouse inner ear: a downstream candidate of hindbrain signaling.* Development, 2005. **132**(10): p. 2309-18.

[185.]Raft, S., S. Nowotschin, J. Liao, and B.E. Morrow, *Suppression of neural fate and control of inner ear morphogenesis by Tbx1.* Development, 2004. **131**(8): p. 1801-12.

[186.]Braunstein, E.M., D.C. Monks, V.S. Aggarwal, J.S. Arnold, and B.E. Morrow, *Tbx1 and Brn4 regulate retinoic acid metabolic genes during cochlear morphogenesis.* BMC Dev Biol, 2009. **9**: p. 31.

[187.]Phippard, D., L. Lu, D. Lee, J.C. Saunders, and E.B. Crenshaw, 3rd, *Targeted mutagenesis of the POU-domain gene Brn4/Pou3f4 causes developmental defects in the inner ear.* J Neurosci, 1999. **19**(14): p. 5980-9.

[188.]Xu, H., A. Viola, Z. Zhang, C.P. Gerken, E.A. Lindsay-Illingworth, and A. Baldini, *Tbx1 regulates population, proliferation and cell fate determination of otic epithelial cells.* Dev Biol, 2007. **302**(2): p. 670-82.

[189.]Yamagishi, H., J. Maeda, T. Hu, J. McAnally, S.J. Conway, T. Kume, E.N. Meyers, C. Yamagishi, and D. Srivastava, *Tbx1 is regulated by tissue-specific forkhead proteins through a common Sonic hedgehog-responsive enhancer.* Genes Dev, 2003. **17**(2): p. 269-81.

[190.]Hebert, J.M. and S.K. McConnell, *Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures.* Dev Biol, 2000. **222**(2): p. 296-306.

[191.]Long, F., X.M. Zhang, S. Karp, Y. Yang, and A.P. McMahon, *Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct*

role in the regulation of chondrocyte proliferation. Development, 2001. **128**(24): p. 5099-108.

[192]Chiang, C., Y. Litingtung, E. Lee, K.E. Young, J.L. Corden, H. Westphal, and P.A. Beachy, *Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function.* Nature, 1996. **383**(6599): p. 407-13.

[193]Madisen, L., T.A. Zwingman, S.M. Sunkin, S.W. Oh, H.A. Zariwala, H. Gu, L.L. Ng, R.D. Palmiter, M.J. Hawrylycz, A.R. Jones, E.S. Lein, and H. Zeng, *A robust and high-throughput Cre reporting and characterization system for the whole mouse brain.* Nat Neurosci, 2010. **13**(1): p. 133-40.

[194]Liao, J., L. Kochilas, S. Nowotschin, J.S. Arnold, V.S. Aggarwal, J.A. Epstein, M.C. Brown, J. Adams, and B.E. Morrow, *Full spectrum of malformations in velo-cardio-facial syndrome/DiGeorge syndrome mouse models by altering Tbx1 dosage.* Hum Mol Genet, 2004. **13**(15): p. 1577-85.

[195]Nissim, S., P. Allard, A. Bandyopadhyay, B.D. Harfe, and C.J. Tabin, *Characterization of a novel ectodermal signaling center regulating Tbx2 and Shh in the vertebrate limb.* Dev Biol, 2007. **304**(1): p. 9-21.

[196]Matise, M.P., D.J. Epstein, H.L. Park, K.A. Platt, and A.L. Joyner, *Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system.* Development, 1998. **125**(15): p. 2759-70.

[197]Martin, P. and D.L. Cockroft, *Culture of postimplantation mouse embryos.* Methods Mol Biol, 2008. **461**: p. 7-22.

[198]Fekete, D.M. and D.K. Wu, *Revisiting cell fate specification in the inner ear.* Curr Opin Neurobiol, 2002. **12**(1): p. 35-42.

[199]Lawoko-Kerali, G., M.N. Rivolta, and M. Holley, *Expression of the transcription factors GATA3 and Pax2 during development of the mammalian inner ear.* J Comp Neurol, 2002. **442**(4): p. 378-91.

[200]Lillevali, K., M. Haugas, T. Matilainen, C. Pussinen, A. Karis, and M. Salminen, *Gata3 is required for early morphogenesis and Fgf10 expression during otic development.* Mech Dev, 2006. **123**(6): p. 415-29.

[201]Fritzsch, B., M. Signore, and A. Simeone, *Otx1 null mutant mice show partial segregation of sensory epithelia comparable to lamprey ears.* Dev Genes Evol, 2001. **211**(8-9): p. 388-96.

[202.]Hammond, K.L. and T.T. Whitfield, *The developing lamprey ear closely resembles the zebrafish otic vesicle: otx1 expression can account for all major patterning differences.* Development, 2006. **133**(7): p. 1347-57.

[203.]Acampora, D., G.R. Merlo, L. Paleari, B. Zerega, M.P. Postiglione, S. Mantero, E. Bober, O. Barbieri, A. Simeone, and G. Levi, *Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5.* Development, 1999. **126**(17): p. 3795-809.

[204.]Merlo, G.R., L. Paleari, S. Mantero, B. Zerega, M. Adamska, S. Rinkwitz, E. Bober, and G. Levi, *The Dlx5 homeobox gene is essential for vestibular morphogenesis in the mouse embryo through a BMP4-mediated pathway.* Dev Biol, 2002. **248**(1): p. 157-69.

[205.]Abello, G., S. Khatri, M. Radosevic, P.J. Scotting, F. Giraldez, and B. Alsina, *Independent regulation of Sox3 and Lmx1b by FGF and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode.* Dev Biol, 2010. **339**(1): p. 166-78.

[206.]Alsina, B., G. Abello, E. Ulloa, D. Henrique, C. Pujades, and F. Giraldez, *FGF signaling is required for determination of otic neuroblasts in the chick embryo.* Dev Biol, 2004. **267**(1): p. 119-34.

[207.]Vazquez-Echeverria, C., E. Dominguez-Frutos, P. Charnay, T. Schimmang, and C. Pujades, *Analysis of mouse kreisler mutants reveals new roles of hindbrain-derived signals in the establishment of the otic neurogenic domain.* Dev Biol, 2008. **322**(1): p. 167-78.

[208.]Klein, P.S. and D.A. Melton, *A molecular mechanism for the effect of lithium on development.* Proc Natl Acad Sci U S A, 1996. **93**(16): p. 8455-9.

[209.]Hamby, J.M., C.J. Connolly, M.C. Schroeder, R.T. Winters, H.D. Showalter, R.L. Panek, T.C. Major, B. Olsewski, M.J. Ryan, T. Dahring, G.H. Lu, J. Keiser, A. Amar, C. Shen, A.J. Kraker, V. Slintak, J.M. Nelson, D.W. Fry, L. Bradford, H. Hallak, and A.M. Doherty, *Structure-activity relationships for a novel series of pyrido[2,3-d]pyrimidine tyrosine kinase inhibitors.* J Med Chem, 1997. **40**(15): p. 2296-303.

[210.]Pachnis, V., B. Mankoo, and F. Costantini, *Expression of the c-ret proto-oncogene during mouse embryogenesis.* Development, 1993. **119**(4): p. 1005-17.

[211.]Cayuso, J., F. Ulloa, B. Cox, J. Briscoe, and E. Marti, *The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity.* Development, 2006. **133**(3): p. 517-28.

[212.]Kenney, A.M. and D.H. Rowitch, *Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors.* Mol Cell Biol, 2000. **20**(23): p. 9055-67.

[213.]Rowitch, D.H., S.J. B, S.M. Lee, J.D. Flax, E.Y. Snyder, and A.P. McMahon, *Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells.* J Neurosci, 1999. **19**(20): p. 8954-65.

[214.]Dessaud, E., A.P. McMahon, and J. Briscoe, *Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network.* Development, 2008. **135**(15): p. 2489-503.

[215.]Lawoko-Kerali, G., M.N. Rivolta, P. Lawlor, D.I. Cacciabue-Rivolta, C. Langton-Hewer, J.H. van Doorninck, and M.C. Holley, *GATA3 and NeuroD distinguish auditory and vestibular neurons during development of the mammalian inner ear.* Mech Dev, 2004. **121**(3): p. 287-99.

[216.]Dahmane, N. and A. Ruiz i Altaba, *Sonic hedgehog regulates the growth and patterning of the cerebellum.* Development, 1999. **126**(14): p. 3089-100.

[217.]Wechsler-Reya, R.J. and M.P. Scott, *Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog.* Neuron, 1999. **22**(1): p. 103-14.

[218.]Freyer, L. and B.E. Morrow, *Canonical Wnt signaling modulates Tbx1, Eya1, and Six1 expression, restricting neurogenesis in the otic vesicle.* Dev Dyn, 2010. **239**(6): p. 1708-22.

[219.]Garg, V., C. Yamagishi, T. Hu, I.S. Kathiriya, H. Yamagishi, and D. Srivastava, *Tbx1, a DiGeorge syndrome candidate gene, is regulated by sonic hedgehog during pharyngeal arch development.* Dev Biol, 2001. **235**(1): p. 62-73.

[220.]Liu, W., G. Li, J.S. Chien, S. Raft, H. Zhang, C. Chiang, and D.A. Frenz, *Sonic hedgehog regulates otic capsule chondrogenesis and inner ear development in the mouse embryo.* Dev Biol, 2002. **248**(2): p. 240-50.

[221.]Iida, K., H. Koseki, H. Kakinuma, N. Kato, Y. Mizutani-Koseki, H. Ohuchi, H. Yoshioka, S. Noji, K. Kawamura, Y. Kataoka, F. Ueno, M. Taniguchi, N. Yoshida, T. Sugiyama, and N. Miura, *Essential roles of the winged helix transcription factor MFH-1 in aortic arch patterning and skeletogenesis.* Development, 1997. **124**(22): p. 4627-38.

[222.]Nakamura, T., M.C. Colbert, and J. Robbins, *Neural crest cells retain multipotential characteristics in the developing valves and label the cardiac conduction system.* Circ Res, 2006. **98**(12): p. 1547-54.

[223]Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

[224]Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

[225]Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists.* Nucleic Acids Res, 2009. **37**(1): p. 1-13.

[226]Xu, P.X., J. Adams, H. Peters, M.C. Brown, S. Heaney, and R. Maas, *Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia.* Nat Genet, 1999. **23**(1): p. 113-7.

[227]Ozaki, H., K. Nakamura, J. Funahashi, K. Ikeda, G. Yamada, H. Tokano, H.O. Okamura, K. Kitamura, S. Muto, H. Kotaki, K. Sudo, R. Horai, Y. Iwakura, and K. Kawakami, *Six1 controls patterning of the mouse otic vesicle.* Development, 2004. **131**(3): p. 551-62.

[228]Zheng, W., L. Huang, Z.B. Wei, D. Silvius, B. Tang, and P.X. Xu, *The role of Six1 in mammalian auditory system development.* Development, 2003. **130**(17): p. 3989-4000.

[229]Chai, R., B. Kuo, T. Wang, E.J. Liaw, A. Xia, T.A. Jan, Z. Liu, M.M. Taketo, J.S. Oghalai, R. Nusse, J. Zuo, and A.G. Cheng, *Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea.* Proc Natl Acad Sci U S A, 2012.

[230]Forrest, D., L.C. Erway, L. Ng, R. Altschuler, and T. Curran, *Thyroid hormone receptor beta is essential for development of auditory function.* Nat Genet, 1996. **13**(3): p. 354-7.

[231]Rusch, A., L. Ng, R. Goodyear, D. Oliver, I. Lisoukov, B. Vennstrom, G. Richardson, M.W. Kelley, and D. Forrest, *Retardation of cochlear maturation and impaired hair cell function caused by deletion of all known thyroid hormone receptors.* J Neurosci, 2001. **21**(24): p. 9792-800.

[232]Pfaff, S.L., M. Mendelsohn, C.L. Stewart, T. Edlund, and T.M. Jessell, *Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation.* Cell, 1996. **84**(2): p. 309-20.

[233.]Tang, L.S., H.M. Alger, F. Lin, and F.A. Pereira, *Dynamic expression of COUP-TFI and COUP-TFII during development and functional maturation of the mouse inner ear.* Gene Expr Patterns, 2005. **5**(5): p. 587-92.

[234.]Tang, L.S., H.M. Alger, and F.A. Pereira, *COUP-TFI controls Notch regulation of hair cell and support cell differentiation.* Development, 2006. **133**(18): p. 3683-93.

[235.]Holley, M., C. Rhodes, A. Kneebone, M.K. Herde, M. Fleming, and K.P. Steel, *Emx2 and early hair cell development in the mouse inner ear.* Dev Biol, 2010. **340**(2): p. 547-56.

[236.]Rhodes, C.R., N. Parkinson, H. Tsai, D. Brooker, S. Mansell, N. Spurr, A.J. Hunter, K.P. Steel, and S.D. Brown, *The homeobox gene Emx2 underlies middle ear and inner ear defects in the deaf mouse mutant pardon.* J Neurocytol, 2003. **32**(9): p. 1143-54.

[237.]Bosse, A., A. Stoykova, K. Nieselt-Struwe, K. Chowdhury, N.G. Copeland, N.A. Jenkins, and P. Gruss, *Identification of a novel mouse Iroquois homeobox gene, Irx5, and chromosomal localisation of all members of the mouse Iroquois gene family.* Dev Dyn, 2000. **218**(1): p. 160-74.

[238.]Bosse, A., A. Zulch, M.B. Becker, M. Torres, J.L. Gomez-Skarmeta, J. Modolell, and P. Gruss, *Identification of the vertebrate Iroquois homeobox gene family with overlapping expression during early development of the nervous system.* Mech Dev, 1997. **69**(1-2): p. 169-81.

[239.]Ahmed, M., E.Y. Wong, J. Sun, J. Xu, F. Wang, and P.X. Xu, *Eya1-Six1 interaction is sufficient to induce hair cell fate in the cochlea by activating Atoh1 expression in cooperation with Sox2.* Dev Cell, 2012. **22**(2): p. 377-90.

[240.]Lehtonen, J., B. Shen, M. Vihinen, A. Casini, A. Scozzafava, C.T. Supuran, A.K. Parkkila, J. Saarnio, A.J. Kivela, A. Waheed, W.S. Sly, and S. Parkkila, *Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family.* J Biol Chem, 2004. **279**(4): p. 2719-27.

[241.]Awakura, Y., E. Nakamura, N. Ito, T. Kamoto, and O. Ogawa, *Methylation-associated silencing of TU3A in human cancers.* Int J Oncol, 2008. **33**(4): p. 893-9.

[242.]Yamato, T., K. Orikasa, S. Fukushige, S. Orikasa, and A. Horii, *Isolation and characterization of the novel gene, TU3A, in a commonly deleted region on 3p14.3-->p14.2 in renal cell carcinoma.* Cytogenet Cell Genet, 1999. **87**(3-4): p. 291-5.

[243]Schmidt, M.V., J.P. Schulke, C. Liebl, M. Stiess, C. Avrabos, J. Bock, G.M. Wochnik, H.A. Davies, N. Zimmermann, S.H. Scharf, D. Trumbach, W. Wurst, W. Zieglgansberger, C. Turck, F. Holsboer, M.G. Stewart, F. Bradke, M. Eder, M.B. Muller, and T. Rein, *Tumor suppressor down-regulated in renal cell carcinoma 1 (DRR1) is a stress-induced actin bundling factor that modulates synaptic efficacy and cognition.* Proc Natl Acad Sci U S A, 2011. **108**(41): p. 17213-8.

[244]Zhang, T., B. Dayanandan, I. Rouiller, E.J. Lawrence, and C.A. Mandato, *Growth-arrest-specific protein 2 inhibits cell division in Xenopus embryos.* PLoS One, 2011. **6**(9): p. e24698.

[245]Cantor, S., R. Drapkin, F. Zhang, Y. Lin, J. Han, S. Pamidi, and D.M. Livingston, *The BRCA1-associated protein BACH1 is a DNA helicase targeted by clinically relevant inactivating mutations.* Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2357-62.

[246]Levran, O., C. Attwooll, R.T. Henry, K.L. Milton, K. Neveling, P. Rio, S.D. Batish, R. Kalb, E. Velleuer, S. Barral, J. Ott, J. Petrini, D. Schindler, H. Hanenberg, and A.D. Auerbach, *The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia.* Nat Genet, 2005. **37**(9): p. 931-3.

[247]Rock, J.R., C.R. Futtner, and B.D. Harfe, *The transmembrane protein TMEM16A is required for normal development of the murine trachea.* Dev Biol, 2008. **321**(1): p. 141-9.

[248]Yoshida, K. and S. Sugano, *Identification of a novel protocadherin gene (PCDH11) on the human XY homology region in Xq21.3.* Genomics, 1999. **62**(3): p. 540-3.

[249]Speevak, M.D. and S.A. Farrell, *Non-syndromic language delay in a child with disruption in the Protocadherin11X/Y gene pair.* Am J Med Genet B Neuropsychiatr Genet, 2011. **156B**(4): p. 484-9.

[250]Lee, E.Y., H. Ji, Z. Ouyang, B. Zhou, W. Ma, S.A. Vokes, A.P. McMahon, W.H. Wong, and M.P. Scott, *Hedgehog pathway-regulated gene networks in cerebellum development and tumorigenesis.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9736-41.

[251]Vokes, S.A., H. Ji, S. McCuine, T. Tenzen, S. Giles, S. Zhong, W.J. Longabaugh, E.H. Davidson, W.H. Wong, and A.P. McMahon, *Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning.* Development, 2007. **134**(10): p. 1977-89.

[252]Soriano, P., *Generalized lacZ expression with the ROSA26 Cre reporter strain.* Nat Genet, 1999. **21**(1): p. 70-1.

[253]Hama, H., H. Kurokawa, H. Kawano, R. Ando, T. Shimogori, H. Noda, K. Fukami, A. Sakaue-Sawano, and A. Miyawaki, *Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain.* Nat Neurosci, 2011. **14**(11): p. 1481-8.

[254]Sienknecht, U.J. and D.M. Fekete, *Comprehensive Wnt-related gene expression during cochlear duct development in chicken.* J Comp Neurol, 2008. **510**(4): p. 378-95.

[255]Hasson, T., P.G. Gillespie, J.A. Garcia, R.B. MacDonald, Y. Zhao, A.G. Yee, M.S. Mooseker, and D.P. Corey, *Unconventional myosins in inner-ear sensory epithelia.* J Cell Biol, 1997. **137**(6): p. 1287-307.

[256]Bermingham-McDonogh, O., E.C. Oesterle, J.S. Stone, C.R. Hume, H.M. Huynh, and T. Hayashi, *Expression of Prox1 during mouse cochlear development.* J Comp Neurol, 2006. **496**(2): p. 172-86.

[257]Mantela, J., Z. Jiang, J. Ylikoski, B. Fritzsch, E. Zacksenhaus, and U. Pirvola, *The retinoblastoma gene pathway regulates the postmitotic state of hair cells of the mouse inner ear.* Development, 2005. **132**(10): p. 2377-88.

[258]Hidalgo-Sanchez, M., R. Alvarado-Mallart, and I.S. Alvarez, *Pax2, Otx2, Gbx2 and Fgf8 expression in early otic vesicle development.* Mech Dev, 2000. **95**(1-2): p. 225-9.

[259]Raft, S., E.J. Koundakjian, H. Quinones, C.S. Jayasena, L.V. Goodrich, J.E. Johnson, N. Segil, and A.K. Groves, *Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells during inner ear development.* Development, 2007. **134**(24): p. 4405-15.

[260]Bell, D., A. Streit, I. Gorospe, I. Varela-Nieto, B. Alsina, and F. Giraldez, *Spatial and temporal segregation of auditory and vestibular neurons in the otic placode.* Dev Biol, 2008. **322**(1): p. 109-20.

[261]Corwin, J.T., *Perpetual production of hair cells and maturational changes in hair cell ultrastructure accompany postembryonic growth in an amphibian ear.* Proc Natl Acad Sci U S A, 1985. **82**(11): p. 3911-5.

[262]Corwin, J.T. and D.A. Cotanche, *Regeneration of sensory hair cells after acoustic trauma.* Science, 1988. **240**(4860): p. 1772-4.

[263]Ryals, B.M. and E.W. Rubel, *Hair cell regeneration after acoustic trauma in adult Coturnix quail.* Science, 1988. **240**(4860): p. 1774-6.

[264.]Pajcini, K.V., S.Y. Corbel, J. Sage, J.H. Pomerantz, and H.M. Blau, *Transient inactivation of Rb and ARF yields regenerative cells from postmitotic mammalian muscle.* Cell Stem Cell, 2010. **7**(2): p. 198-213.

[265.]Chen, P., F. Zindy, C. Abdala, F. Liu, X. Li, M.F. Roussel, and N. Segil, *Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d.* Nat Cell Biol, 2003. **5**(5): p. 422-6.

[266.]Oshima, K., K. Shin, M. Diensthuber, A.W. Peng, A.J. Ricci, and S. Heller, *Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells.* Cell, 2010. **141**(4): p. 704-16.

[267.]Lee, C.S., L.A. Buttitta, N.R. May, A. Kispert, and C.M. Fan, *SHH-N upregulates Sfrp2 to mediate its competitive interaction with WNT1 and WNT4 in the somitic mesoderm.* Development, 2000. **127**(1): p. 109-18.

[268.]Huangfu, D. and K.V. Anderson, *Cilia and Hedgehog responsiveness in the mouse.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11325-30.

[269.]Ross, A.J., H. May-Simera, E.R. Eichers, M. Kai, J. Hill, D.J. Jagger, C.C. Leitch, J.P. Chapple, P.M. Munro, S. Fisher, P.L. Tan, H.M. Phillips, M.R. Leroux, D.J. Henderson, J.N. Murdoch, A.J. Copp, M.M. Eliot, J.R. Lupski, D.T. Kemp, H. Dollfus, M. Tada, N. Katsanis, A. Forge, and P.L. Beales, *Disruption of Bardet-Biedl syndrome ciliary proteins perturbs planar cell polarity in vertebrates.* Nat Genet, 2005. **37**(10): p. 1135-40.

[270.]Jones, C., V.C. Roper, I. Foucher, D. Qian, B. Banizs, C. Petit, B.K. Yoder, and P. Chen, *Ciliary proteins link basal body polarization to planar cell polarity regulation.* Nat Genet, 2008. **40**(1): p. 69-77.

[271.]Heydeck, W., H. Zeng, and A. Liu, *Planar cell polarity effector gene Fuzzy regulates cilia formation and Hedgehog signal transduction in mouse.* Dev Dyn, 2009. **238**(12): p. 3035-3042.

[272.]Park, T.J., S.L. Haigo, and J.B. Wallingford, *Ciliogenesis defects in embryos lacking inturned or fuzzy function are associated with failure of planar cell polarity and Hedgehog signaling.* Nat Genet, 2006. **38**(3): p. 303-11.

[273.]Noda, T., S. Oki, K. Kitajima, T. Harada, S. Komune, and C. Meno, *Restriction of Wnt signaling in the dorsal otocyst determines semicircular canal formation in the mouse embryo.* Dev Biol, 2012. **362**(1): p. 83-93.

[274.]Shi, F., Y.F. Cheng, X.L. Wang, and A.S. Edge, *Beta-catenin up-regulates Atoh1 expression in neural progenitor cells by interaction with an Atoh1 3' enhancer.* J Biol Chem, 2010. **285**(1): p. 392-400.

[275.] Woods, C., M. Montcouquiol, and M.W. Kelley, *Math1 regulates development of the sensory epithelium in the mammalian cochlea.* Nat Neurosci, 2004. **7**(12): p. 1310-8.