COORDINATION OF FGF, WNT, AND HH FOR SENSORY AND NEURAL PATTERNING DURING ZEBRAFISH INNER EAR DEVELOPMENT

A Dissertation

by

AMY LOUISE TAN

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Chair of Committee,	Bruce B. Riley
Committee Members,	L. Rene Garcia
	Christine Merlin
	Rajesh C. Miranda
Head of Department,	Alex C. Keene

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ABSTRACT

During early inner ear development, patterning of the sensory and neural domains is established in response to multiple signaling inputs. Although the importance of Fgf, Wnt, and Hh for inner ear development have been established, coordination of the signals is still poorly understood. Here, we have examined how these signals impact development of the vestibular sensory domain, auditory sensory domain, and neurogenic domain and identified downstream targets which mediate this process.

In zebrafish, the sensory and neural domains form simultaneously in the adjacent medial and lateral regions, respectively, of the otic vesicle. We used transgenic and pharmacological methods to alter Fgf and Wnt signaling during early placodal stages and then examined changes in patterning and growth. Blocking Fgf reduced cell proliferation and eliminated both sensory and neural development. Although Wnt promoted cell proliferation, it was not required for either sensory or neural development. Sustained overactivation of Wnt, however, led to expansion of sensory and medial markers at the expense of lateral and neurogenic markers. We found that *pax2a*, *sp5a*, and *sp5l* are co-regulated by Fgf and Wnt. Additionally, they are required for some aspects of sensory development and for Wnt to properly suppress neurogenesis.

The otic vesicle contains two sensory maculae, the utricle and saccule, which develop concurrently and acquire distinct vestibular and auditory function by 3 and 5 days post fertilization, respectively. We investigated the roles of Fgf and Hh in development of the utricle and saccule using pharmacological and transgenic methods.

pax5, a transcription factor exclusively expressed in the developing utricle, required Fgf and was limited to the anterior domain by Hh. *pou3f3b*, a transcription factor uniquely expressed in the developing saccule, required Hh but also had an early requirement for *fgf8a* and was restricted at later stages by *fgf3*. The Fgf-target gene *pax2a* was required for expression of both *pax5* and *pou3f3b* and expression of *pax2a* was not maintained in the otic vesicle without Fgf and Hh. Thus, Fgf and Hh govern expression of *pax5* and *pou3f3b* and could regulate functional divergence of the utricle and saccule.

DEDICATION

This dissertation is dedicated to my father, Randall Tan, who always had questions.

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CHAPTER I

INTRODUCTION

Development of the inner ear—the endorgan responsible for vestibular and auditory functions in vertebrates—involves interaction of multiple signals and pathways to form its morphologically complex structure. Inner ear development begins with the induction of the otic placode. In zebrafish, this begins around 10 hours post fertilization (hpf) in response to Fgf signals from the hindbrain and otic identity is reinforced by Wnt signaling (Lekven et al., 2001; Lèger and Brand, 2002; Phillips et al., 2001; Phillips et al., 2004). The otic placode is made up of a single epithelial layer which thickens and then cavitates to form the otic vesicle (Haddon and Lewis, 1996). By 22 hpf, mature hair cells appear in the first two sensory maculae near the anterior and posterior poles of the otic vesicle (Riley et al., 1997; Millimaki et al., 2007). The anterior (utricular) domain gives rise to the vestibular system of the inner ear and the posterior (saccular) domain is required for auditory function (Riley et al., 1997; Zeddies and Fay, 2005; Millimaki et al., 2007; Smith et al., 2011; Brietzler et al., 2020). Neural progenitors are specified in the floor of the otic vesicle, between the utricle and saccule, then delaminate and migrate to form the ganglia associated with the inner ear (Haddon and Lewis, 1996). How signals are coordinated to induce the vestibular, auditory, and neurogenic domains in adjacent regions of the otic vesicle is still poorly understood.

Structure of the sensory epithelia

The inner ear becomes functional very early in zebrafish compared to chicks and mice. Pairs of hair cells, called the tether cells, appear by 22 hpf, followed by development of mature hair cells and associated support cells (Riley et al., 1997, Millimaki et al., 2007). The utricle and saccule quickly acquire vestibular and auditory function and zebrafish larvae have full vestibular function (maintaining balance and swimming in a straight line after disturbance) by 3 days post fertilization (dpf) and respond to auditory signals by 5 dpf (Riley and Moorman, 2000; Zeddies and Fay, 2005; Kwak et al., 2006). Other sensory structures, the cristae of the semi-circular canals and the lagena, form much later and the utricle and saccule are the sources of vestibular and auditory input for at least the first month (Haddon and Lewis, 1996; Riley and Moorman, 2000).

The sensory maculae are made up of mechanosensory hair cells intercalated with non-sensory support cells in a “salt-and-pepper” pattern. Hair cells detect sound vibration and acceleration/gravity cues through ciliary bundles which extend into the fluid-filled lumen of the inner ear. In zebrafish, this detection is aided by the movement of otoliths, calcium carbonate aggregates which are associated with the hair cells of the utricle and saccule and help transduce mechanical signals (Haddon and Lewis, 1996; Riley and Moorman, 2000). The support cells provide trophic factors required for hair cell survival and are also capable of acting as a stem cell population so that zebrafish can regenerate damaged hair cells (Haddon et al., 1998; Haddon et al., 1999; Millimaki et al., 2010). Although the sensory epithelia of the utricular and saccular maculae are

structurally similar, they have unique roles and more work is needed to determine how vestibular vs. sensory function is established.

Fgf, Hh, and Wnt during development of the sensory domains

The requirement for Fgf in sensory specification is well established and continues throughout otic development. During the placodal stage, Fgf is required for expression of *atoh1b* and *atoh1a*, the earliest markers of sensory development. *atoh1b* is initially expressed in all cells of the otic placode and becomes restricted to a subset of cells, along with *atoh1a*, through the action of Delta-Notch lateral inhibition. Disruption of Fgf at this stage causes complete loss of sensory epithelium while misexpression of Fgf expands sensory competence and leads to ectopic sensory epithelium (Millimaki et al., 2007; Gou et al., 2018b). In addition to establishing sensory competence, Fgf appears to help govern the anterior-posterior axis for specification of the anterior and posterior sensory maculae. Misexpression of *fgf3* or *fgf8a* leads to ectopic expression of the anterior sensory marker *pax5* into the posterior domain, at the expense of the saccular domain marker *pou3f3b* (Hammond et al., 2011; Sweet et al., 2011; Hartwell et al., 2019). Despite being required for proper development of both sensory epithelia, the effect of Fgf on the anterior and posterior domains is complex. Disruption of *fgf3* causes a reduction in the domain of *pax5* and an anterior expansion of the *pou3f3b* domain. Disruption of *fgf8a*, on the other hand, leads to disrupted formation of the saccule (Kwak et al., 2006; Hammond et al., 2011; Maier et al., 2014; Maulding et al., 2014). Additionally, the utricle and saccule show differential expression of Fgfs by 19 hpf. The

utricle expresses relatively high levels of *fgf3*, *fgf8a*, and *fgf10a* while the saccule has weaker expression of *fgf8a* and *fgf10a*. The Fgf response genes *etv4*, *etv5b*, and *spry4* are expressed in corresponding high and low levels in the two maculae (Feng and Xu, 2010; Kantarci et al., 2020). In general, it appears that a high level of Fgf is necessary for the anterior sensory macula but that Fgf is also required for formation of the posterior sensory macula at some level.

While the anterior sensory domain requires a high level of Fgf, Hh is important for the posterior sensory domain. Beginning around 16.5 hpf, *shh* (*sonic hedgehog*) and *twhh* (*tiggy-winkle hedgehog*) are expressed in the floorplate (both) and notochord (*shh* only) and presence of either is sufficient to provide correct posteriorizing input for the otic vesicle (Hammond et al., 2003). The Hh pathway components *smoothened* (*smo*), *patched1* (*ptc1*), and *patched2* (*ptc2*) are all expressed in the otic vesicle itself. From around 16.5 - 30 hpf, *smo* is expressed throughout the otic vesicle, while *ptc2* is expressed in the ventral region and *ptc1* becomes localized to the medial wall and posterior region by 24 hpf (Hammond et al., 2003; Hammond and Whitfield, 2009). Increased Hh reduces expression of *pax5* in the anterior domain and loss of Hh disrupts the sensory maculae so they appear to be anteriorized (Hammond et al., 2003; Hammond et al., 2010; Sapède and Pujades, 2010). Because of Hh's importance for posterior identity, it could be influencing development of the saccular macula and auditory function. Indeed, in mice, the cochlea requires Shh signaling for proper extension and development of hair cells for hearing (Riccomagno et al., 2002; Carroll Driver et al., 2008; Liu et al., 2010; Brown and Epstein, 2011; Bok et al., 2013; Tateya et al., 2013;

Benito-Gonzalez and Doetzlhofer, 2014). The role of Hh in specification of the saccule in zebrafish is further examined in Chapter III.

Although Wnt is known to be important for otic development and to influence sensory cell formation elsewhere in zebrafish, its targets and interactions during inner ear development are still poorly defined. Following initial induction of the otic placode by Fgf, Wnt reinforces otic specification and influences expression of an early otic placode marker *pax2a*. The combination of Wnt and *pax2a* appears to commit cells to an otic fate and help maintain their otic identity (Lekven et al., 2001; Phillips et al., 2004; McCarroll et al., 2012). *pax2a* also influences expression of prosensory markers and *pax2a* mutants lose hair cells from the sensory maculae, even though they initially produce an excess of hair cells (Riley et al., 1999; Gou et al., 2018a; Millimaki et al., 2007). In the lateral line, Wnt regulates the number of hair cells in the neuromasts by regulating cell proliferation (Wada et al., 2013). Altering Wnt signaling has been shown to modulate expression of several Fgfs, indicating that some of Wnt's role in otic development may be executed through altering Fgf expression (Ladher et al., 2000; Phillips et al., 2004). Work in other vertebrate models indicates that the dual requirement for Fgf and Wnt for otic fate specification is conserved and also reveals that Wnt promotes sensory development while inhibiting neurogenesis (Ladher et al., 2000; Stevens et al., 2003; Wright and Mansour, 2003; Martin and Groves, 2005; Freter et al., 2008; Ohyama et al., 2008; Freyer and Morrow, 2010; Urness et al., 2010). The role of Wnt in the zebrafish inner ear is further examined in Chapter II.

Structure and development of the otic neurons

The hair cells of the inner ear are innervated by the statoacoustic ganglion (SAG). Development of the SAG begins in the floor of the otic vesicle with specification of the neuroblasts. Around 22 hpf, neuroblasts begin delaminating from the otic vesicle and migrating medially to form the transit-amplifying pool (Haddon and Lewis, 1996). The transit-amplifying pool contains proliferating SAG precursors. Cells eventually differentiate and leave this pool to form the SAG, which sits between the hindbrain and otic vesicle (Vemaraju et al., 2012). The SAG itself is spatially divided into an anterior region that is associated with the vestibular portions of the sensory domain and a posterior region that is associated with the auditory regions (Sapède and Pujades, 2010).

Fgf and Wnt have opposing roles in specification of the neuroblasts in the otic vesicle. Fgf is required for both sensory and neural development, while Wnt promotes sensory and inhibits neural development. The neurogenic domain forms in the floor of the otic vesicle and is marked by expression of *neurog1* and *goosecoid* (*gsc*), which are both regulated by Fgf. A subset of cells in the *neurog1* domain upregulate *gsc* and undergo epithelial-to-mesenchymal transition (EMT) to migrate out of the otic vesicle (Korzh et al., 1998; Andermann et al., 2002; Kantarci et al., 2016). *gsc* downregulates *Ecadherin* (*cdh1*), thus controlling which cells are able to delaminate. This action appears to be offset by expression of *pax2a*, which causes an upregulation of *cdh1*, presumably helping to stabilize otic vesicle cells outside the neurogenic domain (Kantarci et al., 2016). The neurogenic domain is specified by a moderate level of Fgf and inhibited if Fgf is increased (Vemaraju et al., 2012). Differentiation of the SAG

neurons is also controlled by Fgf and, as the cells mature and produce more Fgf, it feeds back to delay further differentiation from the transit amplifying pool (Vemaraju et al., 2012; Kantarci et al., 2015; Kantarci et al., 2016). The coordination of Fgf and Wnt in specifying the neurogenic domain is further examined in Chapter II.

Establishment of the otic vesicle's neurogenic domain may also require the influence of pioneer cells. Pioneer cells are first seen around 13 hpf outside the otic vesicle and they migrate into the otic vesicle before *neurog1* is expressed by the otic epithelium (Hoijman et al., 2017). The cells then migrate back out of the otic vesicle and form the first SAG neurons. Although little is known about the pioneer cells, including their origin and what signals control their specification and migration, their presence appears to be essential for establishing the neurogenic domain and ablating them prior to ingression reduces the size of the neurogenic domain. Blocking Fgf results in migrating cells which cannot penetrate the otic vesicle, possibly due to excessive rigidity of the otic epithelium.

The SAG is spatially organized in a manner which corresponds to its innervation of the sensory maculae. Projections from the anterior portion of the SAG connect to the utricular macula while the posterior portion of the SAG connects to the saccular macula. Interestingly, this topological organization appears to be controlled by Hh. When embryos are treated with a Hh inhibiting drug, there is a reduction in the posterior *neurog1* domain in the otic vesicle, a region which gives rise to the auditory neurons of the SAG (Sapède and Pujades, 2010). This indicates that Hh may have a role in

development of the auditory domain, both in the saccular maculae and its associated neurons.

Cooperation of signals during otic development

In zebrafish, the sensory and neurogenic domains are specified simultaneously and adjacent to one another in the otic vesicle (Haddon and Lewis, 1996). The earliest markers of utricular and saccular identity, *pax5* and *pou3f3b*, appear around 20-22 hpf (Kwak et al., 2002; Sweet et al., 2011). Separate functions of the utricle and saccule become apparent by 3 dpf, but how their vestibular and auditory functions are specified is unclear (Riley and Moorman, 2000; Zeddies and Fay, 2005; Kwak et al., 2006). Fgf is required for both sensory and neural development, while Wnt supports sensory but opposes neural development. Meanwhile, Fgf and Hh have opposing roles in the anterior and posterior portions of the otic vesicle and may help establish the differences between the utricle and saccule that lead to differing functions of the maculae. The different responses of these areas to Fgf, Wnt, and Hh within a narrow spatial and temporal window presents questions about how the signals are mediated and how specification of the vestibular sensory domain, auditory sensory domain, and neurogenic domain are achieved. This work addresses these questions and identifies downstream targets which help coordinate specification of the sensory and neural domains.

CHAPTER II

PAX2A, SP5A AND SP5L ACT DOWNSTREAM OF FGF AND WNT TO COORDINATE SENSORY-NEURAL PATTERNING IN THE INNER EAR*

INTRODUCTION

The vertebrate inner ear is a complex organ system that arises from a simple epithelial thickening, the otic placode. The otic placode is induced around the end of gastrulation by Fgf from surrounding tissues (Phillips et al., 2001; Léger and Brand 2002; Maroon et al., 2002; Liu et al., 2003; Wright and Mansour, 2003; Ladher et al., 2005; Martin and Groves, 2006). As the otic placode develops into the otic vesicle, Fgf signaling continues to drive growth and patterning of otic tissue (Léger and Brand, 2002; Mahoney Rogers et al., 2011; Padanad and Riley, 2011; Padanad et al., 2012; Maulding et al., 2014; Urness et al., 2018). After Fgf-dependent induction, Wnt is also required for proper development of the otic placode (Phillips et al., 2004; Ohyama et al., 2006; Freter et al. 2008; Park and Saint-Jeannet, 2008; McCarroll et al., 2012). However, since Wnt often upregulates various Fgf genes during placodal development (Ladher et al., 2000; Phillips et al., 2004), the specific role of Wnt and its relationship to Fgf is still not clear. As the otic vesicle develops further, it produces several patches of sensory epithelia comprising hair cells and support cells, as well as neuroblasts that later delaminate to form neurons

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of the statoacoustic ganglion (SAG). Fgf locally induces formation of both sensory epithelia and neuroblasts in the floor of the otic vesicle (Alsina et al. 2004; Millimaki et al., 2007; Abelló et al. 2010; Brown and Epstein, 2011; Vemaraju et al. 2012; Maier and Whitfield, 2014; Gou et al., 2018; Urness et al., 2018). At the same time, Wnt promotes sensory development while repressing neural development (Stevens et al. 2003; Freyer and Morrow 2010; Brown and Epstein, 2011). How Fgf and Wnt work together to coordinate sensory and neural specification in such close proximity remains poorly understood.

The transcription factor Pax2 (Pax2a in zebrafish) is an early regulator of otic development that potentially mediates the activities of Fgf and Wnt signaling. In response to Fgf, *Pax2*/*pax2a* initially marks all cells in the otic placode and helps maintain otic fate and epithelial integrity (Torres et al., 1996; Hutson et al. 1999; Phillips et al., 2001; Hans et al., 2004; Mackereth et al., 2005; Bouchard et al., 2010; Abelló et al., 2010; Christophorou et al., 2010; Padanad et al., 2012; McCarroll et al., 2012). At later stages, expression of *Pax2/pax2a* marks the medial wall of the otic vesicle and sensory epithelia, regions where Fgf and Wnt are both active. In mouse *Pax2* mutants, early sensory development appears relatively normal, but the saccular epithelium is deficient and extension of the cochlear duct arrests at an early stage, defects attributable to elevated cell death in the ventral otic vesicle (Burton et al., 2004, Bouchard et al., 2010). Knockdown of *Pax2* in chick alters expression of key otic markers and impairs structural integrity of sensory epithelia through loss of cell adhesion and increased cell death (Christophorou et al., 2010). Zebrafish *pax2a* mutants initially produce

supernumerary hair cells due to reduced *delta* gene expression and diminished Notch signaling (Riley et al., 1999). However, hair cells later begin to lose adhesion and are extruded from the sensory epithelium, possibly due to reduced expression of *Ecadherin/cdh2* (Kwak et al., 2006; Kantarci et al., 2016). Loss of *Pax2/pax2a* in mouse and fish does not strongly impact specification of neuroblasts, but misexpression of *pax2a* in zebrafish is sufficient to repress delamination of neuroblasts by eliminating expression of *goosecoid* (*gsc*) (Kantarci et al., 2016).

Sp5-related transcription factors have also been proposed as mediators of Wnt and/or Fgf signaling, though their roles during inner ear development have not been investigated. Human and mouse *Sp5* genes are induced by Wnt and directly regulate transcription of Wnt target genes (Fujimura et al., 2007; Dunty et al., 2014; Kennedy et al., 2016; Huggins et al., 2017; Garriock et al., 2020). In chick and *Xenopus*, *Sp5* is induced by Wnt and Fgf during neural crest development and helps activate neural crest markers (Park et al., 2013; Azambuja and Simoes-Costa, 2021). Zebrafish *sp5a* is regulated primarily by Wnt and helps activate Wnt target genes (Tallafuß et al., 2001; Weidinger et al., 2005). A second zebrafish paralog, *sp5l*, represents a divergent family member that can be activated by Fgf or Wnt (Weidinger et al., 2005; Thorpe et al., 2005), although in some tissues *sp5l* functions primarily through the Fgf pathway (Zhao et al., 2003). In preliminary studies we found that both *sp5a* and *sp5l* are expressed in the medial wall of the otic vesicle in a pattern overlapping with *pax2a*. Thus, *pax2a*, *sp5a* and *sp5l* potentially work together to coordinate Fgf and Wnt signaling during otic development.

Here we altered Fgf or Wnt signaling during placodal stages to determine the effects on later patterning in the otic vesicle. We also examined the effects of disrupting or misexpressing *pax2a*, *sp5a* and *sp5l*. Our findings support a model in which these factors mediate distinct aspects of Fgf and Wnt signaling to promote normal sensory development, whereas they act in a partially redundant manner to repress neurogenesis downstream of Wnt.

MATERIALS AND METHODS

Fish strains and developmental conditions

The wild-type line is derived from the AB strain (Eugene, OR). The *pax2a* mutant allele, *noi*^{tu29a}, is referred to here as *pax2a-/-* (Brand et al., 1996; Lun and Brand, 1998). CRISPR/Cas9 was used to generate mutant alleles for *sp5a*^{x69} and *sp5l*^{x70}, referred to here as *sp5a-/-* and *sp5l-/-*, respectively. Wild-type embryos were injected at the one-cell stage with Cas9 mRNA and guide RNA targeting *sp5a* or *sp5l*. Injected embryos were raised and screened for founders, then crossed to generate *sp5a-/-; sp5l-/-* and *pax2a+/-; sp5a-/-; sp5l-/-* lines. Transgenic lines *TG(hsp70:sp5a)*^{x71} and *TG(hsp70:sp5l)*^{x72}, herein referred to as *hs:sp5a* and *hs:sp5l*, were generated using the Tol2 transposon system. Other transgenes used in this study include *TG(hsp70:hsfgf8a)*^{x17} (Millimaki et al., 2010), *hsp70:dkk1-GFP*^{w32} (Stoick-Cooper et al., 2007), herein referred as *hs:fgf8a*, *hs:dkk1*, which were used for misexpression; and *TG(brn3c:gap43-GFP)*^{s356t} (Xiao et al., 2005) and *TG(top:dGFP)*^{w25} (Dorsky et al., 2002), referred to as *brn3c:gfp*, and *top:gfp*, were used to visualize mature hair cells and cells undergoing canonical Wnt signaling,

respectively. Under standard developmental conditions, embryos were incubated at 28.5ºC in fish water containing methylene blue and staging was based on morphological features (Kimmel et al., 1995). For embryos older than 24 hpf, PTU (1-phenyl 2-thiourea, 0.3 mg/ml, Sigma P-7629) was added to the fish water to prevent melanin formation.

Heat shock misexpression and drug treatments

For heat shock misexpression experiments, transgenic embryos were incubated in a 39ºC water bath for 1 hour at the indicated times. Following heat shock, embryos were incubated at 33ºC until fixing. Embryos undergoing serial heat shock were incubated at 33ºC between heat shocks. Wild-type embryos were heat shocked as controls, under the same conditions as the transgenic embryos. For drug treatments, 10 mM stock solutions were dissolved in DMSO and were then diluted to working concentrations in fish water. Working concentrations used here were 5 µM BIO (Sigma B1686), 10 µM IWR-1 (Sigma I0161), 70 µM SU5402 (Sigma SML0443), and 150 µM SANT-1 (Sigma S4572). Control embryos were treated with the same volume of DMSO in fish water. Embryos treated with SANT-1 had their chorions punctured prior to addition of the drug.

Morpholino injection

The morpholino sequences used here have been previously characterized and validated: For knockdown of *sp5a*, we injected embryos at the 1-cell stage with 2.5 ng of splice-blocking MO with sequence 5'-TTCGGAGTGCGATCCTGGAGCAGAA-3'

(Weidinger et al., 2005); to knockdown *sp5l* we injected 1-cell embryos with translation-blocking MO with sequence 5'-CCCCCTTACACAGCCAGGTGCGTAC-3' (Zhao et al., 2003). Double morphants were co-injected with 2.5 ng of each MO.

Staining and cryosectioning

Wholemount in situ hybridization was performed as previously described (Jowett and Yan, 1996; Riley et al., 1999; Phillips et al., 2001). Immunolocalization was performed using primary antibodies for anti-Phospho-Histone H3 (Sigma H0412, 1:350) to mark proliferating cells, or anti-Islet1/2 (Developmental Studies Hybridoma Bank 39.4D5, 1:75) to mark mature neurons of the SAG, followed by secondary antibody AlexaFluor 488 goat anti-mouse (Invitrogen A11001, 1:100) (Riley et al., 1999; Phillips et al., 2001). AlexaFluor 594 phalloidin (Invitrogen A12381) was used to stain hair cells in lines that did not contain *brn3c:gfp*. For phalloidin staining, embryos were fixed with 4% paraformaldehyde. Washing and staining were performed as described (Feng and Xu, 2010). A 10X stock solution was prepared by dissolving 300 U of Phalloidin conjugated to Alexa Fluor 594 (Invitrogen A12381) in 1.5 ml methanol to a final concentration of 66 µM. Just before staining, aliquots of stock solution were diluted 10-fold in PBT-LS (0.8% sodium chloride, 0.02% potassium chloride, 0.02 M sodium phosphate, pH 7.3, 2 mg/ml bovine serum albumin, 10% lamb serum and 0.1% Triton X-100). For cryosectioning, wholemount-stained embryos were soaked in 30% sucrose until they sank in the solution. Embryos were set in FSC22 Clear Frozen Section

Compound (Leica 3801480) and then cut into 10 µm sections and mounted in 30% glycerol, as previous described (Vemaraju et al., 2012).

Genotyping

Mutants for *sp5a-/-; sp5l-/-* were identified by PCR genotyping using DNA from a fin clip and forward primers specific to the mutant or wild-type sequence. For *sp5a*: forward primers 5'-CACCTGCCCCCATCCCTC-3' (binds wild-type sequence only) and 5'-TACGCAGCACCTGCCCCCAG-3' (binds mutant sequence only); reverse primer 5'-CGGTGTGTGTCCGCAGGT-3' (used with both forward primers). For *sp5l*: forward primers 5'-AGTGGTCCAAACACCCTC-3' (binds wild-type sequence only) and 5'-AAGTGGTCCAAACACCCTTT-3' (binds mutant sequence only); reverse primer 5'-AGCTGCCGTCCCAAGGAGAA-3' (used with both forward primers). With all primers, amplicons are 500 bp in length. Products generated by primers for wild-type vs. mutant alleles were run in separate lanes to distinguish between +/+, heterozygous, and homozygous mutant animals. Mutants for *pax2a* were identified phenotypically or by PCR, followed by a restriction enzyme digest. PCR with primers 5'-GCTCTGCCTCCATGATTGGC-3' (forward) and 5'-CACGTTTTCTCTTTTCGCCGT-3' (reverse) yields an amplicon of 572 bp; digestion with BstB1 (NEB R0519S) results in wild-type amplicons being cut into ~230 bp and 340 bp fragments, while the mutant amplicon remains uncut due to loss of the restriction site. Carriers of *hs:sp5a* and *hs:sp5l* were identified by heat shocking outcrossed

embryos at 24 hpf (39ºC for 1 hour), fixing after 1 hour of recovery, and then conducting an in situ hybridization to detect global misexpression from the transgene in question.

Quantitation, reproducibility and statistics

Data reported herein reflect fully penetrant phenotypes as observed in 15 or more embryos, except where noted in the text. Tests for significance were performed using student's-T (for cases with two groups) or one-way ANOVA with Tukey post-hoc HSD tests for experiments with three or more groups. Cell counts were obtained from directly viewing embryos with a Zeiss compound scope using either brightfield or fluorescence imaging. Hair cell numbers were quantified by counting cells in whole fixed embryos expressing *brn3c:gfp* or embryos stained with phalloidin. Mature neurons in the SAG were quantified in whole mounts by counting cells marked by anti-Islet1/2 staining. Proliferative cells were quantified in whole mounts by anti-Phospho-Histone H3 staining. Cells expressing *gsc* were counted from in situ stained wholemount embryos, and *neurog1*+ cells were counted from serial sections of in situs stained embryos. Otic vesicle length (anteroposterior) was measured from 400X images of *pax2a*-stained embryos. Sample sizes reflect one ear/embryo (wholemounts) or both ears (in sections).

RESULTS

Modulating early Wnt and Fgf.

To examine the role of Fgf and Wnt in early sensory-neural patterning in developing otic tissue, we tested the effects of modulating Fgf or Wnt signaling using pharmacological

and/or transgenic reagents. Treatments to alter these pathways were initiated at 12 hpf, when the nascent otic placode is stably specified but still plastic with respect to regional patterning. To confirm efficacy and specificity of these treatments, we examined expression of the artificial Wnt reporter *top:gfp* (Dorsky et al., 2002) and the Fgf-target gene *etv5b* (previously *erm*) (Raible and Brand, 2001; Roehl et al., 2001) at 14 hpf, two hours after beginning each treatment. To fully block Wnt signaling, embryos were treated with IWR-1 to activate the β-catenin destruction complex, in combination with heat shock activation of transgenic *hs:dkk1* encoding a secreted Wnt antagonist.

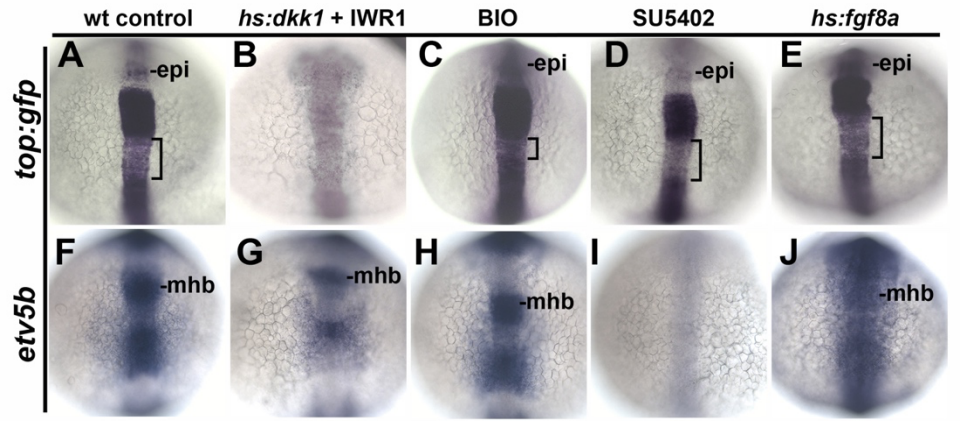


Figure 1. Early responses to modulation of Wnt and Fgf.
(A-J) Dorsal views (anterior to the top) showing expression at 14 hpf of the artificial Wnt reporter *top:gfp* (A-E) and the Fgf-target gene *etv5b* (F-J) following treatment at 12 hpf with DMSO (control), *hs:dkk1* (39ºC for 1 hour) + 10 µM IWR-1 added immediately after heat shock, BIO (5 µM), SU5402 (70 µM), or *hs:fgf8a* (39ºC for 1 hour). Epiphysis (epi) and midbrain-hindbrain border (MHB) are indicated. Brackets mark a zone of weaker *top:gfp* expression in the hindbrain. The same conditions were used in subsequent figures, except for changes in treatment duration and time of fixation as noted. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Inhibiting Wnt from 12 hpf nearly eliminated expression of *top:gfp* (Fig. 1B). Blocking Wnt also caused a moderate reduction of *etv5b* (Fig. 1G), likely reflecting a requirement

for Wnt in maintaining proper expression of *fgf* genes in the hindbrain (Phillips et al., 2004). To activate the Wnt pathway, embryos were treated with BIO to inhibit Gsk3 activity, thereby stabilizing β-catenin. BIO treatment led to strong upregulation and expansion of *top:gfp* but had no effect on *etv5b* (Fig. 1C, H). Blocking Fgf signaling with the pharmacological inhibitor SU5402 eliminated expression of *etv5b* but had little or no effect on *top:gfp* (Fig. 1D, I). Heat shock activation of transgenic *hs:fgf8a* led to global upregulation of *etv5b* but had little effect on *top:gfp* in the hindbrain (Fig. 1E, J). However, *hs:fgf8a* modestly elevated expression of *top:gfp* in the midbrain (Fig. 1E), possibly reflecting upregulation of *wnt* gene expression (Phillips et al., 2004; Urness et al., 2010; Dyer et al., 2014). Thus, treatments to modulate Fgf and Wnt signaling are highly effective within 2 hours and appear relatively specific.

Sensory-neural patterning following altered Wnt and Fgf signaling.

To determine the impact on patterning of the otic vesicle, we altered Wnt and Fgf signaling from 12 hpf and examined expression of various otic markers at 24 hpf. Expression of *pax2a* marks the medial half of the otic vesicle, including nascent sensory epithelia (Fig. 2Aa). *sp5a* and *sp5l* show similar patterns to *pax2a*, but *sp5l* shows maximal expression in the anteromedial region of the otic vesicle where Fgf is most active (Fig. 2Af, Ak). We also examined expression of the prosensory marker *atoh1a* and neurogenic markers *neurog1* and *gsc*. Blocking Wnt reduced the size of the otic placode by 10-15% but had little effect on expression of most of these markers (Fig. 2Ab, Al, Aq, Av, Aaa). However, *sp5a* expression was severely downregulated (Fig.

2Ag), indicating that this gene relies heavily on Wnt. By 30-36 hpf, loss of Wnt signaling reduced hair cell accumulation by 30-35% ($p<0.003$) but had no effect on neurogenesis in the otic vesicle (Fig. 2Ba, Bb, Be, Bf, C, D). Activation of Wnt with

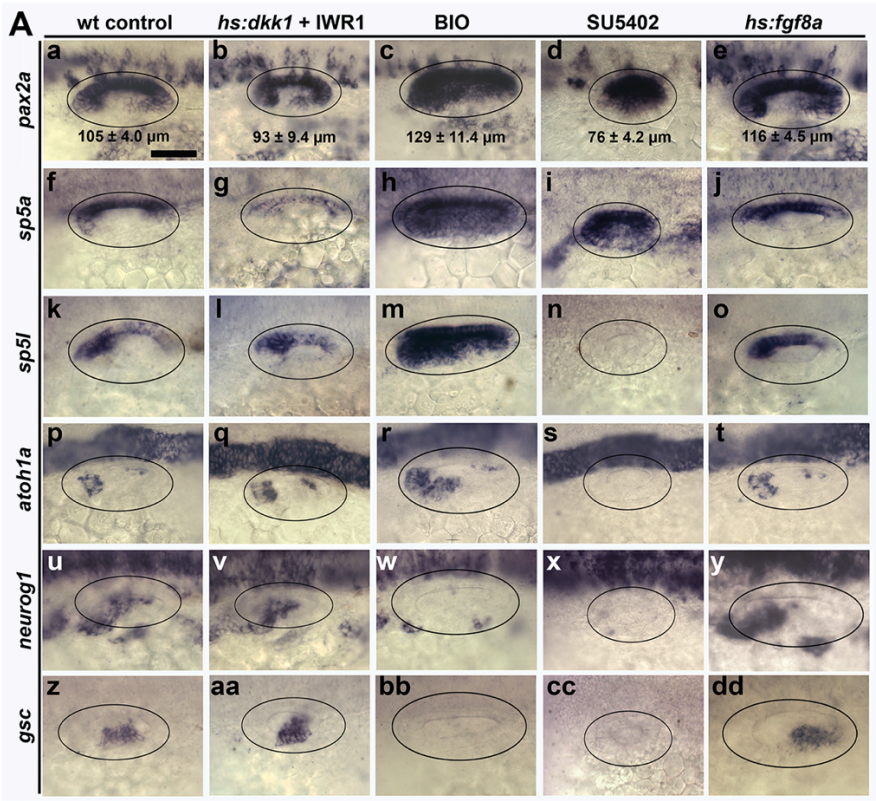
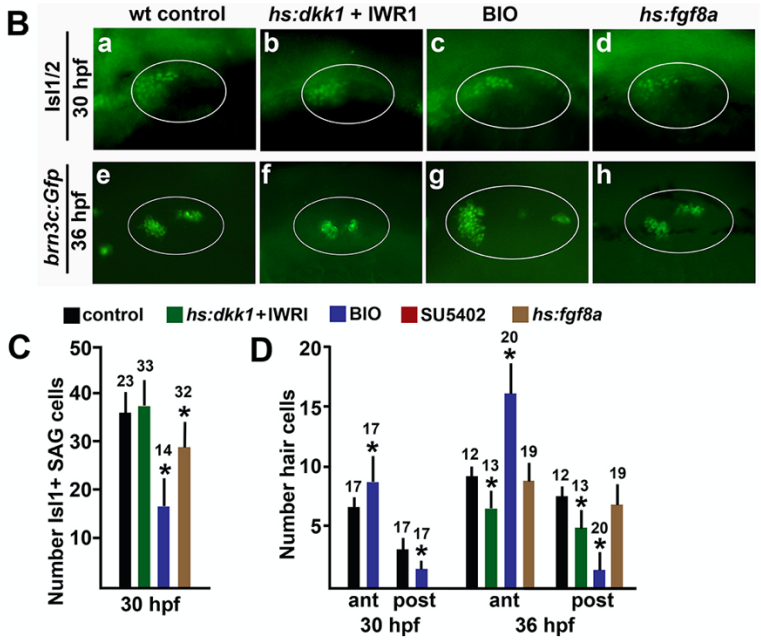


Figure 2. Effects of altered Wnt and Fgf signaling on sensory-neural patterning in the otic vesicle.

(A) Dorsolateral views (anterior to the left) showing expression of otic markers of the medial wall, sensory epithelia and neurogenic domains (indicated on the left) at 24 hpf following treatments to alter Wnt and Fgf signaling at 12 hpf. Oval marquees encircle the otic vesicle. Mean anteroposterior length of the otic vesicle (± SD, $n \geq 8$) is indicated in images of pax2a expression (Aa-e). Scale bar (Aa), 50 µm. (B) Dorsolateral views of anti-Isl1/2 stained SAG neurons at 30 hpf (a-d) and brn3c:Gfp+ hair cells at 36 hpf (e-h) under the indicated conditions. (C, D) Quantification of the effects of signal modulation under conditions indicated by the key at the top. Mean values with errors (standard deviations) and sample sizes are indicated for each group. Asterisks indicate significant differences relative to controls. (C) Mean number of anti-Isl1/2 stained SAG neurons at 30 hpf. (D) Mean number of brn3c:gfp+ hair cells in the anterior and posterior sensory epithelia at 30 and 36 hpf. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Figure 2 cont.



BIO increased the size of the otic vesicle by 20-30% and led to a dramatic lateral expansion of domains of *pax2a*, *sp5a* and *sp5l* (Fig. 2Ac, Ah, Am). There was also a marked lateral expansion of the anterior domain of *atoh1a* (Fig. 2Ar), whereas otic domains of both *neurog1* and *gsc* were lost entirely (Fig. 2Aw, Abb). Similarly, BIO treatment nearly doubled the number of anterior/utricular hair cells at 36 hpf ($p<0.0001$) but reduced the number of SAG neurons by 50-60% ($p<0.0001$) (Fig. 2Bc, Bg, C, D). Interestingly, the number of posterior hair cells remained constant through 36 hpf, suggesting delayed maturation of the saccular macula (Fig. 2Bg, D). These data are consistent with the idea that elevating Wnt expands medial fates, including the anterior sensory epithelium, at the expense of lateral and neurogenic fates in the floor of the otic vesicle. Blocking Fgf at 12 hpf with SU5402 reduced the size of the otic vesicle by nearly 30% (Fig. 2Ad) and led to a complete loss of *sp5l*, *atoh1a*, *neurog1*, and *gsc* (Fig.

2An, As, Ax, Acc), confirming a critical role for Fgf in induction of these genes (Zhou et al., 2003; Millimaki et al., 2007; Vemaraju et al., 2012; Kantarci et al., 2016). Unexpectedly, however, SU5402 also caused lateral expansion of the domains of *pax2a* and *sp5a* (Fig. 2Ad, Ai). Activation of *hs:fgf8a* increased the size of the otic vesicle by 10% and elevated the level of expression of *pax2a* and *sp5l* (Fig. 2Ae, Ao). Domains of *atoh1a* and *neurog1* were close to normal (Fig. 2At, Ay), as was accumulation of hair cells at 36 hpf (Fig. 2Bh, D). However, the number of mature SAG neurons at 30 hpf was reduced by 20% ($p<0.0001$) (Fig. 2Bd, C), likely reflecting complex regulation of later stages of neuroblast development. Together these data support the notion that Fgf promotes sensory and neural specification, whereas Wnt promotes sensory development but eliminates neural specification. The data also show that *sp5l* uniquely requires Fgf whereas *sp5a* uniquely requires Wnt.

Changes in response to drugs based on treatment time.

To explore the temporal requirements for Fgf and Wnt in otic patterning, treatments to modulate signaling were administered at different times and durations. Activating Wnt with BIO from 14-24 hpf increased the size of the otic vesicle and led to lateral expansion of *pax2a* and *sp5a* (Fig. 3A, F), but to a lesser degree than adding BIO at 12-24 hpf (Fig. 2Ac, Ah). Adding BIO at 18 hpf enlarged the otic vesicle and intensified expression of *pax2a* and *sp5a* but caused only modest lateral expansion (Fig. 3B, G).

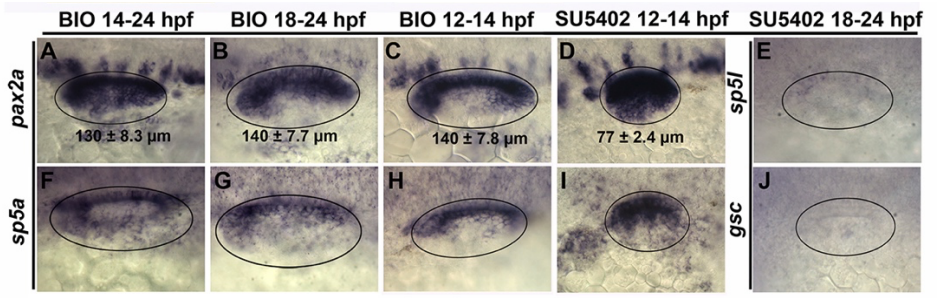


Figure 3. Effects of timing of treatments in altering otic patterning.
(A- J) Dorsolateral views (anterior to the left) showing expression of otic markers (indicated on the left) at 24 hpf following shorter duration treatment times (indicated at the top) to determine temporal requirements for altered patterning by Wnt and Fgf. Oval marquees encircle the otic vesicle; mean anteroposterior length (± SD, n ≥ 8) is indicated in the *pax2a* expression images (A – D). Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Adding BIO at 12 hpf followed by its removal at 14 hpf resulted in enlargement of the otic vesicle, yet expression patterns of *pax2a* and *sp5a* remained normal (Fig. 3C, H). Thus, BIO treatment must be administered early and then maintained to fully affect otic patterning, but even brief early treatment or late addition of BIO was sufficient to significantly increase the size of the otic vesicle. Blocking Fgf with SU5402 from 18-24 hpf completely abolished expression of *sp5l* and *gsc*, with only a slight decrease in otic vesicle size (Fig. 3E, J). Adding SU5402 at 12 hpf followed by its removal at 14 hpf led to lateral expansion of *pax2a* and *sp5a* at 24 hpf (Fig. 3D, I). However, expression of *etv5b* at 24 hpf was eliminated under these conditions, suggesting that a substantial level of SU5402 is retained in embryonic tissues after removal from the medium. Nevertheless, these data show that SU5402 can strongly affects otic patterning even when added relatively late.

Impact on otic cell proliferation.

To explore the mechanism by which Wnt and Fgf affect otic vesicle size, embryos were stained with anti-Phospho-Histone H3 (PH3) to visualize dividing cells (Fig. 4Aa). Blocking or elevating Wnt signaling caused no significant change in numbers of PH3+ cells in the otic region at 14 hpf (Fig. 4Ab, Ac, B). However, BIO-treated embryos showed a significant increase in PH3+ cells in the otic vesicle at 18 hpf (p=0.0158) whereas blocking Wnt significantly decreased the number of PH3+ cells (p=0.0418) (Fig. 4Af, Ag, B). Note, the size of the otic vesicle was not appreciably altered at 18 hpf, suggesting that changes in the number of PH3+ cells did not simply reflect changes in total cell number. Treatment with SU5402 strongly reduced the number of PH3+ cells at both 14 hpf (p=0.0146) and 18 hpf (p<0.0001) (Fig. 4Ad, Ah, B), suggesting that Fgf is required for normal proliferation throughout early otic development.

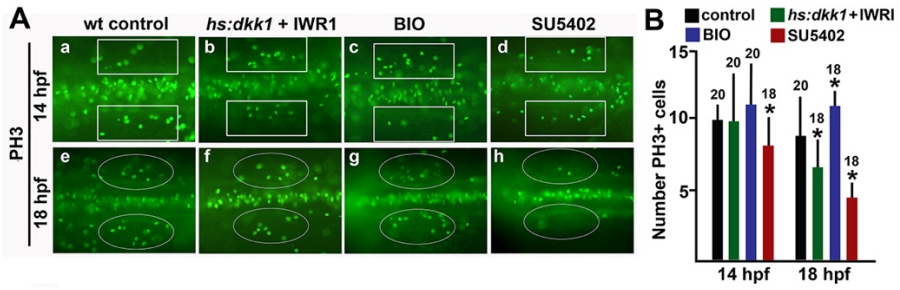


Figure 4. Effects on proliferation.
(A) Dorsal view of embryos stained for phospho-histone H3 to label mitotic cells at 14 and 18 hpf under conditions indicated at the top of the panel. Marquees around the otic placode (200 µm x 50 µm rectangles) or otic vesicle (ovals) were used for quantitation. Ovals delimit the edges of the otic vesicle. (B) Mean number of cells stained with anti-PH3 inside the marquees shown in (A) at 14 and 18 hpf. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Effect on otic regional identity.

To better understand the impact of early Fgf and Wnt signaling, we examined a broader range of regional otic markers at 24 hpf. Blocking Wnt had little effect on any regional markers, except that spatial domains were slightly smaller reflecting overall reduction in otic vesicle size (Fig. 5B, G, L, Q). Expression of *pax5* in the anteromedial region marks the future utricular macula and showed dramatic lateral expansion in BIO-treated embryos (Fig. 5A, C). In contrast, the posteromedial marker *pou3f3b*, which marks the

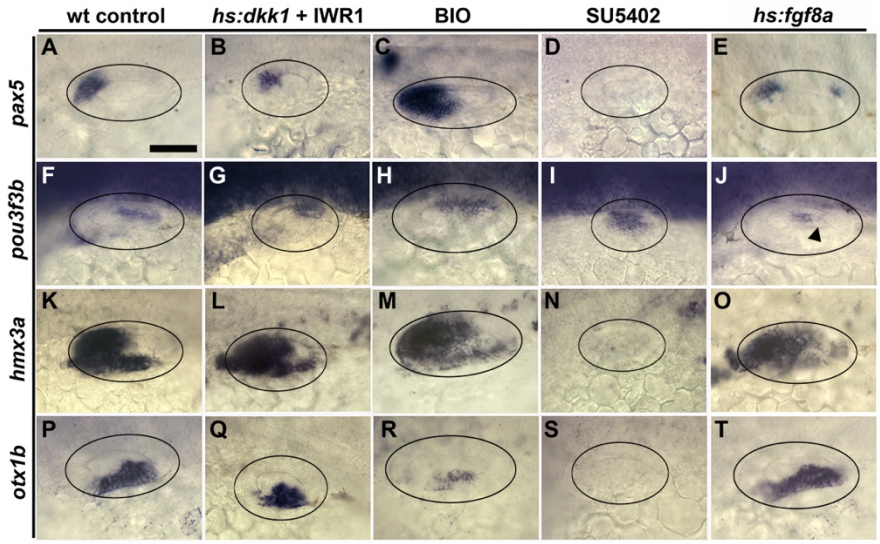


Figure 5. Effects of altered Wnt and Fgf on regional identity.
(A-T) Dorsal views showing regional otic markers (indicated on the left) at 24 hpf following treatments from 12 hpf as indicated at the top. The black arrowhead in (J) marks a posterior domain of *pou3f3b* that is lost following activation of *hs:fgf8a*. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

future saccular macula, showed only a slight lateral expansion following BIO treatment (Fig. 5H). BIO caused little change in expression of the anteroventral marker *hmx3a*, whereas the posterolateral marker *otx1b* was nearly abolished, suggesting contraction of

lateral identity (Fig. 5M, R). SU5402 abolished expression of *pax5*, *hmx3a* and *otx1b* (Fig. 5D, N, S), confirming that these genes are Fgf-dependent (Kwak et al., 2006; Feng and Xu, 2010; Hammond and Whitfield, 2011; Maier and Whitfield, 2014). In contrast, SU5402 caused moderate lateral expansion of the domain of *pou3f3b* (Fig. 5I). Activating *hs:fgf8a* led to ectopic expression of *pax5* in the posterior-medial epithelium as previously reported (Fig. 5E) (Hammond et al., 2011; Sweet et al., 2011; Hartwell et al., 2019), and eliminated the posterior half of the domain of *pou3f3b* (Fig. 5J, arrowhead). *hs:fgf8a* also caused posterior expansion of *hmx3a* but caused little change in *otx1b* (Fig. 5O, T). We next examined Fgf- and Wnt-reporters in the otic vesicle at 24 hpf. Expression of *top:gfp* normally marks the medial wall of the otic vesicle but was nearly eliminated by blocking Wnt, whereas BIO caused pronounced lateral expansion of the *top:gfp* (Fig. 6Aa-c). Expression of *etv5b* normally marks the floor of the otic vesicle with greatest expression near the anterior and posterior poles where Fgf is most active, and weaker expression in the middle of the floor (Fig. 6Af, Ba, Be). Blocking Wnt had little effect on *etv5b* (Fig. 6Ag, Bb, Bf). Unexpectedly, BIO treatment led to pronounced upregulation of *etv5b* throughout the floor (Fig. 6Ah), a finding confirmed by examining sections through the middle of the floor (Fig. 6Bc, Bg). This is likely an indirect effect caused by expansion of otic domains of *fgf3*, *fgf8a* and *fgf10a* (Fig. 6Cb, Cd, Cf). Expression of *fgf3* in adjacent pharyngeal endoderm was also greatly expanded in BIO-treated embryos (Fig. 6Cb), possibly contributing to changes in otic patterning.

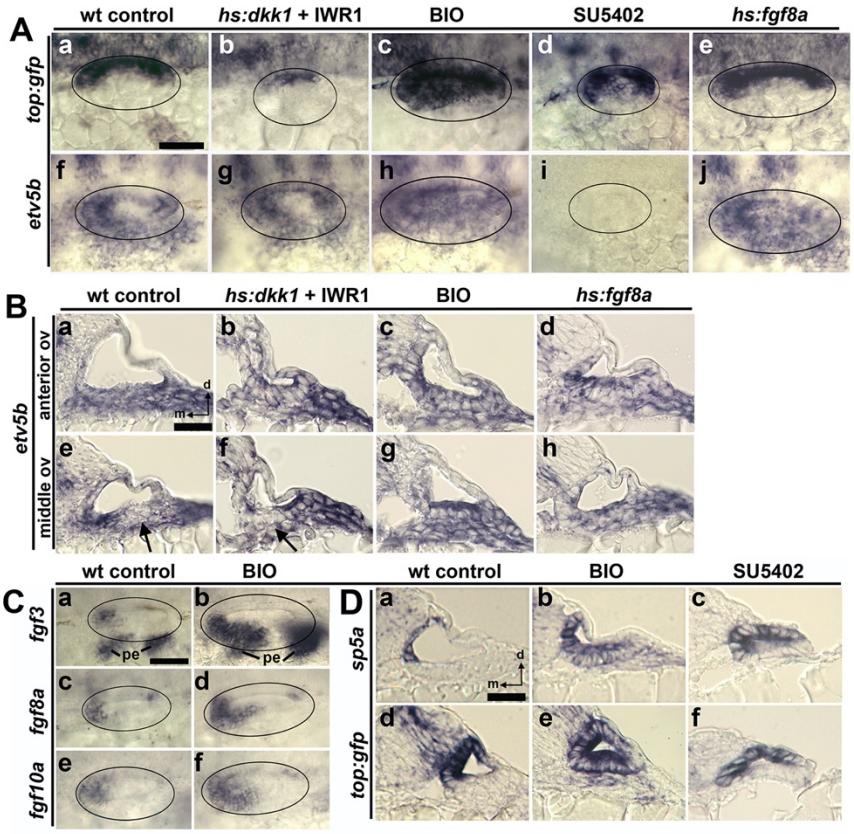


Figure 6. Effects of signal modulation on reporters of Fgf and Wnt signaling.

(A) Dorsolateral views showing expression of Wnt reporter *top:gfp* (a-e) or Fgf-target *etv5b* (f-j) at 24 hpf under conditions indicated at the top. (B) Cross-sections (dorsal to the top, medial to the left) of *etv5b*-stained embryos passing through the anterior or middle the otic vesicle, as indicated on the left. Black arrows (e, f) indicate regions of weaker expression of *etv5b*. (C) Dorsolateral views showing expression of *fgf3*, *fgf8a* and *fgf10a* at 24 hpf in control and BIO-treated embryos. Pharyngeal endoderm (pe) is marked (Ca, Cb). (D) Cross-sections of embryos showing expression of *sp5a* and *top:gfp* at 24 hpf following treatments from 12 hpf, as indicated at the top. Scale bars (Aa, Ba, Ca, Da), 50 µm. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Finally, SU5402 treatment eliminated expression of *etv5b* but caused weak lateral expansion of *top:gfp* (Fig. 6Ad, Ai), and *hs:fgf8a* expanded expression of *etv5b*

throughout the floor but had little effect on *top:gfp* (Fig. 6Ae, Aj, Bd, Bh). Together, these data show that Wnt is not required for proper expression of any of the otic markers examined, but excess Wnt alters most aspects of otic patterning through stimulation of both Wnt and Fgf pathways, leading to expansion of medial fates at the expense of lateral fates. In addition, the greater expansion seen in anterior markers likely reflects expansion of *fgf* gene expression in the otic vesicle and pharyngeal endoderm.

An unexpected finding was that treatment with BIO or SU5402 caused similar patterns of lateral expansion of most medial markers, including *top:gfp* (Fig. 6Ac, Ad). To explore this in more detail, BIO- and SU5402-treated embryos were sectioned to visualize the distribution of Wnt-targets, *sp5a* and *top:gfp*. BIO treatment lead to nearly global expansion of *top:gfp* (Fig. 5De) whereas *sp5a* spread laterally through the floor of the otic vesicle but was excluded from the lateral wall (Fig. 6Db). Conversely, SU5402 treatment led to expansion of both markers into the lateral wall but not the floor of the otic vesicle (Fig. 6Dc, Df). Thus, the effects of excess Wnt signaling are distinctly different from changes caused by blocking Fgf, despite superficial similarities seen in dorsal views of wholemount embryos. The mechanism by which SU5402 expands Wnt target expression into the lateral wall remains unclear.

Role of Pax2a in mediating Wnt.

Previous studies show that Pax2a promotes formation and maintenance of sensory epithelia (Riley et al., 1999; Kwak et al., 2006; Millimaki et al., 2007) and represses aspects of neural development (Kantarci et al., 2016), suggesting it could help mediate

the effects of BIO treatment. To clarify how BIO affects the time course of *pax2a* expression, we sectioned control and treated embryos at various stages. In control embryos, *pax2a* marks all cells in the otic placode at 14 hpf but begins to downregulate in lateral placode cells by 16 hpf (Fig. 7Aa, Ab). By 18 hpf, *pax2a* remains strongly expressed on the medial wall of the otic vesicle and persists at a low level in the floor, but is lost entirely from the lateral wall (Fig. 7Ac). Expression becomes fully restricted to the medial wall by 24 hpf (Fig. 7Ad). In BIO-treated embryos, *pax2a* is maintained in nearly all otic cells through 16 hpf and is retained at high levels in the floor of the otic vesicle at 18-24 hpf (Fig. 7Ae-h). To clarify the function of *pax2a*, we examined various patterning genes in *pax2a-/-* mutants that were treated or untreated with BIO. Untreated *pax2a-/-* mutants show generally reduced levels of *sp5a*, and expression is nearly lost in the anterior medial wall (Fig. 7Ba, arrowhead, compare to control embryo in Fig. 2Af). In contrast, the level of *sp5l* was normal in anterior-medial cells and was upregulated in posterior medial cells (Fig. 7Be). BIO treatment caused lateral expansion of both genes (Fig. 7Bb, Bf), although the level of *sp5a* expression was markedly reduced relative to BIO treated wild-type embryos (Fig 2Ah, Am). This indicates that *pax2a* is required for proper regulation of the level of *sp5a* and *sp5l* expression, but is not required for lateral expansion of *sp5a* and *sp5l* in response to elevated Wnt. Sensory development, marked by *atoh1a*, was slightly expanded in untreated *pax2a-/-* mutants (Fig. 7Bc), reflecting weakened Notch-dependent restriction of sensory domains (Riley et al., 1999). BIO treatment greatly expanded expression of *atoh1a* in *pax2a-/-* mutants, leading to a thin stripe of ectopic expression in the lateral floor of the otic vesicle, spanning from the

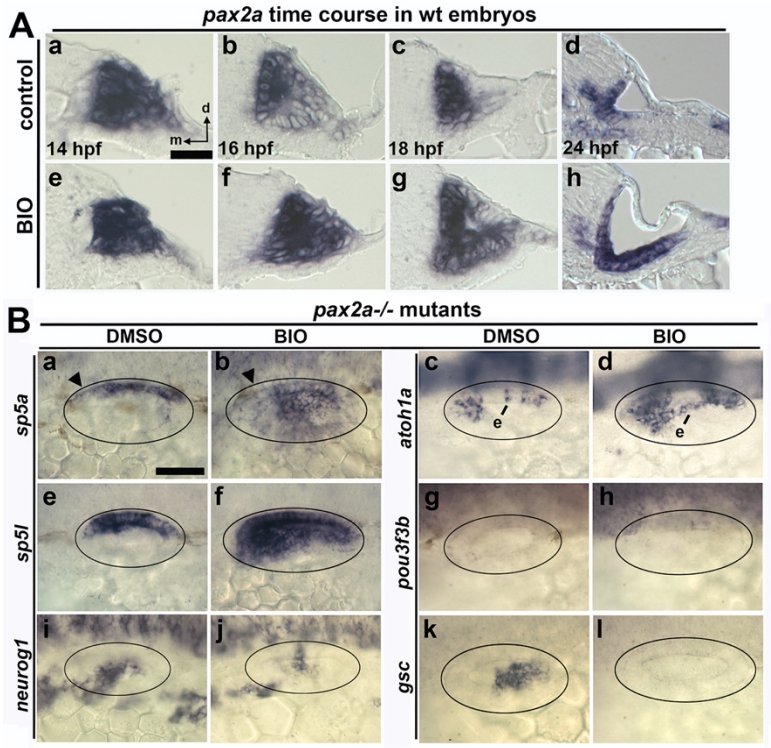


Figure 7. Effects of BIO on *pax2a* expression and otic patterning in *pax2a* mutants.

(A) Cross sections (dorsal to the top, medial to the left) showing expression of *pax2a* at the indicated times in controls and embryos treated with BIO from 12 hpf. (B) Dorsolateral views showing expression of otic markers (as indicated on the left) at 24 hpf after incubation with DMSO (control) or BIO from 12 hpf as indicated at the top. Black arrowheads (Ba, Bb) show regions where *sp5a* expression is downregulated relative to wild-type embryos. Ectopic expression (e) of *atoh1a* is indicated (Bc, Bd). Scale bar, 50 µm. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

anterior to posterior poles (Fig. 7Bd). Despite expanded sensory development, *pax2a-/-* mutants fail to express the regional macular markers *pax5* or *pou3f3b*, regardless of BIO (Fig. 7Bg, Bh) (Pfeffer et al., 1998). Effects of BIO on neural development were mixed: BIO treatment continued to fully repress *gsc* expression in *pax2a-/-* mutants (Fig. 7Bl). However, about half (10/16) of *pax2a-/-* mutants treated with BIO showed appreciable

expression of *neurog1* in the floor of the otic vesicle (Fig. 7Bj), suggesting that *pax2a* is required for full repression of neurogenesis by elevated Wnt.

Role of Sp5a and Sp5l in mediating Wnt.

Involvement of *sp5a* and *sp5l* in early otic development has not been previously examined. We therefore characterized expression of *sp5a* and *sp5l* from the earliest

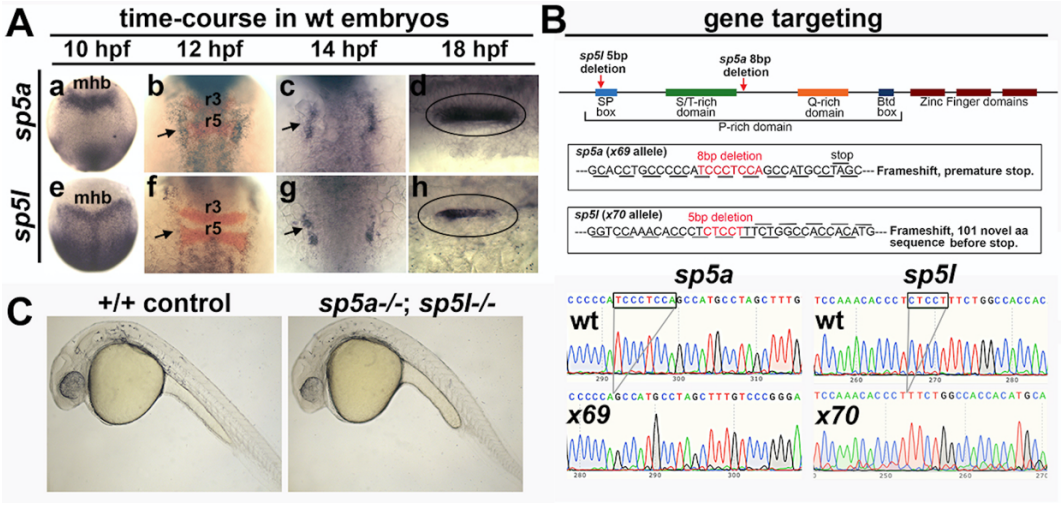
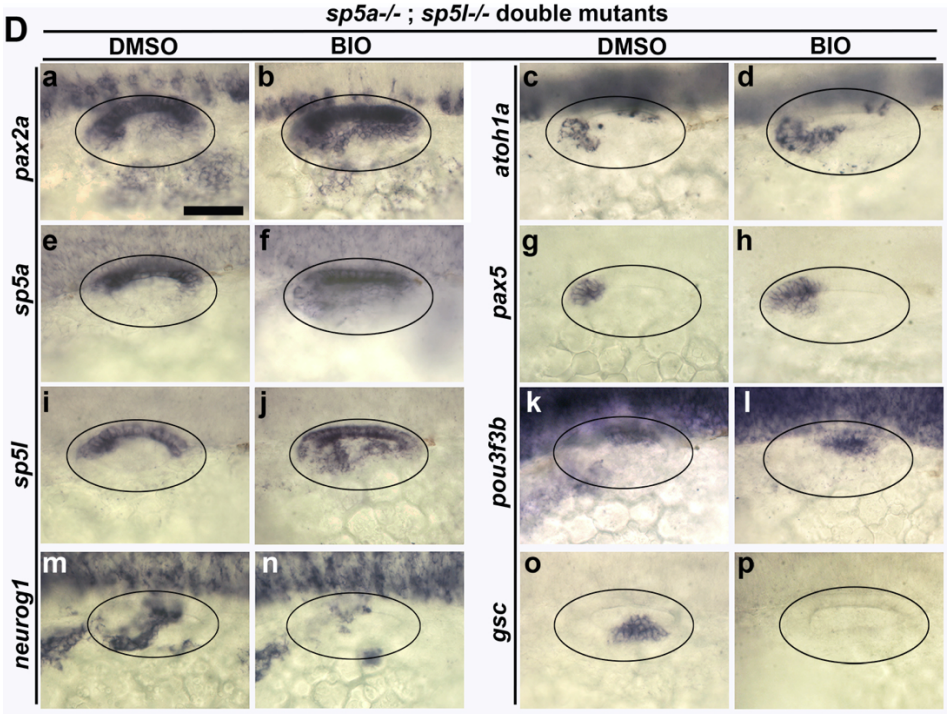


Figure 8. Early expression of *sp5a* and *sp5l* and effects of disruption on otic patterning.
(A) Dorsal views with anterior to the top (Aa-c, Ae-Ag) and dorsal views with anterior to the left (Ad, Ah) showing expression of *sp5a* and *sp5l* in wild type embryos at the times indicated at the top. Co-staining for *krox20* (red) is shown at 12 hpf (Ab, Af) to mark positions of rhombomeres 3 and 5 (r3, r5) in the hindbrain. The midbrain-hindbrain border (mhb) is labeled. Arrows point to otic domains (Ab, Ac, Af, Ag). (B) Targeting of *sp5a* and *sp5l*. The upper diagram represents the general layout of both genes, including regions encoding specific peptide domains. Red arrows show relative positions of deletions induced by CRISPR/Cas9 cutting. Altered sequences in mutant alleles *sp5a*^{x69} and *sp5l*^{x70} are shown in boxed regions and in sequencing traces at the bottom. (C) Lateral views of live wild-type and *sp5a-/-; sp5l-/-* embryos at 24 hpf. (D) Dorsolateral views of *sp5a-/-; sp5l-/-* double mutants showing expression at 24 hpf of various otic genes (as indicated on the left) following treatment with DMSO or BIO from 12 hpf (as indicated at the top). Scale bar, 50 µm. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Figure 8 cont.



stages of otic development. Both genes show broad expression in the neural plate at 10 hpf, possibly including expression in adjacent preplacodal ectoderm (Fig. 8Aa, Ae). Both genes are expressed in the medial edge of the otic domain at 12 and 14 hpf and are restricted to the medial wall of the otic vesicle at 18 hpf (Fig. 8Ab-d, Af-h). Thus, these genes could contribute to early patterning of the otic placode and vesicle.

The zebrafish genome contains a third *sp5* homolog, *sp5b*, but its expression and function have not been described. We therefore examined *sp5b* expression at multiple stages by in situ hybridization. *sp5b* was not detectably expressed until 19 hpf, when the earliest domain appeared as weak variable expression in the medial wall of the otic vesicle (Fig. 9A-C). The level of otic expression increased by 24 hpf (Fig. 9E), and an

additional small domain appeared in the forebrain (Fig. 9D). Because expression was too late and sparse to affect early otic patterning, we did not study *sp5b* further.

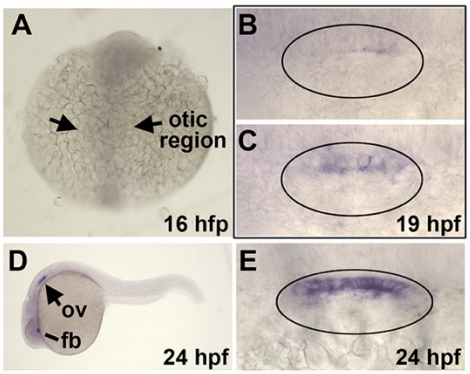


Figure 9. Expression of *sp5b*.
(A) Dorsal view showing absence of *sp5b* staining at 16 hpf. The otic region is indicated. (B, C) Dorsal views (anterior to the left) showing variable weak expression of *sp5b* in the medial wall of the otic vesicle at 19 hpf. (D) Lateral view showing expression of *sp5b* in the otic vesicle (ov) and forebrain (fb) at 24 hpf. (E) Dorsal view showing *sp5b* expression in the medial wall of the otic vesicle at 24 hpf. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

We next targeted *sp5a* and *sp5l* using CRISPR/Cas9 and obtained alleles likely to severely disrupt function (Fig. 8B). Homozygous mutants for either gene showed no obvious phenotype and were viable and fertile. *sp5a-/-; sp5l-/-* double homozygotes, which appeared to develop normally (Fig. 8C) and were viable and fertile, were subsequently analyzed for effects on otic development. Surprisingly, all otic markers appeared normal at 24 hpf, including expression of medial markers *pax2a*, *sp5a* and *sp5l* (Fig. 8Da, De, Di), as well as markers of sensory (Fig. 8Dc, Dg, Dk) and neural development (Fig. 8Dm, Do). However, the impact of BIO treatment on several of these markers was significantly diminished in *sp5a-/-; sp5l-/-* double mutants. For example, lateral expansion of *pax2a*, *sp5a* and *sp5l* into the otic floor was patchy and irregular

(Fig. 8Db, Df, Dj). Although BIO treatment caused full lateral expansion of *atoh1a* (Fig. 8Dd), expansion of *pax5* and *pou3f3b* was substantially reduced (Fig. 8Dh, Dl). Similarly, although BIO treatment eliminated expression of *gsc* (Fig. 8Dp), *neurog1* expression was partially restored in the majority (18/20) of *sp5a-/-; sp5l-/-* double mutants (Fig. 8Dn). These data show that *sp5a* and *sp5l* are required to mediate some of the effects of elevated Wnt.

To validate the *sp5a* and *sp5l* knockout phenotype, we examined the effects of knocking down these genes by injecting morpholino oligomers (MOs). Injection of either *sp5a*-MO or *sp5l*-MO alone had no discernable effect on morphology (Fig. 10Aa, Ab). Coinjection of both MOs into wild type embryos caused slight shortening of the embryonic axis (Fig. 10Ac), but morphology of the otic vesicle appeared normal. The same phenotype was seen when both MOs were injected into *sp5a-/-; sp5l-/-* double mutants (Fig. 10Ad). Expression of otic markers appeared normal in *sp5a-sp5l* double morphants (Fig. 10Ba, Bg, Bm), similar to double mutants. The ability of BIO to expand expression of *sp5l* or *pax5* was strongly suppressed in *sp5a-sp5l* double morphants (Fig. 10Bc, Bi), and *neurog1* expression was partially restored in most (22/24) of BIO-treated double morphants (Fig. 10Bo). BIO's effects on patterning were similarly suppressed in double mutants injected with *sp5a* and *sp5l* MOs (Fig. 10Bf, Bl, Br). Similar effects were also seen in single morphants (Fig. 10Bd, Be, Bj, Bk, Bp, Bq), but suppression of BIO was not as pronounced as in double morphants. Thus *sp5a-sp5l* double morphants strongly mimicked *sp5a-/-;sp5l-/-* double mutants; and MO injection into double mutants did not worsen the phenotype, supporting the idea that both alleles are null or

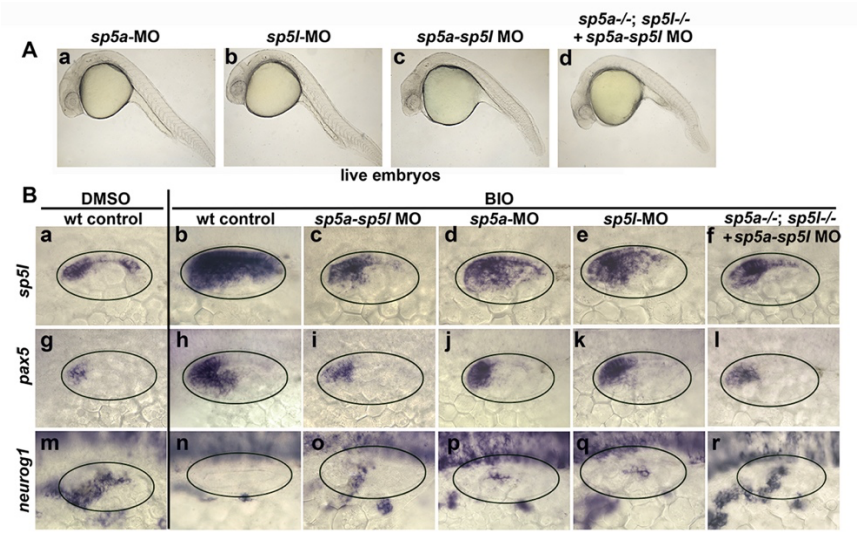


Figure 10. Analysis of sp5a and sp5l morphants.
(A) Lateral views of live embryos at 24 hpf following injection at the one-cell stage with *sp5a*-MO and/or *sp5l*-MO, as indicated at the top. (B) Dorsal lateral views showing expression of genes indicated at the left under conditions indicated at the top. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

strong loss of function. Together, these data further support the conclusion that *sp5a* and *sp5l* are required to mediate some of the effects of Wnt signaling on otic development.

Overlapping functions of *pax2a*, *sp5a* and *sp5l*.

We next examined otic patterning in *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants at 24 hpf. Expression of *pax2a* was reduced at the anterior pole (Fig. 11Aa), and anterior expression of *sp5a* and *sp5l* was nearly abolished (Fig. 11Ae, Ai). In BIO treated triple mutants, lateral expansion of *pax2a*, *sp5a* and *sp5l* was nearly abolished, with only weak irregular patches of expression in the otic floor (Fig. 11Ab, Af, Aj). Additionally, *neurog1* was substantially restored in all BIO-treated triple mutants (n=13) (Fig. 11Al), but *gsc* was abolished entirely (Fig. 11Ap). By 30 hpf, *sp5a-/-; sp5l-/-* double mutants

and *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants produced about 15% fewer SAG neurons than normal ($p<0.0001$) (Fig10Be, Bi, C); BIO treatment reduced accumulation of SAG neurons as expected, but the fold-reduction was less severe in double and triple mutants compared to wild-type embryos, although the difference did not reach statistical significance ($p=0.178$, 0.250, respectively) (Fig. 11Bf, Bj, C). Together, these data suggest that *pax2a*, *sp5a* and *sp5l* are together required for Wnt to promote medial fates and suppress neurogenesis. Although *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants failed to express *pax5* or *pou3f3b* in the otic vesicle (Fig. 11Ac, Ad, Ag, Ah), they showed a dramatic and unexpected expansion of *atoh1a* throughout the medial wall, and this was further expanded into the otic floor by BIO treatment (Fig. 11Am, An). By 48 hpf, the contiguous sensory epithelium in triple mutants contained a total of ~35% more hair cells than in wild-type embryos ($p=0.0102$) (Fig. 11Bk, D). BIO treatment caused an even greater increase of hair cell accumulation, with ~60% more hair cells in triple mutants than in wild-type embryos ($p=0.0001$) (Fig. 11Bl, D). Interestingly, although *sp5a-/-; sp5l-/-* double mutants formed a normal number of hair cells at 48 hpf (Fig. 11Bg, D), they showed an enhanced response to BIO and produced 55% more hair cells than wild-type embryos ($p=0.0009$) (Fig. 11Bh, D), statistically similar to BIO-treated triple mutants.

The expanded expression of *atoh1a* in triple mutants resembles the effects of mutations that disrupt Notch signaling (Haddon et al., 1998; Riley et al., 1999; Millimaki et al., 2007). We previously reported that *pax2a-/-* mutants show minor expansion of

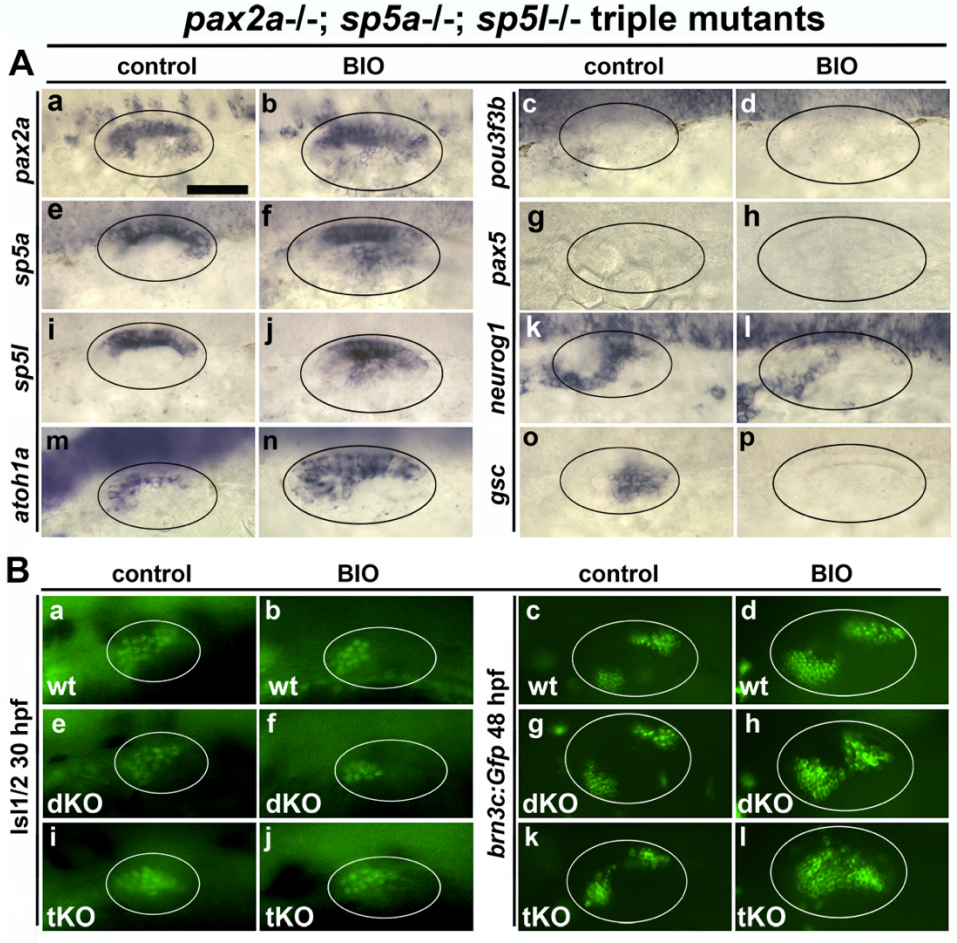
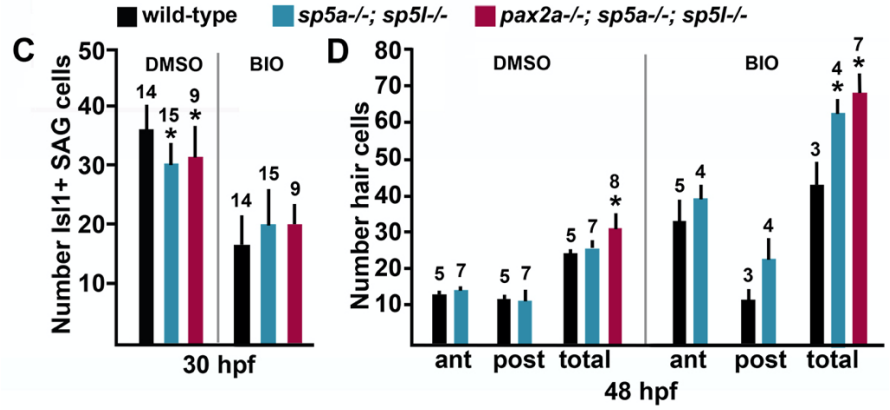


Figure 11. Effects of loss of *pax2a*, *sp5a*, and *sp5l* on otic patterning.
(A) Dorsolateral views of *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants showing expression of otic markers at 24 hpf (as indicated on the left) following treatment with DMSO (control) or BIO from 12 hpf. Scale bar (Aa), 50 µm. (B) Dorsolateral views of anti-Isl1/2 stained SAG neurons at 30 hpf (a, b, e, f, i, j) and brn3c:Gfp+ hair cells at 48 hpf (c, d, g, h, k, l) under the indicated conditions. Ovals delimit the edges of the otic vesicle. Genotypes of +/+ (wt), *sp5a-/-; sp5l-/-* double mutants (dKO, double knockouts) and *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants (tKO, triple knockouts) are indicated. (C, D) Quantification of SAG neurons and hair cells in wild-type embryos, *sp5a-/-; sp5l-/-* double mutants, and *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants (as indicated by the key at the top) following treatment with DMSO or BIO from 12 hpf, as indicated. Data show the mean number of anti-Isl1/2 stained SAG neurons (C) and phalloidin stained hair cells in anterior/utricular and posterior/saccular maculae (D). Error bars (standard deviations) and sample sizes are shown for each group, and asterisks indicate significant differences from wild-type embryos for each treatment. Note, it was not possible to identify anterior and posterior patches of hair cells in triple mutants, which produced an unbroken swath of hair cells along the AP axis. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Figure 11 cont.



sensory development due to reduced *delta* gene expression (Riley at al., 1999). Here we hypothesized that loss of *sp5a*, *sp5l* and *pax2a* might exacerbate impairment of Notch-dependent prosensory restriction. To test this, we examined *atoh1b* expression during placodal stages when the nascent prosensory domain is normally restricted by Notch to form two discrete patches (Millimaki et al., 2007). In wild-type embryos, *atoh1b* is already broken into irregular patches at 12 hpf and was fully restricted to two discrete patches by 14 hpf (Fig. 12A, E). In *pax2a-/-* mutants, *atoh1b* continued to show contiguous expression at 12 hpf, with only partial restriction into discrete patches at 14 hpf (Fig. 12B, F). A similar delay in restriction of *atoh1b* was seen in *sp5a-/-; sp5l-/-* double mutants (Fig. 12C, G), as well as in *sp5a* and *sp5l* single and double morphants (Fig. 12I-P). In *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants, *atoh1b* continued to show strong contiguous expression at 12 and 14 hpf (Fig. 12D, H). Thus, *pax2a*, *sp5a* and *sp5l* are together required for proper restriction of early prosensory domains, explaining the expanded sensory epithelium observed in triple mutants and the enhanced response to BIO in double and triple mutants. Whether such sensory expansion reflects impaired Notch signaling remains uncertain at this time.

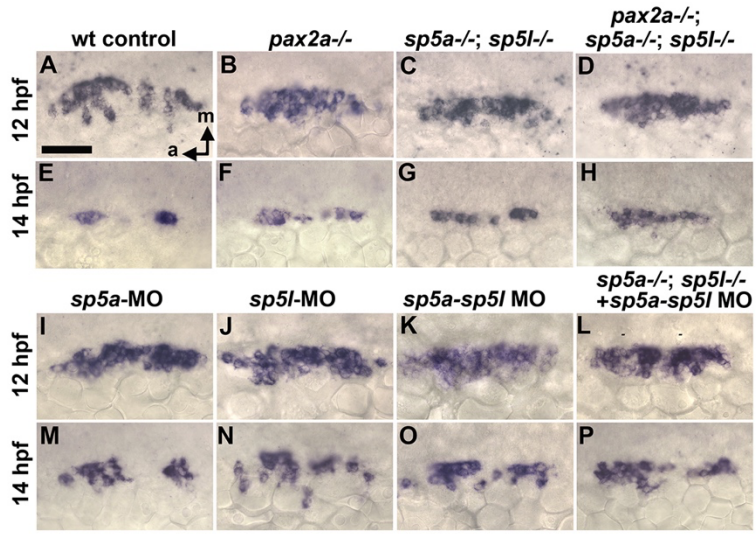


Figure 12. Effects of *sp5a* and *sp5l* knockdown on early prosensory development.
(A-H) Dorsal views (medial to the top, anterior to the left) of otic tissue showing expression of *atoh1b* at 12 and 14 hpf in wild-type control embryos, *pax2a-/-* mutants, *sp5a-/-; sp5l-/-* double mutants, and *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants. Scale bar (A), 50 µm. (I-P) Dorsal views (anterior to left) showing expression of *atoh1b* at 12 and 14 hpf following injection of *sp5a*-MO and/or *sp5l*-MO at the one-cell stage, as indicated at the top. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

Effects of *sp5a* and *sp5l* misexpression.

We previously documented that *pax2a* misexpression is sufficient to fully repress *gsc* in the otic vesicle, although *pax2a* has little effect on expression of *neurog1* (Kantarci et al., 2016). To test whether *sp5a* and *sp5l* are sufficient to mediate some effects of Wnt, we generated heat shock-inducible transgenes, *hs:sp5a* and *hs:sp5l*, to misexpress these factors during early otic development. For full activation, transgenic embryos were heat shocked for 1 hour at 39°C at 12 hpf, followed by a second 1-hour heat shock at 14 hpf. Misexpression of these factors had little effect on sensory development, except that

hs:sp5l uniquely expanded expression of *pax5* into the posterior-medial wall (Fig. 13Ac). Both factors, however, reduced the number of *neurog1*+ cells in the otic vesicle by roughly half ($p=0.0001$) (Fig. 13Ag, Ah, Ai, C), and the number of *gsc*+ cells was reduced by ~70% ($p<0.0001$) (Fig. 13Ad, Ae, Af, B). The effects of serial heat shock on *gsc* expression were similar to, but slightly more severe than a single heat shock at 12 hpf (Fig. 13B). Thus, early misexpression of *sp5a* or *sp5l* is sufficient to substantially repress neurogenesis, supporting a role for these factors in mediating some aspects of Wnt signaling. Additionally, the unique ability of *sp5l* to expand *pax5* expression suggests it also mediates aspects of Fgf signaling.

DISCUSSION

We have investigated mechanisms by which Fgf and Wnt cooperate to regulate sensory-neural patterning in the otic placode and early otic vesicle. Experiments to activate or inhibit each pathway were conducted from 12 hpf, when the otic placode is stably specified, to help discern effects of signaling on patterning and growth independent of placode induction. Both Fgf and Wnt drive proliferative expansion of otic tissue, with Fgf regulating growth throughout placodal development and Wnt promoting growth mostly after the otic vesicle forms at 18 hpf. Analysis of patterning confirms that Fgf is required for specification of both sensory epithelia and neuroblasts in the floor of the otic vesicle, whereas Wnt promotes sensory development while fully repressing neurogenesis. Overall, our data support the summary model shown in Figure 13D: We identified Pax2a, Sp5a and Sp5l as medially expressed transcription factors that mediate

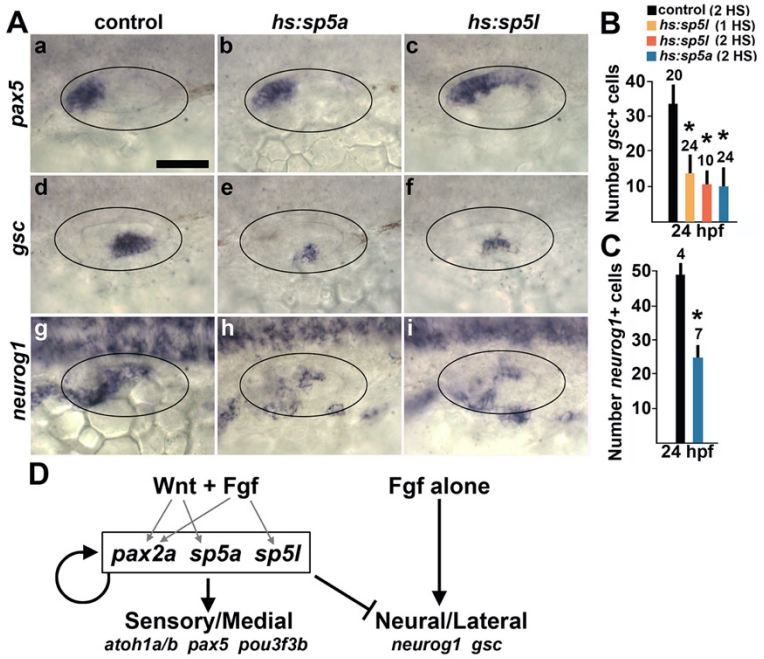


Figure 13. Effect of misexpression of *sp5a* and *sp5l* on sensory and neural development.
(A) Dorsolateral views showing expression of *pax5*, *gsc*, and *neurog1* at 24 hpf following serial activation of *hs:sp5a* or *hs:sp5l* at 12 hpf and 14 hpf. Scale bar (Aa), 50 µm. (B, C) Quantification of the mean number of *gsc*+ cells counted in wholemount embryos (B) and the mean number of *neurog1*+ cells in the otic vesicle counted from serial sections. Embryos were exposed to a single heat shock at 12 hpf (1HS) or serial heat shocks at 12 hpf and 14 hpf (2HS), as indicated in the key at the top. Error bars (standard deviations) and sample sizes are shown for each group. Asterisks indicate a significant difference from control embryos. (D) Summary of regulation of sensory-neural patterning by Fgf and Wnt. Gray arrows show regulation by Fgf and/or Wnt of medial factors *pax2a*, *sp5a* and *sp5l*, which help upregulate and maintain their own expression and promote aspects of sensory development, and are required to repress neurogenesis otherwise activated by Fgf. Figure reprinted with permission from: Tan, A.L., Mohanty, S., Guo, J., Lekven, A. C., and Riley, B. B. 2022. *Pax2a, Sp5a and Sp5l act downstream of Fgf and Wnt to coordinate sensory-neural patterning in the inner ear.* Developmental Biology 492, 139-153. https://doi.org/10.1016/j.ydbio.2022.10.004. Copyright 2022 by Elsevier.

discrete aspects of Fgf and Wnt signaling. Once activated, *pax2a*, *sp5a* and *sp5l* act partially redundantly to reinforce their own expression in the medial wall of the otic vesicle and promote early sensory development, while repressing neurogenesis.

Together, these findings help to explain how sensory epithelia and neuroblasts form concurrently in adjacent, non-overlapping compartments in the floor of the otic vesicle.

A conserved role for Fgf

The role of Fgf has been extensively studied in multiple vertebrate species (reviewed by Riley, 2021), and our findings are consistent with earlier studies. We found that transiently elevating Fgf signaling at 12 hpf enlarges the size of the otic vesicle but does not strongly affect the pattern of sensory and neurogenic development at 24 hpf (Fig. 2). However, elevating Fgf activates ectopic expression of the anterior/utricular sensory marker *pax5* at the posterior pole, with corresponding truncation of the posterior/saccular sensory marker *pou3f3b* (Fig. 5). This appears to reflect a mechanism in which the balance of Fgf vs. Shh establishes the AP axis of the otic vesicle (Hammond et al., 2003; Hammond and Whitfield, 2011; Sapède and Pujades, 2010; Hartwell et al., 2019). In contrast, blocking Fgf from 12 hpf severely reduces the size of the otic vesicle and eliminates specification of both sensory epithelia and neuroblasts (Fig. 2). Blocking Fgf also eliminates expression of *gsc* in the lateral floor of the otic vesicle, which is normally required for delamination of neuroblasts from the otic vesicle (Kantarci et al., 2016). A small number of hair cells still form in SU5402 treated embryos, reflecting earlier Fgf-dependent specification of tether cells at around 10.5 hpf in nascent otic tissue (Riley et al., 1997; Millimaki et al., 2007). Treatment with SU5402 eliminates some regional markers, including anteroventral marker *hmx3a* and posterolateral marker *otx1b*, consistent with earlier studies showing these genes require

Fgf (Feng and Xu, 2010; Maier and Whitfield, 2014). Unexpectedly, however, blocking Fgf also leads to expansion of medial markers into the lateral wall (Fig. 2), including the Wnt reporter *top:gfp* (Fig. 6), indicating disinhibition of canonical Wnt signaling in that region. However, expression of *sp5a* and *top:gfp* do not spread laterally into the floor of the otic vesicle under these conditions, indicating that suppression of neurogenesis is not caused by ectopic Wnt signaling nor conversion to medial identity.

In mouse and chick, too, Fgf plays a critical role in sensory and neural development. Disrupting Fgf fully blocks neurogenesis in both species (Alsina et al., 2004; Abelló et al., 2010; Brown and Epstein, 2011; Urness et al., 2018). In mouse, conditional disruption of Fgf3 and Fgf10 disrupts formation of vestibular sensory epithelia (Urness et al., 2018), and treating cochlear cultures with SU5402 eliminates formation of nearly all hair cells and support cells (Hayashi et al., 2008). Fgf is also required in mouse for proper expression of lateral markers in the otic vesicle as well as restriction of *Pax2* to the medial wall (Urness et al., 2018). Whether Fgf also restricts Wnt signaling in these species is not known, but otherwise the functions of Fgf appear to be highly conserved across vertebrates.

A complex role for Wnt

In contrast to Fgf, much less is known about the role of Wnt in early otic development. For example, we have not identified which specific Wnt ligands regulate patterning in the otic vesicle. Several studies implicate various *Wnt* genes that are expressed in the developing hindbrain (Ladher et al., 2000; Phillips et al., 2004; Ohyama et al., 2006;

Urness et al., 2010). In addition, gene expression studies in zebrafish, chick and mouse reveal a complex pattern of expression of multiple ligands and receptors covering nearly all regions of the otic vesicle (Thisse et al., 2001; Sienknecht and Fekete, 2009; Noda et al., 2012; Duncan et al., 2015). Despite this complexity, expression of *top:gfp*, and related reporters in mouse (Riccomagno et al., 2005; Noda et al., 2012), indicate that canonical Wnt signaling is restricted to the roof and medial wall of the otic vesicle (Fig. 6). We found that activating the Wnt pathway by treating embryos with BIO from 12 hpf expands medial markers into the floor of the otic vesicle at the expense of lateral markers, with corresponding lateral expansion of sensory markers and complete loss of neural markers *neurog1* and *gsc*. In mouse and chick, too, Wnt stimulates sensory development and inhibits neurogenesis, but it is not clear whether similar underlying mechanisms operate in those species (see below). Interestingly, despite complete loss of otic neurogenesis in BIO-treated zebrafish embryos, accumulation of SAG neurons is reduced by only ~50%. A similar fold-reduction is observed in *gsc-/-* mutants, in which newly specified neuroblasts fail to delaminate from the otic vesicle (Kantarci et al., 2016). The source of residual SAG neurons in these settings possibly reflects development of SAG "pioneer cells", which arise from a region well anterior to the otic placode and later undergo complex migration to generate the first SAG neurons in zebrafish (Hoijman et al., 2017). Requirements for development of pioneer cells remain largely unknown, but their distant origins indicate they are not likely to be affected by signals that normally regulate the otic vesicle. Surprisingly, we also found that blocking Wnt by treating embryos with IWR-1, combined with expression of *hs:dkk1*, reduces the

size of the otic vesicle by 10-15% but otherwise has almost no effect on patterning in the otic vesicle. Of particular note, blocking Wnt reduced hair cell production by only 30-35% (Fig. 2D). This is in apparent contrast to mouse, in which conditionally disrupting β-catenin (Rakowiecki and Epstein, 2013; Shi et al., 2014), or treating cochlear cultures with IWR-1 (Jacques et al., 2012; Munnamalai and Fekete, 2016), fully ablates sensory development depending on the stage of disruption. However, gene replacement in mouse with a form of β-catenin lacking transcriptional activity, but retaining its role in cell adhesion, supports nearly normal sensory patterning in the cochlea (Janssen et al., 2019), suggesting that canonical Wnt signaling is not strictly required in that endorgan. On the other hand, the role of Wnt in stimulating proliferation appears highly conserved. In our hands, blocking Wnt from 12 hpf substantially reduces proliferation in the nascent otic vesicle at 18 hpf, explaining the reduction in the size of the otic vesicle at 24 hpf (Fig. 4). Previous studies in zebrafish showed that blocking Wnt from earlier stages yields a much more dramatic reduction in otic tissue (Phillips et al., 2004; McCarroll et al., 2012). However, this is likely attributable to a requirement for Wnt in maintaining proper expression of Fgf ligands (Fig. 1) (Ladher et al., 2000; Phillips et al., 2004). Indeed, misexpression of Fgf can bypass the need for Wnt in otic induction (Phillips et al., 2004). In chick and mouse, blocking Wnt during placodal development also severely reduces the size of the otic vesicle (Ohyama et al., 2006; Freter et al., 2008). In those species, too, Wnt helps regulate expression of Fgf ligands involved in placode induction (Ladher et al., 2000; Vendrell et al., 2013) and prosensory specification (Munnamalai

and Fekete, 2016), but there are as yet no studies examining whether Fgf misexpression can bypass the requirement for Wnt.

Although elevating Wnt is sufficient to expand medial otic fates, redundant mechanisms are clearly able to establish nearly normal mediolateral patterning in the absence of Wnt. One such mechanism possibly involves readout of a mediolateral gradient of Fgf. In the developing otic placode, the prosensory domain of *atoh1b* is restricted to medial cells, i.e. in closest proximity to hindbrain sources of Fgf, and weak activation of *hs:fgf8a* laterally expands *atoh1b* throughout the placode (Gou et al., 2018). Another mechanism identified in mouse involves Shh, which helps regulate the spatial limits of *Pax2* in the otic vesicle (Riccomagno et al., 2005; Brown and Epstein, 2011).

Unique and overlapping functions of *pax2a*, *sp5a* and *sp5l*

Although these genes show overlapping expression in the medial half of the otic vesicle, they are regulated by distinct mechanisms: *sp5l* requires Fgf alone, *sp5a* relies mostly on Wnt, and *pax2a* responds to both Fgf and Wnt (Fig. 2). Through gain- and loss-of-function studies we showed that *pax2a*, *sp5a* and *sp5l* coordinate the effects of Fgf and Wnt on sensory-neural patterning. All three transcription factors expand throughout the floor of the otic vesicle in response to BIO and act redundantly to block neurogenesis. Loss of *sp5l* and *sp5a* together, or *pax2a* alone, restores weak *neurog1* expression in most BIO-treated embryos (Figs. 7 and 8); and loss of all three transcription factors allows more substantial restoration of *neurog1* in BIO-treated embryos (Fig. 11A). Thus, these factors are together required for repression of neurogenesis by BIO. Moreover,

misexpression of *hs:sp5a* or *hs:sp5l* is sufficient to strongly reduce expression of both *neurog1* and *gsc* (Fig. 13), while misexpression of *hs:pax2a* fully represses *gsc* but has little effect on *neurog1* (Kantarci et al., 2016). In contrast to regulation of neurogenesis, lateral expansion of sensory epithelia caused by BIO treatment still occurs in *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants (Fig. 11A). However, these genes are together required for proper restriction of prosensory development (marked by *atoh1b*) during early placodal development (Fig. 12). We previously documented a requirement for *pax2a* in Fgf-dependent induction of *atoh1b* in nascent otic issue, as well as subsequent Notch-dependent restriction of sensory epithelia through regulation of *delta* genes (Riley et al., 1999; Millimaki et al., 2007). Restriction of prosensory development appears to be further supported by early expression of *sp5a* and *sp5l* in the otic placode (Fig. 8A). Accordingly, *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants form an unbroken line of *atoh1b*+ prosensory cells in the otic placode at 14 hpf (Fig. 12H), strongly resembling Notch pathway mutants (Millimaki et al., 2007). Triple mutants also produce a line of ectopic sensory development connecting the utricular and saccular epithelia at 24 hpf, as well as an exaggerated expansion of sensory epithelia in response to BIO (Fig. 11A, D). We also identified several gene-specific functions required for aspects of sensory development. For example, *pax2a* is uniquely required for expression of *pax5* and *pou3f3b* in the utricular and saccular domains, respectively (Fig. 7B) (Pfeffer et al., 1998). In addition, misexpression of *hs:sp5l* uniquely expands the utricular domain of *pax5* into more posterior regions of the otic vesicle (Fig. 13A), consistent with a role in mediating Fgf. Despite its similar structure, *hs:sp5a* lacks this ability.

Whether *Pax2* and *Sp5* genes confer similar functions in birds and mammals remains to be tested. Although *Sp5*-knockout mice are viable and fertile (Harrison et al., 2000), their inner ear function and development have not been examined. *Pax2* has been well studied in the context of sensory development in mouse and chick (Torres et al., 1996; Lawoko-Kerali et al., 2002; Burton et al., 2004; Sánchez-Calerón et al., 2005), but its relationship to neurogenesis remains unclear. Mouse *Pax2* and *Gsc* are expressed in complementary medial and lateral domains in the floor of the otic vesicle (Vitelli et al., 2003), whereas expression of *Neurogenin1* straddles both domains (Burton et al., 2004; Urness et al., 2018). Recently delaminated neuroblasts do not detectably express *Pax2* (Lawoko-Kerali et al., 2002), but it is not known whether they delaminate solely from the *Gsc*+ domain. Another question is how Wnt blocks neurogenesis in mouse. In one study, activating Wnt by treatment with LiCl expands expression of *Tbx1*, a known repressor of *Neurogenin1*, into the neurogenic domain (Brown and Epstein, 2011); a second study reported that constitutively activate β-catenin abolishes *Tbx1*, instead reducing expression of essential cofactors *Eya1* and *Six1* in the floor of the otic vesicle (Freyer and Morrow, 2010). Future studies are clearly needed to answer these questions.

CHAPTER III

DIFFERING REQUIREMENTS FOR FGF AND HH DURING VESTIBULAR AND AUDITORY DOMAIN DEVELOPMENT

INTRODUCTION

Mechanisms of inner ear development are broadly conserved amongst vertebrates, although details concerning early development and morphogenesis of sensory epithelia differs between lineages. In zebrafish, for example, sensory epithelia begin to develop and function at much earlier stages relative to birds and mammals. The first mature sensory hair cells appear near the anterior and posterior poles of the otic vesicle by 22 hours post fertilization (hpf), only a few hours after formation of the lumen. The anterior (utricular) macula is necessary for vestibular function and is fully functional by 3 dpf, while the posterior (saccular) macula serves as the primary endorgan for hearing and becomes functional by 5 dpf (Haddon and Lewis, 1996; Riley and Moorman, 2000; Kwak et al., 2006). Additional sensory epithelia also develop within the inner ear, but the utricular and saccular maculae alone are necessary and sufficient for vestibular and auditory functions, respectively, during the first few weeks of larval growth. The molecular and cellular mechanisms underlying functional divergence of utricular and saccular maculae are of broad interest but are still not well understood. Fgf is required for prosensory specification during placodal stages, and ongoing Fgf signaling is also required for subsequent growth and differentiation of both utricular and saccular maculae (Sweet et al., 2011; Maier and Whitfield, 2014; Gou et al., 2018b). However,

the two maculae experience marked differences in the overall balance of Fgf vs. Shh signaling as they develop. Local upregulation Fgf near the anterior pole of the otic vesicle appears critical for establishing utricular identity, whereas elevated Shh signaling near the posterior pole promotes saccular development (Kwak et al, 2002; Kwak et al., 2006; Hammond et al., 2003; Hammond et al., 2010; Hammond and Whitfield, 2011; Hartwell et al., 2019). Accordingly, conditions that alter levels of Fgf or Shh cause corresponding shifts in utricular vs. saccular development. Identifying downstream target genes that respond to the balance of Fgf and Shh will facilitate better understanding how the unique functional properties of the utricular and saccular maculae are regulated.

In zebrafish, *pax5* and *pou3f3b* uniquely mark the utricular and saccular maculae, respectively, making them useful for examining differential regulation of utricular vs. saccular development. Expression of *pax5* is eliminated by blocking Fgf, and the domain of *pax5* expands into the saccular region under conditions that elevate Fgf signaling (Kwak et al., 2002; Kwak et al. 2006). In contrast, *pax5* is not expressed in the ear under conditions that elevate Hh signaling (Hammond et al., 2010). Knockdown studies show that *pax5* is specifically required for survival of utricular hair cells and vestibular function, whereas saccular hair cells and auditory function remain unaffected (Kwak et al. 2006). Less is known about regulation and function of *pou3f3b*. So far there have been no studies of the role of Hh in regulation of *pou3f3b*, and studies examining the role of Fgf have given contradictory results. For example, loss of *fgf3* expands the domain of *pou3f3b* into the utricular domain (Maulding et al., 2014) whereas expanded

hindbrain expression of *fgf3* in *mafba* mutants (previously *valentino* or *kreisler*) eliminates *pou3f3b* expression in the otic vesicle (Kwak et al., 2002). In contrast, loss of *fgf8* often eliminates formation of the saccular macula (although *pou3f3b* expression has not been examined in this background), whereas early misexpression of *fgf8* from a heat shock-inducible transgene does not strongly affect *pou3f3b* (Kwon et al., 2009; Tan et al., 2022). Thus the role of Fgf remains ambiguous. Although *pou3f3b*'s role in auditory domain development has not been studied in zebrafish, mice that are homozygous for a hypomorphic point mutation in Pou3f3 exhibit hearing deficits (Kumar et al., 2016), and heterozygous loss of human *Pou3f3* causes a characteristic neurodevelopmental syndrome that includes hearing impairment (Blok et al., 2019). Identification of the upstream factor(s) that coordinate expression of *pou3f3b* and *pax5* remains an important unmet goal.

Although *pax5* and *pou3f3b* have some unique requirements for proper expression in the otic vesicle, they also share a common requirement for *pax2a*. Both markers are lost in *pax2a* mutants, despite continuing development of anterior and posterior sensory epithelia (Riley et al., 1999; Tan et al., 2022). Expression of pax2a initially marks all cells in the otic placode in response to inductive Fgf signaling (Phillips et al., 2001; Hans et al., 2004). As the otic placode cavitates to form the otic vesicle, expression of *pax2a* is gradually lost from lateral cells and becomes restricted to medial cells, including nascent utricular and saccular maculae. In mouse, otic expression of *Pax2* is lost entirely in Shh mutants (Riccomagno et al., 2002; Brown and Epstein, 2011), but in zebrafish Hh signaling appears to be dispensable for maintenance of *pax2a* (Hammond et al., 2003).

Resolving how *pax2a* is regulated in zebrafish will likely shed light on coordinate regulation of *pax5* and *pou3f3b*.

Here we have investigated the signaling requirements for localized expression of *pax5*, *pou3f3b*, and *pax2a* during specification of the vestibular and auditory maculae. We confirm that Fgf is necessary and sufficient for expression of *pax5*, while Hh restricts *pax5* from posterior regions of the otic vesicle. Expression of *pou3f3a* requires both Shh and early *fgf8a*, whereas *fgf3* restricts *pou3f3b* at later stages. Expression of *pax5* and *pou3f3b* is regulated independently of sensory development. Fgf and Hh play partially redundant roles in maintaining *pax2a* such that blocking both pathways from placodal stages leads to gradual loss of pax2a expression from the otic vesicle.

MATERIALS AND METHODS

Fish strains and developmental conditions

Wild-type zebrafish were derived from the AB strain (Eugene, OR). The *pax2a* mutant allele, referred to as *pax2a-/-*, is *noi*^{tu29a} (Brand et al., 1996; Lun and Brand, 1998). The Fgf mutants *fgf8*^{x15} and *lia*^{t21142} are referred to here as *fgf8a-/-* and *fgf3-/-*, respectively (Herzog et al., 2004; Kwon and Riley, 2009). The following transgenic lines were used for misexpression: *Tg(hps70:hsfgf8a)*^{x17} is referred to as *hs:fgf8a* (Millimaki et al., 2010); *Tg(hsp70:pax2a)*^{x23} is referred to as *hs:pax2a* (Sweet et al., 2011); *Tg(hsp70:fgf3)*^{x27} is referred to as *hs:fgf3* (Sweet et al., 2011); *Tg(hsp70I:dnfgfr1-EGFP)*^{pd1} is referred to as *hs:dnfgfr1* (Lee et al., 2005); and a new transgenic allele *Tg(hsp70:pax5)*^{x30} was generated for misexpression of *pax5* and is referred to as

hs:pax5. Standard developmental conditions were used: embryos were incubated at 28.5ºC in fish water containing methylene blue. Staging was based on morphological features (Kimmel et al., 1995).

Heat shock misexpression, drug treatments, and morpholino injection

Transgenic embryos were incubated in a 39ºC water bath for 1 hour at the stated times for heat shock misexpression experiments. After heat shock, embryos were incubated at 33ºC until the stated fixing times. As a control, wild-type embryos were heat shocked under the same conditions. Drug stocks were made as 10mM solutions in DMSO and then diluted to working concentration in fish water. Working concentrations were 70 µM SU5402 (Sigma SML0443), 150 µM SANT-1 (Sigma S4572), and 10 µM IWR-1 (Sigma I0161), unless otherwise noted. DMSO was used to treat control embryos with the same volume of DMSO as drug stock in fish water. For embryos treated with SANT-1, the chorions were punctured prior to adding the drug. For experiments involving both heat shock and drug treatment, embryos were first heat shocked and then the drug was added immediately following. Due to the intense color of SU5402, which left embryos stained bright yellow and interfered with attempts to view fluorescent markers, *hs:dnfgfr1* was used in place of SU5402 for such experiments. Heat shock was done as above and any accompanying drug treatments were added immediately after the heat shock. Knockdown of *atoh1a* and *atoh1b* was done using a mixture of 2.5 ng *atoh1a*-MO and 5 ng of *atoh1b*-MO injected at the 1-cell stage (Millimaki et al., 2007).

Staining and reproducibility

Wholemount in situ hybridization was performed as previously described (Jowett and Yan, 1996; Riley et al., 1999; Phillips et al., 2001). Immunolocalization was used to mark hair cells by using a primary antibody for anti-pax2 (Covance PRB-276P, 1:100) and the secondary antibody AlexaFluor 546 goat anti-rabbit (Life Technologies A11010, 1:50) (Riley et al., 1999; Phillips et al., 2001). In situ and live embryo images were taken on a Zeiss compound scope using brightfield imaging at 400X. Fluorescent imaging was done on an Olympus compound scope at 200X. Data shown here represent phenotypes seen in at least 15 embryos, unless otherwise noted.

RESULTS

Expression of *pax5* and *pou3f3b*

Otic expression of *pax5* and *pou3f3b* begins at around 19 hours post fertilization (hpf) and 20 hpf, respectively. To test the requirement for Fgf and Hh, we incubated embryos in inhibitors from 12 hpf. Treatment with the Fgf receptor inhibitor SU5402 at 12 hpf, resulted in a loss of *pax5*, supporting previous work that Fgf is required for expression of *pax5* and other anterior and sensory domain markers (Fig. 14B). *pou3f3b*, however, shifted towards the anterior portion of the otic vesicle when Fgf was inhibited (Fig. 14H). Conversely, loss of Hh, by inhibiting Smoothened using SANT-1, caused a loss of *pou3f3b* but an ectopic expansion of *pax5* along the medial wall, extending back towards the saccule (Fig. 14C, I). Co-treatment with SU5402 and SANT-1 eliminated expression

of both *pax5* and *pou3f3b* (Fig. 14D, J). Loss of *pax2a*—an early marker of the otic placode that is later associated with the sensory epithelia—also caused a loss of *pax5*

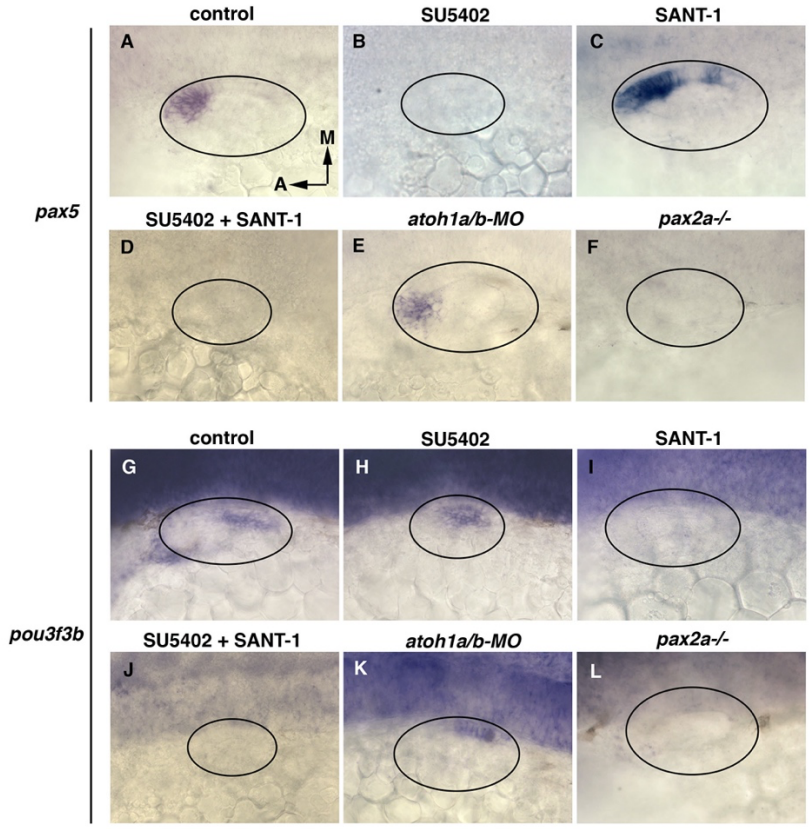


Figure 14. Effects of altering Fgf and Hh on the expression of *pax5* and *pou3f3b*.
Dorsolateral views of *pax5* (A - F) and *pou3f3b* (G - L) at 26 hpf under various conditions (listed at top). (A - D, G - J) Drug treatments were administered at 12 hpf to inhibit Fgf (70 µM SU5402) or Hh (150 µM SANT-1). The same treatment conditions were used in subsequent figures unless otherwise noted. (E, K) The *atoh1a-* and *atoh1b-MO* were co-injected at the one-cell stage. (F, L) *pax2a-/-* completely eliminated expression of both *pax5* and *pou3f3b*. Oval marquee denotes the outer edge of the otic vesicle.

and *pou3f3b*, as has been shown in previous work (Fig. 14F, L). When sensory development was prevented by injection of *atoh1a-MO/atoh1b-MO*, *pax5* and *pou3f3b* expression remained, indicating that the sensory maculae are not required for their expression (Fig. 14E, K). This supports the idea that Fgf is required for expression of the

anterior marker *pax5* and Hh is required for expression of the posterior marker *pou3f3b* and that their expression does not require the sensory maculae.

Maintenance of *pax2a* expression by Fgf and Hh

Since *pax2a* is required for expression of *pax5* and *pou3f3b*, we sought to identify upstream regulators of the medial and sensory domains of *pax2a* in the otic vesicle. *pax2a* initially marks all otic placode cells and is later restricted to the medial half of the otic vesicle, including the sensory domains (Fig. 15A, 2Ga-c). It is unknown what factors regulate the lateral restriction to the medial wall prior to activation of *pax5* and *pou3f3b*. We treated wild-type embryos with pharmacological inhibitors of Fgf, Wnt, and Hh at 12 hpf, before restriction of the *pax2a* domain begins. Inhibiting Hh caused a loss of the saccular domain of *pax2a* at 24 hpf (Fig. 15B). Similarly, blocking Hh by SANT-1 in combination with blocking Wnt by IWR-1, which stabilizes the β-catenin destruction complex, eliminated *pax2a* expression in the saccular domain but did not alter expression of *pax2a* in the utricular domain or the medial wall (Fig. 15D). When Fgf or Fgf and Wnt together were inhibited, *pax2a* expression was retained in the lateral wall (Fig. 15C, E). However, blocking Fgf and Hh eliminated *pax2a* expression from the medial wall and severely reduced its expression in the anterior and posterior sensory domains (Fig. 15F).

To determine how *pax2a* expression is restricted in the absence of Fgf and Hh, embryos were treated with SU5402 and SANT-1 at 12 hpf and expression of *pax2a* was

examined at various points in development. When both Fgf and Hh were blocked at 12 hpf, *pax2a* expression was lighter and patchier by 14 hpf and the otic placode was

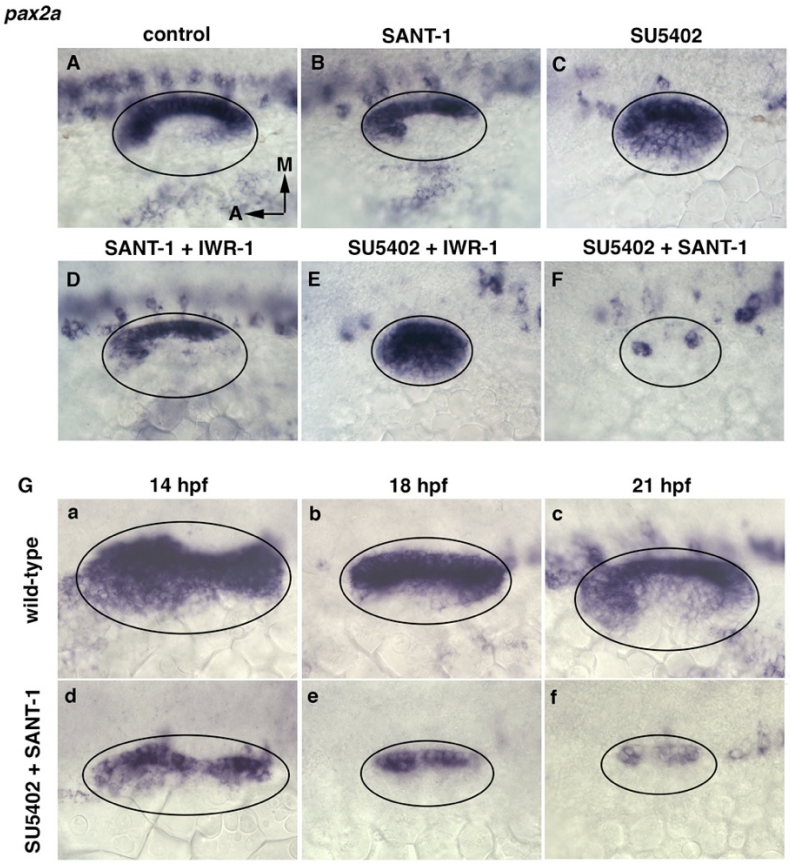


Figure 15. Expression of *pax2a* is maintained by Fgf and Hh.
(A - F) Dorsolateral views of *pax2a* expression at 24 hpf following treatment at 12 hpf with inhibitors for Hh, Wnt, and Fgf (listed at top). IWR-1 (10 μM) was used to inhibit Wnt. (G) Time course of *pax2a* expression in wild-type (DMSO treated) or SU5402 + SANT-1 treated embryos. Drugs were administered at 12 hpf and embryos were fixed at the timepoints listed at top. Oval marquee denotes outer edge of the otic vesicle.

smaller than in wild-type (Fig. 15Ga, d). By 21 hpf, *pax2a* expression was much lighter than in control embryos and inconsistently expressed through the medial wall (Fig. 15Gc, f). This indicates that both Fgf and Hh are required to properly maintain *pax2a* expression in the medial wall and sensory maculae. In the absence of Fgf and Hh, *pax2a*

expression is lost as development progresses, indicating that both the vestibular and auditory sensory domains have been severely disrupted.

In SU5402 + SANT-1 treated embryos, residual *pax2a* expression possibly marks the tether cells, the first pairs of hair cells to develop in the utricular and saccular maculae (Fig. 15F). Therefore, we next assessed the development of the sensory domains in the absence of Fgf and Hh. Co-treatment of SU5402 and SANT-1 resulted in an extremely small otic vesicle that lacked otoliths or otolith precursors (Fig. 16B).

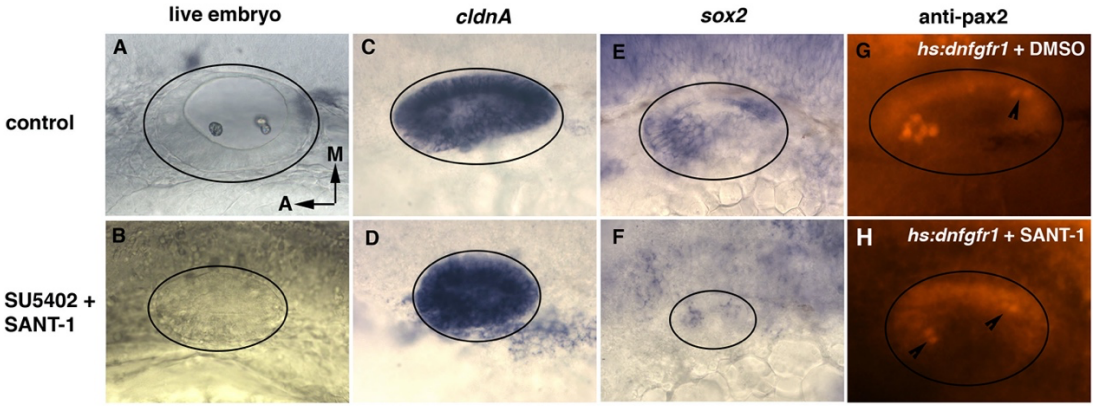


Figure 16. Disruption of sensory development following inhibition of Fgf and Hh.
(A, B) Images of 24 hpf live embryos treated with DMSO (control) or SU5402 + SANT-1. (C, D) Expression of *cldnA* at 24 hpf confirms presence of the otic vesicle in drug treated embryos. (E, F) Expression of *sox2* at 26 hpf shows reduction of sensory epithelium from drug treatment. (G, H) Antibody staining at 31 hpf with *anti-pax2* labels the hair cells. Experiment was done in *hs:dnfgfr1* embryos treated with SANT-1. Embryos were heat shocked at 12 hpf for 1 hour at 39ºC followed by immediate addition of SANT-1 (150 µM). Arrowheads indicate the pairs of two tether cells. Oval marquee denotes outer edge of the otic vesicle.

Expression of *cldnA* confirmed that the otic vesicle was present (Fig. 16C, D). The sensory marker *sox2* was still expressed in small regions associated with the utricle and saccule, similar to the expression of *pax2a* (Fig. 16F). Antibody labeling of hair cells

with anti-pax2 shows that the initial two tether cells were present in the utricle and saccule, but no further sensory development had occurred (Fig. 16H). This indicates that, without Fgf and Hh, the otic vesicle forms but *pax2a* expression is mostly lost, *pax5* and *pou3f3b* are eliminated, and sensory development does not proceed after the development of the tether cells.

Effect of altered Fgf on *pax5* and *pou3f3b*

Although Fgf is required for sensory development, treatment with SU5402 did not affect the sensory markers in the anterior and posterior domains equally so we examined the requirements for individual Fgf ligands. Loss of *fgf3* reduced the *pax5* domain but did not completely eliminate it (Fig. 17Aa). As in SU5402 treated embryos, *pou3f3b* exhibited an anterior shift in *fgf3-/-* and there was also a clearing of *pou3f3b* expression at the very posterior end of its domain (Fig. 17Ab). Homozygous mutants of *fgf8a* always produce an utricular otolith but only some of the homozygous mutants produce both a utricular and saccular otolith. We separated *fgf8a-/-* embryos based on bilateral presence of one or two otoliths prior to fixing and examined them for expression of *pax5* and *pou3f3b. pax5* was expressed in all *fgf8a-/-* regardless of the status of the posterior macula (Fig. 17Ba, d). Unexpectedly, *pou3f3b* expression did not correlate with presence or absence of the posterior macula. *pou3f3b* was still expressed in 41% (7/17) of one otolith embryos despite absence of the saccular macula (Fig. 17Bb). In the remainder of utricle-only *fgf8a-/-*, *pou3f3b* was eliminated (41%; 7/17) or strongly reduced (18%; 3/17) (Fig. 17Bb', c). In *fgf8a-/-* with two maculae, *pou3f3b* was strongly reduced in

29% (6/21) (Fig. 17Bf). Thus, *pou3f3b* expression does not require saccular development, nor does saccular development necessarily correlate with *pou3f3b* expression in *fgf8a-/-*. *pou3f3b* appears to have an early requirement for *fgf8a* but the

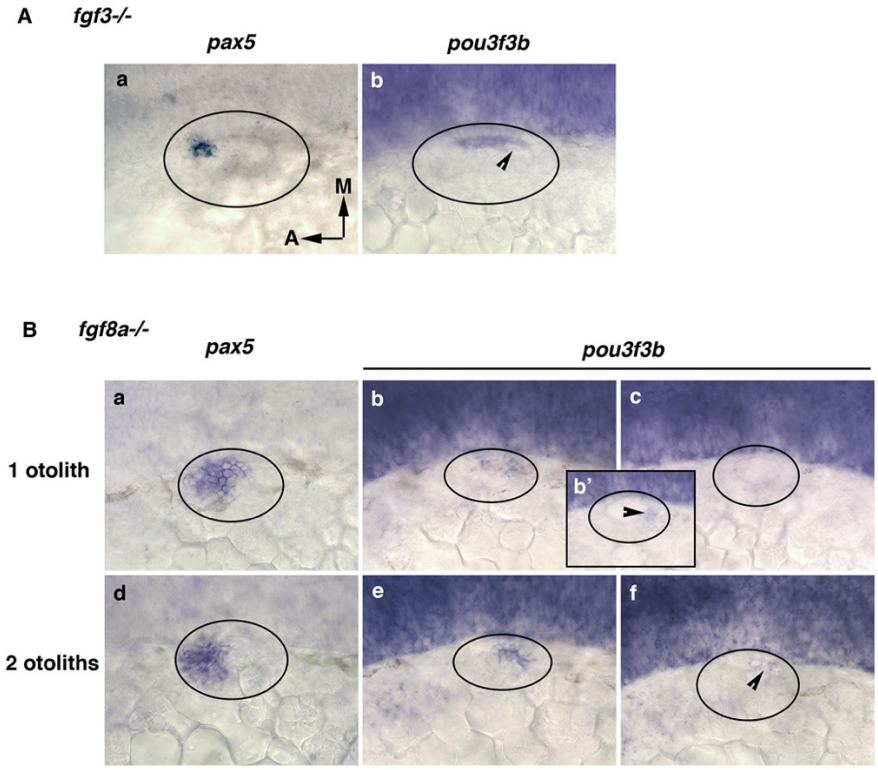


Figure 17. Expression of pax5 and pou3f3b in Fgf mutants.
(A) Expression of *pax5* and *pou3f3b* at 26 hpf in *fgf3-/-* is reduced but not eliminated. Arrowhead in (Ab) shows area of *pou3f3b* clearing at the posterior-most edge of the expression domain. (B) Expression of *pax5* and *pou3f3b* at 26 hpf in *fgf8a-/-*. Embryos were sorted based on the presence (2 otoliths) or absence (1 otolith) of the saccular otolith. (b, c, e, f) Main *pou3f3b* images show the predominant expression patterns present in each otolith number group. (Bb') Inset shows the subset of one otolith embryos with severely reduced, but not absent, *pou3f3b* expression. Arrowheads indicate reduced and constricted *pou3f3b* expression domain. Oval marquee denotes outer edge of the otic vesicle.

effect is likely indirect since blocking all Fgf, even from 12 hpf, does not eliminate its expression. This further supports the idea that the sensory maculae and otoliths are not

required for expression of *pax5* or *pou3f3b* and presence of other Fgfs is sufficient for *pax5* expression in *fgf3-/-* or *fgf8a-/-*.

Finally, we examined how elevated levels of Fgfs and Fgf mediators could influence the expression of *pax5* and *pou3f3b*. Heat shock-induced misexpression of *fgf3* at 12 hpf did not cause an alteration of the *pax5* or *pou3f3b* domains (Fig. 18A, B)

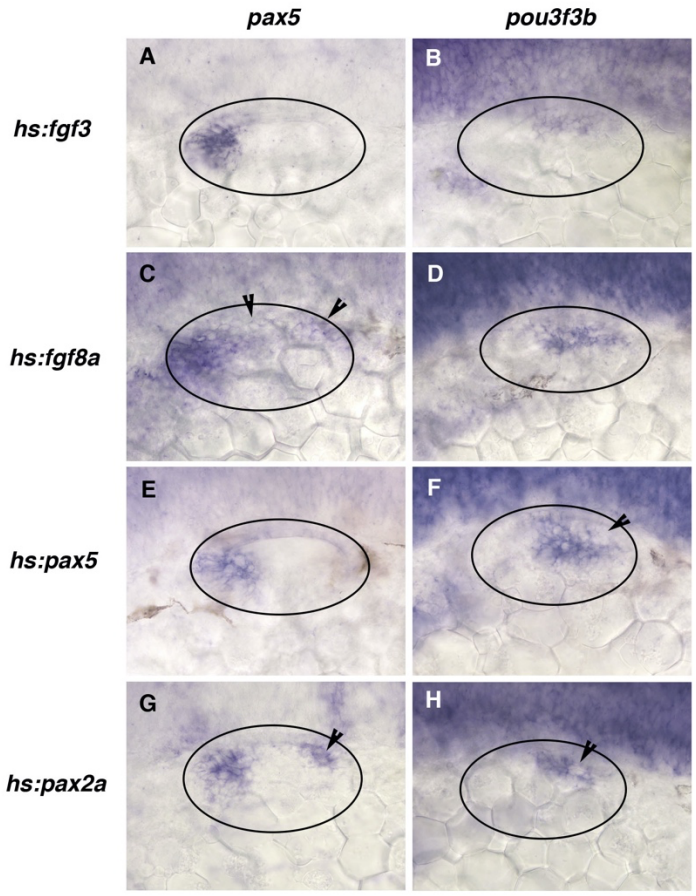


Figure 18. Misexpression of Fgf mediators expand *pax5* and reduce *pou3f3b* saccular expression.
(A - H) Expression of *pax5* and *pou3f3b* at 26 hpf following misexpression of *hs:fgf3* or *hs:pax2a* at 12 hpf or *hs:fgf8a or hs:pax5* at 18 hpf. Arrowheads denote areas of ectopic *pax5* expression and corresponding areas of *pou3f3b* reduction. Oval marquee denotes outer edge of the otic vesicle.

although misexpression of *fgf8a* at 18 hpf caused ectopic expression of *pax5* along through the medial domain and into the saccular domain (Fig. 18C). *pou3f3b* expression shifted slightly medially with misexpression of *fgf8a* (Fig. 18D). Misexpression of *pax5* at 18 hpf caused a universal slight upregulation of *pax5* and caused an expansion of *pou3f3b* laterally into the floor along with a cleared space in the vicinity of the saccular tether cells (Fig. 18E, F). Misexpression of *pax2a* at 12 hpf also caused this saccular clearing of *pou3f3b*, though without the lateral or medial shift or expansion (Fig. 18H) In both *hs:fgf8a* and *hs:pax2a*, this medial shift or saccular clearing of *pou3f3b* is associated with ectopic expression of *pax5* in the posterior sensory domain (Fig. 18C, G). Elevated levels of Fgf or Fgf mediators are capable of inducing saccular expression of *pax5* and a corresponding reduction of *pou3f3b* in this posterior, auditory domain.

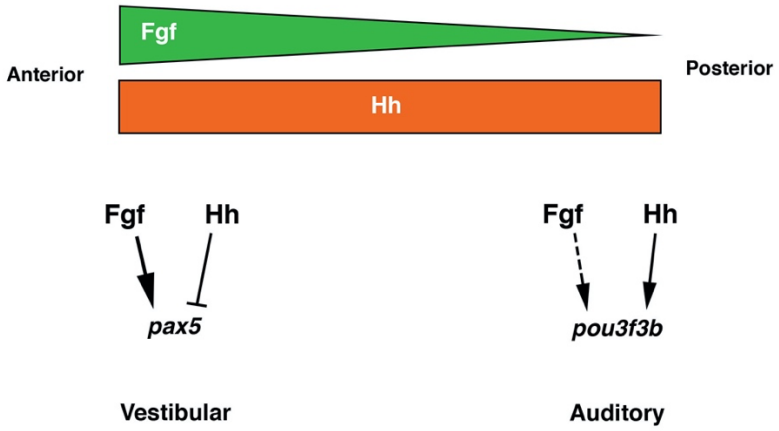


Figure 19. Model of Fgf and Hh regulation of pax5 and pou3f3b.
The utricular *pax5* domain requires a high level of Fgf and Hh limits the expression of *pax5* to this domain. The saccular *pou3f3b* domain requires Hh but Fgf also influences expression of *pou3f3b*.

DISCUSSION

We have examined the roles of Fgf and Hh in specifying the vestibular and auditory regions of the otic vesicle. We confirm previous work that *pax5* requires Fgf and is limited to the anterior domain by Hh while *pou3f3b* requires Hh but is also susceptible to Fgf signaling. *pax2a* is required for expression of both these sensory markers and *pax2a* expression is not maintained if Fgf and Hh are eliminated. In the absence of both Fgf and Hh and consequent disappearance of *pax2a*, *pax5* and *pou3f3b* are not expressed and sensory development does not progress after formation of the tether cells (Fig. 19).

The sensory domains require continuing input of Fgf during sensory development although both increased and decreased levels of Fgf perturb sensory specification. Our findings support the idea that a high level of Fgf promotes *pax5* expression while Hh limits its domain to the anterior portion of the otic vesicle (Fig. 14). Loss of Fgfs, like *fgf3-/-* and *fgf8a-/-*, reduce *pax5* and other anterior sensory markers and the sensory maculae are not properly specified (Kwak et al., 2006; Millimaki et al., 2007; Hammond et al., 2011; Maier and Whitfield, 2014; Maulding et al., 2014). Misexpression of Fgfs results in ectopic expression of anterior sensory markers in the posterior sensory domains (Millimaki et al., 2010; Sweet et al., 2011; Hammond et al., 2011; Hartwell et al., 2019). This ectopic expression of the anterior sensory markers appears to come at the cost of the posterior sensory domain, as in *valentino* mutants where ectopic *fgf3* in the hindbrain results in an expanded domain of *pax5* and a loss of *pou3f3b* (Kwak et al., 2002). Although both sensory domains require continual input of Fgf, a high level of Fgf favors anterior sensory identity over posterior. This is in line with our findings that

SU5402 treatment eliminates expression of *pax5* but not *pou3f3b* and that misexpression of *fgf3* and *fgf8* induce ectopic *pax5* in the posterior domain where there is a corresponding reduction in *pou3f3b* (Figs. 14 and 18). Interestingly, the of the sensory maculae does not correlate with a loss of *pax5* and *pou3f3b*. Previous work has shown that disruption of sensory epithelia formation does not impact expression of *pax5* in the utricle and disruption of *pax5* does not prevent initial formation of hair cells (Kwak et al., 2006; Millimaki et al., 2007). Presence of the saccule is regulated independently from genetic control of *pou3f3b* expression—some *fgf8a-/-* without a saccule still show expression of *pou3f3b* while some with a saccule lack *pou3f3b* (Fig. 17). This highlights the intricacies of Fgf's influence on the sensory domains.

Hedgehog is required for proper posterior identity of the otic vesicle but its requirement for sensory development is unclear. Blocking Hh results in ectopic posterior expression of anterior markers like *pax5*. The sensory maculae are also altered by increased or decreased Hh signaling, often shifting closer together or even merging into a single macula (Hammond et al., 2003; Hammond et al., 2010; Hartwell et al., 2019). We have shown here that Hh is required for expression of *pou3f3b* and that Fgf also influences the expression of *pou3f3b*, though likely indirectly (Figs. 14 and 18). We have also shown that the expression of *pou3f3b* is regulated independently from formation of the saccular epithelium (Figs. 14 and 18). How gain or loss of *pou3f3b* impacts saccular development or auditory function remains to be investigated.

In addition to *pax5* and *pou3f3b*, *pax2a* is also expressed in the sensory domains and through the medial wall of the otic vesicle. The *pax2a* expression domain is

regulated by Fgf, Wnt, and Hh (Phillips et al., 2001; Hans et al., 2004; Tan et al., 2022). While Wnt has a strong influence on the expression domain of *pax2a*, Fgf and Hh had a crucial role in maintaining the expression of *pax2a* following its induction during the otic placode stage (Fig. 15). Inhibiting Fgf and Hh at 12 hpf prevented any further sensory development and, thus, the only hair cells present were the tether cells which are specified prior to 12 hpf (Fig. 16). *pax2a* was also required for the expression of both *pax5* and *pou3f3b* and likely coordinates aspects of Fgf and Hh signaling in these regions (Fig. 14; Kwak et al., 2006; Tan et al., 2022). *pax5* and *pou3f3b* expression was eliminated by loss of *pax2a* and also by treatment with SU5402 + SANT-1. Since the SU5402 + SANT-1 treatment resulted in a gradual loss of *pax2a* expression, the loss of *pax5* and *pou3f3b* could reflect a requirement for *pax2a* in mediating Fgf and Hh. Alternatively, since the SU5402 + SANT-1 treatment resulted in a reduced *pax2a* domain at 18-21 hpf, when *pax5* and *pou3f3b* are initiated, the loss of *pax5* and *pou3f3b* expression could reflect a requirement for a particular level of *pax2a.* Maintenance of *pax2a* expression in the medial wall and anterior and posterior domains requires both Fgf and Hh and *pax2a* likely mediates both pathways in these regions.

Conserved requirements for Fgf and Hh

Studies in mice indicate that the roles of Fgf and Hh in specifying the vestibular and auditory domains may be conserved in mammals. As in zebrafish, there is a requirement for Fgf for proper development of the sensory domains. Disruption of *Fgf3* and *Fgf10* in mice malformation of the cochlea, especially the formation of the organ of

Corti, and the semi-circular canals (Mansour et al., 1993; Hatch et al., 2007; Urness et al., 2018). Disruption of Shh also leads to failed cochlear duct extension. The cochlea extends from the posterior portion of the otocyst, growing towards the Hh source in the notochord and floorplate (Bok et al., 2007; Carroll Driver et al., 2008; Brown and Epstein 2011). Thus, in both mouse and zebrafish, Fgf is required for sensory development and influences both vestibular and auditory domains while Hh acts on posterior regions that form the auditory domains. The failed maintenance of *pax2a* expression in the medial wall and elimination of *pax2a* from the saccule with SANT-1 treatment mirrors findings from mouse where increased Shh caused ectopic expression of *Pax2* and mutation of Shh led to gradual loss of *Pax2* from the medial wall and disrupted development of the auditory structures (Riccomagno et al., 2002). This could support a model where *pax2a* is required to mediate Hh for expression of *pou3f3b* and proper development of the saccule. In addition to cochlear extension, Shh impacts development of the auditory organ by regulating hair cell development, both by maintaining prosensory cell properties and controlling specification of hair cells by regulating cell cycle exit (Bok et al., 2013; Tateya et al., 2013; Benito-Gonzalez and Doetzlhofer, 2014). The type of cochlear hair cell malformations seen in the cochlea with Shh disruption are consistent with low range hearing loss seen in humans with a syndromic condition associated with mutation of *Gli3* (Carroll Driver et al., 2008). Although extensive work has been done on the roles of Fgf and Hh in sensory domain development, examination of the interaction of these two pathways in specifying the vestibular and auditory domains remains to be investigated.

CHAPTER IV

SUMMARY AND DISCUSSION

Summary of findings

Inner ear development has been extensively studied and many of the required signals for specification of sensory epithelia and neuroblasts are well established. However, downstream targets of these signals are still poorly understood, especially when it comes to coordination of the signals. In this work, we have examined the coordination of Fgf, Wnt, and Hh for regulation of the sensory and neural development of the inner ear.

In Chapter II, we examined how Fgf and Wnt are coordinated to specify the sensory and neural domains in adjacent areas of the otic vesicle. We showed that inhibition of Fgf causes a loss of both sensory and neural development, which agrees with previous work (Millimaki et al., 2007; Vemaraju et al., 2012). Overactivation of Wnt caused expansion of the sensory epithelia and inhibited neurogenesis but Wnt was not required for specification of either domain, similar to results seen in mouse and chick (Stevens et al., 2003; Ohyama et al., 2006; Freyer and Morrow, 2010). Wnt also promoted proliferation and, when it was blocked, the otic vesicle's size and the number of hair cells were reduced. To examine the potential coordination of Fgf and Wnt by *pax2a*, *sp5a*, and *sp5l*, we used a mutant of *pax2a* and generated mutant and heat-shock transgenic alleles of *sp5a* and *sp5l*. We found that the three transcription factors have overlapping expression in the otic placode and vesicle and are coregulated by Fgf and

Wnt. In *pax2a-/-; sp5a-/-; sp5l-/-* triple mutants, the ability of Wnt to repress neurogenesis was greatly reduced and there was formation of ectopic hair cells. Loss of *pax2a* alone or *sp5a* and *sp5l* together had similar, but weaker, effects. The lack of prosensory domain restriction and ectopic hair cell production in *pax2a-/-;sp5a-/-;sp5l-/-* triple mutants resembled the loss of lateral inhibition seen in *mind bomb* mutants, but it is unclear why this occurred as Delta expression was not diminished in *pax2a-/-;sp5a-/-; sp5l-/-* triple mutants (Riley et al., 1999; Millimaki et al., 2007). Misexpression of *hs:sp5a* or *hs:sp5l* resulted in reduction of *neurog1* and *gsc*, further supporting their role in mediating Wnt's repression of neurogenesis. We also identified some gene-specific functions, including the requirement of *pax2a* for the utricular and saccular maculae markers *pax5* and *pou3f3b*. Together, these results show that *pax2a*, *sp5a*, and *sp5l* are regulated by a combination of Fgf and Wnt and are required for some aspects of sensory development as well as Wnt's inhibitory effect on neurogenesis.

In Chapter III, we examined the role of Fgf and Hh in specifying the anterior and posterior sensory epithelia. Fgf promoted expression of *pax5* in the anterior domain, as has been previously shown, and blocking Hh allowed *pax5* to expand into the posterior domain (Hammond et al., 2003; Hammond et al., 2010). In keeping with Hh's role as a posteriorizing signal, *pou3f3b* expression required Hh. *pou3f3b* also had an early requirement for *fgf8a* but was restricted at later stages by *fgf3*. Additionally, we found that the expression of *pax5* and *pou3f3b* was regulated independent of sensory development. These results support unique requirements for Fgf and Hh in the utricle and saccule and showed that *pax5* and *pou3f3b* are good markers for investigating this

further. We also showed that, in the absence of Fgf and Hh, *pax2a* expression was not maintained in the otic vesicle and sensory development stalled.

Conserved roles of *pax2a*

pax2a appears to be necessary for proper otic development at multiple stages and it may serve as a mediator for Fgf, Wnt, and Hh. Although *pax2a* requires Fgf for induction, previous research indicates it then becomes self-maintaining (Phillips et al., 2001; Hans et al., 2004). However, we have shown in Chapter III that maintenance of *pax2a* in the otic vesicle was largely dependent on the presence of both Fgf and Hh. In Chapter II, we showed that *pax2a* acted as a mediator of Wnt and Fgf signaling, along with *sp5a* and *sp5l*. Fgf, Wnt, and Shh have been shown to alter *Pax2* expression in mouse and chick, indicating that this function as a mediator of all three pathways is possibly conserved (Ladher et al., 2000; Riccomagno et al., 2002; Riccomagno et al., 2005; Abelló et al., 2010; Brown and Epstein, 2011).

pax2a is required for development of hair cells in all vertebrates. In zebrafish, the absence of *pax2a* causes an initial excess production of hair cells but this is followed by a loss of cell adhesion and death and an overall lower number of hair cells in older embryos (Riley et al. 1999, Kwak et al. 2006, Millimaki et al. 2007). *pax2a* is known to upregulate expression of *cdh1*, though it is unclear whether the hair cell death in *pax2a-/-* is caused or preceded by the loss of cell adhesion (Kantarci et al., 2016). In mouse and chick, as well, *Pax2* is expressed in the hair cells (Lawoko-Kerali et al., 2002; Sánchez-Calderón et al., 2005). Loss of *Pax2* results in severe malformation or

complete agenesis of the cochlea and the organ of Corti, the sensory epithelium contained in the cochlea, does not develop (Torres et al., 1996; Hutson et al., 1999; Burton et al., 2004; Bouchard et al., 2010). There is also a loss of cell adhesion molecules and increased cell death (Burton et al., 2004; Christophorou et al., 2010). The loss of hair cells seen in *Pax2*/*pax2a* mutants could either be caused by a defect in hair cell differentiation or maintenance and studies with a conditional knockout are needed to address this.

In addition to being necessary for proper hair cell specification or maintenance, *pax2a* may play a role in regulating Hh and *pou3f3b* for specification of the auditory regions of the inner ear. In chicks and mice, as in zebrafish, Hh acts on regions of the developing ear which produce the auditory epithelium (Riccomagno et al., 2002; Hammond et al., 2003; Bok et al., 2007; Hammond et al., 2010; Brown and Epstein, 2011; Son et al., 2015). We found that inhibition of Hh eliminated expression of *pou3f3b*, as did loss of *pax2a.* In humans, mutation of *Pax2* causes a syndromic condition which includes hearing loss and there is evidence that hearing loss may also be part of a neurodevelopmental syndrome caused by heterozygous loss of *Pou3f3* (Eccles and Schimmenti, 1999; Bower et al., 2011). Disruption of Pou3f3 in mice causes hearing deficits and loss of either *Pax2* or Shh results in severe malformation of the cochlea (Kumar et al., 2016). This points towards multiple roles of *pax2a* during sensory epithelium development of the inner ear: coordinating input of Fgf, Wnt, and Hh; regulating differentiation or maintenance of hair cells; and regulating expression of downstream targets like *pou3f3b* to control auditory domain fate.

Comparison of auditory organs

Across vertebrates, the organs of the vestibular system include the semi-circular canals and the utricle. In zebrafish and other anamniotes, the saccule provides auditory function of the inner ear while birds and mammals use the cochlea for this purpose. The saccule is present in amniotes, as well, but it serves a vestibular function rather than auditory. Morphologically, the saccule is a much less complex structure than the cochlea and, consequently, it can be difficult to distinguish what drives form vs. function of the auditory endorgan. The mature saccule of zebrafish and other similar fish is ribbon-like (Popper and Platt, 1983). The mammalian cochlea is a coiled structure and the overall tube of the cochlea is divided into three canals. The central tube—the scala media—contains the organ of Corti, which contains four rows of hair cells intercalated with support cells (reviewed in Basch et al., 2016; Carroll Driver and Kelley, 2020).

Although the morphology of the cochlea is more complex than the saccule, the sensory epithelia are similar in many ways. Similar prosensory factors, such as *Atoh1* and *Sox2*, are required for specification of hair cells and support cells (reviewed in Basch et al., 2016; Carroll Driver and Kelley, 2020; Riley, 2021). The cochlea and saccule are tonotopically organized, responding to higher frequencies at one end and lower frequencies at the other. Cochlear hair cells vary in stereocilia length and ion channel expression and the basilar membrane goes from thin, narrow, and stiff at the cochlear base to wide, thick, and more flexible at the apex. The base responds more readily to high frequencies and the apex is more sensitive to low frequencies. Saccular hair cells also vary morphologically and the basal lamina beneath them is thick at the anterior end

and thins at the posterior end. There is a varied response to frequencies, with the anterior end responding to higher frequencies and the posterior end responding to lower frequencies (Lanford et al., 2000; Schuck and Smith, 2009; Smith et al., 2011; Breitzler et al., 2020).

Although the role of Shh for development of the cochlea is well understood, it is still not clear how Hh effects development of the saccule. It is likely that the cochlea and saccule have similar responses to Shh during development but most studies on the auditory organ's requirement for Shh have been done in mice. In response to Shh from the notochord, the cochlea begins to grow from the posterior portion of the ventral otocyst and extension of the cochlea requires both Wnt and Shh (Riccomagno et al., 2002; Brown and Epstein, 2011; reviewed in Carroll Driver and Kelley, 2020). The Shh gradient establishes the tonotopic organization of the organ of Corti (Son et al., 2015; reviewed in Basch et al., 2016; Carroll Driver and Kelley, 2020). Shh downregulation in a gradient from base to apex controls when cells can differentiate. A wave of hair cell differentiation follows this gradient and matches development of the associated auditory neurons (Carroll Driver et al., 2008; Liu et al., 2010; Bok et al., 2013). How the saccule acquires tonotopic organization is not known. It is possible that the role of Hh is conserved, however, more work is needed to understand how a Hh gradient is established across the saccule and influences tonotopy.

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