Development 138, 397-408 (2011) doi:10.1242/dev.056093

Her9 represses neurogenic fate downstream of Tbx1 and retinoic acid signaling in the inner ear

Marija Radosevic[1], Àlex Robert-Moreno[1], Marion Coolen[2], Laure Bally-Cuif[2] and Berta Alsina[1,*]

SUMMARY

Proper spatial control of neurogenesis in the inner ear ensures the precise innervation of mechanotransducing cells and the propagation of auditory and equilibrium stimuli to the brain. Members of the Hairy and enhancer of split (Hes) gene family regulate neurogenesis by inhibiting neuronal differentiation and maintaining neural stem cell pools in non-neurogenic zones. Remarkably, their role in the spatial control of neurogenesis in the ear is unknown. In this study, we identify *her9*, a zebrafish ortholog of *Hes1*, as a key gene in regulating otic neurogenesis through the definition of the posterolateral non-neurogenic field. First, *her9* emerges as a novel otic patterning gene that represses proneural function and regulates the extent of the neurogenic domain. Second, we place Her9 downstream of Tbx1, linking these two families of transcription factors for the first time in the inner ear and suggesting that the reported role of Tbx1 in repressing neurogenesis is in part mediated by the bHLH transcriptional repressor Her9. Third, we have identified retinoic acid (RA) signaling as the upstream patterning signal of otic posterolateral genes such as *tbx1* and *her9*. Finally, we show that at the level of the cranial otic field, opposing RA and Hedgehog signaling position the boundary between the neurogenic and non-neurogenic compartments. These findings permit modeling of the complex genetic cascade that underlies neural patterning of the otic vesicle.

KEY WORDS: HES, Retinoic acid (RA) signaling, Tbx1, Neurogenesis, Proneural, Sensory organs, Zebrafish, Inner ear

INTRODUCTION

The inner ear of vertebrates, one of the main neurosensory structures of the head, conveys auditory and balance sensory information to the brain. Its development commences from the otic placode, an ectodermal structure adjacent to the posterior hindbrain. Extrinsic signals from various surrounding tissues are integrated by otic-fated cells, contributing to the complex three-dimensional organization of the organ and the generation of the stereotyped pattern of sensory neurons, hair cells and supporting cells (Bok et al., 2005; Schneider-Maunoury and Pujades, 2007; Whitfield and Hammond, 2007). The generation of sensory neurons is restricted to the anteromedial subdomain of the otic anlagen and depends on Fibroblast growth factors (Fgfs) (Abello et al., 2010; Adam et al., 1998; Alsina et al., 2004; Kim et al., 2001; Leger and Brand, 2002; Ma et al., 2000; Millimaki et al., 2007). The Tbx1 transcription factor, a candidate for some phenotypes of DiGeorge syndrome (DGS) has been shown to negatively regulate the limits of the ear neurogenic compartment (Raft et al., 2004). In *Tbx1*$^{-/-}$ mice, the neurogenic region of the otic epithelium is expanded, the otocyst is small and the sensory patches do not form (Moraes et al., 2005; Raft et al., 2004; Vitelli et al., 2003; Xu et al., 2007). Our previous studies in chick revealed that *Hairy1* (*Hes1* homolog) is present in the non-neurogenic territory of the otic vesicle (Abello et al., 2007), suggesting that *Hes1* might have an as yet unidentified role in neural patterning of the otic vesicle.

Genes of the Hairy and enhancer of split (Hes) family encode a large and highly conserved subfamily of basic helix-loop-helix (bHLH) proteins that upon homo- or heterodimerization bind to DNA and repress transcription (Kageyama et al., 2007). In the embryo and the adult, Hes proteins play essential roles in a wide variety of biological processes, among which their participation in neural development is one of the best understood. The first studies on these genes were conducted in *Drosophila*. *hairy* represses proneural function in the *Drosophila* ectoderm, acting as a prepatterning gene to promote a non-neural fate over a large territory, whereas the inhibition of proneural function by *Enhancer of split* [*E(spl)*] regulates the number of neuronal precursors in the neuroectoderm (Campos-Ortega and Jan, 1991; Campos-Ortega, 1993). During development of the central nervous system (CNS) in vertebrates, Hes factors maintain similar functions to those in *Drosophila* and antagonize the proneural activities of Atonal (Ath), Achaete scute (Ash) and Neurogenin (Ngn/Neurog) bHLH proteins (Ishibashi et al., 1994; Kageyama et al., 2005; Kageyama et al., 2008; Ross et al., 2003; Sasai et al., 1992; Stigloher et al., 2008). For example, in the mammalian neural tube, the activity of *Hes1*, *Hes3* and *Hes5* inhibits *Mash1* (*Ascl1* – Mouse Genome Informatics) and limits neuronal differentiation (Baek et al., 2006). The zebrafish *hairy/E(spl)*-related gene *her4* is expressed in a complementary fashion to *neurog1* in early proneural clusters where it also inhibits differentiation (Takke et al., 1999). In addition, like *Drosophila* Hairy, vertebrate Hes/Her proteins can also serve as prepatterning factors to define non-neuronal or delayed neuronal fates. Hence, at the mouse embryonic roof plate, Hes1/3/5 act antagonistically to *Ngn2* (*Neurog2* – Mouse Genome informatics) to specify the choroid plexus (Imayoshi et al., 2008), and likewise in zebrafish *her3*/*5*/*9*/*11* inhibit neurogenesis at boundaries and/or interproneuronal stripes within the neural plate (Bae et al., 2005; Hans et al., 2004; Ninkovic et al., 2005). The activity and levels of Hes proteins in some cellular contexts

[1]Developmental Biology Laboratory, Dept. Ciències Experimentals i de la Salut, Universitat Pompeu Fabra-Parc de Recerca Biomèdica de Barcelona, Dr Aiguader 88, 08003 Barcelona, Spain. [2]Laboratory of Neurobiology and Development, Institute of Neurobiology Alfred Fessard, CNRS, Avenue de Terrasse, 91198 cedex, Gif-sur-Yvette, France.

*Author for correspondence (berta.alsina@upf.edu)

Accepted 17 November 2010

have been associated with neural stem cell (NSC) and/or quiescence properties (Imayoshi et al., 2010; Kageyama et al., 2007; Sang et al., 2008; Sang et al., 2010).

In spite of all these data, the regulation of expression of Hes genes remains poorly understood. Although classically proposed to be regulated by Notch signaling (Artavanis-Tsakonas et al., 1999; Yoon and Gaiano, 2005), mounting evidence suggests that Hes genes also integrate other pathways, including Shh (Ingram et al., 2008) and Wnt (Kubo and Nakagawa, 2009), or growth factor signaling via JNK or ERK (Curry et al., 2006; Stockhausen et al., 2005). The full spectrum of their direct targets also remains to be determined; proposed targets include GATA, $p27^{kip1}$ (*Cdkn1b*), *Mash1* and *ath5* (*atoh7*) (Fischer and Gessler, 2007; Ishiko et al., 2005).

In the inner ear, the function of Hes1 has only been assessed during hair cell formation. Mice mutant for *Hes1* display an increased number of inner hair cells of the cochlea as well as in the vestibular epithelia of the saccule and utricle (Zheng et al., 2000; Zine et al., 2001). Co-transfection studies in postnatal explant cultures have demonstrated that *Hes1* is capable of preventing the differentiation of hair cells mediated by *Math1* (*Atoh1* – Mouse Genome Informatics). *Math1*, a bHLH gene and mammalian homolog of *Drosophila atonal*, is essential for the specification and further differentiation of hair cells (Bermingham et al., 1999; Woods et al., 2004). The phenotypes observed in $Hes1^{-/-}$ mice are closely related to those described for the loss of Notch function, indicating that during sensory development *Hes1* is dependent on the Notch pathway (Kiernan et al., 2001; Lanford et al., 1999; Lanford et al., 2000; Zine et al., 2000; Zine et al., 2001). In spite of these studies, no information is yet available on the role of *Hes1* in neuronal development in the inner ear.

In the present study, we address whether Hes1 has an early role in defining the non-neurogenic compartment of the otic placode. We show that a zebrafish *Hes1* ortholog, *her9*, is expressed together with *tbx1* in the non-neurogenic domain of the otic placode and restricts the extent of the neurogenic field: in Her9 morphant embryos, ectopic neurogenesis takes place posterolaterally, leading to the development of a misshapen statoacoustic ganglion (SAG). We further show that *tbx1* and *her9* are co-regulated, both being induced by retinoic acid (RA), inhibited by Hedgehog (Hh) and independent of Notch and Fgf signaling. After exploring the epistatic relationship between *her9* and *tbx1*, we show that in *tbx1* homozygous mutants otic *her9* expression is absent, whereas in *tbx1* overexpression experiments *her9* is ectopically induced. We conclude that Tbx1 is necessary and sufficient for *her9* activation. Altogether, our data demonstrate that the regulatory cascade encoding the non-neuronal otic fate relies on Her9, which represses the neurogenic fate downstream of Tbx1 and RA signaling.

MATERIALS AND METHODS

Zebrafish strains and transgenic lines

Embryos from wild-type (AB), *Tg(isl2:gfp)zc7* [also called *islet3:GFP* (Xiao et al., 2005)] (Pittman et al., 2008) and *Tg(Brn3c:GAP43-GFP)s356t* [also called *brn3c:GFP* (Xiao et al., 2005)] zebrafish were grown at 28°C, staged according to standard protocols (Kimmel et al., 1995) and fixed at the indicated timepoints. vgo^{tm208} (Piotrowski et al., 2003) and val^{b337} (Moens et al., 1996) mutants were obtained by pairwise mating of heterozygous adult carriers.

Morpholino (MO) and capped mRNA injections

The her9-MO (Gene Tools) (Bae et al., 2005) was injected at 0.5 mM. Efficiency was assessed with primers designed to amplify sequences flanking the first exon (125 bp, Ensembl RefSeq peptide, accession number NP_571948) of the *her9* transcript: her9-fwd, 5′-AGGGACTCACACTCTCTCTCGT-3′; and her9-rev, 5′-CTGCCCAAGGCTCTCGTT-3′.

tbx1 capped mRNA was synthesized from *tbx1* full-length cDNA (Piotrowski et al., 2003) using the mMESSAGE mMACHINE Kit (Applied Biosystems) and injected at 250 ng/μl.

Pharmacological treatments

Dechorionated zebrafish embryos were treated with 20 nM all-trans retinoic acid (Sigma), 20 μM 4-(diethylamino)-benzaldehyde (DEAB, Sigma), 60 μM SU5402 Fgf inhibitor (Calbiochem), 150 μM DAPT γ-secretase inhibitor (Calbiochem) or 100 μM cyclopamine A (CyA, LC Laboratories). For control treatments, sibling embryos were incubated in corresponding dilutions of dimethyl sulfoxide (DMSO, Sigma) or of ethanol (Merck) for the CyA treatment.

In situ hybridization and immunohistochemistry

Synthesis of antisense RNA and whole-mount in situ hybridizations were performed as previously described (Thisse et al., 2004). Probes were as follows: *neurog1* and *neurod* (Itoh and Chitnis, 2001), *neurod4* (Wang et al., 2003), *her9* (Leve et al., 2001), *tbx1* (Piotrowski et al., 2003), *atoh1a* (Millimaki et al., 2007), *krox20* (*egr2b*) (Oxtoby and Jowett, 1993), *aldh1a2* (Begemann et al., 2001), *cyp26b1* and *cyp26c1* (White et al., 2007). *cdkn1bl* and *cdkn1c* were cloned by RT-PCR using the following primers: cdkn1bl-fwd, 5′-AAAGTGCGCGTCTCCAAT-3′; cdkn1bl-rev, 5′-GTTTTGGGGTCCGGTTCG-3′; cdkn1c1-fwd, 5′-AGCACTTTTCCTCTCCTCACC-3′; and cdkn1c1-rev, 5′-TCCACGACCCTCTTTCTTTTT-3′.

Immunostaining with rabbit anti-phospho-Histone H3 (pH3; Millipore, 1:400) and anti-rabbit Alexa Fluor 488 (Invitrogen, 1:400) was performed to detect cells in M phase of the cell cycle.

Cell counting

Hair cells were counted from images taken from sagittal and transversal cryostat sections of 48 and 96 hours post-fertilization (hpf) *brn3c:GFP* embryos. For individual neuron counts, coronal sections of 48 hpf *islet3:GFP* embryos were imaged at 40× magnification using a Leica DM6000B fluorescence microscope with DFC300KX camera under the control of LAS-AF (Leica Application Suite Advanced Fluorescence 1.8) software to perform *z*-stacks through GFP^+ otic ganglia (average step of 1 μm).

RESULTS

her9 is expressed in the non-neurogenic domain together with *tbx1*

To reveal the mechanisms that establish the neurogenic and non-neurogenic domains in the zebrafish inner ear, we searched for genes selectively expressed in these domains. Otic neurogenesis at 24 hpf, as depicted by the expression of the proneural genes *neurog1* and *neurod*, is observed in a band that runs from anterolateral to medioposterior (Fig. 1A,B) (Andermann et al., 2002). In parallel, *atoh1a*, a proneural gene required for the specification and maintenance of sensory cells of the otic vesicle (Millimaki et al., 2007), is detected at the anterior macula at 24 hpf (Fig. 1G). By contrast, *her9*, a *Hes1* ortholog, is expressed in the posterolateral region of the otic vesicle (Fig. 1C). Interestingly, *tbx1* is also strongly present in the same domain as *her9* (Fig. 1D,H). Double staining for *neurod* and *tbx1* or for *neurod* and *her9* transcripts revealed that at the ventral otic region *neurod* and *tbx1*/*her9* exhibit an exactly complementary pattern of expression (Fig. 1E,F and see Fig. S1B,B′,D,D′ in the supplementary material). By contrast, at the dorsal part of the otic vesicle, *neurod* was absent and *tbx1* and *her9* displayed a more anterior limit of expression (see Fig. S1A,A′,C,C′ in the supplementary material).

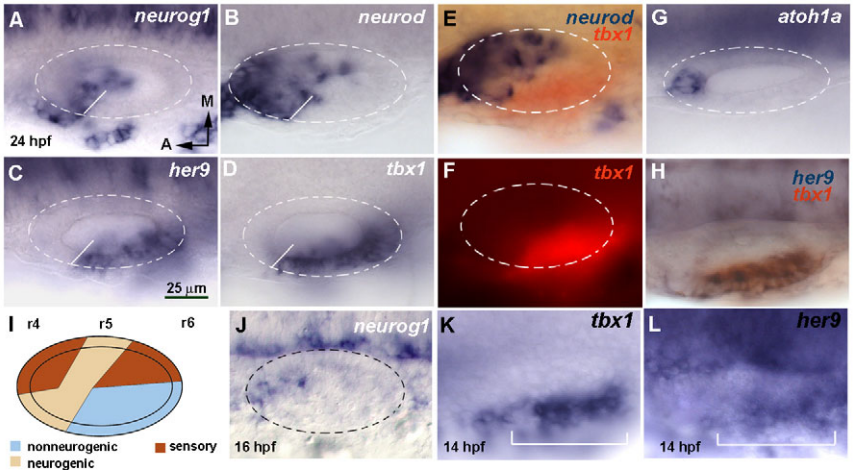

Fig. 1. *her9* and *tbx1* expression is complementary to the neurogenic domain. (**A-H**,**J-L**) Dorsal views of flat-mounted zebrafish embryos at 24 hpf, with anterior to the left and medial to the top. Dashed circles delineate the otic vesicle. In situ hybridization was for *neurog1* (A), *neurod* (B), *her9* (C), *tbx1* (D) and *atoh1a* (G). White lines in A-D indicate the boundary between the neurogenic and non-neurogenic domains. Double in situ hybridization is shown for *tbx1* (red chromogen in E and H, red fluorescence in F) and *neurod* (blue, E) or *her9* (blue, H). (J) *neurog1* expression at 16 hpf. (K,L) Expression of *tbx1* and *her9* in the otic placode at 14 hpf. The bracket indicates the extent of the placodal domain. (**I**) Schematic representation of the neurogenic, sensory and non-neurogenic territories. r, rhombomere. All images are at the same magnification.

These results suggest that *tbx1* and *her9* might together contribute to the definition of the non-neurogenic otic territory (Fig. 1I). To add support to this hypothesis, we explored whether *tbx1* or *her9* was expressed before the initiation of neurogenesis (as revealed by *neurog1* expression starting at 16 hpf; Fig. 1J). We found that *tbx1* is already transcribed in the posterior otic placodal domain at 14 hpf and that *her9* is also expressed at this stage, albeit with a more diffuse pattern (Fig. 1K,L). Thus, expression of *her9* and *tbx1* precedes the initiation of neurogenesis, suggesting that they might act as prepatterning genes.

Loss of function of Her9 causes ectopic expression of otic proneural genes

A prepatterning function would imply that Her9 sets the limits of the neurogenic domain of the ear. To examine the role of *her9* in the otic placode, we injected 1-cell stage embryos with morpholino oligonucleotides (MO) directed against the *her9* transcript. We used the same *her9* splice donor MO (referred to here as her9-MO) that was previously shown to inhibit the development of interproneuronal stripes in the CNS (Bae et al., 2005), a phenotype that was recapitulated (see Fig. S2A-D in the supplementary material). A temporal study of the efficiency of her9-MO in inhibiting *her9* mRNA splicing revealed that at 24 hpf, *her9* splicing was blocked completely. The normally spliced form of *her9* mRNA, which is 125 nt smaller than the intron 1 unspliced form, was first detected at 77 hpf (see Fig. S2E in the supplementary material). Loss of function of Her9 led to the ectopic expression of the proneural genes *neurod* (n=21/41) and *neurod4* (n=22/45) in the non-neurogenic posterolateral domain at 24 hpf (Fig. 2E,F,I,J). For *neurog1*, the expression pattern was not significantly changed in morphant embryos, suggesting a differential response of proneural genes to Her9 function in the otic vesicle (Fig. 2A-D′; n=2/21). The change in cell fate was observed from cells ectopically expressing *neurod* inside the posterolateral wall of the otic vesicle, as visualized by double in situ hybridization with *tbx1* (Fig. 2G-H′). *her9* was also present in the hindbrain, with highest levels at rhombomeric boundaries (Fig. 2K,L, arrowheads), where it is reported that neurogenesis is absent (Amoyel et al., 2005). In morphant embryos, we observed ectopic neurogenesis in the hindbrain concomitant with a reduction of non-neurogenic domains (Fig. 2M,N), but without accompanying patterning defects as shown by unaltered *krox20* expression in rhombomeres 3 and 5 (Fig. 2O,P).

We next assessed the effect of Her9 loss of function on the development of the SAG. Around 22 hpf, the neuroblasts delaminate from the ventral epithelium and coalesce ventrally. In order to image the otic ganglionar neurons, her9-MO was injected into *islet3:GFP* embryos. The SAG of Her9 morphant embryos at 48 hpf displayed a ventral shift accompanied by reduced segregation of its two main branches (Fig. 3A,B, arrowheads). When ganglionar neurons were counted in morphants and wild-type embryos, the numbers of neuronal cells in the SAG as a whole and in the distinct branches of the SAG were not significantly changed (see Fig. S3 in the supplementary material). However, as the otic vesicle was smaller overall (16% reduction in size, as measured by pixel numbers in selected areas), the proportion of the neuronal population might be increased relative to that of non-neuronal cells.

We also addressed the role of *her9* in sensory development. First, the expression of *atoh1a*, which labels the anterior macula at 24 hpf, was explored and neither expansion nor misspecification of *atoh1a*-expressing cells was apparent in Her9 morphants at 24 hpf (Fig. 3C,D; n=0/8). Second, her9-MO was injected into *Tg(brn3c:GFP)* embryos to image and count the number of hair cells that developed at later stages. In these embryos, GFP was visualized in vivo in the sensory maculae and the three sensory cristae at 48 and 96 hpf, respectively. The anterior and posterior maculae of Her9 morphants at 48 hpf contained between 16 and 20 GFP-positive cells, as counted from transverse and sagittal sections. The same number of cells was found in wild-type embryos (Fig. 3E,F). At 96 hpf, ~8-10 cells were counted in each crista of both morphants and wild-type embryos (Fig. 3G,H). We conclude from these results that *her9* regulates neuronal development as observed by improper SAG morphology in Her9 morphants at later stages, whereas *her9* does not control other proneural genes involved in sensory development.

Role of *her9* in cell proliferation

A feature of the otic vesicles of her9-MO-injected embryos was their reduction in size. Hes1 has been reported to prevent differentiation and to maintain cells as precursors or stem cells. In liver, thymus, brain and, as shown recently, in cochlear epithelia, Hes1 promotes precursor cell proliferation through the transcriptional downregulation of the cyclin-dependent kinase inhibitor (Cdkn) $p27^{kip1}$ (Murata et al., 2009). Thus, we assessed the role of *her9* in cell proliferation by analyzing the expression of

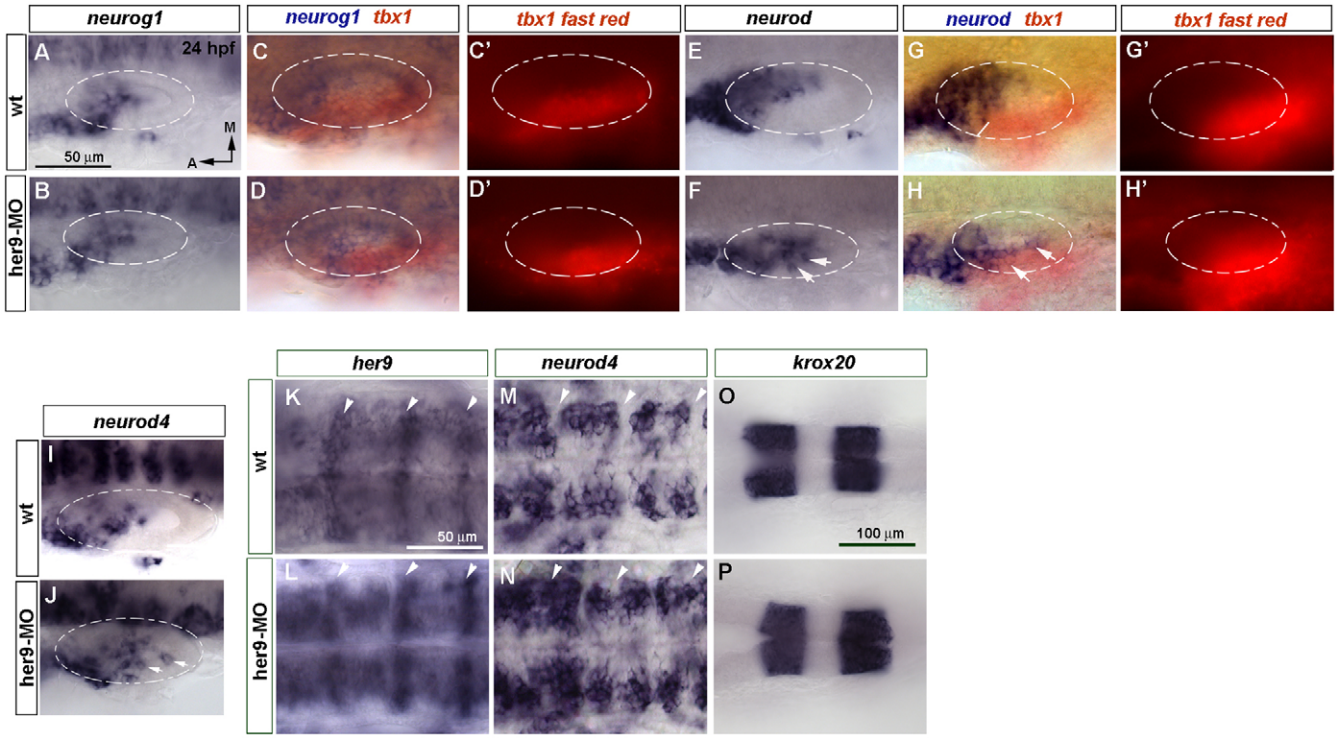

Fig. 2. Blocking *her9* function results in ectopic expression of the proneural genes *neurod* and *neurod4*. (**A-J**) In situ hybridization of 24 hpf wild-type (wt) and Her9 morphant zebrafish embryos to detect changes in *neurog1* (A-D′), *neurod* (E-H′) and *neurod4* (I,J) expression in the inner ear. (C-D′,G-H′) Double in situ hybridization for *tbx*1 (red chromogen in C,D,G,H and red fluorescence in C′,D′,G′,H′) and *neurog1* (blue) or *neurod* (blue). Dashed circles delineate the otic vesicle. Arrows indicate ectopic neurogenesis. (**K**,**L**) Expression of high levels of *her9* at the hindbrain rhombomeric boundaries (arrowheads). (**M**,**N**) *neurod4* expression is expanded in the hindbrain. (**O**,**P**) *krox20* expression in rhombomeres 3 and 5 is unchanged in Her9 morphants. A-J, K-N and O,P are at the same magnification.

Cdkns and determining the number of cells in M phase of cell cycle in wild-type and morphant embryos. Among seven Cdkn genes screened, two were found to be expressed in the zebrafish inner ear. *cdkn1c* (*p57*) was expressed throughout the entire neurosensory domain (Fig. 4C), whereas *cdkn1bl* (*p27-like*) was expressed in only the posterior part (Fig. 4A). None of these genes was expressed in the *her9* territory in wild-type embryos at 24 hpf, suggesting that Her9 might inhibit their expression. In *her9*-MO-injected embryos, ectopic expression of *cdkn1bl* in the *her9* territory was apparent at the posterior epithelium (n=8/12), whereas *cdkn1c* expression did not change significantly (Fig. 4B,D; n=3/12).

To assess the proliferative status of cells in the otic vesicle, cells in M phase were immunostained with anti-phospho-Histone H3 (anti-pH3). We counted the number of cells in mitosis inside and outside the *her9*-positive domain in wild-type and morphant otic vesicles (Fig. 4E-I) and found that the subfraction of mitotic cells in the *her9* domain strongly decreased after Her9 depletion (control, n=16, 0.43±0.22; her9-MO, n=15, 0.15±0.17; P=0.001). Taking into account that (1) no Cdkn genes are endogenously expressed in the *her9*-positive domain, (2) *cdkn1bl* is ectopically induced in otic vesicles of Her9 morphants and (3) there is a loss of mitotic cells in the *her9* territory, we conclude that Her9 has a role in maintaining cells in proliferation in the non-neurogenic domain.

Tbx1 acts upstream of *her9*

As shown in Fig. 2, Her9 blockade does not suppress *tbx1* expression, most probably positioning Tbx1 upstream of Her9. We therefore analyzed *her9* expression in *van gogh* (*vgo*) mutant embryos, which carry a null mutation for the *tbx1* gene (Piotrowski et al., 2003). Indeed, we found that from descendants of *vgo*^{tm208} heterozygote crosses, *her9* expression was abolished throughout its normal expression domain at 16 hpf (data not shown), 18 hpf (Fig. 5B,C; n=9/40) and 24 hpf (Fig. 5J; n=9/32). In parallel to the loss of *her9*, a large number of *neurog1*- and *neurod*-expressing cells appeared in the posterolateral domain, similar to, but stronger than, the effect observed in Her9 morphants (Fig. 5D-I). *tbx1* mRNA staining in *vgo* mutants was still visible in the non-neurogenic territory, revealing ectopic neurogenic cells in the posterolateral wall (Fig. 5E,F,H,I). In *vgo* mutants, some otic vesicles were also smaller and the *tbx1*-expressing domain reduced, but the effect on neurogenesis was similar in small and medium-sized otic vesicles (compare Fig. 5E with 5F).

The capacity of Tbx1 to activate *her9* was further demonstrated by the ectopic induction of *her9* in the anterior neurogenic domain at 24 hpf (Fig. 5L; n=8/24) after injection of *tbx1* capped mRNA into 1- to 2-cell stage embryos. Finally, in Tbx1-overexpressing embryos, *neurod* expression at the epithelium was reduced in about a third of embryos (Fig. 5N; n=10/28). Taken together, these results reveal that both *tbx1* and *her9* are required for the establishment of a non-neurogenic compartment during ear development, and that Tbx1 is necessary and sufficient for *her9* expression in the otic vesicle.

her9 and *tbx1* are Notch independent but are regulated by RA

Hes genes are well-established downstream targets of Notch signaling. To assess whether *her9* is regulated by this pathway, we blocked Notch activity using the γ-secretase inhibitor DAPT

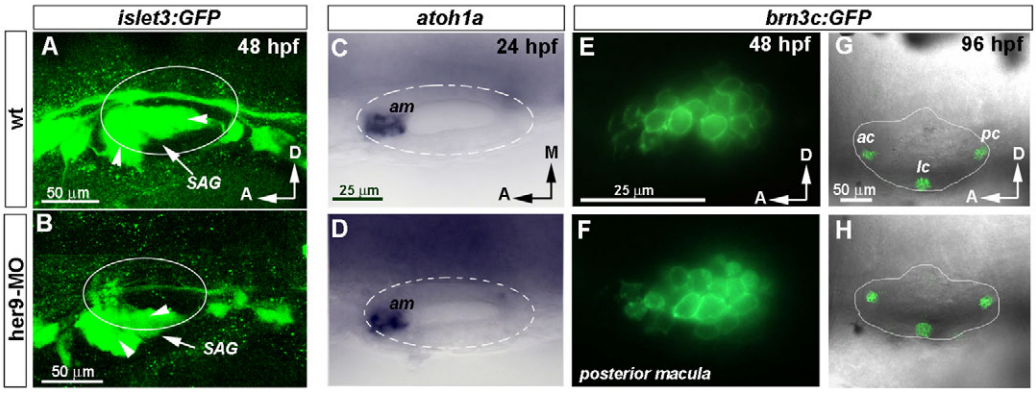

Fig. 3. Depletion of *her9* distorts the development of the SAG but not the generation of hair cells. (**A**,**B**) Three-dimensional reconstruction of the SAG at 48 hpf visualized in wild-type and her9-MO-injected zebrafish embryos carrying the *islet3:GFP* transgene. Arrowheads point to SAG branches. (**C**,**D**) Expression of *atoh1a* in wild-type and Her9 morphant embryos at 24 hpf. (**E**,**F**) Sagittal sections of posterior macula showing GFP fluorescence under the control of *brn3c* (*pou4f3*) reporter in a wild-type and her9-MO-injected embryo. (**G**,**H**) Three-dimensional reconstruction of selected confocal sections showing the otic cristae in wild-type and her9-MO-injected embryos. GFP in adjacent neuromasts of the lateral line has been removed for clarity. C,D, E,F and G,H are at the same magnification. SAG, statoacoustic ganglion; am, anterior macula; ac, anterior crista; lc, lateral crista; pc, posterior crista.

(Geling et al., 2002). Embryos treated from 10.5 until 24 hpf displayed no inhibition of *her9* or *tbx1* expression (Fig. 6A-D; *her9*, *n*=11/11; *tbx1*, *n*=9/9), indicating that expression of both genes is established independently of Notch. As expected from the reported role of Notch signaling in hair cell development (Haddon et al., 1999; Millimaki et al., 2007), we found increased hair cell specification, as revealed by *atoh1a* (Fig. 6E,F; *n*=6/7), arguing for the efficiency of the DAPT treatment.

Several lines of evidence pointed to RA signaling as a good candidate for controlling the expression of posterolateral genes in the otic placode. Loss of RA signaling affects craniofacial patterning (Niederreither et al., 1999; Niederreither et al., 2000) and phenocopies some of the features associated with DGS (Begemann et al., 2001; Vermot et al., 2003). We treated embryos from 10.5 to 24 hpf with DEAB, a potent pharmacological inhibitor of retinaldehyde dehydrogenases (Raldhs/Aldhs). Interestingly, blocking RA signaling abolished the expression of *her9* and *tbx1* (Fig. 6G,H,K,L; *her9*, *n*=9/9; *tbx1*, *n*=6/7) and in parallel caused a posterior expansion of the *neurog1* and *neurod* expression domains (Fig. 6I,J,M,N; *neurog1*, *n*=6/7; *neurod*, *n*=4/6). Conversely, when embryos were incubated with non-teratogenic doses of RA from 10.5 to 12 hpf and left to develop until 24 hpf, the limits of *her9* and *tbx1* expression shifted anteriorly (*her9*, *n*=20/24; *tbx1*, *n*=12/13), concomitant with a medial shift of *neurog1* and *neurod* expression (Fig. 7O-R; *neurog1*, *n*=9/11; *neurod*, *n*=5/5). The anterior shift of *tbx1* expression after RA treatment was not modified by Notch signaling inhibition (data not shown; *n*=3/3). Together, these results further confirm the cross-interaction between the *neurog1* and *tbx1/her9* expression domains, and demonstrate the influence of RA in setting the anteroposterior (AP) boundary of the neurogenic and non-neurogenic domains in the otic vesicle.

Since RA activity influences AP patterning of the neuroectoderm and pharyngeal arches (Linville et al., 2004; Linville et al., 2009) and hindbrain signals pattern the inner ear (Bok et al., 2005; Kwak et al., 2002; Riccomagno et al., 2002), the change in *tbx1* and *her9* expression in DEAB- or RA-treated embryos might be an indirect result of hindbrain misspecification. To assess hindbrain patterning at the level of the otic vesicle, *krox20* expression was analyzed in embryos in which RA was up- or downregulated. *krox20*

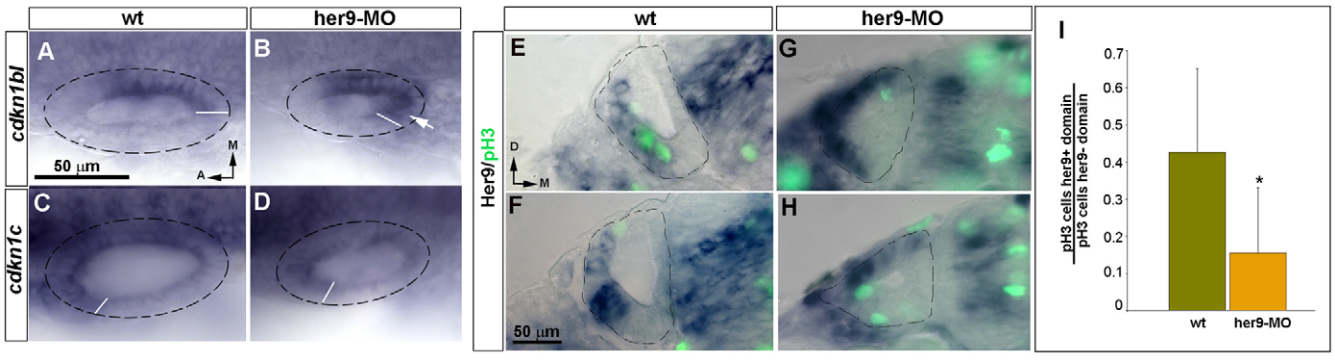

Fig. 4. *her9* inhibits Cdkns and controls cell proliferation. (**A-D**) In situ hybridization of *cdkn1bl* (A,B) and *cdkn1c* (C,D) in wild-type (A,C) and her9-MO-injected (B,D) zebrafish embryos at 24 hpf. Arrow indicates posterolateral expansion of *cdkn1bl* expression. (**E-H**) Transverse sections of wild-type and Her9 morphant embryos stained by in situ hybridization for *her9* (blue) and immunohistochemistry with anti-pH3 (green). (**I**) Proportion of pH3-positive cells counted inside and outside the *her9*-expression domain in wild-type and Her9-depleted embryos. Error bars indicate mean + s.e.m. **P*=0.001. A-D and E-H are at the same magnification.

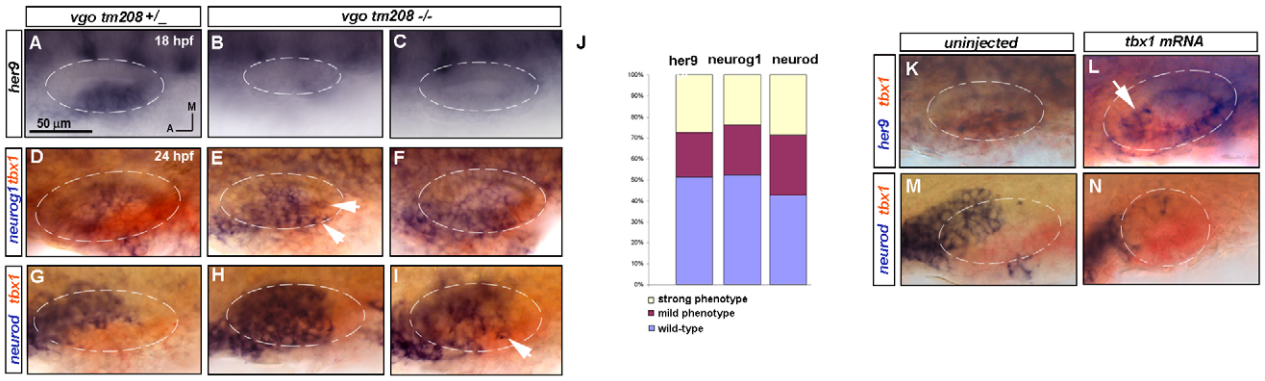

Fig. 5. *tbx1* is genetically upstream of *her9*. (**A-C**) Expression of *her9* in 18 hpf zebrafish embryos from heterozygotic *vgo*^{tm208} crosses. (**D-I**) Double in situ hybridization for *tbx1* (red) and *neurog1* (blue, D-F) or *neurod* (blue, G-I) in 24 hpf embryos from heterozygotic *vgo*^{tm208} crosses. Arrows point to ectopic proneural expression. Two examples of *vgo*^{tm208} mutants are given for each gene in A-I. (**J**) Proportion of descendants from heterozygotic crosses showing a strong phenotype (complete abrogation of *her9* expression, substantial expansion of *neurog1* and *neurod*), mild phenotype or wild-type phenotype at 24 hpf. (**K**,**L**) Double in situ hybridization for *tbx1* (red) and *her9* (blue) shows ectopic expression of *her9* (arrow) after injection of capped *tbx1* mRNA. (**M**,**N**) Double in situ hybridization for *tbx1* (red) and *neurod* (blue) at 24 hpf shows downregulation of *neurod* after overexpression of capped *tbx1* mRNA. All images are at the same magnification.

expression was unchanged in both conditions, as compared with wild-type embryos (see Fig. S4A-C in the supplementary material), thereby excluding hindbrain patterning defects in our manipulations. Moreover, *tbx1* and *her9* expression was unchanged in *valentino* (*mafba*) mutants (*val*^{b337}), which exhibit misspecification of rhombomeres 5 and 6, further indicating that posterior hindbrain patterning is dispensable in establishing the *tbx1/her9* domain (see Fig. S4D-G in the supplementary material).

In conclusion, RA is required for the induction of *tbx1* and *her9* in the posterolateral ear field, which occurs independently of hindbrain patterning and defines the non-neurogenic otic compartment.

Interactions of Fgf and Hh with RA signaling

At the posterior limb, RA signaling cross-talks with Shh (Riddle et al., 1993). Moreover, a head mesenchyme enhancer of *Tbx1* is positively regulated by Shh in mice (Yamagishi et al., 2003). This, together with recent reports that reveal a requirement of Shh/Hh for proper otic patterning (Riccomagno et al., 2002; Hammond et al., 2003; Hammond et al., 2010; Sapede and Pujades, 2010), led us to explore the influence of Hh signaling on *tbx1*. We incubated embryos with the Hh inhibitor cyclopamine A (CyA) for consecutive 3-hour periods from 9 until 21 hpf. Between 12 and 18 hpf, inhibition of Hh caused a strong posteromedial induction of *tbx1* (*n*=10/12; Fig. 7A,C and see Fig. S5 in the supplementary material). These data indicate that Hh has a role in limiting the expression of *tbx1* in the posteromedial domain of the vesicle. Blockade of Hh after 18 hpf had no effect, indicating that at this period the posteromedial domain is already determined (see Fig. S5 in the supplementary material). Incubation of 100 µM CyA together with 20 nM RA at 10.5 hpf had an additive effect and almost the entire vesicle expressed *tbx1* (Fig. 7E; *n*=3/3) and *her9* (data not shown). By contrast, blockade of Notch signaling did not modify the effects of Hh on *tbx1* expression (Fig. 7G; *n*=4/4). These data point to an interplay of positive and negative regulators of the *tbx1* expression domain within the otic vesicle: RA signaling induces *tbx1* expression, which is limited to the posterolateral wall by the inhibitory Hh signal.

As shown above, increasing the levels of RA shifted the anterior limit of *tbx1* expression, even though a small anteromedial domain was always devoid of *tbx1* and still expressed *neurod* (Fig. 7E,F). Does an anterior signal also exist to limit *tbx1* expression? Fgf3 and Fgf8 signals from rhombomere 4 are required for the specification of the sensory territory and for neuronal production (Leger and Brand, 2002; Phillips et al., 2001). We asked whether Fgf influences RA activity and restricts the anterior limit of *tbx1* expression and how a putative role of Fgf in otic patterning could be related to its established roles in otic induction and neurogenesis.

To characterize the temporal requirements for Fgf we treated embryos at different times with 60 µM SU5402, a potent pharmacological inhibitor of Fgf, and assessed its effects on the formation of the otic vesicle, neurogenesis and the expression of *tbx1*. Otic induction was abolished in Fgf-depleted embryos treated from 4.3 to 30 hpf, judging by the lack of any morphological sign of otic vesicles (Fig. 7I,J; *n*=12/12). Fgf blockade from 50% epiboly (5.3 hpf) to 24 hpf led to very tiny otic vesicles, without neurogenesis and with reduced levels of *tbx1* expression (Fig. 7M,N; *n*=7/7). Treatment from 7 hpf onwards resulted in no change in the overall pattern of expression of *tbx1* and *neurod*, although both domains were smaller due to an effect on otic growth (Fig. 7O-R). These data show that Fgf is required for otic induction before the gastrula stage, for neural commitment at the early gastrula and to maintain otic growth after 7 hpf.

Inhibition of Fgf signaling at 50% epiboly resulted in a reduction or loss of *tbx1* expression, suggesting that at this stage Fgf might interfere with RA activity. To assess this, we examined the expression of *aldh1a2* and *cyp26b1/c1*, which encode enzymes involved in the synthesis and degradation of RA, after blocking Fgf signaling. At 13 hpf, the expression of *aldh1a2* is initiated at the cranial mesoderm adjacent to the otic placode (Waxman et al., 2008). Inhibiting Fgf signaling at 50% epiboly suppressed *aldh1a2* expression in the cranial mesoderm and also partially suppressed *cyp26c1* expression at the hindbrain (Fig. 7S, red arrowheads). Since the patterning of the hindbrain is severely disrupted in these embryos, the loss of *aldh1a2* expression might be a direct effect of Fgf signaling or an indirect effect of hindbrain mispatterning. However, blocking Fgf signaling at 10.5 hpf did not have a strong impact on the expression of RA pathway components (Fig. 7T), in agreement with the lack of effect on otic *tbx1* expression at these

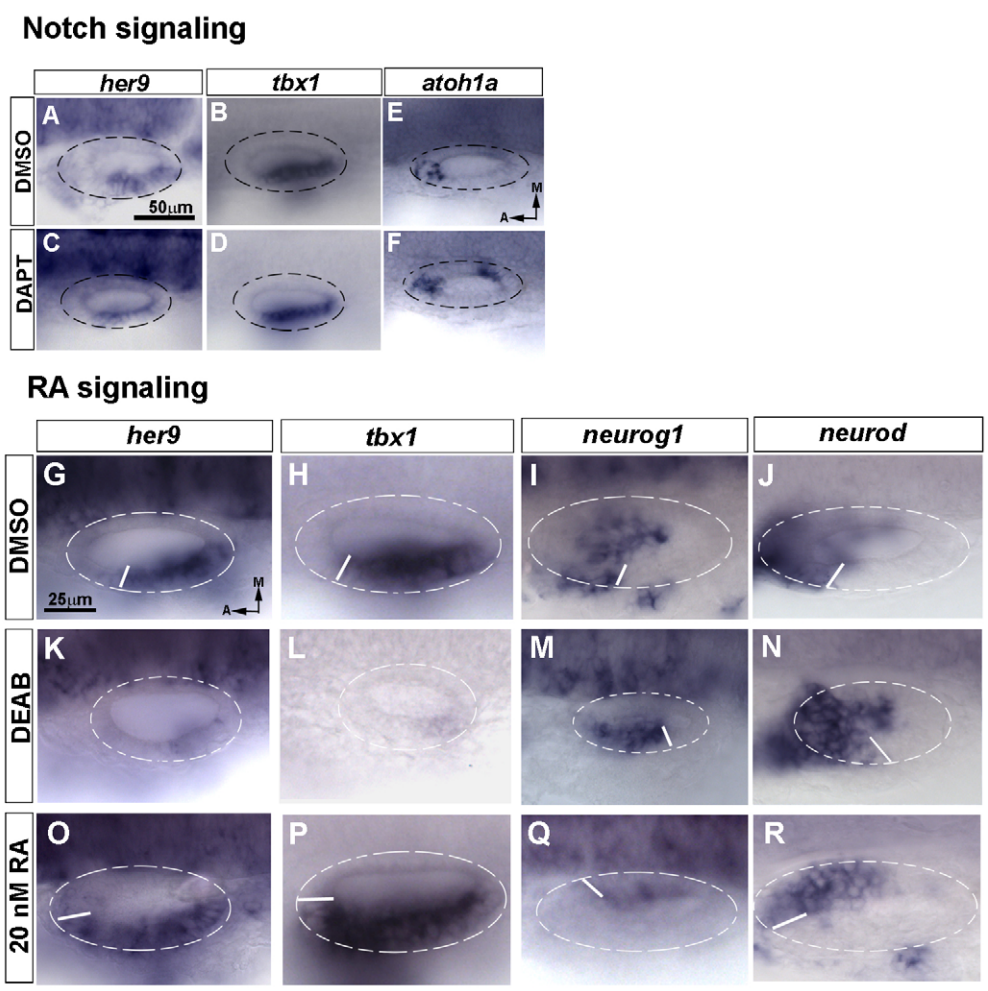

Fig. 6. Retinoic acid, but not Notch, regulates *her9* and *tbx1* expression in the inner ear. (**A-F**) Expression of *her9* (A,C), *tbx1* (B,D) and *atoh1a* (E,F) at 24 hpf in control (DMSO) and Notch-inhibited (DAPT-treated) zebrafish embryos. (**G-R**) Expression of *her9* (G,K,O), *tbx1* (H,L,P), *neurog1* (I,M,Q) and *neurod* (J,N,R) at 24 hpf in embryos treated with DMSO (G-J), the retinaldehyde dehydrogenase inhibitor DEAB (K-N) or retinoic acid (RA) (O-R) from 10.5 hpf. The shift of the boundary of the neurogenic and non-neurogenic compartments is depicted by the white line in each otic vesicle. A-F and G-R are at the same magnification.

stages. Together, these data suggest that Fgf influences RA levels at gastrula stages, whereas RA activity in the otic territory is Fgf independent at later stages.

DISCUSSION

Ear progenitors that further differentiate into neuronal cells and hair cells are born in very precise locations in the ear primordium. Little is known about how the otic vesicle is partitioned such that the neurogenic compartment is generated only in the anteromedial domain of the ear primordium. In this study, we identify *her9*, a zebrafish ortholog of *Hes1*, as a key gene in regulating otic neurogenesis downstream of Tbx1.

her9 is expressed in the otic non-neurogenic domain and represses neurogenic fate

Hes genes antagonize proneural function and maintain progenitor cells in proliferation. In zebrafish, *her9* together with *her3* control primary neurogenesis by repressing *neurod* (Bae et al., 2005). However, a role for Hes/Her genes during inner ear neural development had not been demonstrated. In the zebrafish otic vesicle, *her9* displays a sharp anterior boundary complementary to that of *neurog1* and *neurod*. Loss-of-function experiments indicate that Her9 confers non-neurogenic fate by repressing proneural activity. Her9 therefore plays a role in the inner ear that is equivalent to its role in defining non-neurogenic domains within the developing neural plate. In particular, expression of *neurod* and *neurod4* was strongly affected, whereas *neurog1* was not. The *Neurod* bHLH gene is expressed after *Neurog1* in the inner ear, suggesting that the latter acts as a determination gene and the former as a differentiation gene (Ma et al., 2000; Kim et al., 2001; Adam et al., 1998; Alsina et al., 2004). This raises the question of why *her9* has a differential action over *neurod*/*neurod4* and *neurog1* and whether this has an effect on specific neuronal subtypes or the timing of neuronal differentiation. Further studies should address these questions. Concomitant with the change in *neurod* expression, the SAG was morphologically aberrant, displaying a ventral shift of its medial portion. However, loss of *her9* did not significantly increase the total number of SAG neurons in either of its two branches. This is in agreement with previous reports by Hibi and colleagues, in which expansion of *neurod* into interproneuronal stripes did not result in major changes in the expression of later differentiation markers (Bae et al., 2005).

It is also surprising that sensory development was unaffected by *her9* loss of function, in contrast to the role of *Hes1* in hair cell differentiation in mice (Zheng et al., 2000; Zine et al., 2001). Owing to genomic duplication in the teleost lineage, other Her genes might have taken on this function and the analysis of other members of the family merits further study.

Her9 as the mediator of Tbx1 activity during otic neurogenesis

In mice, it has been shown that overexpression of Tbx1 leads to a suppression of neurogenesis, whereas inhibition of Tbx1 produces ectopic *Neurod*-expressing cells throughout the otic vesicle (Raft et

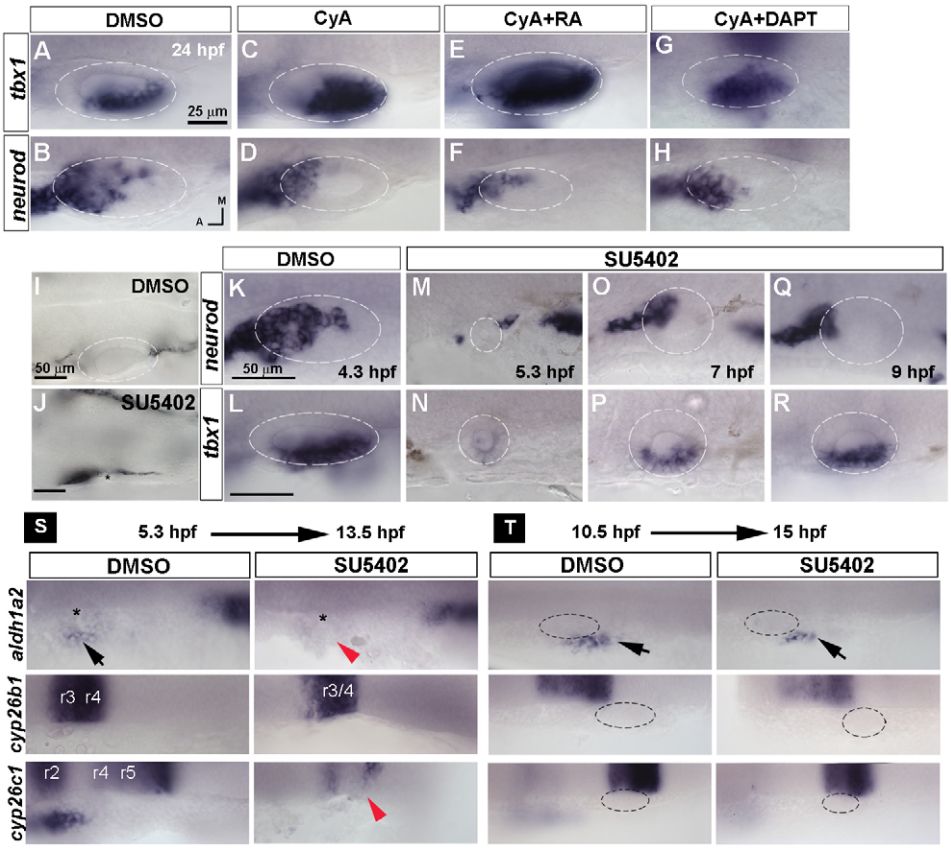

Fig. 7. Temporal requirement for Hh and Fgf signals in otic development. (**A-H**) Expression of *tbx1* and *neurod* in 24 hpf zebrafish embryos treated with DMSO, the Hh inhibitor cyclopamine A (CyA), CyA+RA and CyA+DAPT starting from 10.5 hpf. (**I**,**J**) Bright-field images of 30 hpf embryos treated with DMSO or the Fgf inhibitor SU5402 starting from 4.3 hpf reveals a requirement for Fgf signaling at this stage for otic placode induction. Asterisk indicates putative position of otic vesicle. (**K-R**) Stage-dependent influence of Fgf signaling on *neurod* and *tbx1* expression. (**S**,**T**) In situ hybridization for *aldh1a2*, *cyp26b1* and *cyp26c1* in embryos treated with DMSO or SU5402 starting from 5.3 hpf or 10.5 hpf. Black arrows indicate mesodermal expression of *aldh1a2*; red arrowheads indicate suppression of *aldh1a2* and *cyp26c1* expression; asterisk indicates position of the otic placode. A-H, I,J and K-R are at the same magnification.

al., 2004). Here, we confirm that *tbx1* also regulates neurogenesis in zebrafish and that this is in part mediated by Her9. T-box factors can act as activators or repressors of transcriptional activity; therefore, Tbx1 in the otic vesicle might directly repress proneural activity or activate a repressor of proneural genes. Our data indicate that Tbx1 acts as an activator of *her9* transcription: abrogation of Tbx1 leads to a complete loss of *her9* expression and overexpression of Tbx1 leads to the ectopic expression of *her9* concomitant with the inhibition of *neurod*. The loss of *her9* expression in *tbx1*-null mutants is already observed 2 hours after the expected appearance of Tbx1 in the otic epithelium, suggesting that Tbx1 activates *her9* in a direct manner. To date, few data are available linking these two families of transcription factors. During zebrafish somitogenesis, it has been shown that Tbx24 directly regulates *her1* (Brend and Holley, 2009). Our results therefore suggest that such interactions between these two families might be more general than previously thought.

In *vgo* mutant embryos, *neurog1* is also ectopically expressed, an effect that was not observed in Her9 morphant embryos. How Tbx1 might act on *neurog1* independently of Her9 is still an open question, and further work must be undertaken to identify other putative targets. Along these lines, *vgo* mutants display a severely disrupted semicircular canal formation, with a resulting failure to develop sensory cristae (Piotrowski et al., 2003). Defects in semicircular canals were not found in Her9-depleted embryos (data not shown), which suggests that Tbx1 activates targets other than *her9* that mediate its role in otic morphogenesis.

RA-Tbx1-Her9 cascade in otic proliferation

Interestingly, otic vesicle size was reduced to various degrees in embryos lacking RA signaling as well as in *tbx1*$^{-/-}$embryos and in Her9 morphants. This indicates that this genetic network also regulates cell proliferation. Indeed, we present evidence that Her9 negatively regulates the expression of *cdkn1bl* and the number of mitotic cells. This is similar to the situation reported in the mouse cochlea, where p27^{kip1} expression was upregulated in the developing epithelium concomitant with a reduction in the number of S-phase cells in *Hes1*-null mice (Murata et al., 2009). Interestingly, in that report too, the dysregulation of p27^{kip1} did not lead to a precocious differentiation of hair cells. In mice, it has been suggested that Tbx1 is required for the expansion of a large population of otic epithelia (Vitelli et al., 2003) and for the suppression of neural fate (Raft et al., 2004). Since a massive loss of the Tbx1-positive cell population could lead to a virtual expansion of the neurogenic domain without any real change in cell fate, Xu et al. examined neurogenesis in *tbx1* mutants in which the *tbx1*-expressing population could be traced by X-Gal staining (Xu et al., 2007). It was determined that ectopic neuronal cells were present among *tbx1*-traced cells in spite of a reduction in the mitotic index. Similarly, we also detected ectopic *neurog1*- and *neurod*-expressing cells in the *tbx1* domain of *vgo* mutants, indicating that Tbx1 also has a dual role in zebrafish.

Role of RA in patterning the otic vesicle

Evidence for a Notch-independent regulation of Hes genes is accumulating. In particular, in the floor plate, *her9* expression requires active Nodal signaling (Latimer et al., 2005), whereas in the interproneuronal stripes *her3* and *her9* are controlled by BMP signaling (Bae et al., 2005). We investigated which positional cues might be regulating *her9* and *tbx1* expression in the inner ear and found that loss of RA suppressed both genes. RA signaling is one of the major patterning signals during embryonic development and has a strong influence on the development of craniofacial structures and

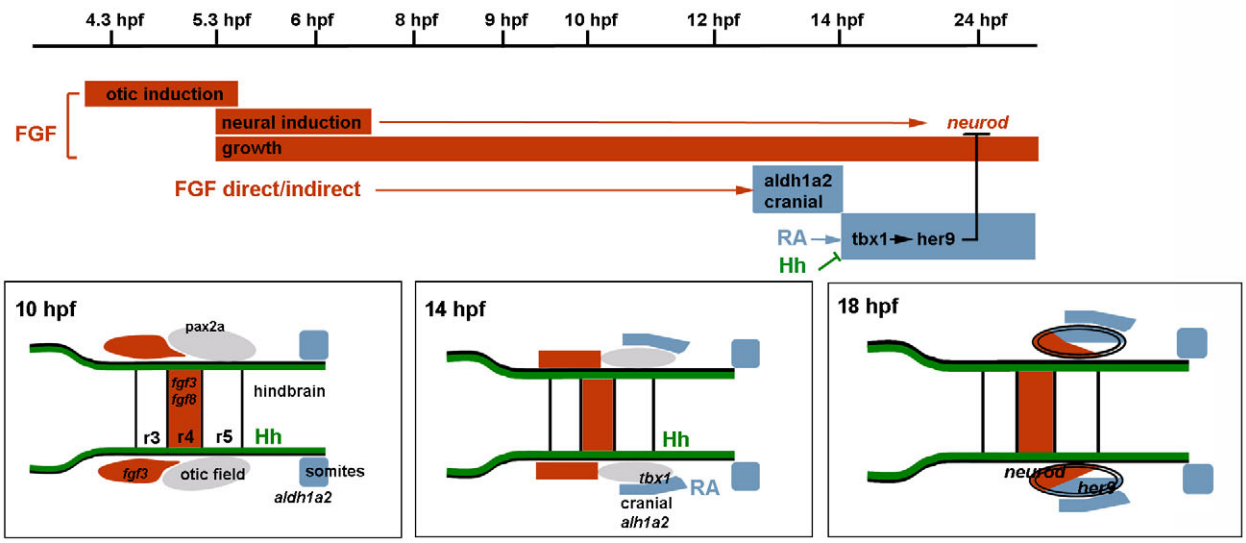

Fig. 8. Integrative model of neurogenic versus non-neurogenic patterning. (Top) The temporal requirements of Fgf (red bars), Hh (green) and RA (blue bars) signaling during otic development are illustrated. Before 5.3 hpf (50% epiboly), Fgf signaling is required for otic induction, whereas from 5.3 until 7 hpf it is required for otic neural induction and for *aldh1a2* expression in the cranial mesoderm. After 7 hpf, Fgf signaling is required for otic growth but not for the establishment of *aldh1a2* and otic *tbx1* expression. RA is required for *tbx1* induction at 14 hpf, leading to the activation of the proneural repressor Her9. In parallel, medial sources of Hh restrict *tbx1* expression. (Bottom) Three key timepoints in otic development, illustrating the dynamics of the extrinsic sources involved in otic neural patterning. From an initially uniform otic field at 10 hpf, the pattern of *tbx1* and *her9* expression established by the opposing actions of RA and Hh at 14 hpf finally results in regionalized *neurod* expression within the otic vesicle at 18 hpf.

the hindbrain (Gale et al., 1999; Gavalas and Krumlauf, 2000; Maden, 2007; Romand, 2003; Schneider et al., 2001). In zebrafish, RA is produced in embryonic tissues by the activity of Aldh1a2 and Aldh1a3, enzymes that oxidize retinaldehyde into RA. At the stages of otic patterning, *aldh1a2* (*raldh2*) is expressed in somites and posterior hindbrain mesenchyme. In amniotes and zebrafish, loss of *aldh1a2* affects craniofacial patterning, with lesser effects on the hindbrain (Niederreither et al., 2000; Begemann et al., 2001). Little is known about the role of RA in otic patterning, but at earlier stages RA is required for otic induction: in amniote embryos, decreased RA signaling from its onset induces supernumerary otic vesicles, whereas in zebrafish an excess of RA causes the same phenotype (Dupe et al., 1999; White et al., 1998; White et al., 2000). Hans and Westerfield have suggested that precise levels of RA are required to determine the extent of otic competence independently of hindbrain signaling (Hans and Westerfield, 2007). Here, we manipulated RA signaling after the period of otic specification to address its role in neurogenesis. Consequently, no effects on hindbrain patterning or supernumerary vesicles were observed. However, we found that abrogation of RA before the onset of neurogenesis did affect the expression of *tbx1* and, consequently, the development of the non-neurogenic domain. These results support a genetic network in which RA activates *tbx1* in the ear, which in turn activates *her9*. This extends previous findings demonstrating that RA is required for *tbx1* expression in the zebrafish pharyngeal arches, and is in keeping with the fact that zebrafish *aldh1a2* mutants recapitulate some phenotypes of DGS (Begemann et al., 2001). Hence, our data strengthen the relevance of RA signaling in inner ear patterning and highlight RA as a novel extrinsic factor controlling the establishment of the neurogenic and non-neurogenic compartments.

In mouse, expression of *Tbx1* in the head mesenchyme is directed by a specific enhancer located 14.3 kb upstream of the coding region (Yamagishi et al., 2003). This cis element is positively regulated by Shh, Foxc1 and Foxc2 transcription factors, but it remains to be explored whether it also regulates *Tbx1* in the inner ear and whether it is influenced by RA. Rather than a loss, we detected an expansion of *tbx1* expression in the otic region upon Hh inhibition. This suggests either that independent enhancer elements control the ear and mesenchyme *tbx1* expression domains or that downstream effectors of the Hh pathway, together with specific ear context transcription factors, inhibit rather than activate *tbx1* transcription. A recent report has shown an inhibition of *neurod* by Hh in the posteromedial part of the neurogenic domain (Sapede and Pujades, 2010). Here, we refine these data by providing evidence that Hh acts first on *tbx1*, which then results in the repression of *neurod*.

Several lines of evidence in the present report demonstrate that the induction of posterolateral otic genes is independent of hindbrain patterning. First, loss of RA signaling at 10.5 hpf completely abolishes *tbx1* and *her9* expression, whereas AP patterning of the hindbrain, as revealed by *krox20* expression, is unchanged under these conditions. Secondly, in *val* mutants, in which rhombomeres 5 and 6 are misspecified, otic *tbx1* and *her9* expression is unaffected.

Otic induction was completely abrogated in Fgf-depleted embryos from 4.3 hpf, whereas otic growth and neural specification were impaired in SU5402-treated embryos from 50% epiboly. In agreement with published data that suggest a sequential requirement of Fgf in inner ear development (Leger and Brand, 2002; Martin and Groves, 2006; Abello et al., 2010), the role of Fgf signals in otic induction could be dissociated from a subsequent role in neural specification at 50% epiboly. Once the anteromedial otic field is neural specified at 7 hpf, Fgf is only required for otic growth and the *tbx1* expression pattern does not change. This indicates that the establishment of *tbx1* expression by RA activity is independent of Fgf signaling, but that the anteromedial limit of

tbx1 expression is set up by the previous step of determination of the neurogenic domain. By contrast, the posteromedial domain is not fully determined to a neurogenic fate until 18 hpf, as *tbx1* can be ectopically induced after Hh inhibition. Interestingly, this reveals spatiotemporal control of the determination of the neurogenic domain.

RA and Fgf signaling counteract each other during somitogenesis, posterior spinal cord neurogenesis and hindbrain development (Diez del Corral et al., 2003; Gonzalez-Quebedo et al., 2010). We found that at gastrula stage, but not at later stages, Fgf signals influence RA pathway components such as *aldh1a2*, as shown previously in *Xenopus* embryos (Shiotsugu et al., 2004). *tbx1* expression is reduced in the otic vesicle when *ald1ha2* from cranial mesoderm, but not from somites, is lost in Fgf-depleted embryos from 50% epiboly, favoring a major role of cranial *aldh1a2* activity in setting up *tbx1* expression. An integrative model of otic development that encompasses the temporal requirements for extrinsic RA, Fgf and Hh signals, as well as their genetic interactions with the transcription factors Neurod, Tbx1 and Her9, is presented in Fig. 8.

After a process of induction of neural competence in the otic field, neurogenesis is restricted to the anterior region of the otic vesicle. This restriction is in part mediated by repression. Here, we have identified *her9* as one of the main genes involved in this process downstream of *tbx1* and RA signaling.

Acknowledgements
We thank Tanya Whitfield for generously providing *vgo* mutant fish; Dora Sapede for experimental support; Bruce Riley for reporter lines; Anna Bigas and James Sharpe for critical reading of the manuscript; and Marta Linares and Miquel Sas for technical assistance. M.R. was supported by an FPI fellowship from MICIIN and A.R.-M. from a JdC postdoctoral contract from MICINN. Grant support was from BFU2008-00714 from MICINN.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.056093/-/DC1

References

Abello, G., Khatri, S., Giraldez, F. and Alsina, B. (2007). Early regionalization of the otic placode and its regulation by the Notch signaling pathway. *Mech. Dev.* **124**, 631-645.
Abello, G., Khatri, S., Radosevic, M., Scotting, P. J., Giraldez, F. and Alsina, B. (2010). Independent regulation of Sox3 and Lmx1b by FGF and BMP signaling influences the neurogenic and non-neurogenic domains in the chick otic placode. *Dev. Biol.* **339**, 166-178.
Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. *Development* **125**, 4645-4654.
Alsina, B., Abello, G., Ulloa, E., Henrique, D., Pujades, C. and Giraldez, F. (2004). FGF signaling is required for determination of otic neuroblasts in the chick embryo. *Dev. Biol.* **267**, 119-134.
Amoyel, M., Cheng, Y. C., Jiang, Y. J. and Wilkinson, D. G. (2005). Wnt1 regulates neurogenesis and mediates lateral inhibition of boundary cell specification in the zebrafish hindbrain. *Development* **132**, 775-785.
Andermann, P., Ungos, J. and Raible, D. W. (2002). Neurogenin1 defines zebrafish cranial sensory ganglia precursors. *Dev. Biol.* **251**, 45-58.
Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **5415**, 770-776.
Bae, Y. K., Shimizu, T. and Hibi, M. (2005). Patterning of proneuronal and inter-proneuronal domains by hairy- and enhancer of split-related genes in zebrafish neuroectoderm. *Development* **132**, 1375-1385.
Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-2476.
Begemann, G., Schilling, T. F., Rauch, G. J., Geisler, R. and Ingham, P. W. (2001). The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain. *Development* **128**, 3081-3094.
Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.
Bok, J., Bronner-Fraser, M. and Wu, D. K. (2005). Role of the hindbrain in dorsoventral but not anteroposterior axial specification of the inner ear. *Development* **132**, 2115-2124.
Brend, T. and Holley, S. A. (2009). Expression of the oscillating gene her1 is directly regulated by Hairy/Enhancer of Split, T-box, and Suppressor of Hairless proteins in the zebrafish segmentation clock. *Dev. Dyn.* **238**, 2745-2759.
Campos-Ortega, J. A. (1993). Mechanisms of early neurogenesis in Drosophila melanogaster. *J. Neurobiol.* **24**, 1305-1327.
Campos-Ortega, J. A. and Jan, Y. N. (1991). Genetic and molecular bases of neurogenesis in Drosophila melanogaster. *Annu. Rev. Neurosci.* **14**, 399-420.
Curry, C. L., Reed, L. L., Nickoloff, B. J., Miele, L. and Foreman, K. E. (2006). Notch-independent regulation of Hes-1 expression by c-Jun N-terminal kinase signaling in human endothelial cells. *Lab. Invest.* **86**, 842-852.
Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M. and Storey, K. (2003). Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. *Neuron* **40**, 65-79.
Dupe, V., Ghyselinck, N. B., Wendling, O., Chambon, P. and Mark, M. (1999). Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. *Development* **126**, 5051-5059.
Fischer, A. and Gessler, M. (2007). Delta-Notch-and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic Acids Res.* **35**, 4583-4596.
Gale, E., Zile, M. and Maden, M. (1999). Hindbrain respecification in the retinoid-deficient quail. *Mech. Dev.* **89**, 43-54.
Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.
Geling, A., Steiner, H., Willem, M., Bally-Cuif, L. and Haass, C. (2002). A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep.* **3**, 688-694.
Gonzalez-Quevedo, R., Lee, Y., Poss, K. D. and Wilkinson, D. G. (2010). Neuronal regulation of the spatial patterning of neurogenesis. *Dev. Cell* **18**, 136-147.
Haddon, C., Mowbray, C., Whitfield, T., Jones, D., Gschmeissner, S. and Lewis, J. (1999). Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. *J. Neurocytol.* **28**, 837-850.
Hammond, K. L., Loynes, H. E., Folarin, A. A., Smith, J. and Whitfield, T. T. (2003). Hedgehog signalling is required for correct anteroposterior patterning of the zebrafish otic vesicle. *Development* **130**, 1403-1417.
Hammond, K. L., van Eeden, F. J. and Whitfield, T. T. (2010). Repression of Hedgehog signalling is required for the acquisition of dorsolateral cell fates in the zebrafish otic vesicle. *Development* **137**, 1361-1371.
Hans, S. and Westerfield, M. (2007). Changes in retinoic acid signaling alter otic patterning. *Development* **134**, 2449-2458.
Hans, S., Scheer, N., Riedl, I., v. Weizsacker, E., Blader, P. and Campos-Ortega, J. A. (2004). her3, a zebrafish member of the hairy-E(spl) family, is repressed by Notch signalling. *Development* **131**, 2957-2969.
Imayoshi, I., Shimogori, T., Ohtsuka, T. and Kageyama, R. (2008). Hes genes and neurogenin regulate non-neural versus neural fate specification in the dorsal telencephalic midline. *Development* **135**, 2531-2541.
Imayoshi, I., Sakamoto, M., Yamaguchi, M., Mori, K. and Kageyama, R. (2010). Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. *J. Neurosci.* **30**, 3489-3498.
Ingram, W. J., McCue, K. I., Tran, T. H., Hallahan, A. R. and Wainwright, B. J. (2008). Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. *Oncogene* **27**, 1489-1500.
Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S. and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *EMBO J.* **13**, 1799-1805.
Ishiko, E., Matsumura, I., Ezoe, S., Gale, K., Ishiko, J., Satoh, Y., Tanaka, H., Shibayama, H., Mizuki, M., Era, T. et al. (2005). Notch signals inhibit the development of erythroid/megakaryocytic cells by suppressing GATA-1 activity through the induction of HES1. *J. Biol. Chem.* **280**, 4929-4939.
Itoh, M. and Chitnis, A. B. (2001). Expression of proneural and neurogenic genes in the zebrafish lateral line primordium correlates with selection of hair cell fate in neuromasts. *Mech. Dev.* **102**, 263-266.
Kageyama, R., Ohtsuka, T., Hatakeyama, J. and Ohsawa, R. (2005). Roles of bHLH genes in neural stem cell differentiation. *Exp. Cell Res.* **306**, 343-348.

Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* **134**, 1243-1251.
Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2008). Roles of Hes genes in neural development. *Dev. Growth Differ.* **50**, S97-S103.
Kiernan, A. E., Ahituv, N., Fuchs, H., Balling, R., Avraham, K. B., Steel, K. P. and Hrabe de Angelis, M. (2001). The Notch ligand Jagged1 is required for inner ear sensory development. *Proc. Natl. Acad. Sci. USA* **98**, 3873-3878.
Kim, W. Y., Fritzsch, B., Serls, A., Bakel, L. A., Huang, E. J., Reichardt, L. F., Barth, D. S. and Lee, J. E. (2001). NeuroD-null mice are deaf due to a severe loss of the inner ear sensory neurons during development. *Development* **128**, 417-426.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Kubo, F. and Nakagawa, S. (2009). Hairy1 acts as a node downstream of Wnt signaling to maintain retinal stem cell-like progenitor cells in the chick ciliary marginal zone. *Development* **136**, 1823-1833.
Kwak, S. J., Phillips, B. T., Heck, R. and Riley, B. B. (2002). An expanded domain of fgf3 expression in the hindbrain of zebrafish valentino mutants results in mis-patterning of the otic vesicle. *Development* **129**, 5279-5287.
Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat. Genet.* **21**, 289-292.
Lanford, P. J., Shailam, R., Norton, C. R., Gridley, T. and Kelley, M. W. (2000). Expression of Math1 and HES5 in the cochleae of wildtype and Jag2 mutant mice. *J. Assoc. Res. Otolaryngol.* **1**, 161-171.
Latimer, A. J., Shin, J. and Appel, B. (2005). Her9 promotes floor plate development in zebrafish. *Dev. Dyn.* **232**, 1098-1104.
Leger, S. and Brand, M. (2002). Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. *Mech. Dev.* **119**, 91-108.
Leve, C., Gajewski, M., Rohr, K. B. and Tautz, D. (2001). Homologues of c-hairy1 (her9) and lunatic fringe in zebrafish are expressed in the developing central nervous system, but not in the presomitic mesoderm. *Dev. Genes Evol.* **211**, 493-500.
Linville, A., Gumusaneli, E., Chandraratna, R. A. and Schilling, T. F. (2004). Independent roles for retinoic acid in segmentation and neuronal differentiation in the zebrafish hindbrain. *Dev. Biol.* **270**, 186-199.
Linville, A., Radtke, K., Waxman, J. S., Yelon, D. and Schilling, T. F. (2009). Combinatorial roles for zebrafish retinoic acid receptors in the hindbrain, limbs and pharyngeal arches. *Dev. Biol.* **325**, 60-70.
Ma, Q., Anderson, D. J. and Fritzsch, B. (2000). Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. *J. Assoc. Res. Otolaryngol.* **1**, 129-143.
Maden, M. (2007). Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat. Rev. Neurosci.* **8**, 755-765.
Martin, K. and Groves, A. (2006). Competence of cranial ectoderm to respond to Fgf signaling suggests a two-step model of otic placode induction. *Development* **133**, 877-887.
Millimaki, B. B., Sweet, E. M., Dhason, M. S. and Riley, B. B. (2007). Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Development* **134**, 295-305.
Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. and Kimmel, C. B. (1996). Valentino: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.
Moraes, F., Novoa, A., Jerome-Majewska, L. A., Papaioannou, V. E. and Mallo, M. (2005). Tbx1 is required for proper neural crest migration and to stabilize spatial patterns during middle and inner ear development. *Mech. Dev.* **126**, 199-212.
Murata, J., Ohtsuka, T., Tokunaga, A., Nishiike, S., Inohara, H., Okano, H. and Kageyama, R. (2009). Notch-Hes1 pathway contributes to the cochlear prosensory formation potentially through the transcriptional down-regulation of p27Kip1. *J. Neurosci. Res.* **87**, 3521-3534.
Niederreither, K., Subbarayan, V., Dolle, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dolle, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.
Ninkovic, J., Tallafuss, A., Leucht, C., Topczewski, J., Tannhauser, B., Solnica-Krezel, L. and Bally-Cuif, L. (2005). Inhibition of neurogenesis at the zebrafish midbrain-hindbrain boundary by the combined and dose-dependent activity of a new hairy/E(spl) gene pair. *Development* **132**, 75-88.
Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Res.* **21**, 1087-1095.
Phillips, B. T., Bolding, K. and Riley, B. B. (2001). Zebrafish fgf3 and fgf8 encode redundant functions required for otic placode induction. *Dev. Biol.* **235**, 351-365.
Piotrowski, T., Ahn, D. G., Schilling, T. F., Nair, S., Ruvinsky, I., Geisler, R., Rauch, G. J., Haffter, P., Zon, L. I., Zhou, Y. et al. (2003). The zebrafish van gogh mutation disrupts tbx1, which is involved in the DiGeorge deletion syndrome in humans. *Development* **130**, 5043-5052.
Pittman, A. J., Law, M. Y. and Chien, C. B. (2008). Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points. *Development* **135**, 2865-2871.
Raft, S., Nowotschin, S., Liao, J. and Morrow, B. E. (2004). Suppression of neural fate and control of inner ear morphogenesis by Tbx1. *Development* **131**, 1801-1812.
Riccomagno, M. M., Martinu, L., Mulheisen, M., Wu, D. K. and Epstein, D. J. (2002). Specification of the mammalian cochlea is dependent on Sonic hedgehog. *Genes Dev.* **16**, 2365-2378.
Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
Romand, R. (2003). The roles of retinoic acid during inner ear development. *Curr. Top. Dev. Biol.* **57**, 261-291.
Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.
Sang, L., Coller, H. A. and Roberts, J. M. (2008). Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. *Science* **321**, 1095-1100.
Sang, L., Roberts, J. M. and Coller, H. A. (2010). Hijacking HES1: how tumors co-opt the anti-differentiation strategies of quiescent cells. *Trends Mol. Med.* **16**, 17-26.
Sapede, D. and Pujades, C. (2010). Hedgehog signaling governs the development of otic sensory epithelium and its associated innervation in zebrafish. *J. Neurosci.* **30**, 3612-3623.
Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev.* **6**, 2620-2634.
Schneider, R. A., Hu, D., Rubenstein, J. L., Maden, M. and Helms, J. A. (2001). Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. *Development* **128**, 2755-2767.
Schneider-Maunoury, S. and Pujades, C. (2007). Hindbrain signals in otic regionalization: walk on the wild side. *Int. J. Dev. Biol.* **51**, 495-506.
Shiotsugu, J., Katsuyama, Y., Arima, K., Baxter, A., Koide, T., Song, J., Chandraratna, R. A. and Blumberg, B. (2004). Multiple points of interaction between retinoic acid and FGF signaling during embryonic axis formation. *Development* **131**, 2653-2667.
Stigloher, C., Chapouton, P., Adolf, B. and Bally-Cuif, L. (2008). Identification of neural progenitor pools by E(Spl) factors in the embryonic and adult brain. *Brain Res. Bull.* **75**, 266-273.
Stockhausen, M. T., Sjolund, J. and Axelson, H. (2005). Regulation of the Notch target gene Hes-1 by TGFalpha induced Ras/MAPK signaling in human neuroblastoma cells. *Exp. Cell Res.* **310**, 218-228.
Takke, C., Dornseifer, P., v. Weizsacker, E. and Campos-Ortega, J. A. (1999). her4, a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling. *Development* **126**, 1811-1821.
Thisse, B., Heyer, V., Lux, A., Alunni, V., Degrave, A., Seiliez, I., Kirchner, J., Parkhill, J. P. and Thisse, C. (2004). Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. *Methods Cell Biol.* **77**, 505-519.
Vermot, J., Niederreither, K., Garnier, J. M., Chambon, P. and Dolle, P. (2003). Decreased embryonic retinoic acid synthesis results in a DiGeorge syndrome phenotype in newborn mice. *Proc. Natl. Acad. Sci. USA* **100**, 1763-1768.
Vitelli, F., Viola, A., Morishima, M., Pramparo, T., Baldini, A. and Lindsay, E. (2003). TBX1 is required for inner ear morphogenesis. *Hum. Mol. Genet.* **12**, 2041-2048.
Wang, X., Emelyanov, A., Korzh, V. and Gong, Z. (2003). Zebrafish atonal homologue zath3 is expressed during neurogenesis in embryonic development. *Dev. Dyn.* **227**, 587-592.
Waxman, J. S., Keegan, B. R., Roberts, R. W., Poss, K. D. and Yelon, D. (2008). Hoxb5b acts downstream of retinoic acid signaling in the forelimb field to restrict heart field potential in zebrafish. *Dev. Cell* **15**, 923-934.
White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, H. F. and Clagett-Dame, M. (1998). Defects in embryonic hindbrain development and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-trans-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95**, 13459-13464.
White, J. C., Highland, M., Kaiser, M. and Clagett-Dame, M. (2000). Vitamin A deficiency results in the dose-dependent acquisition of anterior character and shortening of the caudal hindbrain of the rat embryo. *Dev. Biol.* **220**, 263-284.
White, R. J., Nie, Q., Lander, A. D. and Schilling, T. F. (2007). Complex regulation of cyp26a1 creates a robust retinoic acid gradient in the zebrafish embryo. *PLoS Biol.* **5**, e304.
Whitfield, T. T. and Hammond, K. L. (2007). Axial patterning in the developing vertebrate inner ear. *Int. J. Dev. Biol.* **51**, 507-520.

Woods, C., Montcouquiol, M. and Kelley, M. W. (2004). Math1 regulates development of the sensory epithelium in the mammalian cochlea. *Nat. Neurosci.* **7**, 1310-1318.
Xiao, T., Roeser, T., Staub, W. and Baier, H. (2005). A GFP-based genetic screen reveals mutations that disrupt the architecture of the zebrafish retinotectal projection. *Development* **132**, 2955-2967.
Xu, H., Viola, A., Zhang, Z., Gerken, C. P., Lindsay-Illingworth, E. A. and Baldini, A. (2007). Tbx1 regulates population, proliferation and cell fate determination of otic epithelial cells. *Dev. Biol.* **302**, 670-682.
Yamagishi, H., Maeda, J., Hu, T., McAnally, J., Conway, S. J., Kume, T., Meyers, E. N., Yamagishi, C. and Srivastava, D. (2003). Tbx1 is regulated by tissue-specific forkhead proteins through a common Sonic hedgehog-responsive enhancer. *Genes Dev.* **17**, 269-281.
Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat. Neurosci.* **8**, 709-715.
Zheng, J. L., Shou, J., Guillemot, F., Kageyama, R. and Gao, W. Q. (2000). Hes1 is a negative regulator of inner ear hair cell differentiation. *Development* **127**, 4551-4560.
Zine, A., Van De Water, T. R. and de Ribaupierre, F. (2000). Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. *Development* **127**, 3373-3383.
Zine, A., Aubert, A., Qiu, J., Therianos, S., Guillemot, F., Kageyama, R. and de Ribaupierre, F. (2001). Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. *J. Neurosci.* **21**, 4712-4720.