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

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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 posterior lateral 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 Pujaides, 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.

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have been associated with neural stem cell (NSC) and/or quiescence properties (Imayoshi et al., 2010; Kagayama 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 (Artvan-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, p27kip1 (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 (Zhang 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 (Kierman 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 postero-laterally, 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-neuralotic 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:gfpc)zc7 [also called islet3:GFP (Xiao et al., 2005)] (Pittman et al., 2008) and Tg(Bra3c: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-m230 (Piotrowski et al., 2003) and valp337 (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′-CTGCCAAGGCTCTCGTT-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**

Decohorinated 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 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 (Thiisse 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), atoh1 (Millimaki et al., 2007), krox20 (egr2b) (Oxtoby and Jowett, 1993), alh1a2 (Begemann et al., 2001), cyp26bl and cyp26cl (White et al., 2007), cdkn1bl and cdkn1c were cloned by RT-PCR using the following primers: cdkn1bl-fwd, 5′-AAAGTGGCCGTCTCAAT-3′; cdkn1bl-rev, 5′-GTGTTTGGGTCCGTTGTC-3′; cdkn1c-l-fwd, 5′-AGCACTTTCCTCTCTAC-3′; and cdkn1c-rev, 5′-TCCAGACCCCTTCTTTTT-3′.

Immunostaining with 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 and 96 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 antero-lateral to medio-posterior (Fig. 1A,B) (Andermann et al., 2002). In parallel, atoh1, 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 postero-lateral 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. 1B,1′D,1′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. 1A,1′A’,1′C,1′C’ in the supplementary material).
These results suggest that \textit{tbx1} and \textit{her9} might together contribute to the definition of the non-neurogenic otic territory (Fig. 1I). To add support to this hypothesis, we explored whether \textit{tbx1} or \textit{her9} was expressed before the initiation of neurogenesis (as revealed by \textit{neurog1} expression starting at 16 hpf; Fig. 1J). We found that \textit{tbx1} is already transcribed in the posterior otic placodal domain at 14 hpf and that \textit{her9} is also expressed at this stage, albeit with a more diffuse pattern (Fig. 1K,L). Thus, expression of \textit{her9} and \textit{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 \textit{her9} in the otic placode, we injected 1-cell stage embryos with morpholino oligonucleotides (MO) directed against the \textit{her9} transcript. We used the same \textit{her9} splice donor MO (referred to here as \textit{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 \textit{her9}-MO in inhibiting \textit{her9} mRNA splicing revealed that at 24 hpf, \textit{her9} splicing was blocked completely. The normally spliced form of \textit{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 \textit{neurod} (n=21/41) and \textit{neurod4} (n=22/45) in the non-neurogenic posteralateral domain at 24 hpf (Fig. 2E,F,I,J). For \textit{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 \textit{neurod} inside the posteralateral wall of the otic vesicle, as visualized by double in situ hybridization with \textit{tbx1} (Fig. 2G-H'). \textit{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 \textit{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, \textit{her9}-MO was injected into \textit{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 number 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 \textit{her9} in sensory development. First, the expression of \textit{atoh1a}, which labels the anterior macula at 24 hpf, was explored and neither expansion nor misspecification of \textit{atoh1a}-expressing cells was apparent in Her9 morphants at 24 hpf (Fig. 3C,D, n=0/8). Second, \textit{her9}-MO was injected into \textit{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 cristae of both morphants and wild-type embryos (Fig. 3G,H). We conclude from these results that \textit{her9} regulates neuronal development as observed by improper SAG morphology in Her9 morphants at later stages, whereas \textit{her9} does not control other proneural genes involved in sensory development.

**Role of her9 in cell proliferation**

A feature of the otic vesicles of \textit{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 \textit{her9} in cell proliferation by analyzing the expression of...
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<sup>m208</sup> 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.

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**Fig. 2. Blocking her9 function results in ectopic expression of the proneural genes neurog1 and neurod4.** (A-I) 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 tbx1 (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.
(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 neureg expression domains (Fig. 6I,J,M,N; neurog1, n=6/7; neureg, 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 neureg expression (Fig. 7O-R; neurog1, n=9/11; neureg, 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 neurot 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
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 valentinno (mafba) mutants (valb337), 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 postemeral 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 postemeral domain of the otic vesicle. Blockade of Hh after 18 hpf had no effect, indicating that at this point the postemeral 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. 7L,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 test 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
Her9 regulates otic neurogenesis

**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
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 cdkn1b and the number of mitotic cells. This is similar to the situation reported in the mouse cochlea, where p27kip1 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 p27kip1 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
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 (aldh2) 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 posterosmedial part of the neurogenic domain (Sadpe 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 (Leher 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 postomedial 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 (Díez 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 ald1h1a2, as shown previously in Xenopus embryos (Shiotsugu et al., 2004). tbx1 expression is reduced in the otic vesicle when ald1h1a2 from cranial mesoderm, but not from somites, is lost in Fgf-depleted embryos from 50% epiboly, favoring a major role of cranial ald1h1a2 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.

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