

# Repression of Hedgehog signalling is required for the acquisition of dorsolateral cell fates in the zebrafish otic vesicle

Katherine L. Hammond, Fredericus J. M. van Eeden and Tanya T. Whitfield\*

## SUMMARY

In zebrafish, Hedgehog (Hh) signalling from ventral midline structures is necessary and sufficient to specify posterior otic identity. Loss of Hh signalling gives rise to mirror symmetric ears with double anterior character, whereas severe upregulation of Hh signalling leads to double posterior ears. By contrast, in mouse and chick, Hh is predominantly required for dorsoventral otic patterning. Whereas a loss of Hh function in zebrafish does not affect dorsoventral and mediolateral otic patterning, we now show that a gain of Hh signalling activity causes ventromedial otic territories to expand at the expense of dorsolateral domains. In a panel of lines carrying mutations in Hh inhibitor genes, Hh pathway activity is increased throughout the embryo, and dorsolateral otic structures are lost or reduced. Even a modest increase in Hh signalling has consequences for patterning the ear. In *ptc1<sup>-/-</sup>* and *ptc2<sup>-/-</sup>* mutant embryos, in which Hh signalling is maximal throughout the embryo, the inner ear is severely ventralised and medialised, in addition to displaying the previously reported double posterior character. Transplantation experiments suggest that the effects of the loss of Hh pathway inhibition on the ear are mediated directly. These new data suggest that Hh signalling must be kept tightly repressed for the correct acquisition of dorsolateral cell fates in the zebrafish otic vesicle, revealing distinct similarities between the roles of Hh signalling in zebrafish and amniote inner ear patterning.

**KEY WORDS:** Zebrafish, Otic vesicle, Inner ear, Hedgehog, *dre*, *igu*, *lep*, *uki*, *Dzip*, *Hip*, *Ptc1*, *Ptc2*, *Su(fu)*, Cyclopamine

## INTRODUCTION

The vertebrate inner ear is a complex structure with asymmetries about all three body axes. These asymmetries arise early during ear development; by otic placode and vesicle stages, several otic genes are expressed asymmetrically (reviewed by Whitfield and Hammond, 2007). Patterning defects at these early stages will have consequences for subsequent ear development. To date, a small number of signalling molecules that influence otic patterning at these early stages have been identified, of which Hedgehog (Hh) is one.

Members of the Hh family of signalling molecules act as morphogens during axial patterning of many tissues: for instance patterning the dorsoventral (DV) axis of the neural tube and the anteroposterior (AP) axis of the developing limb bud (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993) (reviewed by Hammerschmidt et al., 1997; Ingham and McMahon, 2001; Ingham and Placzek, 2006). Graded levels of Hh signalling give rise to different fates along the axes of these tissues. Similarly, Hh signalling from ventral midline structures is vital for otic axial patterning in all vertebrates so far examined.

In zebrafish, we have previously shown that Shha and Shhb are necessary and sufficient to specify posterior otic identity (Hammond et al., 2003). When Hh signalling is lost or severely reduced, posterior otic structures are lost and anterior structures are duplicated in their place. Similar double anterior phenotypes have been reported in *Xenopus* embryos overexpressing mRNA encoding the

Hh inhibitor Hip (Waldman et al., 2007). Conversely, when Hh signalling is overactivated by *shh* or *dnPKA* overexpression in the zebrafish embryo, anterior otic structures are absent and posterior regions are duplicated (Hammond et al., 2003). In mouse and chick, however, manipulation of Shh activity predominantly affects otic DV and mediolateral (ML) patterning; AP effects, if present, are not obvious (Bok et al., 2005; Riccomagno et al., 2002). This apparent difference in the role of Hh in otic patterning between amniote and anamniote vertebrates is surprising, as the structure of the inner ear is similar in both groups, except for the presence of the ventrally positioned cochlea, a specialised auditory endorgan, in the amniote ear.

Subsequently, however, we have established that whereas a loss of Hh function does not affect the otic DV and ML axes in zebrafish (Hammond et al., 2003), increasing Hh levels by *shh* mRNA injection causes an expansion of ventromedial (VM) otic territories at the expense of dorsolateral (DL) domains. To investigate further, we analysed the otic phenotypes of a panel of lines carrying mutations in genes encoding inhibitors of the Hh pathway: *ptc1*, *ptc2*, *su(fu)* (*su(fu)* – ZFIN), *dzip1* and *hip*, all of which are expressed in and around the developing otic vesicle. These lines provide a series with increased Hh signalling throughout the embryo (Koudijs et al., 2008; Koudijs et al., 2005). Ptc (Patched), the Hh receptor, represses Hh pathway activity in the absence of Hh ligand (Chen and Struhl, 1996) (reviewed by Ingham and McMahon, 2001). *ptc1* is expressed in a posteroventromedial domain of the zebrafish otic vesicle and *ptc2* in a wider ventral domain (Hammond et al., 2003). Hip (Hedgehog interacting protein) is a membrane-bound protein that binds to the Hh ligand and prevents it binding to the Ptc receptor (Chuang and McMahon, 1999; Ochi et al., 2006). *hip* is expressed in a complex pattern in the zebrafish, initially concentrated towards the anterior of the otic vesicle (Hammond and Whitfield, 2009). *Dzip1* (Daz interacting protein 1) and *Su(fu)* (Suppressor of fused)

MRC Centre for Developmental and Biomedical Genetics and Department of Biomedical Science, University of Sheffield, Sheffield, S10 2TN, UK.

\*Author for correspondence ([t.whitfield@sheffield.ac.uk](mailto:t.whitfield@sheffield.ac.uk))

Accepted 9 February 2010

both act within the Hh-receiving cell to regulate activity of the transcription factor Gli, which mediates the Hh response (Méthot and Basler, 2000; Sekimizu et al., 2004; Wolff et al., 2004) (reviewed by Huangfu and Anderson, 2006). Both *su(fu)* and *dzip1* are expressed ubiquitously throughout the zebrafish embryo (Koudijs et al., 2005; Wolff et al., 2004).

The overriding otic phenotype in these lines is a ventralisation and medialisation of the ear: with increasing Hh activity, dorsolateral structures are progressively lost. In the strongest phenotype, in embryos mutant for *ptc1* and *ptc2*, the otic vesicle is strongly medialised and ventralised as well as posteriorised, and has a stronger phenotype than that generated by *shh* mRNA injection (Hammond et al., 2003). Gene expression pattern changes in the otic vesicle prefigure the defects in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> and *shh* mRNA-injected otic vesicles.

Our data demonstrate that, in addition to a requirement for Hh signalling for AP otic patterning, inhibition of Hh signalling is crucial for the correct development of dorsolateral structures in the zebrafish inner ear. Otic vesicle patterning is very sensitive to small increases in Hh signalling; Hh pathway activity must therefore be tightly regulated for correct inner ear development. In addition, we show that the effects of derepression of Hh signalling on the zebrafish ear are likely to be mediated directly. Our data indicate that a requirement for inhibition of Hh signalling during zebrafish and amniote inner ear patterning is at least partially conserved.

## MATERIALS AND METHODS

### Animals

Wild-type zebrafish strains were AB, Tup Longfin (TL) or WIK. Mutant lines were *dre*<sup>tm146d</sup> (*su(fu)*<sup>-/-</sup>), *igu*<sup>ts294</sup> (*dzip1*<sup>-/-</sup>), *lep*<sup>g222</sup> (*ptc2*<sup>-/-</sup>), *ptc1*<sup>hu1602</sup> (*ptc1*<sup>-/-</sup>) and *uki*<sup>hu418B</sup> (*hip*<sup>-/-</sup>) (Brand et al., 1996; Heisenberg et al., 1996; Karlstrom et al., 1996; Koudijs et al., 2008; Koudijs et al., 2005; Piotrowski et al., 1996; van Eeden et al., 1996; Whitfield et al., 1996). All are recessive loss-of-function alleles. Embryonic stages are given as hours post-fertilisation (hpf) at 28.5°C or as somite stages (S) (Kimmel et al., 1995; Westerfield, 1995).

### In situ hybridisation

Whole-mount *in situ* hybridisation was carried out as described (Hammond et al., 2003). Probes used were *dlx3b*, *eya1*, *fgf8*, *fst1* (*fsta* – ZFIN), *hmx3*, *msxc*, *otx1*, *pax2a*, *pax5*, *ptc1*, *ptc2* (Hammond et al., 2003), *hip*, *pcna* (Koudijs et al., 2005), *tbx1* (Piotrowski et al., 2003), *foxi1* (Solomon et al., 2004) and *raldh3* (*aldh1a3* – ZFIN) (Pittlik et al., 2008).

### PCR genotyping

Genomic DNA was prepared as described (Westerfield, 1995). Primers were: *dre*, F 5'-TTCTGCTGTCAGAGGGTTTC-3', R 5'-CAACTGCAT-TACTGGCAATC-3'; *lep*, F 5'-CACATTAAGATGGAAACCTG-3', R 5'-TATCGAGCCTTTATTTAGCC-3'; *ptc1*, F 5'-GCATATATGTGTG-GAGCTATTCTC-3', R 5'-GAGCTGTGATCTTCAGAAC-3'; *uki*, F 5'-GGGAGGCCCTGGCTTAG-3', R 5'-CCATGGTTAACAGCCTTTGC-3' (Koudijs et al., 2008; Koudijs et al., 2005). Sequencing was carried out at the Genetics Core Facility, University of Sheffield, using an ABI 3730 capillary sequencer. PCR primers were used for sequencing, except for *lep*, where the primer 5'-GTGGTGGTGTAACTTTGC-3' was used.

### FITC-phalloidin and anti-acetylated tubulin antibody staining

Staining was carried out as described (Haddon and Lewis, 1996).

### Collagen type II antibody staining

Embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and stored in methanol. They were washed in PBTw (1×PBS/0.1% Tween), treated with 10 µg/ml proteinase K (1 hour), washed in PBTw, blocked in 10% serum/PBTw (1 hour) and hybridised overnight in 1:500 mouse anti-Collagen type II antibody (II-II6B3 monoclonal, DSHB) at 4°C. After washing in PBTw, anti-mouse IgG-HRP (Sigma; 1:200) was applied before staining with a DAB detection kit (Vector Laboratories).

### Alcian Blue staining

Embryos were fixed overnight in 4% PFA at 4°C, and stained as described (Schilling et al., 1996).

### RNA injection

RNA injection was carried out as described (Hammond et al., 2003).

### Cyclopamine treatment

*ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double-mutant embryos were sorted from siblings at 13–14S based on somite phenotype (Koudijs et al., 2008). Ten to 15 embryos were treated in each well of a 12-well culture dish in 2 ml of embryo medium containing 0.25–50 µM cyclopamine/1% ethanol (Calbiochem) or 1% ethanol alone.

### Acridine Orange treatment

Acridine Orange treatment was carried out as described (Abbas and Whitfield, 2009).

### Microscopy

Microscopy was carried out as described (Hammond et al., 2003).

### Transplants

Donor embryos were labelled with 5% rhodamine-dextran/3% biotin-dextran (Molecular Probes) as described (Piotrowski et al., 2003). Embryos were cooled to 21.5°C overnight to obtain embryos at the correct stage. *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos were identified before embedding based on somite morphology (Koudijs et al., 2008). Embryos were embedded dorsal side up in 1% low melting point agarose in Ringer's solution. Otic vesicles were extirpated from the left-hand side of donor and host embryos using glass microelectrodes and fine-gauge hypodermic needles. Labelled donor otic vesicles were transplanted into unlabelled hosts at 18–19S. Host embryos were cultured overnight in Ringer's solution, and then transferred to embryo medium. Analysis of fluorescence and semicircular canal projection formation was carried out at 3 dpf. To detect biotin-dextran, embryos were fixed overnight in 4% PFA, washed, permeabilised and quenched as in Kane and Kishimoto (Kane and Kishimoto, 2002), and labelled using an ABC kit (Vector Laboratories) followed by a Cy3-tyramide kit (Perkin-Elmer).

## RESULTS

### Mutations in genes encoding inhibitors of Hh signalling cause a spectrum of phenotypes in the zebrafish inner ear, primarily affecting the DL-VM axis

Several genes encoding inhibitors of the Hh signalling pathway are expressed in and around the developing otic vesicle: these include *su(fu)*, *dzip1*, *ptc1*, *ptc2* and *hip* (Hammond et al., 2003; Hammond and Whitfield, 2009; Koudijs et al., 2005; Wolff et al., 2004). We examined otic patterning in embryos carrying homozygous mutations in these genes, both individually and in combination. These were *uki* (*hip*<sup>-/-</sup>), *lep* (*ptc2*<sup>-/-</sup>), *dre* (*su(fu)*<sup>-/-</sup>) (Koudijs et al., 2005), *ptc1*<sup>-/-</sup> (Koudijs et al., 2008) and *igu* (*dzip1*<sup>-/-</sup>) (Sekimizu et al., 2004; Wolff et al., 2004). Hh signalling activity is upregulated in these mutants, ranging from a modest increase in *dre* to maximal Hh pathway overactivation in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants, based on *ptc1* and *gli1* expression levels and phenotypic severity (Koudijs et al., 2008; Koudijs et al., 2005). In *igu* homozygotes, low-level Hh signalling is expanded, and high-level Hh signalling is reduced (Sekimizu et al., 2004; Wolff et al., 2004).

At 4 days post-fertilisation (dpf), inner ear phenotypes ranged from normal (*ptc1*<sup>-/-</sup>), through mild (*dre*, *lep*), moderate (*igu*, *uki*) and substantial (*ptc1*<sup>+/+</sup>; *lep*<sup>-/-</sup>) disruption of dorsolateral structures, to severely ventralised and medialised (*ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup>) (Fig. 1; Table 1). As the severity of the ear phenotype increased, the dorsolateral septum, endolymphatic duct (a dorsomedial structure) and cristae (lateral structures) were progressively lost and the semicircular canal

projections developed increasingly abnormally. All these structures were absent from *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles. We describe how each structure within the ear was affected in detail below.

### Dorsolateral septum

The dorsolateral septum (dls) (Fig. 1A, dls) separates the anterior and posterior semicircular canals. It was normal in *ptc1*<sup>-/-</sup> mutant ears (Fig. 1B), present but malformed and often positioned incorrectly in *lep* (Fig. 1D) and absent from *dre*, *igu*, *uki*, *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> and *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> ears (Fig. 1C,E-H). This did not result from increased cell death: we treated *uki* homozygotes and siblings with Acridine Orange between 42 and 74 hpf, when the dls normally forms (Haddon and Lewis, 1996), and observed no alteration in cell death in the dorsal region of the otic vesicle (data not shown).

### Cristae

In wild-type otic vesicles, the three cristae (anterior, lateral and posterior) develop ventrolaterally, as revealed by *msxc* expression (Fig. 2A) and by staining with FITC-phalloidin (Fig. 2D). Cristae in *dre* embryos were indistinguishable from wild type (Fig. 2U). In *igu*, *lep*, *uki* and *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> embryos, the lateral crista was lost or reduced in 29, 45, 77 and 100% of cases, respectively (Fig. 2B-H,U). In a number of *uki* mutants we also observed an additional small domain of *msxc* expression, possibly representing an incomplete separation of the anterior macula and lateral crista domains (arrowhead, Fig. 2H). Hair cells developed in both this region and the reduced lateral crista (arrowhead, Fig. 2E). No cristae formed in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants (see Fig. 4F).

### Endolympathic duct

The endolympathic duct (ED) forms dorsally from an outpocketing of the otic epithelium and is strongly marked by *foxi1* expression (Abbas and Whitfield, 2009). In *dre*, *lep* and *igu* the ED was not significantly different in length between mutants and stage-matched 3 dpf siblings (*t*-test:  $P=0.39$ ,  $n=8$ ;  $P=0.95$ ,  $n=18$ ;  $P=0.65$ ,  $n=21$ , respectively), whereas in *uki*<sup>-/-</sup> and *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> embryos, ED

length was significantly reduced ( $P=0.0009$ ,  $n=15$  and  $P=0.0052$ ,  $n=8$ , respectively) (Fig. 2K,L). No ED was present in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos (Fig. 2I,J).

### Semicircular canal pillars

In zebrafish, the anterior (ap), posterior (pp) and ventral (vp) (horizontal) semicircular canal pillars form by fusion of epithelial projection tissue by 3 dpf. In *ptc1*<sup>-/-</sup> and *dre* embryos, all three pillars formed normally (Fig. 1A-C). In *lep*, *igu* and *uki*, the ap and pp were often thin and spindly, whereas the vp was enlarged and dysmorphic (Fig. 1D-F). In *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> embryos, all pillars were very reduced (Fig. 1G). In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double-mutant embryos, no canal projections formed (Fig. 1H).

The dysmorphic vp in *lep*, *igu* and *uki* showed several gene-expression abnormalities (arrows, Fig. 2M-T). It expressed *raldh3* and Collagen type II at high levels, and stained positively with Alcian Blue, markers that are all absent from the wild-type vp. All three markers were also occasionally seen in the malformed ap and pp of *lep*, *uki*, *igu* and *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> embryos (data not shown). Expression of *otx1* in the dysmorphic vp was relatively normal, however (arrows, Fig. 2N,R). To test whether the vp in *lep*, *uki* and *igu* was enlarged as a result of overproliferation, we examined expression of proliferating cell nuclear antigen (*pcna*) mRNA in this tissue. However, *pcna* expression levels did not appear to be increased at 60 hpf, when the ventral canal projection formed (data not shown).

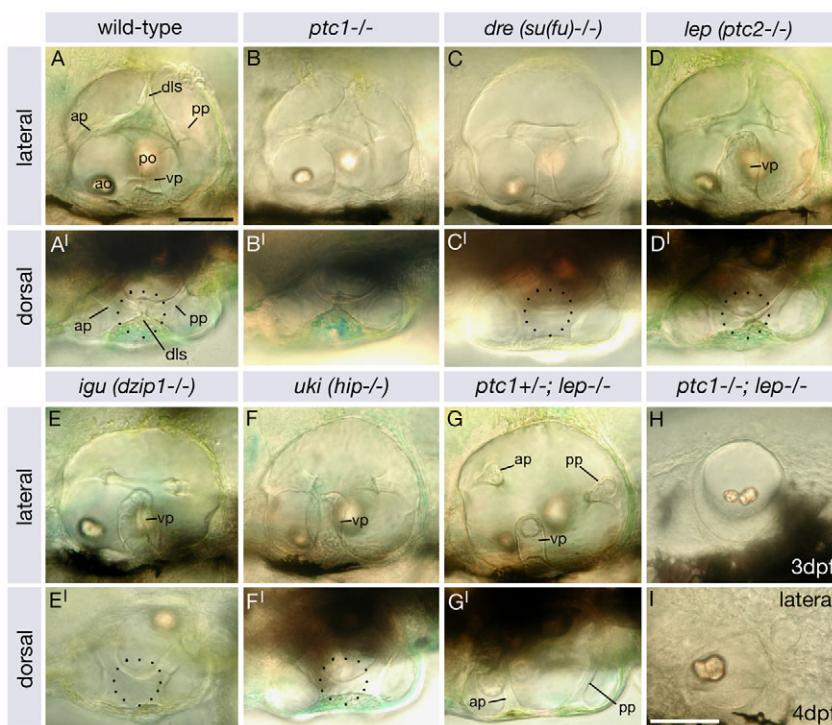
### Maculae

The anterior and posterior maculae were grossly normal in the Hh inhibitor mutants (see, for example, *uki*: Fig. 2D,E), with the exception of *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> embryos, in which the anterior macula was positioned slightly medial to normal (Fig. 2F), *igu*<sup>-/-</sup>, in which the posterior macula was often small and badly formed (data not shown) (Hammond et al., 2003), and *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup>, which had a single medial macula with double posterior character (Fig. 4 and see below).

**Fig. 1. Dorsolateral otic structures are lost with increasing severity of otic phenotype in a panel of Hh pathway inhibitor mutants.**

Images of live zebrafish inner ears taken using DIC optics at 4 dpf, except in H (3 dpf).

(A-I) Lateral views; anterior to left, dorsal to top. (A'-G') Dorsal views; anterior to left, lateral to bottom. (A,A') Wild-type inner ear showing the three pillars around which the semicircular canals form, the dorsolateral septum and the two otoliths. (B,B') *ptc1*<sup>-/-</sup> ears are normal. (C,C') The dls is absent in *dre*. (D,D') In *lep* the dls and ventral canal pillar (vp) are abnormal. (E,F') In *igu* and *uki* the dls is absent and the ventral canal projection is abnormal. (G,G') In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> all three canal projections are reduced and the dls is absent. (H,I) In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos the ear is small, the otoliths fuse and no canal projections or dls form. Dotted circle denotes dls (A',D') or region where it should form (C',E',F'). Scale bars: 50  $\mu$ m (bar in A applies to A-H). ap, anterior semicircular canal pillar; ao, anterior otolith; dls, dorsolateral septum; po, posterior otolith; pp, posterior semicircular canal pillar; vp, ventral semicircular canal pillar.



**Table 1.** Otic phenotypes of the Hh inhibitor mutants

Genotype	Dorsolateral septum	Canal pillars	Cristae	Endolympathic duct	Maculae
Wild type	Present	wt	3	wt	wt
<i>ptc1</i> <sup>-/-</sup>	Present	wt	3	wt	wt
<i>dre</i> ( <i>su(fu)</i> <sup>-/-</sup> )	Absent	wt	3	wt	wt
<i>lep</i> ( <i>ptc2</i> <sup>-/-</sup> )	Present but reduced and abnormally positioned	A, P wt V dysmorphic	3 or 2 (L missing)	wt	wt
<i>igu</i> ( <i>dzip1</i> <sup>-/-</sup> )	Absent	A, P spindly V dysmorphic	3 or 2 (L missing)	wt	P (saccular) macula reduced
<i>uki</i> ( <i>hip</i> <sup>-/-</sup> )	Absent	A, P spindly V dysmorphic	3 or 2 (L missing)	Reduced	wt
<i>ptc1</i> <sup>+/-</sup> ; <i>lep</i>	Absent	All three very reduced	2 (L missing)	Reduced	A (utricle) macula slightly more medial than usual
<i>ptc1</i> <sup>-/-</sup> ; <i>lep</i>	Absent	Absent	0	Absent	Single medial macula

A, anterior; D, dorsal; L, lateral; V, ventral; wt, wild type.

### Severely increased Hh signalling results in DV and ML patterning defects in the zebrafish otic vesicle

To investigate whether the DL otic defects in the Hh inhibitor mutants are due to patterning changes at otic vesicle stages, we examined expression of *tbx1* (a marker of lateral otic epithelium), *pax2a* (medial), *dlx3b* (dorsal) and *eyal* (ventral), between 25S and 30 hpf (Fig. 3). In *uki*, *igu*, *lep* and *dre*, there were no obvious alterations in otic expression of these markers (data not shown). In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles, however, medial and ventral otic territories, marked by *pax2a* and *eyal*, were expanded relative to ear size at the expense of lateral and dorsal domains, marked by *tbx1* and *dlx3b*. Injection of 5 nl of 50 µg/ml *shh* RNA into wild-type (WIK) embryos produced a similar, but variable, effect (Fig. 3).

To test whether the expansion of VM otic fate at the expense of DL identity could be a result of increased cell death in DL otic domains, we stained embryos with Acridine Orange to highlight apoptotic tissue. However, we saw no increase in cell death in DL regions of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles (16S to 29 hpf) compared with age-matched siblings (data not shown). At 16S, the cavitating otic vesicle of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos was similar in size to that of wild-type siblings but by 29 hpf was significantly reduced (see Fig. S1 in the supplementary material). Cell death was, however, increased anteroventrally to the otic vesicle (see Fig. S2 in the supplementary material). This might include the forming statoacoustic ganglion. Interestingly, in zebrafish embryos in which Hh signalling is absent (*smo*, *con*), the otic expression patterns of *pax2a*, *eyal* and *dlx3b* are not altered (Hammond et al., 2003). Therefore, although excessive Hh signalling can perturb DV and ML otic identity, Hh does not appear to be required for correct DV and ML patterning of the zebrafish otic vesicle.

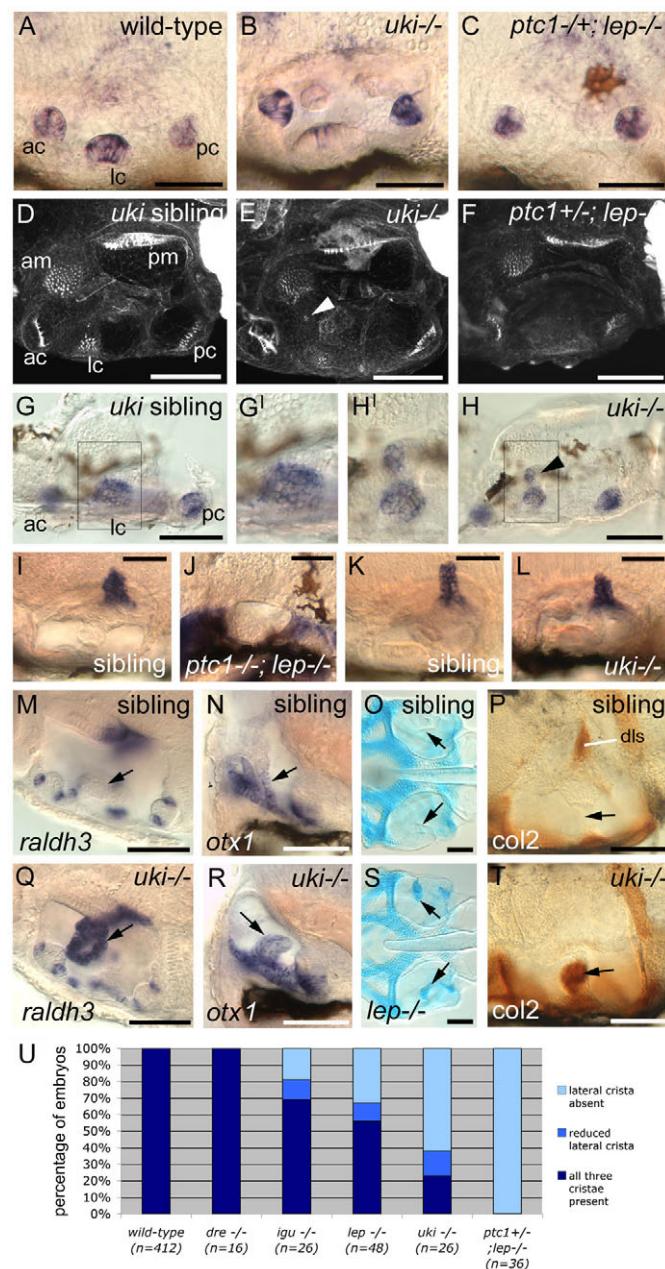
### *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> mutants have a striking double-posterior otic phenotype

In addition to the DV and ML otic defects described above, *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants exhibited a striking posterior otic duplication concomitant with a loss of anterior otic structures. This was similar, but not identical, to the otic phenotype in embryos overexpressing *shh* or *dnPKA* mRNA, in which Hh signalling is upregulated throughout the embryo (Hammond et al., 2003). In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> homozygotes the otic vesicle was small and round, with no cristae or semicircular canal projections (Fig. 1H; Fig. 4B,D). Two separate macular domains of hair cells developed at the anterior and posterior

poles of the vesicle, as in wild-type embryos, but both domains were positioned medially, similar to the posterior domain in wild-type embryos (Fig. 4A-D). Later, these fused to form a single medial macula on the VM wall (Fig. 4F), overlaid by a single dumbbell-shaped otolith, which originated as two separate medial otoliths that later fused (Fig. 1H,I).

We confirmed that the single *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> macula had a double posterior character by examining sensory hair cell polarity patterns (Fig. 4G-J; see Fig. S3 in the supplementary material). Hair cell polarity patterns are stereotypical for each sensory patch, and can be mapped using anti-acetylated tubulin antibody to mark the kinocilia and FITC-phalloidin to mark the hair bundle (Haddon et al., 1999). We mapped polarity in four *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> maculae: hair bundles pointed away from a midline in both the anterior and posterior halves of the maculae with a central region of confused polarity (Fig. 4G,H; see Fig. S3 in the supplementary material). A pattern of hair bundle polarities pointing away from a midline is characteristic of the zebrafish posterior macula (Fig. 4J); this is never seen in the anterior macula (Fig. 4I) (Haddon et al., 1999). We therefore conclude that the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> macula has double-posterior identity. Note, however, that the shape of the macula was almost triangular, differing from the double-posterior ‘bow tie’- or ‘butterfly’-shaped maculae in the ears of embryos injected with *shh* or *dnPKA* mRNA (Fig. 4) (Hammond et al., 2003).

We also examined expression of a panel of otic AP markers in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> homozygotes (Fig. 5). Expression of *hmx3*, *fgf8* and *pax5* was reduced or absent from the anterior region of the otic vesicle (Fig. 5A-F). *otx1* expression was lost or reduced to a small ventrolateral domain, consistent with the absence of tissue between the duplicated halves of the posterior macula (Fig. 5G-J) (Hammond and Whitfield, 2006). Curiously, however, *fst1*, a posterior otic marker, was not expressed in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles ( $n=16$ ), despite being upregulated around the otic vesicle (Fig. 5K,L). This differs from the phenotype in *shh* RNA-injected embryos, in which *fst1* was upregulated at the anterior of 5/22 otic vesicles (Hammond et al., 2003), and from *dre*, *lep*, *igu* and *uki* (see below). The otic vesicles of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos were also significantly smaller than those of *shh* RNA-injected embryos by 4 dpf (Fig. 1H,I). These data, together with the triangular-shaped macula, suggest that the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic phenotype is more severe than that of our *shh*- or *dnPKA*-injected embryos. To confirm this, we applied low doses of cyclopamine, a potent inhibitor of the Hh signalling pathway, to



**Fig. 2. Dorsolateral patterning defects appear as the severity of otic phenotype increases in the Hh inhibitor mutants.**

(A-H') Cristae are lost in the more severe Hh inhibitor mutants. (A-C,G-H) *msxc* *in situ* hybridisation at 3 dpf. (D-F) Confocal z-stacks of ears stained with FITC-phalloidin to reveal actin in the sensory hair bundles at 3 dpf. (A,D,G,G') Three cristae (ac, lc, pc) are present in wild-type ears. (B,E,H,H') *uki* mutants with reduced lateral cristae; the medial region of the lateral crista is sometimes displaced towards the anterior macula (arrowheads, E,H). (C,F) *ptc1*+/-; *lep*-/- mutants with no lateral cristae. (I-L) The endolymphatic duct is reduced in *uki* embryos and absent in *ptc1*+/-; *lep*-/- double mutants: *in situ* hybridisation to *foxi1* at 68 hpf (I,J) and 72 hpf (K,L). (M-T) The ventral semicircular canal pillar (arrows) is abnormal in *lep* and *uki* homozygotes, with ectopic expression of *raldh3* (M,Q) and Collagen type II protein (P,T) at 3 dpf, and ectopic Alcian Blue staining at 5 dpf (O,S). Expression of *otx1* at 3 dpf in the ventral pillar is unaffected (N,R). *igu* embryos show similar defects in the ventral pillar (not shown). (A-C,I-L,P,T) Lateral views; anterior to left, dorsal to top. (D-H,M,Q) Dorsal views; anterior to left, medial to top. (O,S) Dorsal views; anterior to left. (N,R) Transverse hand-cut sections, ~50 µm. Boxes in G,H show the region enlarged in G',H'. Scale bars: 50 µm. (U) Chart showing the proportion of Hh inhibitor mutant embryos with lost or reduced lateral cristae. ac, anterior crista; am, anterior macula; dls, dorsolateral septum; lc, lateral crista; pc, posterior crista; pm, posterior macula.

### Manipulation of Hh pathway activity in *ptc1*+/-; *lep*-/- embryos can phenocopy the weaker Hh inhibitor mutant otic phenotypes

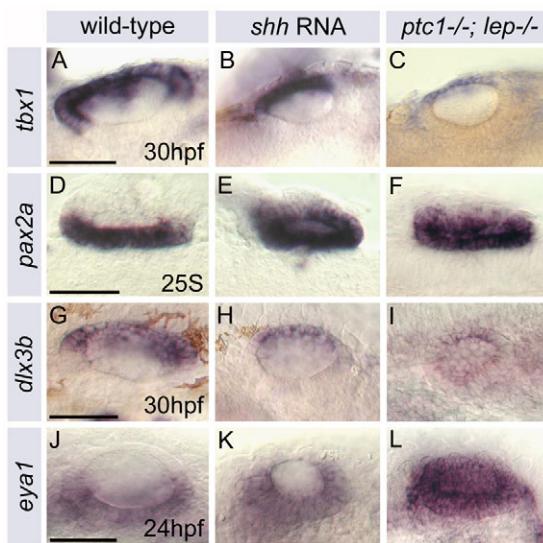
To investigate whether levels of Hh pathway activity in the ear region correspond to the severity of the otic phenotype in the Hh-inhibitor mutants, we used *ptc1* and *ptc2* expression levels as a readout of Hh signalling (Concorde et al., 1996; Goodrich et al., 1996). *ptc1* and *ptc2* RNA levels in the embryo, including the ear region, generally correspond to the severity of otic phenotype. Expression of *ptc1* and *ptc2* is lost or severely reduced in *smo* and *con* mutants, in which Hh signalling is absent or severely downregulated, and is upregulated in *shh* RNA-injected embryos and *ptc1*+/-; *lep*-/- double mutants (Hammond et al., 2003) (see Fig. S5 in the supplementary material; data not shown). In our hands, *ptc1* and *ptc2* RNA levels were not substantially raised in *dre*, *lep* and *uki* mutants, including in the otic vesicle region (data not shown); however, Koudijs et al. (Koudijs et al., 2005) report a slight increase in *ptc1* levels in *lep* and *uki* mutants. This corresponds to the slightly more severe otic phenotypes in these mutants compared with *dre*.

However, in *ptc1*+/- mutants – which have normal ears – *ptc1* and *ptc2* levels were raised throughout the embryo (see Fig. S5 in the supplementary material). Other tissues in the *ptc1*+/- mutant, including the somites, are more severely affected than in *dre*, *lep* and *uki* (Koudijs et al., 2008). The fact that there was no ear phenotype in *ptc1*+/- mutants suggests that although *ptc1* has an important role in many tissues, it is less important than *ptc2* in the otic vesicle. Increased *ptc2* levels in *ptc1*+/- mutants seem to be able to compensate for the absence of *ptc1* function in the otic vesicle.

To avoid complications arising from transcriptional feedback acting on *ptc1* and *ptc2* levels, and to confirm that levels of Hh signalling correspond to severity of the otic phenotype in the Hh inhibitor mutants, we applied graded concentrations of the Hh inhibitor cyclopamine to *ptc1*+/-; *lep*-/- double mutants. This would reduce Hh signalling levels throughout the embryo downstream of the non-functional Ptc receptors, and gradually rescue otic

*ptc1*+/-; *lep*-/- homozygotes from 15S, in order to reduce Hh signalling activity slightly. Using 0.25 µM cyclopamine, the triangular *ptc1*+/-; *lep*-/- macula was rescued to a ‘bow tie’ shape in 3/8 cases (see Fig. S4 in the supplementary material).

In *dre*, *lep*, *igu* and *uki*, there were no morphologically obvious otic AP patterning defects, and expression of *hmx5*, *pax5*, *fgf8* and *otx1* in the ear was normal (data not shown). However, *fst1* was ectopically expressed at the anterior of *dre*, *lep*, *igu* and *uki* otic vesicles. This ectopic expression was variable and was most extreme in *uki* and least severe in *dre* (Fig. 6). A small domain of *fst1* expression was also seen at the anterior of some *dre*, *uki*, *lep* and *igu* siblings (presumed heterozygotes; for example, Fig. 6B). This was not seen in wild-type (TL) embryos (0/26). Note, however, that expression of *fst1* was upregulated in other regions of the embryo in the Hh inhibitor mutants (data not shown). Taken together, these data indicate that Hh inhibitory activity is required for AP patterning of the otic vesicle as well as for DV and ML patterning.

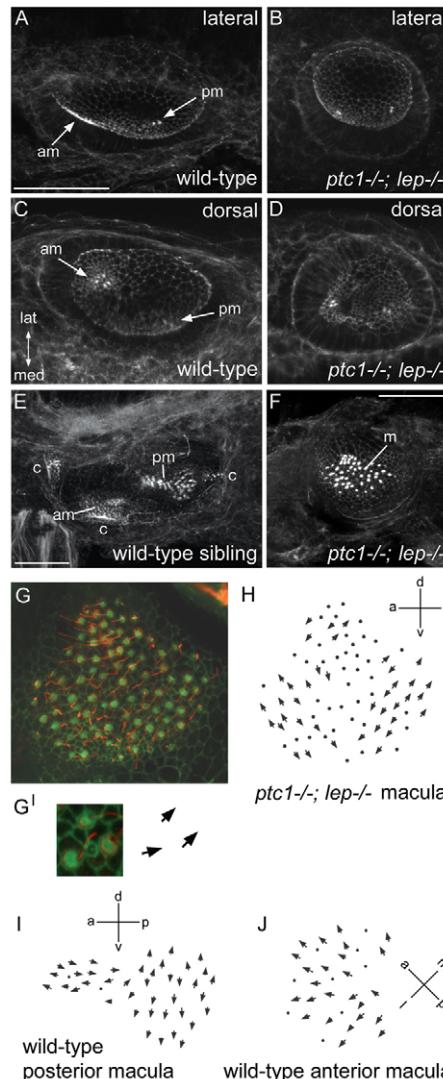


**Fig. 3. Ventromedial otic territories are expanded at the expense of dorsolateral domains in *shh* RNA-injected and *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos.** (A-L) Expression of lateral (*tbx1*), medial (*pax2a*), dorsal (*dlx3b*) and ventral (*eya1*) markers in the zebrafish otic vesicle. Expression domains of dorsolateral markers (*tbx1*, *dlx3b*) are reduced relative to ear size in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> and *shh*-injected embryos, whereas ventromedial markers (*pax2a*, *eya1*) are expanded. Staining in siblings was indistinguishable from wild type. (A-F) Dorsal views; anterior to left, lateral to top. (G-L) Lateral views; anterior to left, dorsal to top. Scale bars: 50 µm (as shown in left-hand image of each trio).

development. We applied cyclopamine at 15–16S to *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double-mutant embryos, and analysed the ear phenotype at 88–90 hpf (Table 2). Using 50 µM cyclopamine, we observed complete or near-complete rescue of the otic phenotype in 25/30 *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos. At 40 µM, as well as *igu*- or *uki*-like ears (with no dls and abnormal semicircular canal projections), we saw both a *dre*-like ear phenotype (no dls) and a *lep*-like ear phenotype (dls present but abnormal, with abnormal canal projections) as well as individuals with a very thin dls and grossly normal canal projections. Between 5 µM and 20 µM cyclopamine, all ears resembled those of *ptc1*<sup>+/-</sup>; *lep*<sup>-/-</sup> mutants, with three spindly canal pillars; at lower concentrations, only the anterior and posterior canal pillars formed; lower still, only the posterior pillar formed. At the lowest concentrations, the vesicle was slightly larger than in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants, and the macula shape was sometimes partially rescued (see above). Overall, the severity of otic phenotype appeared to correlate with the level of Hh pathway activity within the embryo.

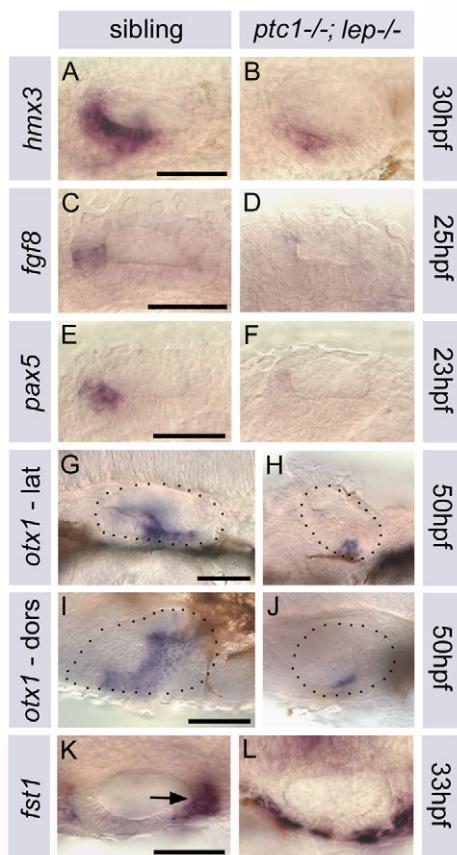
### Inhibition of Hh signalling is required for inner ear patterning between 17S and 48 hpf

To narrow the time interval during which inhibition of Hh signalling is required for otic patterning, we applied 50 µM cyclopamine to *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos from time points between 15S and 48 hpf. We analysed ear and sensory patch morphology at 80 hpf using differential interference contrast (DIC) microscopy and phalloidin staining, respectively. When cyclopamine was applied from 17S, complete rescue of the otic phenotype occurred in 4/6 cases, suggesting that inhibition of Hh signalling is not required before this time (Table 3). Application after 48 hpf had no effect. Cyclopamine applied between these time points led to partial rescue (see Fig. S6



**Fig. 4. Otic vesicles of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos contain a single medial macula with double posterior polarity, which originates as two separate domains of hair cells.** (A-F) Merged confocal z-stacks of otic vesicles at 33 hpf (A-D) and 3 dpf (E,F) stained with FITC-phalloidin. Hair cell bundles appear as bright dots. Two separate sensory patches are seen in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> ears (B,D), as in wild-type ears, but both arise in a medial position like the posterior patch in wild-type ears (compare C with D). By 3 dpf, the patches have merged to form a single macula in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> (F). (G) Merged confocal z-stack of a 70 hpf *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> macula stained with FITC-phalloidin (cuticular plate/stereocilia; green) and anti-acetylated tubulin antibody (kinocilia; red). (G') Enlarged image of three hair cells, showing the planar polarity of each hair cell (arrows). (H) Polarity map of the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> medial macula shown in G. Additional examples are shown in Fig. S3 in the supplementary material. Hair bundles point away from a midline in both the anterior and posterior halves of the macula, resembling the pattern seen in wild-type posterior maculae. (I,J) Polarity maps of wild-type posterior and anterior maculae for comparison. (A,B,E,F) Lateral views; anterior to left, dorsal to top. (C,D) Dorsal views; anterior to left, lateral to top. am, anterior macula; c, crista; lat, lateral; m, medial macula; med, medial; pm, posterior macula. Scale bars: 50 µm (bar in A applies to A-D).

in the supplementary material). This contrasts with a requirement for Hh signalling before 15S for correct AP otic patterning (see Fig. S7 in the supplementary material).

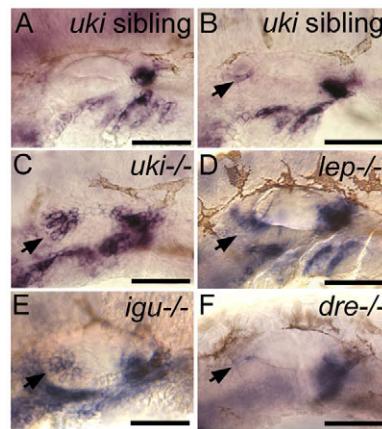


**Fig. 5. Expression of AP markers is altered in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles.** (A-L) Expression of anterior (*hmx3*, *fgf8*, *pax5*), ventral/central (*otx1*) and posterior (*fst1*) otic markers. In *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup>, expression of anterior markers is reduced or absent, *otx1* expression is reduced, and posterior *fst1* expression (arrow, K) is lost in the ear but upregulated in surrounding tissues. (A-H) Lateral views, anterior to left, dorsal to top. (I-L) Dorsal views; anterior to left, lateral to bottom. Scale bars: 50 µm (in left-hand image of each pair).

### Effects of a loss of Hh inhibition on the otic vesicle are likely to be direct

To investigate whether the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> mutant otic phenotype results from the direct action of a gain of Hh signalling activity in the otic epithelium, or is caused indirectly by defects in surrounding tissues, we transplanted *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> or control wild-type (AB) otic vesicles into wild-type (AB) hosts. Donor embryos were labelled with rhodamine- and biotin-dextran at the one- to two-cell stage. At 18S the host otic vesicle was, as far as possible, extirpated and replaced with a donor otic vesicle (Fig. 7A). As described above, treatment with cyclopamine from 18S can almost entirely rescue the otic phenotype of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos; if the effects of a gain of Hh activity are indirect, surrounding tissue should be able to rescue inner ear development from this stage. At 68–70 hpf ears were assayed for rescue of semicircular canal projection development: semicircular canal projection tissue never developed in control *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos (Fig. 7).

Wild-type otic vesicles transplanted into wild-type hosts resulted in abnormal ears, but these contained reasonably well-developed semicircular canal projection tissue in all (6/6) cases (examples are shown in Fig. 7E,G). In two cases, both host and donor tissue was



**Fig. 6. Expression of the posterior marker *fst1* is duplicated at the anterior of the otic vesicle in the Hh inhibitor mutants.**

(A) Posterior otic expression of *fst1* in an *uki* sibling embryo at 30 hpf, indistinguishable from the wild-type pattern. (B-F) In *uki*<sup>-/-</sup>, *lep*<sup>-/-</sup>, *igu*<sup>-/-</sup> and, to a lesser extent, *dre*<sup>-/-</sup> and presumed heterozygous *uki* sibling embryos, *fst1* is ectopically expressed at the anterior of the otic vesicle at 30 hpf (arrows). Lateral views; anterior to left, dorsal to top. E is a composite of two images. Scale bars: 50 µm.

present in the otic vesicles, suggesting incomplete extirpation of the host otic rudiment; host and donor tissue fused, forming a single vesicle in both cases (Fig. 7E). When *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> tissue was transplanted into wild-type hosts, small otic vesicles formed ( $n=16$ ). In contrast to the wild type-to-wild type transplants, if a substantial amount of wild-type otic tissue was present together with the transplanted mutant tissue (7/16 cases), two separate vesicles formed. In most cases, the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic tissue formed a small canal-free otic vesicle (13/16) (Fig. 7F). However, in 3/16 cases, rudimentary semicircular canal projection tissue was formed (Fig. 7H). To investigate further, we labelled the biotin-dextran in the donor tissue using Cy3-tyramide and examined the ears using confocal microscopy. In all three cases, the canal projection tissue appeared to consist entirely of wild-type cells protruding through the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> tissue (Fig. 7I,J). We were unable to assay for any rescue of sensory patch development in these abnormal ears. Nevertheless, the lack of semicircular canal projection development from *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> tissue suggests that the effect of a gain of Hh signalling on otic patterning is predominantly direct.

### DISCUSSION

#### Inhibition of Hh signalling is required for correct acquisition of dorsolateral otic identity in the zebrafish

We have shown that the developing zebrafish ear is exquisitely sensitive to a loss of function of antagonists of the Hh pathway. Even a small increase in Hh pathway activity leads to DV and ML otic patterning defects: we see a spectrum of subtle DL defects in *dre* (*su(fu)*<sup>-/-</sup>), *lep* (*ptc2*<sup>-/-</sup>), *igu* (*dzip1*<sup>-/-</sup>) and *uki* (*hip*<sup>-/-</sup>) embryos, in which Hh signalling is only slightly raised (Koudijs et al., 2005; Wolff et al., 2004). When Hh pathway activity is maximally upregulated, either in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants or when *shh* RNA is overexpressed, the otic vesicle is severely medialised and ventralised. By contrast, the effect of Hh signalling on otic AP patterning is only morphologically obvious at the extremes of Hh

**Table 2. Application of cyclopamine to *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants can phenocopy the weaker Hh inhibitor mutant otic phenotypes**

Cyclopamine ( $\mu$ M)	<i>ptc1</i> <sup>-/-</sup> ; <i>lep</i> -like	Larger vesicle than <i>ptc1</i> <sup>-/-</sup> ; <i>lep</i> /no canal projections	Only P canal projection	Only A and P canal projections	<i>ptc1</i> <sup>-/-</sup> ; <i>lep</i> -like	<i>igu/uki</i> - like	No (or very thin) dls	<i>lep</i> - like	Rescued (wild-type)	Dead	Total
0	21									4	25
0.25		2	5							1	8
1.0		2		6						0	8
2.0				9						0	9
5.0					7					2	9
10						3				0	3
20						2				2	4
40							2	2 (3)	2	4	13
50							1	4	25	0	30

A, anterior; dls, dorsolateral septum; P, posterior.

pathway activity, in *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> double mutants or cyclopamine-treated embryos (this work) or in *shh*- or *dnPKA*-injected embryos or severe Hh loss of function mutants (Hammond et al., 2003).

Evidence from amniotes suggests that, here too, tight regulation of Hh pathway activity is required for correct inner ear development: strictly regulated expression of Gli activator and repressor forms is required for the formation of auditory (ventral) and vestibular (dorsal) areas of the mouse inner ear, respectively, and a precise balance between Shh and Wnt signalling is required for cochlea development (Bok et al., 2007b; Riccomagno et al., 2005). Hh pathway inhibitors could provide the tight regulation of Hh activity required. In mouse otic vesicles, *ptc1* is expressed in a graded fashion, highest ventromedially (Bok et al., 2007b); the expression of *dzip1*, *su(fu)* and *hip* has not yet been described in amniote ears.

### Different Hh inhibitors play different roles during zebrafish inner ear development: Ptc2 has a greater role than Ptc1

Consistent with a graded response to levels of Hh pathway activity throughout the embryo, the severity of the otic defects in the Hh inhibitor mutants form a phenotypic series, corresponding roughly to the severity of the general embryonic phenotype (Koudijs et al., 2008; Koudijs et al., 2005). There are, however, several idiosyncrasies, suggesting that each inhibitor may have a slightly different role. This was not unexpected, as the expression patterns of *ptc1*, *ptc2*, *dzip1*, *su(fu)* and *hip* in and around the developing ear differ from one another (Hammond et al., 2003; Hammond and Whitfield, 2009).

Firstly, the *dre* (*su(fu)*<sup>-/-</sup>) and *lep* (*ptc2*<sup>-/-</sup>) otic phenotypes do not fit together into a smooth series of phenotypic severity: *dre* has no dls but normal semicircular canal pillars, whereas the dls in *lep* is present but malformed, and there are additional canal pillar abnormalities. This could result from expression pattern differences: *su(fu)* is expressed ubiquitously, whereas *ptc2* is expressed in a broad ventral otic domain (Hammond et al., 2003; Hammond and Whitfield, 2009). Interestingly, however, both the *dre* and *lep* phenotypes, as well as intermediate phenotypes, can be produced by treating *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> embryos with 40  $\mu$ M cyclopamine. This may reflect slight spatial and temporal differences in the uptake and effect of cyclopamine between individual embryos.

Secondly, although several indicators (*ptc1* and *ptc2* expression levels and somite phenotype) suggest that the overall phenotype of *ptc1*<sup>-/-</sup> mutants is more severe than that of *dre*, *lep*, *igu* and *uki* mutants, *ptc1*<sup>-/-</sup> embryos have normal ears, whereas *dre*, *lep*, *igu* and *uki* have dorsolateral otic patterning defects. This suggests that although Ptc1 has an important role elsewhere in the embryo, Ptc2 is likely to be the primary Hh receptor in the zebrafish ear. Feedback control on the transcription of *ptc* genes means that *ptc2* levels are raised in *ptc1*<sup>-/-</sup> mutants, and this appears to be able to compensate for the lack of *ptc1* function in the ear. The reverse, however, is not true: *ptc1* is only minimally upregulated in *lep*<sup>-/-</sup> mutants, and is unable to compensate entirely for the absence of *ptc2* function in the ear.

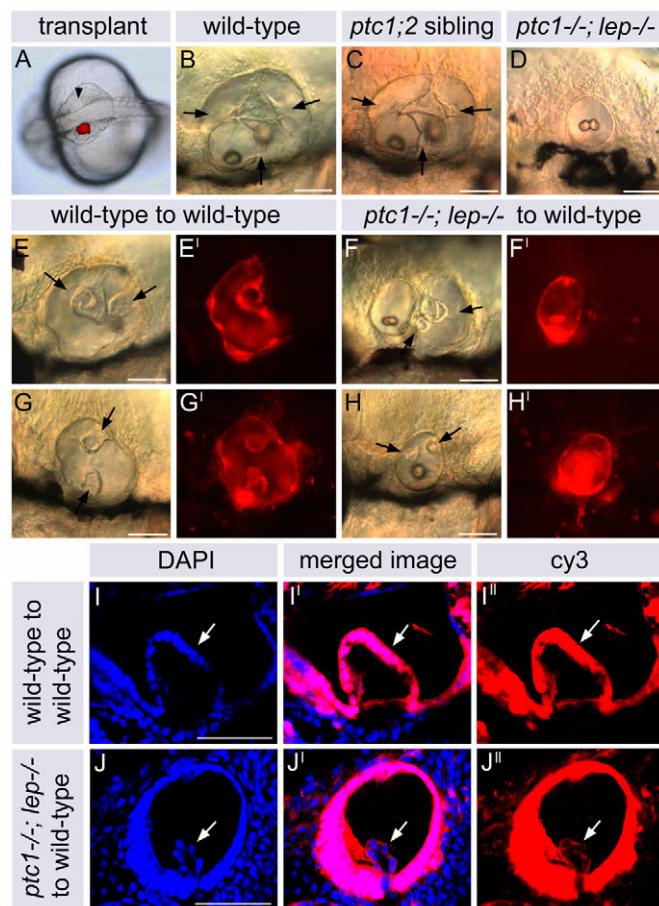
A role for *ptc1* in inner-ear patterning is, however, revealed in combination with *lep*: the otic phenotype of *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> fish is more severe than the *lep* phenotype, and the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> phenotype is yet

**Table 3. Inhibition of Hh signalling is required for otic development between 17S and 48 hpf**

Time cyclopamine applied	Rescue (wild-type)	<i>lep</i> -like: 3 projs and septae present	3 projs + septae/large ear	3 projs/medium ear	3 projs/very small ear	V proj only	<i>ptc1</i> <sup>-/-</sup> ; <i>ptc2</i> <sup>-/-</sup> like	Total
17S +	4		2					6
23S +		10		3				13
26 hpf +				3	5			8
33 hpf +						10		10
38 hpf +						4	6	10
48 hpf +							3	3
Ethanol 17S +							5	5

Corresponding images of each ear phenotype are shown in Fig. S5 in the supplementary material.

3 projs, anterior, posterior and ventral canal pillars/projections present; V proj, only the ventral canal projection is present.



**Fig. 7. Transplanted *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic tissue does not contribute to semicircular canal projections.** (A) Dorsal view of a live 20S embryo immediately post-transplant. Rhodamine-dextran-labelled donor otic tissue was visualised fluorescently and is shown as a red overlay. Transplanted tissue was placed directly opposite the unlabelled host ear (arrowhead). (B–D) DIC images of live, untransplanted ears at 3 dpf. Arrows indicate semicircular canal pillars. Embryos in C and D were injected with rhodamine-dextran at the one- to two-cell stage. (E–H') DIC images of live, transplanted otic vesicles at 3 dpf (E–H) and fluorescent images revealing the rhodamine-dextran-labelled donor tissue (E'–H'). Arrows in E and G indicate development of semicircular canal projection and pillar tissue in transplanted wild-type ears. (F,F') Example of a *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> transplant in which both *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> and wild-type otic tissue are present: the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> tissue has formed a small, separate otic vesicle containing no semicircular canal projection tissue. Arrows in F indicate semicircular canal projection and pillar tissue derived from the unlabelled host. (H,H') Example of a transplanted *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> ear in which a single otic vesicle is present and which contains semicircular canal projection tissue (arrows). This is derived from host tissue (see J–J''). (I–J'') Confocal sections through semicircular canal projection tissue in a wild-type transplant (I–I'') and a *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> transplant (J–J''). (I,J) DAPI stain revealing the nuclei of each cell; (I'',J'') Cy3-tiyamide-labelled biotin-dextran marking transplanted tissue. (I–I'') Semicircular canal projection tissue in transplanted wild-type otic vesicles (arrow) is labelled with Cy3 and derived from transplanted donor tissue. (J–J'') Semicircular canal projection tissue in transplanted *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic vesicles is unlabelled and derived from the host. All lateral views; anterior to left, dorsal to top. Scale bars: 50 µm in A–H; 25 µm in I–J''.

more severe. Similar redundancy between Ptc1 and Ptc2 (which are closely related proteins) has been reported in the development of other organs: for example, the somites (Koudijs et al., 2008).

### Indirect versus direct effects of the loss of Hh inhibition on the zebrafish otic vesicle

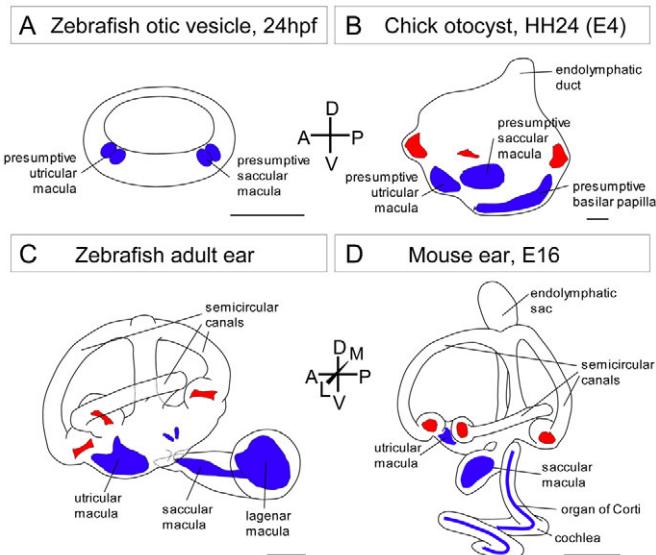
Our transplant experiments suggest that surrounding wild-type tissue cannot effect a rescue of semicircular canal projection development in mutant *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic tissue. This suggests that a loss of Hh inhibition acts directly on the inner ear, as the transplants were performed at 18–19S, from when near-complete cyclopamine-mediated rescue of the *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> otic phenotype is possible (Fig. 7; Table 3). A direct effect of increased Hh signalling in the ear is feasible, as all components of the Hh signalling pathway so far examined, apart from Hh itself, are expressed in the developing ear, and both *ptc1* and *hip* expression levels in the otic epithelium are responsive to Hh pathway activity (Hammond et al., 2003; Hammond and Whitfield, 2009). It is unclear, however, when and to what extent donor tissue integrates sufficiently to receive otic patterning signals from the host. Indeed, when *ptc1*<sup>-/-</sup>; *lep*<sup>-/-</sup> and wild-type otic tissue are both present, small separate otic vesicles form (Fig. 7F).

### The role of Hh signalling in otic patterning is at least partially conserved between zebrafish and amniotes

Previously, we and others have reported that Hh signalling in zebrafish and *Xenopus* is required for otic AP patterning (Hammond et al., 2003; Waldman et al., 2007). In mouse and chick, however,

Hh predominantly affects otic DV patterning (Bok et al., 2005; Liu et al., 2002; Riccomagno et al., 2002; Riccomagno et al., 2005). This was puzzling, as the source of Hh in all these species appears to be the ventral midline tissues (Bok et al., 2005; Hammond et al., 2003), and the relative positioning of the otic vesicle to the midline sources of Hh is similar in both zebrafish and amniotes. Our data now suggest, however, that differences in the role of Hh signalling between amniotes and fish are less extreme than these studies indicated. In zebrafish, as in amniotes (Bok et al., 2007b), derepression of Hh signalling affects specification of dorsal otic domains. There thus appears to be a very similar requirement to keep Hh signalling repressed for the correct patterning of dorsal otic epithelium in both zebrafish and amniotes.

In ventral regions of the ear, there do seem at first sight to be substantial differences in the requirement for Hh signalling between zebrafish and amniotes. When sensory structures are examined in ventral parts of the ear, Hh signalling affects the fish otic AP axis but the amniote DV axis. In many ways, however, patterning of sensory structures along the AP axis of the zebrafish ear is equivalent to patterning along the DV axis of the amniote ear, both functionally and structurally. Auditory reception in zebrafish is performed by posterior otic structures, the saccular and lagena maculae, whereas the major auditory endorgan in amniotes is the ventrally positioned cochlea. Like the saccule and lagena in the fish ear, the amniote cochlear duct and chick basilar papilla arise from a posteroventral region of the otic vesicle (Bok et al., 2007a; Oh et al., 1996; Riccomagno et al., 2002). In the mouse, although all sensory precursors arise from an anteroventral domain in the otocyst, the relative positions of the sensory chambers in the adult ear (utricle-



**Fig. 8. Positions of the sensory patches in the zebrafish and amniote ear relative to the body axes.** (A,B) Sketches of lateral views of a zebrafish otic vesicle (A) and chick otocyst (B), showing the relative positions of the presumptive sensory maculae (blue) and cristae (red). The utricular macula arises anterior to the saccular macula in both species, and the chick basilar papilla (the sensory patch of the cochlea) arises in a ventroposterior region [adapted from data in Oh et al. (Oh et al., 1996)]. Scale bars: 100 µm. (C,D) Sketches of lateral views of the inner ear in the adult zebrafish (D) and E16 mouse embryo (D). In the zebrafish, a third sensory macula, the lagena, develops posteriorly. In the amniote ear, the cochlear duct extends ventrally, but remains connected to the rest of the labyrinth in a relatively posterior position. Scale bars: 500 µm. E, embryonic day; HH, Hamburger-Hamilton stage. Adapted with permission from Whitfield and Hammond (Whitfield and Hammond, 2007).

saccule-cochlea) are equivalent to the AP arrangement (utricule-saccule-lagena) in zebrafish ears (Fig. 8). We conclude that both the requirement for Hh signalling in ventral regions of the otic vesicle, and the need to keep Hh signalling repressed for correct patterning of dorsolateral otic structures, have features that are conserved between zebrafish and amniote vertebrates.

#### Acknowledgements

We thank Laina Murphy and Joanne Spencer for technical assistance, Lisa van Hateren, Claire Allen and the aquarium staff for expert zebrafish care, and many members of the zebrafish community for mutants and probes. This work was funded by grants from the BBSRC (BB/E015875/1) to T.T.W. and the Wellcome Trust (C23207/A8066) to F.v.E. The MRC CDBG zebrafish aquaria and imaging facilities were supported by the MRC (G0400100, G0700091), with additional support from the EU FP6 (ZF-MODELS) and the Wellcome Trust (GR077544AIA). Deposited in PMC for release after 6 months.

#### 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.045666/-DC1>

#### References

- Abbas, L. and Whitfield, T. T. (2009). Nkcc1 (Slc12a2) is required for the regulation of endolymph volume in the otic vesicle and swim bladder volume in the zebrafish larva. *Development* **136**, 2837-2848.
- 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.
- Bok, J., Chang, W. and Wu, D. K. (2007a). Patterning and morphogenesis in the vertebrate inner ear. *Int. J. Dev. Biol.* **51**, 521-533.
- Bok, J., Dolson, D. K., Hill, P., Rüther, U., Epstein, D. J. and Wu, D. K. (2007b). Opposing gradients of Gli repressor and activators mediate Shh signalling along the dorsoventral axis of the inner ear. *Development* **134**, 1713-1722.
- Brand, M., Heisenberg, C.-P., Warga, R. M., Pelegri, F., Karlstrom, R. O., Beuchle, D., Picker, A., Jiang, Y.-J., Furutani-Seiki, M., van Eeden, F. J. M. et al. (1996). Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123**, 129-142.
- Chen, Y. and Struhl, G. (1996). Dual roles for Patched in sequestering and transducing Hedgehog. *Cell* **87**, 553-563.
- Chuang, P.-T. and McMahon, A. P. (1999). Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. *Nature* **397**, 617-621.
- Concordet, J. P., Lewis, K. E., Moore, J. W., Goodrich, L. V., Johnson, R. L., Scott, M. P. and Ingham, P. W. (1996). Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122**, 2835-2846.
- Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.
- Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signalling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.
- Haddon, C. and Lewis, J. (1996). Early ear development in the embryo of the zebrafish, *Danio rerio*. *J. Comp. Neurol.* **365**, 113-123.
- 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.
- Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends Genet.* **13**, 14-21.
- Hammond, K. L. and Whitfield, T. T. (2006). The developing lamprey ear closely resembles the zebrafish otic vesicle: *otx1* expression can account for all major patterning differences. *Development* **133**, 1347-1357.
- Hammond, K. L. and Whitfield, T. T. (2009). Expression of zebrafish *hip*: response to Hedgehog signalling, comparison with *ptc1* expression, and possible role in otic patterning. *Gene Expr. Patterns* **9**, 391-396.
- 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.
- Heisenberg, C.-P., Brand, M., Jiang, Y.-J., Warga, R. M., Beuchle, D., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M. et al. (1996). Genes involved in forebrain development in the zebrafish, *Danio rerio*. *Development* **123**, 191-203.
- Huangfu, D. and Anderson, K. V. (2006). Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. *Development* **133**, 3-14.
- Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.
- Ingham, P. W. and Placzek, M. (2006). Orchestrating ontogeny: variations on a theme by sonic hedgehog. *Nat. Rev. Genet.* **7**, 841-850.
- Kane, D. A. and Kishimoto, Y. (2002). Cell labelling and transplantation techniques. In *Zebrafish: A Practical Approach* (ed. C. Nüsslein-Volhard and R. Dahm), pp. 95-120. Oxford: Oxford University Press.
- Karlstrom, R. O., Trowe, T., Klostermann, S., Baier, H., Brand, M., Crawford, A. D., Grunewald, B., Haffter, P., Hoffmann, H., Meyer, S. U. et al. (1996). Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123**, 427-438.
- 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.
- Koudijs, M. J., den Broeder, M. J., Keijser, A., Wienholds, E., Houwing, S., van Rooijen, E. M., Geisler, R. and van Eeden, F. J. (2005). The zebrafish mutants *dre*, *uki* and *lep* encode negative regulators of the Hedgehog signalling pathway. *PLoS Genet.* **1**, e19.
- Koudijs, M. J., den Broeder, M. J., Groot, E. and van Eeden, F. J. (2008). Genetic analysis of the two zebrafish patched homologues identifies novel roles for the hedgehog signaling pathway. *BMC Dev. Biol.* **8**, 15.
- Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene hedgehog is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.
- Liu, W., Li, G., Chien, J. S., Raft, S., Zhang, H., Chiang, C. and Frenz, D. A. (2002). Sonic hedgehog regulates otic capsule chondrogenesis and inner ear development in the mouse embryo. *Dev. Biol.* **248**, 240-250.

- Méthot, N. and Basler, K.** (2000). Suppressor of fused opposes hedgehog signal transduction by impeding nuclear accumulation of the activator form of Cubitus interruptus. *Development* **127**, 4001-4010.
- Ochi, H., Pearson, B. J., Chuang, P. T., Hammerschmidt, M. and Westerfield, M.** (2006). Hhip regulates zebrafish muscle development by both sequestering Hedgehog and modulating localization of Smoothened. *Dev. Biol.* **297**, 127-140.
- Oh, S.-H., Johnson, R. and Wu, D. K.** (1996). Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J. Neurosci.* **16**, 6463-6475.
- Piotrowski, T., Schilling, T. F., Brand, M., Jiang, Y.-J., Heisenberg, C.-P., Beuchle, D., Grandel, H., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M. et al.** (1996). Jaw and branchial arch mutants in zebrafish II: anterior arches and cartilage differentiation. *Development* **123**, 345-356.
- 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 gofgh* mutation disrupts *tbx1*, which is involved in the DiGeorge deletion syndrome in humans. *Development* **130**, 5043-5052.
- Pittlik, S., Domingues, S., Meyer, A. and Begemann, G.** (2008). Expression of zebrafish *aldh1a3* (*raldh3*) and absence of *aldh1a1* in teleosts. *Gene Expr. Patterns* **8**, 141-147.
- 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.
- Riccomagno, M. M., Takada, S. and Epstein, D. J.** (2005). Wnt-dependent regulation of inner ear morphogenesis is balanced by the opposing and supporting roles of Shh. *Genes Dev.* **19**, 1612-1623.
- 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.
- Schilling, T. F., Walker, C. and Kimmel, C. B.** (1996). The *chinless* mutation and neural crest cell interactions in zebrafish jaw development. *Development* **122**, 1417-1426.
- Sekimizu, K., Nishioka, N., Sasaki, H., Takeda, H., Karlstrom, R. O. and Kawakami, A.** (2004). The zebrafish *iguana* locus encodes Dzip1, a novel zinc-finger protein required for proper regulation of Hedgehog signaling. *Development* **131**, 2521-2532.
- Solomon, K. S., Kwak, S.-J. and Fritz, A.** (2004). Genetic interactions underlying otic placode induction and formation. *Dev. Dyn.* **230**, 419-433.
- van Eeden, F. J. M., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.-P., Jiang, Y.-J., Kane, D. A. et al.** (1996). Genetic analysis of fin formation in the zebrafish, *Danio rerio*. *Development* **123**, 255-262.
- Waldman, E. H., Castillo, A. and Collazo, A.** (2007). Ablation studies on the developing inner ear reveal a propensity for mirror duplications. *Dev. Dyn.* **236**, 1237-1248.
- Westerfield, M.** (1995). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Oregon: University of Oregon Press.
- Whitfield, T. T. and Hammond, K. L.** (2007). Axial patterning in the developing vertebrate inner ear. *Int. J. Dev. Biol.* **51**, 507-520.
- Whitfield, T. T., Granato, M., van Eeden, F. J. M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.-P., Jiang, Y.-J. et al.** (1996). Mutations affecting development of the zebrafish inner ear and lateral line. *Development* **123**, 241-254.
- Wolff, C., Roy, S., Lewis, K. E., Schauerte, H., Joerg-Rauch, G., Kirn, A., Weiler, C., Geisler, R., Haffter, P. and Ingham, P. W.** (2004). *iguana* encodes a novel zinc-finger protein with coiled-coil domains essential for Hedgehog signal transduction in the zebrafish embryo. *Genes Dev.* **18**, 1565-1576.