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

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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+/− and ptc2+/− mutant embryos, in which Hh signalling is maximal throughout the embryo, the inner ear is severely ventralised and mediolateralised, 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)–ZFII), 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
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+/-; lep+/- 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 drez164 (su(fu)), igu294 (dzip1), lep222 (ptc2), ptc1H11032 (ptc1) and ukh223 (hip) (Brand et al., 1996; Heisenberg et al., 1996; Karstemb, 1996; Koudijs et al., 2008; Koudijs et al., 2005; Piotrowski et al., 1996; van Eeden et al., 1996; Whitfield, 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 dixl3b, eya1, fgf8, fat1 (sia – ZFIN), hmx3, msgc, otx1, pax2a, pax3, ptc1, ptc2 (Hammond et al., 2003), hip, pcna (Koudijs et al., 2005), tubl (Piotrowski et al., 2003), fox1 (Solomon et al., 2004) and raldh3 (alldh3 - ZFIN) (Pittlik et al., 2008).

**PCR genotyping**

Genomic DNA was prepared as described (Westerfield, 1995). Primers were: drez, F 5’-TTCTGCTGTCAGGAGGTTTCC-3’, R 5’-CACTGACAAGGCTTACGTA-3’; lep, F 5’-CACATTTAAGGGAACCTTG-3’, R 5’-CATGACCCTTATATTGACC-3’; ptc1, F 5’-GATATAGTGGTGACGTTCTC-3’, R 5’-GAGCTGTGATTCTACGAAAC-3’; uki, F 5’-GGAGGAAGCTCGTCTTAG-3’, R 5’-CCATGTGTTAATAGCTTGTG-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’-GTGGTGGGTTAATTTGGGC-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-I66B3 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+/–; lep+/– 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+/-; lep+/- 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+/-), lep (ptc2+/-), drez (su(fu)+/-) (Koudijs et al., 2005), ptc1+/- (Koudijs et al., 2008) and igu (dzip1+/-) (Sekimizu et al., 2004; Wolff et al., 2004). Hh signalling activity is upregulated in these mutants, ranging from a modest increase in drez to maximal Hh pathway overactivation in ptc1+/-; lep+/- 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+/-), through mild (lep+/-), moderate (igu+/-) and substantial (ptc1+/-; lep+/-) disruption of dorsolateral structures, to severely ventralised and medialised (ptc1+/-; lep+/-) (Fig. 1; Table 1). As the severity of the ear phenotype increased, the dorsolateral septum, endolympathic 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*−/−; *lep*−/−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*−/− mutant ears (Fig. 1B), present but malformed and often positioned incorrectly in *lep*−/− (Fig. 1D) and absent from *dre, igu, uki, ptc1*+/−; *lep*−/− and *ptc1*−/−; *lep*−/− 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 *msx2* 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*+−/−; *lep*−/− 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 separation of the anterior macula and lateral crista domains (arrowhead, Fig. 2E). No cristae formed in *ptc1*+/−; *lep*−/− double-mutant embryos, no canal projections formed (Fig. 1H).

**Endolymphatic duct**

The endolymphatic duct (ED) forms dorsally from an outpocketing of the otic epithelium and is strongly marked by *foxi1* expression, possibly representing an incomplete *msx2* 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*−/− and *ptc1*+/−; *lep*−/− 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*−/−; *lep*−/− embryos (Fig. 2I,J).

**Semicircular canal pillars**

In zebrafish, the anterior (ap), posterior (pp) and ventral (vp) semicircular canal pillars form by fusion of epithelial projection tissue by 3 dpf. In *ptc1*−/− 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*−/−; *lep*−/− embryos, all pillars were very reduced (Fig. 1G). In *ptc1*−/−; *lep*−/− 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 *raldhl3* 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*+/−; *lep*−/− 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 (*pena*) mRNA in this tissue. However, pena 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*−/−; *lep*−/− embryos, in which the anterior macula was positioned slightly medial to normal (Fig. 2F), *iguru*−/−, which had a single medial macula with double posterior character (Fig. 4 and see below).

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**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-H) 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*−/− ears are normal. (C,C’) The dls is absent in *dre*. (D,D’) In *lep* and *dre* the ventral canal pillar (vp) is abnormal. (E-F) In *igu* and *uki* the dls is absent and the ventral canal projection is abnormal. (G,G’) In *ptc1*−/−; *lep*−/− all three canal projections are reduced and the dls is absent. (H,I) In *ptc1*−/−; *lep*−/− 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 μm (bar in A applies to A-H). ap, anterior semicircular canal pillar; ao, anterior otolith; dis, dorsolateral septum; po, posterior otolith; pp, posterior semicircular canal pillar; vp, ventral semicircular canal pillar.
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 *eya1* (ventral), between 25S and 30 hpf (Fig. 3). In *uki*, *igu* and *lep* and *dre*, there were no obvious alterations in otic expression of these markers (data not shown). In *ptc1+/-; lep+/-* otic vesicles, however, medial and ventral otic territories, marked by *pax2a* and *eya1*, 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+/-; lep+/-* otic vesicles (16S to 29 hpf) compared with age-matched siblings (data not shown). At 16S, the cavitating otic vesicle of *ptc1+/-; lep+/-* 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 statocystic ganglion. Interestingly, in zebrafish embryos in which Hh signalling is absent (*dnPKA* or *su fu*), the otic expression patterns of *ptc1*+/−, *lep*−/− and *dre*−/− were very similar, but variable, effect (Fig. 3).

We confirmed that the single *ptc1+/-; lep+/-* 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+/-; lep+/-* 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. 4I); this is never seen in the anterior macula (Fig. 4I) (Haddon et al., 1999). We therefore conclude that the *ptc1+/-; lep+/-* 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+/-; lep+/-* 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+/-; lep+/-* 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+/-; lep+/-* 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+/-; lep+/-* 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Dorsolateral septum</th>
<th>Canal pillars</th>
<th>Cristae</th>
<th>Endolymphatic duct</th>
<th>Maculae</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>Present</td>
<td>wt</td>
<td>3</td>
<td>wt</td>
<td>wt</td>
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<tr>
<td>*ptc1+/-</td>
<td>Present</td>
<td>wt</td>
<td>3</td>
<td>wt</td>
<td>wt</td>
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<tr>
<td><em>dre (su fu)−/−</em></td>
<td>Absent</td>
<td>wt</td>
<td>3</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>*lep (ptc2+/-)</td>
<td>Present but reduced and abnormally positioned</td>
<td>A, P wt V dysmorphic</td>
<td>3 or 2 (L missing)</td>
<td>wt</td>
<td>wt</td>
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<tr>
<td>*igu (dzp1+/-)</td>
<td>Absent</td>
<td>A, P spindly V dysmorphic</td>
<td>3 or 2 (L missing)</td>
<td>wt</td>
<td>P (saccular) macula reduced wt</td>
</tr>
<tr>
<td>*uki (hip+/-)</td>
<td>Absent</td>
<td>A, P spindly</td>
<td>V dysmorphic</td>
<td>3 or 2 (L missing)</td>
<td>Reduced</td>
</tr>
<tr>
<td>*ptc1+/-; lep</td>
<td>Absent</td>
<td>All three very reduced</td>
<td>2 (L missing)</td>
<td>Reduced</td>
<td>A (utricular) macula slightly more medial than usual</td>
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<tr>
<td>*ptc1+/-; lep</td>
<td>Absent</td>
<td>Absent</td>
<td>0</td>
<td>Absent</td>
<td>Single medial macula</td>
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A, anterior; D, dorsal; L, lateral; V, ventral; wt, wild type.

*pptc1+/-; lep+/-* mutants have a striking double-posterior otic phenotype

In addition to the DV and ML otic defects described above, *ptc1+/-; lep+/-* 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+/-; lep+/-* 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).
Manipulation of Hh pathway activity in ptc1<sup>−/−</sup>; lep<sup>−/−</sup> 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 (Concordet 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<sup>−/−</sup>; lep<sup>−/−</sup> double mutants (Hammond et al., 2003) (see Fig. S5 in the supplementary material; data not shown). In our hands, ptc1 and ptc2 levels were not substantially raised in dre, lep and uki mutants, including in the otic vesicle region (data not shown); however, Koudivis et al. (Koudrijs 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<sup>−/−</sup> 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<sup>−/−</sup> mutant, including the somites, are more severely affected than in dre, lep and uki (Koudrijs et al., 2008). The fact that there was no ear phenotype in ptc1<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>; lep<sup>−/−</sup> double mutants. This would reduce Hh signalling levels throughout the embryo downstream of the non-functional Ptc receptors, and gradually rescue otic

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**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) msx3 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,H) Three crista (ac, lc, pc) are present in wild-type ears. (B,E,H) uki mutants with reduced lateral crista; the medial region of the lateral crista is sometimes displaced towards the anterior macula (arrowheads, E,H). (C,F) ptc1<sup>−/−</sup>; lep<sup>−/−</sup> mutants with no lateral crista. (I-L) The endolymphatic duct is reduced in uki embryos and absent in ptc1<sup>−/−</sup>; lep<sup>−/−</sup> double mutants: in situ hybridisation to foxi1 at 68 hpf (I,J) and 72 hpf (K,L). (M-T) The ventral semicircular canal pillars (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,J,L,P,T) lateral views; anterior to left, dorsal to top. (D-H,M,Q) dorsal views; anterior to left, medial to top. (Q,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 crista. ac, anterior crista; am, anterior macula; ds, dorsolateral septum; lc, lateral crista; pm, posterior crista; pm, posterior macula.

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**ptc1<sup>−/−</sup>; lep<sup>−/−</sup>** homozygotes from 1SS, in order to reduce Hh signalling slightly. Using 0.25 μM cyclopamine, the triangular ptc1<sup>−/−</sup>; lep<sup>−/−</sup> 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, 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–/–; lep–/– embryos. (A–L) Expression of lateral (tbx1), medial (pax2a), dorsal (dx3b) and ventral (eya1) markers in the zebrafish otic vesicle. Expression domains of dorsolateral markers (tbx1, dx3b) are reduced relative to ear size in ptc1–/–; lep–/– 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. Scale bars: 50 μm (as shown in left-hand image of each trio).

Inhibition of Hh signalling is required for inner ear patterning between 175 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–/–; lep–/– 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 175 hpf, 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 in the supplementary material). This contrasts with a requirement for Hh signalling before 155 hpf for correct AP otic patterning (see Fig. S7 in the supplementary material).
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 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. 7J). 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 (dzp1<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 mediolateral and ventralised. By contrast, the effect of Hh signalling on otic AP patterning is only morphologically obvious at the extremes of Hh
pathway activity, in ptc1–/–; lep–/– 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 (Koudsijs 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)+) and lep (ptc2+) 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–/–; lep–/– embryos with 40 μM 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–/– mutants is more severe than that of dre, lep, ugi and uki mutants, ptc1–/– embryos have normal ears, whereas dre, lep, ugi 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–/– mutants, and this appears to be able to compensate for the absence of ptc1 function in the ear. The reverse, however, is not true: ptc1 is only minimally upregulated in lep–/– 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–/–; lep–/– fish is more severe than the lep phenotype, and the ptc1–/–; lep–/– phenotype is yet

### Table 2. Application of cyclopamine to ptc1–/–; lep–/– double mutants can phenocopy the weaker Hh inhibitor mutant otic phenotypes

<table>
<thead>
<tr>
<th>Cyclopamine (μM)</th>
<th>ptc1–/+; lep-like</th>
<th>Larger vesicle than ptc1–/+; lep/no canal projections</th>
<th>Only P canal projection</th>
<th>Only A and P canal projections</th>
<th>ptc1–/+; igu/uki-like</th>
<th>No (or very thin) dls</th>
<th>lep-like</th>
<th>Rescued (wild-type)</th>
<th>Dead</th>
<th>Total</th>
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A, anterior; dls, dorsolateral septum; P, posterior.

### Table 3. Inhibition of Hh signalling is required for otic development between 175 and 48 hpf

<table>
<thead>
<tr>
<th>Time cyclopamine applied</th>
<th>Rescue (wild-type)</th>
<th>lep-like</th>
<th>3 pros + septae/large ear</th>
<th>3 pros/medium ear</th>
<th>3 pros/very small ear</th>
<th>V proj only</th>
<th>ptc1–/+; ptc2–/+ like</th>
<th>Total</th>
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<tbody>
<tr>
<td>175 +</td>
<td>4</td>
<td>2</td>
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<td>0</td>
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<td>3</td>
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<td>13</td>
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<td>8</td>
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<tr>
<td>33 hpf +</td>
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<td>10</td>
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<td>38 hpf +</td>
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<td>10</td>
<td>10</td>
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<tr>
<td>48 hpf +</td>
<td>3</td>
<td>3</td>
<td>3</td>
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Corresponding images of each ear phenotype are shown in Fig. S5 in the supplementary material.

3 pros, anterior, posterior and ventral canal pillars/projections present; V proj, only the ventral canal projection is present.
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-/-; lep-/- 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-/-; lep-/- 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 and provide feedback signals from the host. Indeed, when ptc1-/-; lep-/- 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 (utricule-
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 was funded by grants from the BBSRC (BB/E015875/1) to T.T.W. and the many members of the zebrafish community for mutants and probes. This work required for Hh signalling in ventral regions of the otic vesicle, aris (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).

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

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