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Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear

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Organization of the vertebrate inner ear is mainly dependent on localized signals from surrounding tissues. Previous studies demonstrated that sonic hedgehog (Shh) secreted from the floor plate and notochord is required for specification of ventral (auditory) and dorsal (vestibular) inner ear structures, yet it was not clear how this signaling activity is propagated. To elucidate the molecular mechanisms by which Shh regulates inner ear development, we examined embryos with various combinations of mutant alleles for *Shh*, *Gli2* and *Gli3*. Our study shows that Gli3 repressor (R) is required for patterning dorsal inner ear structures, whereas Gli activator (A) proteins are essential for ventral inner ear structures. A proper balance of Gli3R and Gli2/3A is required along the length of the dorsoventral axis of the inner ear to mediate graded levels of Shh signaling, emanating from ventral midline tissues. Formation of the ventral-most otic region, the distal cochlear duct, requires robust Gli2/3A function. By contrast, the formation of the proximal cochlear duct and saccule, which requires less Shh signaling, is achieved by antagonizing Gli3R. The dorsal vestibular region requires the least amount of Shh signaling in order to generate the correct dose of Gli3R required for the development of this otic region. Taken together, our data suggest that reciprocal gradients of GliA and GliR mediate the responses to Shh signaling along the dorsoventral axis of the inner ear.

KEY WORDS: Inner ear, Gli, Sonic hedgehog, Shh, Activator, Repressor, Mouse

INTRODUCTION

The vertebrate inner ear comprises two major components: the dorsal vestibular structures and the ventral cochlear duct, which are responsible for balance and hearing, respectively. Proper patterning of the intricate inner ear from its anlagen, the otocyst, requires signaling from surrounding tissues such as the neural tube, mesoderm and neural crest cells (Barald and Kelley, 2004; Fekete and Wu, 2002; Torres and Giraldez, 1998).

Shh, a member of the Hedgehog family of secreted signaling proteins, is essential for many aspects of vertebrate development (Ingham and McMahon, 2001). Mutations in the *Shh* gene or inappropriate regulation of the Shh signaling pathway causes cancers in humans (Pasca di Magliano and Hebrok, 2003; Taipale and Beachy, 2001), as well as developmental defects in a variety of organs in both humans and mice (Bale, 2002; Hammerschmidt et al., 1997; McMahon et al., 2003; Riccomagno et al., 2002).

Inner ear analyses of *Shh*-knockout mouse embryos, as well as midline ablation experiments in chicken embryos, demonstrated that Shh emanating from the floor plate or notochord is required for the specification of ventral inner ear structures such as the saccule and cochlear duct (Bok et al., 2005; Riccomagno et al., 2002). The expression patterns of *Patched1* (*Ptc1*, also known as *Ptch1* – Mouse Genome Informatics; the receptor for Shh) and *Gli1* (a transcriptional mediator of Shh signaling) within the otic epithelium and periotic mesenchyme, indicate that these cells respond directly to Shh (Riccomagno et al., 2002). However, it was not clear how Shh mediates formation of the various inner ear structures. Deciphering the molecular mechanisms underlying Shh signal transduction in the ear is of fundamental importance considering that mutations in genes associated with this pathway, including *SALL1* and *GLI3*, are known to cause deafness in humans (Kohlhase et al., 1998) (E. C. Driver and M. W. Kelley, personal communication).

Cubitus interruptus (Ci), is the major mediator of Hedgehog (Hh) signaling in *Drosophila* (Aza-Blanc and Kornberg, 1999; Ingham and McMahon, 2001). The three vertebrate Ci homologs, Gli1, Gli2 and Gli3, are also thought to regulate most, if not all, of the transcriptional responses to Hh signaling (Ingham and McMahon, 2001). Gli1 contains a transcriptional activator domain and was shown to positively regulate expression of Hh target genes (Ingham and McMahon, 2001). However, *Gli1*-null mutants are viable and do not show any obvious developmental defects (Bai et al., 2002; Park et al., 2000). Gli2, by contrast, is required for the proper formation of several organs including the lung and CNS (Ding et al., 1998; Matise et al., 1998; Motoyama et al., 1998). Despite the presence of both transcriptional activator and repressor domains, Gli2 is thought to act predominantly as a positive regulator of Hh target gene expression (Bai and Joyner, 2001).

Gli3 functions as either a transcriptional activator or repressor, depending on the availability of Hh protein (Wang et al., 2000). In the absence of any Hh input, the C-terminal region of Gli3 is cleaved, and the truncated N-terminal domain serves to repress the transcription of Hh target genes (Wang et al., 2000). In the presence of Hh, Gli3 is not cleaved and can operate as a transcriptional activator (Dai et al., 1999; Bai et al., 2004). The Hh-dependent regulation of Gli3 processing is essential for many different aspects of embryonic development, including limb digit number and identity, dorsoventral patterning of the neural tube and somites, as well as various aspects of renal, bone and mammary gland development (Ahn and Joyner, 2004; Buttitta et al., 2003; Hatsell and Cowin, 2006; Hu et al., 2006;

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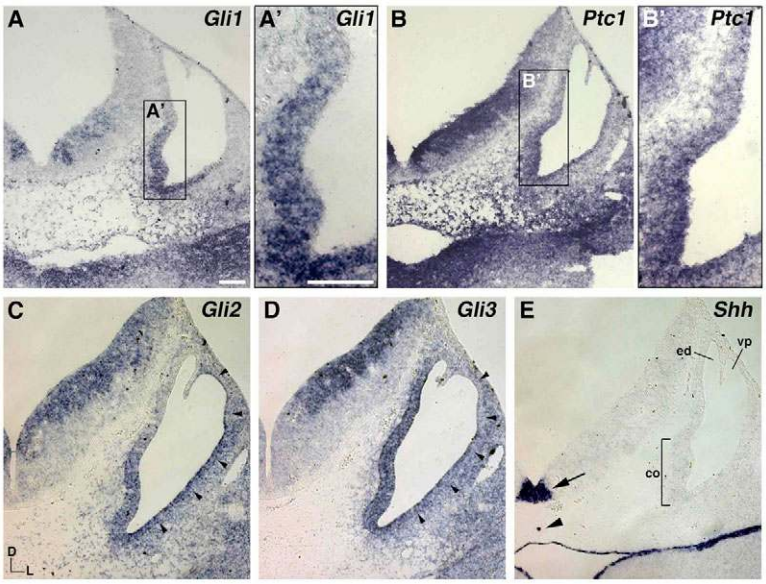

Fig. 1. Expression patterns of *Shh*, *Ptc1* and the Gli family transcription factors in the developing inner ear at 11 dpc. (**A**,**A′**) *Gli1* and (**B**,**B′**) *Ptc1* show a graded expression pattern in the otocyst, strongest in the ventral-medial tip of the otocyst and weaker towards the dorsal region. Weaker hybridization signals are also found in the dorsal-lateral region of the otic epithelium as well as in the mesenchyme. (**C**) Gli2 mRNA is broadly expressed throughout the otic epithelium but its expression in the endolymphatic duct (ed) is barely detectable. (**D**) Gli3 expression is evident in the entire otocyst, with weaker expression in the dorsal-lateral side. Both Gli2 and Gli3 transcripts are also detected in the mesenchymal tissues, especially in the lateral region of the otocyst. Arrowheads (C,D) indicate the margin of the otic epithelium. (**E**) Shh is strongly expressed in the ventral midline structures, the floor plate (arrow) and notochord (arrowhead), but its transcripts are not detectable in the otocyst. vp, vertical pouch; co, cochlea; D, dorsal; L, lateral. Scale bars: in A, 100 μm for A-E; in A′, 100 μm for A′,B′.

Koziel et al., 2005; Litingtung and Chiang, 2000; Litingtung et al., 2002; McDermott et al., 2005; Persson et al., 2002; Rallu et al., 2002).

Mutations in *GLI3* are responsible for several human disorders including Pallister-Hall syndrome (PHS) (Kang et al., 1997), Greig cephalopolysyndactyly syndrome (GCPS) (Vortkamp et al., 1991) and postaxial polydactyly type A (Radhakrishna et al., 1997). Several mouse models with mutations in *Gli3*, similar to those reported in human subjects, are available. For example, *Extra-toes*^{J} (*Xt*^{J}) mutants, in which *Gli3* encodes a non-functional transcript (Hui and Joyner, 1993; Maynard et al., 2002), exhibit characteristic defects similar to human GCPS patients (Hui and Joyner, 1993; Vortkamp et al., 1991). Another mouse mutant, *Gli3Δ699*, which only expresses the truncated N-terminal region of *Gli3*, was modeled after mutations found in PHS patients (Böse et al., 2002).

In order to determine how Shh regulates inner ear patterning through the Gli family of transcription factors, we examined the inner ears of embryos carrying various genetic combinations of mutant alleles including *Shh*, *Gli2*, *Xt*^{J} (*Gli3*), and *Gli3Δ699*. Our results show that several mechanisms are involved in mediating Shh signal transduction in the inner ear. For development of the distal region of the cochlear duct, which is positioned closest to the source of Shh in the notochord and floor plate, Gli2 and Gli3 activator (A) functions are required. Formation of the intermediate regions of the ear including the proximal cochlear duct and saccule were found to be dependent on the removal of Gli3 repressor (R). Interestingly, the vestibular defects observed in *Shh*$^{-/-}$ embryos could be rescued by removing one but not both alleles of *Gli3*, suggesting that Gli3R functions in a dose-dependent manner to mediate vestibular development. Taken together, our data suggest that reciprocal gradients of GliA and GliR mediate the responses to Shh signaling along the dorsoventral axis of the inner ear.

MATERIALS AND METHODS

Mice

Shh$^{+/-}$ and *Gli3*$^{+/Xt\text{-}J}$ mice obtained from Jackson Laboratories (Bar Harbor, ME) and *Gli2*$^{+/-}$ mice obtained from Dr Alex Joyner (New York University School of Medicine, NY) were used to generate the compound heterozygotes and homozygotes described. Genotyping was performed by PCR using primers described previously (Chiang et al., 1996; Matise et al., 1998; Maynard et al., 2002; Böse et al., 2002).

Paint-fill and in situ hybridization

Paint-fill analyses and in situ hybridization experiments were performed as described previously (Morsli et al., 1998). The lengths of cochlear ducts were determined from ventral views of paint-filled cochlear ducts by measuring their outer contours using the ImageJ program (National Institutes of Health). The lengths of the mutant cochlear ducts were compared with those of *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ littermates. Riboprobes were prepared as previously described: *Shh* (Echelard et al., 1993), *Ptc1* (Milenkovic et al., 1999), *Gli1*, *Gli2* and *Gli3* (Hui and Joyner, 1993), *Otx1*, *Otx2* and *Bmp4* (Morsli et al., 1999), and *Msx1* (Satokata and Maas, 1994).

Cell proliferation and programmed cell death assays

Cell proliferation (BrdU labeling) and TUNEL (terminal dUTP nick-end labeling) assays were performed as described (Burton et al., 2004).

RESULTS

Expression patterns of Shh, *Ptc1* and Gli genes in the developing inner ear

To understand how Shh exerts its effect on the development of inner ear structures in mouse, we first examined the expression of Hh signaling components in and around the ear. At the otocyst stage, *Shh* mRNA is not detected in the otic epithelium, but is strongly expressed in the floor plate and notochord (Fig. 1E, arrow and arrowhead) (Bok et al., 2005; Riccomagno et al., 2002). By contrast, both *Ptc1* and *Gli1*, which are transcriptional readouts of Shh signaling (Bai et al., 2002; Dai et al., 1999; Goodrich et al., 1996), displayed graded expression patterns in the otocyst: stronger in the ventral-medial region closest to the source of Shh and weaker in the dorsal-lateral region from 9.5 to 12.5 dpc (Fig. 1A-B′ and see Fig. S1 in the supplementary material). These expression patterns of *Ptc1* and *Gli1* indicate that the otic epithelium responds directly and differentially to Shh signaling. The expression domains of *Gli2* and *Gli3* were not limited to the medial-ventral region but also included the lateral portion of the otocyst as well as the surrounding mesenchyme (Fig. 1C,D). In addition, the expression of *Gli2* or *Gli3* in the otic epithelium does not appear to be regulated by Shh signaling (see Fig. S2 in the supplementary material).

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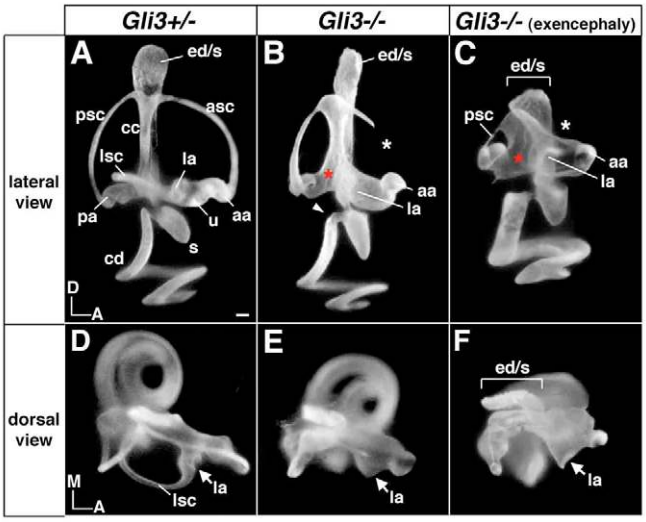

Fig. 2. Inner ear phenotypes of Gli3$^{-/-}$ mutants. Paint-filled inner ears of *Gli3* heterozygous (**A**,**D**) and homozygous (**B**,**C**,**E**,**F**) embryos at 15.5 dpc shown in lateral (A-C) and dorsal (D-F) views. *Gli3*$^{-/-}$ inner ears are missing the lateral canal (red asterisks), but the lateral ampulla is present (arrows in E,F). The anterior canal is usually missing or truncated (white asterisks), and the caliber of the posterior canal is smaller. The saccule and the proximal tip of the cochlear duct are misshapen, but the length of the cochlear duct is normal. Inner ear phenotypes in mutants with exencephaly are more severe (C,F). aa, anterior ampulla; asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed/s, endolymphatic duct and sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle, D, dorsal; A, anterior; M, medial. Scale bar: 100 μm.

Vestibular morphology is altered in *Gli3*$^{-/-}$ embryos

Given the expression patterns of the three Gli transcription factors in the otocyst, we postulated that Shh function in inner ear patterning is mediated through the Gli factors. Since *Gli1*$^{-/-}$ mice are normal (Bai et al., 2002; Park et al., 2000), we focused our attention on *Gli2* and *Gli3* mutant embryos. The inner ears of *Gli2*$^{-/-}$ embryos did not display any obvious morphological defects as assessed by paint-fill analyses (see below). In examining the ears of *Gli3*$^{-/-}$ embryos, a number of vestibular defects were observed, including the loss of the lateral semicircular canal (Fig. 2B,C, red asterisks; *n*=30) and, in 87% of the embryos examined, truncation or absence of the anterior semicircular canal (Fig. 2B,C, white asterisks; *n*=26/30). The lateral and anterior ampullae and associated sensory organs were still present in *Gli3*$^{-/-}$ embryos (Fig. 2B,C,E,F; data not shown). The posterior semicircular canal was usually present, although its diameter was often narrower (Fig. 2; *n*=29/30). The ventral inner ear structures of *Gli3*$^{-/-}$ embryos were largely normal, except for a slight misshaping of the saccule and the most proximal tip of the cochlear duct (Fig. 2B, arrowhead). These results are consistent with the histological analyses published previously (Johnson, 1967).

Approximately one third of the *Gli3*$^{-/-}$ embryos were exencephalic, and their inner ears were usually smaller in size and had widened endolymphatic ducts, common cruses and cochlear ducts (Fig. 2C,F; *n*=11/30). We attributed the more severe inner ear phenotypes to the abnormal brain development in these mutant embryos. Taken together, these results indicate that Gli3 function is important for the development of dorsal vestibular structures.

Gli3R is required for dorsal vestibular development

To examine whether Shh-dependent regulation of Gli3 activities is also important for inner ear development, we analyzed the ears of embryos carrying various combinations of *Shh* and *Gli3* mutant alleles. The inner ears of *Shh*$^{-/-}$ embryos lacked ventral otic derivatives, including the cochlear duct and saccule (Fig. 3B, asterisk), and were missing dorsal vestibular structures such as the lateral semicircular canal/ampulla and the endolymphatic duct (Fig. 3B) (Riccomagno et al., 2002). Even though *Gli3* expression in the inner ear is not regulated by Shh (see Fig. S2 in the supplementary material), when one copy of the *Gli3* gene is mutated on a *Shh*$^{-/-}$ background (*Shh*$^{-/-}$;*Gli3*$^{+/-}$), the morphology of the vestibule was completely rescued to its proper integrity (Fig. 3). By contrast, only a rudimentary knob-like structure was evident ventrally (Fig. 3C, arrow).

We next analyzed the inner ears of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants. Since all of these embryos exhibited exencephaly, they were compared with exencephalic *Gli3*$^{-/-}$ single mutants (Fig. 2C). The dorsal vestibular structures that were rescued in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos were malformed in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (*n*=6), and resembled the dorsal otic defects observed in *Gli3*$^{-/-}$ single mutants (compare Fig. 2C with Fig. 3D). For

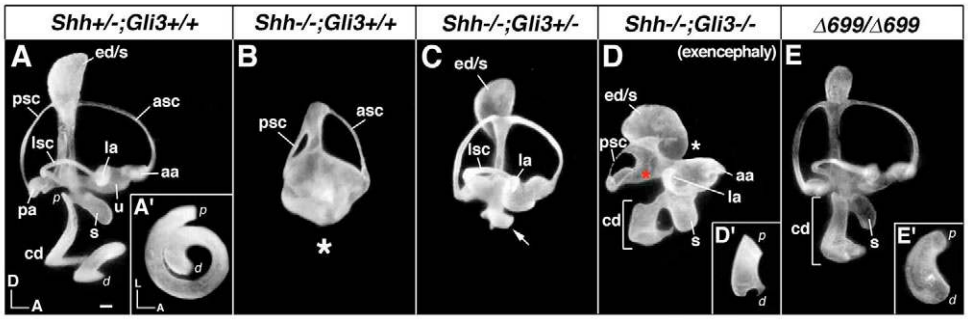

Fig. 3. Inner ears of Shh and Gli3 compound mutants. Paint-filled inner ears of (**A**) *Shh*$^{+/-}$;*Gli3*$^{+/+}$, (**B**) *Shh*$^{-/-}$;*Gli3*$^{+/+}$, (**C**) *Shh*$^{-/-}$;*Gli3*$^{+/-}$, (**D**) *Shh*$^{-/-}$;*Gli3*$^{-/-}$ and (**E**) *Gli3Δ699* mutants at 14.75 dpc. (B) *Shh*$^{-/-}$;*Gli3*$^{+/+}$ inner ears lack ventral structures, including the cochlear duct and saccule (asterisk). Dorsally, the lateral semicircular canal/ampulla and endolymphatic duct are missing. (C) The dorsal structures in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ inner ears are normal, but only a small knob-like structure is present ventrally (arrow). (D) In *Shh*$^{-/-}$;*Gli3*$^{-/-}$ ears, the dorsal structures are malformed, and a shortened cochlear duct is present. (E) Inner ears of *Gli3Δ699* homozygotes show normal dorsal inner ear phenotypes, yet the cochlear duct is shortened. (A′,D′,E′) Ventral views of respective cochlear ducts. For abbreviations refer to Fig. 2; *p*, proximal; *d*, distal. Scale bar: 100 μm.

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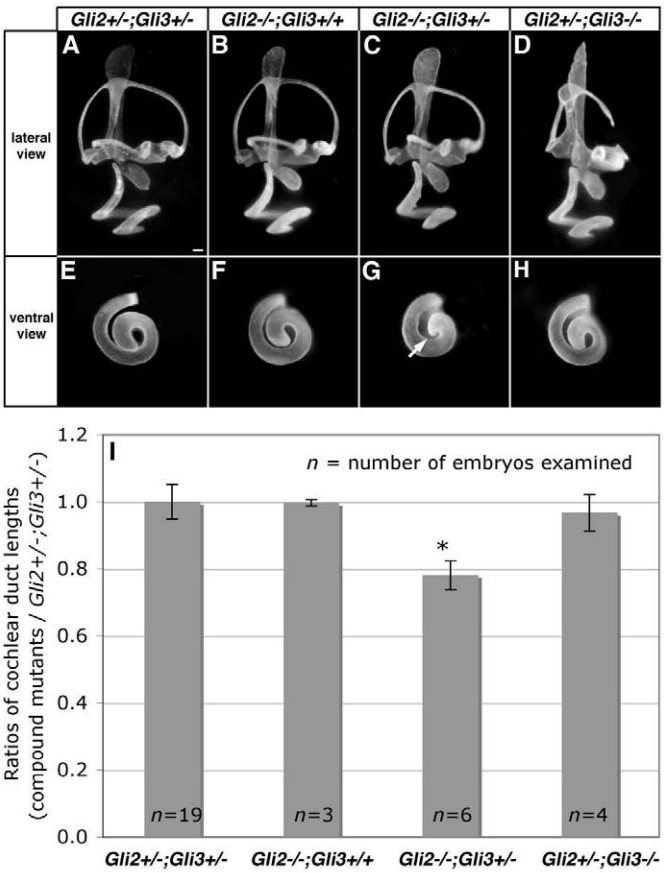

Fig. 4. Inner ears of *Gli2* and *Gli3* compound mutants. (**A-D**) Lateral views of inner ears and (**E-H**) ventral views of cochlear ducts of (A,E) *Gli2*$^{+/-}$;*Gli3*$^{+/-}$, (B,F) *Gli2*$^{-/-}$;*Gli3*$^{+/+}$, (C,G) *Gli2*$^{-/-}$;*Gli3*$^{+/-}$, (D,H) *Gli2*$^{+/-}$;*Gli3*$^{-/-}$ embryos at 14.75 dpc. The dorsal region of the inner ears is normal (A-C), except in *Gli2*$^{+/-}$;*Gli3*$^{-/-}$ (D). By contrast, the length of the cochlear duct in *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ is shortened (G, arrow). (**I**) Bar chart showing ratios of cochlear duct lengths between compound mutants and their *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ littermate controls. Asterisk indicates an average ratio that is significantly different from others (P<0.0001; Student's unpaired t-test). Scale bar: 100 μm.

example, as with the *Gli3*$^{-/-}$ inner ears (Fig. 2C), the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants lacked the lateral and anterior semicircular canals (Fig. 3D, asterisks), but retained their associated sensory organs, the ampullae and cristae (Fig. 3D; data not shown). Moreover, a thinner posterior semicircular canal was also a common phenotype for *Gli3*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (Fig. 2C and Fig. 3D). Ventrally, the saccule and the proximal portion of the cochlear duct, which fail to form in *Shh*$^{-/-}$ mutants, were rescued in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Fig. 3B,D). The partial rescue of inner ear structures in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants is likely to be the result of a dose dependency in the amount of Gli3R that is needed for vestibular and proximal cochlear development, similar to what has been reported for other tissues (Litingtung and Chiang, 2000; Litingtung et al., 2002; Rallu et al., 2002). Our results suggest that vestibular development is particularly sensitive to the level of Gli3R, with embryos that possess too much (*Shh*$^{-/-}$) or too little (*Gli3*$^{-/-}$) succumbing to defects, which can be rescued in a genetic context that restores the proper amount (*Shh*$^{-/-}$;*Gli3*$^{+/-}$).

Inner ear analysis of *Gli3Δ699* embryos, a mouse model of human PHS

The partial rescue of ventral inner ear structures in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants was limited to the saccule and proximal cochlear duct (Fig. 3D), suggesting that eliminating Gli3R alone is insufficient to restore the formation of the entire cochlear duct. Since the distal cochlear duct is presumably receiving higher levels of Shh than other regions, based on the expression patterns of *Ptc1* and *Gli1* (Fig. 1), we reasoned that Shh-mediated GliA function is required for the development of this portion of the cochlea. This hypothesis is supported by the inner ear phenotypes of *Gli3Δ699* mutants, which only express the N-terminal, repressor form of Gli3 (Fig. 3E). In *Gli3Δ699/Δ699* embryos, the cochlear duct was shortened and missing the distal region (Fig. 3E; n=3). Given that the length of the cochlear duct was normal in *Gli3*$^{-/-}$ embryos (Fig. 2B), these findings suggest that the truncated Gli3 protein derived from the *Gli3Δ699* allele might act as a competitive inhibitor of at least some GliA function (see below). The normal vestibular morphology demonstrated by *Gli3Δ699/Δ699* embryos (Fig. 3E) is consistent with our previous findings that dorsal vestibular structures are dependent on Gli3R rather than Gli3A function.

Requirement of GliA during distal cochlear duct development

Absence of the distal cochlear duct in *Gli3Δ699* mutants coupled with the lack of cochlear phenotypes in *Gli2*$^{-/-}$ (Fig. 4B,F,I) and *Gli3*$^{-/-}$ (Fig. 2) single mutants, suggested that the N-terminal truncated Gli3 protein could be acting to inhibit multiple Gli activators. We therefore asked whether GliA function is indeed required for distal cochlear duct development. Since the majority of *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutants die by 14.5 dpc, we first examined inner ear morphology in *Gli2*/*Gli3* compound mutants. Cochlear duct lengths were measured in each of the compound mutants and compared with *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ double heterozygous littermates, which exhibited normal cochlear ducts (Fig. 4A,E,I). Compound mutant embryos carrying one wild-type *Gli3* allele (*Gli2*$^{-/-}$;*Gli3*$^{+/-}$) displayed a modest yet significant reduction in the length of their cochlear ducts (Fig. 4C,G,I, arrow). By contrast, embryos with only one wild-type *Gli2* allele (*Gli2*$^{+/-}$;*Gli3*$^{-/-}$) had normal cochlear duct lengths (Fig. 4D,H,I). These results suggest that Gli2 and Gli3 have redundant functions in cochlear duct outgrowth, yet the activator role of Gli2 is more pronounced than that of Gli3.

Through the course of our studies, a few viable *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutants were recovered at 13.5 dpc (n=3). We compared the inner ears of these double mutants with those of *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ and other compound mutants (Fig. 5). Cochlear duct development was severely impaired in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ embryos, with only the formation of a short proximal region comparable to what was observed in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Fig. 5B,C). This result indicates that, with the exception of the most proximal region, cochlear duct development is dependent on Gli2/Gli3 activator function. With respect to the vestibule, the defects observed in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutants closely resembled those from *Gli3*$^{-/-}$ single and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (Fig. 5B-D), emphasizing once again the importance of Gli3R over Gli3A in dorsal vestibular development.

Shortened cochlear ducts are missing the distal cochlear region

To determine whether the cause of the shortened cochlear ducts observed in various mutants reported here (Fig. 3D,E; Fig. 4C,G; Fig. 5B) results from a specific deficiency in the formation of the

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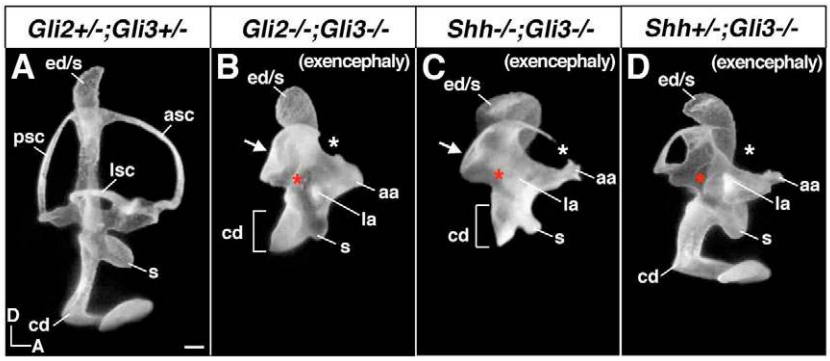

Fig. 5. Inner ears of *Gli2* and *Gli3* compound mutants at 13.5 dpc. Lateral views of inner ears of (**A**) *Gli2*$^{+/-}$;*Gli3*$^{+/-}$, (**B**) *Gli2*$^{-/-}$;*Gli3*$^{-/-}$, (**C**) *Shh*$^{-/-}$;*Gli3*$^{-/-}$, (**D**) *Shh*$^{+/-}$;*Gli3*$^{-/-}$ mutants at 13.5 dpc. (B) *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ inner ear has a shortened cochlear duct that is comparable to that of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (C). The dorsal defects shown in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ inner ears are similar to those in *Gli3*$^{-/-}$ single (D) and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (C). White and red asterisks indicate the lack of anterior and lateral semicircular canals, respectively. There is a developmental delay in inner ears of *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants, as indicated by the lack of resorption of the posterior semicircular canal (arrows). For abbreviations refer to Fig. 2. Scale bar: 100 μm.

distal region rather than a general failure in the growth of the entire duct, we examined the expression of *Msx1*, which marks the distal tip of the cochlear duct (Fig. 6F, arrow), in conjunction with the expression of *Otx2*, a general marker of the ventral otic region. *Otx2* is expressed along the lateral side of the entire saccule and cochlear duct anlagen (Fig. 6A) (Morsli et al., 1999). Neither *Msx1* nor *Otx2* was expressed in the otocysts of *Shh*$^{-/-}$ embryos, consistent with the absence of ventral inner ear structures in these mutants (Fig. 6B,G). Interestingly, the partial restoration of the proximal-most region of the cochlea in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos was coincident with the presence of *Otx2* expression (Fig. 6C,D). By contrast, *Msx1* remained absent from the ventral tip of these inner ears (Fig. 6H,I, red asterisks), indicating that the cochlear duct of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants is indeed missing the distal region. Similar results were found in the inner ears of *Gli3Δ699* and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (Fig. 6E,J,N,R). Despite possessing a slightly

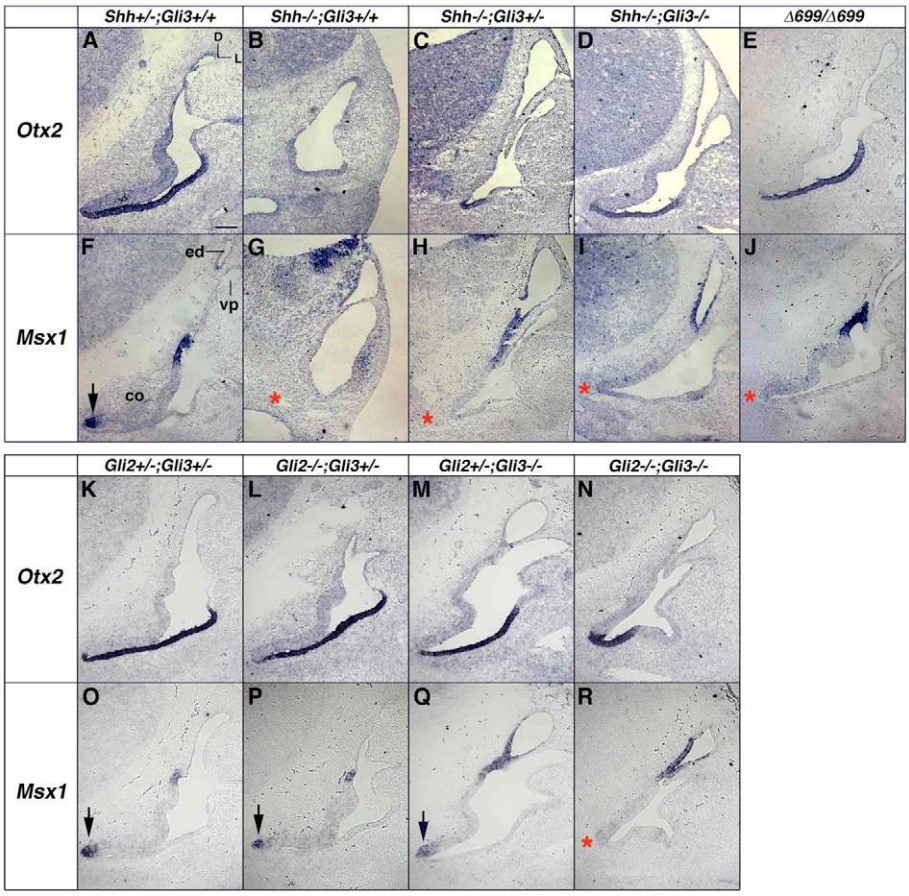

Fig. 6. Expression patterns of *Otx2* and *Msx1* in various *Shh*, *Gli2* and *Gli3* compound mutants. (**A-J**) Expression patterns of *Otx2* (A-E) and *Msx1* (F-J) in the developing cochlear duct of *Shh;Gli3* compound mutants at 12.5 dpc. (A) *Otx2* is expressed in the lateral wall of the developing cochlear duct in wild type. (B) There is no expression of *Otx2* in *Shh*$^{-/-}$ mutants. (C) Otx2 is expressed in the ventral-lateral wall of the *Shh*$^{-/-}$;*Gli3*$^{+/-}$ inner ears. (D,E) Strong *Otx2* expression is observed in the ventral-lateral wall of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (D) and *Gli3Δ699* (E) embryos. (F) *Msx1* is expressed at the distal tip of the cochlear duct (arrow) and the endolymphatic duct of wild-type embryos. (G,H) In *Shh*$^{-/-}$ (G) or *Shh*$^{-/-}$;*Gli3*$^{+/-}$ (H) embryos, in which cochlear duct is not developed, *Msx1* expression is not observed ventrally (asterisk). (I,J) Even though the cochlear duct is partially formed in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (I) and *Gli3Δ699* (J) mutants, *Msx1* is not observed, indicating that these cochlear ducts are missing the distal region. (**K-R**) Expression patterns of *Otx2* (K-N) and *Msx1* (O-R) in the developing cochlear duct of *Gli2;Gli3* compound mutants at 12.5 dpc. Strong *Otx2* expression in the cochlear duct is shown in all the *Gli2;Gli3* compound mutants (K-N). *Msx1* expression at the distal tip of the cochlear duct is found (arrows) in *Gli2*$^{+/-}$;*Gli3*$^{+/-}$ (O), *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ (P) and *Gli2*$^{+/-}$;*Gli3*$^{-/-}$ (Q) embryos, but not in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (asterisk, R). For abbreviations refer to Fig. 1. Scale bar: 100 μm.

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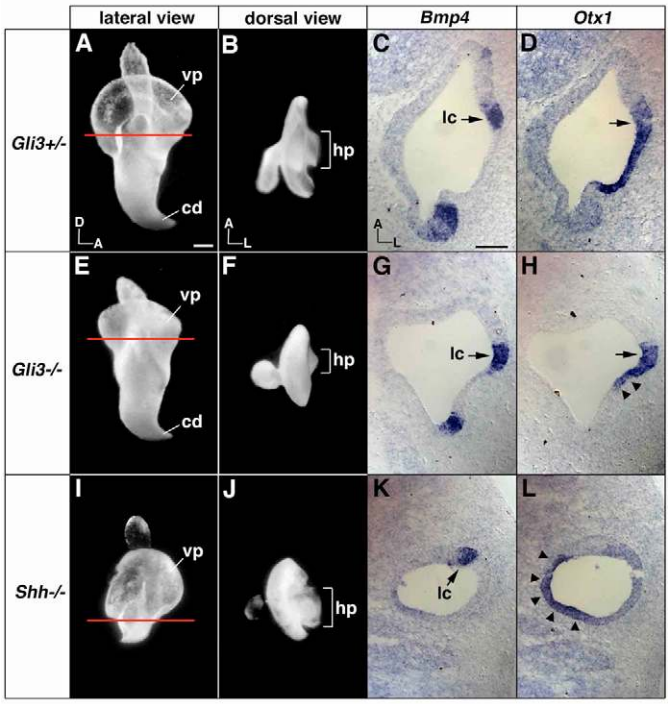

Fig. 7. The lateral canal defects in *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ mutants are caused by different mechanisms. The inner ears of *Gli3*$^{+/-}$ (**A-D**), *Gli3*$^{-/-}$ (**E-H**), and *Shh*$^{-/-}$ (**I-L**) at 11.75 dpc examined by the paint-fill technique (A,B,E,F,I,J) and in situ hybridization (C,D,G,H,K,L). Red lines in A,E,I represent the levels of sections shown in C,D, G,H, and K,L, respectively. (A,B) At 11.75 dpc, the inner ear shows a vertical pouch (vp; develops into the anterior and posterior semicircular canals) dorsally and a horizontal pouch (hp; develops into the lateral semicircular canal) laterally. The developing cochlear duct (cd) is also shown ventrally. (C,D) The location of the prospective lateral crista (lc) is marked by the co-expression of *Bmp4* and *Otx1* (arrows). *Otx1* is also expressed in the prospective lateral canal region. (E,F) The vertical pouch of *Gli3*$^{-/-}$ mutants is smaller than that of heterozygotes. The horizontal pouch is absent or rudimentary. (G,H) The primordial lateral crista is present based on the overlap of expression between *Bmp4* and *Otx1* (arrows). Yet, the *Otx1* expression domain in the canal pouch is much reduced (arrowheads). (I,J) The inner ears of *Shh*$^{-/-}$ mutants show the presence of the horizontal pouch. However, *Otx1* expression, which is normally found in the horizontal pouch, has shifted to the medial side (arrowheads), such that the *Otx1* expression domain no longer overlaps with that of *Bmp4* (K,L). Orientations: D, dorsal; A, anterior; L, lateral. Scale bars: 100 μm.

shortened cochlea (Fig. 4G,I), the ears of *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ embryos expressed *Msx1* (Fig. 6L,P). These results indicate that the reduction in cochlear duct length displayed by the majority of Shh/Gli compound mutants correlates with the specific loss of the distal portion of the cochlear duct.

Lateral semicircular canal defects in *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ mutants arise by different mechanisms

It is intriguing that the same vestibular phenotype, the absence of the lateral canal, manifests in both *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ embryos, given that the dorsal ears of these mutants possess opposing amounts of Gli3R (too much in *Shh*$^{-/-}$ and too little in *Gli3*$^{-/-}$). To better comprehend the nature of these defects we compared the lateral canal phenotypes in *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ embryos at 11.75 dpc. At this stage of development, the primordial structure of the lateral canal, the horizontal pouch, is morphologically evident (Fig. 7B), and the primordial lateral crista can be identified based on the co-expression of *Bmp4* and *Otx1* (Fig. 7C,D) (Morsli et al., 1999).

In *Gli3*$^{-/-}$ mutants, the horizontal and vertical pouches did not form properly and were smaller in size (Fig. 7E,F). Consistently, the *Otx1* expression domain was much smaller, and this is likely to be due to the rudimentary nature of the horizontal pouch (Fig. 7H, arrowheads). Regardless of these defects, the presumptive lateral crista was still present, as evident by the overlapping expression of *Bmp4* and *Otx1* (Fig. 7G,H, arrow). These observations indicate that the lateral canal defect in *Gli3*$^{-/-}$ mutants is already evident at 11.75 dpc, the stage of horizontal pouch outgrowth.

Unlike in the *Gli3*$^{-/-}$ embryos, the horizontal pouch in *Shh*$^{-/-}$ mutants appeared morphologically normal at 11.75 dpc (Fig. 7J). This indicates that the lateral canal defect in *Shh*$^{-/-}$ embryos has a later onset than in *Gli3*$^{-/-}$ embryos (Fig. 7F). Despite the differences in the timing of these phenotypes, the expression of *Otx1* at the level of the lateral crista, which is normally restricted to the lateral region of the horizontal pouch (Fig. 7D), was shifted to the medial side of the otic vesicle in *Shh*$^{-/-}$ embryos (Fig. 7L, arrowheads) and no longer overlapped with *Bmp4* (Fig. 7K). *Otx1* is required for the formation of the lateral crista and canal (Morsli et al., 1999). In *Otx1*$^{-/-}$ mutants, *Bmp4* expression in the presumptive lateral crista was initially normal but failed to be maintained by 12 dpc. Therefore, we attribute the eventual loss of the lateral crista and canal in *Shh*$^{-/-}$ mutants to this misexpression of *Otx1*.

Evidence in further support of the view that the lateral canal phenotypes in *Shh*$^{-/-}$ and *Gli3*$^{-/-}$ mutants are etiologically different, comes from the analysis of cell proliferation and programmed cell death in the lateral canal region at 11.75 dpc. Normally (in *Gli3*$^{+/-}$), there is robust cell proliferation (BrdU labeling) and little cell death (TUNEL) in the horizontal pouch area (Fig. 8A-C). In *Gli3*$^{-/-}$ mutants, we observed a decrease in cell proliferation and an increase in cell death in the lateral wall, where the horizontal pouch normally forms (Fig. 8D-F, arrowheads). By contrast, in *Shh*$^{-/-}$ mutants, there was no obvious change in cell proliferation and programmed cell death in the horizontal pouch region at this stage (Fig. 8G-I), although there was an increase in cell death in the medial and anterior regions of the otic vesicle (Fig. 8I,I′, arrowheads). Together, these observations indicate that the lateral semicircular canal is sensitive to the dose of Gli3R, with too much (*Shh*$^{-/-}$) or too little (*Gli3*$^{-/-}$) causing an arrest in its development, and that there are temporal and etiological differences in these requirements.

DISCUSSION

The distal cochlear duct requires Gli activator function

Our results indicate that the distal region of the cochlear duct located closest to the sources of Shh in the ventral midline requires redundant activator functions of Gli2 and Gli3 proteins, induced by relatively high levels of Shh signaling as compared with other portions of the inner ear (Fig. 9). The cochlear duct develops normally in the *Gli2* or *Gli3* single mutant, and in compound mutants of *Gli2* and *Gli3* provided that one wild-type allele of *Gli2* is present. The most significant roles assumed by Gli2 and Gli3 in cochlear duct morphogenesis are realized in *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ mutants, in which the distal portion of the cochlear duct is malformed and absent, respectively. The fact that the activator role of *Gli2* is more important than that of *Gli3* in cochlear duct formation differs from the situation in somites, where the

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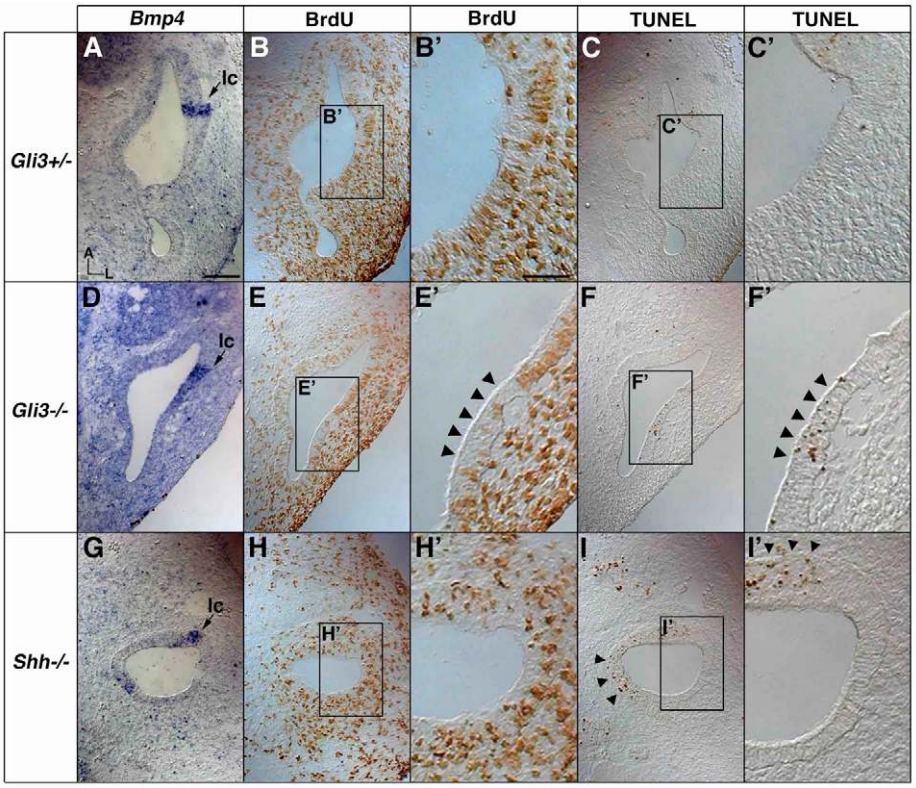

Fig. 8. Cell proliferation and programmed cell death in the developing lateral canal region of *Gli3*$^{-/-}$ and *Shh*$^{-/-}$ mutants. *Bmp4* expression (**A**,**D**,**G**), BrdU labeling (**B**,**E**,**H**) and TUNEL staining (**C**,**F**,**I**) in *Gli3*$^{+/-}$ (A-C), *Gli3*$^{-/-}$ (D-F), and *Shh*$^{-/-}$ (G-I) inner ears at 11.75 dpc. (A-C) Robust cell proliferation and little programmed cell death are observed in the primordial lateral canal region in *Gli3*$^{+/-}$ ears. The presumptive lateral crista is *Bmp4* positive. (D-F) Markedly decreased cell proliferation and increased programmed cell death (arrowheads) are found in the lateral wall of the inner ears of *Gli3*$^{-/-}$ mutants. (G-I) In *Shh*$^{-/-}$ mutants, no changes in cell proliferation (H) and programmed cell death (I) are observed in the primordial lateral canal area. Yet, there is increased cell death in the medial and anterior wall of the inner ear (I,I′, arrowheads). Orientations: A, anterior; L, lateral. Scale bar: 100 μm.

presence of one allele of either *Gli2* or *Gli3* is sufficient to induce *Shh* target genes in presomitic mesoderm (Buttitta et al., 2003) and *Myf5* in epaxial muscle progenitor cells (McDermott et al., 2005).

It is our contention that the removal of Gli3R is insufficient to mediate normal distal cochlear duct outgrowth because the lack of Gli3R in the absence of Shh signaling (*Shh*$^{-/-}$;*Gli3*$^{-/-}$) fails to restore full cochlear development. Moreover, the presence of a shortened cochlear duct in *Gli3Δ699/Δ699* but not *Gli3*$^{-/-}$ embryos supports our claim that the N-terminal truncated Gli3 protein encoded by the *Gli3Δ699* allele is acting in a competitive fashion to block other Gli activators in the cochlear duct. Similar dominant-negative effects of Gli3R have been postulated to occur in the gastrointestinal tract of *Gli3Δ699* mutants (Böse et al., 2002).

Genes such as *Otx2* and *Pax2* are required for ventral patterning of the inner ear (Burton et al., 2004; Morsli et al., 1999) and both of these genes are regulated by Shh signaling (Riccomagno et al., 2002). It has been shown in other systems that little, if any, activator function of Gli proteins exists in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants to induce Shh target genes (Litingtung et al., 2002; te Welscher et al., 2002). The presence of *Otx2* and *Pax2* in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos suggests that these genes do not require Gli activator function and can be induced by alleviating the repressive action of Gli3R. The presence of *Otx2* (Fig. 6) and *Pax2* (data not shown) in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ inner ears is consistent with this notion.

Based on the above findings, we propose that cochlear duct morphogenesis is dependent on two phases of Shh signaling regulated by different Gli intermediaries. In the first phase, Shh secreted from the ventral midline promotes ventral identity in the otic vesicle by antagonizing Gli3R, thus leading to the activation of *Pax2*, *Otx2* and other target genes. In the second phase of signaling, Shh recruits Gli2/Gli3A function to mediate the distal outgrowth of the cochlear duct. This second phase of Shh signaling could be provided by other tissues, including the cochleovestibular ganglion (Liu et al., 2002). Interestingly, *Msx1* was used in our study as a marker of the distal cochlear duct region; however, it might actually be a Shh-responsive gene that participates in the extension of the cochlear duct. Whereas the cochlear duct is normal in *Msx1*$^{-/-}$ embryos (Satokata and Maas, 1994), it is shortened in *Msx1*$^{-/-}$;*Msx2*$^{-/-}$ double mutants, consistent with the idea that these genes are downstream targets of Shh and Gli activation pathways (Yiping Chen and D.K.W., unpublished).

Formation of the saccule and proximal cochlear duct requires a balance of Gli activator and repressor functions

The saccule and the proximal-most region of the cochlear duct are located further from the sources of Shh than other regions of the cochlea and might therefore depend on lower levels of Shh for their development (Fig. 9). Shh appears to regulate the formation of the saccule and proximal cochlear duct by antagonizing Gli3R. The initial indication that a principal function of Shh is to alleviate Gli3R activity was demonstrated by the partial suppression of *Shh* mutant phenotypes in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Litingtung and Chiang, 2000; Litingtung et al., 2002; te Welscher et al., 2002). For example, in the spinal cord, a number of ventral neuronal subtypes that are absent in *Shh*$^{-/-}$ embryos are rescued in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (Litingtung and Chiang, 2000). By the same token, the restoration of the saccule and proximal cochlear duct in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos indicates that a primary function of Shh in this region of the ear is to effect the release of Gli3R from Shh target genes.

Surprisingly, the saccule and proximal part of the cochlear duct develop normally in *Gli3Δ699* mutants, which possess a form of Gli3R whose activity is thought to be independent of Shh regulation. How then does the intermediate region of the inner ear form in the *Gli3Δ699* mutants? Since Shh signaling persists in *Gli3Δ699/Δ699* embryos, we reason that other Gli activators, such as Gli2 and/or

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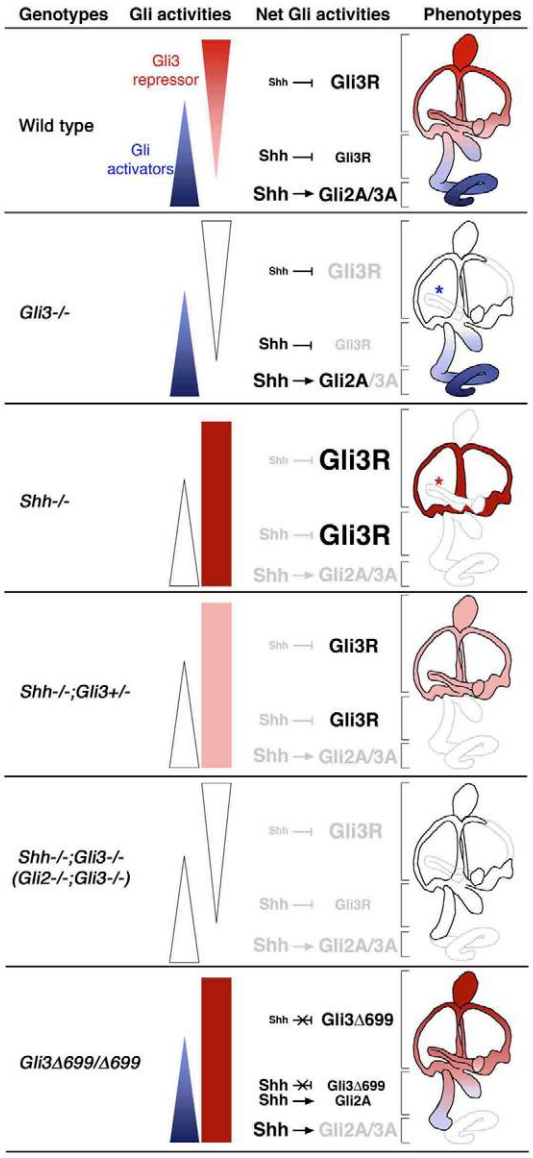

Fig. 9. A summary of how different levels of Shh mediate the formation of inner ear structures in the wild type and various mutants. A wild-type inner ear receives graded levels of Shh protein during development, highest in the ventral region and decreasing towards the dorsal region. This graded Shh signaling results in various levels of Gli activator (blue triangle) and repressor (red triangle) activities within the otocyst that are responsible for mediating the formation of different inner ear structures. The distal region of the cochlear duct (blue) requires the activator function of Gli proteins that is redundantly shared by Gli2 and Gli3, and possibly Gli1. The proximal region of the cochlear duct and the saccule (pale red and pale blue) requires relatively low levels of Shh signaling, as compared with the distal region, to remove Gli3R. The dorsal region of the inner ear (red) requires a correct dose of Gli3R balanced by the least amount of Shh signaling. In the various mutants, the missing inner ear structures are outlined in grey. The absence of GliA (blue) affected the distal region of the cochlear duct in all mutants analyzed. For most mutants, the formation of the saccule and the proximal region of the cochlear duct are affected in those situations in which Gli3R is not properly alleviated by Shh signaling (dark red rectangle, *Shh*$^{-/-}$; pink rectangle, *Shh*$^{-/-}$;*Gli3*$^{+/-}$). In addition, the semicircular canal region is also partially affected in the absence of Gli3R (white, *Gli3*$^{-/-}$ and *Shh*$^{-/-}$;*Gli3*$^{-/-}$) or in the presence of too much Gli3R (dark red, *Shh*$^{-/-}$). The exception is the normalcy of the vestibule and proximal cochlear duct region in the *Gli3Δ699* mutant, in which Gli3R levels cannot be alleviated. We attributed the absence of vestibular and proximal cochlear defects in this mutant to the presumably normal function of Gli1/Gli2A. There is a temporal difference in the requirement of Gli3R and Shh for lateral canal formation (asterisks).

Gli1, can compete with Gli3*Δ699* to promote the formation of the saccule and proximal cochlear duct. In support of this claim, we observe a rudimentary saccule and the absence of a definite cochlear duct in *Shh*$^{-/-}$;*Gli3Δ699*/– mutants, in which no GliA function is present (n=3; see Fig. S3 in the supplementary material). Although GliA may be able to override the repressive effects of Gli3R to promote saccule and proximal cochlear duct development in *Gli3Δ699/Δ699* embryos, it is not clear whether GliA normally plays any role in the formation of these structures.

Dose dependency of the Gli3 repressor in vestibular formation

Our data demonstrate that in dorsal regions of the inner ear, Gli3R functions in a dose-dependent manner to mediate vestibular development (Fig. 9). Dorsal inner ear structures such as the semicircular canals and endolymphatic duct are malformed in *Gli3*$^{-/-}$ embryos but are normal in *Gli3Δ699* mutants, highlighting the importance of Gli3R over Gli3A in mediating normal vestibular development. The finding that dorsal vestibular defects arise in embryos with an excess (*Shh*$^{-/-}$) or shortage (*Gli3*$^{-/-}$) of Gli3R suggests that the absolute amount of Gli3R generated in the dorsal otocyst is crucial for vestibular development. This contention is best supported by results showing that the dorsal vestibular phenotypes seen in *Shh*$^{-/-}$ embryos are corrected in the presence of one, but not two, mutated copies of *Gli3*. One conclusion that can be drawn from these findings is that Shh signaling in dorsal otic tissue serves to block the processing of a set amount of full-length Gli3 into Gli3R.

Alternatively, the dorsal otic defects observed in *Shh*$^{-/-}$ embryos might be caused indirectly through the disruption of signals derived from the dorsal hindbrain. Several lines of evidence prompted this notion. First, there is normally little expression of *Ptc1* and *Gli1* in the dorsal region of the inner ear. Second, dorsal structures, including the endolymphatic duct and lateral canal, are induced properly in *Shh*$^{-/-}$ embryos but fail to develop later in development when the juxtaposition of the neural tube and otocyst is altered by the abnormal morphology and size reduction of the *Shh* mutant hindbrain (Fig. 7) (Riccomagno et al., 2002). In this scenario, one could postulate that malformations of dorsal otic structures in *Shh*$^{-/-}$ embryos are caused by the loss of dorsal neural tube signals at a critical time of development when signaling from the hindbrain is still required for inner ear patterning (Riccomagno et al., 2005). If this is the case, then hindbrain defects should be rescued in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants, in which the dorsal otic structures are well formed. Even though there is no substantial improvement in the morphology of the hindbrain in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ mutants, the positioning of the hindbrain with respect to the otocyst is better in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ as compared with *Shh*$^{-/-}$ mutants at 11 dpc when the primordial endolymphatic duct and lateral canal structures are forming (Litingtung and Chiang, 2000). Distinguishing which of the two interpretations discussed above is correct will necessitate the conditional inactivation of Shh signaling in the dorsal otocyst.

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Regardless of how Shh signaling is mediated in the dorsal vestibular region, analyses of the lateral canal defects in the various mutants indicates that two temporally distinct processes regulate its formation: an earlier process that requires Gli3R and a later process that requires Shh. The role of Shh in the latter case is to repress Gli3R, as indicated by the restored lateral canal in *Shh*$^{-/-}$ mutants possessing only one wild-type copy of *Gli3*. Notably, in *Gli3Δ699/Δ699* embryos, the lateral canal forms normally, suggesting that neither of these processes is disrupted in these mutants. Although the early requirement for Gli3R may not be perturbed, it is somewhat surprising that no canal phenotype resembling that seen in *Shh*-null mutants occurs in *Gli3Δ699/Δ699* embryos. However, unlike *Shh* mutants, other Gli activators are still present in *Gli3Δ699/Δ699* mutants. These Gli activators might override Gli3R (Δ699) function, resulting in normal lateral canal formation, as was described above for the saccule and proximal cochlear duct region.

In summary, our study reveals that multiple Shh/Gli-mediated signaling mechanisms are operating during inner ear development, with Gli3R required dorsally for vestibular formation and GliA functioning ventrally to form the cochlear duct (Fig. 9). The distribution of the various Shh/Gli signaling mechanisms along the dorsoventral axis of the ear is similar to what has been described for the spinal cord, where Gli3R functions in dorsal regions of the neural tube to restrict the dorsal limits of p1 and p0 interneuron subtypes, and Gli2/Gli3A function redundantly to specify ventral neuronal cell fates (Bai et al., 2004; Lei et al., 2004; Persson et al., 2002). What is particularly novel about Shh/Gli signaling in the inner ear is the dosage requirement of Gli3R in the vestibular region. Although partial restitution of *Shh*$^{-/-}$ limb, neural tube and somite defects are observed in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos when the dose of Gli3R is limiting (Litingtung and Chiang, 2000; Litingtung et al., 2002; McDermott et al., 2005; Rallu et al., 2002), only the dorsal ear shows an absolute dependency on Gli3R dosage as vestibular structures are completely rescued in *Shh*$^{-/-}$;*Gli3*$^{+/-}$ embryos.

Furthermore, despite the necessity of the cochlear duct for hearing, not much is known about the regulation of its outgrowth and coiling. Our results indicate for the first time that different regions of the coiling cochlear duct are patterned by different mechanisms, and that this patterning is established early at the otocyst stage primarily by Shh emanating from the ventral midline (Bok et al., 2005; Riccomagno et al., 2002). The association of human deafness with mutations in genes involved in the Shh/Gli signaling pathway warrants a more thorough understanding of how these genes function in inner ear development. Future studies will focus on identifying the downstream targets regulated by Shh and Gli proteins.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/9/1713/DC1

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