Ildr1b is essential for semicircular canal development, migration of the posterior lateral line primordium and hearing ability in zebrafish: implications for a role in the recessive hearing impairment DFNB42

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Immunoglobulin-like domain containing receptor 1 (ILDR1) is a poorly characterized gene that was first identified in lymphoma cells. Recently, ILDR1 has been found to be responsible for autosomal recessive hearing impairment DFNB42. Patients with ILDR1 mutations cause bilateral non-progressive moderate-to-severe sensorineural hearing impairment. However, the etiology and mechanism of ILDR1-related hearing loss remains to be elucidated. In order to uncover the pathology of DFNB42 deafness, we used the morpholino injection technique to establish an ildr1b-morphant zebrafish model. Ildr1b-morphant zebrafish displayed defective hearing and imbalanced swimming, and developmental delays were seen in the semicircular canals of the inner ear. The gene expression profile and real-time PCR revealed down-regulation of atp1b2b (encoding Na+/K+ transporting, beta 2b polypeptide) in ildr1b-morphant zebrafish. We found that injection of atp1b2b mRNA into ildr1b-knockdown zebrafish could rescue the phenotype of developmental delay of the semicircular canals. Moreover, ildr1b-morphant zebrafish had reduced numbers of lateral line neuromasts due to the disruption of lateral line primordium migration. In situ hybridization showed the involvement of attenuated FGF signaling and the chemokine receptor 4b (cxcr4b) and chemokine receptor 7b (cxcr7b) in posterior lateral line primordium of ildr1b-morphant zebrafish. We concluded that Ildr1b is crucial for the development of the inner ear and the lateral line system. This study provides the first evidence for the mechanism of Ildr1b on hearing in vivo and sheds light on the pathology of DFNB42.
INTRODUCTION

Hearing loss is among the most common sensory impairment affecting several million people around the world. The global prevalence of hearing loss is ~1 per 1000, and half of all cases are caused by genetic factors (1–3). A total of 121 loci associated with non-syndromic deafness have been mapped in the human genome, and 62 genes have been identified as the responsible genes (http://hereditaryhearingloss.org).

Among the genes associated with deafness, the gene encoding immunoglobulin-like domain containing receptor 1 (ILDR1) was found to be associated with the autosomal recessive hearing impairment DFNB42 in consanguineous Pakistani and Iranian families (4). This suggests a pivotal role for ILDR1 in maintaining normal hearing. ILDR1 was first identified in lymphoma and was shown to be associated with the development and/or progression of cancer (5). In mice, ildr1 is expressed at low-to-intermediate levels in hair cells and at higher levels in the Pillar cells and Hensen cells (4). There is evidence that ILDR1 localizes at tricellular contacts and is required for the establishment of a strong epithelium in the organ of Corti. While this implies a role in the pathogenesis of deafness, the function of ILDR1 is largely unknown and the molecular mechanism of ILDR1 in deafness remains to be elucidated.

Zebrafish is an excellent model to study hearing development and deafness (6). Zebrafish have two sensory organs that can sense changes in the water: the inner ear and the lateral line system. Zebrafish have a typical vertebrate inner ear—but no sense of balance (11). Thus, this model allowed us to figure out the role of ILDR1 in hearing development.

There are two paralogs for ildr1 in zebrafish (ildr1a and ildr1b). We demonstrated the genomic structure of ildr1 in Figure 1. By knockdown of ildr1b in zebrafish through morpholino injection, we investigated the function of Ildr1b and its potential role in DFNB42 deafness. Behavioral studies suggested that the hearing and balance system was severely disturbed in ildr1b-knockdown zebrafish. Injection of ildr1b antisense morpholino oligonucleotides resulted in the developmental delay of the semicircular canals in the inner ear and a reduction in the number of lateral line neuromasts in the lateral line. Influence of ildr1b on the inner ear is partly through the regulation of atp1b2b, while disruption of the lateral line primordium and attenuation of the FGF signaling pathway were associated with phenotypes of lateral line in ildr1b-morphant fish. Taken together, these results indicate that Ildr1b is essential for the development of the inner ear and the lateral line neuromasts to maintain normal hearing and balance in zebrafish.

RESULTS

Developmental expression of ildr1b in zebrafish

Because zebrafish ildr1b encoding 551 amino acids shared a higher protein sequence identity with human full length ILDR1 protein (546 amino acids), our analysis focused on the expression of ildr1b in zebrafish. To characterize the developmental expression of ildr1b, we performed in situ hybridization using ildr1b antisense probes. At 24 hpf and 34 hpf, ildr1b was mainly detected in the otic vesicle, and the posterior lateral line primordium (PLLp) (Fig. 2A and B). At 48, 72 and 96 hpf, ildr1b was highly expressed in the inner ear and the anterior lateral line neuromasts (Fig. 2C–E). The expression pattern of ildr1b in the developing zebrafish implies that ildr1b might play a role in hearing development.

Ildr1b-morphant zebrafish display defective hearing and imbalanced swimming

In order to explore the effect of ildr1b on hearing, we designed morpholino oligonucleotides to reduce the expression of ildr1b. The ildr1b-MO efficiently blocked the translation of ildr1b (Supplementary Material, Fig. S1). Control experiments showed that the viability of zebrafish injected with 6 ng ildr1b-MO was comparable to that of those injected with conMO (Supplementary Material, Fig. S2A). We tested the fast escape reflex, the C-shaped startle response, using near-field pure tone stimulation. Importantly, we found that the probability of the C-startle response was significantly lower for ildr1b-morphant zebrafish than control zebrafish or wild-type zebrafish (P < 0.001, Fig. 3), which demonstrated that the ildr1b-morphant zebrafish have hearing problems.

Development of semicircular canals was delayed in ildr1b-morphant zebrafish

There is evidence that the inner ear plays crucial roles in zebrafish hearing and balance. Therefore, we first examined morphological defects of the inner ear in the ildr1b-morphant zebrafish at 96 hpf. The percentage of zebrafish that cannot form mature semicircular canals were counted. We found that the development of the semicircular canals was significantly delayed in 80% of the ildr1b-morphant zebrafish compared with the controls at 96 hpf. Protrusions from the otic epithelial failed to fuse into functional semicircular canals in these fish (Fig. 4B). The phenotype can be rescued by co-injection of ildr1b-MO and ildr1b mRNA (Fig. 4C and D).

It has been extensively reported that there are five sensory cristae in the inner ear—each of which is composed of hair cells and the surrounding supporting cells—we then tested the hypothesis that knockdown of ildr1b would affect the
development of sensory cells in the inner ear. However, we found no differences in morphology or number of supporting cells and hair cells between the controls and the ildr1b-morphant zebrafish (Supplementary Material, Fig. S3). The hair cells were arranged regularly in the middle of the anterior macula (Supplementary Material, Fig. S3C and D) and were surrounded by regular supporting cells (Supplementary Material, Fig. S3A and B). Transduction channels of hair cells are also have normal function, which was confirmed by injecting of FM1-43FX into the inner ear of zebrafish (Supplementary Material, Fig. S3G and H). Similarly, stereocilia protruding from the hair cells in the posterior cristae, medial cristae, and anterior cristae were normal in the ildr1b-morphant zebrafish (Supplementary Material, Fig. S4).

We also used morpholino oligonucleotides of ildr1a to investigate its role in semicircular canal development. However, knockdown of ildr1a has no effect on the development of semicircular canals (Supplementary Material, Fig. S5G and H).

Influence of ildr1b on the inner ear is partly through the regulation of atp1b2b

In order to find out the possible pathways that ildr1b might participate in, we compared gene expression profiles between control zebrafish and ildr1b knockdown zebrafish. The profiles reveal down-regulation of several genes by ildr1b (Supplementary Material, Table S1). Among the down-regulated genes, atp1b2b has been reported to be associated with the development of the semicircular canals (12,13). We found that injection of atp1b2b mRNA into ildr1b-knockdown zebrafish could rescue 30% of the phenotype associated with developmental delay of the semicircular canals (Fig. 4E). It is possible, therefore, that the influence of ildr1b on the inner ear is partly through the regulation of atp1b2b.

The number of hair cells and supporting cells in the lateral line is significantly reduced in ildr1b-morphant zebrafish

In situ hybridization data show significant expression of ildr1b in the zebrafish lateral line PLLp (Fig. 2). Because neuromasts are important sensory organs in the lateral line and are composed of hair cells and supporting cells, we first asked whether knockdown of ildr1b in zebrafish affected the formation and maturation of hair cells or supporting cells in lateral line neuromasts. We stained hair cells in the neuromasts with the vital dye FM1-43FX and found that the number of hair cells in neuromasts labeled with FM1-43FX was significantly reduced in the lateral line system of ildr1b-morphant zebrafish (Fig. 5B and D). The phenotype was further confirmed by injection of ildr1b-spMO (Supplementary Material, Fig. S5A–C). In addition, the average number of lateral line neuromasts in ildr1b-knockdown zebrafish was markedly increased in fish co-injected with

Figure 2. Whole-mount in situ hybridization with the ildr1b probe in wild-type zebrafish at five different stages as indicated in the bottom left corner. (A–F) Lateral views of zebrafish with the anterior end to the left. The insets in (A–E) show the magnification of the ildr1b-positive otic vesicle or ear. The black arrowheads in (A and B) point to the otic vesicle, while gray arrowheads point to the PLLp. Black arrowheads in (C–E) point to the ear. Arrows in (D and E) point to the anterior lateral line neuromasts. OV: otic vesicle; e: ear; PLLp: posterior lateral line primordium.
ildr1b-MO and ildr1b mRNA (Fig. 5F and Fig. 6H). FM1-43FX is believed to label hair cells by traversing the mechanosensitive channels, and this provides an index of mechanotransduction (14,15). Thus, only hair cells with high vitality can be stained by FM1-43FX. To rule out the possibility that the reduction of hair cells labeled by FM1-43FX resulted from low vitality of the hair cells rather than the disappearance of the hair cells in ildr1b-morphant zebrafish, we performed immunofluorescence with sox2 antibody and myosin-7a antibody, which label supporting cells and hair cells, respectively. Consistent with the results of FM1-43FX labeling, the number of hair cells in the lateral line neuromasts were significantly reduced (P < 0.001) (Fig. 5H and K). This indicated that the disappearance of the hair cells in the lateral line neuromasts of ildr1b-knockdown zebrafish was not resulted from the low vitality of the hair cells. In addition, the number of supporting cells was also significantly reduced (P < 0.001, Fig. 5G and J). To rule out the possibility that with existence of ildr1a, ildr1b-morphant fish may have incomplete phenotypes of lateral line system, we also produced ildr1a-morphant zebrafish. Results demonstrate that knockdown of ildr1a have no effects on the number of lateral line neuromasts (Supplementary Material, Fig. S5D–F and I).

Knockdown of ildr1b has distinct effects on the migration of the PLLp and neuromast deposition

To test the hypothesis that the disappearance of the hair cells and supporting cells in the lateral line system is due to the disappearance of the neuromasts, we used the transgenic zebrafish Et(gata2:EGFP)mp189b to trace the migration of the PLLp and the deposition of neuromasts in control and ildr1b-morphant zebrafish. In control zebrafish injected with conMO, the PLLp formed at 25 hpf and the first neuromast was deposited at 30 hpf. The PLLp migrated normally and successfully deposited six neuromasts ~45 hpf (Fig. 6A). In ildr1b-knockdown zebrafish, the PLLp also formed at 25 hpf and the pro-neuromasts began to segregate from the ildr1b-knockdown primordium at 30 hpf (Fig. 6B). Over time, however, the primordium failed to generate new rosettes and became disorganized leaving a small trail of leading cells that finally disappeared at 35 hpf (Fig. 6C and D). At 72 hpf, there were ~8 neuromasts in the wild-type zebrafish lateral line that consisted of supporting cells and hair cells (Fig. 6H). However, the average number of neuromasts was reduced to ~2 in the ildr1b-knockdown zebrafish (Fig. 6H). About 77% of the ildr1b-knockdown zebrafish developed 1–3 neuromasts (Fig. 6G). Moreover, the development of neuromasts in 20% of the ildr1b-knockdown zebrafish was completely disrupted and no mature neuromasts were deposited (Fig. 6G). On average, 5 and 6 ng morpholino injections resulted in a reduction in neuromasts to 4 and 2, respectively, showing the dose-dependent nature of the reduction (Supplementary Material, Fig. S2B). The conclusion that we drew was that the disappearance of hair cells and supporting cells in the lateral line system is indeed the result of the disappearance of the neuromasts.

To further confirm the disruption of PLLp migration in ildr1b-knockdown zebrafish, we conducted in situ hybridization using TACSTD probe, a lateral line marker that is primarily expressed...
in the migrating primordium and the deposited neuromasts. Similarly, the PLLp formed at 22 hpf in ildr1b-knockdown zebrafish but migrated slowly and then collapsed between 30 and 48 hpf leaving only one neuromast deposited near the otic vesicle (Fig. 7B, D, and F). In control zebrafish, eight complete neuromasts had been deposited by 48 hpf (Fig. 7A, C and E). Therefore, we can further conclude that the reduced numbers of lateral line neuromasts in the ildr1b-knockdown zebrafish are due to the progressive collapse of the PLLp during migration.

It has been reported that defects observed in the morpholino-injected fish might derive from the activation of \textit{p}53 and subsequent \textit{p}53-induced apoptosis (16). To verify the specificity of the phenotype that results from ildr1b knockdown, we conducted \textit{p}53 injection and rescue experiments. Co-injection of ildr1b-MO and \textit{p}53-MO resulted in a similar phenotype as injection with ildr1b-MO alone (Supplementary Material, Fig. S6). The result demonstrated that the phenotype of reduced neuromasts observed upon injection of ildr1b-MO was indeed the result of the knockdown of ildr1b.

To determine whether deposited neuromasts in the ildr1b-deficient zebrafish have normal morphology, we first assessed the neuromast positioning relative to somite number in control zebrafish and ildr1b-morphant fish. Approximately 77% of the ildr1b-knockdown fish deposited the first three neuromasts, and these are deposited at regular distances that are comparable to control zebrafish (Fig. 6E and F). Assessment of the morphology of the supporting cells and hair cells in the last deposited neuromasts showed that these cells were intact in the ildr1b-knockdown zebrafish (Supplementary Material, Fig. S7), and this suggested that the few neuromasts that were deposited still underwent full maturation.

\textbf{Attenuation of the FGF signaling pathway, cxcr4b and cxcr7b expression is involved in the disruption of PLLp migration}

The formation and migration of the lateral line primordium requires the mutual interaction of the FGF and Wnt signaling pathways (9,10). To elucidate the molecular mechanism responsible for the progressive disruption of the PLLp migration in the ildr1b-knockdown fish, we investigated the expression levels of key members in the FGF signaling pathway (\textit{fgf3}, \textit{fgf10}, \textit{pea3}, \textit{fgfr1} and \textit{sef}) and the Wnt signaling pathway (\textit{axin2} and \textit{lef1}). We initially chose zebrafish at 30 hpf and found no significant difference in any of the probes between the control fish and the ildr1b-knockdown fish (data not shown). However, in 34 hpf fish we found that the expression of \textit{fgf3}, \textit{fgf10}, \textit{pea3} and \textit{fgfr1}, but not \textit{sef}, in the FGF signaling pathway were significantly decreased in the primordium of zebrafish injected with ildr1b-MO compared with control fish (Fig. 8A–J). At this time point, there was no difference in expression of \textit{axin2} and \textit{lef1} in the Wnt signaling pathway between the ildr1b-knockdown fish.

\textbf{Figure 4. Morphology of semicircular canals in ildr1b-knockdown zebrafish. (A–C) ildr1b-knockdown resulted in the developmental delay of semicircular canals. (D and E) Histograms showing the morphology of the semicircular canals rescued by co-injection of ildr1b-MO with ildr1b mRNA or \textit{atp1b2b} mRNA. The black arrowheads in (A and C) point to the epithelial pillar which fused into normal semicircular canals. The asterisks in (B) show incomplete epithelial pillar, which cannot fuse into normal semicircular canals. EP: epithelial pillar; \textit{***} \textit{P} < 0.001.
and the control fish (Fig. 8K–N). In addition, we found that the expression of the two chemokine receptors \textit{cxcr4b} and \textit{cxcr7b}—which are required for proper migration of the PLLp—were significantly reduced in the \textit{ildr1b}-morphant fish (Fig. 8O–R).

**DISCUSSION**

In the present study, we report an essential role for \textit{ildr1b} expression in the development of the inner ear and posterior lateral line in zebrafish. Because \textit{ildr1a} has no effects on the formation of the inner ear and development of the lateral line system, therefore, effects of \textit{ildr1} on hearing can be entirely indicated by investigating \textit{ildr1b} only. \textit{Ildr1b} knockdown impaired normal sensory responses, delayed the development of the semicircular canals, reduced neuromast deposition, and disrupted PLLp migration. Influence of \textit{ildr1b} on the inner ear is partly through the regulation of \textit{atp1b2b}. Attenuation of FGF signaling and expression of chemokine receptors was involved in the disruption of PLLp migration in the lateral line of \textit{ildr1b}-morphant zebrafish. This study provides the first evidence that \textit{ildr1b} plays an important role in the development of the inner ear, and its effect on the migration of the PLLp, which shed light on the pathology of \textit{ildr1b} deficiency-related deafness.

![Figure 5. Morphology and number of hair cells and supporting cells in \textit{ildr1b}-knockdown and control zebrafish. (A–F) Reduced numbers of neuromast hair cells in \textit{ildr1b}-morphant fish labeled by FM1-43FX at 72 hpf (n = 80). (G–L) Immunostaining of zebrafish lateral line hair cells and supporting cells at 72 hpf. Hair cells were stained with myosin-7a antibody and supporting cells were stained with sox2 antibody. (G and J) Zebrafish stained with sox2 antibody. (H and K) Zebrafish stained with myosin-7a antibody. (I and L) are merged images. The arrows point to the position of posterior lateral line neuromasts.](https://academic.oup.com/hmg/article-abstract/23/23/6201/2900787)
The function of ILDR1 is less well characterized. It was first cloned in follicular lymphoma (5), and protein structure analysis indicates that it is a member of the protein family containing an Ig-like extracellular N-terminal domain and a cysteine-rich region in the C-terminal intracellular domain. It is widely expressed in a variety of tissues, including the prostate, testes, pancreas, kidneys, etc. (5). Recently, Borck et al. (4) identified several loss-of-function ILDR1 mutations in the autosomal recessive hearing impairment DFNB42. In addition, in situ hybridization detected early expression of *ildr1* in development in the vestibule, hair cells and supporting cells of the mouse cochlea (4). However, until now the functional role of ILDR1 in hearing was largely unknown.

We found that *ildr1b* is highly expressed in the otic vesicle and in the primordium of the posterior lateral line, and this implies that it might have a role in hearing development. To investigate the function of Ildr1b, we produced *ildr1b*-morphant zebrafish that displayed a lack of sensitivity to sound stimulation and an imbalanced swimming motion that mimics the human hearing impairment DFNB42. We did not observe any obvious differences in the size of the otoliths or the morphology of the sensory cells between *ildr1b*-morphant fish and controls.

However, we found that the development of the semicircular canals was severely delayed in *ildr1b*-morphant zebrafish. Large amounts of *ildr1b* were expressed in the otocyst. The outgrowth of otocyst finally forms the zebrafish semicircular canals (7,17,18). Therefore, knockdown of *ildr1b* in zebrafish might affect the growth of the otocyst and this might result in the developmental delay seen in the semicircular canals.

Several lines of evidence suggest that Na\(^{+}/K^{+}\)-ATPase genes such as *atp1a1a* and *atp1b2b* are required for the development of the inner ear of zebrafish. The Na\(^{+}/K^{+}\)-ATPase participates in the secretion of the otolith matrix proteins from ionocytes that contribute to the ionic endolymph environment in which the otoliths form (19,20). Knockdown of *atp1a1a* or *atp1b2b* has been shown to lead to various otolith defects and to the disruption of semicircular canal development (12,13). Gene expression profile reveals down-regulation of *atp1b2b* and several calcium ATPase genes in *ildr1b*-knockdown zebrafish both at 48 and 72 hpf compared with zebrafish injected with conMO. The phenotype we observed in the inner ear in *ildr1b* knockdown zebrafish is similar to that in *atp1b2b*-knockdown zebrafish. Furthermore, injection of *atp1b2b* mRNA could partly rescue the phenotype that resulted from the *ildr1b* dysfunction. There is

![Figure 6. Reduced number of neuromasts in *ildr1b* knockdown zebrafish. (A and B) Time-lapse analysis of PLLp migration in Et(gata2:EGFP)\(^{tm1884}\) embryos (n = 85). Images were taken every 5 h. (C and D) Epithelial rosettes are disorganized in PLLp of *ildr1b*-morphant fish. White arrowheads in (C) point to the center of epithelial rosettes. (E and F) Histograms showing the number and positions of neuromasts in control and *ildr1b*-morphant fish. Colors denote different populations of sequentially deposited neuromasts. (G) Percentage of zebrafish depositing 0–4 neuromasts. (H) Histograms showing the number of neuromasts rescued by co-injection of *ildr1b*-MO and *ildr1b* mRNA.](https://academic.oup.com/hmg/article-abstract/23/23/6201/2900787)
evidence that Na\(^+\)/K\(^+\)-ATPase activity creates an osmotic gradient and establishes the transepithelial ion potential in the developing otic cysts, which regulates protrusion and morphogenesis of the semicircular canal (12). Thus, knockdown of ildr1b decreased atp1b2b expression, which in turn destroys osmotic gradient and ion potentials, and finally may delay semicircular canal development. A possible mechanism for atp1b2b mRNA rescuing the phenotype of delayed development of the semicircular canals is that injection of atp1b2b mRNA restores Na\(^+\)/K\(^+\)-ATPase activity and keeps normal osmotic gradient and ion potential, which sustains normal semicircular canal development. Therefore, it is possible that the delay in semicircular canal development in the ildr1b-knockdown fish was through the regulation of atp1b2b expression in the inner ear.

Furthermore, we found that the migration of the PLLp and the deposition of the neuromasts were significantly affected in the ildr1b-knockdown fish. The lateral line organ is a mechanosensory organ that detects changes in water flow. For the development of the lateral line system, the primordium arises from the lateral line placode near the developing ear between 19 and 25 hpf (21,22). Then the primordium migrates caudally followed by the formation of proneuromasts (23). This process is mainly controlled by two mutually antagonistic signaling centers: the Wnt signaling center in the leading zone of migrating primordia and the FGF signaling center in the trailing zone (9,10). FGF signaling is critical for the generation of proneuromasts from the primordium, and Wnt signaling regulates such patterning partly by modulating FGF signaling (24). In addition, Wnt and FGF signaling have been suggested to regulate primordium migration by controlling the expression of the cxcr4b and cxcr7b (10).

To investigate the molecular mechanism behind the reduced neuromast deposition and disrupted migration of the PLLp, we performed in situ hybridization experiments with probes of key members in the FGF and Wnt signaling pathways. Our results demonstrated that the expression of a few key genes in the FGF signaling pathway—fgf3, fgf10, fgfr1 and pea3—were significantly decreased in the lateral line primordium of the ildr1b-morphant zebrafish (Fig. 8A-I). It has been reported that blocking FGF signaling will result in a disorganized primordium after the deposition of a single neuromast and a cessation of primordium migration (21), and this can explain why attenuation of FGF signaling is involved in the failure of neuromast formation and primordium migration in ildr1b-morphant fish. Chemokine receptors cxcr4b and cxcr7b are important guidance signals for the directional migration of the PLLp (25,26). Pharmacological FGF pathway inhibition with SU5402 causes ectopic expression of cxcr4b and loss of cxcr7b, and this affects the migration of the PLLp (10). However, the expression of cxcr4b and cxcr7b were both significantly reduced in the ildr1b-morphant zebrafish. We speculated that other mechanism associated with the regulation of cxcr4b may exist except the involvement of FGF pathway and cxcr7b in the regulation of PLLp migration.

**Figure 7.** Whole-mount in situ hybridization with TACSTD probe in wild-type and ildr1b-morphant fish at different stages. Stages are indicated in the bottom right corners. Arrows in (A and B) show the PLLp. PLLp: posterior lateral line primordium.

**Figure 8.** Whole-mount in situ hybridization in 34 hpf fish with probes for FGF signaling pathway genes (fgf3, fgf10, pea3, fgfr1 and sef); Wnt signaling pathway genes (axin2 and lef1); two chemokine receptors (cxcr4b and cxcr7b).
ILDR1 is localized at the tricellular tight junctions in the organ of Corti in mice (27). DFNB42-associated ILDR1 mutant proteins cannot recruit tricellulin to the tight junction in vitro, and tricellulin-deficient mice, which have abnormal tight junction structures, rapidly develop hearing loss (27,28). This suggests that the proper formation of tTJs is required for hearing. It has been shown that FGF3 and FGF10 are key members of the FGF signaling pathway, and they act synergistically to mediate FGF signaling in the PLLp. fgf3:fgf10 double mutant primordia showed reduced speed of migration and adopted an extended conformation (24,29). Moreover, the epithelial tight junction protein ZO-1 was undetectable in ildr1b knockdown of PLLp, which led ultimately to defective hearing (Fig.9). Further experiments should be pursued to investigate the relationship between tight junction and development of PLLp. The signaling pathway depicted as a solid line was adapted from (10).

Figure 9. Schematic models showing the function of ildr1b in the inner ear and the PLLp. The signaling pathway depicted as a solid line was adapted from (10). The dotted line shows the possible pathway of ildr1b function in inner ear development and PLLp migration.

Materials and methods

Zebrafish strains and breeding

Zebrafish (Danio rerio) were reared and maintained at 28.5°C as described by Westerfield (30). The wild-type strain was derived from the AB line. Embryos were collected at natural spawns at different stages of the experiment. Embryos were moved to embryo medium containing 0.2 mm phenylthiourea at ~20 h post-fertilization (hpf) to prevent pigmentation. Time-lapse recordings were made with Et(gata2:EGFP)mp189b embryos gifted from Dr Dong Liu of Peking University.

Morpholino-mediated gene knockdown

To knockdown the expression of ildr1a and ildr1b, we designed ildr1a translation blocking morpholino (ildr1a-MO), ildr1a splicing blocking morpholino (ildr1a-spMO), ildr1b translation blocking morpholino (ildr1b-MO), ildr1b splicing blocking morpholino (ildr1b-spMO). In order to confirm the specificity of the phenotype we observed, standard control morpholino oligonucleotides (conMO) and p53 morpholino oligonucleotides (p53-MO) were also designed. All morpholinos were synthesized from a commercial company Gene Tools. All of the morpholinos were injected into 1 or 2-cell-stage zebrafish embryos. The morpholino sequences and the injected amounts are provided below.

ildr1a-MO (7 ng): 5′-CACACCTGATCATTCCCTCATCAT-3′
ildr1a-spMO (4 ng): 5′-AGCAGGAGGCAGTGATCTACCT-3′
ttc-3′
ildr1b-MO (6 ng): 5′-GTATTTTAATGTGCTACTTCTCTCCTCCATTCTCAT-3′
ildr1b-spMO (6 ng): 5′-TACTACACAGCACTCCAGCGGA-3′
conMO (6 ng): 5′-CTCCTATCCATCTGCAACAGCTGTA-3′
P53-MO (8 ng): 5′-GCAGCCATGCTTGGCAAGAATG-3′

mRNA synthesis and phenotypic rescue

Full-length zebrafish ildr1b or atp1b2b cDNA was amplified and cloned into the pGEMT vector (Tiangen). The cloned vectors were linearized with the Sall restriction enzyme, and the linearized product was purified as template and transcribed in vitro using the mMESSAGE mMACHINE High Yield Capped RNA Transcription kit (Ambion). The synthesized ildr1b or atp1b2b mRNA was co-injected with ildr1b-MO into 1 or 2-cell-stage zebrafish embryos. The rate of rescued zebrafish after injection of ildr1b or atp1b2b mRNA was analyzed at 72 hpf.

Startle response test

Sound-evoked C-shaped startle response was tested at 6 dpf fish in 96-well plastic plates and recorded with a high-speed camera (Redlake, MotionScope M3, 1000 fps) under infrared light illumination. Pure tone stimulations (10 ms, 500 Hz) were given through a plastic board mounted on a voice box (HiVi, D1080MKII). Each larva was tested 10 times with an interval of 10 seconds between trials.
of 5 min and the percentage of C-startle responses was calculated for each larva. The probability of the C-startle response for a group of larvae was the average percentage of C-startle reflexes.

Semicircular canal morphology analysis

The structure of semicircular canal of 6 dpf fish was analyzed by light microscope. We defined fish that can form epithelial pillars but cannot fuse into functional semicircular canals as semicircular canal defects.

Hair cell labeling

Functional mechanotransducing hair cells were labeled with the vital dye FM1-43FX (Molecular Probes, Invitrogen), a fixable analog of FM1-43. To label hair cells in the neuromasts, larvae were placed in embryo medium containing 3 μM FM1-43FX for 30 s and rinsed three times in fresh embryo medium. Fish were then anesthetized with 0.02% Tricaine (MS-222; Sigma) for visualization. To label hair cells in the inner ear, 12 μM FM1-43FX was directly injected into the inner ear and hair cells were captured immediately.

Fluorescein–phalloidin staining

Larvae were fixed in 4% formaldehyde in phosphate buffered saline (PBS) overnight. The larvae were then rinsed in several changes of 2% Triton X-100 in PBS over several days until the otoliths were dissolved. Larvae were then stained with 2.5 μg/mL FITC-labeled phalloidin in PBS for 2 h in the dark and then rinsed several times with PBS over 2 h. The larvae were embedded in agar and visualized using a Leica TCS SP2 confocal microscope. All fixing, staining and rinsing was done at 4°C.

Whole-mount immunofluorescence

Zebrafish were fixed in 4% paraformaldehyde at 4°C overnight. After fixation, the solution was replaced with an equivalent volume of phosphate buffer. Prior to labeling, the embryos were washed 5 × 5 min with PBS to remove all traces of fixative. A total of 20–30 embryos were incubated in 0.5 mL 10% (v/v) serum in PBS plus 0.8% Triton X-100 (PBT) for 1 h at room temperature on a rocking table. The primary antibody was diluted to the appropriate concentration in PBT plus 1% goat serum at 4°C overnight. As much of the primary antibody as possible was removed and replaced with a large volume of washing solution (PBT) for 5 × 15 min. The secondary antibody was diluted as recommended by the supplier in PBT plus 1% goat serum and incubated with the washed embryos at 4°C overnight. The antibodies were removed by washing the embryos in PBT for 5 × 15 min. In the final step, the embryos were transferred to PBS for detection. Anti-sox2 antibody (Abcam) and anti-myosin-7a antibody (kindly provided by Dr Duan Ma from Fudan University) were used at 1:1000 dilutions. Either Alexafluor 488 or Alexafluor 594 secondary antibodies (Invitrogen, Molecular Probes) were used at 1:200 dilutions.

Time-lapse imaging

E(tgata2:EGFP)mp189b embryos were anesthetized at ~25 hpf in 600 μM Tricaine (Sigma), and the time-lapse images were acquired immediately. After imaging, the fish were returned to the dish for feeding. Every 5 h, images of the same zebrafish were captured using an inverted Leica SP5 confocal microscope with a ×20 objective lens.

Probe synthesis and in situ hybridization

In situ hybridization was performed at 65°C as described previously (31,32). RNA probes were synthesized using a DIG labeling kit (Roche). BCIP/NBT substrate (Roche) was used as a chromogen. Digoxygenin-labeled antisense RNA probes were generated for the following genes: fgf3, fgf10, pea3, fgfr1 and sef of the FGF signaling pathway; axin2 and lef1 of the Wnt signaling pathway; the two chemokine receptors cxcr4b and cxcr7b.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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