Grhl2 deficiency impairs otic development and hearing ability in a zebrafish model of the progressive dominant hearing loss DFNA28

Yanchao Han¹, Yu Mu², Xiaouan Li², Pengfei Xu¹, Jingyuan Tong¹, Zhaoting Liu³, Tingting Ma¹, Guodong Zeng¹, Shuyan Yang¹, Jiulin Du² and Anming Meng¹,³,*

1 Developmental Genetics Laboratory of Tsinghua University, School of Life Sciences, Tsinghua University, Beijing 100084, China

2 Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

3 State Key Laboratory of Biomembrane and Membrane Engineering, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

* Correspondence Author

Anming Meng

Developmental Genetics Laboratory of Tsinghua University, School of Life Sciences, Tsinghua University, Beijing 100084, China

Tel: +86-10-62772256

Fax: +86-10-62794401

E-mail: mengam@mail.tsinghua.edu.cn or mengam@ioz.ac.cn

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ABSTRACT

Congenital and progressive hearing impairment is a common distressing disease. The progressive dominant hearing loss DFNA28 in human is associated with a frameshift mutation of Grainyhead-like 2 (GRHL2) but its etiology and mechanism remain unknown. Here we report a zebrafish grhl2b\textsuperscript{T086} mutant line, in which grhl2b expression is interrupted by an insertion of a Tol2 transposon element. The mutants exhibit enlarged otocysts, smaller or eliminated otoliths, malformed semicircular canals, insensitiveness to sound stimulation, and imbalanced swimming motion. Since Grainyhead-like family members can regulate epithelial adhesion, we examined the expression of some genes encoding junction proteins in mutants. We show that the expression of Claudin b (cldnb) and epcam is abolished or dramatically reduced and apical junctional complexes are abnormal in otic epithelial cells of mutant embryos. Coinjection of cldnb and epcam mRNA could largely rescue the mutant phenotype. Injection of human wild-type GRHL2 mRNA but not the mutant GRHL2 mRNA derived from DFNA28 patients into grhl2b\textsuperscript{T086} mutant embryos could rescue the inner ear defects. Furthermore, we demonstrate that Grhl2b directly binds to the enhancers and promotes the expression of cldnb and epcam. Thus, this work reveals an evolutionarily conserved function of Grhl2 in otic development and provides a fish model for further studying mechanisms of Grhl2-related hearing loss.
INTRODUCTION

Congenital and progressive hearing loss is one of the most common diseases that disturb the daily life of patients. According to the statistics of American National Institute on Deafness and Other Communication Disorders (NIDCD), 2 to 3 per thousand neonates have hearing disorders in the United States and the disorders are hereditary in at least half of the cases. According to the Hereditary Hearing loss Homepage (http://hereditaryhearingloss.org), among 138 loci associated with non-syndromic deafness, 121 loci have been mapped to the chromosomes and only 55 loci have been defined for the affected genes. Many of the deafness genes have been found to play important roles in inner ear development during embryogenesis. An analysis of an American pedigree (LMG45) with dominant progressive nonsensory hearing impairment DFNA28 for the first time discloses an association of Grainyhead-like 2 (GRHL2) gene mutation with hearing loss (1). The appearance of hearing loss in patients heterozygous for the GRHL2 mutation suggests a haploinsufficiency of GRHL2. Van Laer et al. (2008) demonstrated that several single nucleotide polymorphisms (SNPs) at the GRHL2 locus are associated with age-related hearing impairment (2). However, it is still unclear how the mutation of GRHL2 causes hearing loss and whether Grhl2 is necessary for the vertebrate inner ear development.

Although the inner ear structures exhibit considerable diversities across vertebrate species, the molecular bases underlying inner ear development are relatively conserved (3). The zebrafish is an excellent model organism to study the mechanisms of inner ear development for reasons such as large clutch size, embryonic transparency and easy genetic manipulations. The zebrafish otic vesicles are comprised of sensory hair cells and other non-sensory epithelial cells. The integrity and barrier function of the otic epithelial cells are crucial to maintain the homeostasis of the otic lumen, the endolymph and the perilymph, which provides normal conditions required for the development of the inner ear structures, such as
otoliths, semicircular canals and hair cells as well as for the establishment of the mechanotransduction (4). Deficiency of the tight junction components could disrupt the endolymph homeostasis, affect otolith growth and hair cell survival and cause deafness in zebrafish (5), mouse (6-8) and human (9, 10) due to increased paracellular permeability. Ion channels present in the membranes of the otic epithelial cells secrete ions and other components into the otic lumen and endolymph (11); and their abnormal functions can alter the endolymph homeostasis and disrupt otolith formation (12-15). Therefore, both junction proteins and ion channels in the otic epithelial cells are important for the homeostasis maintenance and inner ear development.

Members of the Grainyhead-like (Grhl) transcription factor family have been found to play essential roles in epithelial morphogenesis and epidermal development in several organs and tissues in flies (16, 17), mice (18-24), Xenopus (25, 26) and zebrafish (27). These factors can regulate the epidermal barrier (19, 24, 28) and the expression of desmosomal cadherin (18) and apical junctional proteins in flies and mice (29, 30). Despite the discovery of GRHL2 correlation to hearing loss in human (1, 2), none of the Grhl family members have been found to be implicated in otic development and hearing in animals. It is worth generating an animal model of human GRHL2-related hearing loss for studying its underlying molecular mechanisms.

In a Tol2 transposon-mediated insertional mutagenesis in zebrafish (31, 32), we identified a mutant line, grhl2bT086, which carries the transposon element in the grhl2b locus. The homozygous embryos exhibit enlarged otic vesicles, decreased or eliminated otoliths and malformed semicircular canals. Behavioral and electrophysiological studies suggest that the hearing and balance system is severely disrupted in the mutants. Mechanistically, grhl2b may regulate the differentiation of otic epithelial cells by directly promoting the expression of cldnb and epcam in the otic vesicles.
RESULTS

grhl2b<sup>T086</sup> transgenic embryos express EGFP in otic vesicles and homozygotes have inner ear defects

During an insertional mutagenesis screen employing the Tol2 transposon-mediated gene trap strategy (31, 32), we identified a trapped line, named grhl2b<sup>T086</sup> after the identification of the interrupted gene, in which the marker EGFP was zygotically expressed in specific tissues under the control of the trapped gene’s promoter. The EGFP expression was initiated in the telecephalon, otic primordia, prechordal mesoderm, and pronephric ducts at 10-12 somite stages (Figure 1A). At 24 hours postfertilization (hpf), EGFP was prominent in the hatching glands, pronephric ducts, epidermis and many placodal tissues, including olfactory placodes, lens, otic vesicles, anterior and posterior lateral line primordia and branchial arches (Figure 1B-C); the same expression pattern remained at 48 hpf except that EGFP was observed in the pectoral fins (Figure 1D-E). Confocal microscopy indicated that the hair cells and surrounding cells appeared to express lower levels of EGFP compared to the other otic epithelial cells (Figure S1). Heterozygous fish (F<sub>1</sub>) intercrosses produced three classes of F<sub>2</sub> embryos, sorted according to EGFP levels, i.e., no EGFP expression, weak (Figure 1B, D) and strong EGFP (Figure 1C, E). Among 1249 F<sub>2</sub> embryos observed, 297 (23.8%) were negative for EGFP, 636 (50.9%) showed weak EGFP, and 316 (25.3%) displayed strong EGFP. This segregation pattern agrees with Mendel’s Law of Segregation, suggesting that a single locus in this line has been trapped by the Tol2 element. Accordingly, no EGFP, weak EGFP and strong EGFP in an F<sub>2</sub> family should represent no insertion (wild-type), heterozygous and homozygous insertions, respectively.

We then examined morphological defects in the homozygous embryos. The homozygotes were indistinguishable in gross morphology from the wild-type or heterozygous siblings except for the otic vesicles at 24 hpf (Figure 1F-H). The otic defects of 24-hpf homozygotes
(hereafter referred to as mutants) included larger otocysts, thinner otic epithelia, and smaller
or eliminated otoliths (Figure 1I-K). At 36 hpf, otoliths in mutants showed varying degrees of
size reduction. According to the otolith size, we categorized mutants into 4 groups: normal
otolith group (accounting for 1.2%, n = 497), which had otoliths in size comparable to
wild-type embryos (on average 184 ± 18 μm², n = 15); small otolith group (11.1%), which
had otoliths retaining 35 - 75% of the average wild-type otolith size; tiny otolith group
(63.4%), which had otoliths retaining 10 - 34% of the average wild-type otolith size; and
absent otolith group (24.3%), which had otoliths with a size below 10% of that of the
wild-type embryos. At or after 50 hpf, mutant embryos with tiny or without otoliths were
referred to as severe mutants hereafter. At 50 hpf, the semicircular canal protrusions in
wild-type and heterozygous siblings had grown into the otic lumen (Figure 1L), while the
protrusions in the severe mutants just began to outgrow at this time (Figure 1M). At 5 days
postfertilization (dpf), the hubs of the semicircular canals in the inner ears of the wild-type
and heterozygous larvae exhibited regular shapes (Figure 1N); in contrast, those in the severe
mutants sprouted arbitrary spikes or even discontinuous canals (Figure 1O). These data
suggest that the transposon insertion disrupts the normal development of the inner ear and
affects otolith and semicircular canal growth.

The viability of mutants was comparable to that of wild-type and heterozygous embryos
in the first five days of development. After 8 dpf, the survival rate of mutants started
decreasing; and only about 72% of mutants could survive after 1 month under normal feeding
conditions, which contrasted with a survival rate of 95% for heterozygous siblings. This
decreased surviving rate might be caused by dietary deficiency due to the defective balance
system of mutants (33). Surviving adult mutants were fertile, but mutant males had difficulties
chasing females due to a swimming problem, as described below.
grhl2b<sup>T086</sup> mutants display defective hearing and imbalanced swimming

The inner ear plays crucial roles in zebrafish hearing and balance (34). Because of the predominant defects in the inner ears of grhl2b<sup>T086</sup> mutants, we set out to investigate swimming behavior and hearing ability of the mutants. The wild-type and heterozygous larvae and adult fish usually swam or rested with back upwards and swam at the same depth. However, mutant larvae and adult fish usually swam and rested in abnormal positions; they swam up and down or in a circular manner sometimes (Movie 1). The abnormal swimming behavior of mutants indicated a defective balance system.

To test the hearing ability, we performed electrophysiological experiments to record the excitatory postsynaptic currents (EPSC) in the Mauthner cells (M-cell) of 5 - 6 dpf larvae. Severe mutant embryos were selected at 54 hpf and then used for the assays at 5 - 6 dpf. Both wild-type and heterozygous siblings showed robust EPSC in the M-cells after the sound stimulation (Figure 2A) and the amplitudes and the total charges between the two groups of larvae were statistically indistinguishable under different sound intensities ($p>0.11$, Figure 2B and C). However, the sound stimulation induced little EPSC in the M-cells of the severe mutant larvae (Figure 2A). Compared to wild-type and heterozygous siblings, the severe mutants exhibited significant decrease in the amplitudes and total charges when the sound intensity was above 14 dB ($p<0.005$, Figure 2B, C). These data indicate that the hearing of the severe mutants was severely impaired.

To further confirm the impairment of hearing, we tested the fast escape reflex, the C-shaped startle response mediated predominately by M-cells (35), using near-field pure tone stimulation with two different levels of sound intensity (36). Consistent with the electrophysiological results, the probability of the C-startle response (see the Methods for definition) between wild-type and heterozygous larvae was indistinguishable for either sound intensity ($p=0.57$ for weak intensity and $p=0.44$ for strong intensity). Although the probability
for mutants with smaller otoliths was not statistically different from their wild-type or heterozygous siblings (Figure S2), it was significantly lower for the severe mutants than that for wild-type or heterozygous siblings ($p = 1.1 \times 10^{-9}$ or $5.9 \times 10^{-13}$, Figure 2D-E). These data support the idea that those $grhl2b^{T086}$ mutants with tiny or without otoliths have a hearing problem.

*grainyhead-like 2b (grhl2b) gene was trapped in the grhl2b$^{T086}$ line*

In order to identify the trapped gene, we first performed thermal asymmetric interlaced polymerase chain reactions (TAIL-PCR) (37) to amplify the flanking sequences of the *Tol2* element using genomic DNA extracted from grhl2b$^{T086}$ heterozygous fish. The amplified sequences were all found to locate about 1 kb upstream of the reported *grainyhead-like 2b (grhl2b)* transcription start site. Sequence alignment between existing zebrafish Grhl2b and other vertebrate Grhl2 proteins revealed that the zebrafish Grhl2b deposited in the GenBank database lacks about 50 amino acid residues at the N-terminus, suggesting that the reported *grhl2b* sequence is incomplete. We then performed 5’-RACE and identified another exon upstream of the reported *grhl2b* sequence. The *Tol2*-flanking sequence located downstream of the newly identified exon, i.e., in the first intron of the *grhl2b* locus (Figure 3A). Linkage analysis showed that the *Tol2* transposon element inserted in the *grhl2b* locus was closely linked with the EGFP expression as well as the phenotypes in the grhl2b$^{T086}$ line consequently (Figure S3).

The insertion of the *Tol2* transposon element into the *grhl2b* locus would produce a fused transcript containing the first exon of *grhl2b* and the EGFP coding sequence (Figure 3B). Because the putative translation start codon of *grhl2b* was located in the first exon and was in frame with the EGFP coding sequence, the fusion transcript was predicted to code for a fusion
protein containing the first six amino acid residues of Grhl2b. To confirm the existence of the fusion construct, we performed reverse transcription-PCR (RT-PCR). We found that the fusion transcript (using the primer set fl/r4) was detected only in the heterozygotes and homozygotes, while the wild-type transcript (using primer set fl/r3) was undetectable in the homozygotes (Figure 3C). Real-time RT-PCR indicated that the expression level of the wild-type grhl2b transcript in 24-hpf heterozygotes and homozygotes decreased to 72% and 1.4% of that of wild-type embryos, respectively (Figure 3D). Taken together, these data suggest that the transposon insertion in grhl2bT086 embryos disrupts normal splicing of grhl2b primary transcript and consequently leads to a dramatic reduction of the level of normal grhl2b transcripts.

We next investigated the expression of grhl2b by whole-mount in situ hybridization using an antisense probe against the grhl2b transcript downstream of the transposon insertion site. The expression pattern of grhl2b in wild-type embryos (Figure 3E-H), which resembled the pattern reported previously (27), was almost identical to the EGFP pattern in grhl2bT086 embryos (Figure 1A-C). The expression level of grhl2b in grhl2bT086 heterozygous embryos was slightly reduced (Figure 3I), but dramatically reduced or abolished in homozygotes (Figure 3J and K). These results confirm that the interrupted gene in grhl2bT086 line is grhl2b.

grhl2b is required for inner ear development

To confirm the implication of grhl2b in inner ear development, we examined the effect of grhl2b knockdown by a morpholino in zebrafish embryos with a focus on otoliths for easy observation. We initially noted extensive cell death at 24 hpf after morpholino injection at the one-cell stage, and therefore we co-injected grhl2b morpholino with p53-MO to avoid off-target activation of p53 gene (38). As shown in Figure S4, a grhl2b-specific morpholino
(G2MO) targeting the ATG region of grhl2b mRNA efficiently repressed EGFP expression of grhl2b<sup>T086</sup> embryos, whereas a mismatch control morpholino (G2cMO) did not, which suggests effectiveness and specificity of G2MO. Compared to uninjected or G2cMO-injected embryos, G2MO-injected wild-type embryos exhibited significantly enlarged otic vesicles and size-reduced or eliminated otoliths (Figure 4A). To better illustrate the effect of grhl2b knockdown, we calculated the ratio of embryos in different categories at 36 - 40 hpf (Figure 4B). As the G2MO injection dose increased, ranging from 0.5 to 2 ng, the proportion of injected wild-type embryos with tiny and absent otoliths rose (Figure 4C). The distribution of different categories after injection with 2 ng G2MO resembled that of grhl2b<sup>T086</sup> mutants (Figure 4C). We also individually knocked down grhl1, grhl2a and grhl3, the other members of the Grainyhead-like family, and did not find otic defects (data not shown). These results support the notion that grhl2b is crucial for inner ear development.

To confirm the accountability of grhl2b deficiency for otic defects of grhl2b<sup>T086</sup> mutants, we tested whether injection of synthetic wild-type grhl2b mRNA could rescue the mutant phenotype. One-cell embryos derived from crosses between grhl2b<sup>T086</sup> homozygous females and heterozygous males were injected with 200 pg of zebrafish grhl2b mRNA, and homozygous mutant embryos were sorted based on EGFP intensity at around 24 hpf and classified based on the otolith size at 36 - 40 hpf as mentioned above. Compared to 87.9% (58/66) of uninjected mutants with tiny or absent otoliths, only 11.9% (7/59) of grhl2b-injected mutants fell into those two categories while 66.1% (39/59) had otoliths of normal size (Figure 4D). Apparently, overexpression of zebrafish grhl2b mRNA in mutant embryos was able to effectively rescue otolith defects. We noted that the same dose of grhl2b mRNA injection into wild-type embryos resulted in unaltered or slightly reduced otic volumes (data not shown). These observations confirm that the defects in inner ears of grhl2b<sup>T086</sup> are caused by grhl2b deficiency.
Overexpression of mammalian *Grhl2* can rescue otic defects of zebrafish *grhl2b*<sup>T086</sup> mutants

*Grhl2* is highly conserved in amino acid sequence among different vertebrate species (Figure S5). The zebrafish *Grhl2b* protein shares 71% identical amino acid residues with the human *GRHL2* protein. Higher sequence homologies between zebrafish and mammalian *Grhl2* proteins were found in the N-terminal transcriptional activation domain, the middle CP2 DNA binding domain and the C-terminal dimerization domain (Figure 5A), which are functional domains of the Grhl family members (1, 39).

Since a mutation of the human *GRHL2* gene has been reported to be associated with the dominant progressive hearing impairment DFNA28 (1), we hypothesized that mammalian *Grhl2* may play a role similar to fish *Grhl2b* in inner ear development. To test this, we injected human *GRHL2* or mouse *Grhl2* mRNA into *grhl2b*<sup>T086</sup> mutant embryos at the one-cell stage. Like ectopic zebrafish *grhl2b*, human or mouse *Grhl2* expression in mutants rescued the otolith defect (Figure 5B). This result implies that the role of *Grhl2* in inner ear development is conserved across vertebrate species. In human progressive deafness DFNA28, the 1609-1610insC frameshift mutation of *GRHL2* causes a premature stop codon, resulting in a protein lacking the C-terminal dimerization domain (Figure 5A) (1). Overexpression of the corresponding human mutant *GRHL2* mRNA, *GRHL2m*, in *grhl2b*<sup>T086</sup> mutant embryos failed to rescue the otolith defects. This suggests that the *GRHL2* mutation in DFNA28 patients is a loss-of-function mutation.

*grhl2b* deficiency does not affect otic patterning and hair cell development

In order to clarify the molecular mechanism underlying the function of *grhl2b* in otogenesis, we first examined the expression pattern of marker genes involved in otic
patterning in grhl2b<sup>T086</sup> mutant embryos. The expression of the medial marker pax2a, the dorsal marker dlx3b and the anterior marker foxj1b in the otic vesicle was unaltered in mutant embryos (Figure S6). The expressions of otomp and starmaker (stm), two matrix protein genes required for otolith formation (40, 41), were also unaffected in the mutant embryos (Figure S6). These data suggest that otic patterning and specification are normal in mutants.

Next, we examined otic hair cells that play essential roles in the development and function of the inner ear. The expression of hair cell marker atp1b2b (Figure S7A) and kinocilium growth, indicated by anti-acetylated tubulin antibody staining (Figure S7B), appeared normal in mutant embryos at 24 hpf. Further visualization of otic hair cells using anti-Myo6 antibody revealed that the number of hair cells remained unchanged in either utricular or saccular maculae at 24 hpf and 36 hpf (Figure S7C-D). Phalloidin staining for stereociliary bundles (Figure S7E) also demonstrated correct differentiation of the sensory maculae and cristae in mutant embryos at 5 dpf. In addition, the neuronal marker neurod showed a normal expression pattern in the developing cranial ganglia, including the developing statoacoustic ganglion (Figure S7F). Taken together, these data suggest that the patterning and specification of the otic vesicles and differentiation of hair cells are normal in the grhl2b-deficient embryos.

cldnb and epcam are down-regulated in otic vesicles of grhl2b<sup>T086</sup> mutants

To identify the downstream molecules of Grhl2b, we searched and examined markers co-expressed with grhl2b, focusing on junction proteins since Grainyhead-like family members have been shown to be involved in epithelial adhesion (18, 29, 42). Consequently, we found that the expression of the apical junction protein gene claudin-b (cldnb) disappeared in the otic vesicles of mutants at 24 hpf, but its expression in other domains was retained with decreased levels (Figure 6A). The expression of epcam encoding a basolateral junction
protein was severely reduced in mutant embryos (Figure 6A). In contrast, the otic expression of several other Claudin family members, including \textit{cldna}, \textit{cldnj} and \textit{cldn7}, appeared normal or slightly decreased (Figure S8). Given that embryos deficient for Epcam showed abnormal apical junctional complexes (AJCs) in cells of the enveloping layer (43), we further investigated the integrity of the otic epithelial AJCs in \textit{grhl2b}^{T086} mutants. The membranous localization of \(\beta\)-catenin and the apical localization of the tight junction protein ZO-1 appeared normal in mutant otic epithelial cells (Figure 6B-C). However, the length of otic epithelial AJCs of 24-hpf mutants, as detected by transmission electronic microscopy, exhibited a dramatic decrease compared to their wild-type siblings (Figure 6D). Interestingly, the length of the epidermal AJCs covering the otic vesicles in mutant embryos was comparable to that in wild-type embryos (Figure 6D). These data indicate that loss of Grhl2b affects the AJCs of the otic epithelial cells specifically.

We further investigated the essential role of \textit{grhl2b} in \textit{cldnb} and \textit{epcam} expression using \textit{Tg(hsp70:grhl2b-EGFP);grhl2b}^{T086/T086} embryos, which could express Grhl2b-GFP fusion protein in a \textit{grhl2b} mutant background following heat shock stimulation (see Materials and Methods). We found that heat shock stimulation restored \textit{cldnb} and \textit{epcam} expression levels in all of the treated \textit{Tg(hsp70:grhl2b-EGFP);grhl2b}^{T086/T086} embryos (n >50), which confirms that Grhl2b positively regulates \textit{cldnb} and \textit{epcam} expression. Overexpression of zebrafish \textit{grhl2b} mRNA in wild-type embryos was able to enhance \textit{cldnb} and \textit{epcam} expression at the bud stage, as detected by in situ hybridization (data not shown), allowing us to test whether \textit{Grhl2} mRNA of different species have similar activity on \textit{cldnb} and \textit{epcam} transcription in an easier way. Quantitative RT-PCR analysis indicated that injection of zebrafish, mouse or human \textit{Grhl2} mRNA at the one-cell stage could increase \textit{cldnb} or \textit{epcam} transcript levels at the bud stage (Figure 6F), suggesting a conserved function of Grhl2 in vertebrates. This result is consistent with previous findings that mouse Grhl2 protein is essential for \textit{Cldn4} and
Epcam expression during inner ear and/or neural tube development (30, 44). Interestingly, overexpression of the mutant form of human GRHL2, GRHL2m, did not alter their expression (Figure 6F), further suggesting that this mutant is a null allele of GRHL2.

cldnb and epcam overexpression rescues otolith defects of grhl2b^{T086} mutants

Previous studies have shown that epcam and the Claudin family member cldnj are required for otic development in zebrafish (5, 43). We asked whether cldnb and epcam were the downstream mediators of Grhl2b in inner ear development and whether their overexpression in grhl2b^{T086} mutant embryos could rescue the otolith defects. In mutant embryos injected with either 200 pg cldnb or epcam mRNA alone, the ratio of embryos with tiny or absent otoliths decreased to 59.2% (42/71) or 61.6% (125/203) from 88.4% (206/233) for uninjected mutants, respectively (Figure 6G). Importantly, coinjection of cldnb and epcam mRNAs at 100 pg or 200 pg each led to a further decrease of the ratio of mutant embryos with tiny or absent otoliths to 40.6% (39/96) or 19.0% (24/126) (Figure 6G) respectively. These data suggest that cldnb and epcam co-mediate grhl2b function in the epithelial differentiation and inner ear development.

Grhl2b directly regulates the transcription of cldnb and epcam

The next issue to be addressed was whether cldnb and epcam gene transcription was directly regulated by Grhl2b. Based on the binding sequences of Drosophila grainy head protein (29), we identified a potential Grhl2b binding site (G2BS) (AACCGGTT) 1392 bp upstream of the transcription start site of the cldnb locus and another G2BS (AACTGGTT) 140 bp downstream of the transcribed region of epcam (Figure 7A), each of which might act as an enhancer for transcription of the corresponding gene. Then, we performed chromatin
immunoprecipitation (ChIP) experiment with anti-GFP antibody using wild-type or
*Tg(hsp70:grhl2b-EGFP)* transgenic embryos at 24 hpf. ChIP-PCR results showed that the
G2BS-containing DNA regions were enriched in the immunoprecipitated chromatin mixes of
the *Tg(hsp70:grhl2b-EGFP)* transgenic embryos (Figure 7B), implying that Grhl2b could
bind to these sites in embryos. We further investigated the role of the identified G2BS of the
cldnb locus in transcription by a transgenic approach. The *cldnb* promoter sequence between
positions -2305 and +37 was used to drive EGFP expression in the *Tol2* transposon-based
transgene construct *Tol2(cldnb:EGFP)* (Figure 7C). The resulting *Tg(cldnb:EGFP)* transgenic
embryos expressed strong EGFP in the otic vesicles, olfactory placodes, hatching glands,
pharyngeal arches and pronephric ducts at 30 hpf (Figure 7D), which mimicked the
endogenous *cldnb* expression pattern (Figure 6A). We then introduced 4 mutations into the
Grhl2b binding site in *Tol2(cldnb:EGFP)* background to generate the construct
*Tol2(cldnb-mut:EGFP)*. In *Tg(cldnb-mut:EGFP)* transgenic embryos, EGFP expression was
undetectable in the otic vesicles but retained in other domains at 30 hpf, suggesting that the
Grhl2b binding site in the *cldnb* promoter is essential for specific expression of *cldnb* in otic
vesicles. Furthermore, the otic expression of *EGFP* in *Tg(cldnb:EGFP)* transgenic embryos
was specifically abolished when *grhl2b* was knocked down (Figure 7C). Taken together, these
results indicate that Grhl2b directly regulates *cldnb* and *epcam* transcription in otic vesicles
and the Grhl2b-binding site in the *cldnb* promoter is essential for *cldnb* expression.

**DISCUSSION**

In this study, we generated the zebrafish *grhl2b*-deficient line *grhl2b* *T086* by *Tol2*
transposon-based gene trap mutagenesis. *grhl2b* *T086* homozygous mutant embryos show
enlarged otocysts, reduced or absent otoliths, malformed semicircular canals, insensitiveness
to sound stimulation and imbalanced swimming motion, while hair cells of their inner ears
appear normal. Genetically, *grhl2b* regulates inner ear development by directly promoting the expression of the junction protein-coding genes *cldnb* and *epcam*, which are essential for the apical junctional complex formation and epithelial differentiation.

The *Drosophila* Grainyhead/Elf -1 is the founding member of the Grainyhead-like transcription factor family and its mutation results in embryonic lethality with defects in head skeleton and cuticular structures (17). Later on, three homologues of Grainyhead, Grainyhead-like 1, Grainyhead-like 2 and Grainyhead-like 3, have been identified in vertebrate species (45-47). An autosomal dominant form of progressive nonsyndromic sensorineural hearing loss in a large American family, which shows varying degrees of severity and variable age of onset among the affected individuals, has been shown to be linked with a single nucleotide insertion (1609-1610insC) in the coding region of the *GRHL2* locus that ultimately results in a truncated form of GRHL2 (1). A recent association study revealed that several SNPs at the *GRHL2* locus are associated with age-related hearing impairment and the SNPs presumably change the expression level of this gene (2). In mouse *Grhl2*-knockouts, however, abnormal development of the inner ear and hearing inability are not observed probably due to embryonic lethality occurring around day E11.5 (30, 48). In this study, we report that zebrafish *grhl2b*T086 mutant embryos exhibit inner ear defects with variable expressivity among individuals. Thus, this mutant line becomes the first animal model of the human progressive hearing loss DFNA28, and can be used to further study the mechanisms underlying *GRHL2*-associated deafness. The mammalian model could be generated by conditionally knocking out *Grhl2* in the otic epithelial cells of mice, which will help understand the role of Grhl2 in mammalian inner ear development.

Zebrafish *grhl2b* is expressed in the otic vesicles, olfactory placodes, pharyngeal arches, pronephric ducts and other tissues/organs during embryogenesis. However, developmental abnormalities in the *grhl2b*-deficient mutant embryos appear restricted to the inner ears in the
first few days during development. The human progressive hearing loss DFNA28 is non-syndromic without other detectable defects, although GRHL2 is also expressed in multiple human tissues/organs, including the prostate, thymus, kidney, lung, salivary gland, mammary gland and digestive tract (1). The similarity in the phenotype between zebrafish and human Grhl2-deficient individuals suggests that Grhl2 is essential for the development of the inner ear and the acquisition of the hearing ability. Interestingly, Grhl2-knockout mouse embryos have defects in neural tube and other epithelial organs, while defects in otic development are not reported (30, 48). It may be predicted that human GRHL2-null embryos could exhibit various defects in multiple tissues and organs. Moreover, grhl2b T086 mutants need to be examined at later stages of development to make sure whether other tissues and organs are affected.

The Grhl family members play important roles in epithelial differentiation which could lead to cell cycle arrest. It has been reported that the Drosophila grainyhead mutants have more abdominal post-embryonic neuroblasts than the wild-type flies, suggesting hyperproliferation of the abdominal neuroblasts (42). The epidermis of Grhl3-null mice also exhibits cellular hyperproliferation (49). The zebrafish grhl2b T086 mutants manifest enlarged otocysts at 24 hpf and 50 hpf (Figure 1J, M). Immunostaining for phospho-Histone H3 (pH3) using a anti-pH3 antibody identified more positive cells in the otic vesicles of the mutants compared to those in the heterozygous siblings (Figure S9), suggesting hyperproliferative otic epithelial cells of mutants. Therefore, it is likely that Grhl2b regulates proliferation and differentiation of the otic epithelial cells by mechanisms similar to other Grhl family members.

We found that Grhl2b is required for the expression of cldnb and epcam, both of which encode junction proteins. A previous study showed that a mutation in the zebrafish epcam locus causes expanded otocysts and reduced otoliths (43), which is similar to the
grhl2b-deficient phenotype. Although the involvement of cldnb in inner ear development has not been reported, the defective inner ears or deafness due to the deficiency or mutations of other Claudin family genes, e.g., cldnj (5), Cldn9 (8), Cldn11 (7) and Cldn14 (6, 9, 10), have been documented in various vertebrate species. In addition, overexpression of exogenous cldnb and epcam could rescue the otolith defects in grhl2bT086 mutants. Thus, it is not exceptional that Grhl2b in zebrafish exerts its effect on otic development through Cldnb and Epcam. However, we cannot exclude the possibility that Grhl2b regulates the otic development through other downstream targets.

The cellular junctions formed by junction proteins play crucial roles in the homeostasis of the otic lumen and the endolymph. Abnormal expression of genes encoding junction proteins or ion channels can cause severe defects in inner ear development (4). For instance, Cldn4 has been shown to regulate the epithelial barrier function in rat submandibular gland cells (50). In grhl2bT086 mutants, the contracted apical junctional complexes may affect the barrier function of the otic epithelial cells and alter the ionic composition and homeostasis of the otic lumen and the endolymph. Consequently, the loss of homeostasis could hamper the volume control of the otocysts and the growth of otoliths and semicircular canals. Collectively, these defects lead to hearing and balance problems (Figure 7E), although whether vestibular dysfunction exists in the DFNA28 patients still needs to be determined. Grhl2b may also directly regulate the expression of some ion channels in a manner similar to mouse CP2L1 (51). Further analysis of the ionic composition of the otic lumen and endolymph in grhl2bT086 mutants will help illustrate the role of Grhl2b in ion channel formation.
MATERIALS AND METHODS

Zebrafish strains and transgenesis

The zebrafish AB line was used in this study. For Tol2 transposon-based insertional mutagenesis, the transposon vector TSG was modified from a previous vector, T2BGS (32, 52), by replacing the Tol2 element with shorter ones from pDestTol2pA2 (53). Two TGA stop codons upstream of the EGFP sequence were introduced into the two reading frames that were different from the EGFP frame to terminate unwanted translations. Injection of TSG DNA and transposase mRNA, screening of gene trapping lines and identification of mutant lines were carried out as previously described (31). grhl2bT086 line, one of the trapped lines, was maintained by crossing the heterozygotes with wild-type fish to avoid inbreeding depression. To obtain more homozygous embryos for analyses, homozygous females were mated to heterozygous males. Stronger EGFP fluorescence of homozygous embryos allowed their separation from heterozygous embryos (weak EGFP).

For cldnb promoter analysis, a sequence located between -2305 and +37 bp of the cldnb locus was amplified by PCR, and first cloned into the pEGFP-N3 vector. Then the expression cassette was subcloned into the vector pT2AL200R150G (54) to generate the transgene construct Tol2(cldnb:EGFP). The construct Tol2(cldnb-mut:EGFP) was identical to Tol2(cldnb:EGFP) except mutations within the putative Grh1lb binding site in the cldnb promoter (see Figure 7H). Tol2(cldnb:EGFP) or Tol2(cldnb-mut:EGFP) DNA was co-injected with Tol2 transposase mRNA into one-cell wild-type embryos. The founder fish were mated to wild-type fish, and their progenies expressing EGFP were sorted out for growing up. Different lines of Tg(cldnb:EGFP) showed similar EGFP pattern, but the fluorescence intensity varied. The same phenomenon for Tg(cldnb-mut:EGFP) transgenic lines was observed.
To establish the \textit{Tg(hsp70:grhl2b-EGFP)} transgenic line, the zebrafish \textit{hsp70} promoter and the grhl2b-EGFP fusion coding sequence were cloned into a \textit{Tol2} transposon-based vector, and recombinant plasmid was co-injected with \textit{Tol2} transposase mRNA to produce the founder fish. The transgenic founder fish were mated to \textit{grhl2b}^{T086} homozygous fish, allowing production of \textit{Tg(hsp70:grhl2b-EGFP); grhl2b}^{T086/T086} mutant embryos thereafter. For heat shock experiment, embryos at 20 hpf were raised at 38°C for 30 min and then fixed at 24 hpf for further examination.

**TAIL-PCR, RT-PCR and 5’ RACE**

The thermal asymmetric interlaced PCR (TAIL-PCR), reverse transcription PCR and real-time PCR were performed as previously described (31, 52). 5’ RACE was performed using the FirstChoice® RLM-RACE Kit (Ambion) according to the manufacturer’s instructions. Sequence information of all primers used in this study will be provided upon request.

**Chromatin immunoprecipitation**

Wild-type and \textit{Tg(hsp70:grhl2b-EGFP)} embryos were heat-shocked at 20 hpf and collected at 24 hpf for chromatin precipitation with anti-GFP antibody (7G9, Abmart Inc.). ChIP experiments were performed as previously described (55). To amplify the regions surrounding the putative Grhl2b binding site (Figure 7A) from the precipitated chromatin, the following primers were used: cldnb-forward (5’-GAACAGACATGCCTGAGCAATGG-3’), cldnb-reverse (5’-ACTTTATGGCTCCTGGACATGAC-3’), epcam-forward (5’-CGAAGTCAGCAGAGATTCAAACC-3’), epcam-reverse (5’-CGAAGTGTCAGCAGATTCAAACC-3’), control-forward
(5’-GTTTGCAGGACTTGAAAAGCAGATG-3’) and control-reverse (5’-CCACGGTACAAAAGAACATTTGACC-3’).

**Mauthner cell recording and startle response tests**

Embryos were raised in Hank’s solution with 100 μM phenylthiourea (PTU, Sigma) and 5 - 6 dpf larvae were paralyzed with 0.1% α-Bungarotoxin (Tocris Bioscience) and mounted in 1% low melting agarose gel for Mauthner cell recording. Break-through whole-cell recording of Mauthner cell was made under visual control. The micropipette was made from borosilicate glass capillaries (WPI). The internal solution contained 110 mM K-gluconate, 6 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 10 mM EGTA (pH 7.3) and the reversal potential of chloride ion (E_Cl⁻) was about -60 mV. Recording was made with patch-clamp amplifiers (MultiClamp 700B, Axon Instruments). For sound stimulation, brief pure tones (10 ms, 500 Hz) with several intensities were given through the air from a voice box (Edifier, R1800TII).

Sound-evoked C-shape startle response was tested in 96-well plastic plates, and recorded through a high speed camera (Redlake, MotionScope M3, 1000 fps) under infrared light illumination. Pure tone stimulations (10 ms, 500 Hz) with two different intensities were given through plastic board mounted on a voice box (HiVi, D1080MKII). Each larva was tested 14-16 times and the percentage of C-startle times was calculated for each larva. The probability of the C-startle response for a group of larvae was the average percentage of C-startle reflects.
In situ hybridization, immunofluorescence and phalloidin staining

The whole mount in situ hybridization and immunofluorescence were performed as previously described (32, 52) with modifications. The embryos were bathed in 1 mM EDTA (pH 8.0) at 94-100°C for 10 min and cooled down to room temperature before adding serum block to repair antigen. Mouse anti-acetylated tubulin antibody was purchased from Sigma (T6793), rabbit anti-phospho-Histone H3 (Ser10) antibody from Cell Signaling (9701S), rabbit anti-ZO-1 antibody from Invitrogen (61-7300), mouse anti-GFP antibody from Santa Cruz Biotechnology (sc-9996), and rabbit anti-Myosin 6 antibody was a kind gift from Dr. Dong Liu. Phalloidin staining was performed according to Haddon and Lewis’ description (56). The Z-projections of the confocal stacks were generated using either FluoView or ImageJ softwares.

Transmission electronic microscopy

Embryos at 28 hpf were fixed with 2.5% glutaraldehyde and 1% OsO₄ followed by dehydration with graded ethanol solution and acetone. Then, embryos were filtered and embedded with Epoxy resin 812. The inner ears were sectioned at the sagittal plane.

Morpholinos and mRNA injection

Three morpholinos were used in this study: G2MO1 (5'-CTGTGACATATTCTTCCTCCGC-3’), which targets the translational starting region of zebrafish *grhl2b*; the control morpholino G2cMO (5'-CTGTGACTTCCAAAGTCTTCCTCCGC-3’), which contains six mismatched nucleotides (underlined); and p53-MO (5’-GACCTCCTCTCCACTAAACTACGAT-3’), which targets
p53. The coding sequence of a gene was cloned into the vector pXT7 and the linearized plasmid was used as a template for in vitro transcription using the mMessage Machine Kit (Ambion). The plasmid containing human or mouse Grhl2 coding sequence was kindly provided by Dr. S. M. Jane (45). The coding sequence of the human GRHL2 1609-1610insC mutant (GRHL2m) was generated by PCR using site-specific mutagenesis. Morpholino or mRNA was injected at the one-cell stage at the indicated dose.

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REFERENCES


*Development, 136*, 2837-2848.


differentiation-associated Grainyhead gene Get1/Grhl3 also regulates urothelial

22 Ting, S.B., Wilanowski, T., Auden, A., Hall, M., Voss, A.K., Thomas, T., Parekh, V.,

23 Gustavsson, P., Greene, N.D., Lad, D., Pauws, E., de Castro, S.C., Stanier, P. and Copp,

regulates epidermal terminal differentiation and interacts functionally with LMO4. *Dev.

Grainyhead-like 3, a transcription factor identified in a microarray screen, promotes the

26 Tao, J., Kuliiev, E., Wang, X., Li, X., Wilanowski, T., Jane, S.M., Mead, P.E. and
Cunningham, J.M. (2005) BMP4-dependent expression of Xenopus Grainyhead-like 1 is
27 Janicke, M., Renisch, B. and Hammerschmidt, M. (2010) Zebrafish grainyhead-like1 is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a Foxi3-dependent manner. *Int. J. Dev. Biol.*, 54, 837-850.


Methods, 118, 177-187.

amplification and sequencing of insert end fragments from P1 and YAC clones for 

38 Robu, M.E., Larson, J.D., Nasevicius, A., Beiraghi, S., Brenner, C., Farber, S.A. and 

Grainyhead contains novel DNA-binding and dimerization domains which are conserved 

40 Sollner, C., Burghammer, M., Busch-Nentwich, E., Berger, J., Schwarz, H., Riekel, C. and 
Nicolson, T. (2003) Control of crystal size and lattice formation by starmaker in otolith 

Neurobiol.*, 68, 209-222.

42 Almeida, M.S. and Bray, S.J. (2005) Regulation of post-embryonic neuroblasts by 
30

43 Slanchev, K., Carney, T.J., Stemmler, M.P., Koschorz, B., Amsterdam, A., Schwarz, H.


LEGENDS TO FIGURES

Figure 1. EGFP expression and phenotypic changes in grhl2b\textsuperscript{T086} embryos. (A-E) EGFP expression in grhl2b\textsuperscript{T086} heterozygous (hetero) (B, D) and homozygous (homo) (A, C, E) embryos at 12 s (A), 24 hpf (B, C) and 48 hpf (D, E). Embryos of different genotypes showed different levels of EGFP. al: anterior lateral line primordium; ba: branchial arches; hg: hatching gland; l: lens; ll: lateral line neuromast; ol: olfactory placode; ot: otic vesicle; pd: pronephric duct; pf: pectoral fin; pl: posterior lateral line primordium. (F-H) Gross morphology of embryos of different genotypes at 24 hpf. WT, wild-type. (I, J) Otic vesicles at 24 hpf were visualized by DIC microscopy. Scale bars: 40 μm. (K) Statistical analysis of the otic lumen area, otolith area and epithelia width in different types of embryos at 30 hpf. Individual embryos were randomly picked up from each type. The otic lumen and otolith areas were measured in the focal plane representing the maximal area. The epithelia width of a single otic vesicle was defined as the average width of the otic epithelial layer and was estimated to be the area of the otic epithelial cells divided by the mean value of the inner and outer perimeters of the otic epithelial layer. The parameters were measured with SPOT Advanced software (version 4.6). Error bars: standard deviation (also applies to other figures); ns: nonsignificant; ***: p<0.0001 (student’s \( t \)-test, also applies to other figures unless indicated otherwise). (L, M) Otic vesicles of heterozygous and homozygous embryos at 50 hpf showing the semicircular canal protrusions (black arrow) and otoliths (yellow arrowhead). (N-O) Otic vesicles of wild-type (N) and severe homozygous (O) embryos at 5 dpf. The otoliths and semicircular canals were indicated by yellow arrowheads and red arrows respectively.
Figure 2. Hearing disability of grhl2b<sup>T086</sup> severe mutants at 5 - 6 dpf. (A-C) Excitatory postsynaptic currents (EPSC) of Mauthner cells (M-cells) were recorded under sound stimuli with 500 Hz frequency and 10 ms duration. The severe mutants (with tiny or without otoliths) were used. (A) Examples of the recorded EPSC in wild-type (WT), heterozygous (hetero) or homozygous (homo) individuals. The sound stimuli (80 dB) started at t = 0 and lasted 10 ms, during which hair cell signals could be induced several times and multiple action potentials in the ganglion cells could be produced. Besides, a single M-cell could receive input from many different hair cells through ganglion cells, and as a result, multiple peaks were produced. (B, C) The EPSC amplitudes (B) and total charges (C) were shown for different sound levels. n = 7 for each group. (D) Examples of C-shaped startle responses of different larvae in response to the sound stimuli with 500 Hz frequency and 10 ms duration. The typical escape response of a wild-type larva was initiated 5-7 ms after the sound stimulation. (E) The average C-startle response probability. 87-88 larvae were tested for each group. The sound intensities were designated with "+" and "++" to avoid confusion because the sound stimulations were given by different ways in the M-cell recording and startle response experiments (see the MATERIALS and METHODS section). ns: nonsignificant; **: p<0.01; ***: p<0.0001.

Figure 3. Grhl2b was trapped in the grhl2b<sup>T086</sup> line. (A) Genomic organization of the zebrafish grhl2b locus. The Tol2 transposon element was inserted into the first intron in the grhl2b<sup>T086</sup> line. f1, f2, f3, r2, r3 and r4 represented the positions of the primers used for linkage analysis and RT-PCR. (B) The predicted mature wild-type (WT) transcript and mutant
grhl2b-EGFP fusion transcript. (C) RT-PCR analysis using the indicated primers. RNA was isolated from 24 hpf embryos. The f1/r3 primer pair amplified a wild-type 342 bp fragment and the f1/r4 pair amplified a mutant 434 bp fragment. (D) Real-time PCR analysis of grhl2b wild-type transcripts in different types of embryos at 24 hpf. β-actin was used as an internal control. *: p<0.05; **: p<0.001. (E-K) Expression pattern of grhl2b in different types of embryos at indicated stages, which was detected by in situ hybridization. al: anterior lateral line primordium; ll: lateral line neuromast; ol: olfactory placode; ot: otic vesicle; pd: pronephric duct; pf: pectoral fin; pl: posterior lateral line primordium.

Figure 4. Effect of grhl2b knockdown in wild-type embryos and rescue effect of grhl2b overexpression in mutants. (A) Morphology of 36-hpf wild-type embryos injected with different morpholinos. One-cell embryos were injected with 1 ng of G2MO or G2cMO (control). Uninj: uninjected. Note the change in otolith size. (B) The variable otolith size of mutants (grhl2bT086 homozygotes) embryos at 36 hpf. Based on the otolith size, the embryos were classified into four categories: normal, small, tiny and absent. (C) Percentages of embryos in each category. One-cell embryos were injected with indicated morpholinos at indicated dose and categorized at 36 - 40 hpf. Uninj, uninjected wild-type embryos; Homo, un.injected grhl2bT086 homozygous embryos. (D) Percentages of mutant embryos in each category. One-cell grhl2bT086 homozygous (mutant) embryos were uninjected or injected with 200 pg of zebrafish grhl2b mRNA and categorized at 36 - 40 hpf. n, the number of observed embryos.
Figure 5. Grhl2 is conserved in structure and function. (A) Schematic representation of the three functional domains of zebrafish, mouse and human Grhl2 proteins. The percentage of identity/similarity was indicated for the transactivation domain (AD), the CP2 DNA binding domain (CP2) and the dimerization domain (DD). hGRHL2m is the truncated form of hGRHL2 in DFNA28 patients. (B) Effect of mouse or human Grhl2 overexpression in grhl2b<sup>T086</sup> mutants. One-cell grhl2b<sup>T086</sup> mutant embryos were uninjected (Uninj) or injected with 200 pg of mouse (mG2) or human (hG2) Grhl2 mRNA or human mutant GRHL2 (hG2m), and categorized at 36 - 40 hpf based on the otolith size. n, the number of observed embryos.

Figure 6. Grhl2b regulates inner ear development by promoting the otic expression of cldnb and epcam. (A) Expression of cldnb and epcam in wild-type (WT) and mutant (homo) embryos at 24 hpf, detected by in situ hybridization. Insets show the enlarged images of the otic vesicles. (B-C) Subcellular localization of β-catenin and ZO-1 proteins in heterozygous and homozygous embryos at 28 hpf. Pan-cadherin antibody was used to label the cell membrane. Scale bars: 20 μm. Dorsal to the top and anterior to the left. (D) Transmission electronic microscopic images showing otic epithelial and epidermal apical junctional complexes (AJCs). The AJCs size between two adjacent cells was marked between two arrows. Four wild-type (WT) and four mutant (homo) embryos at 24 hpf were examined. Scale bars: 200 nm. The statistical data were shown on the right for the average length of AJCs. n, total number of the AJCs. ns: non-specific, ***: p<0.0001. (E) Expression of cldnb and epcam in grhl2b<sup>T086/T086</sup> mutant (homo) and in Tg(hsp70:grhl2b-EGFP<sub>mutant</sub>) at 24 hpf after heat shock induction. (F) Real-time PCR results showed the effects of zebrafish, mouse, or human Grhl2 mRNA
overexpression in wild-type embryos on cldnb and epcam transcript levels. Note that hGRHL2m, the mutant form of human GRHL2, had no effect while others increased cldnb and epcam expression. (G) Statistical data showing distribution of mutant embryos with different otolith size after injection of cldnb or/and epcam mRNAs. Embryos were injected at the one-cell stage and categorized at 38 - 40 hpf based on otolith size. The number of observed embryos was indicated. Chi-square ($\chi^2$) values were calculated with a 2×4 contingency table for each rescue experiment.

**Figure 7. Grhl2b directly regulates the otic expression of cldnb and epcam.** (A) Schematic representation of the putative Grhl2b binding site (G2BS) in the cldnb or epcam locus. The orange boxes indicate the transcribed region. The lines below indicate the corresponding regions amplified in ChIP-PCR. (B) ChIP-PCR results. Heat shock-induced Tg(hsp70:grhl2b-EGFP) or wild-type embryos were used to perform chromatin immunoprecipitation with or without anti-GFP antibody. Note that only G2BS-containing regions were enriched in the chromatin mixes pulled-down by the antibody. The control region (ctr) from the cldnb locus could not be enriched. (C) Schematic representation of the transgenic vectors. The G2BS-containing sequence in the putative cldnb promoter is shown. The mutated nucleotides were in blue. (D) EGFP expression in the Tg(cldnb:EGFP) and Tg(cldnb-mut:EGFP) transgenic fish at 30 hpf. Note that EGFP was expressed in the otic vesicle of Tg(cldnb:EGFP) but not Tg(cldnb-mut:EGFP) embryos. Injection of 2 ng G2MO into the Tg(cldnb:EGFP) embryos eliminated otic EGFP expression while G2cMO injection had no effect. The otic vesicles were enlarged in the insets. (E) A model of Grhl2 functions in inner ear development and hearing. Grhl2 directly regulates the expression of the junction
proteins such as Cldnb and Epcam at the transcriptional level, which in turn control the integrity and differentiation of the otic epithelium. The correct differentiation and function of the otic epithelium provide appropriate conditions for the development of the statoacoustic organs like otoliths and vestibular canals, which are essential for the hearing and balance systems.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A

mGrhl2  AD  CP2  DD  70% / 86%
zGrhl2b AD  CP2  DD  71% / 86%
hGRHL2 AD  CP2  DD  96% / 98%
hGRHL2m AD  CP2

B

% of different types of embryos

Uninj  mG2  Uninj  hG2  Uninj  hG2m

n= 81  90  80  74  74  97

Absent  Tiny  Small  Normal
Figure 6.
Figure 7.

A

\[ \text{G2BS} \quad \text{cldnb} \quad \text{ctr} \quad \text{epcam} \quad \text{G2BS} \]

B

\begin{tabular}{ccc}
Embryos & WT & hsp70:grh12b-EGFP \hline
Anti-GFP & + & - & + & + & - & + & + \\
ChIP & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} & \includegraphics[width=0.5\textwidth]{chips.png} \\
Input & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} & \includegraphics[width=0.5\textwidth]{inputs.png} \\
\end{tabular}

C

\[ \text{To12 3'} \quad \text{cldnb promoter} \quad \text{EGFP} \quad \text{pA} \quad \text{To12 5'} \]

\begin{align*}
\text{Wild-type:} & \quad \text{AGCAGAAACCGTTTGACCA} \\
\text{Mutant:} & \quad \text{AGCAGAAATCGACTTGACCA} \\
-1398 & \quad -1378
\end{align*}

D

\[ \text{Tg(cldnb:EGFP)} \quad \text{Tg(cldnb:EGFP)} \]

\[ \text{Uninj} \quad \text{G2cMO} \]

\[ \text{Tg(cldnb:EGFP)} \quad \text{Tg(cldnb-mut:EGFP)} \]

\[ \text{G2cMO} \quad \text{Uninj} \]

E

\[ \text{Grh12} \quad \text{Cldnb} \quad \text{Epcam} \quad \text{Tight junction} \quad \ldots \quad \text{Otocyst volume} \quad \text{Otolith} \quad \text{vestibular canals} \quad \ldots \quad \text{Hearing balance} \]