Int. J. Dev. Biol. 59: 417-423 (2015)
doi: 10.1387/ijdb.140230FL

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Grhl1 deficiency affects inner ear development in zebrafish

FEI LIU[1,#], FAN YANG[1,#], DANPING WEN[1], WENJUN XIA[1], LILI HAO[1], JIONGJIONG HU[2], JIE ZONG[3], XIAOFANG SHEN[1], JING MA[1], NAN JIANG[1], SHAOYANG SUN[1], JIN ZHANG[1], HUIJUN WANG[4], XU WANG[1], ZHAOXIN MA*[,2] and DUAN MA*[,1,4]

[1]Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Institute of Biomedical Sciences, School of Basic Medical Sciences, Fudan University, [2]Department of Otorhinolaryngology, Shanghai East Hospital, Tongji University, [3]Novel bioinformatics Co., Ltd and [4]Children's Hospital of Fudan University, Fudan University, Shanghai, China

ABSTRACT Many genes that have been found to contribute to deafness are currently being studied. Some 87 non-syndromic hereditary deafness genes have been confirmed. Proteins associated with cochlear development have also been confirmed. Some of these proteins have important relationships with gap junctions (GJ) and tight junctions (TJ). However, the desmosome junction has received little attention due to controversy over whether it could be detected in the inner ear. *GRHL1* is a conserved transcriptional regulator, and it is key to vertebrate desmosome formation. *GRHL2* has been confirmed as a deafness gene at the DFNA28 locus. These two homologous proteins have similar sequences and functions. Here, a *grhl1* down-regulated zebrafish model exhibited inner ear developmental malformations, including missing otoliths, disordered and abnormal numbers of hair cells in the inner ear and lateral line, and sound insensitivity. The mutant zebrafish swam in circles. Hair cell apoptosis was evident. Under electron microscopy, desmosomes in the otic sensory epithelium were found to be damaged. These defects were partially rescued by treatment with either *GRHL1* or its target gene, *DSG1*. Collectively, these data are the first to indicate that *grhl1* is important to the developing inner ear epithelia in zebrafish and that it acts via desmosome junction regulation.

KEY WORDS: *GRHL1, zebrafish, desmosom, hair cell, DSG1*

Introduction

Federal surveys estimate that up to 36 million Americans have hearing loss, and these numbers are expected to increase worldwide over time. Approximately 50% of hearing impairments are attributable to genetic causes (Ganapathy *et al.*, 2014), and 80% of hereditary hearing loss is non-syndromic (lacking other clinical symptoms). Up to Jan 2015, approximately 87 NSHL deafness genes have been identified (http://hereditaryhearingloss.org/main) and previously unknown genes that contribute to deafness are under investigation. As more genes are identified and confirmed to be associated with hearing loss, gene distribution patterns suggest that they cluster in one family (Table 1). This paper concerns the function of genes in the same families as known deafness genes. *GRHL1* was chosen as the target gene and its role in the inner ear of zebrafish was studied in depth. The grainy head (GRH) family of transcription factors is one of the most conserved genes found in vertebrates. It is critical to various types of system development (Cenci and Gould, 2005, Gao *et al.*, 2013, Han *et al.*, 2011, Janicke *et al.*, 2010, Sanchez-Calderon *et al.*, 2007). For example,

Abbreviations used in this paper: DAPI, 4',6-diamidino-2-phenylindole; DEPC, diethy pyrocarbonate; DFNA, autosomal dominant non-syndromic sensorineural hearing loss; dpf/hpf, days/hours post-fertilization; DSG1, desmoglein 1, *Homo sapiens*; GJ, gap junctions; grhl1, grainyhead-like 1 (*Drosophila*); HC, hair cell; Mcon, mismatch morpholino oligonucleotides; MO, morpholino oligonucleotide; PBST, phosphate buffer; PTU, Propylthiouracil; SC, supporting cell; SE, sensory epithelium; TEM, transmission electron microscopy; TJ, tight junction; TUNEL, terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; WISH, whole mount *in situ* hybridization.

***Address correspondence to:** Duan Ma. Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Institutes of Biomedical Science, Shanghai Medical College, Fudan University, Shanghai 200032, China. Tel/Fax: +86-21-5423-7441. E-mail: duanma@fudan.edu.cn or Zhaoxin Ma. Department of Otorhinolaryngology, Shanghai East Hospital,Tongji University, Shanghai, 200120, China. Tel/Fax: +86-21-5882.2171. E-mail: mzhx114@163.com

[#]These authors contributed equally to this work.

Supplementary Material (six figures) for this paper is available at: http://dx.doi.org/10.1387/ijdb.140230FL

Accepted: 5 February 2015.

ISSN: Online 1696-3547, Print 0214-6282

TABLE 1

MANY GENES RELATED TO DEAFNESS ARE FROM THE SAME FAMILY

GJB2	GJB3	GJB6		
MY01A	MY06	MY07A	MY015A	MY03A
SLC17A8	SLC26A4	SLC26A5		
DIAPH1	DIAPH3			
MYH9	MYH14			
POU3F4	POU4F3			
COL4A6	COL11A2			
.....				

the GRH family is important to epithelial barrier development and post-wounding barrier repair via regulation of junction protein expression and to epithelial adhesion, which is essential to organ development (Hama) (Forge *et al.*, 2013, Zhu *et al.*, 2013). Within this family, *GRHL2* has been confirmed to be related to DFNA28. Two genes in the same family, *GRHL1* and *GRHL3*, have yet to be characterized (Vona *et al.*, 2013). *GRHL1* was studied here because it is evolutionarily more similar to *GRHL2* than to *GRHL3* (Fig. 1).

Zebrafish have an integrated inner ear consisting of HCs, supporting cells, and cilia and principles of inner ear development in the zebrafish seem to be the same as in other vertebrates desmosomal(Bok *et al.*, 2007, Haddon and Lewis, 1996, Mogass *et al.*, 2000, Waterman and Bell, 1984). During embryogenesis, special neuromasts within HCs form along the embryo head and lateral line. Zebrafish share 84.87% gene homology with humans, making then excellent models for genetic and molecular, analyses (Wu *et al.*, 2010). Here, zebrafish were used to verify the influence of *grhl1* in hearing and to identify the mechanism underlying this process.

Morpholino-modified antisense oligonucleotides were used in this study to down-regulate *grhl1* in zebrafish. Zebrafish lacking *grhl1* exhibited abnormal phenotypes consisting of small and eliminated otoliths, disorded and abnormal numbers of HCs in the inner ear and lateral line, and insensitivity to sound stimulation. Injection of morpholino oligonucleotides (MO) can also cause apoptosis of HCs. At the subcellular level, desmosomes in the sensory epithelium were seriously damaged. These abnormal defects could be rescued by coinjecting the mRNA of either *GRHL1* or its target gene *DSG1*.

Results

Multi members associated with hearing loss in one protein family

To discover the candidate deafness genes, stocks of known genes in genome were scanned for regularity. As shown (Table 1), some genes were found to cluster in one family.

Grhl1 is expressed in the inner ear and is important for auditory development in zebrafish

During auditory development, the embryonic zebrafish inner ear appears as a solid otic placode near the hindbrain by 13.5 hpf (Colantonio *et al.*, 2009). RT-PCR (0–96 hpf) and WISH were used to investigate the timing of grhl1 mRNA emergence. The *grhl1* gene was expressed at the 6th hour onward (Fig. 1B). WISH was performed in 12 and 24 h samples (Fig. 1D). Results showed that *grhl1* expressed in inner ear and is important for auditory development in zebrafish. This pattern of *grhl1* expression suggests that the gene may place a role in inner ear development.

MO was designed with Gene Tools as described previously to block the splice site between the 3rd exon and the 3rd intron (Janicke *et al.*, 2010). This produced mature mRNA with a terminator codon (Sup. Fig. 1). RT-PCR was performed to evaluate MO knock-down efficiency and specific cDNA products were amplified. MO zebrafish had 400 bp products and morphants had no product. These assays confirm that grhl1-MO is highly efficient (Fig. 1C, Sup. Fig. 2).

Phenotypes of otoliths, hair cells, and neuromasts in the antisense morpholino oligonucleotide (MO) group

To determine whether *grhl1* is essential to otic development in zebrafish, the MO zebrafish phenotype was examined and compared to that of wild-type embryos. The overall morphology of wild-type embryos, mismatch control MO (Mcon MO), and *grhl1* MO at 60 hpf were used to exclude nonspecific phenotypes. Zebrafish in the MO group had abnormal otoliths with respect to size, number, and location, and a malformed semicircular canal was observed (Fig. 2A). An optimal concentration of MO was 3 ng; this was less lethal but offered significant numbers of abnormal phenotypes (Fig. 2C). Next, auditory system damage was assessed after MO injection with FM staining to detect HCs and phalloidin-labeled FITC was used

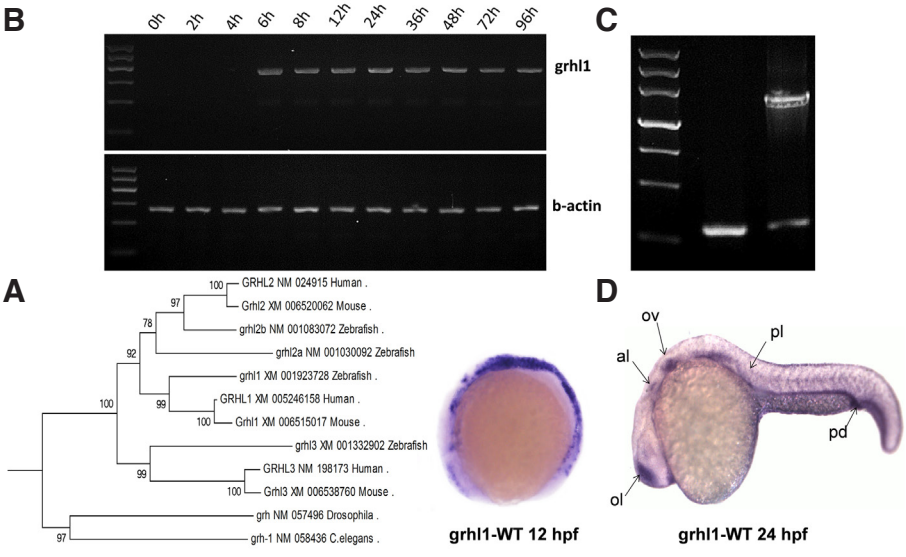

Fig. 1. *grhl1* mRNA expression in embryonic zebrafish. (A) *Phylogenetic analysis indicates that GRHL1 is evolutionarily nearer to GRHL2 than to GRHL3.* **(B)** Grhl1 *expression persists from 6 hpf onward in zebrafish.* **(C)** *Reverse-transcription PCR indicates considerable knockdown efficiency in the MO group. Primers were designed to work in intron 3 and exon 3.* **(D)** *WISH indicates a ubiquity of* grhl1 *in embryos at 12 and 24 hpf. The signal was detected in the ear region, al, anterior lateral line primordium; ll, lateral line neuromast; ol, olfactory placode; ov, otic vesicle; pd, pronephric duct; pf, pectoral fin; pl, posterior lateral line primordium.*

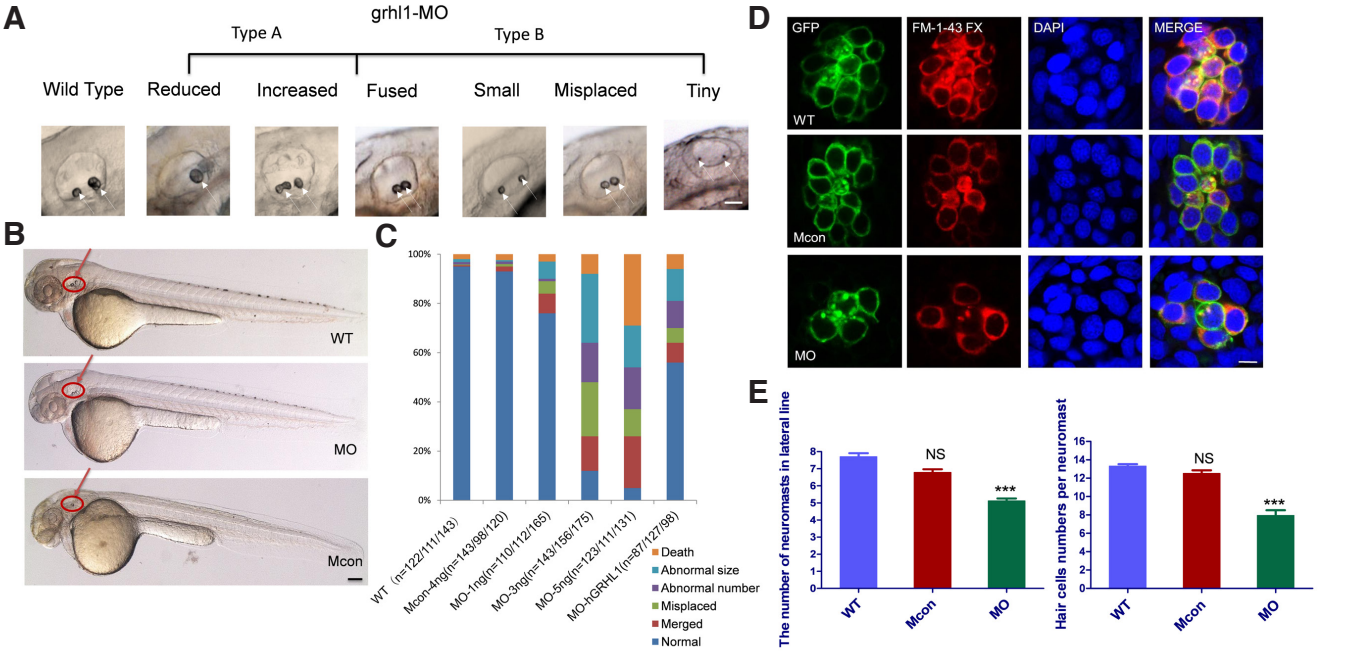

Fig. 2. Knockdown of *grhl1* induces otolith and neuromast defects in larval zebrafish. (A) *Different types of malformed otoliths in the MO group.* **(B)** *No significant exterior differences among wide-type embryo and zebrafish injected with MO or mismatch control MO.* **(C)** *Statistical results of different types of malformed otoliths over all MO concentrations (3 ng was the optimal concentration).* **(D–F)** *Lack of* grhl1 *led to decreased HCs/neuromasts. Bar: 200 μm in (A,B); 10 μm in (D).*

to stain and image cilia.

Zebrafish have a lateral line system composed of neuromasts within HCs and supporting cells. Neuromasts, originating from a primordium near the head, are neatly arranged from L1 to L8 (Sup. Fig. 5). However, the primordium is homologous to the neuromasts of the inner ear. A transgenic zebrafish model in which the *pou4f3* promoter drives GFP expression specifically in HCs was used to quantify neuromasts in the lateral line and HCs in a single neuromast (Sup. Fig. 5) (He *et al.*, 2013).

As shown, *grhl1* MO decreased the number of neuromasts in

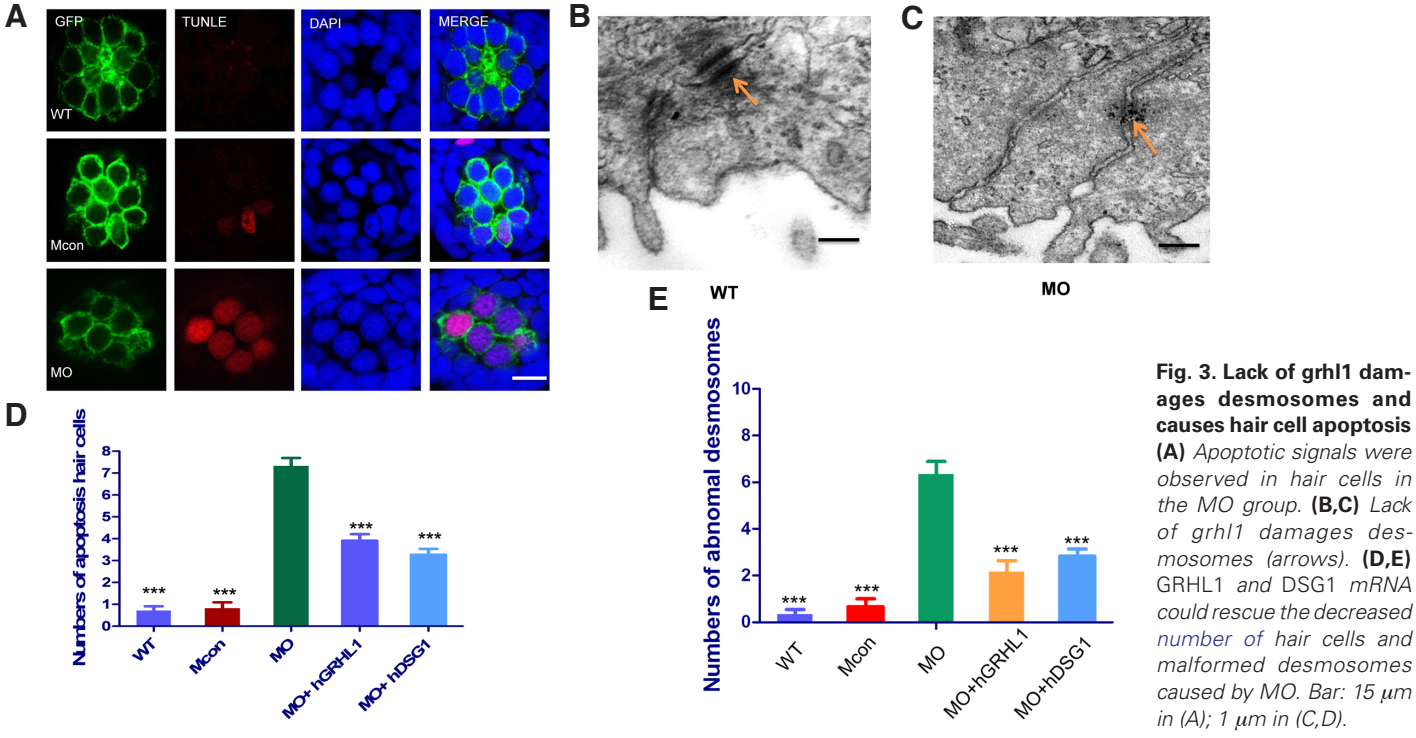

Fig. 3. Lack of grhl1 damages desmosomes and causes hair cell apoptosis (A) *Apoptotic signals were observed in hair cells in the MO group.* **(B,C)** *Lack of grhl1 damages desmosomes (arrows).* **(D,E)** GRHL1 *and* DSG1 *mRNA could rescue the decreased number of hair cells and malformed desmosomes caused by MO. Bar: 15 μm in (A); 1 μm in (C,D).*

the lateral line and disturbed their order (Fig. 2E). The neuromasts were also brighter in the WT group (Sup. Fig. 4). Changes in the numbers of HCs numbers in a single neuromasts were studied using confocal microscopy (Fig. 2 D,F).

Lack of grhl1 damages desmosomes and causes hair cell apoptosis

We observed red signal (TMR-RED) in most living HCs (Fig. 3A). Prior to TUNEL assays, TEM was used to visualize desmosomes in the auditory sensory epithelium (Sup. Fig. 6). Desmosomes were observed in the inner ear neuromasts and saw few integrated desmosomes in the MO group (Fig. 3C, D).

Down-regulation of grhl1 was found to cause otic developmental defects in zebrafish. *GRHL1* mRNA was injected with MO to correct these defects. *GRHL1* did rescue the MO phenotype, restoring sound sensitivity (Fig. 3D) and decreased HCs/neuromasts (Fig. 3F). These data confirm the phenotype specificity.

Otic defects could be rescued by* DSG1 *mRNA

Desmoglein-1 has been shown to be the downstream mediator of *GRHL1* in previous studies. For this reason, the issue of whether phenotypes in the MO group actually originate from decreased regulation of grhl1 with respect to desmosome formation was addressed here. The rescue efficiency of *DSG1* in *grhl1* mutant embryos was tested using a rescue assay with human *DSG1* mRNA. Data show that *grhl1* affects desmosomes by regulating *dsg1* in zebrafish (Fig. 4 A–D).

Discussion

This study suggests that down-regulation of *grhl1* affects their hearing. Then different catergories of otolith phenotype were established for easy observation. The zebrafish in the MO group showed fewer HCs and neuromasts in the inner ear and lateral line than control zebrafish (Figs. 3 and 4). As expected, TEM revealed that desmosomes, as in the Grhl1-deficient mouse model, were damaged in knock-out zebrafish (Wilanowski *et al.*, 2008).

GRHL1 can affect construction of desmosomes by regulating *DSG1*, so a rescue experiment was performed. Results showed that both the abnormal desmosomes and the apoptotic hair cells could be significantly rescued by either *GRHL1* or its target gene *DSG1*, suggesting that *grhl1* regulates inner ear development by promoting the otic expression of "desmoglein-1" in zebrafish.

Inner ear defects were observed in mutant embryos and the underlying mechanisms were explored. Morphogenesis of the zebrafish inner ear has been described in detail in previous works (Haddon and Lewis, 1996, Kimmel *et al.*, 1995). Precursor dispersal particles in the otic vesicle are attracted to vortices caused by cilia motility. These cilia and particles together facilitate otolith formation (Colantonio *et al.*, 2009, Wu *et al.*, 2011). The formation of otolith requires robust cilia; cilia morphology depends on HCs. In this way, loss of HC function could account for observed abnormities in otoliths (Fig. 3).

Auditory sensory epithelia are mosaic of HCs and supporting cells wherein mechanical sound vibrations are converted to chemical signals (Hawkins *et al.*, 2007). The procedure is highly reliant on the integrality of the circumferential band of cell junctions in epithelia (Deans, 2013, Groves and Fekete, 2012). Considerable integrity is necessary to allow the structure to withstand mechanical stress and maintenance of homeostasis in the inner ear. Physiologically, the barrier is strong enough to maintain ion exchange equilibrium in the otic lumen and the endolymph, but once the barrier is compromised, auditory signal transduction is paralyzed (Han *et al.*, 2011). A typical barrier complex includes tight junctions, adherens junctions, and desmosomes. Previous studies have suggested that different types of intercellular junctions are important to normal ear function (Forge *et al.*, 2013, Hardison *et al.*, 2005, Zhu *et al.*, 2013). Desmosome function in the organ of Corti is a largely unexplored area. As reported, desmosomes develop at the same time as HCs. They then disappear from HC junctional complexes but remain in the intercellular junctional complexes of other types of cells in the membranous labyrinth (Raphael and Altschuler, 1991, Raphael

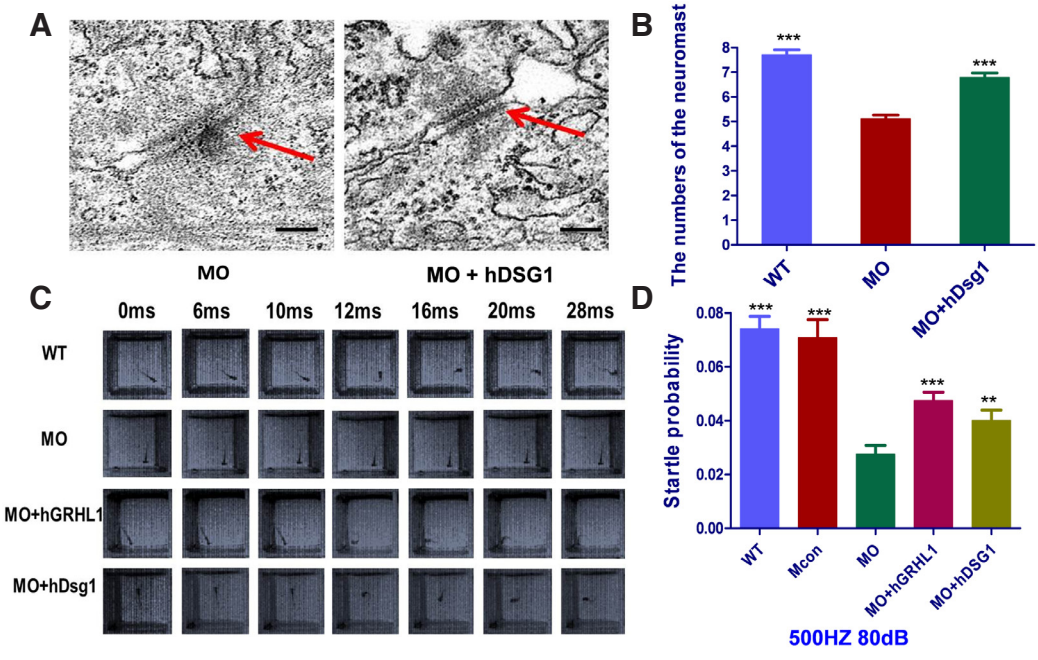

Fig. 4. Hearing disability in grhl1 knockdown zebrafish which could be rescued by *DSG1* mRNA. (A) *Poorly organized desmosomes (arrows) in the MO group could be rescued by* DSG1 *mRNA.* **(B)** *Dsg1 rescued decreasing numbers of neuromasts in the lateral line.* **(C)** *Examples of C-shaped startle responses of different larval groups in response to a 500 Hz sound of 10 ms duration. Typical escape response of wild-type larva initiated 10 ms after stimulation.* **(D)** *Average C-startle response probability after sound stimulation. At least 40 larvae were tested for each group. Bar, 1 μm.*

et al., 1988). Gulley and Reese reported that desmosomes are prominent in junctions between support cells (Gulley and Reese, 1976). Some researchers believe that the specific desmosome distribution pattern is not merely the result of differentiation but may rather regulate tissue morphogenesis and function (Kimura *et al.*, 2012, Wilanowski *et al.*, 2008). Similarly, the current study showed abnormal desmosomes to be correlated with defects in the auditory system.

Desomosome deficiency in sensory epithelium, which was caused by the absence of *grhl1*, led to lesions in the cell junction barrier. Decreased junction barrier integrity compromised membrane permeability, which is important to homeostasis and signal transduction in HCs (Han *et al.*, 2011). Specifically, the barrier maintained membrane tension to permit transmission of mechanical vibration to HCs in the sensory epithelium (Kakehata and Santos-Sacchi, 1995). This renders the MO embryo sound-insensitive (Fig. 4C).

However, the relationship between desmosome defects and HC apoptosis remains poorly understood. As shown in Fig. 3A, apoptotic signals appeared to be restricted to the HCs in the MO group. One possible explanation for this is that HCs may depend on supporting cells for survival within the organ of Corti. HCs may be unable to survive without a supply of nutritients and morphologic-maintenance cells. However, the manner in which SCs are supported requires additional research. Desmosomes may participate in HC development. For this reason, some deficient HCs may be less tolerant to apoptotic-inducing factors.

In this way, the function of *grhl1* was confirmed in the context of hindering normal development of sensory epithelia in zebrafish via desmosome construction. These data offer a foundation for the study and identification of novel deafness genes.

Materials and Methods

Phylogenetic tree mapping

To identify a candidate gene from 2 members of the *GRHL* family, a phylogenetic tree was mapped to determine which genes were closer to *GRH*L2. Clustal X1.83 was used to compare human wild-type GRHL1 mRNA sequence with the orthologs from mouse, *Drosophila*, Danio, Caenorhabditis, and *Xenopus* (sequences obtained from http://www.ensembl.org/).

Zebrafish lines

Wild-type (AB strain) zebrafish were obtained from the International Zebrafish Research Center (University of Oregon, Eugene, OR, U.S.). Embryos were obtained from natural spawning of wild-type adults. Transgenic pou3f4-GFP zebrafish embryos were provided by Dr. Huawei Li. Zebrafish were kept at 28.5°C and 0.003% 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, St. Louis, MO, U.S.) MO was used to suppress pigmentation (Karlsson *et al.*, 2001). Developmental stages were measured by hours after fertilization (hpf) and days after fertilization (dpf). All zebrafish experimental protocols were approved by the Institutional Animal Care and Use Committee of Fudan University.

Morpholino antisense oligonucleotides (MO) and mRNA microinjection

One *grhl1* MO was designed as described in a previous work (Janicke *et al.*, 2010). MO blocked the splice site between the 3rd exon and the 3rd intron, which led to an excrescent 3rd intron with a terminator codon remaining in the mature mRNA. All related MO sequences were obtained from Gene Tools (http://www.gene-tools.com/Oligo_Design) and MO injections were injected into one cell-stage embryos as follows:

grhl1 In3 MO	5′-CTTTGATGAGAGCTTCACCTTTTGT-3′	3 ng
Mcon MO	5′- CCTCTTACCTCAGTTACAATTTATA-3′	3 ng
p53-MO	5′- GCGCCATTGCTTTGCAAGAATTG -3′	3 ng

Both *GRHL1* and *DSG1* cDNA, obtained from the HeLa cell cDNA library using RT-PCR and amplified with a forward primer that was designed with a consideration for incorporating a T7 forward promoter sequence (5′-GATCAC TAATACGACTCACTATAGGG-3′). The PCR product served as a template after sequence confirmation. Then, capped and polyadenylated RNA of *GRHL1* and *DSG*1 were synthesized *in vitro* by transcription with T7 RNA polymerase using the mMessage mMachine T7 Ultra Kit (Ambion, Austin, TX, U.S.) (final concentration of co-injection mixture = 180 ng/μl). All morpholinos and mRNA depicted were diluted to the indicated concentrations with RNase-free water (TaKaRa, Tokoyo, Japan).

Reverse-transcription PCR

RNA was extracted from zebrafish embryos at various developmental stages using Trizol (Ambion). First-strand cDNA was then prepared using a SuperSciprt III First-Strand Synthesis System (Invitrogen, U.S.) with 1 μg RNA as template. cDNA was diluted (1:50) for each PCR reaction. To detect grhl1 expression in zebrafish, semi-quantitative PCR was performed in triplicate with the primers below:

z-beta-actin-F, 5′-ATGCCCCTCGTGCTGTTTTC-3′;
z-beta-actin-R, 5′-GCCTCATCTCCCACATAGGA-3′;
z-grhl1-F, 5′- TCAATGCAGCTAGGGGACAA -3′;
z-grhl1-R, 5′-GTTCGGGACAGTGACAGAGA-3′.

To evaluate MO knock down efficiency, one pair of primers was used:

kd-F, in exon 3, 5′-CTGGAAGTCGTTCCTGGAGA-3′;
kd-R, in intron 3, 5′-AGCCTTTAAACAGCATGAAAAA-3′. The MO group had a 400 bp product, indicating intron 3 was inserted in mature mRNA.

Whole-mount in situ *hybridization (WISH)*

Whole-mount *in situ* hybridization of grhl1 was performed as previously described (Janicke *et al.*, 2010). Zebrafish grhl1 complementary DNA was subcloned into pGEM®-T vector by PCR amplification as follows: forward: 5′-CCCGTTCTCGTACAGGCAATTATAACG-3′ and reverse 5′-GCTTCAGTTGGTCATCTCTGGATTTC-3′. After sequence confirmation, pGEM-grhl1-T plasmid was linearized using SalI and antisense digoxigenin-UTP-labeled full-length riboprobe was transcribed using T7 RNA polymerase (Promega, Madison, WI, U.S.). Then, grhl1 mRNA expression was detected 6 h after fertilization by RT-PCR. Embryos (N=30) were collected at 12 and 24 h, washed three times with PBST in DEPC, and fixed in 1 ml 4% paraformaldehyde for 5 min at room temperature. Embryos were then fixed in 1 ml of paraformaldehyde for 12–16 h at 4°C prior to WISH, which was carried out as described previously (Chen and Fishman, 1996, Tassin *et al.*, 1994). Anti-digoxigenin antibody (Roche, Basel, Switzerland) was used to detect grhl1 mRNA signals.

Staining of FM-1-43FX and phalloidin

For 96 hfp embryos, neuromast HCs were labeled by immersing larvae in a solution of FM1-43FX (Invitrogen) for 1–2 min at room temperature. They were washed in PBS. Zebrafish were immobilized in 1% low-melting-point agarose gel and labeled HCs were viewed under a fluorescent microscope.

Before phalloidin staining, 96 hpf embryos were fixed in 4% PFA overnight at 4°C. Larvae were permeated with 2% Triton X-100 (Sigma-Aldrich) in PBS and then washed twice with PBS. Then the embryos were immersed 2.5 mg/ml fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma-Aldrich) in PBS for 1 h in the dark at room temperature. After incubation, samples were washed with PBST for 2 h and cilia were marked with green fluorescence (Leger and Brand, 2002).

Apoptosis detection by TUNEL and acridine orange staining

A TUNEL assay was performed with a TMR-RED *in situ* cell death detection kit (Roche). Embryos (5 dpf) with HCs marked with GFP were washed

in PBS and fixed in 4% paraformaldehyde overnight at 4°C, immersed in methanol for 1 h at room temperature and then washed twice with PBST. Samples were incubated in acetone at -20°C for 20 min, permeabilized with 0.1% sodium citrate and 0.1% TritonX-100 for 15 min at room temperature and embryos were incubated with the reaction mixture (5 μl enzyme solution + 45 μl labelling solution) for 1 h in the dark at 37°C after washing twice with PBST. Then the reaction was stopped with three washes in PBST. A fluorescent signal was imaged and digitized using a confocal microscope (TCS SP8, Leica, Wetzlar, Germany).

Acridine orange staining was performed at 24 hpf to exclude MO off-target effects. Membranes were stripped from embryos and fish were immersed in 2 mg/l acridine orange. The embryos were washed five times in PBS, and photos were taken.

Startle response tests

The inner ear is important to zebrafish hearing and balance. Developmental defects have been observed in inner ears of *grhl1* MO knockdown zebrafish. Here, swimming behavior and hearing were assessed in MO knockdown zebrafish. For hearing tests, zebrafish were randomly placed in blocks and exposed to a 10 ms audio stimulus at 500 Hz. The C-startle response (named for the embryonic shapes mediated by M-cells in response to sound) was measured using near-field pure tone stimulation at two different intensities. The C-startle response was measured in 96-well plastic plates, and recorded with a high-speed camera. Larvae were considered positive for the C-startle response if this response occurred within 20 ms of the application of stimuli. At least 25 larvae were tested in each group. The probability of the C-startle response for a group of larvae was the average percentage of all C-startle effects (Han *et al*., 2011).

Transmission electron microscopy (TEM)

A group of 120 hpf embryo types were fixed with 2.5% glutaraldehyde and 1% OsO4 followed by dehydration with a graded ethanol and acetone solution. Then the embryos were infiltrated with epoxy resin for more than 24 h. The otic sensory epithelium was examined under TEM. The figure in (Sup. Fig. 6) shows a systematic sketch for identifying the sensory epithelium. The anatomy of zebrafish in detail could be found from http://www.zebrafish.uni-freiburg.de/anatomy/120hrs/120hrs.html.

Statistical analysis

Data are shown as means ± SEM. All data were processed by SPSS, mono factor analysis of variance was used for analysis. Differences were considered statically significant at $P<0.01$.

Acknowledgments

This study was supported by the National Basic Research Program of China (2011CB504502), the Foundation of the Ministry of Health (201202005), and the Shanghai Science and Technology Commission (11dz1950300). We thank Yang Liu from Elsevier for his linguistic assistance during the preparation of this manuscript.

Author contributions

Conceived and designed the experiments: Duan Ma, Jin Zhang, Huijun Wang, and Xu Wang. Performed the experiments: Fan Yang, Fei Liu, Danping Wen, Jiongjiong Hu, and Nan Jiang. Analyzed the data: Wenjun Xia, Xiaofang Shen, and Jie Zong. Contributed reagents, materials, or analytical tools: Zhaoxin Ma, Xu Wang. Composed the manuscript: Fan Yang and Fei Liu.

References

BOK, J., CHANG, W. and WU, D.K. (2007). Patterning and morphogenesis of the vertebrate inner ear. *Int J Dev Biol* 51: 521-533.

CENCI, C. and GOULD, A.P. (2005). *Drosophila* Grainyhead specifies late programmes of neural proliferation by regulating the mitotic activity and Hox-dependent apoptosis of neuroblasts. *Development* 132: 3835-3845.

CHEN, J.N. and FISHMAN, M.C. (1996). Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. *Development* 122: 3809-3816.

COLANTONIO, J.R., VERMOT, J., WU, D., LANGENBACHER, A.D., FRASER, S., CHEN, J.N. and HILL, K.L. (2009). The dynein regulatory complex is required for ciliary motility and otolith biogenesis in the inner ear. *Nature* 457: 205-209.

DEANS, M.R. (2013). A balance of form and function: planar polarity and development of the vestibular maculae. *Semin Cell Dev Biol* 24: 490-498.

FORGE, A., JAGGER, D.J., KELLY, J.J. and TAYLOR, R.R. (2013). Connexin30-mediated intercellular communication plays an essential role in epithelial repair in the cochlea. *J Cell Sci* 126: 1703-1712.

GANAPATHY, A., PANDEY, N., SRISAILAPATHY, C.R., JALVI, R., MALHOTRA, V., VENKATAPPA, M., CHATTERJEE, A., SHARMA, M., SANTHANAM, R., CHADHA, S. *et al.,* (2014). Non-syndromic hearing impairment in India: high allelic heterogeneity among mutations in TMPRSS3, TMC1, USHIC, CDH23 and TMIE. *PLoS One* 9: e84773.

GAO, X., VOCKLEY, C.M., PAULI, F., NEWBERRY, K.M., XUE, Y., RANDELL, S.H., REDDY, T.E. and HOGAN, B.L. (2013). Evidence for multiple roles for grainyheadlike 2 in the establishment and maintenance of human mucociliary airway epithelium. *Proc Natl Acad Sci USA* 110: 9356-9361.

GROVES, A.K. and FEKETE, D.M. (2012). Shaping sound in space: the regulation of inner ear patterning. *Development* 139: 245-257.

GULLEY, R.L. and REESE, T.S. (1976). Intercellular junctions in the reticular lamina of the organ of Corti. *J Neurocytol* 5: 479-507.

HADDON, C. and LEWIS, J. (1996). Early ear development in the embryo of the zebrafish, Danio rerio. *J Comp Neurol* 365: 113-128.

HAMA, K. (1980). Fine structure of the afferent synapse and gap junctions on the sensory hair cell in the saccular macula of goldfish: A freeze-fracture study. *J. Neurocytol.* 9: 845-860.

HAN, Y., MU, Y., LI, X., XU, P., TONG, J., LIU, Z., MA, T., ZENG, G., YANG, S., DU, J. *et al.,* (2011). Grhl2 deficiency impairs otic development and hearing ability in a zebrafish model of the progressive dominant hearing loss DFNA28. *Hum Mol Genet* 20: 3213-3226.

HARDISON, A.L., LICHTEN, L., BANERJEE-BASU, S., BECKER, T.S. and BURGESS, S.M. (2005). The zebrafish gene claudinj is essential for normal ear function and important for the formation of the otoliths. *Mech Dev* 122: 949-958.

HAWKINS, R.D., BASHIARDES, S., POWDER, K.E., SAJAN, S.A., BHONAGIRI, V., ALVARADO, D.M., SPECK, J., WARCHOL, M.E. and LOVETT, M. (2007). Large scale gene expression profiles of regenerating inner ear sensory epithelia. *PLoS One* 2: e525.

HE, Y., YU, H., SUN, S., WANG, Y., LIU, I., CHEN, Z. and LI, H. (2013). Trans-2-phenylcyclopropylamine regulates zebrafish lateral line neuromast development mediated by depression of LSD1 activity. *Int J Dev Biol* 57: 365-373.

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.

KAKEHATA, S. and SANTOS-SACCHI, J. (1995). Membrane tension directly shifts voltage dependence of outer hair cell motility and associated gating charge. *Biophys J* 68: 2190-2197.

KARLSSON, J., VON HOFSTEN, J. and OLSSON, P.E. (2001). Generating transparent zebrafish: a refined method to improve detection of gene expression during embryonic development. *Mar Biotechnol (NY)* 3: 522-527.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMANN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253-310.

KIMURA, T.E., MERRITT, A.J., LOCK, F.R., ECKERT, J.J., FLEMING, T.P. and GARROD, D.R. (2012). Desmosomal adhesiveness is developmentally regulated in the mouse embryo and modulated during trophectoderm migration. *Dev Biol* 369: 286-297.

LEGER, S. and BRAND, M. (2002). Fgf8 and Fgf3 are required for zebrafish ear placode induction, maintenance and inner ear patterning. *Mech Dev* 119: 91-108.

MOGASS, M., BRINGAS, P., JR. and SHULER, C.F. (2000). Characterization of desmosomal component expression during palatogenesis. *Int J Dev Biol* 44: 317-322.

RAPHAEL, Y. and ALTSCHULER, R.A. (1991). Reorganization of cytoskeletal and junctional proteins during cochlear hair cell degeneration. *Cell Motil Cytoskeleton*

18: 215-227.

RAPHAEL, Y., VOLK, T., CROSSIN, K.L., EDELMAN, G.M. and GEIGER, B. (1988). The modulation of cell adhesion molecule expression and intercellular junction formation in the developing avian inner ear. *Dev Biol* 128: 222-235.

SANCHEZ-CALDERON, H., MILO, M., LEON, Y. and VARELA-NIETO, I. (2007). A network of growth and transcription factors controls neuronal differentation and survival in the developing ear. *Int J Dev Biol* 51: 557-570.

TASSIN, M.T., BEZIAU, A., GUBLER, M.C. and BOYER, B. (1994). Spatiotemporal expression of molecules associated with junctional complexes during the *in vivo* maturation of renal podocytes. *Int J Dev Biol* 38: 45-54.

VONA, B., NANDA, I., NEUNER, C., MULLER, T. and HAAF, T. (2013). Confirmation of GRHL2 as the gene for the DFNA28 locus. *Am J Med Genet A* 161A: 2060-5.

WATERMAN, R.E. and BELL, D.H. (1984). Epithelial fusion during early semicircular canal formation in the embryonic zebrafish, Brachydanio rerio. *Anat Rec* 210: 101-114.

WILANOWSKI, T., CADDY, J., TING, S.B., HISLOP, N.R., CERRUTI, L., AUDEN, A., ZHAO, L.L., ASQUITH, S., ELLIS, S., SINCLAIR, R. *et al.,* (2008). Perturbed desmosomal cadherin expression in grainy head-like 1-null mice. *EMBO J*27: 886-897.

WU, D., FREUND, J.B., FRASER, S.E. and VERMOT, J. (2011). Mechanistic basis of otolith formation during teleost inner ear development. *Dev Cell* 20: 271-278.

WU, G.F., HOU, Y.L., HOU, W.R., SONG, Y. and ZHANG, T. (2010). Giant panda ribosomal protein S14: cDNA, genomic sequence cloning, sequence analysis, and overexpression. *Genet Mol Res* 9: 2004-2015.

ZHU, Y., LIANG, C., CHEN, J., ZONG, L., CHEN, G.D. and ZHAO, H.B. (2013). Active cochlear amplification is dependent on supporting cell gap junctions. *Nat Commun* 4: 1786.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Zebrafish *grainyhead-like1* is a common marker of different non-keratinocyte epidermal cell lineages, which segregate from each other in a Foxi3-dependent manner
Martina Jänicke, Björn Renisch and Matthias Hammerschmidt
Int. J. Dev. Biol. (2010) 54: 837-850
http://dx.doi.org/10.1387/ijdb.092877mj

Patterning and morphogenesis of the vertebrate inner ear
Jinwoong Bok, Weise Chang and Doris K. Wu
Int. J. Dev. Biol. (2007) 51: 521-533
http://dx.doi.org/10.1387/ijdb.072381jb

A network of growth and transcription factors controls neuronal differentation and survival in the developing ear
Hortensia Sánchez-Calderón, Marta Milo, Yolanda León and Isabel Varela-Nieto
Int. J. Dev. Biol. (2007) 51: 557-570
http://dx.doi.org/10.1387/ijdb.072373hs

Characterization of desmosomal component expression during palatogenesis
M Mogass, P Bringas and C F Shuler
Int. J. Dev. Biol. (2000) 44: 317-322
http://dx.doi.org/10.1387/ijdb.10853828

Spatiotemporal expression of molecules associated with junctional complexes during the *in vivo* maturation of renal podocytes
M T Tassin, A Beziau, M C Gubler and B Boyer
Int. J. Dev. Biol. (1994) 38: 45-54
http://dx.doi.org/10.1387/ijdb.8074995