Analysis and functional evaluation of the hair-cell transcriptome

Brian M. McDermott, Jr.*, Jessica M. Baucom, and A. J. Hudspeth†

Howard Hughes Medical Institute and Laboratory of Sensory Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399

Contributed by A. J. Hudspeth, May 17, 2007 (sent for review March 31, 2007)

An understanding of the molecular bases of the morphogenesis, organization, and functioning of hair cells requires that the genes expressed in these cells be identified and their functions ascertained. After purifying zebrafish hair cells and detecting mRNAs with oligonucleotide microarrays, we developed a subtractive strategy that identified 1,037 hair cell-expressed genes whose cognate proteins subserve functions including membrane transport, synaptic transmission, transcriptional control, cellular adhesion and signal transduction, and cytoskeletal organization. To assess the validity of the subtracted hair-cell data set, we verified the presence of 11 transcripts in inner-ear tissue. Functional evaluation of two genes from the subtracted data set revealed their importance in hair bundles: zebrafish larvae bearing the *seahorse* and *ift 172* mutations display specific kinociliary defects. Moreover, a search for candidate genes that underlie heritable deafness identified a human ortholog of a zebrafish hair-cell gene whose map location is bracketed by the markers of a deafness locus.

auditory system | balance | hearing | vestibular system | zebrafish

The hair cell is a mechanoreceptor that is essential not only for hearing but also for balance and for the detection of water movement by some aquatic vertebrates. This sensitive detector transduces mechanical stimuli into electrical responses and forwards the information to the brain across chemical synapses onto afferent nerve fibers. Although much is known about how hair cells function in hearing, we have only a limited understanding of their protein constituents (1). Eliminating this disparity requires the enumeration of the genes expressed in hair cells and the assignment of their roles in these highly specialized cells.

Functional analyses of the hair-cell transcriptome would improve our understanding of the hair cell's unique and defining organelle, the mechanosensitive hair bundle, and of such specializations as its ribbon synapse. Such studies also hold the promise of insights into human heritable deafness (2). Although much of our knowledge of the proteins involved in hair-cell function stems from the analysis of the genes underlying nonsyndromic deafness (3), many of the genes required for hair-cell development and operation are unlikely to be identified by that approach.

Our understanding of the molecular bases of sensation has benefited substantially from transcriptional analyses of photoreceptors (4), touch receptors (5), thermoreceptors (6), Merkel cells (7), and olfactory neurons (6, 8). We have used a functional-genomics approach to identify genes whose products are involved in the development, maintenance, and function of hair cells in the zebrafish.

Results and Discussion

Identification of the Zebrafish Hair Cell's Transcriptome. We used a three-step subtractive strategy to identify genes expressed preferentially in hair cells [supporting information (SI) Fig. 4*A*]. In the first step, we detected a large number of transcripts by probing DNA oligonucleotide microarrays with RNA amplified from hair cells. To generate the necessary RNA, we isolated a pure cellular population by picking individual hair cells dissociated from the lagena, a receptor organ of the zebrafish's ear. Linear amplification of the RNA from 200 hair cells yielded ≈40 μg of aRNA, an enhancement of ≈1 millionfold. The resultant labeled aRNA was hybridized to an Affymetrix microarray (Affymetrix, Santa Clara, CA) containing ≈15,000 oligonucleotide probe sets. Averaging the outcomes of three experiments (SI Data Set 1) resulted in the identification of 6,472 transcripts scored as "present" (SI Data Set 2).

In the second step, we defined the transcriptome from cells of a nonsensory organ, the liver (SI Data Set 1). Hepatocytes were selected for the subtraction process for three reasons: they are nonneuronal and thus unlikely to express synaptic factors; they lack cilia (http://members.global2000.net/bowser/cilialist.html) and therefore the constituents of the axoneme; and, like hair cells, they are epithelial cells and consequently express an overlapping set of housekeeping genes. The liver transcriptome encompassed 4,335 transcripts scored as "present" (SI Data Set 3). To assess the reproducibility of both hair cell and liver microarray data, we compared gene expression between replicate homotypic samples (SI Fig. 5). The unnormalized correlation coefficients for two such comparisons were 0.95 and 0.99, indicating robust consistency between experiments.

In the third step, we eliminated genes that were expressed by both cell types from the hair-cell transcriptome to yield 1,037 hair-cell genes (SI Fig. 4*B* and SI Data Set 4). This filtering methodology resulted in an 84% reduction of the hair-cell transcriptome. Included among the genes after the subtraction are 12 that have been previously shown to be expressed in hair cells (SI Table 2). Of these, four genes are known to be expressed in the zebrafish: *cacna1d* (9), *cadherin 4* (10), *parvalbumin 3a* (11), and *ribeye b* (12). The hair-cell set also contains seven confirmed or candidate genes that, when mutated, cause syndromic or nonsyndromic deafness in humans or other vertebrates; these include *cacna1d* (13) and the genes encoding proteins similar to *Otof* (14), *Pmca2* (15), *SALL4* (16), *Ush1c* (17), *USH1G* (18), and *MYO15A* (19). By contrast, the supporting-cell markers *claudin b* (20), *hes5* (21), and $p27^{kip1}$ (22) are not included in the hair-cell transcriptome. Furthermore, the probe for the ganglionic neuronal marker *Hu antigen C* (23) and that for the efferent-axonal marker *acetylcholinesterase* (24) are negative in the hair-cell data set. Taken together, these results indicate that the hair-cell isolation process suffered no detectable contamination from either supporting cells or neurons.

To confirm that the hair cell-expressed genes are absent from liver, we performed BLAST searches using a subset of hair-cell

Author contributions: B.M.M. and A.J.H. designed research; B.M.M. and J.M.B. performed research; B.M.M. and A.J.H. analyzed data; and B.M.M. and A.J.H. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

*Present address: Department of Otolaryngology–Head and Neck Surgery, Case Western Reserve University School of Medicine, 11100 Euclid Avenue, Cleveland, OH 44106.

†To whom correspondence should be addressed. E-mail: hudspaj@rockefeller.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0704476104/DC1.

www.pnas.org/cgi/doi/10.1073/pnas.0704476104

Table 1. Hair cell-expressed genes

GenBank no.	Gene symbol
Membrane transport	
BC045489.1	*slc16a3*
BI980828	{*SLC6A8*}
BC045304.1	*kcnd3*^{H}
Synaptic transmission	
AW170964	*nsf*
BI981058	*rims2*^{H}
BI670921	{*DMXL2*}
BQ264112	*synj1*^{H}
Transcriptional control	
BM181845	{*SALL4*}
AF375872.1	*meis2.2*
Cellular adhesion and signal transduction	
AI721951	{*Sema5b*}^{M}
BQ073847	*epha4b*^{M}
AW116925	*zgc:112212*^{M}
AB086625.1	*pcdh10b*^{H}
AW058804	*magi1*
Cytoskeletal organization	
Actin-associated	
BC049461.1	{*Capg*}
BC045462.1	*slmap*^{H}
AW281644	{*CTTNBP2*}^{M}
AW077331	{*KIDINS220*}
BI533674	{*SYNE1*}
BG883293	{*CORO1B*}^{M}
AW116918	*smarca5*
BE201818	*cotl1*
BM316681	{*Mybpc3*}^{M}
Microtubule- or cilium-associated	
BI671160	*bbs7*
AW076841	{*DYNC2H1*}
AW077770	*lrrc6l*
AW076580	{*EML1*}^{M}
BQ450283	{*Cetn2*}
AL924416	*ift172*

This list contains transcripts that, have not been described heretofore in hair cells. PCR confirmation was conducted for selected transcripts with amplified hair-cell cDNA (^{H}) or macular cDNA (^{M}). A bracketed symbol for a human or mouse gene indicates a zebrafish transcript for which the corresponding protein's amino acid sequence most resembles that encoded by the indicated gene.

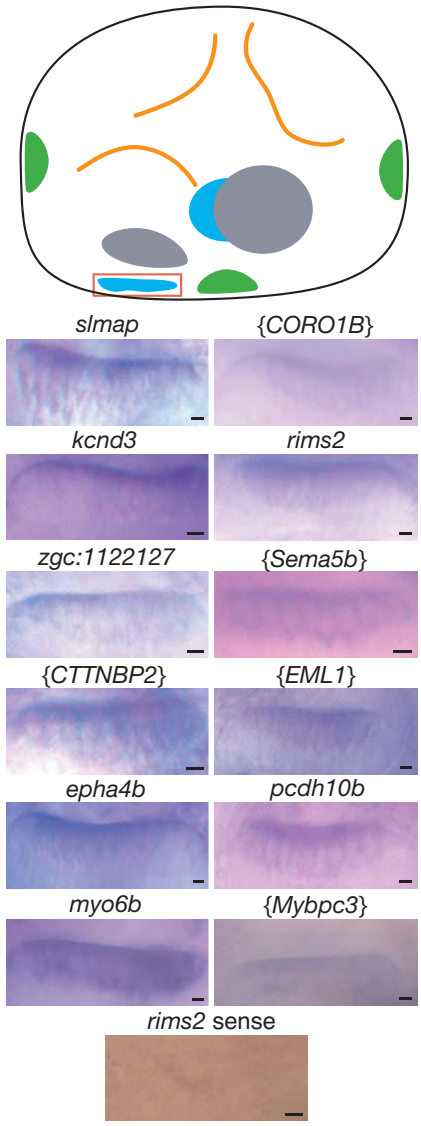

Fig. 1. Expression of hair-cell transcripts in the anterior macula. The schematic diagram of the left ear of a 4-day-old zebrafish larva depicts the sensory maculae (blue), cristae of the semicircular canals (green), and otoliths (gray). A red box delimits the anterior macula. *In situ* hybridization was performed on whole-mount embryos for selected genes identified in Table 1. A bracketed symbol for a human or mouse gene indicates a probe designed to hybridize with a zebrafish transcript for which the corresponding protein's amino acid sequence most resembles that encoded by the indicated gene. The *rims2* sense probe and the *myo6* probe constituted, respectively, a negative and a positive control. (Scale bars, 5 μm.)

transcripts in Table 1 and SI Table 2 and two libraries of expressed-sequence tags from the zebrafish liver. For 40 of the 41 tentative consensus sequences analyzed, none of the hair-cell transcripts was identified in the liver. For the single exception of *coactosin-like 1*, the expression was minimal, with only one transcript identified in a total liver library (NIH_ZGC_25) and none in a library of hepatic expressed-sequence tags (NIH_ZGC_8).

Classes of Hair-Cell Transcripts. Many of the genes in the subtracted hair-cell data set have not been annotated. We therefore selected transcripts randomly and performed BLAST analysis to identify significant similarities with homologous proteins and recognized protein motifs. Hair-cell genes from the subtracted data set are organized in Table 1 and SI Table 2 according to known or putative function. These tables contain 41 genes whose cognate proteins have functions falling into the broad categories of membrane transport, synaptic transmission, transcriptional control, cellular adhesion and signal transduction, and cytoskeletal organization.

Validation of the Data Set. The principal purpose of our molecular screen was to identify genes transcribed in hair cells. To confirm that our procedure succeeded in identifying appropriate transcripts, we used RT-PCR to examine the presence of 13 transcripts in otolithic organs or more exclusively in hair cells. This procedure demonstrated the expression of all of the genes tested (Table 1 and SI Table 2). Furthermore, we performed *in situ* hybridization on larval zebrafish and analyzed the anterior maculae using probes prepared for 11 hair-cell genes representing several functional classes (Fig. 1). None of the genes analyzed by *in situ* hybridization showed expression in the liver (data not shown).

Membrane-Transport Genes. The hair-cell data set contains transcripts for five channels, transporters, and pumps. Included on

the list is the voltage-gated potassium channel, *Shal-related subfamily*, *member 3* (*kcnd3*). Hair cells display numerous ionic conductances (25), including an A-type K^+ conductance that underlies the principal outward currents of certain amphibian, avian, and mammalian hair cells (26). In fishes, this conductance plays a role in the maintenance of the hair cell's resting potential and in the generation of the membrane-potential oscillations that underlie frequency tuning (27). Our data indicate that Kcnd3 is a candidate subunit of the A-type K^+ channels in zebrafish hair cells. Consistent with this possible function, the presence of this transcript in anterior-macular tissue was confirmed by RT-PCR and *in situ* hybridization (Fig. 1).

Members of the solute-linked carrier family are required for mammalian hearing and have been shown to occur in hair cells (28, 29). We determined that hair cells express genes encoding two additional solute-linked carriers, both of which have roles in the retina. One such gene is *slc16a3*, whose product is an H^+-coupled transporter of monocarboxylic acids important for lactate transport in the retinal pigment epithelium (30). The presence of this transporter in hair cells suggests that they have energy requirements similar to those of photoreceptors, for which lactate released from Müller cells provides the primary energy source during oxidative metabolism (31).

Also present in the hair-cell data set is a transcript encoding a protein similar to SLC6a8, a Na^+- and Cl^--dependent creatine transporter. The SLC6a8 protein is localized to the inner segments of photoreceptors, the site of energy production (32). During intervals of energy depletion, creatine accumulated by SLC6a8 serves as a buffer for high-energy phosphate, thus maintaining a stable concentration of ATP in the outer segments (33). It is possible that the SLC6a8-like transporter and the creatine energy shuttle play a comparable role in stereocilia during such metabolically taxing processes as myosin-based adaptation and the extrusion of Ca^{2+}.

Synaptic-Transmission Genes. The proteins encoded by seven genes identified in this screen participate in synaptic transmission at conventional or ribbon synapses. One of the genes identified in our screen, *synaptojanin 1*, which has yet to be described in hair cells and functions in clathrin-mediated endocytosis and cytoskeletal dynamics. Disruption of the corresponding gene in mice produces deficits in vesicle uncoating and fission during synaptic-vesicle recycling at conventional synapses (34). Mutation of *synaptojanin 1* in zebrafish yields the *no optokinetic response c* (*nrc*) phenotype characterized by unanchored ribbons and abnormal synaptic transmission in cone photoreceptors (35). Some *nrc* mutants lack the acoustic startle response, an assay used to identify deaf zebrafish (our unpublished results and S. E. Brockerhoff, personal communication). In conjunction with the expression of *synaptojanin 1* by hair cells, this result implies a role for phosphoinositide metabolism at the ribbon synapses of hair cells.

Transcriptional-Control Genes. The data set contains genes encoding two putative transcription factors, Meis2.2 and another similar to human Sal-like protein 4 (SALL4). Human *SALL4*, whose zebrafish ortholog occurs in the hair-cell data set, encodes a putative zinc-finger transcription factor. Mutations in this gene result in Okihiro–Duane radial-ray syndrome, an autosomal dominant condition characterized by strabismus and radial-ray defects of the hand and arm. Among the associated abnormalities is sensorineural hearing loss (16), which our results suggest originates at the level of the hair cell.

Cellular-Adhesion and Signal-Transduction Genes. The data set also contains eight transcripts that encode proteins putatively involved in cellular adhesion and signal transduction. Five of these mRNAs have not previously been detected in hair cells; we have verified the presence of four by RT-PCR and *in situ* hybridization (Fig. 1). We identified two genes whose products are likely to be involved in axonal guidance or cell movement: *ephrin A4b* and a gene encoding a protein similar to human semaphorin 5b.

Cytoskeletal-Organization Genes. Nineteen transcripts associated with cytoskeletal function occur in the hair-cell data set. Including genes that encode proteins associated with actin filaments, microtubules, and cilia, this group is of note because its gene products are potentially involved in the morphogenesis and maintenance of the hair bundle and cuticular plate.

Eleven genes encode actin-associated proteins; nine of these have not previously been reported in the hair cell. Included in the data set is a gene that encodes a gelsolin-like capping protein similar to murine CapG. Such capping proteins bind the barbed ends of actin filaments and in some instances promote assembly. The actin-binding activity of CapG is activated by micromolar concentrations of Ca^{2+} and is inhibited by phosphoinositides. At submicromolar Ca^{2+} concentrations, actin-associated CapG rapidly dissociates from the barbed ends of microfilaments (36). The capacity of this protein for reversible binding has implicated it in the regulation of actin filament length in response to changes in the intracellular Ca^{2+} concentration. CapG is required for receptor-mediated plasma-membrane ruffling, a reputedly Ca^{2+}-sensitive process resulting from the localized assembly of actin filaments (37).

The data set also contains genes encoding cytoskeletal proteins whose function is less well understood. One such gene encodes a protein similar to cortactin-binding protein 2. The orthologous cortactin-binding protein 90 associates with cortactin, a multidomain scaffolding protein involved in actin polymerization (38). In the rat, the known expression of cortactin-binding protein 90 is restricted to the brain (39). *In situ* analysis in the zebrafish revealed gene expression in both the brain (data not shown) and the anterior macula of the developing inner ear (Fig. 1).

Another gene included in the subtracted hair-cell data set encodes a protein similar to the mammalian phosphoprotein coronin 1b. This protein associates with cortical actin and regulates leading-edge dynamics and cellular motility through interaction with the Arp2/3 complex (40). The zebrafish protein is expressed in the anterior macula (Fig. 1), eye, and brain (data not shown). Like the foregoing protein, the hair cell's coronin may contribute to the morphogenesis of the stereocilia and cuticular plate.

This screen identified six genes that encode ciliary or microtubule-associated proteins. In *Chlamydomonas*, intraflagellar-transport particles comprise some 15 polypeptides forming two protein complexes, A and B (41). We encountered in hair cells the transcript for *ift172*, whose product is a member of the B complex responsible for the turnover of ciliary constituents (42). In view of the importance of IFT 172 for algal cilia, we investigated the requirement for this protein in hair cells. The zebrafish mutant strain *moe*, which was generated by retroviral insertional mutagenesis, is defective in IFT 172 and displays polycystic kidneys (43). Upon examining 10 mutant zebrafish larvae at 4 days of age, we found that the anterior maculae, cristae, and neuromasts possessed hair cells nearly devoid of kinocilia (Fig. 2). Of the 50 sensory epithelia investigated from the mutant cohort, only 10 displayed even a single kinocilium, even though the number of hair cells remained normal.

The subtracted hair-cell data set contains another gene, *leucine-rich repeat-containing 6-like* (*lrrc6l*), that is required for the proper development of renal glomeruli and tubules. Because mutation of this gene in the zebrafish *seahorse* strain results in polycystic kidneys (43), a phenotype commonly associated with defective cilia, we examined the kinocilia of hair cells in *seahorse* mutants. Fluorescence microscopy of 14 larvae showed that the

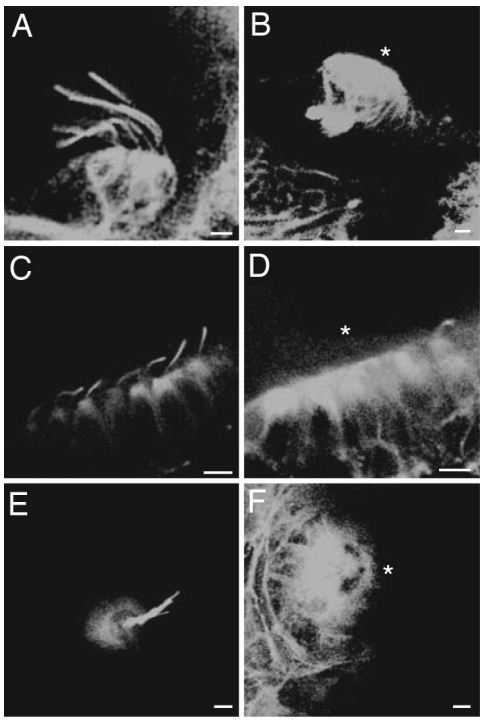

Fig. 2. Kinocilia in wild-type and *ift172* mutant zebrafish. (*A*) In a confocal image of the crista from a semicircular canal in a wild-type animal, immunolabeling of acetylated tubulin shows prominent kinocilia. (*B*) The corresponding image from an *ift172* mutant reveals no kinocilia. For this and subsequent instances in which a receptor organ lacks kinocilia, an asterisk indicates the apical hair-cell surface. (*C*) Numerous kinocilia are apparent in a control preparation from the anterior macula. (*D*) Although the outlines of hair cells are clearly visible in the anterior macula of a mutant, all of the kinocilia but one are missing. (*E*) A neuromast from the lateral line of a wild-type animal displays a cluster of kinocilia. (*F*) The kinocilia are absent from a mutant. (Scale bars, 5 μm.)

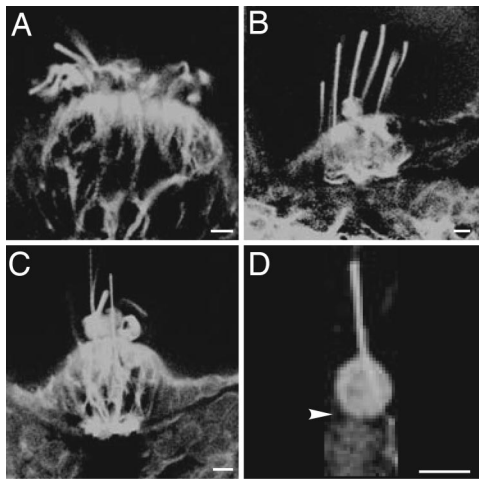

Fig. 3. Effect of the *seahorse* mutation on the kinocilia of 4-day-old zebrafish. (*A*) Numerous kinocilia, labeled by an antiserum against acetylated α-tubulin, protrude from the crista of semicircular canal in a wild-type animal. (*B*) The kinocilia of a *seahorse* mutant larva displays enlarged kinocilia, one with a basal swelling. (*C*) In another mutant animal, several kinocilia are bloated at their bases. (*D*) A higher-magnification view of one hair cell from a *seahorse* larva depicts the swollen base of a kinocilium; the arrowhead indicates the position of the hair cell's apical surface. (Scale bars, 5 μm.)

kinocilia in the cristae of half the animals had swollen bases filled in part with acetylated tubulin (Fig. 3).

Deafness Genes. Many of the genes responsible for sensorineural deafness are expressed in hair cells, and their deficiency causes hair-cell defects. Moreover, when the zebrafish homologs of human deafness genes are mutated, the animals often display deafness and vestibular dysfunction (9). Among the hair cell-expressed genes are several associated with heritable human deafness. The complete, subtracted hair-cell data set (SI Data Set 4) is therefore likely to contain candidate deafness genes. A preliminary screen of human deafness loci identified such a gene at the locus for *DFNB47*, an autosomal recessive nonsyndromic hearing loss that has been mapped to a 13.2-cM interval in the chromosomal region 2p25.1-p24.3. This region contains well over 20 genes, three of which having been sequenced without identification of the causative mutation (44). By comparison of the deafness locus with the corresponding region on chromosome 20 of the zebrafish genome and our expression database, we have identified *KIDINS220* as a candidate for DFNB47. In both genomes, these orthologs are flanked on either side by the *ID2* and *MBOAT2* genes. *KIDINS220* is expressed in human cochleae (www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=9873). A substrate of protein kinase D, Kidins220, contains 11 ankyrin repeats, four transmembrane domains, a SAM motif, and a PDZ-binding domain (45, 46). In some cells, this protein colocalizes with filamentous actin at the leading edge of cellular protrusions (47). Sequence analysis of *KIDINS220* in deaf humans and phenotypic analysis of zebrafish that are devoid of the orthologous gene's cognate protein should facilitate understanding of this gene's role in the hair cell.

Conclusion

Our molecular screen identified several known hair-cell transcripts as well as numerous previously undescribed genes. Two of these, *seahorse* and *ift172*, have proven to be necessary for kinociliary development or maintenance. We have additionally identified a candidate deafness gene that is expressed in hair cells. This data set should serve as a resource to assist the community of hearing researchers in the identification of genes relevant to hair-cell development and operation. For instance, to identify genes with genetic lesions responsible for hereditary deafness in humans or mice, a comprehensive comparison of the zebrafish hair-cell transcriptome with all murine and human deafness loci might provide valuable insights into hair-cell dysfunction.

Materials and Methods

Cell Isolation. Adult zebrafish of the wild-type Tübingen strain were anesthetized in a 2 mg/ml solution of 3-aminobenzoic acid ethyl ester methanesulfonate. The maculae, or sensory epithelia, were isolated from the paired lagenae, which are receptor organs thought to subserve the senses of balance and perhaps audition. These samples and liver lobes were placed separately into oxygenated standard saline solution containing 110 mM Na^+, 2 mM K^+, 4 mM Ca^{2+}, 118 mM Cl^-, 3 mM D-glucose, and 5 mM Hepes at pH 7.3. The specimens were treated for 20 min at room temperature with 150 μg/ml of protease (type XXIV; Sigma–Aldrich, St. Louis, MO) in the same solution. They were then dissociated by trituration with a Pasteur pipette. The products were treated with 10% FBS (HyClone, Logan, UT) to inactivate the protease. Under $\times$40 magnification and differential-interference-contrast optics, individual hair cells or hepatocytes were captured and dispensed with an adjustable pipette (CellTram; Eppendorf, Hamburg, Germany) held in a micromanipulator. To avoid contamination, each cell selected was subsequently reisolated with a second pipette. Hair cells with

abnormalities, such as apical blebs, pronounced organellar diffusion, or unidentifiable attached material, were discarded.

RNA Amplification, Microarray Hybridization, and Data Analysis. The total RNA from 200 isolated hair cells or liver cells was purified (PicoPure RNA Isolation kit; Molecular Devices, Sunnyvale, CA). RNA amplification was initiated (RiboAmp HS RNA Amplification Kit; Molecular Devices) and continued for one-and-a-half rounds. After second-strand cDNA synthesis, labeled and amplified RNA (aRNA) was produced and fragmented (BioArray HighYield RNA Transcript Labeling kit; ENZO Life Sciences, Farmingdale, NY). The quantities and size distributions of the labeled RNA samples were determined by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE) and capillary electrophoresis (2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA), respectively. The labeled aRNA was hybridized to the Zebrafish Genome Array in a GeneChip Hybridization Oven 320, then processed with a GeneChip Fluidics Station 450 and scanned with a GeneChip Scanner 3000 (all from Affymetrix). Three completely independent experiments apiece were performed for hair-cell and liver-cell samples.

Each potentially detectable transcript of the hybridization trials was scored as "present" or "absent" by the detection algorithm (GeneChip Operating Software, Affymetrix) with default settings. Subsequent data analysis was performed with the GeneSpring GX 7.3.1 software package (Agilent Technologies). The data were globally normalized. The signals from each microarray were normalized to the median measurement taken from that chip. The hybridization signal for each gene was then normalized to the median for that gene on all like-sample chips. Finally, the signal for each probe set was averaged across the three experiments.

To identify transcripts expressed in hair cells, we selected probes that yielded a "present" result in at least two experiments (SI Data Set 2). This process was repeated for liver-cell data to recognize liver-cell transcripts (SI Data Set 3). To produce the subtracted hair-cell data set (SI Data Set 4), we required a "present" result in at least two hair-cell experiments and an "absent" result in at least two liver-cell experiments for retention. The data set was then thinned by choosing genes that yielded an averaged normalized signal intensity at least twice as great for hair-cell probes as for liver-cell probes. Moreover, we selected genes displaying significantly different expression by ANOVA ($P < 0.055$); this criterion implies that the expression of 94.5% of the genes identified was significantly different between the two cellular populations. Finally, the probe sets of the hair-cell replicates that yielded t test P values >0.15 were discarded. This P value indicates the probability that the replicate probe-set values differ significantly from the baseline. This cutoff value, which was selected empirically on the basis of the P value for *ift172* ($P = 0.148$), allowed the inclusion of all previously identified hair-cell transcripts shown in SI Table 2.

Partial annotation of the hair cell data set was performed by using the following bioinformatics tools and procedures. The GenBank accession number associated with each Affymetrix probe set was used to search for annotated or putative gene descriptions from UniGene, Entrez Gene, and TIGR Zebrafish Gene Index (ZGI) databases. If the gene had not been annotated previously, then TBLASTN analysis was performed on the GenBank sequence and the available assembled cDNA sequences from nonredundant databases (www.ncbi.nlm.nih.gov/blast). The human or mouse gene with the highest amino acid sequence similarity was used for the annotation of each zebrafish gene. If a probe could not be assigned by these methods, we performed BLASTN analysis (www.ensembl.org/Multi/blastview and http://vega.sanger.ac.uk/Danio_rerio/blastview) against the zebrafish-genome assemblies using the sequence associated with the GenBank accession number. The resulting sequence was subjected to BLAST analysis against the nonredundant databases; the human or murine gene with the highest score was used in annotation. For transcripts requiring this procedure, the BLAST expectation value (E) is shown in SI Table 2: the lower the E value, the more significant is the score. All of the transcripts shown yielded E values well below the suggested BLAST cutoff of 10^{-3} (48).

The functions of human and murine gene products were investigated with the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and Genecard (www.genecards.org/background.shtml) unification tools. Comparisons of human deafness loci and corresponding regions of the zebrafish genome were performed by using the Ensemble Genome Browser (www.ensembl.org/index.html). The abundances of transcripts in liver EST libraries NIH_ZGC_8 and NIH_ZGC_25 were determined by searches using the DFCI Zebrafish Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=zfish).

RT-PCR with Hair-Cell or Lagenar RNA. Hair-cell RNA was isolated as described above and used to produce cDNA (Super SMART PCR cDNA Synthesis kit, Clontech, Mountain View, CA). Lagenar RNA was isolated (RNeasy Mini kit; Qiagen, Valencia, CA) and used to synthesize randomly primed cDNA (SuperScript III Reverse Transcriptase; Invitrogen, Carlsbad, CA). PCRs were performed (Ex TaqDNA Polymerase Premix; Takara Bio, Madison, WI) with the primer pairs displayed in SI Table 3. All procedures involving kits were performed according to manufacturers' protocols.

***In Situ* Hybridization.** Whole-mount *in situ* hybridizations (49) were conducted on wild-type Tübingen zebrafish larvae treated with 1-phenyl-2-thiourea to reduce pigmentation (11). SI Table 4 describes all of the probes save that for *myo6b* (49).

Immunohistochemistry. Larvae were immunolabeled by conventional techniques (20). Monoclonal anti-acetylated tubulin (6–11B-1; Sigma) was used at a dilution of 1/1,000, and Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) was used at 1/200.

We thank Dr. N. Hopkins (Massachusetts Institute of Technology, Cambridge, MA) for the provision of mutant zebrafish strains, Mr. A. Afolalu and Ms. P. Espitia for fish husbandry, and the members of the Rockefeller Genomics Resource Center for assistance with processing microarray samples. Dr. K. Alagramam, Dr. J. Xiang, and the members of our research group provided insightful comments on the manuscript. This work was supported by National Institutes of Health (NIH) Grant DC00241 and by the National Organization for Hearing Research. B.M.M. was supported by NIH National Research Service Award DC006539. A.J.H. is an Investigator of the Howard Hughes Medical Institute.

1. Heller S (2002) *J Neurobiol* 53:265–275.
2. Collins FS, Green ED, Guttmacher AE, Guyer MS (2003) *Nature* 422:835–847.
3. Petit C, Levilliers J, Hardelin JP (2001) *Annu Rev Genet* 35:589–646.
4. Blackshaw S, Fraioli RE, Furukawa T, Cepko CL (2001) *Cell* 107:579–589.
5. Zhang Y, Ma C, Delohery T, Nasipak B, Foat BC, Bounoutas A, Bussemaker HJ, Kim SK, Chalfie M (2002) *Nature* 418:331–335.
6. Colosimo ME, Brown A, Mukhopadhyay S, Gabel C, Lanjuin AE, Samuel AD, Sengupta P (2004) *Curr Biol* 14:2245–2251.
7. Haeberle H, Fujiwara M, Chuang J, Medina MM, Panditrao MV, Bechstedt S, Howard J, Lumpkin EA (2004) *Proc Natl Acad Sci USA* 101:14503–14508.
8. Tietjen I, Rihel J, Dulac CG (2005) *Int J Dev Biol* 49:201–207.
9. Nicolson T (2005) *Annu Rev Genet* 39:9–22.
10. Novince ZM, Azodi E, Marrs JA, Raymond PA, Liu Q (2003) *Gene Expr Patterns* 3:337–339.
11. Starr CJ (2003) PhD thesis (The Rockefeller Univ, New York).
12. Wan L, Almers W, Chen W (2005) *J Neurosci* 25:941–949.

13. Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J (2000) *Cell* 102:89–97.
14. Yasunaga S, Grati M, Cohen-Salmon M, El-Amraoui A, Mustapha M, Salem N, El-Zir E, Loiselet J, Petit C (1999) *Nat Genet* 21:363–369.
15. Street VA, McKee-Johnson JW, Fonseca RC, Tempel BL, Noben-Trauth K (1998) *Nat Genet* 19:390–394.
16. Hayes A, Costa T, Polomeno RC (1985) *Am J Med Genet* 22:273–280.
17. Verpy E, Leibovici M, Zwaenepoel I, Liu XZ, Gal A, Salem N, Mansour A, Blanchard S, Kobayashi I, Keats BJ, *et al.* (2000) *Nat Genet* 26:51–55.
18. Weil D, El-Amraoui A, Masmoudi S, Mustapha M, Kikkawa Y, Laine S, Delmaghani S, Adato A, Nadifi S, Zina ZB, *et al.* (2003) *Hum Mol Genet* 12:463–471.
19. Liburd N, Ghosh M, Riazuddin S, Naz S, Khan S, Ahmed Z, Riazuddin S, Liang Y, Menon PS, Smith T, *et al.* (2001) *Hum Genet* 109:535–541.
20. Lopez-Schier H, Starr CJ, Kappler JA, Kollmar R, Hudspeth AJ (2004) *Dev Cell* 7:401–412.
21. Zine A, Aubert A, Qiu J, Therianos S, Guillemot F, Kageyama R, de Ribaupierre F (2001) *J Neurosci* 21:4712–4720.
22. Chen P, Segil N (1999) *Development (Cambridge, UK)* 126:1581–1590.
23. Lee SA, Shen EL, Fiser A, Sali A, Guo S (2003) *Development (Cambridge, UK)* 130:2669–2679.
24. Sugihara I (2001) *Hear Res* 153:91–99.
25. Hudspeth AJ (1989) *Nature* 341:397–404.
26. Hudspeth AJ, Lewis RS (1988) *J Physiol* 400:237–274.
27. Sugihara I, Furukawa T (1995) *J Physiol* 489(Pt 2):443–453.
28. Bok D, Galbraith G, Lopez I, Woodruff M, Nusinowitz S, BeltrandelRio H, Huang W, Zhao S, Geske R, Montgomery C, *et al.* (2003) *Nat Genet* 34:313–319.
29. Liu XZ, Ouyang XM, Xia XJ, Zheng J, Pandya A, Li F, Du LL, Welch KO, Petit C, Smith RJ, *et al.* (2003) *Hum Mol Genet* 12:1155–1162.
30. Chidlow G, Wood JP, Graham M, Osborne NN (2005) *Am J Physiol* 288:C416–428.
31. Poitry-Yamate CL, Poitry S, Tsacopoulos M (1995) *J Neurosci* 15:5179–5191.
32. Acosta ML, Kalloniatis M, Christie DL (2005) *Am J Physiol* 289:C1015–C1023.
33. Speer O, Neukomm LJ, Murphy RM, Zanolla E, Schlattner U, Henry H, Snow RJ, Wallimann T (2004) *Mol Cell Biochem 256-* 257:407–424.
34. Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, *et al.* (1999) *Cell* 99:179–188.
35. Van Epps HA, Hayashi M, Lucast L, Stearns GW, Hurley JB, De Camilli P, Brockerhoff SE (2004) *J Neurosci* 24:8641–8650.
36. Schafer DA, Cooper JA (1995) *Annu Rev Cell Dev Biol* 11:497–518.
37. Witke W, Li W, Kwiatkowski DJ, Southwick FS (2001) *J Cell Biol* 154:775–784.
38. Bryce NS, Clark ES, Leysath JL, Currie JD, Webb DJ, Weaver AM (2005) *Curr Biol* 15:1276–1285.
39. Ohoka Y, Takai Y (1998) *Genes Cells* 3:603–612.
40. Cai L, Holoweckyj N, Schaller MD, Bear JE (2005) *J Biol Chem* 280:31913–31923.
41. Cole DG, Diener DR, Himelblau AL, Beech PL, Fuster JC, Rosenbaum JL (1998) *J Cell Biol* 141:993–1008.
42. Pedersen LB, Miller MS, Geimer S, Leitch JM, Rosenbaum JL, Cole DG (2005) *Curr Biol* 15:262–266.
43. Sun Z, Amsterdam A, Pazour GJ, Cole DG, Miller MS, Hopkins N (2004) *Development* 131:4085–4093.
44. Hassan MJ, Santos RL, Rafiq MA, Chahrour MH, Pham TL, Wajid M, Hijab N, Wambangco M, Lee K, Ansar M, *et al.* (2006) *Hum Genet* 118:605–610.
45. Iglesias T, Cabrera-Poch N, Mitchell MP, Naven TJ, Rozengurt E, Schiavo G (2000) *J Biol Chem* 275:40048–40056.
46. Kong H, Boulter J, Weber JL, Lai C, Chao MV (2001) *J Neurosci* 21:176–185.
47. Riol-Blanco L, Iglesias T, Sanchez-Sanchez N, de la Rosa G, Sanchez-Ruiloba L, Cabrera-Poch N, Torres A, Longo I, Garcia-Bordas J, Longo N, *et al.* (2004) *Eur J Immunol* 34:108–118.
48. Baxevanis AD, Ouellette BFF (2005) *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins* (Wiley, Hoboken, NJ).
49. Kappler JA, Starr CJ, Chan DK, Kollmar R, Hudspeth AJ (2004) *Proc Natl Acad Sci USA* 101:13056–13061.