Sp8 regulates inner ear development

Hyeyoung A. Chung, Sofia Medina-Ruiz, and Richard M. Harland

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200

Edited by Donald D. Brown, Carnegie Institution for Science, Baltimore, MD, and approved March 14, 2014 (received for review October 23, 2013)

A forward genetic screen of N-ethyl-N-nitrosourea mutagenized Xenopus tropicalis has identified an inner ear mutant named eclipse (ecl). Mutants developed enlarged otic vesicles and various defects of otocyst development; they also showed abnormal circular and inverted swimming patterns. Positional cloning identified specificity protein 8 (sp8), which was previously found to induce thickened sensory epithelium, and otoconia, all of which are associated with inner ear malformations (2). However, the study of hearing and balance impairment in humans is limited by the inability to follow inner ear development. Vertebrates share similarities in the sequence of developmental events that form the inner ear: the formation of an otic placode from an ectodermal thickening, morphogenesis to form the otocyst, and regional patterning of the otic vesicle (OV), resulting in the 3D membranous labyrinth (3, 4). Multipotent sensory progenitor cells are induced in the ectoderm surrounding the anterior neural plate, a domain termed the preplacodal region (PPR), and sixl has been characterized as marking this panplacodal domain. Signals from hindbrain and the regional expression of different transcription factors differentiate the PPR into the otic placode. The OV is partitioned by asymmetrical expression of various developmental regulators to pattern subdomains of the developing inner ear.

The abilities of Xenopus to elucidate the cellular and molecular aspects of developmental processes position it as a valuable model organism (5–7). Earlier studies of lineage analysis and spatiotemporal expression of transcription factors during inner ear development led to construction of an inner ear fate map in Xenopus and this fate map allows us to interpret gene expression patterns within the context of the anatomy (8, 9). In recent years, genetic and genomic approaches have been developed in Xenopus tropicalis (10–13). To advance our understanding of inner ear development, we have screened N-ethyl-N-nitrosourea (ENU) mutagenized X. tropicalis colonies and recovered an inner ear mutant named eclipse (ecl). The ecl mutant perturbs specificity protein 8 (sp8) expression and leads to aberrant sensory organ development, un compartmentalized and enlarged OVs, and otocional defects. The zinc-finger transcription factor sp8 is related to Drosophila buttonhead. In mice, mutation of sp8 caused severe truncations of the limbs and tail and defective brain and abnormal olfactory development (14–17). Limb/fin outgrowth in chick and zebrafish embryos also employs sp8 (18), suggesting the conserved function of sp8 across phylogeny. In Xenopus, the gene was identified as a target of sax17 (19). Here, we demonstrate a role for sp8 in inner ear development.

Results

In a screen of ENU-mutagenized X. tropicalis, we identified eclipse mutants. Mutant tadpoles were usually immobile and often swam upside down or in circles, whereas WT tadpoles actively swam with a dorsal-up position and linear trajectory, suggesting swimming and balance defects in the mutants. When the culture dish was tapped, a WT embryo responded quickly and swam, whereas the mutant showed a slower response (Movies S1 and S2). Mutant embryos developed normally until the mid-20 stage but showed enlarged OV from stage 28. By stage 40, the gross external phenotypes of mutants are comparable to those of WT siblings but various inner ear defects including enlarged OV and complete or partial loss of otoconia are seen in ecl embryos (Fig. 1A–C). At stage 45, when otocyst are mature, the following otocional defects were observed: devoid of otoconia, reduced otocyst, or scattered otocyst (Fig. 1 A–C′). We classified the ecl phenotypes into three classes: ecl 0, with two inner ears of enlarged OV and no otoconia; ecl 1, with enlarged OV and devoid of otocyst on one side ear and a relatively normal size of OV with otoconia defects on the other side; and ecl 2, with two inner ears with otocyst defects but relatively normal OV size (Fig. S1). After feeding stage 45/46, ecl embryos declined rapidly and never reached metamorphosis. To determine the genetic lesion in ecl mutants, we genotyped genomic DNA from mutants and WT siblings using published or self-designed simple sequence-length polymorphism (SSLP) markers (11, 20). Linkage analysis using pools of genomic DNA from gynogenetic mutants and WT siblings assigned the ecl mutation to linkage group 6 (LG6) (old LG2), corresponding to scaffold in X. tropicalis genome assembly 7.1 (www.xenbase.org) (Fig. S2). We genotyped ~4,000 F3 ecl tadpoles from a mapcross and placed ecl between two flanking markers of 25B04 and SSLP 52.5, an interval of 353.2 kb (Fig. 1D). We further generated an

Significance

Deficits in hearing or balance are common and result from both developmental and environmental causes. Model organisms have contributed many fundamental insights into embryonic development and we have added Xenopus tropicalis as a new genetically tractable organism in the field of inner ear development. As a result of a forward genetic screen in X. tropicalis, we have identified specificity protein 8, a new initiator of ear development, and analyzed mutant phenotypes and molecular interactions with genes that are involved in inner ear development. Given morphological and genetic similarities between inner ears of frog and mammals, the establishment of a new in vivo model system amenable to genetic manipulation will provide an important new tool to study vertebrate ear development.

Author contributions: H.A.C. and R.M.H. designed research; H.A.C. performed research; H.A.C., S.M.-R., and R.M.H. analyzed data; and H.A.C. and R.M.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence of sp8 mRNA reported in this paper has been deposited in the GenBank database (accession no. KJ158464).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1319301111/-/DCSupplemental.
intervening SSLP 52.3 marker and confirmed the mapping interval of ecl. The predicted gene models in the published assembly include specificity protein 8, ATP-binding cassette subfamily B, hypothetical protein, and putative hydroxlate. Among these genes, sp8 is transiently expressed in the OV in mouse embryos (15) so we analyzed expression patterns of sp8 in X. tropicalis by in situ hybridization in WT and mutant embryos. As also reported for EST10 (19), staining for sp8 mRNA was found in the olfactory bulb, midbrain, hindbrain, and the OV in WT embryos, whereas no such signals were detected in ecl mutants (Fig. 1 E–H). Interestingly, sp8 is expressed in the entire OV at early stages but becomes dorsally restricted in the OV at stage 33/34. Quantitative PCR analysis revealed that expression of sp8 in mutants was drastically reduced compared with WT siblings at stage 33/34 and at stage 40. Compared with the expression of sp8 in WT at stage 40, there was ~30% and 95% reduction of sp8 expression in ecl 2 and ecl 1, respectively, (Fig. 1I). A more extreme difference was found in RNA-sequence data using dissected OVs of WT and ecl 0 embryos at stage 37 (2,772/108 versus 1.45/108 reads, respectively, (Fig. 2F and Fig. S3). A standard control MO (Con MO) did not show any effects. The inner ear defects induced by sp8 ATGMO can be partially restored by the addition of rescuing form RNAi sp8 mRNA, which also phenocopied ecl mutants when overexpressed. This experiment indicates that Sp8 is depleted in a gene-specific manner by ATGMO and the precise level of Sp8 is important for proper ear development. In addition, a splice-blocking MO (SBMO) also phenocopied inner ear defects found in the mutants (Fig. S3). These gene knockdown and rescue results confirm the requirement of sp8 in inner ear development, supporting the genetic mapping and expression results that sp8 is reduced or eliminated in the ecl mutant.

The availability of targeted genome editing methods such as zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) permits independent mutations to be made in genes of interest (22, 23). Inner ear defects in sp8 TALEN-injected embryos can be observed from stage 28 and we classified TALEN-induced phenotypes as for ecl mutants (Fig. 2 B–E and G). Compared with the un.injected controls, sp8 TALEN-injected embryos showed abnormal swimming and startle behavior at the swimming tadpole stage (Movie S3), with a dose-dependent severity (Fig. 2G). Sp8 TALEN also elevated the penetrance of mutant phenotypes from matings of ecl heterozygotes. Sp8 TALEN-injected embryos will undergo double-strand breaks (DSBs) at the TALEN target sites and the repair of DSBs can lead to a spectrum of indels within this region. To

**Fig. 1.** Sp8 mRNA is reduced in ecl mutants. Gross morphology at stage 40 in WT (A) and ecl mutants (B and C) at stage 45. (Scale bar, 500 μm.) (D) Mapping interval of ecl mutants on the scaffold/chromosome 6. Parentheses indicate numbers of recombinants from 4,000 genotyped ecl tadpoles. Whole-mount in situ hybridization in WT (E and G) and ecl mutants (F and H). (Upper) Stage 26; (Lower) stage 33/34. (Inset) sp8 expression pattern in the OV of WT embryos. (J) qPCR analysis in WT and ecl embryos at stages 33/34 and 40. Error bars indicate SEM.

**Fig. 2.** Depletion of Sp8 by MO and TALEN phenocopy ecl mutants. (A) Sp8 gene model and sequences of MO and TALEN target. Translation start site is marked in red. Sequences of MO, 5’ UTR, and TALEN binding sites are denoted in italic uppercase, lowercase, and underline, respectively. Domains of Sp, buttonhead box, and zinc finger are colored orange, blue, and green, respectively. (B–E) Phenotypes of sp8 TALEN-injected embryos. (F and G) Phenotypes of sp8 MO, mRNA, and TALEN-injected embryos. Number of injected embryos is shown in parentheses. (H) sp8 TALEN target amplicon. (I) Cel 1 assay for nonhomologous end joining events induced by sp8 TALEN. Red arrows indicate mismatch cleavages due to mutation at the target. L is the DNA size marker. (J) Regional injection of sp8 TALEN leads to loss of limbs. UC, un.injected control.
characterize TALEN-induced mutations at targeted sp8 loci, we amplified genomic DNA fragments of 520 bp from sp8 TALEN-injected individuals (Fig. 2H) and used CEL I endonuclease as previously to detect mutations induced by ZFNs (22). The CEL I assay produced two smaller fragments of ~200 bp and 300 bp, respectively, only in sp8 TALEN-injected individuals, whereas only a larger fragment of 520 bp was found in controls (Fig. 2I). Even injected embryos with a WT inner ear structure showed evidence of cleavage, suggesting nearly all sp8 TALEN-injected embryos contain targeted mutations. We sequenced PCR amplicons and identified a wide range of variants at the targeted region from embryos. We found only a larger fragment of 520 bp in controls (Fig. 2I). This result suggests that TALEN-induced mutations at targeted loci do not survive, the mosaic nature of TALEN-induced mutations allows a way to observe later defects in Xenopus. Indeed, unilateral injection of TALEN at the two-cell stage allowed survival of tadpoles to metamorphosis (n = 25, starting from n = 98) and in many cases these animals lacked limbs on the injected side (n = 16) (Fig. 2J). We also made TALENs to target candidate regulatory sequences near sp8 (Fig. S4). Compared with the sp8 TALEN that targets the coding region, each of these TALENs induced only moderate inner ear phenotypes. Thus, the enhancer activity is probably distributed and we cannot specify with certainty which region is associated with the ecl mutation.

The inner ear is a membranous labyrinth whose fluid-filled tubes and chambers facilitate senses of hearing and balance. In vertebrates, the spherical OV undergoes extensive invaginations of the vesicle walls to form a multichambered inner ear with vestibular and auditory end organs (24). Wax sections, stained with hematoxylin and eosin (H&E), showed the initial compartmentalization of the utricle, horizontal canal, and sacculus in WT embryos at stage 46/47. In anterior sections, the thickening of lateral membrane in the utricular compartment indicates the initial structure of anterior canal cristae, with otoconia located near the utricular membrane (Fig. 3A, arrow). In middle sections,
the protrusion of the vesicle wall leads to separation of saccule and horizontal canal (Fig. 3 B and C, arrowhead). Previous studies demonstrated that the extensive invaginations and fusion of these vesicle membrane protrusions give rise to the formation of semi-circular canal (SCC) compartments (24–26). A saccular macula (SM), cochlovestibular ganglion (CVG), otocyst particles in the saccule, and endolympathic duct (ED) are visible in these sections. CVG cells are present between the OV and hindbrain. The protrusion of the vesicle wall is still obvious in the posterior section (Fig. 3D, arrowhead). In the ecl mutant, however, there is only a small membranous protrusion (Fig. 3F, arrowhead); the inner ear remains as a single large vesicle with no otocyst particles or ED (Fig. 3 F–I). Due to enlarged OV, the neural tube is compressed toward the medial axis (Fig. 3H, open arrow) and the OV and archenteron wall even touch, with no intervening cartilage (Fig. 3H, open arrowhead). More strikingly, the SM was extremely thin and the CVGs were dispersed, in contrast to those of WT, which are clustered together between the hindbrain and SM (Fig. 3 E and J). These observations are consistent with increased volume and pressure of OV fluid, in addition to disorganization of the sensory structures.

To address the structure of sensory organs, we examined the distribution of cell membranes, sensory ganglia, and end organ innervations. Cross-sections were taken from WT and mutant embryos at stage 45. In WT embryos, α-catenin, localized to adherens junctions in columnar sensory epithelium (27), was enriched in the apical membrane of SM (Fig. 3 K–K′′, arrow). However, apically biased distribution of α-catenin was not detected in mutant embryos and otic epithelium failed to establish a columnar morphology, indicating that the tissue integrity is lost in mutant SM (Fig. 3 N–N′′). Next, we examined CVG and neurofilament development. In WT, we found that Isl1 marks auditory and vestibular neurons in CVG, between the hindbrain and OV, as well as in cells within the SM (Fig. 3 L–L′′, arrows). In ecl mutants, Isl1-positive CVG cells are not positioned properly and the number of Isl1-expressing cells is reduced by ~60% (Fig. 2 O–O′, arrows and Fig. S5). Highly organized neurofilament-positive sensory projections into the CVGs and the SM are evident in WT embryos, whereas these projections immunostained by acetylated-α-tubulin antibody are misrouted around the SM in mutants (Fig. 3 M–M′′, arrows). Although we observed apical accumulation of α-tubulin in some cells of SM and protrusions from these cells in WT, these are largely absent in mutant embryos. Abnormal distribution of these proteins due to sp8 down-regulation indicates that the neuronal connection of the inner ear into the central nervous system was functionally impaired in ecl. We further observed sensory hair cell patches in whole embryos or dissected OVs using confocal microscopy. The actin-rich stereociliary bundles and kinocilium of mechanosensory hair cells that are important to detect sound and gravity were visualized with Alexa-conjugated phalloidin, a probe of F-actin, and acetylated-α-tubulin antibody, respectively. In the inner ear, stereociliary bundles can be observed by stage 31 (24). Stereociliary bundles are readily stained in sensory end organs of the utricle, saccule, and three cristae by stage 45, and lagena at stage 46/47 in WT embryos. Basal accumulation of actin in the stereociliary bundles of these sensory end organs, and perpendicularly protrusions of stereociliary bundles from the basal membrane are observed in WT OVs (Fig. 3 Q–Q′′). The kinocilia in the three cristae can be easily visualized at this stage. To our surprise, we failed to detect any of these in the cristae of ecl mutants, or in sp8 depleted embryos at stage 45. By stage 46/47, hair cells in the utricle, saccule, and cristae were not only reduced in numbers but also developed abnormally in shape in sp8-depleted embryos (Fig. 3 T–U″). No lagena was found in these embryos. Whereas we observed stereociliary bundles, kinocilia, and thickened epithelium in all three cristae of WT embryos, we could only detect partially developed and multidirectional stereociliary bundles in the cristae of sp8-depleted embryos.

Although previous studies have shown that sp8 is important during embryogenesis, it is unknown how sp8 regulates inner ear development. The expression of six1 was examined to determine whether sp8 affects PPR development. Six1 expression in PPR was unchanged in sp8-depleted embryos compared with uninjected controls (Fig. S6). We assessed hindbrain patterning, especially focusing on rhombomere (r)3–6, adjacent to the developing inner ear. At stage 18, krox20 and epha4 were normally expressed in r3 and r5 in uninjected control embryos. In TALEN- or MO-injected sides, r5 expression of krox20 and epha4 was reduced, especially near the presumptive otic placode (Fig. 4 A–D). As in mice (28), mafb is expressed in r5 and r6 in X. tropicalis embryos. In TALEN- or MO-injected embryos, the mafb expression domain was elongated laterally and especially expanded in the future otic territory (Fig. 4 E and F). A posterior shift of ghn2 expression was observed, consistent with the previous study of sp8 as a midbrain–hindbrain boundary regulator (16). Although the ecl mutation did not show the severe head defects reported for the mouse, these expression analyses reveal that sp8 still has a conserved function to regulate brain development. We examined foxj1.2 expression in TALEN-injected embryos and uninjected control embryos. Foxj1.2 expression was detected in the presumptive OV, as previously reported in X. tropicalis and zebrasfish embryos (29, 30), and this expression was reduced on the sp8 TALEN-injected side (Fig. 4 I and J), suggesting sp8 regulates hair cells via foxj1 and the hair cell defects found in sp8-reduced embryos are likely to be primary effects. We then examined markers of ear development (Fig. 4 K–T). Pax2, whose function along with pax8 is critical for otic placode formation and cochlear and vestibular development (31, 32), was expressed in the whole OV with strong dorsomedial expression in WT but its expression was reduced in mutants. We also observed loss of pax2 expression in TALEN- or MO-injected embryos at stage 19/20 embryos and these findings are similar to the effects of loss of sp5 in zebrasfish (33). Msx1, implicated in ED formation (26), is restricted to the dorsal part of the OV in WT, whereas this dorsal expression was absent in mutants. Notably, sp8 expression is also restricted to the dorsal OV and overlapped with msx1 expression.

Fig. 4. Molecular markers of the inner ear in sp8 MO- or TALEN-injected embryos. (A–J) Expression patterns of krox20, epha4, mafb, gbx2, and foxj1.2 in uninjected and sp8 TALEN- or MO-injected embryos. Arrows, uninjected side. Arrowheads, injected side. Anterior is up. (K–T) Expression domains of pax2, msx1, tbx1, sox2, and oc90 in WT and mutant embryos at stage 33/34.
and ectopically expressed or up-regulated genes include expression of \( pax2 \) (in size compared with the endogenous OV. Of injected embryos 5 embryos. (posterior to the endogenous OV. The expression domain of various locations anterior to ventral or dorsal but very rarely additional placodal domains were located around the head with 44/45, multiple ectopic microvesicles were often seen. These and \( oc90 \) types. At stage 28, we observed different classes of aberrant dorsal patterning was particularly perturbed in the \( sp8 \) expression was reduced in \( ecl \) mutants despite expansion of OV. Sox2 was depleted (Fig. S9). We asked next whether inhibiting canonical Wnt signaling or transient inactivation of Bmp signaling classification by TALEN and MO knockdown of \( sp8 \) or \( fgf8 \) expression was also observed in sections (Fig. 5N′′′), confirming that \( sp8 \) is sufficient to induce fully differentiated inner ear structures within the placodal field. Wnt signaling is known to regulate \( sp8 \) expression in the limb (18). To determine whether this is similar in inner ear development, embryos were treated from stage 13 to late stage 28 with 6-bromoindirubin-3′,4′-dihydroxynitrobenzene, an inhibitor of GSK3\( \alpha \) and \( \beta \)-tubulin were used as before. This approach demonstrated the presence of both kinosilia and innervation in the ectopic OV (Fig. 5N–N′′′), confirming that \( sp8 \) is sufficient to induce fully differentiated inner ear structures within the placodal field. Wnt signaling has a conserved and selective role in compared with untreated embryos and the expression level of \( sp8 \) was increased by BIO treatment (Fig. 5 O–Q and T). Increased or ectopic \( oc90 \) expression was also observed in BIO-treated embryos and these changes were reversed when \( sp8 \) was depleted (Fig. S9). We asked next whether inhibiting canonical Wnt signaling affects \( sp8 \) expression. We unilaterally injected 80 pg of \( dkk1 \), an extracellular Wnt antagonist, at the two-cell stage, and analyzed \( sp8 \) and \( oc90 \) expression. We observed down-regulation of \( sp8 \) expression but not \( oc90 \) expression in the presumptive OV compared with the control embryos (Fig. 5 R and S and Fig. S9). These data indicate Wnt signaling has a conserved and selective role in \( sp8 \) expression.

Discussion

In this study, we have characterized a novel function of \( sp8 \) during inner ear development (Fig. 1). Although we could not find the specific lesion in the eclipse locus here, genome modification by TALEN and MO knockdown of \( sp8 \) function phenocopied the mutation, providing confidence that \( sp8 \) disruption is the cause of the \( ecl \) phenotype (Fig. 2). We demonstrate that \( sp8 \) is sufficient to induce ectopic OVs possessing differentiated sensory organs (Fig. 5). Recent studies showed that either activation of Wnt signaling or transient inactivation of Bmp signaling can lead to ectopic digit/fin formation in other vertebrates and increased \( sp8 \) expression was evident in both situations (18, 37). Although \( ecl \) mutants did not survive until metamorphosis, we found that embryos injected unilaterally with \( sp8 \) TALEN showed limb outgrowth defects (Fig. 2F) and this finding supports a conserved function of \( sp8 \) in the limb context. Similarly, it is likely that conserved functions of \( sp8 \) apply to inner ear development. During inner ear development, many genes are expressed asymmetrically and orchestrate region-specific development of the inner ear. Although initially uniform, expression of \( sp8 \) becomes restricted.

![Fig. 5. \( sp8 \) can induce ectopic OVs. (A–D) Bright field images of the inner ear at stage 45 in control and \( sp8 \) mRNA-injected embryos. Expression of \( pax2 \) (E–H) and \( oc90 \) (I–L) at stage 28. (M) H&E staining of \( sp8 \)-injected embryos possessing ectopic OV. (N–N′′′) Confocal images of ectopic OV stained with phalloidin 488, anti-acetylated-\( \alpha \)-tubulin antibody, and merged image. Inset is enlarged image of SE and hair cells in the ectopic OV. (Scale bar, 100 \( \mu m \)) (O–S) Expression patterns of \( sp8 \) in BIO-treated or \( dkk1 \)-injected embryos. (T) qPCR analysis of \( sp8 \) expression for BIO-unjected or 1 \( \mu m \) and 10 \( \mu m \) treated embryos. Error bars indicate SEM. *P < 0.05 between BIO-treated and control-treated embryos. Black arrows, ectopic OVs; white arrows, endogenous OVs.

Chung et al.
to the dorsal region of the OV, which will give rise to SCC and ED. Loss of sp8 resulted in otic dysmorphogenesis, similar to mouse mutants of fgf3, mafb, and gbx2 (28, 38, 39): absence of ED, abnormal SCC, swelling of the membranous labyrinth, abnormal sensory organs, accompanied by epithelial dilation (Fig. 5), the most common phenotypes of endolympathic hydrops. The loss of ED in the ecl mutant may lead to retention of fluid, enlargement of the OV, and its various consequences as demonstrated in this study. Grainyhead-like 2 (ghl2) mutants in zebrafish (40) showed a range of otic dysmorphogenesis very similar to the ecl mutant; however, the molecular mechanism is likely to be dissimilar. Whereas ghl2 directly regulates epithelial tissue integrity, sp8 causes more complex effects and the epithelial defects addressed in this study may be a subset of the cause of the defects. Genome sequence data suggest the causative lesion in ecl may lie in regulatory elements. Indeed, 5 genomic sequences of sp8 contain putative T-cell factor and lymphoid enhancing factor binding elements, conserved sequences of WWCAAG, suggesting that sp8 expression might be directly regulated by Wnt/β-catenin signaling in the ear. Notably, Wnt expression is active in the dorsal OV (41) where sp8 is also expressed, and sp8 responds to Wnt manipulation (Fig. 5 O–S). Genetic and embryological analyses in other contexts have revealed that sp8 reciprocally regulates Fgf signaling (14, 18). Indeed, a recent study has shown that sp8 is up-regulated by Fgf signaling during otic placode development (42).

Abundant bioinformatic and genetic tools are now available in X. tropicalis. The optical clarity of tadpoles at the stage of OV and otocyst formation enables direct observation of inner ear defects. Together our approaches will exploit the full potential of X. tropicalis as an inner ear model system, increase knowledge of otic development and otocyst formation, and enhance our understanding of diseases and disorders affecting hearing and balance in vertebrates, including humans.

Materials and Methods

Forward Genetic Screen, Injection, and Imaging. Detailed information is described in SI Materials and Methods.

5’ RACE. 5’ RACE was performed using the FirstChoice RLM-RACE kit (Ambion) and 5’ RACE kit (Invitrogen) according to the manufacturers’ instructions. Sp8 sequence information is deposited in GenBank (accession no. KJ158464).

ACKNOWLEDGMENTS. We thank Divya Gupta, supported by the Undergraduate Research Apprentice Program, for her contribution to mapping and R.M.H. laboratory members for helpful discussion. This research was initiated with support from the University of California Berkeley Center for Integrative Genomics and completed with the support of National Institutes of Health’s National Institute on Deafness and Other Communication Disorders (R21DC010210 and R01DC011901).

Supporting Information

Chung et al. 10.1073/pnas.1319301111

SI Materials and Methods

Forward Genetic Screen, Mapping, RNA Sequencing, and Xenopus tropicalis Embryos. N-ethyl-N-nitrosurea (ENU) mutagenesis of the Nigerian strain was carried out as previously described (1). Mature F1 females were screened by gynogenesis as previously described (2) and simultaneously mated to Ivory Coast males to generate an F2 mapcross. In addition, F1 males were screened by mating to Ivory Coast females and their F2 mapcross daughters were screened by gynogenesis. We screened ∼300 mutagenized females and diploid gynogenotes were inspected under the dissecting microscope for morphological features, including ear morphology, otoconia development, and swimming/balance behavior. For genotyping, at stage 45, genomic DNA was prepared after lysis in 150 μL of 1× TE buffer containing proteinase K (250 μg/mL). Genomic DNA lysed as previously described (2) was further purified by phenol/chloroform extraction. After 2 h (or overnight) incubation at 55 °C, samples were heat inactivated at 85 °C. For PCR amplification, DNA samples were diluted to 20 ng/μL and 1 μL was used per PCR in most cases. We assessed centromere linkage as previously described (2). For detailed mapping, ~4,000 phenotypically ecl F3 tadpoles from mapcrosses of 8 ecl carriers were analyzed using published simple sequence-length polymorphism (SSLP) markers (2, 3) and new SSLP markers designed from Xenopus tropicalis genomic sequences. PCR amplicons were analyzed on 6–12% polyacrylamide gels and visualized by staining with ethidium bromide. X. tropicalis embryos were obtained and maintained as described (2). RNA sequencing was carried out as previously described (4). Embryonic development was staged according to Nieuwoop and Faber (5). This work was done with approval of University of California, Berkeley’s Animal Care and Use Committee. University of California, Berkeley’s assurance number is A3084-01, and is on file at the National Institutes of Health Office of Laboratory Animal Welfare.

PCR. Genomic PCR, reverse transcription PCR, and qPCR were as previously described (2, 6). Sequence information of all primers used in this study will be provided upon request.

Morpholino, Transcription Activator-like Effector Nucleases, mRNA, and Microinjection. Three morpholinos (MOs) from Gene Tools were used in this study: sp8 ATGMO, 5′-CTTCCCCAGTGAGATGGTGCCCAT-3′; sp8 splice-blocking MO (SBMO), 5′-GAAAATCTCCAGACTCAACCCCTAATG-3′, and a standard control MO with 3′ fluorescent modification, 5′-CCTTACCTCAGGTACATTATA-3′. Six silent mutations were introduced in the rescue form of sp8 mRNA, 5′-ATGCGACCATCTGCTAG-GaGAAG-3′, as indicated by lowercase nucleotides. Golden Gate transcription activator-like effector nuclease (TALEN) and TAL Effector kit 2.0 were purchased from Addgene. Sp8 TALENs were constructed as described (7). We designed four sets of TALEN within the sp8 coding region and mRNAs of TALEN constructs were injected into both blastomeres of two-cell-stage embryos. All of these TALEN sets produced ecl-like phenotypes to some extent, but the one that binds to the first zinc-finger domain region induced the highest frequency of ecl 0-like phenotypes. This set of TALEN was used for the experiments. The amplified genomic DNA fragments from sp8 TALEN-injected individuals were subject to the Cel 1 enzyme assay. Am- plicons from sp8 TALEN-targeted loci were cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen) and the sequences were determined using M13 forward and reverse primers.

Histological Studies and Immunohistochemical Analyses. The whole mount in situ hybridization and immunohistochemical analyses were as described (8, 9). For paraffin sections, fixed embryos were dehydrated, cleared, embedded in wax, and sectioned tissues were H&E stained using Varistain Gemini ES (Thermo Scientific). Antibodies are as follows: Mouse anti-α-catenin antibody (1:100; Invitrogen), Islet 1 (1:37.5; Developmental Studies Hybridoma Bank), antiacetylated tubulin antibody (1:100; Sigma T6793), and anti-rabbit anti-pH3 (Ser10) (1:1,000; Millipore). Alexa Fluor 488 or 555 conjugated anti-mouse or anti-rabbit IgG (1:100; Invitrogen) and Alexa Fluor 488 phalloidin (1:100), DAPI, and Hoechst (1:1,000) were from Invitrogen. Terminal deoxynucleotidyl transferase (TdT) (Invitrogen) was used for the TUNEL assay, along with dig-dUTP and staining as for in situ hybridization. Cryostat sections were prepared as described (6) and photographed with fluorescence microscopy (Zeiss). Whole embryos or dissected otic vesicles (OVs) were observed using confocal microscopy (Zeiss 700). Embryos were decalcified for the hair cell image. Z-stack images were generated using built-in software or Fiji. Adobe Photoshop was used for image processing.

Fig. S1. Otic vesicle sizes of wild type, ecl 0, and ecl 2 were measured at stage 40. To compare otic vesicle sizes of mutant embryos and wild-type siblings, embryos were anesthetized with 0.005% benzocaine and OV images were taken under the dissection microscope using Image-Pro 5.1.

Fig. S2. ecl mutation is linked to "old" linkage group 2 of Wells et al. (3). Pooled diploid gynogenotes were genotyped using published SSLP markers and analyzed on 6–12% polyacrylamide gels. This figure shows the cosegregation of the linkage group (LG) 2 centromere-linked marker with the eclipse mutant embryos. Previous linkage group 2 is linkage group 6 in the new X. tropicalis linkage map (3). Whereas eclipse (ec) female possesses polymorphisms from both parents of Nigerian (N) and Ivory Coast (IC) for other linkage groups, half-tetrad (diploid) pools of mutant embryos show only the Nigerian-derived allele.
Both *sp8* ATGMO and *sp8* SBMO phenocopy *ecl* mutation. When injected bilaterally at the two-cell stage, both a translation blocking MO (ATGMO) and a splice-blocking MO (SBMO) phenocopied *ecl* mutants. *Sp8* SBMO is designed to bind the *sp8* exon 1/intron 1 junction. *Sp8* MO represents these two MOs. The gross external morphology of these MO-injected embryos was normal but showed otic dysmorphogenesis with various penetrance (A’–D’). Enlarged otic vesicles were devoid of otoconia, whereas relatively normal sized otic vesicles had reduced, scattered, or fused otoconia. These embryos also showed circular and ventral swimming behaviors compared with their uninjected control or standard control MO (Con MO)-injected embryos that exhibit linear and dorsal-up swimming patterns. Phenotypes are summarized in E.
(A) Sequences of TALEN-modified coding sequences in sp8. (B and C) Targeted modification of sp8 evolutionarily conserved regions (ECRs) using TALENs showed mild but ecl-like phenotypes. We identified ECRs using ecrbrowser.dcode.org, with human base genome chr7:20811894–20836508. (B) There are six ECRs within 20 kb upstream of sp8 transcripts; we identified eight SNPs around these six ECRs, with two located within ECR1 and ECR2, which we targeted with sp8 ECR TALEN_1, and sp8 ECR TALEN_2, respectively. To test whether a T-cell factor (TCF)/lymphoid enhancing factor (LEF) binding site in ECR1 is required for mediating Wnt signaling, we also generated a TALEN targeting a TCF/LEF binding site in ECR1 (sp8 ECR TALEN_3). (C) We targeted these three regions by bilaterally injecting 100 pg, 250 pg, and 500 pg TALENs at the two-cell stage and scored phenotypes at stage 45. Compared with the TALEN that targets the sp8 coding region, these ECR TALENs showed moderate inner ear phenotypes.

Fig. S5. (A) Number of Islet-1 positive cells in the sensory epithelium of wild-type and ecl embryos. Islet-1 positive cells were counted in cryosections of wild-type (n = 5) and ecl embryos (n = 5).
Fig. S6. Gene expression patterns in sp8 depleted or overexpressing embryos. Sp8 MO/TALENs or mRNA were injected unilaterally at the two-cell stage with \textit{lacZ} lineage tracer. To analyze expression patterns of marker genes, the embryos were fixed at the desired stages, and subjected to in situ hybridization. White arrows indicate endogenous expression of marker genes. Black arrows indicate ectopic or up-regulated expression of marker genes. (A and B) \textit{six1} expression, (C and D) \textit{msx1} expression, (E and F) \textit{neurod} expression, and (G and H) \textit{sox2} expression. UC, uninjected control.

Fig. S7. Number of pH3 positive cells and TUNEL positive cells in wild-type and \textit{ecl} embryos. Wild-type and mutant embryos were collected at stage 30 and fixed in 4 morpholine propane sulfonic acid, EGTA, magnesium sulfate, and formaldehyde and subject to phospho histone 3 antibody staining and TUNEL assays as described (6).
Fig. S8. Genetic interaction of sp8 and pax2, pax8, and fgf8 was evaluated by oc90 expression in single-injected or coinjected embryos. No strong interactions or synergies were observed. The number of analyzed embryos is in parentheses. Serial dilution of each factor determined the lowest dose of mRNA, which induced ectopic otic tissues. Microinjection of pax2, pax8, or fgf8 alone resulted in the development of OVs that were reduced or enlarged in size, but did not induce ectopic otic tissues. A higher concentration of pax2 has a potential to produce ectopic OV, which partially agrees with previous studies (1). When 12.5 pg of pax2 was coinjected with sp8 mRNA (12.5 pg), there was an approximately fourfold increase in the number of embryos that show ectopic oc90 expression domain, compared with a single injection of 12.5 pg of either sp8 or pax2 (which usually led to otic vesicle reduction). At higher pax2/sp8 combined concentrations, however, the domain of oc90 expression more frequently developed enlarged otic tissues. Unlike pax2/sp8 coinjection, 7% of pax8/sp8-coinjected embryos showed ectopic otic tissues and this is similar to sp8 (6%) single injections. Pax8/sp8 coinjection resulted in reduction of oc90 expression in 33% of embryos, which is higher than sp8 (18%) and pax8 (12%) single injections. Fgf8/sp8 coinjection most frequently reduced oc90 expression, more frequently than single injections of sp8 and fgf8, 18% and 15%, respectively.


Fig. S9. Ectopic otic vesicle formation by activation of Wnt signaling is reduced when sp8 expression is down-regulated. Ectopic otic vesicle formation was evaluated by oc90 expression. Embryos incubated with 6-bromoindirubin-3′-oxime (BIO) from stage 13/14 to stage 28 were subject to in situ hybridization analysis. Whereas control embryos showed normal oc90 expression (A), BIO-treated embryos showed increased or ectopic oc90 expression (B–D). To address whether Wnt-induced ectopic otic vesicle formation requires sp8, embryos receiving sp8 ATGMO or sp8 TALEN injection at the two-cell stage were incubated with (E) or without (F) BIO from stage 13/14 to stage 28. Interestingly, BIO-induced ectopic otic vesicles were not observed in sp8 MO or TALEN injected embryos (E), indicating sp8 is required for the Wnt-mediated ectopic otic vesicle formation. Black arrows indicate ectopic oc90 expression. In addition, we observed that oc90 expression was retained on the dkk1-injected side (H) in comparison to the uninjected side (G), suggesting that the absence of sp8 expression in dkk1-injected embryos is not due to the loss of otic vesicle tissue.

Chung et al. www.pnas.org/cgi/content/short/1319301111 6 of 9
Movie S1. Swimming and balance motions of a wild-type embryo were recorded. A wild-type feeding stage tadpole was placed on an agarose-coated culture dish with 1/9 modified ringer medium. Swimming patterns and startle response were recorded using an iphone 4S. When the culture dish was tapped, a wild-type tadpole showed a fast response and swam with a linear trajectory.
Movie S2. Swimming and balance motions of eclipse were recorded. Swimming patterns were recorded for an ecl mutant tadpole that was maintained in the same condition as wild-type tadpoles. The mutant tadpole occasionally showed circular swimming patterns but was mostly immobile. When the culture dish was tapped, the ecl mutant embryo did not respond.
Movie S3. Swimming and balance motions of sp8 TALEN-injected embryos were recorded. Both blastomeres received sp8 TALEN injection at the two-cell stage and were raised until swimming tadpole stage. Swimming/balance behavior and startle response of these tadpoles were recorded. Note that one sp8 TALEN-injected embryo remained immobile (and upside down) although the culture dish was tapped. The other three sp8 TALEN-injected tadpoles showed different degrees of abnormal circular swimming patterns.

Movie S3