Expression of Neurog1 Instead of Atoh1 Can Partially Rescue Organ of Corti Cell Survival

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Abstract

In the mammalian inner ear neurosensory cell fate depends on three closely related transcription factors, Atoh1 for hair cells and Neurog1 and Neurod1 for neurons. We have previously shown that neuronal cell fate can be altered towards hair cell fate by eliminating Neurod1-mediated repression of Atoh1 expression in neurons. To test whether a similar plasticity is present in hair cell fate commitment, we have generated a knockin (KI) mouse line (Atoh1<sup>KI</sup>Neurog1) in which Atoh1 is replaced by Neurog1. Expression of Neurog1 under Atoh1 promoter control alters the cellular gene expression pattern, differentiation and survival of hair cell precursors in both heterozygous (Atoh1<sup>f/KINeurog1</sup>) and homozygous (Atoh1<sup>KI</sup>Neurog1<sup>/KINeurog1</sup>) KI mice. Homozygous KI mice develop patches of organ of Corti precursor cells that express Neurog1, Neurod1, several prosenyn genes and neurotrophins. In addition, these patches of cells receive afferent and efferent processes. Some cells among these patches form multiple microvilli but no stereocilia. Importantly, Neurog1 expressing mutants differ from Atoh1 null mutants, as they have intermittent formation of organ of Corti-like patches, opposed to a complete ‘flat epithelium’ in the absence of Atoh1. In heterozygous KI mice co-expression of Atoh1 and Neurog1 results in change in fate and patterning of some hair cells and supporting cells in addition to the abnormal hair cell polarity in the later stages of development. This differs from haploinsufficiency of Atoh1 (Pax2cre; Atoh1<sup>f/f</sup>), indicating the effect of Neurog1 expression in developing hair cells. Our data suggest that Atoh1<sup>KI</sup>Neurog1 can provide some degree of functional support for survival of organ of Corti cells. In contrast to the previously demonstrated fate plasticity of neurons to differentiate as hair cells, hair cell precursors can be maintained for a limited time by Neurog1 but do not transdifferentiate as neurons.

Introduction

Basic Helix-Loop-Helix (bHLH) transcription factors are essential for cell fate determination and differentiation in a wide range of tissue [1]. In the retina, spinal cord and forebrain, a mixture of bHLH expression profiles form complex cross-regulatory interactions [2,3,4,5,6]. In certain cases a cell population dependent upon a single bHLH gene can be replenished through a change in the fate of another population dependent on a different bHLH gene, as observed in spinal dorsal root ganglial development [7]. Transgenic misexpression of another bHLH gene under the promoter control of another bHLH gene results in diverse phenotypic outcomes depending on the tissue and the gene replaced [8,9,10]. In the retina, the bHLH gene Neurod1 is needed for differentiation of amacrine cells and Atoh7 for differentiation of retinal ganglion neurons [5]. However, misexpressing Neurod1 under Atoh7 promoter control rescues developing ganglion neurons [11]. This indicates a switch in context specific action of this misexpressed bHLH gene [8], possibly related to a sophisticated bHLH gene cross-regulation [4,12] that may differ in the targeted tissue [11] or during certain developmental steps [13]. This variability of one bHLH gene to functionally replace another seems to relate in part to the similarities in the DNA binding domains, i.e., the E-boxes [14] and the complexity of the cis enhancer elements [3] for the different bHLH genes, but may also relate to the availability and type of the E-box associated protein binding partners [15,16].

The inner ear is simpler developing system compared to the retina or the brain. The ear develops just two neurosensory cell types, hair cells for mechanotransduction and sensory neurons to conduct the information from the ear to the brain. Two bHLH transcription factors, Atoh1 (formerly Math1) and Neurog1 (formerly Ngn1) are necessary for neurosensory development in the inner ear. Eliminating either Atoh1 or Neurog1 leads to the absence of differentiated hair cells or neuron development in the mouse, respectively [17,18]. In addition, several other bHLH genes [14] are also expressed in the inner ear and provide the molecular basis for the heterogeneity of a given neurosensory cell type [19]. While many cells in the inner ear will undergo apoptosis in the absence of their specific required bHLH gene, [20,21,22], under certain circumstances a transformation of one cell type into another cell type has been reported [23,24,25]. For example, in Neurod1 conditional knockout mutants some cells in inner ear ganglia can...
differentiate as hair cells [19] through upregulation of Atoh1 that is normally suppressed by Neurod1. These knockout data raise the possibility that other inner ear neurosensory cells could also react plastically when one bHLH gene is replaced by another through the altered cross-regulation of bHLH genes. Given that absence of Neurog1 affects Atoh1 mediated hair cell differentiation [23], we wanted to test the potential of fate changes for hair cell precursors to differentiate as neurons when Atoh1 was replaced with Neurog1 under Atoh1 promoter control. To achieve this, we generated a knockin (KI) mouse where heterozygous KI mice (Atoh1+/KINeurog1) allowed us to assess the effect of co-expression of two different bHLH genes on hair cell development. We also bred these KI mice to homozygosity (Atoh1KINeurog1/KINeurog1) to test whether Neurog1 could functionally replace Atoh1 by either initiating differentiation of hair cell precursors or altering the fate of these precursors.

Our data show that Neurog1 is expressed in hair cells of heterozygous KI mice and in clusters of undifferentiated organ of Corti precursors cells of homozygous KI mice where it regulates expression of Neurod1 and several other hair cell-associated genes. In homozygous KI mice the patches of organ of Corti-like cells form microvilli and preserve afferent and efferent innervation instead of transforming into a 'flat epithelium' without any innervation as observed in Atoh1 null or conditional knockout mice [17,22]. We also show subtle but compelling evidence in heterozygous KI mice in altering the fate and patterning of both hair cell and supporting cells with gradual increasing severity of the defect with age. The phenotypes in the heterozygous KI mice indicate that Atoh1 and Neurog1 co-expression influence the extent of the morphological and histological defects. The data in homozygous KI mice suggest that replacement of Atoh1 by Neurog1 cannot fully rescue hair cell differentiation but can provide functional support for limited survival of organ of Corti-like patches without altering their fate to differentiate into neurons.

Materials and Methods

All animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC) guidelines for use of laboratory animals in biological research (ACURF #1103057).

Generation of knockin (KI) mouse model (Atoh1KINeurog1)

Plasmids used. Six plasmid clones were utilized for the construction of the KI targeting vector. The pCS2-MT-Ngn1 plasmid was kindly provided by Dr. Quifu Ma. This plasmid contains the full-length coding region of mouse Neurog1 that was cloned into the pCS2-MT [26] vector from its original cDNA Bluescript plasmid (pBS) [27]. Both the pPGKneo-II (GenBank ID:AF335420) [28] and the PGKstartA [29] plasmids were obtained from the University of Nebraska Medical Center Mouse Genome Engineering Core Facility, Omaha, NE. pIRES2-DsRed2 was purchased from Clontech (Mountain View, CA). Two pBSII KS clones containing genomic fragments isolated from a mouse 129/SvEv genomic DNA library (Stratagene) were kindly made available by Dr. Huda Zoghbi [30]. The pMath1-5’-9 plasmid contained an EcoRI/Apal 10.5 kb fragment of the Atoh1 locus. This fragment contains the 5’ flanking fragment (5.06 kb), the Atoh1 open reading frame (~1.06 kb) and a 4.38 kb 3’ flanking sequence. The second plasmid, pApa4.2, contains a 4.54 kb Apal fragment that represents ~164 bp of the 3’ end of the Atoh1 coding sequence and a 4.38 kb sequence of the downstream 3’ fragment.

Construction of the Atoh1KINeurog1 plasmid. An initial plasmid was produced where a 1.29 kb IRES2-DsRed2 sequence was inserted 3’ to the 6XMy-c-mNeurog1 sequence in the pCS2-MT-Ngn1 plasmid. The single Ncol site in the original pIRES2-DsRed2 plasmid was replaced with a XbaI restriction enzyme recognition site. This was done by linearizing the plasmid by NotI enzymatic digestion, followed by blunt-ending using T4 polymerase. A XbaI adapter was then ligated with T4 DNA ligase onto the linearized plasmid and sticky ends were obtained through subsequent XbaI digestion. This modified plasmid was circularized by ligation with T4 DNA ligase. The resulting plasmid was then digested with BamHI, blunt-ended with T4 DNA polymerase and then digested with XbaI. Subsequently a 1.29 kb fragment containing the IRES2-DsRed2 sequence was gel purified. The pCS2-MT-Ngn1 plasmid was prepared for insertion of the IRES2-DsRed2 fragment by linearizing through XhoI digest, then blunt-ended with T4 DNA polymerase, followed by XbaI digestion, and then dephosphorylated using shrimp alkaline phosphatase. After gel purification this linearized plasmid was ligated with the blunt/sticky-ended IRES2-DsRed2 fragment to generate a pCS2-MT-6xMyc-mNeurog1-IRES2-DsRed2 plasmid.

The next step involved the insertion of the 5’ Atoh1 genomic fragment into the pCS2-MT-6xMyc-mNeurog1-IRES2-DsRed2 plasmid. A unique single EcoRV site was created in pCS2-MT-6xMyc-mNeurog1-IRES2-DsRed2 plasmid by partial DraI digestion to yield a linearized plasmid with only one of the four DraI sites (nucleotide positions 93, 3817, 3836 and 4528) being cut. EcoRV adapters were next ligated onto the ends of the linearized plasmids, followed by digested with EcoRV and then circularized by ligation. A clone containing an EcoRV site at position 95 was selected for further cloning steps. A linearized plasmid with sticky/blunt ends was produced by a double digestion using the ClaI and EcoRV restriction enzymes, which was then dephosphorylated with shrimp alkaline phosphatase. The ClaI site was immediately upstream of EcoRV site. The pMath1-5’-9 plasmid was initially digested with SphI and blunt-ended with T4 DNA polymerase, which was then followed by complete digestion with ClaI to create ClaI sticky ends. The 5.07 kb fragment, which included 15 bps of the Atoh1 coding sequence, was ligated into pCS2-MT-6xMyc-mNeurog1-IRES2-DsRed2 linearized plasmid to create a pCS2-MT-5’Atoh1-6xMyc-mNeurog1-IRES2-DsRed2 plasmid.

A plasmid, pBSII-loxP-pGKneo-3’Atoh1, was constructed that contained the floxed PKGneo cassette in reverse orientation to the 3’ Atoh1 sequence. The pGKneo-II plasmid was partially digested with XbaI and blunt-ended by T4 DNA polymerase treatment. The 4.77 kb linearized pGKneo-II plasmid was then digested with BamHI and treated with shrimp alkaline phosphatase. A 4.60 kb fragment containing the 3’ Atoh1 sequence was generated by a partial digestion with KpnI to linearize the pApa4.2 fragment, which was then blunt-ended by T4 DNA polymerase. This product was then digested with BamHI and the fragment was then gel purified, followed by ligation into the dephosphorylated linearized pGKneoII plasmid. The multilinker sequence in this construct was removed by partial digestion with Sall to yield a linearized plasmid and then digested with BamHI. The overhanging ends were blunt-ended using T4 DNA ligase to generate the pBSII-loxP-PGKneo-3’Atoh1 plasmid.

The final knockin (KI) construct was created by linearizing the pBSII-loxP-PGKneo-3’Atoh1 through digestion of the adjacent ClaI and EcoRV sites with both their respective enzymes to create a sticky/blunt linearized plasmid. This DNA was then dephosphorylated with shrimp alkaline phosphatase. A 7.63 kb fragment containing the 5’Atoh1-6XMy-c-mNeurog1-IRES2-DsRed2 sequence was prepared for insert into the prepared pBSII-loxP-
PKNeo-3’Atoh1 dephosphorylated plasmid. The pCS2-MT-5’Atoh1-6XMyc-mNeurog1-IRES2-DSRed2 plasmid was linearized by NotI digestion and then treated with T4 DNA polymerase to create blunt ends, followed by digestion with Clal. The resulting 7.63 kb fragment was then ligated into the pBSII-loxP-PGKneo-3’Atoh1 to generate a pBSII-5’Atoh1-6XMyc-mNeurog1-IRES2-DSRed2- loxP-PGKneo-3’Atoh1 plasmid and was designated as pAtoh1PKNeog.

The Atoh1PKNeog sequence was then cloned into PGKdtabpA. The Atoh1PKNeog insert was prepared by digestion of pAtoh1PKNeog with Clal followed by the blunting using T4 DNA polymerase and the desired fragment was excised with NotI digestion. This fragment was gel purified. The PGKdtabpA vector was prepared by partial SpeI digestion to linearize the plasmid, blunting with T4 DNA polymerase, followed by NotI digestion and then dephosphorylated by shrimp alkaline phosphatase. The Atoh1PKNeog sequence was then ligated with T4 DNA ligase into the gel purified linearized PGKdtabpA plasmid. The final pPGKdtabpA-Atoh1PKNeog plasmid was linearized by NotI digestion and gel purified. This fragment was used for transformation of the embryonic stem cells. All plasmid constructs were generated. A complete sequence analysis of the Neurog1 insert was done to verify that no changes had occurred during the production of the pPGKdtabpA-Atoh1PKNeog plasmid.

Atoh1PKNeog production. Two 129/SvJ ES cell lines, E14 and R1, were used for targeting [31,32]. The linearized pGKdtabpa-KI construct was injected into ES cell lines. The number of ES cell DNA after positive (Neo) and negative (HSV thymidine kinase and PGK-Neo) were used. Digested DNAs were electrophoresed on a 0.8% agarose gel. The gel was treated by soaking in 1.5 M NaCl/0.1 M NaOH solution, followed by a 10X SSC solution (1.5 M NaCl/130 mM sodium citrate, pH 7.0) and then blotted onto Hybond-N filters (Amersham, Piscataway, NJ) using a vacuum blotter system (BioRad, Hercules, CA) following the manufacturer’s instructions. Probes were amplified by two primer sets specific for genomic DNA up- and downstream of the mouse Atoh1 gene. 5’ probe: gmAtoh1 5’-FOR (5’-CTGAGGAATCCGGATGCG-GAG-3’); and gmAtoh1 5’-REV (5’-CTACTTCCCTTTAGCCACCAGTTCC-3’) with an 1102 bp ampiclon. 3’ probe: gmAtoh1 3’-FOR (5’-GATGCTGACTGGTTCC-TTCTGCTTC-3’) and gmAtoh1 3’-REV (5’-GCTCTGGC-TCDTGAAACTCCTGC-3’) that yielded 1195 bp product. Purified PCR amplicons were then random primer labeled using 6000 CI/ml dCTP (α32P-dCTP) and dATP (α32P-dATP, as per manufacturer’s protocol (Roche Applied Science, Indianapolis, IN). Filters were hybridized overnight at 65°C [33] and washed using high stringent conditions (0.1X SSC, 1% SDS) at 65°C. The filters were developed using a Storm Phosphorimager and the resulting images analyzed to determine the number and sizes of the detected RFLs.

Genotyping. Genotyping of Atoh1KNeog mice was completed using tail DNA for standard PCR amplification. The PCR conditions consisted of an initial denaturing step at 94°C for 2 minutes, an amplification step for 32 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes. EconoTaq plus green 2X master mix (Lucigen, 30033) and a three primer set were used for these reactions. All resultant products were electrophoresed and visualized on a 2% agarose gel. The three primer set consisted of KI-PKG-9225 (3’ forward) with a sequence 5’-CTA CCCC GTCTTCCATT CCG C-3’; GT gmAtoh1-9179 (3’ reverse) with a sequence of 5’-ACT CTC CGT CAC TGG TGT GGA-3’ and Atoh1 S1 with a sequence 5’-CTT CGC ACC-3’. These primers were specific for genomic DNA up- and downstream of the mouse Atoh1 gene. The wild-type Atoh1 allele (Atoh1*) was determined by the primer set Atoh1 S1 and GT gmAtoh1-9179 and produced a ~300 bp product. The KNeog allele was detected with the primer set KI-PKG-9225 and GT gmAtoh1-9179 and produced a ~600 bp product. Atoh1 conditional knockout (CKO, Pax2-cre; Atoh1+/−) mice were generated by crossing the floxed Atoh1 with the Tg (Pax2-cre) line described previously [17,22].

In situ hybridization. In situ hybridization was performed using the RNA probe labeled with digoxigenin. The plasmids containing the cDNAs were used to generate the RNA probe by in vitro transcription. Locked nucleic acid (LNA) probes for microRNAs (miR-96 and miR-124) were purchased and used as described previously (miRCURY LNA probes; Exiqon, Woburn, MA; [34]). Nf3 antisense probe was made using the IMAGE clone 1177925 and EcoRI restriction enzymes and T3 RNA polymerase was used. After being anesthetized with Avertin, mice were perfused in 4% paraformaldehyde (PFA) and fixed overnight in 4% PFA. The ears were dissected in 0.4% PFA and dehydrated and rehydrated in graded methanol series and then digested briefly with 20 μg/ml of Proteinase K (Ambion, Austin, TX, USA) for 15-20 minutes. Then the samples were hybridized overnight at 60°C to the riboprobe in hybridization solution containing 50% (v/v) formamide, 50% (v/v) 2X saline sodium citrate (Roche) and 6% (w/v) dextran sulphate. After washing off the unbound probe, the samples were incubated overnight with an anti-digoxigenin antibody (Roche Diagnostics GmbH, Mannheim, Germany) conjugated with alkaline phosphatase. After a series of washes, the samples were reacted with nitroblue phosphate/5-bromo, 4-chloro, 3-indolyl phosphate (BM purple substrate, Roche Diagnostics, Germany) which is enzymatically converted to a purple colored product. The ears were mounted flat in glycerol and viewed in a Nikon Eclipse 800 microscope using differential...
interference contrast microscopy and images were captured with Metamorph software. The ears of the littermate of different genotype for the same gene expression were performed in the same reaction tubes to maintain the reaction accuracy.

Immunohistochemistry
The mice for immunofluorescent staining were collected as mentioned previously and fixed in 4% PFA overnight, the ears were dissected and dehydrated in 100% ethanol and then rehydrated in graded ethanol series and in PBS and blocked with 2.5% normal goat serum in PBS containing 0.5% Triton-X-100 for 1 hour. Then the primary antibodies for Myo7a (Myosin 7a, Proteus Biosciences), Tubulin (Sigma), Sox2 (Millipore), activated Caspase3 (Cell Signaling Technology), DiRed2 (anti-red fluorescent protein; Genway) and Prox1 (Covance) were used in dilutions of 1:200, 1:500, 1:1000, 1:10000 and 1:2000, respectively and incubated for 24–48 hours at 4°C. After several washes with PBS, corresponding secondary antibodies (1:500) (Alexa fluor molecular probes 647 or 532 or 488; Invitrogen) were added and incubated overnight at 4°C. Hoechst nuclear stain (Polysciences; 10mg/ml) was used at room temperature for 1 hour. The ears were washed with PBS and mounted in glycerol and images were taken with a Leica TCS SP5 confocal microscope.

Scanning electron microscopy (SEM)
The mice for scanning electron microscopy were perfused and fixed in 2.5% glutaraldehyde in 1% PFA after sedating with Avertin. Ears of postnatal mice were decalcified with EDTA. Following osmication in 2% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for up to 1 hour, the ears were microdissected including removal of the Reissners membrane and the tectorial membrane. The samples were then washed several times with distilled water to remove ions, dehydrated in a graded ethanol series and in propylene oxide. The samples were then rehydrated in graded ethanol series and in PBS and blocked with 1% protease inhibitor cocktail (Roche, Germany) and was homogenized in radio immune precipitation assay (RIPA) buffer with 1% protease inhibitor cocktail (Roche, Germany) and was clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration in the brain lysate was measured using a Pierce BCA protein assay kit (Thermo scientific, Rockford, IL), protein was denatured at 95°C for 5 minutes and 30 μg of protein samples were then loaded in a 10% polyacrylamide gel. The samples were then blotted onto nitrocellulose membranes and probed with the primary antibodies of Neurog1 (Neurogenin1; 1: 500; Abcam, ab89461), Myc-tag (1:500; Millipore; clone 9E10-05-419) and β-catenin (1:500; BD Transduction Laboratories) overnight at 4°C. Species specific HRP secondary antibodies (1: 5000; Thermo scientific, Rockford, IL) were used for 1 hour at room temperature. The protein bands were identified using Chemiluminescent substrates (Thermo scientific, Rockford, IL).

Western Blot Analysis
P5 and P9 heterozygous mice were decapitated after a lethal dose of Avertin and brains (cerebella) of the mice were snap frozen in liquid nitrogen and stored at -80°C. Then the cerebella were homogenized in radio immune precipitation assay (RIPA) buffer with 1% protease inhibitor cocktail (Roche, Germany) and was clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration in the brain lysate was measured using a Pierce BCA protein assay kit (Thermo scientific, Rockford, IL), protein was denatured at 95°C for 5 minutes and 30 μg of protein samples were then loaded in a 10% polyacrylamide gel. The samples were then blotted onto nitrocellulose membranes and probed with the primary antibodies of Neurog1 (Neurogenin1; 1:500; Abcam, ab89461), Myc-tag (1:500; Millipore; clone 9E10-05-419) and β-catenin (1:500; BD Transduction Laboratories) overnight at 4°C. Species specific HRP secondary antibodies (1:5000; Thermo scientific, Rockford, IL) were used for 1 hour at room temperature. The protein bands were identified using Chemiluminescent substrates (Thermo scientific, Rockford, IL).
lead to normal hair cell differentiation (Fig. 1g). In contrast to this expectation, Neurog1 was expressed in E18.5 homozygous KI mice only in clusters of organ of Corti cells, except for continuous expression in the apical tip demonstrated by in situ hybridization (Fig. 2f). Atoh1 expression in the homozygous KI mice was eliminated as expected but was also reduced in the heterozygous KI cochlea, specifically in the basal half compared to the wild-type littermate (Fig. 2a, b, c). Both homozygous KI and Atoh1 CKO mice show expression of Neurog1 in the delaminating neuroblasts and in the epithelia of the utricle and saccule (g, g', h, h'). In addition, Neurog1 is expressed in the canal cristae and the mid-base of the cochlea of homozygous KI mice (white arrow in g) which is not observed in the Atoh1 CKO cochlea (h). The expression of Neurog1 in the sensory epithelia recapitulates Atoh1 expression below the level encountered in wild-type, homozygous KI and Atoh1 CKO mice (Fig. 2g–i). Co-expression of Atoh1 with Neurog1 in the developing hair cells restricts Neurog1 expression in delaminating neurons in the heterozygous KI mice below the level encountered in wild-type, homozygous KI and Atoh1 CKO mice (Red arrows indicate the delaminating neuroblasts; asterisks indicate the sensory epithelia; yellow dotted lines demarcate the boundaries of the utricle and saccule in e,e', e'', f, f', f'', g, g', h, h'). AC, anterior crista; Co, Cochlea; HC, horizontal crista; PC, posterior crista; S, saccule; U, utricle. Bar indicates 100 μm. doi:10.1371/journal.pone.0030853.g001

Figure 1. Replacement of Atoh1 with Neurog1 mimics spatio-temporal expression of Atoh1. In situ hybridization at E14.5 shows the expression of Atoh1 in a wild-type (Atoh1+/+) mouse (a) which is less profound in the heterozygous KI (Atoh1+/Neurog1−/−) littermate (b). There is no in situ signal for Atoh1 mRNA in the homozygous KI (Atoh1−/Neurog1−/−) (c) and in the Atoh1 conditional knockout mice (CKO, Pax2cre; Atoh1−/−) (d). Neurog1 in situ hybridization in the wild-type control mice represents expression only in the delaminating neurons emanating from the utricle and saccule but not in the sensory epithelia (e, e'). In heterozygous KI mice, Neurog1 is expressed in the vestibular sensory epithelia, but not yet in the cochlea and is diminished in the delaminating neuroblasts near the utricle and saccule compared to the wild-type littermate (compare red arrows in e, e', e'' and f, f'). Both homozygous KI and Atoh1 CKO mice show expression of Neurog1 in the delaminating neuroblasts and in the epithelia of the utricle and saccule (g, g', h, h'). In addition, Neurog1 is expressed in the canal cristae and the mid-base of the cochlea of homozygous KI mice (white arrow in g) which is not observed in the Atoh1 CKO cochlea (h). The expression of Neurog1 in the sensory epithelia recapitulates Atoh1 expression (compare a, g) whereas the more obvious expression of Neurog1 in delaminating neurons is observed in Atoh1 CKO mice (h). Co-expression of Atoh1 with Neurog1 in the developing hair cells restricts Neurog1 expression in delaminating neurons in the heterozygous KI mice below the level encountered in wild-type, homozygous KI and Atoh1 CKO mice (Red arrows indicate the delaminating neuroblasts; asterisks indicate the sensory epithelia; yellow dotted lines demarcate the boundaries of the utricle and saccule in e,e', f, f', g, g', h, h'). AC, anterior crista; Co, Cochlea; HC, horizontal crista; PC, posterior crista; S, saccule; U, utricle. Bar indicates 100 μm. doi:10.1371/journal.pone.0030853.g001
Atoh1 expression in the KI mice that have no homozygous KI mice advanced toward the apex in the cochlea of heterozygous KI mice than Neurod1 expression of the heterozygous KI cochlea (e). Homozygous KI mice show a strong expression in discontinuous cluster of cells in the organ of Corti of the heterozygous KI cochlea (e). Homozygous KI mice show a strong expression of Neurog1 in discontinuous cluster of cells in the organ of Corti, except for a continuous expression in the apex of the cochlea (f). Neurod1, a downstream gene to Neurog1 and Atoh1 is relatively more advanced toward the apex in the cochlea of heterozygous KI mice than the wild-type littermate (inserts in g, h; compare the distance in g,h). In homozygous KI mice Neurod1 is strongly expressed in discontinuous patches of organ of Corti cells and some spiral neurons (i) and almost reaches the apical tip (compare g, h, i). The distance measured from the apical tip of the cochlea to the Neurod1 expression is marked with yellow dotted lines (g,h,i). Immunohistochemistry in E18.5 homozygous KI mice demonstrates both Neurog1 and Myc-tag immunopositivity in the hair cells as well as in the spiral ganglia (j,k). Neurog1 and Myc-tag immunopositivity in hair cells is shown together with Myo7a, a marker for hair cells (j-k). DsRed2, a protein marker for Neurog1 expression showed localization only in the hair cells in the heterozygous KI mice which are innervated by the radial fibers labeled with anti-Tubulin antibody (l). OC, organ of Corti; RF, radial fibers; Spg, spiral ganglia. Bar indicates 100 μm except j and k where it indicates 10 μm.

Figure 2. Neurog1 and Neurod1 expression is retained in the organ of Corti cells in the KI mice that have no Atoh1 expression. Atoh1 in situ hybridization shows a reduced expression in E18.5 heterozygous KI mice compared to the wild-type littermate (a, b) but complete absence in homozygous KI mice (c). Neurog1 is absent in the wild-type cochlea by E18.5 (d) but present in the organ of Corti of the heterozygous KI mouse (e). Homozygous KI mice show a strong expression in discontinuous cluster of cells in the organ of Corti (f). Neurod1, a downstream gene to Neurog1 and Atoh1 is relatively more advanced toward the apex in the cochlea of heterozygous KI mice than the wild-type littermate (inserts in g, h; compare the distance in g, h). In homozygous KI mice Neurod1 is strongly expressed in discontinuous patches of organ of Corti cells and some spiral neurons (i) and almost reaches the apical tip (compare g, h, i). The distance measured from the apical tip of the cochlea to the Neurod1 expression is marked with yellow dotted lines (g, h, i). Immunohistochemistry in E18.5 heterozygous KI mice demonstrates both Neurog1 and Myc-tag immunopositivity in the hair cells as well as in the spiral ganglia (j, k). Neurog1 and Myc-tag immunopositivity in hair cells is shown together with Myo7a, a marker for hair cells (j-k). DsRed2, a protein marker for Neurog1 expression showed localization only in the hair cells in the heterozygous KI mice which are innervated by the radial fibers labeled with anti-Tubulin antibody (l). OC, organ of Corti; RF, radial fibers; Spg, spiral ganglia. Bar indicates 100 μm except j and k where it indicates 10 μm.

Myo7a, in the heterozygous KI mice (Fig. 2j-k'). DsRed2 was co-labeled with anti-Tubulin antibody showing the projection of radial fibers to these DsRed2-positive hair cells (Fig. 2l). In addition, we identified Neurog1 and Myc-tag proteins at the approximate expected size (~25 kDa and ~36 kDa, respectively) by western blot analysis using the cerebella of P5 and P9 heterozygous KI mice (Fig. S2a). However, the same sized protein bands for Neurog1 and Myc-tag were also found in the wild-type samples which suggested non-specific protein detection (Fig. S2b). Antibodies against several bHLH transcription factors are generally problematic, requiring novel approaches to overcome these problems [35].

Distribution of Neurog1 in cells within patches of organ of Corti in homozygous KI mice indicated either a differential expression of Neurog1 in hair cells precursors or loss of some hair cell precursors. Neurog1 distribution in these mice resembled the discontinuous distribution of remaining Atoh1lacZ-positive cells reported in E18.5 Atoh1lacZ/lacZ null mutant [36] but was more patchy and showed more rows of cells within the patches. Besides inner ear, misexpression of Neurog1 revealed comparable replacement of Atoh1 expression by Neurog1 in brains as well where Neurog1 recapitulated Atoh1-pattern in the homozygous KI mice both in the cochlear nucleus and in proliferating external granule cells of the cerebellum (Fig. S3c,c',f,f',f').

Neurog1 misexpression in homozygous KI mice rescued clusters of organ of Corti precursors.

Homozygous KI mice had no detectable Myo7a expression (Fig. 3b) in comparison to the wild-type cochlea showing normal organization of Myo7a immunofluorescence labeled hair cells (Fig. 3a). To investigate the morphology of Neurog1 positive cells within the organ of Corti-like patches and their associated ultrastructure in the homozygous KI mice, we examined newborn (P0) mice with scanning electron microscopy (SEM) (Fig. 3c-g). Previous reports on Atoh1 null mice [17] and our observations on Atoh1 CKO mice (Fig. 3h, h') [22] showed a uniform ‘flat epithelium’ that is not covered by a tectorial membrane. In contrast, we found formation of single or cluster of cells with long microvilli or rudimentary stereocilia without any staircase patterned stereocilia in homozygous KI mice (Fig. 3c-g). These organ of Corti cells were localized in patches (Fig. 3c-g) and in most cases were associated with an expansion of the tectorial membrane that was otherwise confined to the greater epithelial ridge (GER) (Fig. 3c-g). These cells were always in the center of Claudius-like cells (Fig. 3c, d, e) indicating some cellular interaction to organize cells around the cells bearing microvilli. Our data suggested that misexpression of Neurog1 can rescue some organ of Corti cells to obtain some degree of differentiation (Fig. 3c-g) instead of forming a ‘flat epithelium’ as in the Atoh1 CKO mice (Fig. 3h, h').

Co-expression of Atoh1 and Neurog1 in heterozygous KI mice resulted in progressive disorientation of hair cells and supporting cells including deviation of hair cell polarity.

We next examined if the co-expression of Neurog1 and Atoh1 could affect hair cell differentiation. Myo7a and Tubulin immunohistochemistry indicated near normal hair cell differentiation with normal appearing organ of Corti cytoarchitecture at E18.5, except for some occasional misalignment of inner hair cells (data not shown). As these mice are viable, we analyzed later stages of development. At P7, immunohistochemistry of Myo7a revealed loss of some outer hair cells but near normal organization of inner hair
cells (Fig. 4a). Disorganization became more apparent in supporting cells at this stage. Immunochemistry of Prox1 and Tubulin showed pillar and Deiters' cells were displaced in the rows of inner hair cells and/or outer hair cells which were associated with the loss of outer hair cells (Fig. 4a'-a''', b,b'). This disorganization in heterozygous KI mice was more obvious at P9 showing ectopic Myo7a positive outer hair cell-like cells in the rows of inner and outer hair cells including loss of some pillar cells (Fig. 4c–c''). The extent of the organ of Corti disruption appeared more at P26 and involved also in loss of some inner hair cells (Fig. 4d–d''). Closer investigation with SEM revealed further disorganization in hair cell polarity and stereocilia (Fig. 4f–l). These phenotypes in the heterozygous KI mice were compared with the Pax2cre; Atoh1f/+ mice which confirmed the defect was not caused by the haploinsufficiency of Atoh1 but rather the co-expression of Neurog1 (Fig. 4e). Kinocilia have been implicated to act as a receptor for planar cell polarity signals to determine the correct polarity of the cell and the stereocilia [37,38]. During development, kinocilia progressively move from the center of the bundle to the edge of the tall stereocilia providing the symmetry of the bundle [37,38]. In heterozygous KI mice we found that in many hair cells, predominantly in the outer hair cells the stereocilia bundle was not arranged symmetrically in relation to the kinocilia (Fig. 4f, f'). At P1, occasionally inner hair cells were found with formation of additional stereocilia bundle (Fig. 4g) and formation of ectopic hair cells adjacent to the inner hair cells without formation of supporting cells in between (Fig. 4h,h'). At P9, the outer hair cell stereocilia asymmetry became more profound including formation of extra rows of outer hair cells in the cochlear apex (Fig. 4i, j), which was not observed in the base (Fig. 4k). In addition, we found occasional formation of ectopic stereocilia adjacent to inner hair cells at P9 (Fig. 4k,k') resembling the formation of Myo7a positive ectopic hair cells in the gaps of pillar cells (Fig. 4a''',a''''',c–c'') as well as later loss of some inner hair cells at P26 (Fig. 4l, d,d''). The heterozygous KI mice affected the patterning of both hair cells and supporting cells suggesting cellular interaction with adjacent supporting cells.

Expression of Neurog1 could regulate downstream gene expression and influence patterning of the organ of Corti by differential regulation of some molecular markers

We next examined how the altered phenotype in organ of Corti cells was associated with the gene expression patterns in the E18.5 KI mice.
Figure 4. Neurog1 expression causes disorganization of both hair cells and supporting cells in the heterozygous KI mice. Immunochemistry of Myo7a shows normal rows of hair cells with loss of some outer hair cells in P7 heterozygous KI mice (white arrows in a, a', a'''). Co-labeling of Myo7a with the supporting cell-specific markers, Prox1 and Tubulin shows the loss of few outer hair cells in areas of misaligned and/or ectopic pillar cell formation as well as disorganization of Deiters’ cells (white arrows in a’-a''', b,b'). Some ectopic hair cells are found in the position of inner hair cells more apparently at P9 and also in between the rows of outer hair cells which are associated with the disorganization of supporting cells (yellow arrows in a’-a''', c-c'') followed by loss of some inner hair cells and pillar cells by P26 (arrows in d-d''). Anti-Prox1 specifically labels outer
pillar cells and third rows of Deiters’ cells at P7 whereas anti-Tubulin strongly labels outer pillar and all three rows of Deiters’ cells. Anti-Tubulin more weakly labels inner pillar cells in heterozygous (a‘’), c,c,d,d') and wild-type cochlea (b, b'). Prox1 is shown in cyan in a’ and lilac in a'' and b for better contrast. Scanning electron microscopy P1 control (Pax2-cre; Atoh1<sup>+<sup>+<sub>ki</sub></sup></sup>) mice reveal four parallel rows of hair cells in the organ of Corti with a staircase-pattern of stereocilia (e). SEM in E18.5 to P26 heterozygous KI mice reveals defects in hair cell stereocilia development and patterning (f,i). Hair cells show kinocilia that are not centered in relation to the symmetry of the stereocilia bundle (shown with yellow arrows and red asterisks in f,i,g). At P1, some abnormal patches of stereocilia form in the inner hair cells (red arrow in g) as well as some ectopic hair cells adjacent to inner hair cells (red arrows in h and h'). At P9, occasionally extra rows of outer hair cells form with unequal length of the ‘W’ end of the stereocilia (i, red asterisk in j). In addition, the stereocilia of inner hair cells are sporadically disturbed or fused abnormally (k, k') and some inner hair cells at P26 are missing (arrow in l) consistent with gaps seen with the Myo7α immunocytochem. Bar indicates 10 μm except 2 μm in e’, f’, g’, j’.

provided Neurog1 expression but also altered the expression of several inner ear specific genes in organ of Corti cells which differed from Atoh1 CKO mice (Fig. 5; Fgf8, a gene selectively expressed in the inner hair cells, was reduced comparable to Atoh1 expression (Fig. 5a, a’)) or absent in some inner hair cells in the heterozygous KI cochlea (Fig. 5b, b’). Hair cells co-expressing Atoh1 and Neurog1 displayed heterogeneity in the extent of Fgf8 expression. In the homozygous KI mice, Fgf8 was not expressed in the cochlea like Atoh1 (Fig. 5b‘’, b’’, a‘’, a’’). In contrast, a few Fgf8 positive inner hair cell precursors were identified in the Atoh1 CKO cochlea in absence of Atoh1 (Fig. 5a‘’’, b‘’’, a‘’). In the heterozygous and homozygous KI mice (Fig. 5b‘, b’’, b’’’) Neurog1 showed a more profound, albeit patchy expression in the homogenous KI cochlea comparable to Neurog1 expression (Fig. 5e‘’, e’’, d‘’, d’’). In contrast, Neurod1 was undetectable by in situ hybridization in the Atoh1 CKO cochlea (Fig. 5d‘’’), indicating that either Atoh1 or Neurog1 is needed to drive the expression of Neurod1 in hair cells. Another bHLH gene, Nhlh1, which has been claimed to be regulated by both Neurod1 and Atoh1 [35,39], was expressed in patches of organ of Corti cells in the apical half of the cochlea but was absent in the base of homozygous KI mice (Fig. 5e‘’’). Nhlh1 was absent in the Atoh1 CKO mice (Fig. 5ec‘’’). Clearly, the expression of Neurod1 and Nhlh1 in the organ of Corti cells of the homozygous KI cochlea was driven by misexpression of Neurog1 under the endogenous Atoh1 promoter. These data showed that expression of Neurog1 affected normal gene expression in differentiating hair cells or in undifferentiated hair cell precursors and could thus progressively alter the organ of Corti differentiation.

Since Neurog1 misexpression in hair cell precursors lacking Atoh1 can elicit expression of some genes within organ of Corti precursors (Fig. 5d‘’, d’’’, e‘’, e’’), we next wanted to evaluate a more extensive panel of genes that affect the prosensory development of the inner ear. We investigated Jag1, a Notch ligand, which is required for prosensory formation and the function of Notch [40] downstream of Rbpj [41,42]. Jag1 displayed a patchy expression pattern with a gradient from apex to base forming a ‘rossette-like’ structure in the homogenous KI organ of Corti (Fig. 5f‘’, f’’’, insert in f’‘ and f’’’). The expression of Jag1 in a cluster of cells may indicate the potential for formation of mosaic cellular pattern of the organ of Corti. However, unlike the other Notch ligands, early Jag1 expression specifies the prosensory domain with a subsequent expression in the adjacent supporting cells [40,43,44]. In contrast, Jag1 expression in the Atoh1 CKO mice was nearly absent (Fig. 5f’’’), except for a limited expression in the apical tip (data not shown). Hes5, a downstream mediator of Notch [45,46], was also differentially expressed in the heterozygous and homozygous KI mice (Fig. 5g‘’, g’’). Homozygous KI mice showed expression of Hes5 only in the apex of the cochlea (Fig. 5g‘’, g’’). Absence of Jag1 and Hes5 in the Atoh1 CKO (Fig. 5f’’’, g’’’) indicated that, removal of Atoh1 limited prosensory epithelium formation and ultimately resulted in the formation of ‘flat epithelium’. In the homozygous KI mice Neurog1 initiated some degree of prosensory development resulting in survival of some organ of Corti cells, including a limited, patchy expression of Jag1 and Hes5.

We next examined the expression of Gata3, a zinc finger transcription factor expressed in the neurosensory precursors of the otic epithelium [47]. Some disparity in the Gata3 expression pattern was observed between homozygous KI and Atoh1 CKO mice (Fig. 5h–h’’’’) such as patchy expression of Gata3 next to organ of Corti precursors in the homozygous KI cochlea (Fig. 5h’’’’). In addition, Gata3 was moderately increased in the basal half of the cochlea in homozygous KI mice (Fig. 5h’’’’). This patchy Gata3 expression indicated some intercellular signaling between the organ of Corti-like clusters of cells that expressed Neurog1, Neurod1, Nhlh1 and Jag1 to surrounding cells. Therefore, Neurog1 misexpression had a broader impact than just alterations of fate in the hair cell precursor and extended to the adjacent supporting cells. We next wanted to understand if the effects of Neurog1 misexpression impact other genes that affect the development of the organ of Corti. Fgf10 is expressed in the GER, medial to the developing organ of Corti [48]; whereas, Bmp4 defines the lateral boundary of the organ of Corti destined to become the Hensen’s and Claudius cells [49,50]. It was reported previously that the expression of Fgf10 and Bmp4 changed and downregulated in the absence of a differentiated organ of Corti, approximated toward each other as the organ of Corti cells degenerate [22]. We therefore wanted to investigate the expression pattern of these two organ of Corti flanking molecular markers in the presence of Neurog1 positive organ of Corti-like cells in the homozygous KI mice (Fig. 5i–i’’, j–j’’’’). We found that the differentiated organ of Corti in wild-type and in heterozygous KI cochlea were flanked by Fgf10 medially (neural side) and Bmp4 laterally (abnormal side) (Fig. 5i, i’ , j, j’). In the homozygous KI cochlea, in situ data demonstrated lateral expansion of Fgf10 and medial expansion of Bmp4, extending into the areas of the organ of Corti between the patches of organ of Corti cells (Fig. 5i’’’, i’’’’, j’’’’, j’’’’). These data suggested that presence of Neurog1 positive organ of Corti cells in homozygous KI mice affected the expression patterns of these molecular markers in more distant cells belonging to the GER and the Claudius cells, indicating the existence of a thus far uncharacterized feedback loop emanating from the organ of Corti. This pattern of Bmp4 expression correlated with medial expansion of Claudius-like cells between remaining Neurog1 positive organ of Corti cells observed in the SEM (Fig. 5e,d,c,e). As previously reported [22], in Atoh1 CKO mice, Bmp4 and Fgf10 expression was also decreased in a base to apex gradient but no mediolateral expansions into the shrinking organ of Corti were observed (Fig. 5m, m’). We also investigated expression of miR-96 which is essential for inner ear neurosensory development [51]. MiR-96, a miR-183 family member, is expressed in inner ear neurons at an early stage and later in sensory epithelia [34]. Mutation of miR-96 results in
|                  | Atoh1<sup>+/+</sup> | Atoh1<sup>+/+</sup>/Neurog1<sup>−/−</sup> | Atoh1<sup>−/−</sup>/Neurog1<sup>−/−</sup> | Atoh1<sup>−/−</sup> CKO |
|------------------|---------------------|----------------------------------|----------------------------------|-------------------|
| **Atoh1**        | mid                 | mid                              | mid                              | base              |
| **Fgfl**         | b                   | IHC                              | b'                               | b''               |
| **Neurog1**      | c                   | c'                               | c''                              | c'''              |
| **Neurod1**      | d                   | d'                               | d''                              | d'''              |
| **Nhhl1**        | e                   | e'                               | e''                              | e'''              |
| **Jag1**         | f                   | f'                               | f''                              | f'''              |
| **Hes5**         | g                   | g'                               | g'' apex                         | g'''              |
| **Gata3**        | h                   | h'                               | h''                              | h'''              |
| **Fgf10**        | i                   | i'                               | i''                              | i'''              |
| **Bmp4**         | j                   | j'                               | j''                              | j'''              |

Note: Red arrows indicate OC.
nonsyndromic progressive hearing loss in humans and mice [51].

We observed retention of miR-96 expression in the organ of Corti precursor cells in the apex of homozygous KI mice in contrast to diminished expression in Atoh1 CKO mice (Fig. S4c,c',d,d'). We then examined miR-124 expression, which is normally associated with neuronal differentiation [52], to determine whether it is misexpressed in hair cells in KI mice. In situ hybridization of miR-124 showed many positive neurons were present in homozygous KI mice, particularly in the basal half compared to Atoh1 CKO spiral ganglia (Fig. S4g and h). However, miR-124 expression was not found in hair cells of heterozygous or homozygous KI mice.

In conclusion, while Gata3, Jag1, Fgf10 and Bmp4 were expressed prior to and independent of Atoh1 in and around the developing organ of Corti, misexpression of Neurog1 in cochlear hair cells generated a considerable amount of expression changes within and outside the organ of Corti. This is vastly different from changes in the cochlea associated with the simple loss of Atoh1 and indicates the impact of signaling by the Neurog1 positive cells to the surrounding cells in the cochlea.

Replacement of Atoh1 with Neurog1 led to patchy degeneration of the organ of Corti in homozygous KI mice

Since the gene expression and SEM data revealed patches of microvilli bearing organ of Corti cells, we next wanted to investigate the fate of the cells between these patches. Previous work had shown that most organ of Corti cells die via apoptosis in Atoh1 CKO or in Atoh1 null mice [22,53]. Immunohistochemistry revealed the presence of activated-Caspase3 in patches of organ of Corti cells mostly in the basal half of the cochlea around E16.5 (Fig. 6a, a', b). The degeneration in the base of the cochlea correlated with the finding of fewer Neurog1 positive patches of organ of Corti cells in the base of homozygous KI mice (Fig. 6d).

We also found limited activated-Caspase3 positive cells at E18.5 in homozygous KI mice which were localized medial to Sox2 immunopositive presumed supporting cells (Fig. 6c). At E18.5 Sox2 positive cells were present both in the GER and in the supporting cells in control mice (Fig. 7a). These activated-Caspase3 positive cells were also innervated by the radial fibers shown with Tubulin immunohistochemistry (Fig. 6b, c). Neurog1 positive organ of Corti-like cells in the homozygous KI cochlea gradually declined from E16.5 to E18.5 (Fig. 6d,c) as probably the cells negative for Neurog1 died, similar to the fate of most organ of Corti cells in Atoh1 null mice [53,54]. Due to postnatal lethality of the mice we could not determine the fate of the remaining Neurog1 positive cells. We suspect that continued reduction of Neurog1 expressing organ of Corti cells will happen in neonates comparable to Atoh1 null mice, which result in the eventual death of all Atoh1-negative hair cell precursors. Interestingly, this process of organ of Corti cell death was apparently delayed compared to simple Atoh1 null mice where this was nearly completed at birth [53,54], suggesting a possibly transient but limited rescue of organ of Corti cells by Neurog1.

As whole mount data suggested that particularly the more medial (neural side), likely representing inner hair cell progenitors, die first in a given patch (Fig. 6b,c, arrows in b, c, d), we sectioned a Neurog1 in situ reacted cochlea to better understand the distribution of Neurog1 positive cells in the homozygous KI mice. As with Atoh1 null mutants that express lacZ across a 1-2 cell wide region of the organ of Corti, outside the boundaries of the Atoh1 positive areas, Neurog1 behaved differently from those cells of the ‘flat epithelium’. Tubulin immunohistochemistry demonstrated the distribution of radial fibers projecting to the patchy organ of Corti cells with the progressively increasing gradient from base to apex being associated with patches of the homozygous KI mice (Fig. 7c,c',d,d',e,e'). In contrast to the Atoh1 CKO mice, where the majority of the fibers did not reach the presumed organ of Corti (Fig. 7f, f'), homozygous KI mice showed a substantial increase in radial fiber density in similar regions of the cochlea with their terminals reaching deep into the organ of Corti-like cells (Fig. 7c', d', e'). Sox2 is an early marker of the prosensory domain of the developing cochlea and required for organ of Corti development.
Sox2 immunochemistry showed distribution of Sox2 positive cells in the GER and in the supporting cells in E18.5 wild-type mice (Fig. 7a). We found clusters of Sox2 immunopositive cells in the cochlea of E18.5 homozygous KI mice with a gradient from apex to base (Fig. 7c–d) which contrasted to the occasional Sox2 positive cells found in the apex of Atoh1 null mutants [57] or in Atoh1 CKO mice (Fig. 7f; [22]). Radial fiber terminals projected to the Sox2 positive areas in the homozygous KI cochlea; whereas, in Atoh1 CKO mice these fibers formed loops before reaching the proximity to GER with very occasional fibers reaching the few Sox2 positive cells of the organ of Corti (Fig. 7c–d) which contrasted to the occasional Sox2 positive cells found in the apex of Atoh1 null mutants [57] or in Atoh1 CKO mice (Fig. 7f; [22]). The radial width of the Sox2 positive patches in the GER was reduced in the homozygous KI mice compared to wild-type littermate (Fig. 7a, c–e), which corresponded to the loss of other markers of the GER such as Fgf10 (Fig. 5h'', h''', S4). The possible interactions between Sox2, Fgf10 and Bmp4 remain to be explored.

Interestingly, Sox2 immunofluorescence in the heterozygous KI mice revealed disorganization in the supporting cell layer morphology along with reduced intensity of Sox2 in the GER (Fig. 7b). Tubulin immunostaining also showed the disorganization of the type II fibers with aberrant projections of radial fibers both toward the apex and base of the cochlea (Fig. 7b'). This aberrant fiber projection was related to the abnormalities found in the outer hair cells and supporting cells in the heterozygous KI mice (Fig. 4). This phenotype resembled somewhat the defects reported in Prox1 null mice [58] and could indicate interaction of ingrowing fibers with supporting cells. However, Prox1 expression in the E18.5 heterozygous KI mice showed a near normal pattern by in situ hybridization (Fig. S3b') but displayed aberrant expression in a later stage (Fig. 4a', a''). In contrast, the homozygous KI mice showed diminished levels of both Prox1 mRNA and protein where the signal was limited to only a few patches in the cochlea (Fig. S3c, c', d). Furthermore, Prox1 was clearly expressed in the basal turn of homozygous KI cochlea compared to complete absence in the Atoh1 null cochlea [57].

Previous work has established that efferent fiber growth is more truncated than afferent fiber growth in Atoh1 null mice [22] suggesting that neurons with limited support from a non-existing organ of Corti cannot sustain proper fiber extension [59]. Efferent innervation was examined using Neurovue dye injections into the brainstem in the homozygous KI mice. We found profound outgrowth of efferent fibers, including well-formed intra-gangli-
Figure 7. Neurog1 expression in organ of Corti cells affects afferent and efferent innervation. In E18.5 wild-type mice, Sox2 is present in the GER and in all supporting cells (a) and many radial fibers project to inner hair cells. Anti-Tubulin antibody labeling shows Type II fibers projecting in a regular pattern to outer hair cells (a’) between the Sox2 immunopositive supporting cells (a”). Heterozygous KI mice reveal some disorientation in Sox2 positive supporting cells with disorganized projection of type II fibers between the disorganized supporting cells (arrows in b, b’, b”). In homozygous KI littermates Sox2 positive cells are organized as multiple patches along the organ of Corti with a gradient of decreased density from apex to base (c-e’). Tubulin immunolabeling reveals fibers reaching to these patches and extending between Sox2 positive cells (arrows in e’ and e”). Atoh1 CKO mice have few occasional patches of Sox2 positive cells (f) and tubulin positive fibers mostly form loops with some reaching with rare projections to Sox2 positive cells of the organ of Corti (compare e’ and f’). Neurovue dye tracing of efferent innervation from the brainstem shows formation of intraganglionic spiral bundles (IGSB) in homozygous KI mice comparable to wild-type littermate (compare g and h). Multiple efferents in the homozygous KI mice enter to the undifferentiated organ of Corti (h-h”) where fibers ramify apparently between the remaining patches of organ of Corti cells (arrows in h’, h”). GER, greater epithelial ridge; OC, organ of Corti; ‘OC’, presumed organ of Corti in Atoh1 CKO; RF, radial fibers; SC, supporting cells; ‘SC’, putative supporting cells expressing Sox2; ‘[’ indicates position of the putative organ of Corti. Bar indicates 50 μm in all except 100 μm in d-d”, g, h and 10 μm in h’ and h”.

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Neurons. We therefore investigated the expression of Ntf3 whereas responsible for the innervation of embryonic vestibular epithelia Bdnf support to developing inner ear sensory neurons [60].

Two types of neurotrophins, Ngfβ and Bdnf, provide trophic support to developing inner ear sensory neurons [60]. Bdnf is responsible for the innervation of embryonic vestibular epithelia whereas Ngfβ predominately supports the basal turn spiral neurons. We therefore investigated the expression of Ngfβ and Bdnf in both heterozygous and homozygous KI mice by in situ hybridization (Fig. 8, S6). A profound expression of Ngfβ was found in heterozygous KI cochlea at E18.5 compared to wild-type littermates, as well as Ngfβ being localized to the clusters of organ of Corti precursors in homozygous KI cochlea (Fig. 8a–c'). These results contrasted with the previous report of lack of Ngfβ expression in the absence of hair cell differentiation in Atoh1 CKO [22]. Misexpression of Neurog1 enhanced the Ngfβ expression.

Figure 8. Residual Ntf3 expression in the patchy organ of Corti cells may support restricted innervation. Ntf3 expression is uniform throughout the organ of Corti but appears more prominent in heterozygous KI mice compared to the wild-type littermate (a, a', b, b'). In contrast, homozygous KI mice exhibit patchy Ntf3 expression in the middle and base, but continuous in the apex and in basal tip of the cochlea (c,c'), consistent with Sox2 distribution and the pattern of residual innervation. In contrast, Bdnf which is predominantly expressed in the hair cells is somewhat elevated in heterozygotic KI mice compared to wild-type littermates (d,e) but shows expression only in the apical turn in homozygotic KI mice (f; insert in f). 'p' indicates the area of differentiated organ of Corti 'p' indicates the putative organ of Corti in the homozygous KI mice. Bar indicates 100 μm.

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in the organ of Corti precursors. The presence of Ngfβ apparently provided support to radial fiber growth in those patches of cells in the homozygous KI mice (Fig. 7c,d,e', 8c,c') consistent with the functional role of Ngfβ in spiral ganglion cell development and maintenance [60,61,62]. In contrast, the innervation was greatly reduced in the Atoh1 CKO, a fact that corresponded well to reduced expression of Ngfβ (Fig. 7f, [22]).

Unlike Ngfβ, Bdnf expression remained unchanged in E18.5 homozygous KI mice (Fig. 8f) as Bdnf expression is possible even in the absence of Atoh1 mediated differentiation of hair cells [36] and is likely due to its complex promoter system that does not appear to be exclusively dependent on Atoh1 expression [63,64]. Bdnf expression persisted only in the apex of the homozygous KI cochlea which was consistent with the dense apical innervation reported in in the Atoh1 null [36] and in Atoh1 CKO mutants [22]. Analysis of earlier stages (E15.5) revealed Bdnf expression in the delaminating neurons as well as in the hair cells of the vestibular epithelia, being most profound in the canal crista and in the apex of the cochlea in wild-type mice (Fig. S6d, d'). Likewise, both the homozygous KI and Atoh1 CKO mice showed expression of Bdnf in the canal cristae and in the apex of the cochlea (Fig. S6e–f'). Bdnf was absent in the utricular and saccular hair cells, whereas it was profoundly expressed in the delaminating neurons near the utricle and saccule in both homozygous KI and Atoh1 CKO mice (Fig. S6e–f'). This is consistent with previous work which showed that in Neurog1 null mice no neurons delaminated and Bdnf-lacZ positive cells were restricted to the sensory epithilia of utricle and saccule [25]. Homozygous KI mice showed no apparent alteration in expression of Bdnf relative to Atoh1 CKO (Fig. S6e–f', 7f, [22]) or Atoh1 null mice [36]. Therefore, we demonstrated that expression of Ngfβ particularly at E18.5 in the clusters of organ of Corti-like cells in homozygous KI mice (Fig. 8a–c', S6h,h'), provided the molecular basis for the enhanced afferent and efferent innervation of these patches. Future work in viable adult mice with at least one allele carrying the Neurog1 KI combined with a floxed Atoh1 allele is needed to investigate the long term fate of cochlear hair cell innervation.

In summary (Fig. 9), our data suggested that we obtained expression of Neurog1 under the control of the Atoh1 promoter as predicted. Our expression, morphological and histological data suggested that co-expression of Neurog1 with Atoh1 in the heterozygous KI mice resulted in mild aberrations of hair cell and supporting cell development, indicating that Neurog1 altered the hair cell phenotype beyond a haploinsufficiency of Atoh1. Homozygous KI mice showed rescue of some undifferentiated organ of Corti cells at least until P0. These cells had a distinct gene expression profile, showed an unusual morphology, attracted more afferent and efferent neurites than simple Atoh1 null mice, but did not differentiate either as neurons or as hair cells in the organ of Corti.

Discussion

Past work has shown that replacement of certain bHLH genes with other paralogs can reveal functional equivalence [1,63,66]. This was largely attributed to redundancy of signaling possibly due to the similarity of E-box sequences recognized by the different bHLH proteins [14,67]. In essence, it was proposed that limited redundancy of expression in the PNS requires a key-lock action of a specific bHLH gene whereas in the CNS the general bHLH gene action combined with channeling effects of the cellular context suffice. Among bHLH genes, Atoh1 is unique in that it is expressed in both proliferating precursors and/or postmitotic differentiating cells [16,25,65,68]. In addition, Atoh1/tonal1 is the only bHLH
gene known to be functionally conserved across phyla: the fly atonal gene functions in mouse and the mouse Atoh1 gene functions in flies [69]. Hair cells belong to an ancient group of neurosensory cells that have split in vertebrate ancestors into two cells, a neuron and an axonless hair cell [70]. There is a functional bifurcation with the derived bHLH gene, Neurog1, driving neuronal development [18,23] whereas the conserved bHLH gene, Atoh1, driving hair cell development [14]. Generating a mouse in which Neurog1 and Atoh1 can be co-expressed in the same cell through their regulation by the endogenous Atoh1 promoter (Atoh1+/KI) could result in three possible phenotypes:

1) Hair cells could be primed to respond exclusively to Atoh1 and will develop normally, like in a simple Atoh1+/− mouse [17].
2) All or a subset of hair cells (in particular those who have a lineage relationship with neurons [24,25]) could respond to Neurog1 to differentiate as neurons.
3) All or a subset of hair cells (in particular those who have a lineage relationship with neurons [24,25]) could differentiate as a hybrid between neurons and hair cells, forming essentially primary sensory cells with their own axon, much like sensory cells in many invertebrates, including the atonal dependent fly mechanosensory cells [71].

Figure 9. Atoh1 promoter driven Neurog1 differentially regulates downstream genes and affects organ of Corti cell differentiation. This diagram shows the interactions of Atoh1 protein with its enhancer to increase levels of Atoh1 mRNA expression (a). Atoh1 in part regulates Neurod1 and presumably also Fgf8 (a’, [74]) whereas Neurod1 in turn suppresses Atoh1 and Fgf8 as reported previously in Neurod1 null mice [24] and shown here. In wild-type mice inner hair cells (IHC) express all three genes (color coded as, red, Atoh1; blue, Neurod1 and lilac, Fgf8; a) and outer hair cells (OHC) express Atoh1 and Neurod1 but not Fgf8 (red and blue; a’, a’). In heterozygous KI mice (Atoh1+/KI) (b-b’) enhancer activation of Atoh1 as well as activation of the Neurod1 and Fgf8 will be retained with expression of Atoh1. However, Neurog1 protein mediated enhanced expression of Neurod1 suppresses Atoh1 and Fgf8 more strongly. Consistent with the proposed regulation changes are our observed changes in mRNA expression pattern and the effects on hair cell development in heterozygous KI mice such as extra rows of outer hair cells, misalignment and progressive loss of inner hair cells (b”,b’’). In heterozygous KI mice, all four genes including Neurog1 are expressed in IHC (green) but have reduced expression of Atoh1 and Fgf8 (b”, b’’) and the three genes are expressed in OHC (b”, b’’). In homozygous KI mice (Atoh1+/KI) there is no Atoh1 expression and thus no activation of the Atoh1 enhancer (c). Therefore, enhanced expression of Atoh1 driven by the Atoh1 protein binding to the enhancer will be disrupted, leaving only non-Atoh1 protein mediated activation to drive Neurog1 expression. Neurog1 protein mediated Neurod1 expression apparently suppresses all Fgf8 expression (c’). Homozygous KI mice show undifferentiated organ of Corti cells (which express only Neurog1 and Neurod1) that may survive but form only micravilli (c”, c’’) and are surrounded by a reduced expression of Bmp4 (c’). In the Atoh1 CKO mice (d) the recombined Atoh1 locus (At) does not produce a protein able to activate the enhancer or expression of Neurod1. However, a limited expression of Fgf8 is possible indicating that other factors are involved in regulating that gene. The reduced expression domains of Fgf10 and Bmp4 approximate each other (d” and d’”) and no organ of Corti cell remains in the ‘flat epithelium’ (d”, d’’).
Beyond these possible outcomes, we wanted to understand further the necessity of availability of Atoh1 protein to drive hair cell differentiation without activation by a positive feed-forward mechanism that utilizes a cis-enhancer element. We choose to eliminate Atoh1 protein (Fig. 9) through its substitution by another bHLH gene presumably unable to bind to the Atoh1-specific enhancer E-box [3]. In our KI mouse the Atoh1 coding sequence is replaced by Neurog1, a closely related bHLH gene [14]. We analyzed the effects of Neurog1 misexpression in the homozygous and heterozygous KI mice on the development of the ear. Our data show that not only hair cell development is impaired by substitution of Atoh1 with its Neurog1 paralogue but Neurog1 does modify the hair cell phenotype even when Atoh1 is present. The impact of Neurog1 misexpression is not just localized to the hair cells, but there is also a wider disturbance of both the morphology and expression pattern in the adjacent supporting cells as well as the more distal cells in the organ of Corti, GER and spiral ganglion.

**Neurog1 protein can transiently support the viability of some organ of Corti and surrounding cells, but cannot initiate their full differentiation**

In homozygous KI mice, patchy Neurog1 positive cells remain in the base and middle turn of the organ of Corti, distinct from the Atoh1-lacZ distribution in Atoh1-null cochlea where all organ of Corti cells seem to die over time [22,23]. Previous work had identified cross-regulation between Atoh1 and Neurog1 in the spinal cord [2] and the ear [22,24]. Specifically, in Atoh1 null mice there is an expansion of Neurog1 [24] and Neurod1 [22] in the utricule and saccule. We also found a more profound expression of Neurog1 in the delaminating neuroblasts in the utricule and saccule in the E14.5 homozygous KI mice as well as in the vestibular sensory epithelia and in the mid-base region of the cochlea. As expected, absence of a putative negative feedback loop in neurons, due to the absence of Atoh1 protein, resulted in enhanced expression of Neurog1 in the delaminating neuroblasts or common neurosensory precursors. However, the reduced differentiation of the organ of Corti appears to be unable to sustain neuron retention.

SEM investigations reveal the development of tall microvilli among some of these clusters of organ of Corti cells in homozygous KI mice, a novel feature in the absence of hair cell differentiating factor Atoh1. The appearance of these cells in more lateral areas of the organ of Corti is suggestive of outer hair cells precursors. This assumption is consistent with the more medial loss of cells in the organ of Corti observed with activated caspase3. Neurog1 misexpression supports these patches of cells to survive as a modified precursor cell with some rudimentary stereocilia. Whether these surviving cells relate to a specific hair cell precursor type has not yet been established.

The Neurog1 positive patches of organ of Corti precursor cells can drive expression of downstream genes like Neurod1 and Nhlh1 [18]. Comparable to other mutants like Pou4f3, where some pillar cells form due to interaction with short-lived hair cells [72], replacement of Atoh1 with Neurog1 in homozygous KI mice can apparently modulate interactions with surrounding cells, possibly through expression of delta/notch ligands and effector genes such as Jag1 and Hes5, resulting in enhanced viability of the additional non-Neurog1 positive cells in these organ of Corti patches. This extent of differentiation has not been reported in Atoh1 null [17] or Atoh1 CKO [22] mice where Jag1 shows faint expression only in the apex of the cochlea. In addition to Jag1, the expression of Sox2 and Prox1, presumably in supporting cell precursors in the center of a rosette-like cluster of organ of Corti cells, indicates intercellular interactions to specify to some extent organ of Corti cell differentiation. In contrast to the basal expression in the homozygous KI mice, Sox2 and Prox1 are only found in the apex of Atoh1 null mice [22,57] with very few occasional patches of Sox2 in the middle turn.

**Neurog1 misexpression also alters expression of Fgf10 and Bmp4 which are defining the medial and lateral boundaries of the cochlea, respectively [49,49]. BMP signaling is necessary for progeny specification as shown in double-mutant mice of BMP receptors, Alk3/Alk6 [50]. A previous report in Atoh1 CKO mice showed that subsequent to organ of Corti degeneration, Fgf10 and Bmp4 expression diminished and more closely spaced to each other [22] by reducing the area of the putative organ of Corti as revealed by Gata3 expression. In homozygous KI mice survival of organ of Corti-like patches of cells modify the expression of Bmp4, Fgf10 and Gata3. In situ hybridization of these markers revealed undulation of the boundaries adjacent to the remaining organ of Corti patches. Combined with the SEM data, this suggests that Bmp4 positive domains of non-sensory cells (mostly Claudius cells) expand medially between the remaining sensory patches, transforming the cells between patches into a ‘flat epithelium’ [73]. These data suggest a short range interaction of the organ of Corti cells with surrounding cells expressing Bmp4 and Fgf10 that has not been recognized thus far.

**Neurog1 protein interferes with Atoh1 signaling in developing hair cells of heterozygous KI mice**

In heterozygous KI mice, Neurog1 recapitulates the spatiotemporal expression of Atoh1 resulting in co-expression of Atoh1 and Neurog1 in hair cells. We took advantage of the viability of these mice to analyze the extent and diversity of hair cell differentiation at post-natal stages. Immunocytochemistry of Myo7a, Prox1 and Tubulin revealed the progressive disorganization of both hair cells and supporting cells in heterozygous KI mice. SEM investigations correlated with the finding of immunocytochemistry. We found disoriented organ of Corti cell formation. Occasionally some inner hair cells were replaced by pillar cells with a gradual loss of some inner hair cells and pillar cells in later stage (P26). The phenotype in the heterozygous KI mice differs from haploinsufficient effects of Atoh1<sup>+/−</sup> [17] or Pax2-cre; Atoh1<sup>+/−</sup> mice [22]. This suggests that Neurog1 protein alters Atoh1 signaling. This might occur prior to E-box binding, through reduced availability of E-proteins, at E-box binding by sterically hindering Atoh1-E-protein dimer binding, or by regulating different downstream genes that interfere with Atoh1 downstream signals. Our data provides evidence that co-expression of Atoh1 and Neurog1 prematurely upregulates expression of Neurod1 and Nhlh1 in hair cells but delays Atoh1 and Fgβ expression in hair cells and alters Neurog1 expression in delaminating sensory neurons. Clearly, co-expression of Neurog1 with Atoh1 can modulate Neurog1 downstream gene expression, indicating that perhaps the latter aspect is the most prominent. Our data suggest a negative feedback loop of Neurog1 on hair cells, somewhat comparable to that observed in other systems [19] and possibly in part mediated by Neurod1 (Fig. 9). Consistent with this is the effect of KI insertion on Fgβ expression in inner hair cells. Fgβ becomes prematurely upregulated in the absence of Neurod1 [19] but is downregulated in the heterozygous KI mice and eliminated in KI homozygous mice (Fig. 9). These data are in agreement with the presence of Neurod1 specific E-box sequence in an Fgβ intronic enhancer region that requires further functional verification [74]. Our data suggest that Neurog1 contributes to and alters the expression profile of inner hair cells resulting in a modified hair cell phenotype. How these expression changes relate causally to the later defects in hair cell ultrastructure and viability remains to be established.
Patches of organ of Corti cells in homozygous KI mice maintain some innervation through enhanced neurotrophin expression

It has been long hypothesized that inner ear hair cells are the attractor for the growth of afferent innervation by secreting neurotrophins [62,75]. However, other reports suggest that initial afferents can grow in the absence of differentiated hair cells and without the presence of hair cell- and supporting cell-derived neurotrophins [22]. Nevertheless most of the afferents and efferents are lost before birth in the absence of hair cells [22]. We show that if Neurog1 is substituted into the Atoh1 locus, organ of Corti-like patches of cells remain that do not fully differentiate as hair cells but are densely innervated by both afferents and efferents. In situ hybridization shows the presence of Neurog1 expressing clusters of organ of Corti cells which may be the factor responsible for the remaining innervation, consistent with the more prominent role of Neurog1 in early cochlear afferent support [60,61]. However, Bdbf expression persists in the apex as reported previously in Atoh1 null mice, and may provide the additional support of dense innervation that remains in the apex of Atoh1 null mice [22] and in homozygous KI mice. Analysis of neuronal markers such as Fig10, Proxl, mir124 in the KI mice demonstrates survival of the spiral ganglia in middle turn of the cochlea which are almost completely lost in Atoh1 null [36] and in Atoh1 CKO mice [22].

Expression of Neurog1 under Atoh1 promoter control cannot change hair cell precursors to neurons

The limited ability of the Neurog1 expression to transiently rescue patches of organ of Corti-like cells that show altered gene expression patterns in these and surrounding cells is a clear indication that the KI allele results in a protein that has some transcription factor signaling ability. However, this knockin of Neurog1 into the Atoh1 locus cannot initiate hair cell differentiation, but can modulate the organ of Corti precursors to survive as clusters of undifferentiated cells instead of leading to rapid and complete apoptosis as in Atoh1 null [36] and in Atoh1 CKO mice [22]. Despite this retention of immature patches of the organ of Corti, it is obvious that Neurog1 cannot change the fate of organ of Corti precursors to differentiate as neurons. It remains to be seen if the Neurog1 effects are due to limited signaling ability of Neurog1, limited accessibility of Neurog1 specific E-boxes, the channeling of development through factors that control the expression of Neurog1 (and thus Neurog1 in the KI allele) or partial overlapping in Atoll-associated downstream gene regulation.

In summary, our data show that Neurog1 expression under endogenous Atoh1 promoter control interferes with normal Atoh1 signaling in the hair cells of heterozygous KI mice (Fig. 9). This is likely due to the negative feedback provided by the early and profound regulation of Neurog1 in the heterozygous KI mice and a concomitant suppression of Fig10 and Atoh1 expression, leading to disorganization and loss of hair cells. In homozygous KI mice the absence of Atoh1 protein likely limits the level of expression of Neurog1 which nevertheless can drive the expression of several downstream genes that result in partially viable organ of Corti cells with high density of innervation. These cells signal on their immediate and distant neighbors, changing expression profiles of several genes in patches of organ of Corti cells. What initiates cell death in between the patches of surviving organ of Corti cells requires further molecular characterization. Understanding why some organ of Corti precursors remain at least until birth while others die rapidly suggests a surprising level of genetic heterogeneity inherent within the hair cell population that could, if understood, help to minimize hair cell damage and thus delay hearing loss. Mice that combine a conditional knockout of the Atoh1 allele with the Neurog1 KI allele are currently being bred to verify the long term effects of the Neurog1 expression on hair cells of adult mice.

Supporting Information

Figure S1 Atoh1KI/Neurog1 construct. This illustration shows the knockin construct where mmy epitope-tagged Neurog1 coding sequence was introduced in place of Atoh1 coding region. An IRES-DsRed2 fragment was inserted 3' to the coding region of Neurog1 to show its expression with this reporter. The polyadenylation sequence (PAS) was preserved after the DsRed2. A second fragment that includes the pGK-neo selection cassette flanked by two loxp sites was inserted downstream to the Neurog1 coding sequence (a, b). The restriction enzyme sites are shown by vertical lines. (E: EcoRI, H: HindIII) and the two probes used in Southern blotting are also indicated in b.

(TIF)

Figure S2 Neurog1 and Myc-tag Protein expression.

Western blot analysis of Neurog1 and Myc-tag protein in the postnatal (P5 and P9) cerebella of heterozygous KI mice shows approximately correct molecular weight ~25 and ~36 kDa, respectively (a). However, wild-type littermates show similar sized bands, indicating some degree of non-specificity (b). β-Catenin antibody was used as the loading control in the western blot analysis (a,b).

(TIF)

Figure S3 Atoh1 expression is replaced by Neurog1 in the cochlear nucleus and in cerebellum in Neurog1 KI mice. In situ hybridization shows downregulation of Atoh1 expression in the cochlear nucleus and in cerebellum of the heterozygous KI mice (b, b') and complete absence in the homozygous KI mice (c, c') compared to wild-type littermate (a, a'). Atoh1 is expressed in the proliferating precursors of the outer part of the external granule cell layer (insert in a') which is maintained in some lobules in the heterozygous KI mice (b'). Neurog1 is completely absent in the cochlear nucleus and very faintly expressed in the deep nuclei of the cerebellum of wild-type mice without any expression in the cerebellar cortex (d-d'). In contrast, both heterozygous and homozygous KI mice show Neurog1 expression in the cochlear nucleus and in the proliferating external granule cell layer in cerebellum imitating the Atoh1 expression (e-e", f-f""). Replacement of Atoh1 with Neurog1 successfully recapitulates Atoh1- pattern both peripherally (e' and e'') and centrally (cochlear nucleus and cerebellum). The smaller sized cerebellum in homozygous KI mice is demarked with black dotted line in c' and d'. Red dotted lines in d", e", f" demonstrate the area of external granule cell layer (EGL), CB, cerebellum; CN, cochlear nucleus; CP, choroid plexus; IC, inferior colliculus. Bar indicates 500 μm.

(TIF)

Figure S4 Neurog1 KI mice show basal turn spiral ganglia and enhanced organ of Corti gene expression compared to Atoh1 CKO mice. miR-96 in situ hybridization shows expression in the hair cells in both wild-type and heterozygous KI mice at E18.5 (a,a',b, b'), miR-96 is expressed only in the apex of the homozygous KI cochlea whereas severely diminished in Atoh1 CKO mice (c,c',d'). miR-124 is a neuronal marker expressed in all spiral ganglion cells (e-h). Homozygous KI mice retain neurons in the base that are almost absent in Atoh1 CKO mice (g,h). Neurog1 does not drive expression of miR-124 in

(TIF)
hair cells. Another marker, Fgf10, is uniform in wild-type heterozygous knockin mice (i,j) and shows presence of neurons in the base of homozygous knockin cochlea (k) compared to Atoh1 CKO cochlea (l). Fgf10 is also expressed in the GER in wild-type and heterozygous KI mice (i,j). Homozygous KI mice show reduction of Fgf10 expression in the base, which is more profound in Atoh1 CKO (k,l). Another marker, Bmp4 is expressed in the Claudius cells defining the lateral (abneural) side of the developing organ of Corti. Simultaneous in situ hybridization of both Fgf10 and Bmp4 flank medial and lateral to the organ of Corti in wild-type and heterozygous KI cochleae (m,n). In homozygous KI mice, the patchy distribution of organ of Corti cells correlate with medial un hålultations of the Bmp4 expression in the base of the cochlea. Both Bmp4 and Fgf10 in situ signal are nearly absent in the Atoh1 CKO base (o,p). Note that the spiral ganglia in the homozygous KI mice are removed to allow complete non-overlapping mounting of the cochlea (q). Spg, spiral ganglia. ‘OC’, putative organ of Corti in Atoh1 CKO mice. ‘‘OC’’ marks the differentiated organ of Corti and ‘‘C’’ marks the presumptive organ of corti in the homozygous KI mice. Green and yellow bar indicates Fgf10 and Bmp4 positive area, respectively. Bar indicates 100 μm. (TIF)

Figure S5 Prox1 expression exists in patches of supporting cell progenitors in homozygous KI mice. In situ hybridization of Prox1 in E18.5 mice demonstrates the expression of Prox1 in the spiral ganglia as well as in the supporting cells in wild-type (a,a’), and heterozygous KI mice (b,b’). In homozygous KI mice (c,c’), Prox1 is expressed in patches of organ of Corti cells, except some continuity in the apex. Prox1 in situ signal also confirmed presence of spiral ganglia in the base of homozygous KI mice (a,a,b,b,c,c’). Immunohistochemistry of Prox1 and tubulin shows patches of Prox1 positive organ of Corti cells in the base of cochlea which receive projection of the radial fibers to those patches in the homozygous KI mice (d,d’). This supports formation of some supporting cells in clusters of organ of Corti cells. SC, supporting cells; SC, probable supporting cells; Spg, spiral ganglion cells. Bar indicates 100 μm. (TIF)

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Author Contributions

Conceived and designed the experiments: IJ KWB BF. Performed the experiments: IJ NK JK LEG KAM BK JSD BF. Analyzed the data: IJ BF. Contributed reagents/materials/analysis tools: IJ NK JK. Wrote the paper: IJ KWB BF.

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