



# A spontaneous mouse deletion in *Mctp1* uncovers a long-range cis-regulatory region crucial for NR2F1 function during inner ear development

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## ABSTRACT

Hundreds of thousands of cis-regulatory DNA sequences are predicted in vertebrate genomes, but unlike genes themselves, few have been characterized at the functional level or even unambiguously paired with a target gene. Here we serendipitously identified and started investigating the first reported long-range regulatory region for the *Nr2f1* (*Coup-TF1*) transcription factor gene. NR2F1 is temporally and spatially regulated during development and required for patterning and regionalization in the nervous system, including sensory hair cell organization in the auditory epithelium of the cochlea. Analyzing the deaf wanderer (*dwnd*) spontaneous mouse mutation, we traced back the cause of its associated circling behavior to a 53 kb deletion removing five exons and adjacent intronic regions of the poorly characterized *Mctp1* gene. Interestingly, loss of *Mctp1* function cannot account for the hearing loss, inner ear dysmorphology and sensory hair cell disorganization observed in *dwnd* mutants. Instead, we found that the *Mctp1*<sup>*dwnd*</sup> deletion affects the *Nr2f1* gene located 1.4 Mb away, downregulating transcription and protein expression in the embryonic cochlea. Remarkably, the *Mctp1*<sup>*dwnd*</sup> allele failed to complement a targeted inactivation allele of *Nr2f1*, and transheterozygotes or *Mctp1*<sup>*dwnd*</sup> homozygotes exhibit the same morphological defects observed in inner ears of *Nr2f1* mutants without sharing their early life lethality. Defects include improper separation of the utricle and saccule in the vestibule not described previously, which can explain the circling behavior that first brought the spontaneous mutation to attention. By contrast, mice homozygous for a targeted inactivation of *Mctp1* have normal hearing and inner ear structures. We conclude that the 53 kb *Mctp1*<sup>*dwnd*</sup> deletion encompasses a long-range cis-regulatory region essential for proper *Nr2f1* expression in the embryonic inner ear, providing a first opportunity to investigate *Nr2f1* function in postnatal inner ears. This work adds to the short list of long-range regulatory regions characterized as essential to drive expression of key developmental control genes.

## 1. Introduction

Nuclear receptor subfamily 2, group F, members 1 and 2 (NR2F1 and NR2F2, also known as COUP-TFI and COUP-TFII) are well-characterized orphan nuclear receptors belonging to the superfamily of steroid/thyroid hormone receptors. They function as ligand-related transcription factors that can both activate and repress target gene expression. Their evolutionarily conserved DNA sequences and the early lethality associated with their loss-of-function mutations suggest that they play vital roles in many developmental processes (Pereira et al., 2000). Most mice that are homozygous for a null mutation of *Nr2f1* die perinatally, with only a few surviving until 3–4 weeks of age. The mutant mice exhibit defects of neuronal differentiation and axon

guidance (Armentano et al., 2006; Qiu et al., 1997; Yamaguchi et al., 2004; Zhou et al., 1999), irregularities in the patterning and regionalization of the neocortex (Armentano et al., 2007; Bertacchi et al., 2018; Zhou et al., 2001), and inner ear abnormalities (Tang et al., 2006).

*Nr2f1* expression is spatially and temporally regulated during mouse development, suggesting that it may be involved in tissue-specific organogenesis. *Nr2f1* is highly expressed in the nervous system, especially in organs that develop by epithelial proliferation and differentiation like the ectoderm of the inner ear (Pereira et al., 2000). *Nr2f1* is expressed early in the development of the otic vesicle and later in the developing cochlear duct and maturation of the organ of Corti (Tang et al., 2005). Inner ears of developing *Nr2f1*-null mice exhibit a short cochlear duct with supernumerary hair cells in the

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apical turn, which led the authors to suggest that *Nr2f1* may control Notch regulation of cochlear hair cell differentiation (Tang et al., 2006). Human clinical studies also support a role for NR2F1 in cochlear development and function. A patient with a paracentric chromosomal inversion accompanied by a deletion of *NR2F1* exhibited syndromic deafness (Brown et al., 2009), and 20% of individuals with pathogenic *NR2F1* variants were shown to have associated hearing defects (Chen et al., 2016). *NR2F1* (COUP-TFI) mutations underlie autosomal-dominant Bosch-Boontra-Schaaf optic atrophy syndrome (BBSOAS), which is characterized by delayed development, intellectual disability, and optic atrophy (OMIM #615722). Clinical features may also include hypotonia, seizures, autism spectrum disorder, oromotor dysfunction, thinning of the corpus callosum, and hearing defects (Bosch et al., 2014; Chen et al., 2016).

Although NR2F1 has been extensively studied and many of its transcriptional targets have been identified (Montemayor et al., 2010; Pereira et al., 2000), the mechanisms that underlie its own spatial and temporal expression patterns are largely unknown. Distally located cis-regulatory elements (enhancers) can function at large distances from their target genes and are key contributors to the spatiotemporal expression patterns of transcription factors that guide morphogenesis during development (Bulger and Groudine, 2011; Long et al., 2016; Ong and Corces, 2011; Shlyueva et al., 2014). Advances in DNA sequencing have led to estimates of hundreds of thousands of enhancers in the vertebrate genome, but few have been identified with known target genes or phenotypic consequences, and these usually involve the dysregulation of key developmental control genes (Kleinjan and van Heyningen, 2005; Spitz, 2016). Although *NR2F1* is an important developmental control gene, no long-range regulators have yet been identified that modulate its expression or that are associated with any of its disease phenotypes (Zhang et al., 2018).

Here, we report on the discovery of a long-range regulator of *Nr2f1* expression with specific effects on inner ear development in the mouse. We identified a new spontaneous mouse mutation, named “deaf wanderer” (symbol *dwnd*), by its associated hearing and balance deficits and found that it is a 53 kb deletion within the *Mctp1* gene, but that *Mctp1* dysfunction is not responsible for its mutant phenotype. Because the inner ear abnormalities of *Mctp1<sup>dwnd/dwnd</sup>* mutant mice are very similar to those reported in mice homozygous for a null mutation of the nearby *Nr2f1* gene (Tang et al., 2006), we hypothesized and later confirmed that the loss of cis-regulatory sequences located in the deleted region of the *Mctp1<sup>dwnd</sup>* allele severely reduces *Nr2f1* expression in the inner ear and disrupts its development. The mammalian inner ear is a very complex and intricate structure that requires a highly coordinated pattern of cell-type and stage-specific transcriptional regulation for its development (Doetzlhofer and Avraham, 2017; Fritsch et al., 2015; Groves and Fekete, 2012; Wu and Kelley, 2012). Our discovery of a critical regulatory region for *Nr2f1* within the *Mctp1* gene adds to our understanding of how such a precise patterning of gene expression is achieved.

## 2. Materials and methods

### 2.1. Mice

All mice in this study were obtained from research or production colonies and housed in the Research Animal Facility at The Jackson Laboratory (JAX) in Bar Harbor, ME. JAX is

accredited by the American Association for the Accreditation of Laboratory Animal Care. All procedures involving the use of mice were approved by the JAX Institutional Animal Care and

Use Committee and were performed in accordance with the guidelines and regulations of the US National Institutes of Health Office of Laboratory Animal Welfare and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

The recessive *dwnd* mutation occurred spontaneously in the

C57BL/10SnJ colony of mice at The Jackson Laboratory (JAX) and was shown to be an intragenic deletion of the *Mctp1* gene. To alleviate breeding problems, a congenic inbred strain was developed by repeated backcrossing of mutant mice to the C57BL/6J inbred strain until achieving the equivalent of 6 backcross generations, followed by inbreeding to generate the B6.B10Sn-*Mctp1<sup>dwnd</sup>*/Kjn congenic strain, which is homozygous for the *Mctp1<sup>dwnd</sup>* allele and available from JAX as Stock # 9690. All studies of the *Mctp1<sup>dwnd</sup>* mutation described here were done with mice of this congenic strain. Homozygous mutant mice (*Mctp1<sup>dwnd/dwnd</sup>*) can sometimes be identified by their moderate circling behavior, but penetrance of this phenotype is only about 35% compared with full penetrance of elevated ABR thresholds.

Three additional mouse strains were used in this study. The B6N(Cg)-*Nr2f1<sup>tm1.1(KOMP)Mbp</sup>*/J strain with a knockout mutation of the *Nr2f1* gene was generated by the Knockout Mouse Phenotyping Program (KOMP<sup>2</sup>) at The Jackson Laboratory. Cryopreserved sperm from the C57BL/6NCrl *Mctp1<sup>em1(IMPC)Mbp</sup>*/Mmucd strain was imported from the Mutant Mouse Resource & Research Center (MMRC) at the University of California, Davis, and mice were recovered for research use at The Jackson Laboratory. The B6.129-*Lhfp15<sup>tm1Kjn</sup>*/Kjn strain (JAX Stock # 5434) contains a *LacZ* reporter gene in the *Lhfp15* gene that is expressed specifically in hair cells of the inner ear (Longo-Guess et al., 2007).

Embryonic stages of mice were determined from timed matings by observing females for vaginal plugs. The day of the plug was considered embryonic day 0.5 (E0.5).

### 2.2. ABR measurements

Hearing in mice was assessed by ABR threshold analysis. Mice were anesthetized with an intraperitoneal injection of tribromoethanol (0.2 ml of 20 mg/ml stock per 10 g of body weight), and then placed on a 37 °C temperature-controlled pad (FHC Inc., Bowdoin, ME) in a sound-attenuating chamber (Acoustic Systems, Austin, TX). Needle electrodes were placed just under the skin, with the active electrode placed between the ears just above the vertex of the skull, the ground electrode placed ventrolateral to the right ear, and the reference electrode underneath the left ear. High-frequency transducers were placed just inside the ear canal and computer-generated sound stimuli were presented at defined intervals. Thresholds were determined for broad-band clicks and 8-, 16-, and 32-kHz pure-tone stimuli by increasing the sound pressure level (SPL) in 10-dB increments followed by 5-dB increases and decreases to determine the lowest level at which a distinct ABR wave pattern could be recognized. Stimulus presentation and data acquisition were performed using the Smart EP evoked potential system (Intelligent Hearing Systems, Miami, FL).

### 2.3. Inner ear whole mounts and X-gal staining of hair cells

To better visualize cochlear lengths in whole mount preparations, we crossed B6.B10Sn-*Mctp1<sup>dwnd</sup>*/J mice with mice of the B6.129-*Lhfp15<sup>tm1Kjn</sup>*/Kjn knockout strain, which contains a *LacZ* reporter gene for *Lhfp15* that is expressed specifically in hair cells of the inner ear (Longo-Guess et al., 2007). *Lhfp15<sup>+/-</sup>* and *Mctp1<sup>+/dwnd</sup>* doubly heterozygous mice were intercrossed, and *Mctp1<sup>+/+</sup>* and *Mctp1<sup>dwnd/dwnd</sup>* progeny with *Lhfp15<sup>+/-</sup>* genotypes were selected for cochlear hair cell visualization by X-gal detection of the *LacZ* reporter gene for *Lhfp15* expression, performed essentially as described (Oberdick J et al., 1994). Briefly, inner ears were dissected from the mouse and fixed overnight in 2% PFA in 0.1 M modified PIPES buffer. After fixation, the bony shell of the cochlea was removed and the inner ears were stained overnight at 37 °C in X-gal buffer. Whole mount inner ears were visualized using a Leica MZ12.5 stereomicroscope and Leica Application Suite software v3.6.0 (Leica Microsystems GmbH, Wetzlar, Germany).

#### 2.4. Scanning electron microscopy of cochlear hair cells

SEM was performed essentially as described (Furness and Hackney, 1986). Inner ears were dissected out of the skull, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3–4 h at 4 °C, and then washed three to four times in 0.1 M phosphate buffer. Hair cells of the organ of Corti were exposed by carefully dissecting away the overlying bone and membranes. The tissues were then processed in osmium tetroxide-thiocarbohydrazide (OTOTO), dehydrated with ethanol, and dried with hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA). Samples were mounted onto aluminum stubs, sputter-coated to produce a 15-nm gold coat, and examined at 20 kV with a Hitachi 3000 N VP Scanning Electron Microscope with an EDAX X-ray Microanalysis unit and PCI Quartz Image Management software (Vancouver, BC).

#### 2.5. Hair cell counts and measurements of cochlear length

Hair cell counts and cochlear lengths were determined from rhodamine phalloidin stained surface preparations of P0 cochlea. After staining, each of the three turns: apical, middle, and basal were separated from the modiolus, mounted onto glass slides, and imaged with an Olympus BX51 microscope and a DP72 camera with cellSens standard software (Olympus, Tokyo, Japan). Cochlear measurements were taken using the same software. Inner and outer hair cells were counted separately from three random sections of each turn of an individual cochlea. Hair cell density per 100  $\mu\text{m}$  was calculated for each of the two hair cell populations. Lengths for each of the three sections of the cochlear duct were measured from digital images of the phalloidin preps, and these partial lengths were added together to give a total cochlear length for each ear.

#### 2.6. Immunofluorescence and phalloidin stainings

E16.5 inner ears or eyes were fixed for 1 h in PFA 4%, equilibrated in sucrose 20% overnight and embedded in a 1:1 mixture of sucrose 20%: OCT (Tissue-Tek). Cryosections (12  $\mu\text{m}$ ) were stored at -80C, thawed, and processed for citrate antigen retrieval (boiled five times for 1 min in 10 mM citrate with cooling intervals). The sections were then permeabilized and blocked in 0.5% Triton X-100 and 1% BSA, and incubated with the primary antibodies overnight (NR2F1: R & D Systems PPH813299, mouse monoclonal clone H8132 (1:100); SOX2: Santa Cruz Biotechnology sc-17320, goat polyclonal (1:100)). Secondary antibodies were donkey anti-mouse conjugated to Alex Fluor 555 and donkey anti-goat conjugated to Alex Fluor 647 (ThermoFisher). Nuclei were stained with Hoechst 33342 (ThermoFisher). For the quantifications in Fig. 3D, a 25  $\times$  25  $\mu\text{m}$  region of interest was defined to match the organ of Corti, and signal intensity was measured separately for the SOX2 and NR2F1 channels using ImageJ (IntDens).

For whole-mounts at birth (Fig. 5), inner ears were fixed 1 h in 4% PFA, the sensory epithelia were exposed, permeabilized in 0.5% Triton X-100, stained with phalloidin conjugated to Alexa Fluor 488 (ThermoFisher), and mounted flat. Images were acquired with a Zeiss LSM800 confocal microscope.

#### 2.7. Inner ear paint fills

E15.5 embryos (*Mctp1*<sup>dwnd/dwnd</sup>, n = 4; *Mctp1*<sup>-/-</sup>, n = 3; *Nr2f1*<sup>-/-</sup>, n = 4; *Mctp1*<sup>+/- dwnd</sup> *Nr2f1*<sup>+/-</sup>, n = 5; and *Mctp1*<sup>+/- dwnd</sup>, n = 6) and P1 mice (*Mctp1*<sup>dwnd/dwnd</sup>, n = 3; *Mctp1*<sup>+/- dwnd</sup>, n = 3) were used for inner ear paint fills. E15.5 embryos were decapitated and whole heads were fixed in Bodian's fixative. P1 mice were decapitated and half-heads were fixed in Bodian's fixative after the brain was removed. Heads were fixed overnight and then dehydrated with 75% ethanol (2  $\times$  2 h), 95%

ethanol (2  $\times$  2 h), and 100% ethanol (2  $\times$  2 h). Heads were then rinsed once with methyl salicylate and cleared overnight by placing specimens in methyl salicylate. Inner ears were filled with 1% Wite-Out correction fluid in methyl salicylate using a Hamilton syringe with a pulled glass capillary needle broken to a tip diameter of 20–40  $\mu\text{m}$ . E15.5 inner ears were injected through the middle turn of the cochlea. Two injections were done for P1 inner ears with one injection in the cochlear middle turn and the second injection in the common crus, as previously described (Kiernan, 2006; Tian et al., 2017).

#### 2.8. PCR methods for mutation analysis and genotyping

DNA samples from backcross progeny were isolated from mouse tail tips using the hot sodium hydroxide and Tris (HotSHOT) method (Truett et al., 2000). For linkage mapping, microsatellite markers were selected for each chromosome, genotyped by PCR using the HotMaster Taq DNA Polymerase kit (5 PRIME Inc., Gaithersburg, MD) according to manufacturer's instructions, and analyzed for cosegregation with the mutant phenotype, which was assessed by ABR.

PCR primers for sequencing *Mctp1* exons and to genotype mice for the *Mctp1*<sup>dwnd</sup> mutation were designed using Primer3 software (<http://primer3.ut.ee>) and synthesized by Integrated DNA Technologies (Coralville, Iowa). Pairs of primers flanking each of the 21 exons of *Mctp1* were used to amplify and sequence DNA products from *dwnd/dwnd* and *+/+* mice. Three primers were used in a single PCR reaction to distinguish *+/+*, *+/dwnd* and *dwnd/dwnd* genotypes of mice from genomic DNA: a forward primer located outside the deletion (ACAGGAGGTCAGGGTGTGTC), a forward primer located within the deletion (TGCCAAAGAGCATTAGTGTGAT), and a reverse primer located outside the deletion (TTCTCACTGGTTTCACACCATC), as indicated in Fig. 2A. PCR products were separated on 3% NuSieve gels (Lonza, Rockland, ME) containing ethidium bromide and visualized by UV illumination.

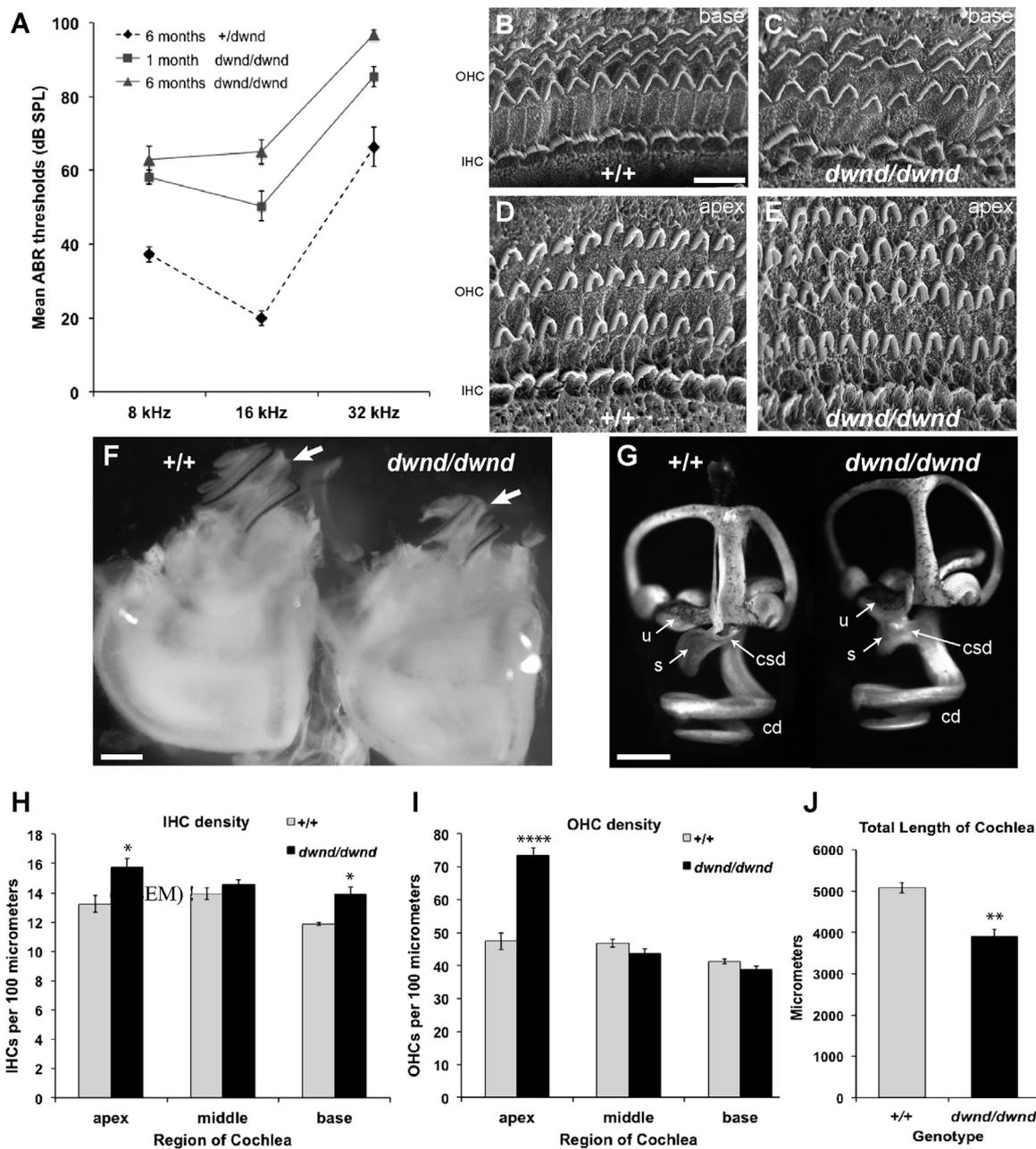
#### 2.9. RT-qPCR

Whole cochleae of E16.5 embryos were microdissected from the condensed mesenchyme shell and flash frozen in liquid nitrogen. Following genotyping of the litter, samples of the same genotype (heterozygous or homozygous for *Mctp1*<sup>dwnd</sup>) were pooled and RNA extracted using the RNeasy Plus mini kit (Qiagen). Two litters were processed in this way, with each genotype group containing between 6 and 10 cochleae per litter. RNA was then reverse-transcribed using an oligo-dT primer to obtain first-strand cDNA using standard methods, and qPCR performed using a SYBR Green PCR Master Mix (ThermoFisher #4309155) and a Viia 7 Real-Time PCR System (ThermoFisher). Primers used were as follows: GAPDH F: AGGTCGGTGTGAACGGATTG, GAPDH R: TGTAGACCATGTAGTTGAGGTCA, Nr2f1 F: CGAGTACAGCTGCCTCAAAG (exon 2), Nr2f1 R: CTTTCGATGTGGGCAGCATC (exon 3), *Mctp1* F: GCTGATCCC GGAATGTACCA (exon2/first coding), *Mctp1* R: CACATACGGATCGCTTGTC (exon 3). Reactions were run in triplicate and fold changes for target transcripts versus the *Gapdh* housekeeping gene were calculated using the  $\Delta\Delta\text{Ct}$  method (Livak and Schmittgen, 2001) on the averaged Ct values from the triplicate reactions. Data points represent the samples pooled by genotype for each of the two litters collected.

### 3. Results

#### 3.1. The phenotype of *dwnd/dwnd* mutant mice

A new recessive mutation was discovered at The Jackson Laboratory, first identified by the moderate circling behavior of homozygous individuals. Because circling behavior is characteristic of

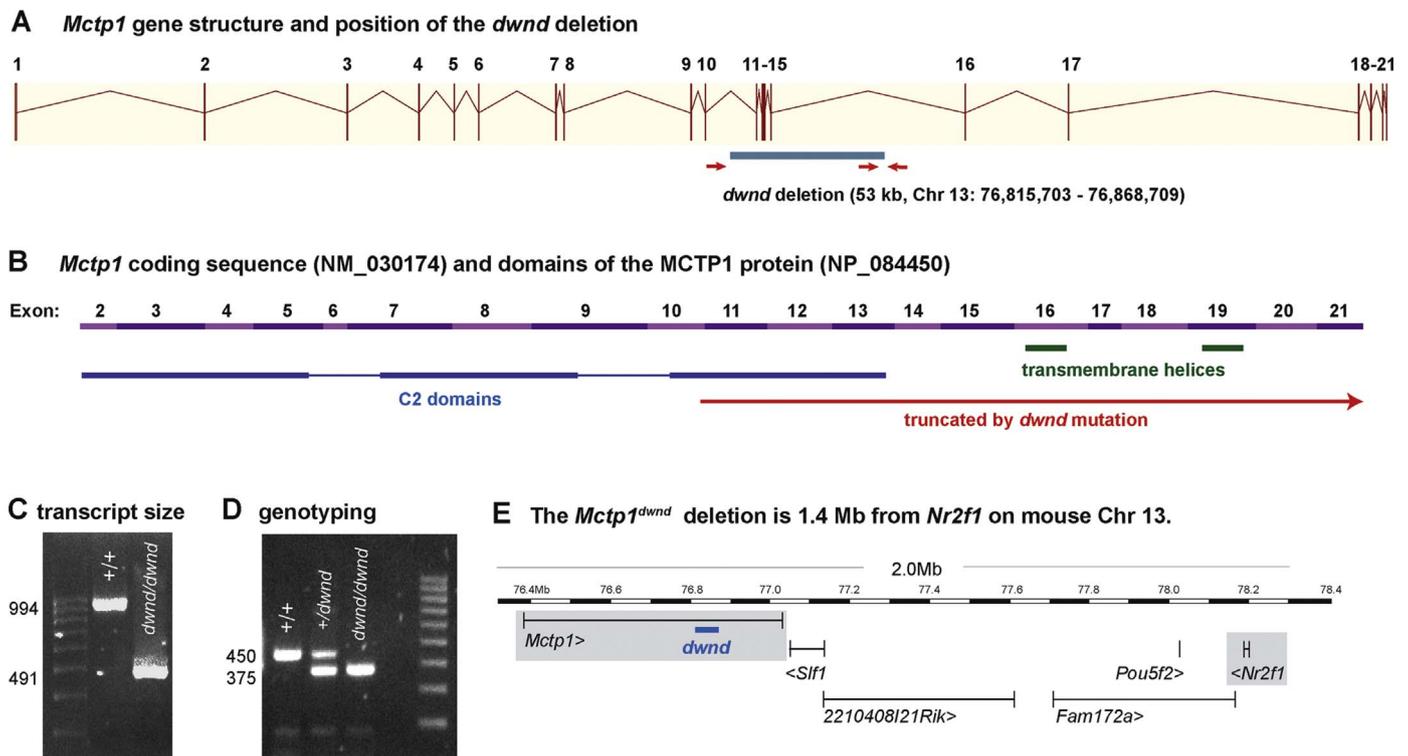


**Fig. 1. Auditory dysfunction and inner ear abnormalities of *dwnd/dwnd* mice.** (A) Mice homozygous for the *dwnd* mutation are hearing impaired. Means ( $\pm$  SEM) of ABR thresholds of 16 *dwnd/dwnd* mice tested at one month of age and 12 tested at six months of age were compared with those of 7 *+/+* control mice tested at six months of age for 8, 16, and 32 kHz test stimuli. Thresholds of mutant mice were about 20–30 dB higher than those of controls at one month of age and about 25–45 dB higher at six months of age. (B–E) Cochleae of *dwnd/dwnd* mice have regions with supernumerary hair cells. SEM images of organ of Corti surface preparations from cochleae of P8 mice show extra inner hair cells (IHC) at the cochlear base (C) and an extra row of outer hair cells (OHC) at the apex (E) of *dwnd/dwnd* mice as compared with corresponding *+/+* controls (B, D). Scale bar for B–E (shown in B), 10  $\mu$ m. (F) The overall size of the cochlea (indicated by arrows) in *dwnd/dwnd* mice is smaller than that of *+/+* controls. Dissected inner ears of adult mice that also carry a copy of an *Lhfp15-LacZ* reporter gene were cleared and the cochlear spiral exposed, showing rows of hair cells stained with LacZ (see Methods). Scale bar, 0.5 mm. (G) Inner ear paintfills of P1 (newborn) mice. In addition to a shorter cochlear duct (cd), the *dwnd/dwnd* inner ear has a smaller sacculus (s) that is not separated from the utricle (u) and a much larger cochleosaccular duct (csd) than the *+/+* control. Scale bar, 0.5 mm. (H–J) Cochlear lengths and hair cell densities in *dwnd/dwnd* and control mice. The density of inner hair cells near the apex and near the base of the cochlea is greater in *dwnd/dwnd* mutants than controls (H), and the density of outer hair cells near the cochlear apex is much greater in *dwnd/dwnd* mice than controls (I). Although hair cell densities are greater, the length of the cochlear duct is shorter in *dwnd/dwnd* mutants than controls (J), suggesting that total hair cell counts are similar. Means and standard error bars are shown for the average measurements of individual mice of each genotype (ten ears of *+/+* mice and nine ears of *dwnd/dwnd* mice). Statistical significance of mean differences was determined using a student's *t*-test. \**p* < 0.01; \*\*\*\**p* < 0.0001.

inner ear vestibular dysfunction, mutant mice were evaluated for hearing acuity by auditory brainstem response (ABR) measurements and found to be hearing impaired, with ABR thresholds 25–45 dB above those of age-matched control mice (Fig. 1A). The new mutation was named “deaf wanderer” (symbol *dwnd*) because of its associated auditory and vestibular dysfunctions. From intercrosses of obligate *+/dwnd* mice, we deduced that hearing impairment, which occurred in

about 25% of the progeny, was fully penetrant in *dwnd/dwnd* mice, but that only about 40% of the hearing-impaired *dwnd/dwnd* progeny exhibited circling behavior.

To search for cochlear abnormalities that may be responsible for the hearing impairment of mutant mice (*dwnd/dwnd*), we compared scanning electron microscopy (SEM) images of organ of Corti surface preparations from cochleae of mutant and control mice (Fig. 1B–E).



**Fig. 2. The *dwnd* mutation of the *Mctp1* gene.** (A) The structure of the *Mctp1* gene is shown as numbered vertical lines representing exons and horizontal connecting lines representing introns. The *dwnd* mutation (represented by the blue-gray bar beneath the gene diagram) is a 53 kb intragenic deletion of *Mctp1*, which includes exons 11–15 and adjoining intronic regions. The red arrows indicate positions and directions of PCR primers designed to distinguish wildtype and *dwnd* alleles. (B) Alternating pink and purple line segments indicate the exon-encoded regions of the protein-coding portion of the *Mctp1* transcript, with corresponding protein domains shown below. The out-of-frame *dwnd* mutation is expected to create a premature stop codon and truncate the protein after exon 10, thereby eliminating the third C2 domain and the two transmembrane helices of the encoded MCTP1 protein, as shown by the horizontal red arrow. (C) The *dwnd* mutation in the *Mctp1* gene was first identified by RT-PCR. Amplification of cDNA from brain tissue of mutant mice with primers in exons 9 and 17 of the *Mctp1* transcript (NM\_03174) produced a product that was 503 bp smaller than that of the *+/+* control, corresponding to the loss of exons 11–15. (D) A simple PCR assay of genomic DNA was designed to genotype mice with the *dwnd* mutation. The PCR assay (using three primers positioned as shown in A) amplifies a 450 bp product from the wildtype *+* allele and a 375 bp product from the mutant *dwnd* allele. Genotypes can be determined by the presence or absence of these PCR products as shown in the gel image for *+/+*, *+/dwnd*, and *dwnd/dwnd* individuals. (E) The site of the *Mctp1*<sup>*dwnd*</sup> deletion at the 76.8 Mb position on mouse Chromosome 13 is 1.4 Mb from the *Nr2f1* gene at the 78.2 Mb position. The *Mctp1* (21 exons) and *Nr2f1* (3 exons) genes are shaded in gray, and a blue horizontal bar beneath the *Mctp1* gene designates the position and extent of the *dwnd* deletion. Genome coordinates are shown above. Panels A, B, and E were derived from the mouse Ensemble Genome Browser (GRCm38).

Compared with *+/+* controls (Fig. 1B,D), the cochleae of *dwnd/dwnd* mice have additional, disorganized inner hair cells (IHCs) near the base (Fig. 1C) and an extra row of outer hair cells (OHCs) near the apex (Fig. 1E). Cross-sectional analysis by light microscopy revealed no additional cochlear abnormalities; however, examinations of dissected and cleared whole-mounts of inner ears showed that the cochlea of *dwnd/dwnd* mice is smaller and shorter than that of *+/+* controls (Fig. 1F). For better visualization of the cochlear duct, whole mounts were stained with X-gal to detect expression of a hair cell-specific *Lhfpl5-LacZ* reporter gene, as detailed in Materials and Methods.

To examine global inner ear morphology, paintfills of inner ears from newborn *dwnd/dwnd* mice were compared with those of *+/+* controls (Fig. 1G). The cochlear duct of *dwnd/dwnd* mice appeared shorter than controls, as seen in the whole mount inner ear preparations (Fig. 1F); however, additional abnormalities were revealed in other inner ear structures. The sacculus of the mutant inner ear is noticeably smaller than that of the control and is not fully separated from the utricle, and the duct connecting the sacculus with the cochlea is much larger in the mutant (Fig. 1G).

Hair cell counts and cochlear length measurements in mutant and control mice supported the histological observations. The cochleae of *dwnd/dwnd* mutants have a greater density of IHCs near the apex and near the base (Fig. 1H), a much greater OHC density near the apex (Fig. 1I), and a shorter length (Fig. 1J) compared with cochleae of *+/+* controls. Although hair cell densities are greater in *dwnd/dwnd* mutants than controls, the shorter cochlear length suggests that the total number of hair cells is similar.

### 3.2. Genetic mapping of the *dwnd* mutation

The chromosomal map position of the *dwnd* mutation was determined by genetic analysis of 159 progeny from a (B6. B10Sn-*dwnd/dwnd* × C3HeB/FeJ) F1 × B6. B10Sn-*dwnd/dwnd* backcross. Because of the incomplete penetrance of circling behavior, each of the backcross mice was assigned a genotype (*dwnd/dwnd* or *+/dwnd*) according to its ABR thresholds (Fig. 1A). Genotypes of mice were analyzed for cosegregation with polymorphic microsatellite markers located throughout the genome, and linkage was found with markers on Chromosome 13 in a region between markers *D13Mit26* (72.2 Mb) and *D13Mit193* (91.8 Mb), GRCm38 Mb positions. This 20 Mb region contains more than 60 protein-coding genes, but only three are known to affect inner ear development or function: *Nr2f1*, *Adgrv1*, and *Slc12a7*, according to the Mouse Genome Informatics website (<http://www.informatics.jax.org/marker>) results for this region using “inner ear” as the phenotype search term.

The *Nr2f1* gene stood out as a likely candidate because a mouse knockout of this gene (Tang et al., 2006) has an inner ear phenotype that is very similar to what we observed for *dwnd/dwnd* mice. However, we initially eliminated *Nr2f1* as a candidate gene because genomic DNA from *dwnd/dwnd* mutants and *+/+* control mice showed no sequence differences in any of the *Nr2f1* exons or their adjacent splice sites. Although *Slc12a7*<sup>-/-</sup> and *Adgrv1*<sup>-/-</sup> mice are deaf, their inner ear phenotypes are very distinct from that of *dwnd/dwnd* mice; neither has supernumerary cochlear hair cells nor exhibits behaviors characteristic of vestibular dysfunction. *Slc12a7*<sup>-/-</sup> mutant

mice exhibit rapid outer hair cell degeneration but have no other inner ear abnormalities (Boettger et al., 2002), and inner ear defects of *Adgrv1*<sup>-/-</sup> mice are limited to disorganized stereocilia bundles of cochlear hair cells (McGee et al., 2006). Because of their distinctively different mutant phenotypes, *Slc12a7* and *Adgrv1* were not further considered as candidate genes for the *dwnd* mutation.

Information on each of the remaining genes in the *dwnd* candidate interval was accessed from the Mouse Genome Database (<http://www.informatics.jax.org/marker>), but none of the well-characterized genes in the interval have functions or expression patterns that we deemed relevant to inner ear development. Of the poorly characterized genes, we selected *Mctp1* as a possible candidate because of its proposed function in calcium signaling at the plasma membrane (Shin et al., 2005), which we thought could relate to the defect in cochlear convergent extension observed in *dwnd* mutant mice (Fig. 1), a process that involves intercellular calcium signaling. *Mctp1* spans more than 540 kb and is comprised of 21 exons (Fig. 2A), which encode a 694 amino acid protein containing three C2 calcium-binding domains and two transmembrane helices (Shin et al., 2005), as shown in Fig. 2B. The presence of multiple C2 domains suggested to us that MCTP1 might function as a Ca<sup>2+</sup> sensor in membranes of the inner ear similar to otoferlin, a multi-C2 domain protein that is essential for hearing (Pangrsic et al., 2012). In addition, transcriptome analyses using mouse expression arrays (Hertzano et al., 2011) and RNA-seq (Maass et al., 2016) suggested that *Mctp1* is expressed in auditory sensory cells of P0–P6 mice. For these reasons and because no associated phenotypes had been reported for *Mctp1* mutations, we investigated the possibility that *Mctp1* dysfunction may underlie the *dwnd/dwnd* phenotype.

### 3.3. Molecular analysis of the *Mctp1* candidate gene

To evaluate the *Mctp1* candidate gene, three sets of PCR primers were designed to amplify overlapping cDNA products (reference sequence NM\_030174). Using primers located in exons 9 and 17, a much smaller 491 bp product was amplified from *dwnd* cDNA as compared to the 994 bp product amplified from the control cDNA (Fig. 2C). Sequencing of the smaller product showed that *dwnd* cDNA is missing exons 11–15 of the *Mctp1* transcript (Fig. 2B). No size differences were observed for products amplified with the other *Mctp1* cDNA primer sets. The *dwnd* deletion is predicted to cause a frameshift and premature stop codon at the beginning of exon 16 and therefore is expected to eliminate the third C2 domain and the two transmembrane helices of the MCTP1 protein, thus inactivating normal MCTP1 function (Fig. 2B).

To confirm the *dwnd* mutation at the genomic level, primers specific to each of the 21 *Mctp1* exons were used to amplify and sequence products from mutant and control DNA. The primers for exons 11–15 did not amplify any products when *dwnd/dwnd* DNA was used as the template, but all other primer sets produced the expected wildtype product sizes with no sequence differences between mutant and control DNA. The extent of the *dwnd* deletion was refined by PCR analysis using multiple primer pairs distributed throughout intron 10–11 and intron 15–16. The presence or absence of the PCR products further delimited the boundaries of the deleted region. Sequencing of genomic DNA of *dwnd/dwnd* mice amplified with primers flanking the deletion showed that 53,008 bp are precisely deleted without any accompanying DNA alterations. In addition to the 4830 bp that includes exons 11–15 and the introns between these exons, the deletion includes 7270 bp of flanking intron 10–11 and 40,908 bp of flanking intron 15–16.

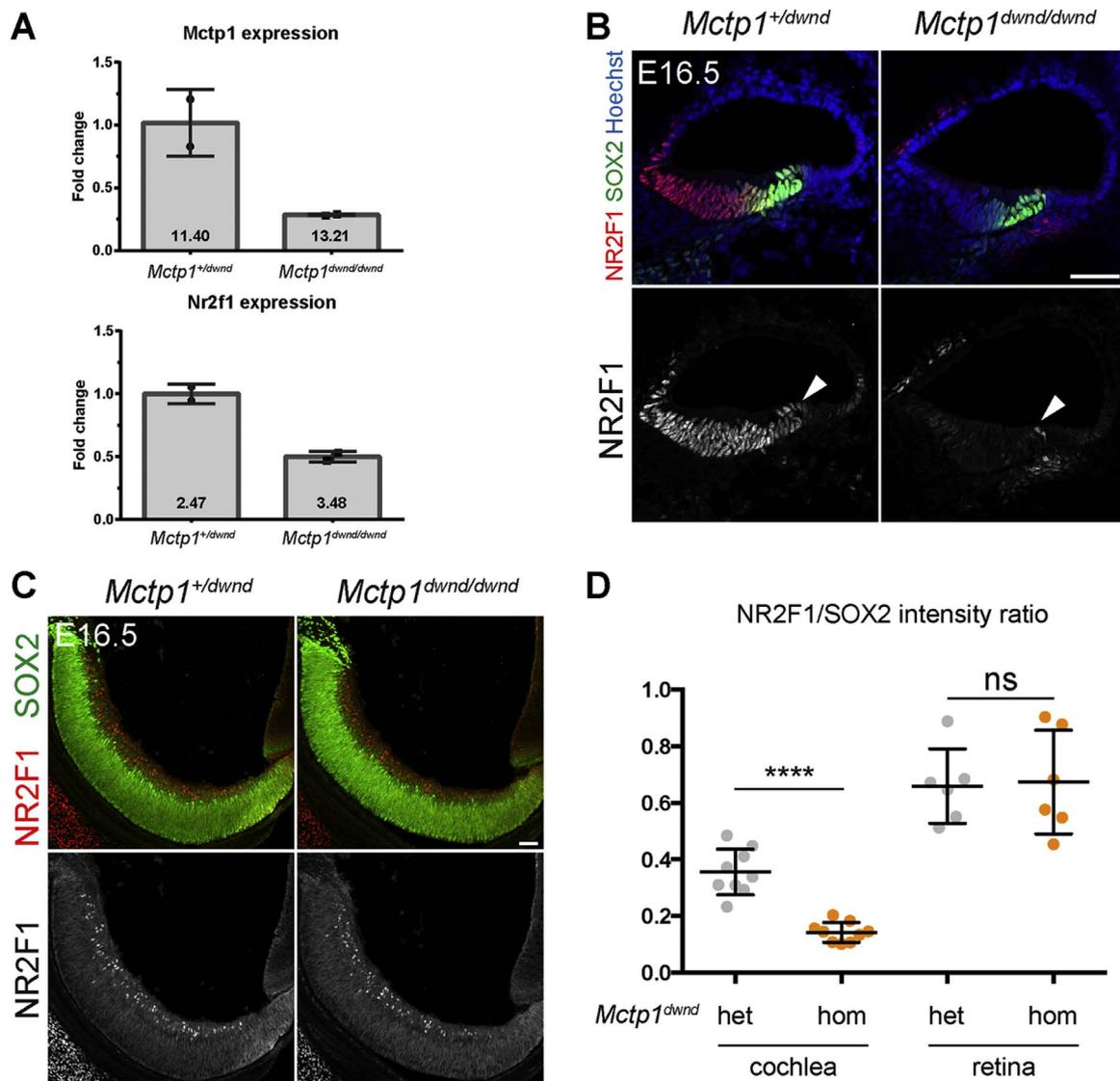
To distinguish between the genotypes of *dwnd/dwnd*, *+/dwnd*, and *+/+* mice, a three-primer genotyping assay was devised using a common reverse primer located outside of the deletion and a forward primer that spans across the deletion (to amplify the mutant product) combined with a forward primer located within the deletion (to amplify

the wildtype product), as shown in Fig. 2A. A 450 bp product was obtained from DNA of wild type mice, and a 375 bp band was obtained in *dwnd/dwnd* mice, with heterozygotes (*+/dwnd*) exhibiting both bands (Fig. 2D). The *Mctp1* genotypes obtained from DNA analysis corresponded with wildtype (*+/+* and *+/dwnd*) and mutant (*dwnd/dwnd*) phenotypes of all mice examined in this study, supporting the causative nature of the *Mctp1*<sup>*dwnd*</sup> mutation. Because the spontaneous *dwnd* mutation arose recently in the C57BL/10SnJ inbred mouse strain, it is expected to be the only DNA difference between the C57BL/10SnJ strain and the congenic region of the *dwnd* mutant strain (B6.B10Sn-*Mctp1*<sup>*dwnd*</sup>/Kjn, see Materials and Methods), providing further confirmation that the *Mctp1* intragenic deletion we discovered, and not some other undetected mutation, is responsible for the *dwnd/dwnd* phenotype.

### 3.4. *Nr2f1* transcript and protein are downregulated in the *Mctp1*<sup>*dwnd/dwnd*</sup> cochlea

We could not localize or quantitate *Mctp1* expression in the embryonic cochlea because, in our hands, *in situ* hybridization and immunohistochemistry revealed only faint *Mctp1* transcript and protein signals close to background levels. The site of the *Mctp1*<sup>*dwnd*</sup> deletion at the 76.8 Mb position on mouse Chromosome 13 is 1.4 Mb from the *Nr2f1* gene at the 78.2 Mb position (Fig. 2E). Four intervening genes separate *Mctp1* from *Nr2f1* (*Pou5f2*, *Fam172a*, *2210408I21Rik*, *Slf1*) and no distant regulatory sequence for *Nr2f1* has been reported so far. Relative physical proximity and the similarities in the inner ear abnormalities of *Mctp1*<sup>*dwnd/dwnd*</sup> (Fig. 1) and *Nr2f1*<sup>-/-</sup> (Tang et al., 2006) mice nevertheless prompted us to test whether the *Mctp1*<sup>*dwnd/dwnd*</sup> mutant phenotype could be caused by the loss of *cis*-regulatory elements for *Nr2f1* transcription located in the *Mctp1*<sup>*dwnd*</sup> deletion, and not by the loss of MCTP1 function itself. To compare transcript levels, the membranous labyrinths of E16.5 cochlea from *+/dwnd* and *dwnd/dwnd* fetuses were pooled by genotype, cDNA prepared and RT-qPCR performed with primer pairs for *Mctp1* (exon 2–3) and *Nr2f1* (exon 2–3). Fold change calculated using the  $\Delta\Delta Ct$  method revealed that *Mctp1* expression was reduced ~75% in the *Mctp1*<sup>*dwnd/dwnd*</sup> cochlea, indicating that truncated transcripts are less stable than wildtype (Fig. 3A), an unsurprising finding because the premature stop codon is likely to activate nonsense-mediated decay of the mutant transcript. In comparison, *Nr2f1* expression was reduced ~50% in the *Mctp1*<sup>*dwnd/dwnd*</sup> cochlea, a dramatic change given that the *Nr2f1* gene itself and more than 1 Mb of surrounding sequence were left intact. Furthermore, normalization to *Gapdh* levels revealed that *Mctp1* is expressed at much lower levels than *Nr2f1* (controls: Ct<sub>*Mctp1*</sub>-Ct<sub>*Gapdh*</sub>= 11.4 cycles, versus Ct<sub>*Nr2f1*</sub>-Ct<sub>*Gapdh*</sub>=2.47 cycles).

We next sought to confirm the functional impact of decreased gene expression by assessing NR2F1 protein distribution in the embryonic cochlea with immunofluorescence. Sections of the E16.5 cochlea of *Mctp1*<sup>*+/dwnd*</sup> control mice revealed that NR2F1 was broadly located in the cochlear floor, overlapping with SOX2 that labels the prosensory domain, but also extending more neurally (Fig. 3B). In *Mctp1*<sup>*dwnd/dwnd*</sup> mutants, however, NR2F1 antibody signal in the cochlear floor was severely reduced, and only the most abneural cells as well as non-epithelial cells outside the cochlear duct retained normal protein expression (Fig. 3B, arrowheads). By contrast, NR2F1 signals appeared unchanged in the E16.5 *Mctp1*<sup>*dwnd/dwnd*</sup> retina (Fig. 3C), and quantification of the NR2F1/SOX2 intensity ratio in both organs confirmed a significant reduction of NR2F1 in the cochlear floor but not in the retina (Fig. 3D). Overall, these results suggest that *dwnd* compromises a regulatory region located in *Mctp1* that enhances *Nr2f1* transcript and protein expression in the embryonic cochlea. This long-range regulatory region exhibits cell specificity even within the cochlear duct, probably explaining why *Mctp1*<sup>*dwnd/dwnd*</sup> mutants share the organ of Corti disorganization reported in *Nr2f1*<sup>-/-</sup> mutants (Fig. 1B–D) (Tang et al., 2006), but not the associated postnatal lethality, for example.



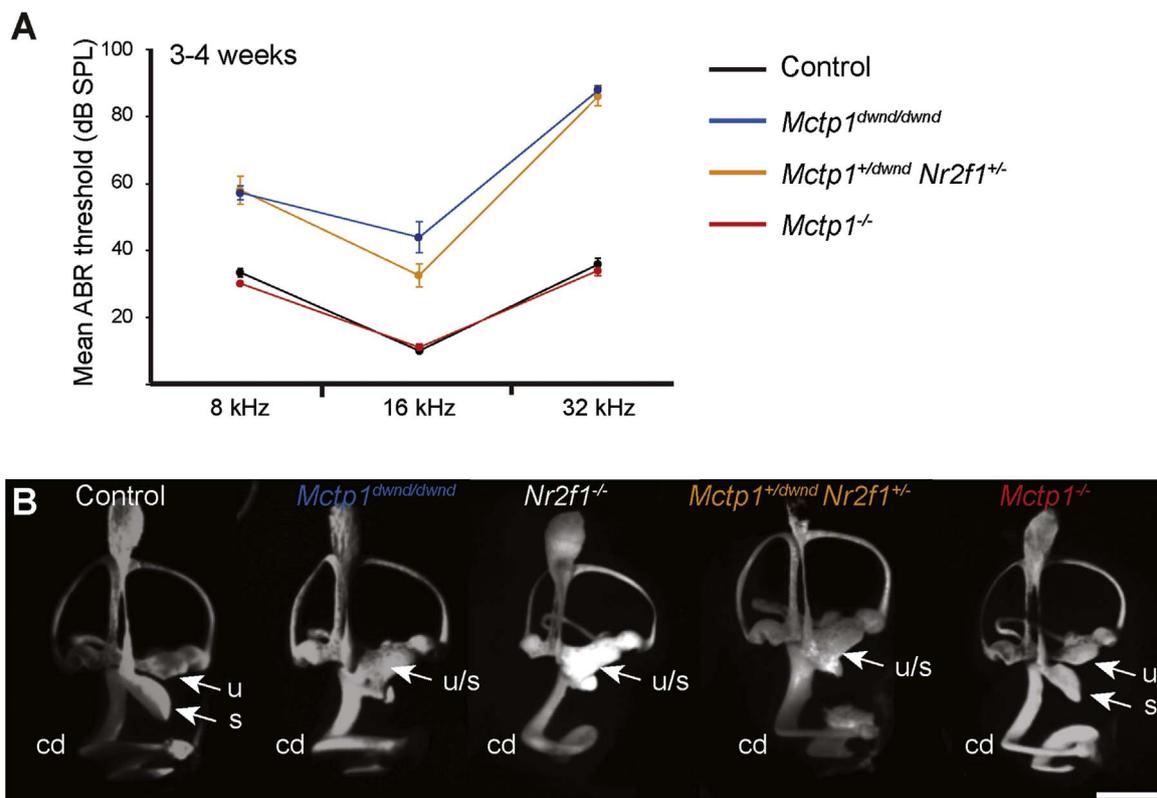
**Fig. 3.** *Nr2f1* transcript and protein downregulation in *Mctp1*<sup>*dwnd*</sup> cochlea. (A) RT-qPCR on E16.5 cochleae pooled by genotype. *Mctp1* expression is reduced by about 75% in *Mctp1*<sup>*dwnd*/*dwnd*</sup> mutants, and *Nr2f1* expression is reduced by about 50%. Data points represent samples pooled by genotype for each of two litters collected (see Methods). Numbers inside the bars indicate the average  $\Delta Ct = Ct_{\text{target}} - Ct_{\text{Gapdh}}$ , and reveal that *Nr2f1* is much more abundantly expressed at E16.5 than is *Mctp1*. (B–C) E16.5 immunodetection of NR2F1 (red) and SOX2 (green) proteins in cochlear cryosections in the basal turn (B) and in half-retina at the optic nerve (C). In the cochlear floor (B), NR2F1 protein is enriched at much lower levels in *Mctp1*<sup>*dwnd*/*dwnd*</sup> compared to heterozygote controls, whereas SOX2 is comparable. Arrowheads point to abnormal cells in the sensory domain that interestingly appear to retain normal NR2F1 protein in homozygotes. In the retina (C), NR2F1 is by contrast enriched in a comparable manner in *Mctp1*<sup>*dwnd*/*dwnd*</sup> and controls. (D) Quantification of the immunostaining intensity ratio between co-localized NR2F1 and SOX2 proteins in the E16.5 cochlea and retina. Using SOX2 as an internal control, NR2F1 is significantly downregulated in the cochlea but not in the retina. Each point represents one section (10 cochlea or 6 retina sections per genotype), two animals represented per genotype; average  $\pm$  SD; unpaired *t*-test with Welch's correction, ns, not significant; \*\*\*\*  $p < 0.0001$ . Scale bars 50  $\mu\text{m}$ .

### 3.5. Non-complementation between *Mctp1*<sup>*dwnd*</sup> and *Nr2f1*<sup>-</sup> alleles

To formally confirm that *Mctp1*<sup>*dwnd*</sup> is a regulatory allele of *Nr2f1*, we next asked whether the *Mctp1*<sup>*dwnd*</sup> allele could complement a null allele of *Nr2f1* produced by the KOMP consortium (*Nr2f1*<sup>*tm1.1(KOMP)*</sup> *Mbp*, hereafter *Nr2f1*<sup>-</sup>). We generated transheterozygous animals where one parental Chromosome 13 carries *Mctp1*<sup>*dwnd*</sup> and *Nr2f1*<sup>-</sup> alleles and the other carries *Mctp1*<sup>+</sup> and *Nr2f1*<sup>-</sup> alleles (hereafter denoted as *Mctp1*<sup>+/*dwnd*</sup> *Nr2f1*<sup>+/-</sup>). Even though both genes were heterozygous, some of the transheterozygotes exhibited signs of vestibular dysfunction (2 out of 10 exhibited a noticeable circling behavior), and all suffered from hearing loss, with ABR thresholds recorded at 8, 16 and 32 kHz raised to similar levels as the *Mctp1*<sup>*dwnd*/*dwnd*</sup> mutants (Fig. 4A). The *Mctp1*<sup>+/*dwnd*</sup> *Nr2f1*<sup>+/-</sup> transheterozygotes also shared the defective organ-scale features observed earlier in *Mctp1*<sup>*dwnd*/*dwnd*</sup> homozygotes: improperly separated utricle and saccule, and a shorter

cochlear duct (Fig. 4B). Of note, these defects were also observed in *Nr2f1*<sup>-</sup> inner ears (Fig. 4B), as expected if they originate strictly from the loss of NR2F1 function.

At the cellular level, the basal region of the cochlea in neonatal *Mctp1*<sup>+/*dwnd*</sup> *Nr2f1*<sup>+/-</sup> transheterozygotes showed hair cell disorganization including an excess of IHCs, mirroring our results in P8 *Mctp1*<sup>*dwnd*/*dwnd*</sup> mutants (Fig. 5A top; compare with Fig. 1B–C). Furthermore, the apex region of the neonate cochlea in transheterozygotes exhibited extra rows of OHCs previously noted in *Mctp1*<sup>*dwnd*/*dwnd*</sup> mutants (Fig. 5A bottom, arrowheads; compare with Fig. 1D–E). In *Mctp1*<sup>+/*dwnd*</sup> *Nr2f1*<sup>+/-</sup> transheterozygotes, but not in *Mctp1*<sup>+/*dwnd*</sup> *Nr2f1*<sup>+/+</sup> littermate controls, the utricular macula was reduced from an ovate to a trapezoid shape (Fig. 5B), and the saccular macula appeared disrupted, being often attached with the utricular macula (not shown) and the cochlear base (Fig. 5C). Altogether, these results strongly suggest that the loss of NR2F1 function in the developing *Mctp1*<sup>*dwnd*/*dwnd*</sup>



**Fig. 4.** Hearing thresholds and inner ear paintfills of *Mctp1*<sup>dwnd/dwnd</sup>, *Mctp1*<sup>-/-</sup>, *Nr2f1*<sup>-/-</sup>, and *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> mice. (A) *Mctp1*<sup>dwnd/dwnd</sup> mice and *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> transheterozygotes are hearing impaired. Mean ( $\pm$  SEM) ABR thresholds of 11 *Mctp1*<sup>dwnd/dwnd</sup> mice, 10 *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> transheterozygotes and 12 *Mctp1*<sup>- / -</sup> mice tested at one month of age were compared with those of 12 age-matched *Mctp1*<sup>+ / dwnd</sup> heterozygous control mice for 8, 16, and 32 kHz test stimuli. Thresholds of *Mctp1*<sup>dwnd/dwnd</sup> and *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> mice are not statistically significantly different from each other but are about 25–50 dB higher than those of *Mctp1*<sup>+ / dwnd</sup> control mice. In contrast, thresholds of *Mctp1*<sup>- / -</sup> mice are not statistically significantly different than those of control mice. *Nr2f1*<sup>- / -</sup> mice are not included in the hearing test comparisons because they die after birth. (B) Inner ear paintfills of E15.5 embryos clearly show the fusion of the utricle (u) and saccule (s) in inner ears of *Mctp1*<sup>dwnd/dwnd</sup>, *Nr2f1*<sup>- / -</sup>, and *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> mice (u/s) but not in the *Mctp1*<sup>- / -</sup> mice. A smaller and shortened cochlear duct (cd) can also be seen in inner ears of the three mutant strains with u/s fusions. Scale bar, 0.5 mm.

*dwnd* inner ear fully accounts for physiological and morphological defects observed in both the cochlea and vestibular organs.

### 3.6. Targeted *Mctp1* knockout mice have normal inner ear morphology and function

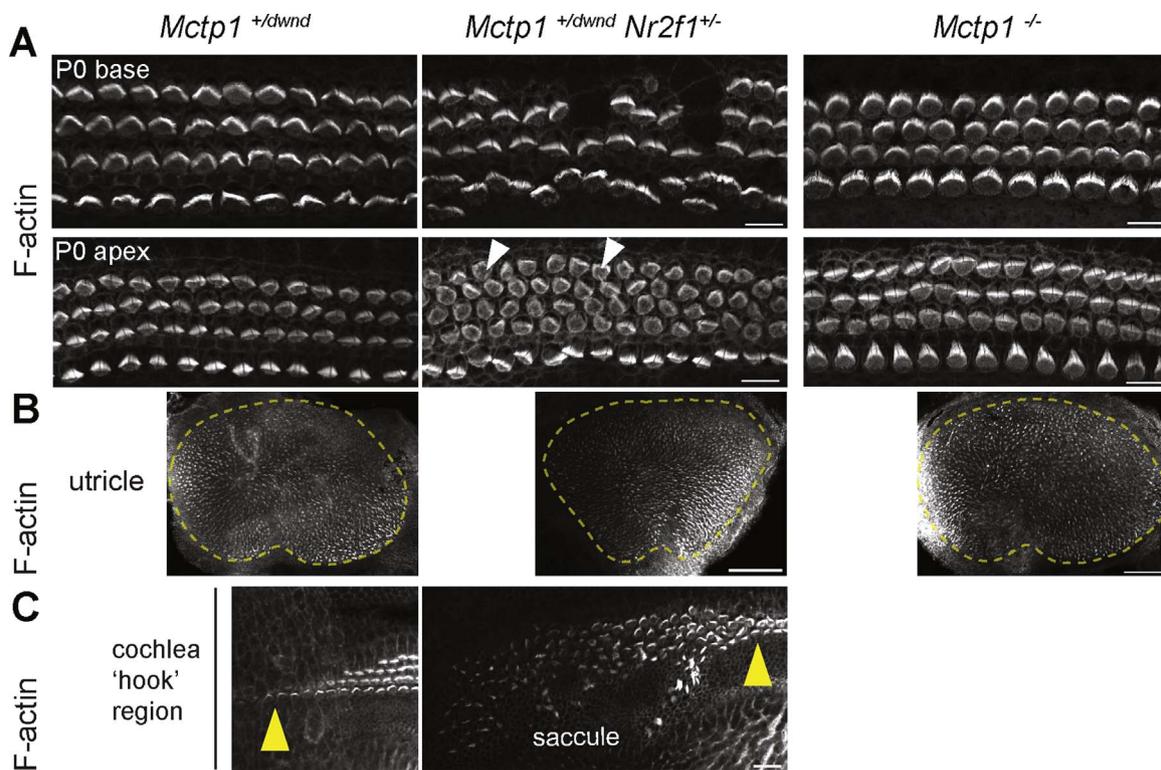
The phenotype of mice with *Mctp1* loss-of-function mutations has not been reported. To definitively rule out an involvement of MCTP1 dysfunction in the *dwnd/dwnd* phenotype we obtained mice with a targeted *Mctp1* knockout mutation (*Mctp1*<sup>em1(IMPC)Mbp</sup>, hereafter *Mctp1*<sup>- / -</sup>). *Mctp1*<sup>- / -</sup> mice appear healthy and are able to breed, with no overt behavioral or morphological abnormalities. They have normal ABR thresholds comparable to *Mctp1*<sup>+ / dwnd</sup> control mice at one month of age (Fig. 4A), and do not exhibit the moderate circling behavior that first exposed the spontaneous *Mctp1*<sup>dwnd</sup> mutation. Furthermore, paintfills at E15.5 revealed an overall normal morphology of inner ear ears in *Mctp1*<sup>- / -</sup> mutants (Fig. 4B). Finally, the hair cell arrangement in neonatal *Mctp1*<sup>- / -</sup> mutant cochlea was unaffected (Fig. 5A), and the shape and size of the utricular and saccular maculae were normal (Fig. 5B). Along with the lack of complementation between *Mctp1*<sup>dwnd</sup> and *Nr2f1*<sup>- / -</sup> alleles, these results confirm that the loss of MCTP1 function is not the cause of the hearing loss or inner ear dysmorphology associated with the *Mctp1*<sup>dwnd</sup> mutation.

## 4. Discussion

We provide several lines of evidence supporting our conclusion that the inner ear phenotype of *dwnd/dwnd* mutant mice is caused by the deletion of cis-regulatory sequences located within the *Mctp1* gene that enhance *Nr2f1* expression in the inner ear: (1) A deletion within the

*Mctp1* gene is the causative DNA lesion underlying the abnormal inner ear phenotype of mice homozygous for the spontaneous *dwnd* mutation. (2) MCTP1 deficiency cannot be the cause of the *Mctp1*<sup>dwnd/dwnd</sup> phenotype because *Mctp1*<sup>- / -</sup> knockout mice are viable and have normal inner ear morphology and function; therefore, deletion of non-coding regulatory sequences in the *Mctp1* gene must instead be responsible. (3) The transcription factor gene *Nr2f1* is located 1.4 Mb from *Mctp1*, and the inner ear phenotype of *Nr2f1*<sup>- / -</sup> mice (Tang et al., 2006) is very similar to what we observed in *Mctp1*<sup>dwnd/dwnd</sup> mice. (4) Transcript and protein expression of *Nr2f1* is greatly reduced in inner ears of *Mctp1*<sup>dwnd/dwnd</sup> mice. (5) The mutant phenotype of *Mctp1*<sup>+ / dwnd</sup> *Nr2f1*<sup>+ / -</sup> transheterozygotes is similar to that of *Nr2f1*<sup>- / -</sup> and *Mctp1*<sup>dwnd/dwnd</sup> mice, indicating genetic non-complementation of *Mctp1*<sup>dwnd</sup> and *Nr2f1*<sup>- / -</sup> alleles and demonstrating a cis-regulatory effect of the *Mctp1*<sup>dwnd</sup> deletion on *Nr2f1* expression. The region deleted by the *Mctp1*<sup>dwnd</sup> mutation is necessary for the normal expression of *Nr2f1* during inner ear development, but the phenotypic effect of the deletion is not necessarily due to the loss of a single enhancer element. The deletion may eliminate multiple enhancer elements, or it may disrupt the 3D chromosomal conformation necessary for proper enhancer interaction with the *Nr2f1* promoter. Further experiments, including reporter assays for enhancer activity, are required to determine the underlying mechanism of this long-range, cis-regulatory region.

The *Mctp1*<sup>dwnd</sup> deletion causes loss of *Nr2f1* expression in a tissue-specific manner. We found no effect of the deletion on NR2F1 expression in the retina, although expression was dramatically reduced in the inner ear (Fig. 3). *Mctp1*<sup>dwnd/dwnd</sup> mutants did not exhibit the lethality of *Nr2f1*<sup>- / -</sup> mutants, indicating that their defects are restricted to organs, such as the inner ear, that are not critical to post-natal



**Fig. 5.** Hair cell patterning in inner ears of *Mctp1*<sup>dwnd/dwnd</sup>, *Mctp1*<sup>+/dwnd</sup> *Nr2f1*<sup>+/-</sup>, and *Mctp1*<sup>-/-</sup> mice. All panels show phalloidin-stained flat mounts. (A) P0 organ of Corti for the genotypes and positions indicated. *Mctp1*<sup>+/dwnd</sup> *Nr2f1*<sup>+/-</sup> transheterozygotes show hair cell disorganization at the base (top) and extra OHC rows at the apex (bottom, arrowheads), as seen in the *Mctp1*<sup>dwnd/dwnd</sup> mutants (see Fig. 1B–E). In contrast, hair cell organization in targeted *Mctp1* mutants (*Mctp1*<sup>-/-</sup>) looks normal. (B) P0 utricle with the sensory region (macula) outlined. Transheterozygotes show a dysmorphic macula, unlike *Mctp1*<sup>-/-</sup> mutants. (C) P0 cochlear base showing failed separation of the sensory epithelia of the cochlea and the saccular macula in transheterozygotes. Arrowheads indicate the basal most position of the organ of Corti. *Mctp1*<sup>-/-</sup> have a normal saccule (not shown). *Mctp1*<sup>+/dwnd</sup> is used as control. Scale bars, 10  $\mu$ m (A), 100  $\mu$ m (B) and 20  $\mu$ m (C).

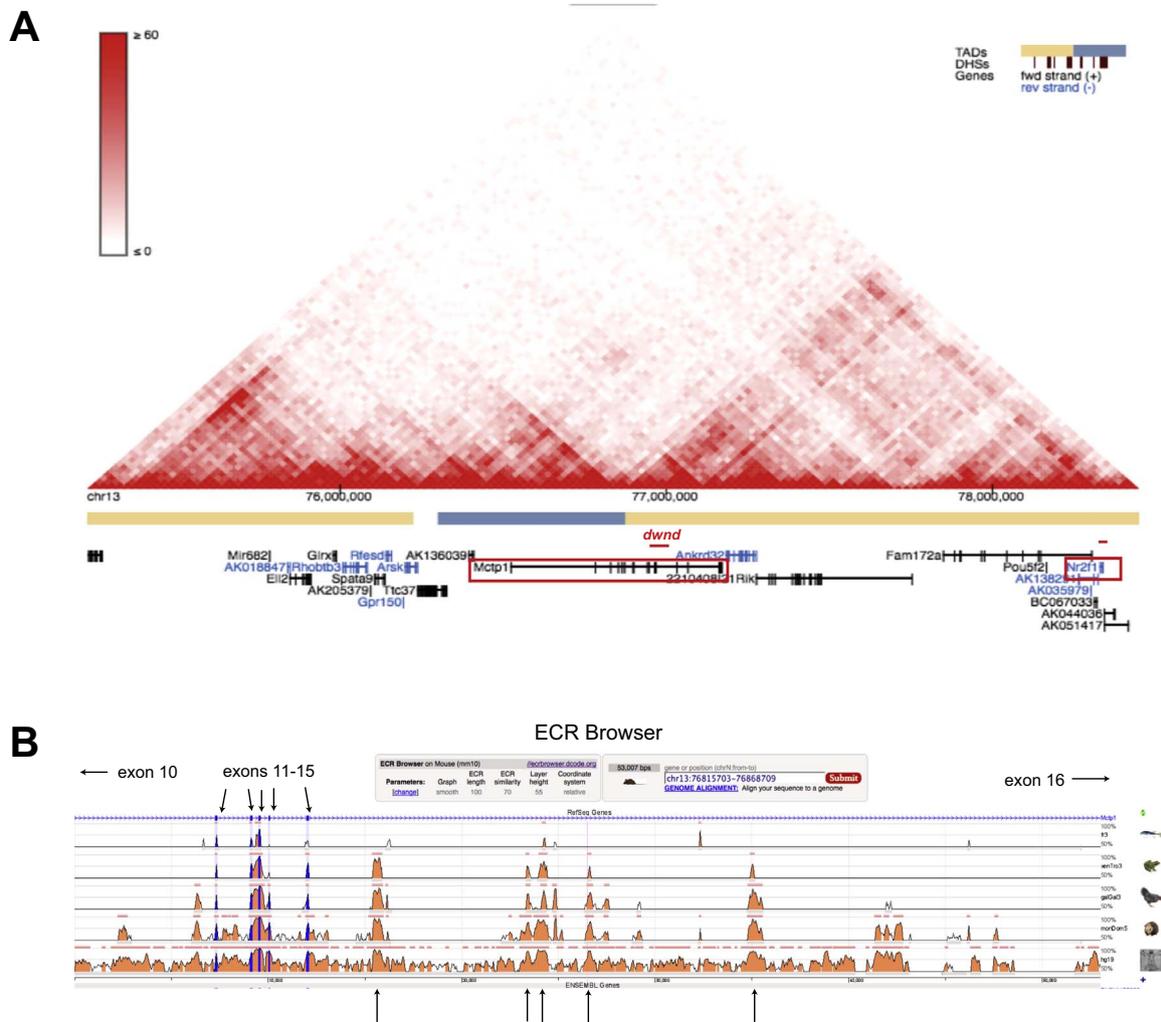
viability. The non-lethality of *Mctp1*<sup>dwnd/dwnd</sup> mutant mice is useful for studying the effects of *Nr2f1* deficiency specifically on inner ear function without the restrictions imposed by the perinatal lethality of *Nr2f1*<sup>-/-</sup> knockout mice. This advantage allows for studies of *Nr2f1* deficiency in adults, including examinations of age-related changes in inner ear histology and auditory and vestibular physiology, which are not possible in embryos or neonates.

The cis-regulatory region we discovered within the *Mctp1* gene, which is 1.4 Mb from its target gene *Nr2f1*, may represent the most distantly located mammalian enhancer thus far reported with an associated phenotypic consequence. The most distant regulatory element we could find in a search of previous publications is an enhancer associated with preaxial polydactyly that is located in intron 5 of the *Lmbr1* gene, which is 1 Mb from its target gene *Shh* (Lettice et al., 2003). Large regulatory domains of chromosomes have been shown to correspond with topologically associating domains (TADs), self-interacting physical domains that limit the extent of enhancer interactions with target genes (Symmons et al., 2014). In mammals, TAD sizes are estimated to vary from 40 kb to 3 Mb, with a median size of 185 kb (Rao et al., 2014). The 1.4 Mb distance between the *Mctp1*<sup>dwnd</sup> regulatory region and its *Nr2f1* target thus falls within the expected size range of TADs.

To identify TAD boundaries for the *Mctp1*<sup>dwnd</sup> regulatory region, we used the 3D Genome Browser: a web-based browser for visualizing 3D genome organization and long-range chromatin interactions (<http://promoter.bx.psu.edu/hi-c/>). Although the Hi-C dataset accessed from this website was generated from mouse B-lymphoblast cells (Rao et al., 2014), TAD boundaries are typically invariant across different cell types and between species and should also apply to cells of the embryonic inner ear (Dixon et al., 2015; Jin et al., 2013; Lonfat et al., 2014). According to the Hi-C heatmap generated from this dataset, the *Mctp1*<sup>dwnd</sup> deletion and the *Nr2f1* gene are located in the

same TAD (Fig. 6A), supporting the possibility of a long-range physical interaction between these loci, as was deduced from our genetic analysis and expression results. The 1.55 Mb TAD containing the *Mctp1*<sup>dwnd</sup> regulatory region and *Nr2f1* spans the 76,900,000–78,450,000 bp region of Chr 13 and includes four other protein-coding genes: *Pou5f2*, *Fam172a*, *2210408I21Rik*, *Slf1*. Because enhancer activity is not necessarily gene specific, the *Mctp1*<sup>dwnd</sup> enhancer region may also activate one of these four genes; however, inner ear expression has not been reported for any of them.

The 53 kb *Mctp1*<sup>dwnd</sup> deletion spans the 76,815,703–76,868,709 bp region of Chr 13 (GRCm38), which contains exons 11–15 of *Mctp1* and surrounding intronic regions (Fig. 6B). As a first approach to identifying potential regulatory elements within this region, we used a comparative genomic strategy because regulatory sequences, like protein-coding sequences, are often under evolutionary constraint (Visel et al., 2009). We used the ECR Browser (<https://ecrbrowser.dcode.org/>) to visualize evolutionarily conserved regions, comparing DNA sequence similarities of mouse with other vertebrate species (human, opossum, chicken, frog, and fish). In addition to conserved sequences corresponding to exons 11–15, five non-coding sequences located in the large intron between exons 15 and 16 are highly conserved in all of the vertebrates examined except fish (Fig. 6B) and thus could be considered candidates for enhancer elements. Homozygosity for the *Mctp1*<sup>dwnd</sup> deletion reduces *Nr2f1* expression during inner ear development, which causes malformation of the utricle and saccule as well as the cochlea. Because *Nr2f1* is expressed early in the developing otic vesicle of mice (Tang et al., 2005), sequences regulating its expression are likely to be evolutionarily conserved in other vertebrate species, which share many aspects of inner ear development (Groves et al., 2012). Regulatory sequences affecting *Nr2f1* expression in inner ears of mice, however, may not be conserved in fish ears, which use vestibular sensory organs rather than



**Fig. 6. Supplementary information accessed from public database browsers.** (A) *Mctp1<sup>dwnd</sup>* and *Nr2f1* are in the same TAD. The *Mctp1<sup>dwnd</sup>* deletion and the *Nr2f1* gene (shown by horizontal red bars above the gene diagrams enclosed by red rectangles) are located in the same topologically associating domain (TAD), supporting the possibility of long-range chromatin interactions. The Hi-C heatmap was obtained from the 3D Genome Browser: a web-based browser for visualizing 3D genome organization and long-range chromatin interactions (<http://promoter.bx.psu.edu/hi-c/>). Although the heatmap shown in this figure was generated from Hi-C analysis of mouse B-lymphoblast cells (Rao et al., 2014), TADs are relatively constant between different cell types and are highly conserved across species, and so should be similar in the mouse embryonic inner ear. (B) Conserved DNA sequences suggest possible enhancer elements in the *Mctp1<sup>dwnd</sup>* deleted region. Multiple phylogenetically conserved DNA sequences are present in the 53 kb *Mctp1<sup>dwnd</sup>* deletion (Chr13: 76,815,703-76,868,709; GRCm38). The ECR Browser (<https://ecrbrowser.dcode.org/>) was used to compare DNA sequence similarities of mouse with other vertebrate species (human, opossum, chicken, frog, and fish). In addition to the sequences corresponding to exons 11–15 (indicated by downward pointing arrows), five non-coding sequences are highly conserved in all of the vertebrates except fish (indicated by upward pointing arrows).

a distinct auditory epithelium to respond to sound. In addition to DNA sequence conservation, potential enhancer elements also can be detected by their chromatin signatures, which require high throughput DNA sequencing often combined with chromatin immunoprecipitation (ChIP-seq) for their identification, and include transcription factor and co-factor binding sites, chromatin accessibility, histone modifications, and 3C-based methods to identify physical interactions of enhancers and promoters (Shlyueva et al., 2014). The DNA sequences selected as enhancer candidates will require functional studies to determine if they increase *Nr2f1* expression in the inner ear or if their loss diminishes expression.

The cochlear abnormalities we observed in *Mctp1<sup>dwnd/dwnd</sup>* mice are nearly identical to those reported in *Nr2f1<sup>-/-</sup>* mice (Tang et al., 2006). The shorter cochlear duct (Fig. 1F,G, J) and the extra hair cells at the cochlear apex (Fig. 1E) in the inner ears of these mutant mice may be inter-related and could be the result of incomplete intercalation of post-mitotic prosensory cells during cochlear convergent extension (Driver et al., 2017). We also discovered vestibular abnormalities in inner ears of *Mctp1<sup>dwnd/dwnd</sup>* and *Nr2f1<sup>-/-</sup>* mice, a finding that was not reported by Tang and colleagues (Tang et al., 2006), although a

sacculus abnormality is apparent in Fig. 1B of their paper. We observed that the sacculus is much reduced in size and fused with the utricle in inner ears of *Nr2f1<sup>-/-</sup>* and *Mctp1<sup>dwnd/dwnd</sup>* mice and in *Mctp1<sup>+/-dwnd</sup>* *Nr2f1<sup>+/-</sup>* transheterozygotes (Fig. 4B), similar to the fused utriculosacculus chambers reported in inner ears of mice with mutations of other developmental control genes, including *Lmx1a* (Nichols et al., 2008; Steffes et al., 2012), *Otx1* (Morsli et al., 1999), *Hmx2* (Wang et al., 2001), and *Mycn* (N-myc) (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). We also observed a large cochleosacculus duct (ductus reuniens) in the mutant inner ear (Fig. 1G) and a failed separation of the saccular macula from the cochlear base (Fig. 5C), which may be related to the disorganization of hair cells at the cochlear base (Fig. 1C, Fig. 5A). These observations are similar to, but less pronounced than, those reported for *Lmx1a<sup>-/-</sup>* and *Mycn<sup>-/-</sup>* mutant mice, in which the utricle, sacculus, and cochlea appear continuous with one another.

In contrast to *Lmx1a<sup>-/-</sup>*, *Otx1<sup>-/-</sup>*, *Hmx2<sup>-/-</sup>*, and *Mycn<sup>-/-</sup>* mutant mice, development of the dorsal otic epithelium appears unaffected in *Nr2f1*-deficient mice, with grossly normal endolymphatic sac and duct, semicircular canals, and ampullae (Fig. 4B). A recently proposed model of inner ear sensory patch formation (Mann et al., 2017) suggests that

sensory patch boundaries are determined by the antagonistic effects of Notch signaling, which promotes *Sox2* expression and sensory domain formation, and *Lmx1a* expression, which promotes cellular commitment to a non-sensory fate. According to this model, loss of *Lmx1a* expression in boundary regions would lead to an expansion and fusion of sensory compartments. Our results suggest that *Nr2f1* may have a more limited role in the establishment of sensory patch boundaries than *Lmx1a*, being restricted to boundaries separating utricle, saccule, and cochlea.

Enhancers that affect the expression of other transcription factors and signaling molecules involved in inner ear development have been described. Loss of a long-range enhancer region located 900 kb upstream of the POU-domain transcription factor *POU3F4* gene was found to be associated with instances of X-linked deafness type 3 (de Kok et al., 1996). Multiple potential enhancers in a 1-Mb region upstream of *POU3F4* were later identified by the evolutionary conservation of their DNA sequences and their ability to enhance otic expression of a reporter gene in transgenic *Xenopus* and zebrafish (Naranjo et al., 2010). Evolutionarily conserved sequences and transgenic reporter assays have been used to identify potential inner ear enhancers of other developmentally important genes, including *Fgf10* (Ohuchi et al., 2005), *Sox9* (Bagheri-Fam et al., 2006), and *Six1* (Sato et al., 2012). More recently, the deletion of a long-range enhancer region located within the *Sle25a13* gene was shown to reduce *Dlx5* expression and disrupt inner ear development in the mouse (Johnson et al., 2018). Our discovery of a long-range regulator of *Nr2f1* expression adds to the growing list of cis-regulatory elements that determine the spatiotemporal expression patterns of key genes involved in the development of the inner ear.

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