The chromatin remodelling factor Chd7 protects auditory neurons and sensory hair cells from stress-induced degeneration

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Neurons and sensory cells are particularly vulnerable to oxidative stress due to their high oxygen demand during stimulus perception and transmission. The mechanisms that protect them from stress-induced death and degeneration remain elusive. Here we show that embryonic deletion of the chromodomain helicase DNA-binding protein 7 (CHD7) in auditory neurons or hair cells leads to sensorineural hearing loss due to postnatal degeneration of both cell types. Mechanistically, we demonstrate that CHD7 controls the expression of major stress pathway components. In its absence, hair cells are hypersensitive, dying rapidly after brief exposure to stress inducers, suggesting that sound at the onset of hearing triggers their degeneration. In humans, CHD7 haploinsufficiency causes CHARGE syndrome, a disorder affecting multiple organs including the ear. Our findings suggest that CHD7 mutations cause developmentally silent phenotypes that predispose cells to postnatal degeneration due to a failure of protective mechanisms.
Sensorineural hearing loss (SNHL) is a common feature of CHARGE syndrome and affects 50–70% of individuals. Mice with heterozygous Chd7 mutations are an excellent model for CHARGE syndrome and, like humans, they exhibit SNHL. Chd7 plays an important role during neurogenesis both in the brain and the inner ear. At embryonic day (E) 9.5-E10.5, neuronal progenitors are reduced in the inner ear of Chd7+/− mutants, and Chd7 is necessary for their proliferation. However, by E11.5, the number of neuronal progenitors is restored and inner ear neurons, as well as the hair cells they innervate, appear normal after birth. Therefore, the cellular function of Chd7 and the mechanisms underlying SNHL have yet to be elucidated.

In the cochlea, inner hair cells are responsible for sound perception, while outer hair cells modulate the sound amplitude. They are innervated by type I and type II spiral ganglion neurons, respectively, which project to the auditory nuclei in the brain stem. As the hair cells (and spiral ganglion neurons) lack the capacity to regenerate, oxidative stress caused by loud noise, ageing, or ototoxicity leads to cell death and permanent hearing loss.

Results
In mice, hair cell specification mediated by the transcription factor Atoh1 occurs between E12.5 and E16.5. However, their development continues postnatally and they reach maturity just before the onset of hearing between postnatal day 10 (P10) and P14. Chd7 is expressed in hair cells and neurons throughout embryonic and postnatal stages. To investigate its function in hair cells, we deleted Chd7 using the hair cell-specific Atoh1Cre driver (Atoh1Cre+/−;Chd7flox). We compared Cre-positive and Cre-negative control with Chd7 heterozygous and homozygous littermates. In Atoh1Cre+/−;Chd7fl ox mice, Chd7 deletion was initiated in hair cells from E12.5 onwards and we confirmed loss of Chd7 expression in the basal and middle turns of the cochlea at E16.5. Surprisingly, hair cells appeared normal at P8 (n = 10/10; Fig. S2a, b) suggesting that Chd7 function may not be required for their development.

We, therefore, wished to confirm hair cell integrity and functionality in the early postnatal cochlea. A key feature of hair cells is their ability to transform mechanical force into electrical energy that in turn elicits action potentials in spiral ganglion neurons. This requires functional mechanotransduction channels located in the hair cell stereocilia. We visualised uptake of the styryl membrane dye FM1-43 through these channels to assess their activity. Cochleae isolated from P3 mice were exposed to FM1-43 for 30 s and imaged using confocal microscopy. Hair cells from both Atoh1Cre+/− (or Chd7+/+) control and Atoh1Cre+/−;Chd7fl ox homozygous mutant mice showed robust FM1-43 uptake, indicating that mechanotransduction in mutant hair cells was relatively normal. To investigate whether mutant inner hair cells established connectivity with spiral ganglion neurons, we assessed the presence and density of ribbon synapses; there was no difference between controls and mutants at P7. Together, these results suggest that although Chd7 is deleted at embryonic stages as hair cells are specified, hair cells develop with normal morphology until the first postnatal week with intact mechanotransduction channels and neuronal connections.

Thereafter we observed rapid degeneration of inner hair cells at P10 (n = 6/8) and P14 (n = 15/16), while outer hair cells degenerated more slowly. By P21, most inner hair cell nuclei were missing, pyknotic or fragmented, and outer hair cells were still present.

Fig. 1 Chd7 expression in wildtype and Atoh1Cre+/−;Chd7fl ox mutant organ of Corti. a Immunohistochemistry in wildtype cochlea showing Chd7 expression in hair cells at E16.5 and P7. Expression is weaker at P7 compared to E16.5. Hair cells are stained with F-actin and Calbindin 1. Tectorial membrane is stained with Satb2. b In-situ hybridisation showing loss of Chd7 expression in Atoh1Cre+/−;Chd7fl ox mutant hair cells within the middle region of the cochlea at E16.5. Atoh1Cre+/− or Chd7fl oxed siblings were used as controls (indicated at the top of the panel). IHC inner hair cells; OHC outer hair cells; TM tectorial membrane. Scale bars 25 μm.
indicating progressive degeneration and cell death \((n = 8/8;\) Fig. 3m, n, Fig. S2b). Chd7 heterozygous mutants showed equally severe phenotypes (Fig. S2c) but at a reduced frequency \((n = 5/24).\) To establish when Chd7 function is critical during hair cell formation, we used inducible \(Atoh1^\text{CreERT2}\) to temporally delete Chd7. Tamoxifen was administered at E16, with loss of Chd7 protein, particularly in inner hair cells, evident at E18.5 (Fig. S3a, b). Unlike early embryonic Chd7 deletion as soon as hair cells were specified, loss of Chd7 after E16.5 did not cause postnatal hair cell degeneration \((n = 6/6;\) Fig. S3c). These observations suggest that Chd7 loss during early hair cell development predisposes cells to postnatal degeneration which leads to SNHL.

Postmitotic neural progenitors arise in the otic vesicle from ~E9 onwards under the control of NeuroD1 and differentiate into spiral ganglion neurons by E14.23–28. However, the peripheral auditory circuit is only established in the first 10 days after birth (P0-P10), prior to the onset of hearing.24,29,30 To assess Chd7 function in spiral ganglion neurons, we analysed \(NeuroD1^\text{Cre}\);\(Chd7^{flx}\) mutants. In homozygous mutants, ganglion size and neuronal numbers were indistinguishable from controls at P1 \((n = 3/3;\) Fig. 3o, Fig. S4a), but neurons degenerated rapidly to 50% by P7 \((n = 3/3;\) Fig. 3o, p). This phenotype was also observed in Chd7 heterozygous mutants, although neurodegeneration occurred gradually with 50% loss by P21 \((n = 3/3;\) Fig. S4b). Thus, Chd7 controls the survival of a subset of spiral ganglion neurons. Like in hair cells, Chd7 deletion at embryonic stages does not appear to affect neuronal development but leads to delayed neurodegeneration postnatally.

To determine whether Chd7 deletion results in hearing loss, we measured the auditory brainstem response (ABR) of 4- and 8-week-old mutant and control animals. Most \(Atoh1^\text{Cre}\);\(Chd7^{flx}\) homozygous mutants exhibited severe-profound hearing loss across all frequencies \((n = 6/7;\) Fig. 4a, Fig. S5), while only 1/7 heterozygous mutants showed a similar ABR profile (Fig. S5). In contrast, \(NeuroD1^\text{Cre}\);\(Chd7^{flx}\) mutants exhibited moderate hearing loss \((n = 6;\) Fig. 4b, Fig. S6), presumably due to surviving neurons. Nonetheless, the ABR tests confirm that SNHL correlates with hair cell or neuronal degeneration.

Chd7 controls transcription through regulation of chromatin architecture,9,11 but how it exerts its function in auditory hair cells and neurons is poorly understood. We, therefore, examined the earliest transcriptional changes resulting from Chd7 deletion by comparing gene expression of FAC-sorted hair cells or spiral ganglia neurons from mutant and control animals (Fig. 5a, Supplementary Data 1, 4). Using the non-inducible \(Atoh1^\text{Cre}\) and inducible \(Atoh1^\text{CreERT2}\) mediated deletion of Chd7 enabled us to pinpoint the earliest developmental window in which Chd7 function is required \((i.e.,\) between E12.5 and E16.5). By E16.5, most hair cells have formed,20,21 by this time point Chd7 expression was lost in mutant cochlea. Therefore, we analysed changes in hair cell gene expression at E16.5. We were unable to determine the precise time point for Chd7 function in neurons due to the lack of an inducible \(NeuroD1^\text{CreERT2}\) transgenic line. We, therefore, chose P4 for transcriptional profiling to capture the time point when spiral ganglion neurons actively undergo cell death in mutants (Fig. 3o) and when they normally establish synaptic connections with hair cells.14,29,30

Differential gene expression analysis revealed significant changes \((FDR ≤ 0.05,\) fold change >2) in 2437 transcripts in hair cells and 1653 transcripts in neurons (Fig. 5b, Fig. S7a–d, Supplementary Data 1–4). We selected genes with high fold-change in expression (see Supplementary Data 2) as well as genes unaffected by Chd7 deletion \((e.g.,\) Atoh1) and transcripts that are normally not expressed \((e.g.,\) Satb2) for validation by qRT-PCR (Fig. 5c) and protein by immunohistochemistry (Fig. S8). These results confirmed our RNAseq data. Analysis of Disease Ontology terms for all differentially expressed genes showed enrichment of Chd7-associated syndromes as well as hearing loss. Surprisingly, there was also a strong association with neurodegenerative diseases including dementia (Fig. 5d, Supplementary Data 2, 4, 5, 6) pointing towards a common mechanism underlying these conditions.

Gene Ontology terms for RNA processing and stress pathways were strongly associated with all differentially expressed genes (Fig. 5e, Supplementary Data 3, 4), while RNA-binding proteins were among the most prominent transcripts deregulated by Chd7 deletion (Fig. 6a, b). Interestingly, previous reports showed that Chd7 binds to many of their promoters in neuronal progenitors (Fig. S7e, Supplementary Data 9; ref. 7) suggesting that they may be direct Chd7 targets. RNA-binding proteins are critical regulators of cellular stress, controlling the assembly and disassembly of stress granules.31–33 As transient membrane-less compartments, they assemble in the cytoplasm under oxidative stress conditions to allow cells to survive, while their prolonged persistence triggers apoptosis31–35. The metabolic demands of sound...
detection and amplification elicits oxidative stress in neurons and hair cells that causes cell death unless tightly regulated\textsuperscript{16,36}. We, therefore, tested the hypothesis that Chd7 mutant hair cells are hypersensitive to oxidative stress by exploiting a cochlear explant system in which oxidative stress can be induced by treatment with aminoglycosides\textsuperscript{37–39}. When P6 control explants from Atoh1\textsuperscript{Cre/+};mTmG mice were exposed to gentamicin for 5 h (100 µM), hair cells were intact along the entire length of the cochlea, as were untreated Atoh1\textsuperscript{Cre/+};Chd7\textsuperscript{fl/fl} homozygous mutant hair cells (n = 5/5 each; Fig. 7a, b). In contrast, gentamicin treatment of Atoh1\textsuperscript{Cre/+};Chd7\textsuperscript{fl/fl} mutant explants resulted in a reduction of hair cells by more than 50% across all regions of the cochlea (n = 6/6; Fig. 7a, b). These findings show that Chd7 mutant hair cells are hypersensitive to oxidative stress, causing degeneration in
controls at 4 weeks and 8 weeks reveals profound hearing loss in homozygous and mild-moderate hearing loss in heterozygous

frequencies.

****< 0.000005). See Figs. S5 and S6 for ABR pro

Fig. 4 Postnatal degeneration of hair cells and neurons in Chd7 mutants. a Auditory brainstem response (ABR) tests of Atoh1Ctrl/+;Chd7flox mutants and controls at 4 weeks and 8 weeks reveals profound hearing loss in homozygous and mild-moderate hearing loss in heterozygous Chd7 mutants across all frequencies. b ABR tests of NeuroD1Ctrl/+;Chd7flox mutants and controls at 4 weeks and 8 weeks reveals mild-moderate hearing loss in homozygous mutants. Frequencies where significant threshold elevations were observed are indicated by asterisks (P-values: *< 0.05, **< 0.005, ***< 0.0005, ****< 0.000005). See Figs. S5 and S6 for ABR profiles of each mouse. Error bars represent the standard error of mean (see Figs. S5 and S6).

CTRL = control; HET = heterozygote; HOM = homozygote.

response to stress inducers. Thus, in vivo sound exposure at the onset of hearing may trigger cell death in Chd7-deficient hair cells. Our data suggest that SNHL in CHARGE syndrome may partly be due to misregulation of RNA-binding proteins as key regulators of stress granules thereby altering the response of neurons and hair cells to normal sound.

**Discussion**

In humans, CHD7 haploinsufficiency is the major cause of CHARGE syndrome, a complex developmental disorder associated with growth retardation and defects of the eye, heart, genitals and the ear. 50–70% of CHARGE patients present with sensorineural hearing loss, however, the cellular and molecular mechanisms involved are poorly understood. Here we demonstrate that Chd7 plays an essential role in the survival of neurons and hair cells in the murine inner ear. Deleting Chd7 at early developmental stages, when hair cells or neurons are being specified and start to differentiate, does not result in overt developmental phenotypes but rather has longlasting effects on their ability to survive postnatally. What are the underlying molecular mechanisms? Other members of the Chd family are important regulators of stress and key components of these stress response pathways which demand a balanced response to stress inducers. Thus, in vivo sound exposure at the onset of hearing may trigger cell death in Chd7-deficient hair cells. Our data suggest that SNHL in CHARGE syndrome may partly be due to misregulation of RNA-binding proteins as key regulators of stress granules thereby altering the response of neurons and hair cells to normal sound.

Another recent study corroborates our finding that Atoh1Ctrl/+;Chd7f/f mutant hair cells develop with seemingly normal morphology at early postnatal stages. We show that from the second postnatal week when hair cells begin to detect sound and encounter oxidative stress, they rapidly degenerate. While a critical role for Chd7 in promoting neural progenitor expansion has been demonstrated using Neurog1Cre+;Chd7f/f mutants, the deletion of Chd7 using ShhCre+ in maturing spiral ganglion neurons has no effect on their ongoing development, at least until P14. Whether abnormalities in spiral ganglion neurons arise thereafter is unknown. Our results show that despite normal morphology at P1 in NeuroD1Ctrl/+;Chd7f/f mutants, rapid neurodegeneration is observed between P2–P7. This is a crucial and metabolically demanding period for neurons as they establish synaptic connections with hair cells and refine their activity in preparation for the onset of hearing (i.e., P10+).

Single-cell transcriptomic data reveal that type 1 spiral ganglion neurons can be divided into molecularly distinct subtypes. Identifying whether specific subtypes undergo neurodegeneration following Chd7 deletion and gaining insights into the disruption of tonotopy may guide improvements in cochlear implants for CHARGE syndrome patients. Interestingly, ~50% of NeuroD1Ctrl/+;Chd7f/f mutant spiral ganglion neurons escape degeneration. Although neurons project to the cochlear epithelium and navigate towards hair cells, our transcriptomic data indicate that subtle axonal phenotypes may

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Fig. 5 Transcriptome analysis of control and Chd7 mutant hair cells and neurons reveals misregulation of cellular stress pathways. a Schematic showing the experimental approach used for RNA sequencing. DP = double positive. n = 6 cochleae or ganglia were pooled for RNA-seq in three independent experiments per genotype. b Comparison of the number of differentially expressed genes between hair cells and neurons in homozygotes. c qPCR expression analysis of genes in controls and homozygous FAC-sorted hair cells at E16.5. Error bars represent the standard error. P-values: * = <0.05, ** < 0.005, ***< 0.0005. ns = not significant. d Plot of Odds ratio by −log10 of the P-value for human diseases identified by disease ontology. Odds ratio is used to determine the relative odds of the occurrence and magnitude of disease, given exposure to the risk factor (e.g., genes). Complete disease ontology is provided in Supplementary Data 5 and 6. e Gene ontology for differentially regulated genes. Complete gene ontology is provided in Supplementary Data 7 and 8.
contribute to hearing loss since a number of genes encoding proteins involved in axon guidance, axon elongation and fasciculation are altered in the mutants.

In summary, Chd7 emerges as a key coordinator of cellular stress proteins. Its embryonic deletion leads to an imbalance in stress pathways that does not affect the normal development of neurons or hair cells. Axons navigate towards hair cells and ribbon synapses are correctly established. However, as cells mature and encounter environmental stress, they begin to degenerate. Our findings suggest that some neurodegenerative diseases arise from neurodevelopmental abnormalities that go undetected, and that SNHL may be an early indicator for these conditions.

**Methods**

**Animals.** The Atoh1Cre (B6.Cg-Tg(Atoh1-cre)1Bfri)\(^{88}\), Chd7\(^{fl}\)ox (B6.tm1c(EUCOMM)Wtsi\(^8\)) and NeuroD1Cre (B6.Cg-Tg(NeuroD1-cre)RZ24Gsat49 mice were maintained on a C57BL/6J genetic background. The mTmG (tm4(ACTB-tdTomato,-EGFP)Luo)\(^{50}\) mice were maintained on a 129S6/SvEv background. The Atoh1CreERT2 (Tg(Atoh1-cre/Esr1\(*)14Fsh, JAX #007684) used in Fig. S4 was maintained on an FVB/NJ background. The Chd7\(^{fl}\)oxed mice were crossed with the relevant Cre/reporter lines and backcrossed to C57BL/6J for three generations. All mice were maintained in either C57BL/6J or mixed genetic background. For tamoxifen-induced Cre recombination, a single dose of 20 mg/ml tamoxifen (Sigma, T5648) dissolved in corn oil (Sigma, C8267) was administered to pregnant Atoh1CreERT2;Chd7\(^{fl}\)ox;mTmG dams (80 mg/kg of body weight) by intraperitoneal injection. To minimise abortion, 10 mg/ml of progesterone (Sigma: P0130) was simultaneously administered at half tamoxifen dose (40 mg/kg of body weight). One injection gave a consistent recombination efficiency of 95–99%. Upon Cre-mediated recombination, targeted cells expressed membrane GFP. All animal work was performed in accordance with UK Home Office regulations. Experiments were performed on male and female littermates maintained in the same environment to avoid bias. Animals were randomly allocated to experimental groups.

**Immunohistochemistry.** Dissected inner ear tissue was fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and processed for whole-mount immunostaining, following permeabilisation with 0.2% Triton X-100/PBS for 20 min. For immunostaining on cryoprotected sections, tissues were fixed in 4% PFA in PBS and permeabilised in 0.1% Triton X-100/PBS (3 × 10 min) and blocked with 0.2% Triton X-100/5% serum/PBS (1 h at room temperature). After staining with DAPI, the cochlear tissues were washed extensively prior to mounting onto slides in 50% glycerol/PBS. For immunostaining on cryoprotected sections, tissues were fixed in 4% PFA in PBS and permeabilised in 0.1% Triton X-100/PBS (3 × 10 min) and blocked with 0.2% Triton X-100/5% serum/PBS (30 min).
sections were incubated overnight at 4 °C with primary antibodies. After several washes in 0.1% TritonX-100/PBS, the sections were incubated for 1 h at room temperature with fluorescent secondary antibodies, subsequently washed in PBS, stained with DAPI and mounted onto slides with 50% glycerol/PBS. Primary antibodies used were: rabbit Myo7a (1:100, Proteus, 25-6790); rabbit NeuN (1:1000, Abcam, ab177487); mouse NF-M (1:200, ThermoFisher Scientific, 13-0700); rabbit Sphin1 (1:500, Bethyl Laboratories, A300-936A); rabbit Lmx1a (1:100, Abcam, ab139726); rabbit Epha8 (1:100, St John’s Laboratory, ST110712); mouse Calbindin (1:50, Abcam, ab28012); mouse Parvalbumin (1:100, Sigma, P3088); mouse Satb2 (1:100, Abcam, ab281); rabbit Myo7a (1:1000, Invitrogen, A12379) or 546 (1:500, Invitrogen, anti-mouse Alexa Fluor 488 (1:1000, Invitrogen, A11001). F-actin were stained with Phalloidin 488 (1:1000, Invitrogen, A12379) or 546 (1:500, Invitrogen, A22283).

**In-situ hybridisation.** E16.5 inner ears were fixed in 4% PFA overnight at 4 °C followed by cochlea dissection in DEPC-treated PBS. In-situ hybridisation was performed using dig-labelled mouse Chd7 riboprobe.

**Auditory brainstem response (ABR).** ABR measurements were performed as described in ref.31. An audiometric profile for each mouse at 4 and 8 weeks old was obtained across a range of sound frequencies (3, 6, 12, 18, 24, 30, 36 and 42 kHz). The mice were on a mixed genetic background (C57BL/6J x 129S6/SvEv). Statistical significance was obtained using Kruskal–Wallis non-parametric ANOVA and Bonferroni-corrected significance in GraphPad Prism 9.0.0.121.

**Isolation of hair cells and neurons by FAC-sorting.** For RNA-sequencing, samples were collected for three biological replicates on independent occasions. E16.5 cochlear duct from Atoh1Cre;Chd7flox mice or P4 spiral ganglia neurons from NeuroD1Cre;Chd7mouse were isolated from inner ears in cold L-15 medium (ThermoScientific, PA5-72964); mouse C57BL/6 (1:100, BD Biosciences, 612044). Secondary antibodies were goat anti-rabbit Alexa Fluor 635 (1:500, Invitrogen, A31576); goat anti-mouse Alexa Fluor 488 (1:1000, Invitrogen, A11001). F-actin were stained with Phalloidin 488 (1:1000, Invitrogen, A12379) or 546 (1:500, Invitrogen, A22283).

**Differential gene expression analysis.** Differential gene expression analysis was performed using DESeq2 version 2.10.40.6, applying parametric fit55. Prior to differential gene expression analysis, a number of filters were applied. We considered the RPKM values of genes that are not normally expressed in E16.5 hair cells (i.e., Satb2) or P4 spiral ganglia neurons (i.e., Atoh1) and removed all genes with an RPKM value equivalent to or less than Satb2 or Atoh1. This resulted in 6910 transcripts for genes expressed in hair cells and 11,293 genes expressed in neurons. We performed a pairwise comparison between controls and homozygotes and controls and heterozygotes. Considering an adjusted p-value (FDR) of ≤0.05 and linear fold change of >2 in either direction, we found a total of 2437 genes in neurons (609 upregulated, 1423 downregulated) and 1653 genes in neurons (609 upregulated, 1044 downregulated) that were differentially expressed in Chd7 homozygous mutants compared to controls (Fig. 3b, Supplementary Data 1, E7335S/2L was used for linear regression (Agilent, 2100). Additional library quality control was performed by the Oxford Genomic Centre at the Wellcome Centre for Human Genetics (funded by the Wellcome Trust, grant 203141/Z/16/Z) and sequenced using Illumina HiSeq 4000 75 bp paired-end reads. Following quality control, paired reads were aligned to mouse MM10 genome assembly. Alignment was performed using HiSAT2 version 2.1.0 with the default parameters in Galaxy version 2.1.055. To facilitate quantitative gene expression analysis, aligned reads for each sample were counted using featureCounts version 1.6.2.54.

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**RNA purification, library preparation and RNA Sequencing.** FACS-sorted cells were processed using the NEB Monarch kit (T2010S) for polyA+ RNA isolation and NEBNext Single Cell/Low Input RNA Library Prep Kit for Illumina (E6420S/ L) and NEBNext Multiplex Oligos for Illumina (Index Primers Set 1, E7335S/L) was used for library preparation (as per kit instructions). RNA and cDNA quality was analysed using Agilent Total RNA 6000 Pico or High Sensitivity DNA Assay on a Bioanalyzer (Agilent, 2100). Additional library quality control was performed by the Oxford Genomic Centre at the Wellcome Centre for Human Genetics (funded by the Wellcome Trust, grant 203141/Z/16/Z) and sequenced using Illumina HiSeq 4000 75 bp paired-end reads. Following quality control, paired reads were aligned to mouse MM10 genome assembly. Alignment was performed using HiSAT2 version 2.1.0 with the default parameters in Galaxy version 2.1.055. To facilitate quantitative gene expression analysis, aligned reads for each sample were counted using featureCounts version 1.6.2.54.
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Acknowledgements

We thank Karen Steel, Claudio Stern and Jemeen Sreedharan for critical reading of the manuscript, Owen Harrison and Kimberley Riegman for excellent technical assistance, Zoe Mann and members of the Streit group for discussions. We thank Mary Beth Hatten for the NeuroD1Cre line. This work was supported by the MRC MR/R004625/1 and by Action on Hearing Loss (S39).

Author contributions

M.A. conceptualised and designed the study together with M.A.B. and A.S. M.A. performed most experiments and data analysis; R.M. analysed the neuronal Chd7 phenotype, while E.J. performed ABR tests. R.P. assisted in sequencing alignment and bioinformatics. M.A., A.S. and M.A.B. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s42003-021-02788-6.

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**Peer review information** *Communications Biology* thanks Angelika Doetzlhofer and the other, anonymous, reviewer for their contribution to the peer review of this work. Primary Handling Editor: Karli Montague-Cardoso.

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