Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d

Ping Chen*, Frederique Zindy†, Caroline Abdala‡, Feng Liu§, Xiankui Li*, Martine F. Roussel† and Neil Segil*§¶#

*Gonda Department of Cell and Molecular Biology, ‡Children's Auditory Research and Evaluation Center, House Ear Institute, 2100 W. 3rd St., Fifth Floor, Los Angeles, CA 90057, USA
†Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
§Neuroscience Program, University of Southern California, Los Angeles, CA 90089, USA
¶Department of Cell and Neurobiology, University of Southern California School of Medicine, Los Angeles, CA 90033, USA
#e-mail: nsegil@hei.org

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Maintenance of the post-mitotic state in the post-natal mammalian brain is an active process that requires the cyclin-dependent kinase inhibitors (CKIs) $p19^{Ink4d}$ (Ink4d) and $p27^{Kip1}$ (Kip1)[1]. In animals with targeted deletions of both *Ink4d* and *Kip1*, terminally differentiated, post-mitotic neurons are observed to re-enter the cell cycle, divide and undergo apoptosis. However, when either *Ink4d* or *Kip1* alone are deleted, the post-mitotic state is maintained, suggesting a redundant role for these genes in mature neurons[1]. In the organ of Corti — the auditory sensory epithelium of mammals — sensory hair cells and supporting cells become post-mitotic during embryogenesis[2] and remain quiescent for the life of the animal. When lost as a result of environmental insult or genetic abnormality, hair cells do not regenerate, and this loss is a common cause of deafness in humans[3]. Here, we report that targeted deletion of *Ink4d* alone is sufficient to disrupt the maintenance of the post-mitotic state of sensory hair cells in post-natal mice. In $Ink4d^{-/-}$ animals, hair cells are observed to aberrantly re-enter the cell cycle and subsequently undergo apoptosis, resulting in progressive hearing loss. Our results identify a novel mechanism underlying a non-syndromic form of progressive hearing loss in mice.

Starting between embryonic day 12.5 (E12.5) and E13.5, the cells in the dorsal wall of the embryonic cochlear duct that will go on to form the organ of Corti exit the cell cycle in a relatively synchronous wave to form a zone of non-proliferating cells (ZNPC)[2,4]. Establishment of the ZNPC is dependent on the onset of *Kip1* expression, which coincides spatially and temporally with its formation[5]. Subsequently, between E15.5 and E17.5, cell differentiation patterns the ZNPC into the stereotyped mosaic of hair cells and surrounding supporting cells that characterize the mature organ of Corti (Fig.1a–c). At post-natal day 5 (P5), the appearance of the hair cell mosaic is identical in $Ink4d^{+/+}$ (Fig. 1a) and $Ink4d^{-/-}$ (Fig. 1d) animals, with complete rows of both inner and outer hair cells present. This indicates that embryonic patterning of the organ of Corti occurs normally in the absence of Ink4d. However, by 2.5 weeks post-natal, hair cell loss was observed in the $Ink4d^{-/-}$ organ of Corti among both inner and outer hair cells, although inner hair cells and the innermost row of outer hair cells in the basal to mid-basal regions of the cochlear duct were most affected (Fig. 1e, arrowheads; see Table 1 for quantification). In contrast, no hair cell loss was observed in $Ink4d^{+/+}$ littermates (Fig.1b). By seven weeks post-natal, hair cell loss in $Ink4d^{-/-}$ animals had progressed (Fig. 1f, arrowheads), and now affected all rows of hair cells, although hair cell loss was greater among inner hair cells (43.3%) when compared with all three rows of outer hair cells (27.8%; 8.1% and 8.5%, respectively; see Table 1). At 7 weeks, the inner and outer hair cells in $Ink4d^{+/+}$ littermates remained intact (Fig. 1c).

To assess whether the *Ink4d* mutant animals experienced a progressive hearing loss, we recorded both distortion product otoacoustic emissions (DPOAE), which specifically evaluate outer hair cell function, as well as the click-evoked auditory brain stem response (ABR). DPOAEs and ABRs have been used to compare hearing in inbred strains of mice[6,7]. DPOAE amplitudes in $Ink4d^{+/+}$ animals were measured from 1,500–20,000 Hz. Between 9,000 to 20,000 Hz, DPOAEs in wild-type littermates averaged 11 dB sound pressure level (SPL) in amplitude (Fig. 2a). In contrast, in $Ink4d^{-/-}$ animals at both 7 and 15 weeks of age, DPOAEs were absent or occurred at very low levels across the entire frequency range tested (Fig. 2a).

At seven weeks of age, ABR thresholds in $Ink4d^{-/-}$ mice were unaffected relative to $Ink4d^{+/+}$ littermates, with a threshold between 10 and 20 dB SPL (Fig.2b), probably reflecting the relatively limited hair cell loss at this time. However, by 15 weeks of age, $Ink4d^{-/-}$

Table 1 Quantification of hair cell loss in the basal and mid-basal region of the cochlear duct of *Ink4d*-mutant animals.

	Inner hair cells	Row 1 outer hair cells	Row 2 outer hair cells	Row 3 outer hair cells
5 days post-natal (n = 3)	0	0	0	0
2.5 weeks post-natal (n = 4)	6.4% (± 2.9)	7.5% (± 4.5)	1.4% (± 1.7)	0
7 weeks post-natal (n = 5)	43.3% (± 7.1)	27.8% (± 3.0)	8.1% (± 3.0)	8.5% (± 5.3)

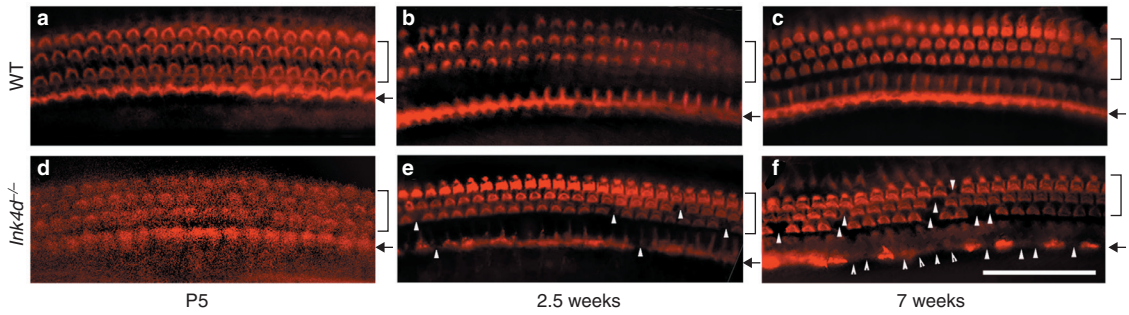

Figure 1 **Progressive hair cell loss in *Ink4d*$^{-/-}$ post-natal animals. a–f**, Representative pictures from whole mounts of the organ of Corti in the basal to mid-basal region of the cochlear duct. Whole mounts were labelled with rhodamine–phalloidin to identify the actin-rich stereocilia and cuticular plates of the hair cells. Black arrows indicate the row of inner hair cells and brackets indicate the three rows of outer hair cells. Organs of Corti were isolated from wild-type (**a**–**c**) and *Ink4d*$^{-/-}$ (**d**–**f**) embryos at post-natal day 5 (P5; **a** and **d**), 2.5 weeks post-natal (**b** and **e**) and 7 weeks post-natal (**c** and **f**). White arrowheads in **e** and **f** indicate missing hair cells. Loss of hair cells in *Ink4d*$^{-/-}$ animals was confirmed by staining with additional hair cell markers, Myosin VIIa and Math1 (data not shown). Scale bar represents 50 μm.

mice had undergone a shift in ABR threshold to ~40 dB SPL, indicative of significant hearing loss. At seven months of age, *Ink4d*$^{-/-}$ animals showed a continuing increase in ABR threshold (~50 dB SPL; Fig. 2b) when compared with age-matched inbred C57BL/6 animals, which showed the previously reported 20–30 dB SPL threshold shift characteristic of this cell line[8,9] (see Methods). All heterozygous animals tested had normal ABRs (data not shown), suggesting a recessive-type mutation. These measures indicate that hearing is significantly compromised, starting between 7 and 15 weeks of age, and that hearing loss progresses into adulthood as a result of mutation in the *Ink4d* gene.

As abnormal mitotic activity results in cell death in mature neurons of *Ink4d Kip1* double-mutants[1], we tested whether similar abnormal mitotic activity in mature hair cells correlated with their disappearance in *Ink4d*$^{-/-}$ animals. Ten-day-old *Ink4d*$^{+/+}$ and *Ink4d*$^{-/-}$ animals were injected with bromodeoxyuridine (BrdU) to label cells undergoing DNA synthesis. Although BrdU incorporation was never detected in the hair cells of wild-type animals (Fig. 3a), it was detected in the hair cells of *Ink4d*$^{-/-}$ mice (Fig. 3b). Supporting cells remained unlabelled, as in wild-type animals. This contrasts with the situation in *Kip1*$^{-/-}$ mice, where cells in the supporting cell region of the organ of Corti were observed to incorporate BrdU, whereas hair cells remained quiescent (Fig. 3c; also see refs 5, 10). To assess the mechanism of hair cell loss, sections through the organ of Corti were stained with an antibody against activated Caspase 3 (Casp3), a marker of cells dying by apoptosis[11]. Casp3-positive hair cells were observed in *Ink4d*$^{-/-}$ animals (Fig. 3e). Quantitative analysis in each of two complete whole-mount preparations of individual organs of Corti indicated that a small number of BrdU-positive cells (three and seven, respectively) were labelled after an 8-h BrdU pulse. A comparably small number of Casp3-positive cells were observed in two complete whole-mount preparations (six and eight, respectively). No BrdU- or Casp3-positive cells were ever observed in control wild-type whole mounts or sections. The small number of BrdU-positive and Casp3-positive hair cells observed in *Ink4d*$^{-/-}$ mice at single times is consistent with the progressive nature of hair cell loss observed in these mice and suggests that loss of control of the post-mitotic state results in death of hair cells.

In situ hybridization of the developing cochlea indicates that *Ink4d* mRNA is co-expressed with p27^{Kip1} in the sensory primordium at the time of cell cycle exit (E14.5; compare Fig. 4a with 4c), before differentiation of hair cells and supporting cells[4]. Expression persists in the differentiating organ of Corti at E16.5 as hair cells continue to differentiate (compare Fig. 4b with 4d). Expression of *Ink4d* mRNA in differentiated hair cells was confirmed by RT-PCR using purified hair cells obtained by FACS from a transgenic mouse expressing enhanced green fluorescent protein (EGFP) under the control of the Math1 promoter[4] in hair cells (Fig. 4e, f; see Methods). Interestingly, although expression of *Ink4d* in differentiated hair cells is consistent with its role in maintaining the post-mitotic state, its earlier expression in the sensory primordium does not seem to be essential. This is shown by the fact that formation of the ZNPC and the subsequent patterning of sensory hair cells in the organ of Corti is normal in *Ink4d*-null embryos (compare Fig. 5a with 5b, and Fig. 5d with 5e, respectively). These observations are in contrast to expression of *Kip1*, which is required for cell cycle exit (Fig. 5c) and normal morphogenesis (Figs 3c and 5f) of the developing organ of Corti[5,10].

Our data emphasize the importance of CKIs in active maintenance of the post-mitotic state and the role of cell-type-specific developmental CKI regulation in this process. A growing number of studies indicate that neuronal apoptosis associated with several neuro-degenerative diseases is preceded by re-activation of the G1 cell cycle machinery (for review, see refs 12–14). If cell cycle re-entry results in apoptosis in these cases, the level and type of CKI expression could determine the sensitivity of terminally differentiated cell types, such as neurons and hair cells, to apoptotic stimuli. Whereas p19^{Ink4d} and p27^{Kip1} seem to have redundant roles in maintenance of the post-mitotic state in the brain [1], our data from the inner ear suggest a model in which hair cells are dependent on p19^{Ink4d} alone, as a result of the developmentally controlled down-regulation of p27^{Kip1} in these cells[5].

The level and cell-type specificity of CKI expression in the human inner ear is not currently known. However, individual differences in CKI expression levels could result in a predisposition to hair cell loss, perhaps by influencing the relative sensitivity of hair cells to apoptotic stimuli. Although no currently mapped mutations resulting in hearing loss have been linked to the *Ink4d* locus in humans (*CDKN2D*), progressive hearing loss is a common disorder in the general population[15]. Our data suggest that a mutation in the functional homologue of *Ink4d* or a disturbance of its regulated expression, such as gene silencing, has the potential to be the underlying cause of hearing loss in a subset of these patients. Therefore, mutational analysis of this locus in patients experiencing hearing loss is warranted.

The fact that Ink4d and Kip1 are involved in maintaining cellular quiescence suggests that CKIs may also influence the regenerative potential of the inner ear. In birds, if the auditory sensory epithelium is damaged, robust regeneration of hair cells occurs through the stimulated proliferation and differentiation of normally quiescent supporting cells[16,17]. However, in the mammalian organ of Corti, loss of hair cells does not result in proliferation of supporting

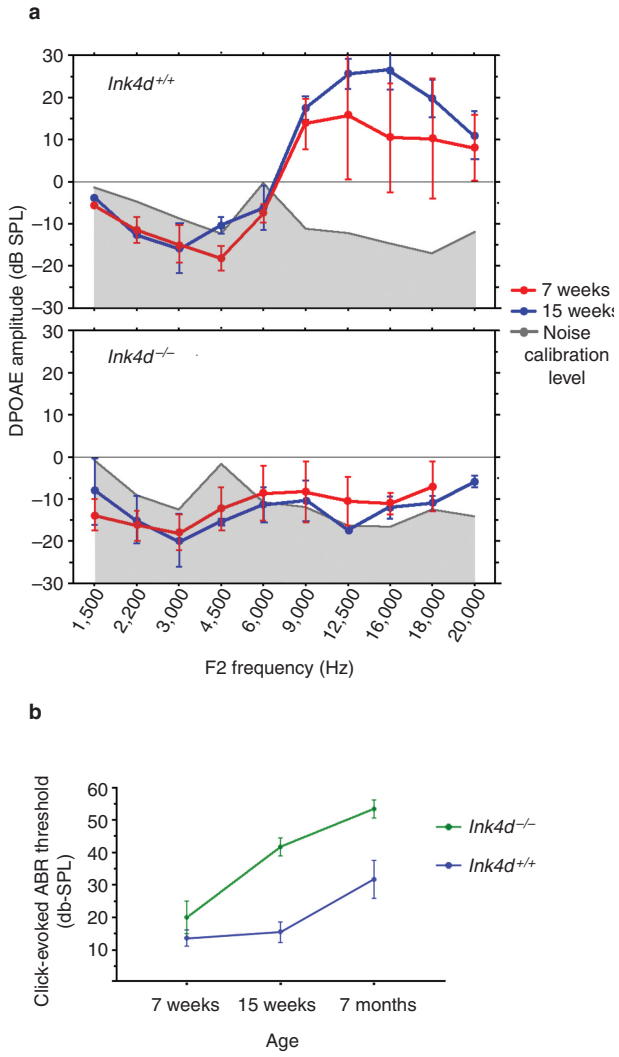

Figure 2 **Progressive hearing loss in *Ink4d*$^{-/-}$ animals. a**, Distortion Product Otoacoustic Emissions (DPOAEs). At both 7 and 15 weeks of age, DPOAE amplitudes in *Ink4d*$^{+/+}$ animals averaged between 10 and 30 dB SPL. In *Ink4d*$^{-/-}$ animals, however, DPOAEs were either absent or abnormally low. **b**, Click-evoked ABRs were measured in wild-type and mutant animals at 7 weeks, 15 weeks and 7 months of age. At 7 weeks of age, *Ink4d*$^{-/-}$ animals had ABR thresholds within the normal range. By 15 weeks, however, mutant animals showed a 20-dB SPL threshold shift that worsened by an additional 10–15 dB SPL at 7 months, compared with age-matched C57BL/6 controls. Error bars indicate standard deviation.

cells[18]. The high levels of p27^{Kip1} maintained in these cells suggests that CKIs could be appropriate targets for gene therapy aimed at reducing CKI levels and bringing about limited supporting cell proliferation within the organ of Corti to replace lost hair cells. However, our results suggest that in some terminally differentiated, post-mitotic cell populations, CKIs are crucial for maintaining cellular homeostasis, and so their manipulation should be carried out with caution. □

Methods

Animals

Targeted disruption of the *Ink4d* locus and confirmation of the lack of p19^{Ink4d} protein in homozygous mutant mice was previously described[19]. *Ink4d*-mutant mice used in experiments were maintained on a mixed genetic background (129/SvJ × C57BL/6 × CD-1). Production of *Kip1*-mutant mice was as previously described[5,20]. Genotyping of the animals was performed by PCR using primers diagnostic of the wild-type *Ink4d* locus: 5′-CAAGATGCCTCCGGTACTAG-3′ and 5′-TCCCTTCTTCAATG-GACAGG-3′ and the mutant locus containing the neomycin insert: 5′-AAGCTGACCACTGAGCTATG-3′ and 5′-CACGAGATTTCGATT CCA-3′, respectively. Animal care was in accordance with institutional guidelines.

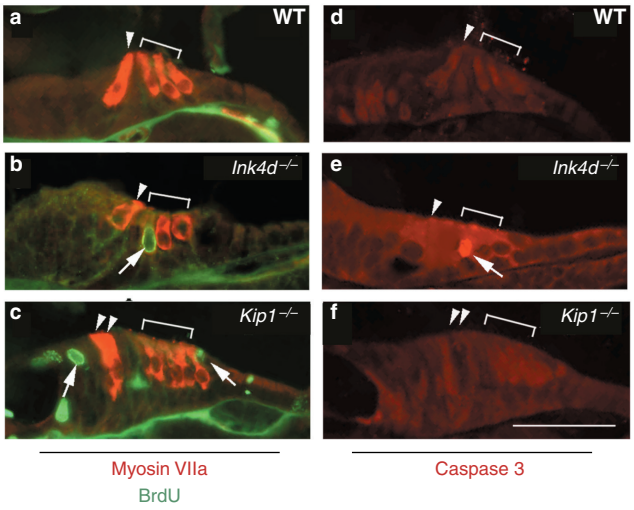

Figure 3 **Ink4d is essential for actively maintaining the post-mitotic state of hair cells. a–f,** Sections through representative P10 organ of Corti from wild-type (**a** and **d**), *Ink4d*$^{-/-}$ (**b** and **e**) and *Kip1*$^{-/-}$ (**c** and **f**) animals. **a–c**, Sections were double-labelled with antibodies against BrdU (green) and Myosin VIIa (red). **d–f**, Comparable sections were stained with an antibody against activated Caspase 3, a marker of apoptotic cells. Arrowheads indicate inner hair cells and brackets indicate outer hair cells. Arrows indicate a BrdU-positive hair cell (**b**) and an activated Caspase 3-positive hair cell (**e**) in *Ink4d*$^{-/-}$ animals, and BrdU-positive supporting cells in a *Kip1*$^{-/-}$ animal (**c**). Scale bar represents 50 μm.

Hearing tests

Animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (80 mg kg^{-1}) and xylazine hydrochloride (10 mg kg^{-1}) and maintained at 37 °C during the procedure. ABRs were recorded as previously described[5]. For the measurement of DPOAEs, stimuli were generated and data acquired using an Ariel DSP16+ signal processing and acquisition board (Ariel, Highland Park, NJ) housed in a Compaq Prolinea 590 personal computer. The Ariel board was connected to an Etymotic Research ER-10C probe system (Etymotic Research, Elk Grove Village, IL), and to an analogue high-pass filter (12 dB per octave; 710-Hz high-pass cut-off). Data were accepted only if the measured DPOAE level was at least 5 dB above the average noise measured for three frequency bins (12.2 Hz wide) on either side of the 2f1-f2 frequency. The average level of system distortion was −21 dB SPL and the recording system noise floor ranged between −22 and −27 dB SPL, depending on frequency. All tests of hearing were performed in a sound-attenuated booth. DPOAE amplitude (2f1-f2) was recorded for each animal at 10 f2 frequencies ranging from 1500 to 20,000 Hz. Stimulus levels were kept constant at 65–55 dB SPL with an f2:f1 ratio of 1:2. Between 75 and 100 sweeps were averaged to obtain each DPOAE data point. Further details of recording procedures are available on request.

Because some inbred strains of mice show age-related changes in ABR and DPOAE, we first compared *Ink4d*$^{+/+}$ animals (littermates), which have a mixed genetic background, with age-matched animals from the three constituent inbred strains (129SvJ, C57BL/6 and CD-1). On the mixed genetic background used here, no significant difference between *Ink4d*$^{+/+}$ littermates and the inbred strains were observed either for DPOAE or ABR at the ages tested (data not shown). This suggests that the inbred strains are adequate controls for comparison to the mixed background littermates. Although this observation does not preclude the possibility that strain differences may affect the severity of the *Ink4d*$^{-/-}$ phenotype, the penetrance of the phenotype was 100% in mixed background crosses.

Cell proliferation

To examine cell proliferation, mice were injected intraperitoneally with a BrdU solution (5 mg ml^{-1} in PBS at pH 7.0) four times at 2-h intervals (final concentration 50 μg BrdU per gram of body weight per injection). Two hours after the final injection, animals were euthanized and cochlear tissues were dissected and fixed with 4% paraformaldehyde in PBS. Frozen cryostat (Leica, Bannockburn, IL) sections were prepared and stained for the presence of BrdU incorporation.

Histochemistry

Immunohistochemistry using frozen tissue sections were prepared by standard procedures[21]. Antibodies used included: anti-p27(Kip1) (1:100; NeoMarkers, Freemont, CA), anti-BrdU (1:100; Chemicon, Temecula, CA), anti-activated Caspase 3 (1:100; R&D Research, Minneapolis, MN),

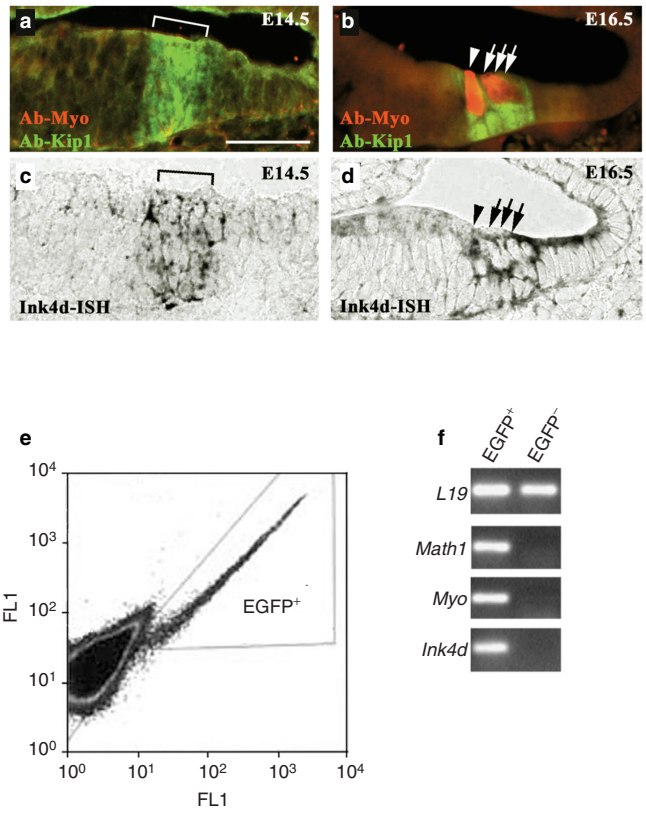

Figure 4 Ink4d in the cochlear sensory primordium and differentiated hair cells. **a**, **b**, Sections through the embryonic organ of Corti were double-labelled with antibodies against $p27^{Kip1}$ (green) and Myosin VIIa (red) to identify the $p27^{Kip1}$-positive sensory primordium at E14.5 before hair cell differentiation (indicated by the bracket in **a**), and the same region at E16.5 after Myosin VIIa-positive inner and outer hair cells have begun to differentiate (**b**, arrowhead and arrows, respectively). Note that although $p27^{Kip1}$ is present throughout the sensory primordium at E14.5, it is not present in differentiated hair cells at E16.5 (as previously described[5,10]). **c**, **d**, *In situ* hybridization using an anti-sense *Ink4d* cRNA probe. Bracket, arrows and arrowhead are as in **a** and **b**. No signal was present when a sense *Ink4d* cRNA probe was used as control (data not shown). At E14.5, expression is present in the sensory primordium (**c**, bracket), and at E16.5, expression is present in the region of differentiating hair cells (arrowhead and arrows as in **a** and **b**). The presence of signal near the lumen of the cochlear duct adjacent to the inner sulcus is unconfirmed. **e**, **f**, Differentiated hair cells (EGFP-positive) were purified by FACS (**e**) from dissociated E17.5 cochlea dissected from transgenic mice expressing EGFP in the cochlea, exclusively in hair cells (see text and ref. 4). Total RNA from equal numbers of EGFP-positive and EGFP-negative cells was analysed by RT-PCR (**f**). *Ink4d* was expressed in hair cells along with the hair-cell-specific markers Myosin VIIa and Math1. The ribosomal gene *L19* was used as a positive control. Scale bar represents 50 μm in **a**–**d**.

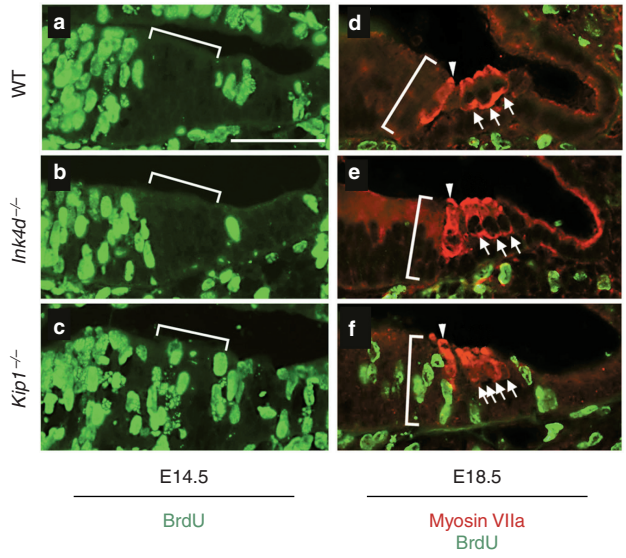

Figure 5 **Normal development of the organ of Corti in *Ink4d*$^{-/-}$ mice.** All animals were treated with BrdU for 8 h before euthanasia (see Methods). **a**–**c**, Sections through E14.5 organ of Corti from wild-type (**a**), *Ink4d*$^{-/-}$ (**b**) and Kip1$^{-/-}$ (**c**) animals stained with a BrdU antibody. At E14.5 in the wild-type animals (**a**), a zone of non-proliferating cells (ZNPC) is indicated by the lack of BrdU incorporation at the site of the sensory primordium within the cochlear duct (bracket)[4]. Note that in *Ink4d*$^{-/-}$ animals, the ZNPC forms normally (**b**), whereas in *Kip1*$^{-/-}$ animals it does not (**c**). **d**–**f**, E18.5 organs of Corti from wild type (**d**), *Ink4d*$^{-/-}$ (**e**) and *Kip1*$^{-/-}$ (**f**) animals. Sections were double-labelled with BrdU antibody (green) to detect cells in the cell cycle, and anti-Myosin VIIa to detect differentiated hair cells (red). Note that the *Ink4d*$^{-/-}$ animals have a normal complement of hair cells at this time, and no BrdU-positive cells are observed in the organ of Corti (**e**, bracket), whereas the *Kip1*$^{-/-}$ animal has an abnormal complement of outer hair cells and increased BrdU labelling in supporting cell regions (**f**, as described previously[5,10]). Arrowheads, inner hair cells; arrows, outer hair cells. Scale bar represents 50 μm.

anti-myosin VIIa (1:1000; a gift from C. Petit, Pasteur Institute). Antigen retrieval (as per manufacturer's recommendation) was required for successful use of the $p27^{Kip1}$ antibody. For BrdU staining, the tissues or sections were treated with 50% formamide/2×SSC at 65 °C for 2 h before washing with 2×SSC and treatment with 2 N HCl at 37 °C for 30 min. After neutralization with 0.1 M Boric acid at pH 8.5 for 10 min, the samples were ready for immunostaining with the antibody against BrdU. Fluorescently labelled secondary antibodies were from Jackson ImmuoResearch, West Grove, PA. Sections were photographed on a Zeiss Axiophot microscope (Zeiss, Thornwood, NY) with a digital camera. Digital images were processed in Adobe Photoshop for reproduction.

Quantification of cell loss and staining

Whole-mount preparations of the organ of Corti from *Ink4d*$^{+/+}$ and *Ink4d*$^{-/-}$ animals were stained with rhodamine–phalloidin or an antibody against Myosin VIIa. Hair cell loss in each specimen was counted by averaging missing hair cells in three or more fields, 40 cells in length from the basal to mid-basal region of each specimen. Percentage cell loss and standard deviation were calculated from 3–5 animals per experimental group. BrdU-positive and activated Caspase 3-positive cells were counted in total from whole mount preparations.

In situ hybridization

In situ hybridization was performed according to ref. 22. Briefly, 8-μm frozen sections were fixed in 4% paraformaldehyde before treatment with 50 μg ml^{-1} proteinase K and triethanolamine-HCl at pH 8.0. Sections were then subjected to prehybridization at 65 °C for 4 h and hybridized with digoxigenin (Roche Diagnostics, Basel, Switzerland)-labelled RNA probes (full-length *Ink4d* cDNA (sense or anti-sense); 2 μg ml^{-1}) overnight at 65 °C. After stringent washing, hybridization signals were visualized with an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Basel, Switzerland).

Purification of hair cells by FACS

E17.5 mouse embryos expressing EGFP under the control of the *Math1* enhancer were used[4]. Expression of EGFP in the inner ear sensory epithelium of this mouse is limited to hair cells at this stage[4]. Cochleas were dissected from embryonic inner ears, treated with trypsin-EDTA (Invitrogen, Carlsbad, CA) and 1 mg ml^{-1} collagenase (Sigma, St Louis, MO) for 1–2 h at room temperature. After removal of the enzyme solution, DMEM containing 10% foetal calf serum (FCS) was added before gentle trituration to disperse the tissues. Cell clumps and debris were removed by passing the tissue suspension through a 100-μm Falcon cell strainer (Becton Dickinson, Franklin Lakes, NJ) before FACS sorting on a MoFlo (DakoCytomation, Fort Collins, CO). Cells were sorted into EGFP-positive and EGFP-negative populations.

RNA extraction and RT–PCR

Intact cochlea (12–16) from E17.5 embryos were dissociated and used to isolate EGFP-positive cells by FACS. Approximately 10,000 EGFP-positive and EGFP-negative cells were sorted directly into Trizol solution and total RNA was isolated as per manufacturers recommendations (Promega, Madison, WI). cDNA was made using Ominiscript reverse transcriptase (Qiagen, Valencia, CA). Resulting cDNA populations were analysed by PCR to detect expression of the hair-cell markers (Math1 and MyosinVIIa), CKI (*Ink4d*), and a ribosomal gene (*L19*) as a positive control. Primers used for PCR were as follows: *Math1*: 5′-CAGATGGCCCAGATCTACATCA-3′ and 5′-CCCGCACCTCCTTCATAGG-3′; *MyosinVIIa:* 5′-CCAGGTGGTGGATGATGAAGA-3′ and 5′-ATGCCTGCCTCGTTGAGATC-3′; *Ink4d:* 5′-GCTCCGTGGTCAGCTTCCTA-3′ and 5′-CCTGCAGAATGTCCATGAGGTT-3′; *L19*: 5′-GGTCTGGTTGGATCCCAATG-3′ and 5′-CCCGGGAATGGACAGTCA-3′.

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1. Zindy, F. *et al.* Post-natal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc. Natl Acad. Sci. USA* **96,** 13462–13467 (1999).
2. Ruben, R. J. Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol* **Suppl.** 1–44 (1967).
3. Schuknecht, H. F. *Pathology Of The Ear* (Lea and Febiger, Philadelphia, 1993).
4. Chen, P., Johnson, J. E., Zoghbi, H. Y. & Segil, N. The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* **129,** 2495–2505 (2002).
5. Chen, P. & Segil, N. p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* **126,** 1581–1590 (1999).
6. Schrott, A., Puel, J. L. & Rebillard, G. Cochlear origin of 2f1-f2 distortion products assessed by using 2 types of mutant mice. *Hear. Res.* **52,** 245–253 (1991).
7. Zheng, Q. Y., Johnson, K. R. & Erway, L. C. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. *Hear. Res.* **130,** 94–107 (1999).
8. Jimenez, A. M., Stagner, B. B., Martin, G. K. & Lonsbury-Martin, B. L. Age-related loss of distortion product otoacoustic emissions in four mouse strains. *Hear. Res.* **138,** 91–105 (1999).
9. Ohlemiller, K. K. Reduction in sharpness of frequency tuning but not endocochlear potential in aging and noise-exposed BALB/cJ Mice. *J. Assoc. Res. Otolaryngol.* **3,** 444–456 (2002).
10. Lowenheim, H. *et al.* Gene disruption of p27(Kip1) allows cell proliferation in the post-natal and adult organ of corti. *Proc. Natl Acad. Sci. USA* **96,** 4084–4088 (1999).
11. Kouroku, Y. *et al.* Detection of activated Caspase-3 by a cleavage site-directed antiserum during naturally occurring DRG neurons apoptosis. *Biochem. Biophys. Res. Commun.* **247,** 780–784 (1998).
12. Liu, D. X. & Greene, L. A. Neuronal apoptosis at the G1/S cell cycle checkpoint. *Cell Tissue Res.* **305,** 217–228 (2001).
13. Herrup, K. & Yang, Y. Pictures in molecular medicine: contemplating Alzheimer's disease as cancer: a loss of cell-cycle control. *Trends Mol. Med.* **7,** 527 (2001).
14. Nguyen, M. D., Mushynski, W. E. & Julien, J. P. Cycling at the interface between neurodevelopment and neurodegeneration. *Cell Death Differ.* **9,** 1294–1306 (2002).
15. Morton, N. E. Genetic epidemiology of hearing impairment. *Ann. NY Acad. Sci.* **630,** 16–31 (1991).
16. Corwin, J. T. & Cotanche, D. A. Regeneration of sensory hair cells after acoustic trauma. *Science* **240,** 1772–1774 (1988).
17. Ryals, B. M. & Rubel, E. W. Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science* **240,** 1774–1776 (1988).
18. Chardin, S. & Romand, R. Regeneration and mammalian auditory hair cells. *Science* **267,** 707–711 (1995).
19. Zindy, F., van Deursen, J., Grosveld, G., Sherr, C. J. & Roussel, M. F. Ink4d-deficient mice are fertile despite testicular atrophy. *Mol. Cell. Biol.* **20,** 372–378 (2000).
20. Fero, M. L. *et al.* A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85,** 733–744 (1996).
21. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
22. White, P. M. & Anderson, D. J. *In vivo* transplantation of mammalian neural crest cells into chick hosts reveals a new autonomic sublineage restriction. *Development* **126,** 4351–4363 (1999).

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Correspondence and requests for materials should be addressed to N.S.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.