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Lhx3, a LIM domain transcription factor, is regulated by Pou4f3 in the auditory but not in the vestibular system

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Abstract

A dominant mutation of the gene encoding the POU4F3 transcription factor underlies human non-syndromic progressive hearing loss DFNA15. Using oligonucleotide microarrays to generate expression profiles of inner ears of *Pou4f3*$^{ddl/ddl}$ mutant and wild-type mice, we have identified and validated Lhx3, a LIM domain transcription factor, as an *in vivo* target gene regulated by Pou4f3. Lhx3 is a hair cell-specific gene expressed in all hair cells of the auditory and vestibular system as early as embryonic day 16. The level of *Lhx3* mRNA is greatly reduced in the inner ears of embryonic *Pou4f3* mutant mice. Our data also show that the expression of Lhx3 is regulated differently in auditory and vestibular hair cells. This is the first example of a hair cell-specific gene expressed both in auditory and in vestibular hair cells, with differential regulation of expression in these two closely related systems.

Introduction

The mammalian inner ear consists of six sensory organs that contain the sensory epithelia responsible for transducing sound waves and head movements into neuronal impulses, coding for hearing and balance information. The sensory epithelium of the organ of Corti is responsible for auditory processing, while five sensory organs in the vestibular system are responsible for balance. These five sensory epithelia consist of three cristae ampullaris, in the semicircular canals, and two maculae, the macula utriculi and the macula sacculi. All sensory epithelia consist of a mosaic of sensory and supporting cells. The sensory cells are named 'hair cells' due to their apical actin-rich projections named stereocilia, which deflect in response to sound or movement. This deflection results in a mechanical opening of an ion channel at the apical tip of each stereocilium. An ion influx through the opened channels results in a depolarization of the hair cells and a release of neurotransmitter at the base of the hair cell. There are at least five different supporting cell types in the cochlea, at least one in the vestibular system, and their function is still poorly understood. It has been shown that the supporting cells can recycle potassium ions and form a sealed barrier between the endolymph- and perilymph-filled spaces, thus maintaining hair cell function. The transcriptional cascades governing these processes are largely unknown. Genetic mutations leading to defects in hearing or balance may help elucidate these transcriptional pathways.

Pou4f3 (Brn3.1, Brn3c) is a class IV POU domain transcription factor that has a central function in the development and survival of all hair cells in the human and mouse inner ear sensory epithelia (Erkman *et al*., 1996; Xiang *et al*., 1997). A mutation of *POU4F3* underlies human autosomal dominant, non-syndromic progressive hearing loss DFNA15 (Vahava *et al*., 1998). In the mouse, hair cells initially differentiate in the absence of Pou4f3, but begin to undergo apoptosis at embryonic day 17 (E17), leading to a complete depletion of hair cells from all inner ear sensory epithelia by early postnatal stages (Xiang *et al*., 1998; Hertzano *et al*., 2004). We previously used the dreidel allele of *Pou4f3* (*Pou4f3*$^{ddl/ddl}$), a functional null-allele that arose on a C57BL/6J genetic background, to search for downstream targets of Pou4f3 by expression profiling of mouse inner ears. We identified *Gfi1*, Growth factor independence 1, a hair cell-specific gene, as the first bona-fide downstream target gene of Pou4f3. In the *Pou4f3*$^{-/-}$ or *Pou4f3*$^{ddl/ddl}$ mice, *Gfi1* mRNA could not be detected. It appears that outer hair cell degeneration in the *Pou4f3* mutant mice results from the lack of expression of Gfi1 (Hertzano *et al*., 2004). We have now extended our analysis to other differentially expressed genes in the ears of the *Pou4f3*$^{ddl/ddl}$ mice and identified Lhx3 as a hair cell-specific gene regulated by Pou4f3. Lhx3 is a member of the LIM homeodomain transcription factor family. We studied the developmental expression pattern of Lhx3 in the cochlea, the expression of Lhx3 in the inner ears of the *Pou4f3* and *Gfi1* mutant mice, and evaluated it as a candidate for a deafness gene in humans.

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Materials and methods

Breeding, phenotyping and genotyping of mice

All procedures involving animals met the guidelines described in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and had been approved by the Animal Care and Use Committees of the National Institutes of Health and Tel Aviv University (M-00-65). Breeding, phenotyping and genotyping of the *Pou4f3*$^{ddl/ddl}$, *Pou4f3*$^{-/-}$, *Gfi1*$^{-/-}$ and *Lhx3*$^{-/-}$ mice were described previously (Sheng *et al*., 1996; Karsunky *et al*., 2002; Hertzano *et al*., 2004). To study the inner ears of *Lhx3*$^{-/-}$ mice, cochlear cultures of inner ears from E15.5 embryos were made as described (Montcouquiol & Kelley, 2003). Time-mated female mice were killed by cervical dislocation. E18.5 mice were killed by decapitation.

RNA expression analysis

Microarray analyses, RNA extraction, reverse transcription and real time reverse transcriptase PCR (RT-PCR) were performed as described (Hertzano *et al*., 2004). The following primers and FAM-labeled probes were used: Lhx3 (F) 5′-GCAGAATTGTGCACCGTGAA-3′, (R) 5′-CCAGCCTCCTCCAGTGGAA-3′, (probe) 6FAM- CACCTTTGCCAAACAGACTGTGGTCCC-TAMRA; Scinderin (F) 5′-CATGTCCGCGTTTCTGACTGT-3′, (R) 5′-AAATAATGAGTGGTTTGTCTTTGAACA-3′, (probe) 6FAM-TGCAGGTCCGTGTCTCTCAAGGCAA-TAMRA; Stat5b (F) 5′-TGGATCCCTCACGCACAGT-3′, (R) 5′-TCCACGAGGGCGATGAA-3′, (probe) 6FAM- TGACCAGACCTCACCACCTGCAGCT-TAMRA; Calbindin2 (F) 5′-AGAACTCAGATGGGAAAATTGAGATG-3′, (R) 5′-CCCACGTGCTGCCTGAAG-3′, (probe) 6FAM-TCCTGCCGACCGAAGAGAATTTCCTTT-TAMRA; FoxA2 (F) 5′-GGAAAAGTAGCCACCACACTTCA-3′, (R) 5′-AGATCACTGTGGCCCATCTATTTAG-3′, (probe) 6FAM-CCTCAAGGGAGCAGTCTCACCTGTCTG-TAMRA; Rtkn (F) 5′-TGGAGGCACTGTTGTGATGAA-3′, (R) 5′-GATGTCGTCTAGCGGCTCAAT-3′, (probe) 6FAM-CCACCCCAAGCCCTGGTCAAGC-TAMRA; Dlgh3 (F) 5′-CCCACCCCACTATGCTCCTT-3′, (R) 5′-TGCGTCATCCCTCCAGTGT-3′, (probe) 6FAM-AAGCCAA CGTGCCTCCCTCACCTC-TAMRA. The primers and probes for the amplification of *Myo6*, *Myo7a*, *Pou4f3* and *Gfi1* were previously described (Hertzano *et al*., 2004).

For *in situ* hybridization, 647 bp from the Lhx3 3′ untranslated region (UTR) (L38248, nucleotides 1428–2074) were cloned into pGEM-T Easy (Promega, Madison, WI, USA) for the generation of digoxigenin-labeled sense and antisense probes. *In situ* hybridization of whole mounts and sections of mouse cochleae were performed as previously described, with minor modifications (Wu & Oh, 1996; Lanford *et al*., 2000).

Immunohistochemistry, peroxidase immunostaining and immunofluorescence

Immunohistochemistry, peroxidase immunostaining and immunofluorescence immunohistochemistry, peroxidase staining and immunofluorescence of whole cryoprotected inner ears were performed as described (Hertzano *et al*., 2004). For protein detection, samples were incubated with a myosin VI antibody at a dilution of 1 : 100 (Sigma, St. Louis, MO, USA) or a monoclonal antibody against Lhx3 at a dilution of 1 : 5 (DSHB, The University of Iowa).

For immunofluorescence of paraffin sections, dissected inner ears were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in a graded series of ethanol followed by a 1-h and a 2-h incubation in isopropanoland toluene. Samples were then embedded in paraffin using a Leica Histoembedder. Sections (10 μm) were dewaxed with xylene and rehydrated with a decreasing ethanol gradient. Antigen unmasking was performed using a citric acid-based antigen unmasking solution (Vector, Burlingame, CA, USA). Following a 2-h incubation in a PBSTG blocking solution (0.2% Tween 20 and gelatin in PBS), immunofluorescence was carried out as described above. The immunohistochemistry was repeated on 36 slides from eight litters in eight independent experiments.

Family enrolment, genomic DNA isolation and genotyping

Institutional Review Board approval (OH93-N-016) and written informed consent were obtained for all subjects in this study. Peripheral blood samples were obtained from participating subjects. DNA was extracted from peripheral blood or buccal swabs (BuccalAmp™ DNA Extraction Kit, Epicentre Technologies, Madison, WI, USA). Linkage analyses were performed using markers D9S1826 and D9S158 and the data were analysed using ABI GeneMapper (v.3.7) software.

Results

Lhx3 mRNA levels are decreased in the Pou4f3 mutant ears

In order to identify *in vivo* target genes of the Pou4f3 transcription factor, we hybridized RNA extracted from whole inner ears of E16.5 *Pou4f3*$^{ddl/ddl}$ and their wild-type littermates to the Affymetrix Murine Genome U74Av2 oligonucleotide microarrays. From the 12 000 probes present on the array, *Gfi1* (Gilks *et al*., 1993) was the only gene that was identified as 'present' in all samples from the wild-type inner ears while being identified as 'absent' in all samples from the mutant inner ears (Hertzano *et al*., 2004). In order to extend our search for differentially expressed genes, we performed a *t*-test analysis requiring an absolute *t* score above 1.8 and a fold change greater than 1.5. Ninety-one genes were detected as down-regulated in the samples from the *Pou4f3*$^{ddl/ddl}$ mice, as compared with samples from the wild-type mice, and 122 genes showed the opposite pattern of expression (full data set available as a supplementary table linked to Hertzano *et al*., 2004). We then selected a total of nine potential target genes for validation based on their function, *t*-test score and fold change. These genes were *Gfi1*, *Bdnf*, *Lhx3*, *Scinderin*, *Stat5b*, *Calbindin2* and *FoxA2*, all of which the microarray detected as down-regulated in the ears of the *Pou4f3* mice, as well as *Rtkn* and *Dlgh3*, which were detected as up-regulated.

At E16 and E18.5, real-time semi-quantitative RT-PCR confirmed that *Gfi1*, *Bdnf*, *Lhx3* and *Calbindin2* transcript abundance were significantly lower in the samples from the *Pou4f3*$^{ddl/ddl}$ inner ears as compared with the wild type. The expression of all four genes declined at least two-fold for one or more of these time points, in contrast to hair cell-specific genes not regulated by Pou4f3 that retained 63–88% of their mRNA abundance in the wild-type mice (Table 1) (Clough *et al*., 2004; Hertzano *et al*., 2004). Conversely, the measured decreases in the mRNA levels of *Stat5b*, *FoxA2*, *Rtkn* and *Dlgh3* were less than two-fold, and the semi-quantification of their RNA showed some variability from the Affymetrix microarray results. When we extended the validation to RNA samples from *Pou4f3*$^{-/-}$ mice, we confirmed the down-regulation of *Gfi1*, *Lhx3*, *Bdnf* and *Calbindin2* but not of *Scinderin* (data not shown).

Lhx3 is a transcription factor previously found to be associated with activation of pituitary hormone genes and, through a combinatorial control, with assignment of subtype identity of motor neurons in the spinal cord (Sheng *et al*., 1996; Sharma *et al*., 1998; Sloop *et al*., 1999). Our results indicate that *Lhx3* mRNA is expressed in the wild-type mouse inner ear. *Lhx3* mRNA levels appear to increase from E16

TABLE 1. Real time RT-PCR results

Gene		Array results		Real time RT-PCR	
Gene name	Affy ID	Fold change	*t*-score	E16	E18.5
Myo6	162245_f_at	1.2	1.73	−1.13	−1.134
	92382_at	1.03	0.43		
Myo7a	94713_at	−1.04	−0.64	−1.58	−1.18
Pou4f3	Not on array			−1.39	−1.35
Gfil†*	103259_at	−2.07	−12.17	−20.34†	−11.9
*Bdnf**	102727_at	−1.48	−4.76	−1.89	−2.01
*Lhx3**	102902_at	−1.4	−2.65	−6.3	−5.1
Scinderin‡	103715_at	−1.43	−2.52	+5.7	−1.32
Stat5b	92199_at	−1.17	−1.29	−1.5	+1.02
*Calbindin2**	95036_at	−1.48	−2.93	−9.8	−1.49
FoxA2	93950_at	−1.34	−0.97	−1.149	+1.23
Rtkn‡	162243_f_at	+1.34	1.23	−1.517	+1.11
Dlgh3	160892_at	+1.38	2.82	+1.61	−1.13

*An asterisk indicates a significant difference between wild-type and mutant mRNA transcript abundance ($P < 0.0001$). †The expression of *Gfil* in the E16 *Pouf43*$^{ddl/ddl}$ mice did not reach the detection threshold. ‡Genes having more than one probe on the array.

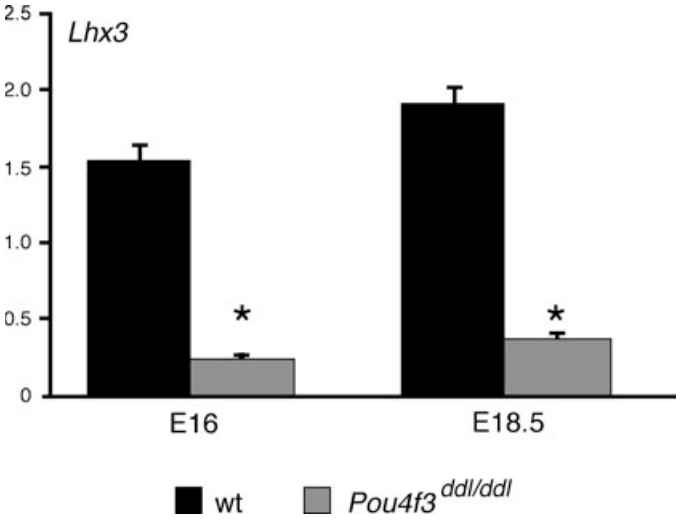

FIG. 1. *Lhx3* mRNA is 5–6-fold down-regulated in the ears of E16 and E18.5 *Pou4f3*$^{ddl/ddl}$ mice. Black bars indicate mRNA levels in wild-type inner ears and grey bars indicate mRNA levels in the *Pou4f3*$^{ddl/ddl}$ inner ears. Ordinate: mRNA levels, arbitrary units; abscissa: developmental time points. Error bars represent one standard error, $n = 6$. *$P < 0.0001$.

to E18.5 but were 6.3- and 5.1-fold reduced in the ears of the *Pou4f3*$^{ddl/ddl}$ mice at both time points, respectively ($P < 0.0001$) (Fig. 1). Similar results were obtained in the *Pou4f3*$^{-/-}$ mice (data not shown). Interestingly, although these results indicate a dramatic reduction in mRNA abundance, *Lhx3* transcripts were clearly detected in RNA extracted from whole inner ears of the mutant mice.

Lhx3 *is a hair cell-specific gene in the mouse inner ear*

The genomic structure of the mouse *Lhx3* gene, a LIM and homeodomain transcription factor, was previously characterized (Zhadanov *et al*., 1995b). 5′ rapid amplification of cDNA ends revealed that *Lhx3* encodes two alternatively spliced first exons (146 and 195 bp long), defining two isoforms, *Lhx3a* and *Lhx3b*, encoding unique 5′ UTRs and 29 or 31 unique amino acids in Lhx3a (NM_010711) and Lhx3b (L38248), respectively. Lhx3a and Lhx3b are differentially expressed in specific pituitary cell lines (Zhadanov *et al*., 1995a), and differ in their DNA binding and transcription activation properties (Sloop *et al*., 1999; Yaden *et al*., 2005). A third isoform of *Lhx3* is formed from an internal start codon (Sloop *et al*., 2001). By RT-PCR, using primer pairs specific for *Lhx3a* and *Lhx3b* isoforms of the gene, we identified expression of both in the E16.5 mouse inner ear (data not shown). Using an antibody that detects both isoforms of the gene, we showed that Lhx3 is expressed in the nuclei of all hair cells of the auditory and vestibular systems in postnatal day 1 (P1) wild-type mice (Fig. 2). No expression was observed in any other cell type of the inner ear, indicating that in the mouse, Lhx3 is a hair cell-specific protein. Whole-mount and section *in situ* hybridization using a digoxygenin-labeled probe showed similar results in E16 and P0 wild-type inner ears (data not shown). These data correlate with the results of Sage *et al*. (2005), who used Lhx3 as a marker for nuclei of differentiated hair cells in a study of hair cell development in a targeted deletion of Rb1.

The expression pattern of Lhx3 *follows the expression pattern of* Pou4f3 *in the developing auditory sensory epithelium*

To study the expression pattern of *Pou4f3* and *Lhx3* in the developing organ of Corti, we extracted RNA at seven time points between E12 and P3 as described (Hertzano *et al*., 2004). The semi-quantitative mRNA profile of *Pou4f3* indicated that *Pou4f3* mRNA is detectable at a low level as early as E12, with increasing levels of expression up to P3. *Lhx3* mRNA transcripts could be detected slightly before E16, but not before E15, and increased in abundance up to P0 followed by a down-regulation between P0 and P3 (Fig. 3). The onset of expression of *Lhx3*, as well as its pattern of expression, was very similar to that of *Gfi1*, a gene that we reported to be regulated by Pou4f3 (Hertzano *et al*., 2004). By comparison, *Myo6* and *Myo7a* are two hair cell-specific genes whose expression is not dependent on Pou4f3 (Xiang *et al*., 1998; Hertzano *et al*., 2004), while *Bdnf* is a gene that is partially regulated by Pou4f3 (Fig. 3) (Clough *et al*., 2004). The reduction in *Lhx3* expression levels between P0 and P3 in the wild type suggests that other transcription factors are required to down-regulate the expression of Lhx3 postnatally.

Lhx3 *is differentially regulated in the mouse auditory and vestibular systems*

Although *Lhx3* mRNA abundance was significantly reduced in the inner ears of the *Pou4f3*$^{ddl/ddl}$ and *Pou4f3*$^{-/-}$ mice, we could still detect it by semi-quantitative RT-PCR analyses at E16 and E18.5 (Fig. 1). This is in sharp contrast to the complete loss of expression of *Gfi1* in the ears of the *Pou4f3* mutant mice (Hertzano *et al*., 2004). Given that in the mouse inner ear Lhx3 is uniquely expressed in the hair cells, we tested the hypothesis that Pou4f3 contributes to the expression of Lhx3 but does not solely regulate its expression in all hair cell types. Figure 4 shows that all the hair cells of E18.5 wild-type mice express Lhx3, in both the auditory and the vestibular epithelia (Fig. 4A and D). By contrast, Lhx3 was not expressed throughout the cochlear duct of *Pou4f3* mutant mice (Fig. 4B), but was maintained in all hair cells of the vestibular sensory epithelia (Fig. 4E).

The hair cells of the *Pou4f3* mutant mice do not express *Gfi1* (Hertzano *et al*., 2004). We stained inner ears of *Gfi1* mutant mice with an antibody that detects Lhx3 in order to rule out the possibility that the loss of expression of Lhx3 in the cochleae of the *Pou4f3* mutant mice results from a deficiency in Gfi1. In the E18.5 *Gfi1*$^{-/-}$ mice, the expression of Lhx3 is maintained in both auditory and vestibular hair cells (Fig. 4C and F), indicating that the loss of expression of Lhx3 in

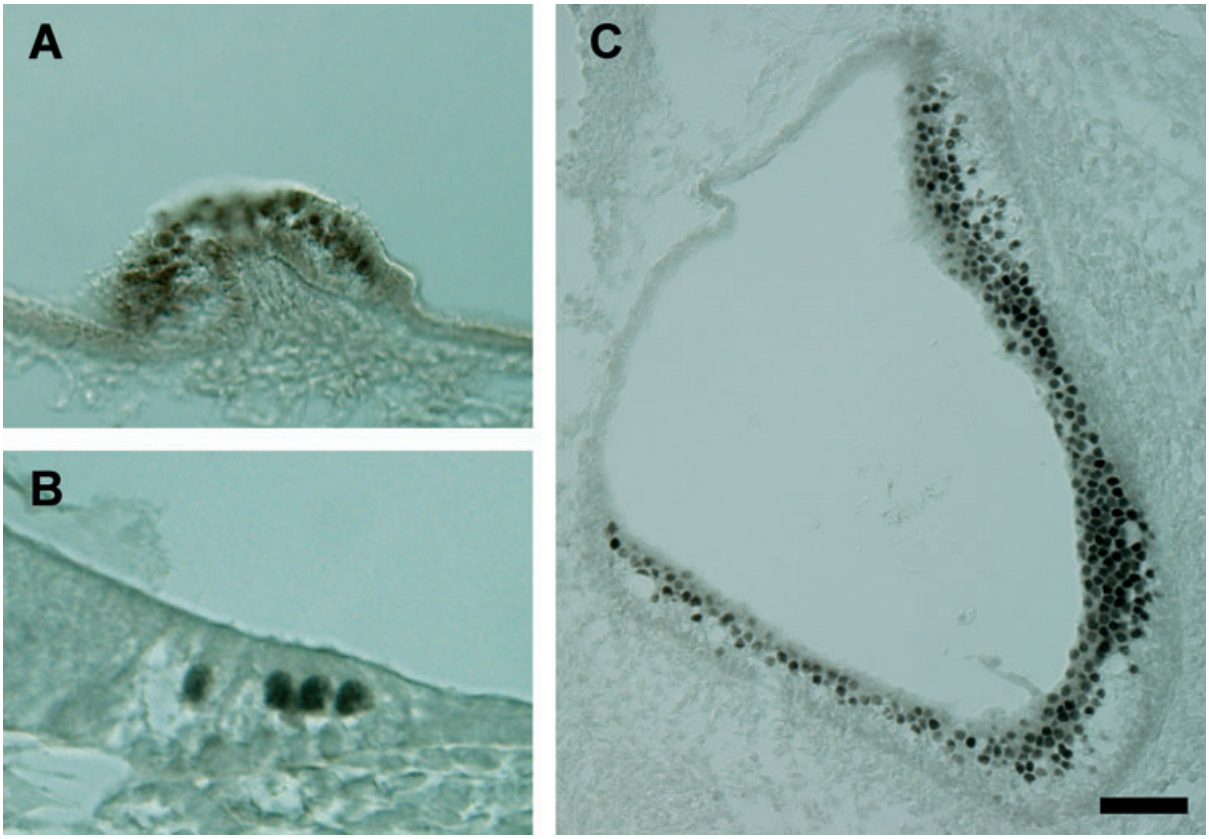

FIG. 2. *Lhx3* is a hair cell-specific gene in the mouse inner ear. Immunohistochemistry on cryosections of P1 mouse inner ears localize Lhx3 to the nuclei of all hair cells in the cristae ampullaris (A), cochlear duct (B) and utricle (C). Scale bars: A, 25 μm; B and C, 50 μm.

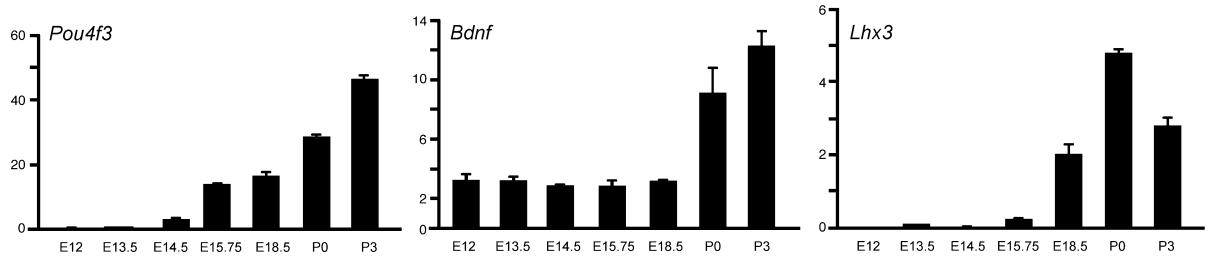

FIG. 3. Developmental semi-quantitative expression patterns of mRNA abundance for *Pou4f3*, *Bdnf* and *Lhx3*. mRNA abundance was determined by semi-quantitative RT-PCR on cDNA extracted from thermolysin-treated cochlear epithelia at seven developmental time points. Whereas *Lhx3* follows the expression pattern of *Pou4f3* up to P0, *Bdnf* can be detected before *Pou4f3* and does not seem to follow the quantitative expression pattern of *Pou4f3*. Ordinate: mRNA levels, arbitrary units; abscissa: developmental time points. Error bars represent one standard error, $n = 3$.

the cochleae of the *Pou4f3* mutant mice does not result from the loss of expression of Gfi1. Furthermore, as the outer hair cells of the *Pou4f3* and *Gfi1* mutant mice have a similar pattern of degeneration, the expression of Lhx3 in the outer hair cells of the *Gfi1* mutant mice indicates that a lack of expression of Lhx3 in the cochleae of the *Pou4f3* mutant mice is not a consequence of cell death.

Auditory hair cells of the mouse inner ear can develop and differentiate in mice with a targeted deletion of Lhx3

Lhx3 homozygous mutant mice are embryonically lethal or die soon after birth (Sheng *et al.*, 1996). In order to determine whether Lhx3 is necessary for hair cell development and survival, E15.5 embryos were obtained from a time mated intercross of $Lhx3^{+/-}$ mice. Inner ears were dissected and cultured for 4 days *in vitro*. Cultured ears were then stained with an antibody for myosin VI and for actin using phalloidin. No developmental abnormalities in the inner ear cochlear sensory epithelium were observed (data not shown).

Screening of deaf families for linkage to the LHX3 *locus*

In humans recessive mutations of *LHX3* have been previously reported to underlie combined pituitary hormone deficiency and rigid cervical spine syndrome (Netchine *et al.*, 2000). These mutations included a missense mutation in one of the LIM domains and an intragenic deletion that results in a truncated protein that lacks the DNA binding

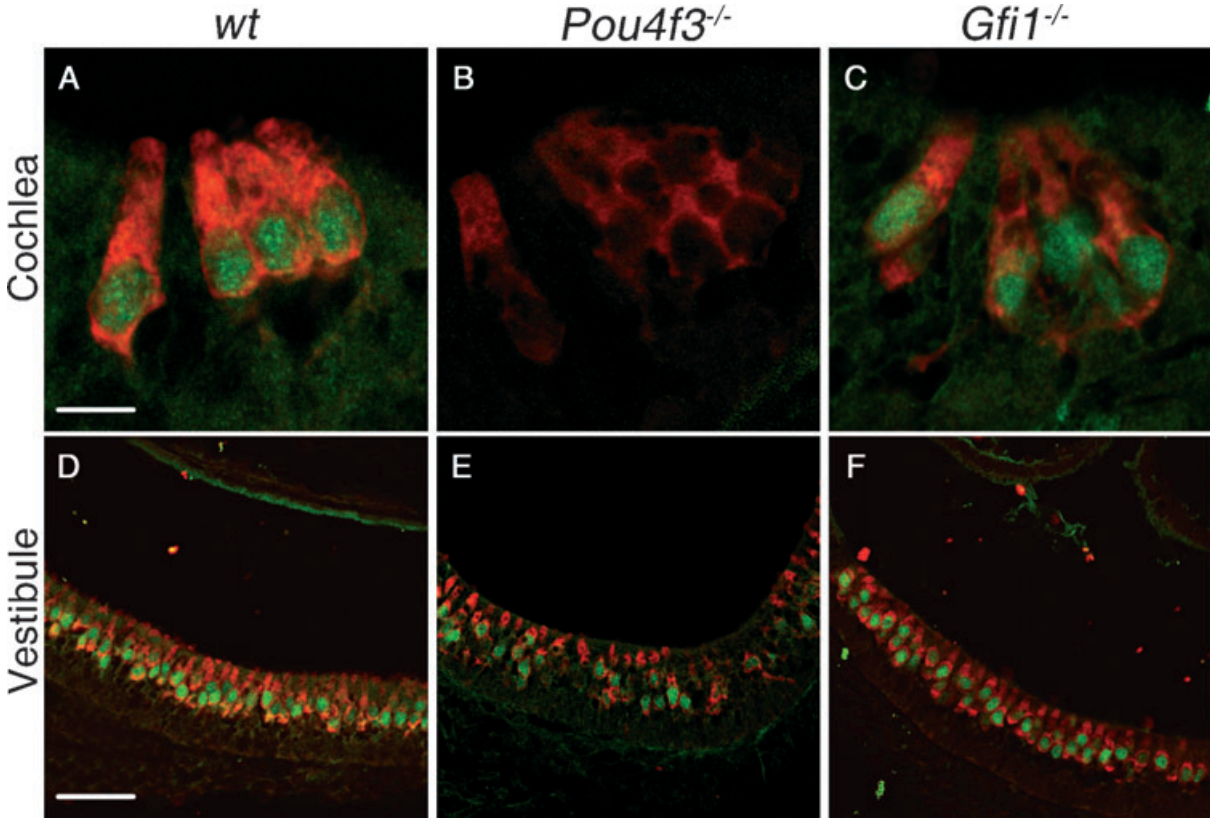

FIG. 4. Expression of Lhx3 (green) and myosin VI (red) in the cochleae (A–C) and the vestibular system utricle (D–F) on paraffin sections of mouse inner ears of E18.5 wild-type (A and D), *Pou4f3*$^{-/-}$ (B and E) and *Gfi1*$^{-/-}$ (C and F) mice. Whereas Lhx3 is expressed in the nuclei of all hair cells in the wild-type and *Gfi1*$^{-/-}$ inner ears, Lhx3 expression could be detected only in the vestibular system of the *Pou4f3*$^{-/-}$ but not in any of the nuclei of the cochlear hair cells. Scale bars: 9.4 μm (A–C), 37.5 μm (D–F).

homeodomain. Patients with the described mutations were not reported to present with hearing loss. To determine whether *LHX3* could be involved in human hereditary non-syndromic hearing loss, we searched the Hereditary Hearing Loss Homepage (http://webhost.ua.ac.be/hhh/) for deafness loci that map to the region of human chromosome 9q34, the location of the *LHX3* gene. We found that *LHX3* localized within the linkage interval for non-syndromic hearing loss DFNB33 (Medlej-Hashim *et al*., 2002) between short tandem repeat (STR) markers D9S1826 and D9S1838. This region spans roughly two million base pairs. Using D9S158, a marker located 4000 bp upstream of *LHX3*, we screened 452 families segregating recessively inherited non-syndromic deafness, 120 families from India and 332 families from Pakistan (Friedman *et al*., 2002). We found one consanguineous family, consisting of five affected individuals in two sibships, with homozygosity in affected individuals for DFNB33 markers. We performed a mutation analysis on DNA derived from this family and no mutations were found in the coding region and splice sites of *LHX3* (data not shown; primers available upon request).

Discussion

The LIM-homeobox genes encode a family of proteins that consist of a highly conserved homeodomain that functions to bind DNA and two LIM domains, each containing two zinc- fingers, which are necessary for protein–protein interactions (reviewed in Retaux & Bachy, 2002; Gill, 2003). The LIM-homeobox proteins bridge molecules to form multimeric complexes that bind to DNA to activate or repress transcription. This family of proteins is well conserved with homologues in *Drosophila*, *Caenorhabditis elegans*, *Xenopus*, fish and mammals (Hobert & Westphal, 2000).

Lhx3 plays a pivotal role in the specification of pituitary cell lineages during development (Sheng *et al*., 1996), activating expression of pituitary hormone genes (Sloop *et al*., 1999), and in concert with Isl1 determines whether spinal cord motor neurons will differentiate into motor neurons or V2 interneurons (Sharma *et al*., 1998). Lhx3 also shares high similarity with mec-3, which is essential for touch neuron differentiation in *C. elegans*. UNC-86 in *C. elegans*, a Pou4f3 homolog (Chalfie *et al*., 1981; Ninkina *et al*., 1993), promotes mechanosensory neuron differentiation by first binding to the *mec-3* promoter and then activating transcription of mechanosensory neuron-specific genes together with the protein product of *mec-3*. We have previously described a similar conserved genetic hierarchy with the *C. elegans* homologue of Gfi1 (Hertzano *et al*., 2004). This suggests a possible role for Lhx3 or another Lhx molecule in the specification of hair cell differentiation in mammals (Lichtsteiner & Tjian, 1995; Duggan *et al*., 1998; Rohrig *et al*., 2000).

We demonstrate that in the ear, Lhx3 is a hair cell-specific protein that in the cochlear hair cells is regulated either directly or indirectly by Pou4f3. However, the role of Lhx3 in the inner ear remains elusive. Cultured cochleae from *Lhx3*$^{-/-}$ mice were indistinguishable from those of their wild-type littermate controls, indicating that Lhx3 is not necessary for early hair cell specification, survival or differentiation. Patients with recessive mutations in *LHX3* that cause combined pituitary hormone deficiency and rigid cervical spine syndrome have

not been diagnosed with hearing loss (Netchine *et al*., 2000). These mutations of *LHX3* included a truncating allele that results in a short and inactive form of the protein. It is possible that another LIM-homeodomain protein, such as Islet-1, a LIM domain protein that is expressed in the inner ear, can compensate for the loss of expression of LHX3/Lhx3 (Meyer, 2003; Radde-Gallwitz *et al*., 2004). However, *Isl1* mRNA levels did not show a statistically significant change in expression levels between the wild-type and the $Pou4f3^{ddl/ddl}$ samples in our microarray results (fold of change 1.09, $P > 0.3$). Moreover, based on a recent microarray experiment performed in our laboratory, only Lhx3 but not Lhx1, 2, 4, 5, 6, 8 or 9 are expressed in the early postnatal auditory or vestibular sensory epithelia (data not shown). Future studies assessing each of these genes individually throughout development might reveal a candidate gene that could compensate for the role of Lhx3. Alternatively, another different molecule might be compensating for the loss of Lhx3, or Lhx3 might not be necessary for the proper function of the auditory epithelium.

Hair cells in the auditory and vestibular systems share common as well as unique morphological features. There are two types of hair cells of the cochlea, inner and outer hair cells (IHCs and OHCs), which differ in their structure, pattern of innervation and function. The IHCs receive most of the afferent innervation of the organ of Corti and very little efferent innervation. In the vestibular system, there are two types of hair cells, type I and type II. Both contain 50–100 stereocilia and a kinocilium that is maintained throughout life. Type I hair cell cell-bodies are pear shaped, similar to the IHCs in the cochlea, and contain a single large calyx engulfing the hair cell body. Type II hair cells have a tubular shape, similar to the OHCs in the cochlea, and multiple nerve endings synapse directly onto the basal end of their cell body. Nevertheless, the same basic mechanism of mechanotransduction operates in all four types and they do share many morphological as well as molecular features.

Although Pou4f3 is a pan-hair cell-specific transcription factor, we show that Lhx3, in contrast to Gfi1, is regulated by Pou4f3 in the auditory but not in vestibular system hair cells. Lhx3 is the first example of a hair cell-specific gene that is expressed in all hair cells of the mouse inner ear, but is regulated differently in auditory and vestibular hair cells. We are clearly just at the beginning of identifying the underlying mechanisms responsible for variation in the regulation of a gene in different hair-cell types, and target genes of Pou4f3 appears to be one such pivotal factor in distinguishing between these two systems.

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Abbreviations

E, embryonic day; IHCs, inner hair cells; OHCs, outer hair cells; P, postantal day; PBSTG, PBS with 0.2% Tween and gelatin; RT-PCR, reverse transcriptase-polymerase chain reaction; STR, short tandem repeat; UTR, untranslated region.

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