

Mutations in *CDC14A*, encoding a protein phosphatase

involved in hair cell ciliogenesis, cause

autosomal recessive severe to profound deafness

Sedigheh Delmaghani,^{1,2,3} Asadollah Aghaie,^{2,3,4} Yosra Bouyacoub,^{5,6} Hala El Hachmi,⁷
Crystel Bonnet,^{2,3,4} Zied Riahi,^{2,3,4} Sebastien Chardenoux,^{1,2,3} Isabelle Perfettini,^{1,2,3} Jean-
Pierre Hardelin,^{1,2,3} Ahmed Houmeida,⁷ Philippe Herbomel,^{3,8,9} and Christine Petit^{1,2,3,4,10,*}

¹Unité de Génétique et Physiologie de l'Audition, Institut Pasteur, 75015 Paris, France

²UMRS 1120, Institut National de la Santé et de la Recherche Médicale (INSERM), 75015 Paris, France

³Sorbonne Universités, UPMC Université Paris 06, Complexité du Vivant, 75005 Paris, France

⁴Syndrome de Usher et Autres Atteintes Rétino-Cochléaires, Institut de la Vision, 75012 Paris, France

⁵Institut Pasteur de Tunis, LR11IPT05, Biomedical Genomics and Oncogenetics Laboratory, 1002, Tunis, Tunisia

⁶Université de Monastir, Institut Supérieur de Biotechnologie, BP 56 Monastir 5000, Tunisia

⁷Laboratoire de Biochimie et Biologie Moléculaire, Faculté des Sciences et Techniques, BP 5026, Nouakchott, Mauritania

⁸Unité des Macrophages et Développement de l'Immunité, Institut Pasteur, 75015 Paris, France

⁹UMR 3738, Centre National de la Recherche Scientifique (CNRS), 75015 Paris, France

¹⁰Collège de France, 75005 Paris, France

*Correspondence: christine.petit@pasteur.fr

Abstract

By genetic linkage analysis in a large consanguineous Iranian family with eleven individuals affected by severe to profound congenital deafness, we could define a 2.8 Mb critical interval (at chromosome 1p21.2-1p21.1) for an autosomal recessive nonsyndromic deafness locus (DFNB). Whole-exome sequencing allowed us to identify a biallelic nonsense mutation in *CDC14A*, c.1126C>T (p.Arg376*), which was present in the eight clinically affected individuals still alive. Subsequent screening of 115 unrelated individuals affected by severe or profound congenital deafness of unknown genetic cause led us to identify another *CDC14A* biallelic nonsense mutation, c.1015C>T (p.Arg339*), in an individual originating from Mauritania. *CDC14A* encodes a protein tyrosine phosphatase. Immunofluorescence analysis of the protein distribution in the mouse inner ear showed a strong labeling of the hair cells' kinocilia. By using a morpholino strategy to knockdown *cdc14a* in zebrafish larvae, we found that the length of the kinocilia was reduced in inner ear hair cells. Therefore, deafness caused by loss-of-function mutations in *CDC14A* likely arises from a morphogenetic defect of the auditory sensory cells' hair bundles, whose differentiation critically depends on the proper growth of their kinocilium.

Almost 90% of all cases of nonsyndromic, severe to profound congenital deafness display an autosomal recessive mode of transmission (DFNB forms). Sixty genes have already been identified, but many others remain to be discovered according to the much larger number of DFNB loci reported (Hereditary Hearing Loss website).¹ With high-throughput sequencing techniques becoming available and the whole exome sequencing approach in affected individuals, the pace of gene discovery has accelerated. We report here the identification of two different nonsense mutations in *CDC144*, using a combination of genetic linkage analysis and whole exome sequencing.

Informed consent was obtained from all participants to the study. Of the eight affected individuals still alive in a consanguineous Iranian family (Figure 1A), the six that could be tested for auditory function (V.1, V.6, V.8, V.14, V.15, V.18), aged 21 to 69 years, all suffered from prelingual, severe to profound deafness of cochlear origin, as shown by the markedly increased detection thresholds in pure-tone audiometry (both with air- and bone-transmitted sounds) and auditory brainstem responses, and by the absence of transient evoked otoacoustic emissions (Figure 1B and data not shown).^{2,3} Otoscopic examination and tympanometry with acoustic reflex testing did not show evidence of a conductive hearing impairment. General clinical examination did not find any feature of syndromic deafness, and normal age of walking onset allowed us to exclude severe congenital vestibular dysfunction. Genetic linkage analysis was carried out on 21 family members. SNP array analysis (700k Illumina OmniExpress-12) and homozygosity mapping defined a single critical region of 2.8 Mb between rs7537296 and rs950060 at chromosome 1p21.2-1p21.1 (Figure 1A). This locus (DFNB105 [OMIM: 616958]) does not match any of the previously reported human deafness loci, and the murine syntenic region at chromosome 3qF3-3qG1 does not contain a reported deafness locus either. We then carried out whole exome sequencing in three affected individuals (V.6, V.8, and V.14), and identified a biallelic nonsense mutation in exon 11 of

CDC14A (*cell division cycle 14A*; NCBI ID: 8556), c.1126C>T (p.Arg376*) (NCBI RefSeq: NM_033312.2). Incidentally, a nonsynonymous and six synonymous sequence variants were also found in the critical interval, all of which were present in the HapMap, 1000 genomes, and Exome Variant Server databases. Sanger sequencing of *CDC14A* exon 11 confirmed the presence of the biallelic nonsense mutation in the eight clinically affected individuals only. In addition, all the clinically unaffected individuals tested except three (V.4, V.10, and V.17) carried the mutation at the heterozygous state, as expected from the genetic linkage analysis (Figure 1A). This mutation was absent from the 1000 genome and Exome Variant Server databases, and was not detected in 150 Iranian individuals from the general population. Whole exome sequencing of 115 unrelated individuals originating from Maghreb and affected by severe or profound congenital deafness allowed us to identify another biallelic nonsense mutation in the same *CDC14A* exon, c.1015C>T (p.Arg339*), in a Mauritanian individual affected by profound deafness (Figure 1C). This mutation was not detected in 105 normal-hearing individuals from this geographical region, including 50 Mauritanian individuals. Incidentally, neither of these nonsense mutations was detected in 195 additional congenitally deaf individuals originating from Maghreb or Iran.

CDC14A consists of 18 exons. It encodes a widely expressed protein tyrosine phosphatase (NCBI database of the transcriptome, UniGene). Six different transcripts resulting from alternative splicing have been reported (NCBI RefSeq: NM_003672.3, NM_033312.2, NM_033313.2, NM_001319210.1, NM_001319211.1, and NM_001319212.1). The transcript containing the largest open reading frame encodes a 623 amino acid protein (NCBI RefSeq: NP_201569.1, Figure 1D) involved in DNA repair, mitosis, meiosis, cell migration and adhesion, and ciliogenesis.⁴⁻¹⁶ Both mutations are expected to result either in nonsense-mediated mRNA decay or in a significantly truncated protein.¹⁷ We produced and purified two rabbit polyclonal antibodies directed against overlapping peptides (aa 412-489 and aa

412-522) from the C-terminal region of the mouse CDC14A (NCBI RefSeq: NP_001074287.1). Immunofluorescence experiments on the mouse inner ear between embryonic day 18.5 (E18.5) and postnatal day 21 (P21) showed the presence of CDC14A along both the transient kinocilia of developing cochlear hair cells and the persistent kinocilia of vestibular hair cells with either antibody. The protein was therefore detected from early stages of hair bundle differentiation onward (Figure 2 and data not shown). Functional studies in zebrafish have shown a role of *cdc14a* in ciliogenesis in Kupffer's vesicle, the ciliated organ of body asymmetry.¹⁶ We investigated the role of *cdc14a* in the zebrafish auditory organ by a knockdown strategy, using an antisense morpholino oligonucleotide (MO) targeting the splice donor site of intron 2 and a mismatch oligonucleotide (MI) as negative control. MO injection in one to two-cell stage embryos resulted in two abnormal mature transcripts in the larvae: one lacking the exon 2 sequence and the other one retaining intron 2. No abnormal transcripts were detected in MI-injected larvae (Figure 3A). In the *cdc14a* knockdown larvae at 3 days post fertilization, we did not observe gross morphological defects of the inner ear. However, there was a shortening of the hair cells' kinocilia: the mean length of the kinocilium was $4.89 \pm 0.12 \mu\text{m}$ in MO-injected larvae versus $6.31 \pm 0.17 \mu\text{m}$ in MI-injected and $6.17 \pm 0.18 \mu\text{m}$ in non-injected control animals (unpaired two-tailed Student's t-test, $p < 0.001$) (Figure 3B). No hair bundle shape anomalies including fragmentation were detected by confocal microscopy (data not shown).

Together, these results establish that loss-of-function mutations of *CDC14A* cause autosomal recessive severe to profound congenital deafness, and suggest that the hearing impairment arises from abnormally short kinocilia in the differentiating hair bundles of cochlear sensory cells. The absence of an associated balance disorder, despite the detection of the defective protein also in vestibular hair cells in the mouse, is common in genetic forms of human deafness. It presumably results from the fact that equilibration involves multimodal

perception, including vision and proprioception, which both can compensate for vestibular dysfunction. In addition, compensatory molecular mechanisms present until the full maturation of the hair cells^{18,19} may continue to operate in the vestibular hair cells which, contrary to the auditory hair cells, can regenerate. To our knowledge, *CDC14A* is the second reported DFNB gene involved in the control of kinocilium growth.^{20,21} During hair cell development, the growing hair bundle is connected to the kinocilium by fibrous links, which contribute to the hair bundle integrity.²² If these links are defective, the mature hair bundle may have an abnormal structure, therefore affecting the mechanoelectrical transduction process.²³ Alternatively, the absence of CDC14A, a protein phosphatase involved in cell signaling²⁴, might directly affect the hair cells, as previously reported for the suppression of different ciliary proteins in other tissues.²⁵ The absence, in the Iranian and Mauritanian affected individuals, of clinical symptoms of ciliopathies other than deafness, such as recurrent respiratory infections, kidney disorders, retinal degeneration, or obesity^{26,27}, suggests that the lack of CDC14A in other tissues is efficiently compensated by another protein phosphatase, possibly CDC14B.^{14,16}

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Web Resources

The URLs for data presented herein are as follows:

Exome Variant Server, NHLBI Exome Sequencing Project (ESP),

<http://evs.gs.washington.edu/EVS/>

Hereditary hearing loss homepage, <http://hereditaryhearingloss.org/>

NCBI database of the transcriptome, UniGene, www.ncbi.nlm.nih.gov/uniGene

Online Mendelian Inheritance in Man (OMIM). www.omim.org/

The 1000 Genomes Project, <http://www.1000genomes.org/>

The International HapMap Project, <http://hapmap.ncbi.nlm.nih.gov/>

UCSC Human Genome Database Build hg19, February 2009, <http://www.genome.ucsc.edu>

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Legends to figures:

Figure 1. Phenotypic and genotypic analysis in the Iranian and Mauritanian families

(A) Segregation analysis with SNP markers at chromosome 1p21.2-1p21.1 in the Iranian family. Deaf individuals and unaffected individuals are indicated by filled symbols and open symbols, respectively. The haplotype associated with the affected allele is boxed. The physical distances (in megabases, Mb) between the SNP markers and the centromere are indicated on the left (human genome reference sequence build UCSC hg19/GRCh37). Right panels: partial DNA sequence chromatograms of *CDC14A* exon 11 containing the c.1126C>T (p.Arg376*) nonsense mutation at heterozygous state in the normal hearing individual IV.6, and at homozygous state in the deaf individual V.18. (B) Right and left ear audiograms in a normal hearing individual (V.11, 29 years old) and six hearing-impaired individuals (V.1, 69 years; V.6, 40 years; V.8, 34 years; V.14, 21 years; V.15, 38 years; V.18, 31 years) of the Iranian family. (C) Pedigree of the Mauritanian family and partial DNA sequence chromatograms of *CDC14A* exon 11 in a normal-hearing control and in the deaf proband, showing the c.1015C>T (p.Arg339*) biallelic nonsense mutation in the latter. (D) Schematic representation of the CDC14A longest isoform (623 amino acids). The dual specificity protein phosphatase, N-terminal (DSPn) and protein tyrosine phosphatase (PTPase) domains are shown in purple and in blue, respectively. Vertical arrows indicate the positions of the two nonsense mutations.

Figure 2. CDC14A in the mouse inner ear

The distribution of CDC14A was analyzed in the cochlear sensory epithelium (organ of Corti) and one of the vestibular sensory epithelia (utricle macula) on E18.5, P3, P12, and P21, by whole-mount immunofluorescence. An anti-acetylated tubulin monoclonal antibody (Sigma T7451) and phalloidin (Sigma 94072) were used to label the hair cells' kinocilia and F-actin filled stereocilia (the modified microvilli that make the hair cells' hair bundles, which are at the core of the mechanoelectrical transduction process), respectively. CDC14A is present

along the kinocilia of cochlear and vestibular hair cells at all stages analyzed. In the cochlea, the kinocilium is present only in developing hair cells, not in mature (P21) hair cells, whereas in the vestibular end organs the kinocilium persists in mature hair cells. On P12, CDC14A-immunoreactive kinocilia were still detected in inner hair cells (IHCs) of the cochlear apical region, but not in outer hair cells (OHCs), which have already lost their kinocilia at this stage (data not shown). Note that CDC14A is also detected in the primary cilia of supporting cells on E18.5 (asterisks). No labeling was observed at any stage with the rabbit pre-immune serum (data not shown). Scale bars represent 5 μ m.

Figure 3. Knockdown of *cdc14a* in zebrafish

(A) One to two-cell stage embryos of *Danio rerio* were injected with 4 ng of either a *cdc14a* splice blocking morpholino (5'-GTTTGGGCAGACTCACTTTTCATAA-3') or a mismatch morpholino (5'-GTaTcGGCAcACTCAgTTTTgATAA-3') as negative control (Gene Tools), and larvae were analyzed at 3 days post fertilization (dpf). The blocking morpholino oligonucleotide (MO, red bar) was designed to encompass the splice donor site of *cdc14a* (NCBI: NM_201149.1, 15 exons) intron 2. This resulted in abnormally spliced *cdc14a* transcripts either retaining intron 2 (transcript b) or lacking exon 2 (transcript c) in the larvae injected with the blocking morpholino (knockdown), instead of the normal transcript (transcript a) present in the larvae injected with the mismatch morpholino (control), as shown by RT-PCR analysis (with primers located in exons 1 and 5) followed by sequencing of the amplicons. M, DNA size marker: 100 bp ladder. (B) Whole-mount immunofluorescence analysis of the inner ear anterior macula in 3 dpf control larvae (i.e., injected with the mismatched morpholino, MI) and *cdc14a* knockdown larvae (i.e., injected with the blocking morpholino, MO) with an anti-acetylated tubulin monoclonal antibody and phalloidin to label the hair cells' kinocilia and F-actin filled stereocilia, respectively (confocal microscopy). In

the knockdown larva, the overall structure of the hair bundles is preserved, but the kinocilia have reduced lengths. The bar chart shows the quantitative analysis of kinocilia lengths, measured with ImageJ software, in 4 non-injected (n = 91 kinocilia), 5 MI-injected (n = 119), and 10 MO-injected (n = 227) larvae. Data are represented as the mean $\pm$ SEM. Scale bar represents 10 μ m. n.s., not significant; ***, $p < 0.001$.

A

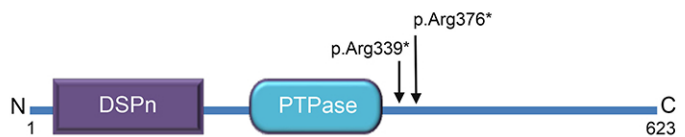
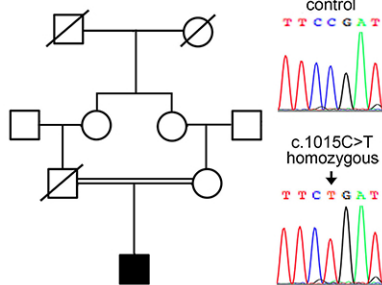
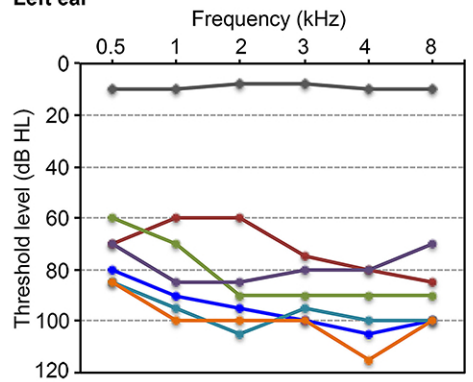


Figure 2

F-actin / CDC14A / acetylated tubulin

organ of Corti (cochlea)

utricle macula (vestibule)

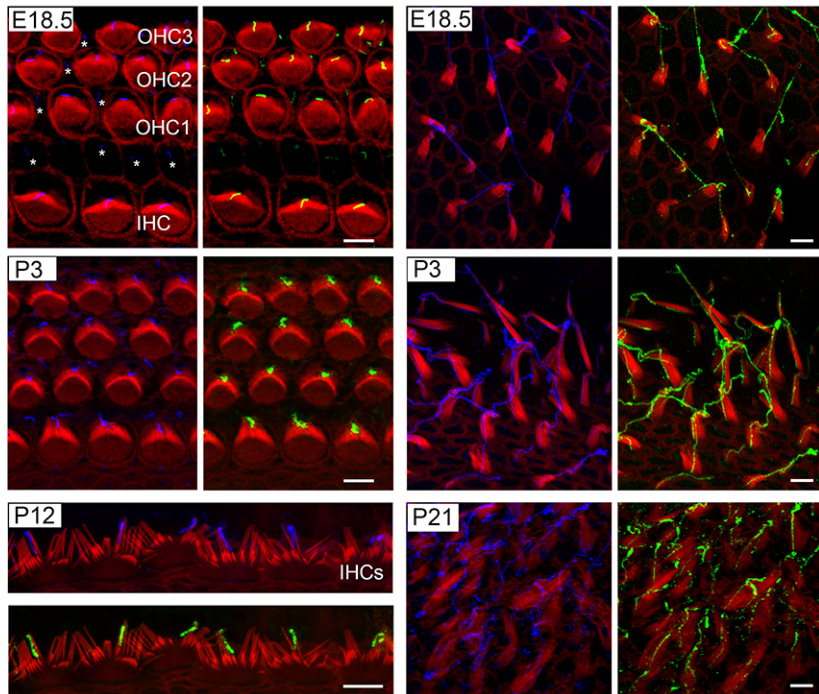
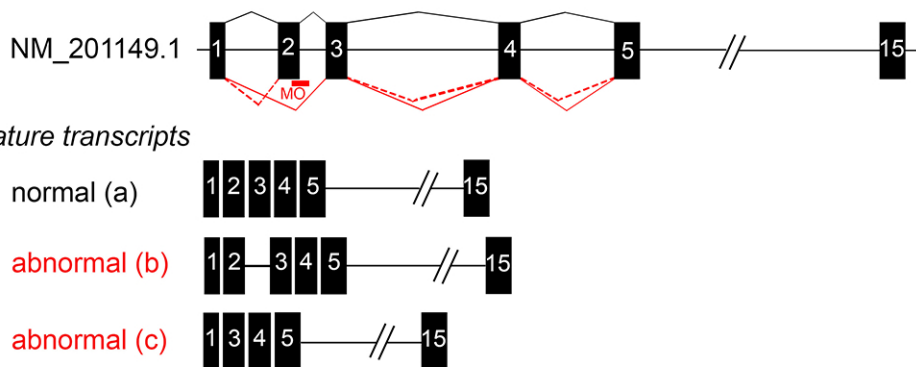
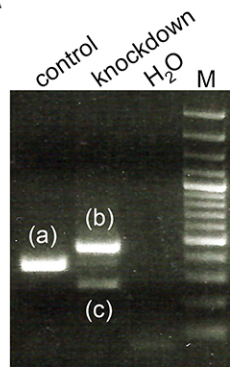


Figure 3

A



B

