

- hybrid 46-pole undulator (wavelength range of 0.3 to 1.8 Å) were used simultaneously to further enhance the x-ray intensity at the crystal. Single x-ray pulses were isolated with a microsecond shutter train (14) from the ESRF storage ring operating in single-bunch mode at a machine current of 15 mA. Laue diffraction intensities were collected on an image-intensified charge-coupled device detector (Thompson; ESRF). To minimize systematic errors such as those related to x-ray absorption, we collected a dark data set for each crystal in addition to the photoexcited data sets. Data sets consist of 30 images taken at crystal angular settings separated by 2° through a range of 60°. Each image is a composite of 10 150-ps exposures accumulated on the detector before readout, to enhance the relatively weak diffraction data collected in single-bunch mode. No significant radiation damage was detected over the course of data collection. Data were reduced and scaled with the LaueView software package (28). Laue reflections stimulated by several energies (multiples) were successfully deconvoluted and included in the final data set, which improved the completeness at low resolution and the overall redundancy.
18. Extent of photoinitiation. Optical thickness directly affects the extent of photoexcitation in the crystal. Crystals that are too thick will have a lower extent of photoinitiation and a highly nonuniform excitation throughout their volume (9). With 7-ns laser pulses at longer wavelengths, the possibility of photoexcitation of molecules in the [pR] state is encountered, which may stimulate reisomerization of the [pR] state back to pG. We demonstrated (V. Srajer and B. Perman, unpublished results) that the photocycle can be successfully initiated in the crystal under our experimental conditions, and estimate that 12% of the molecules are converted to the [pB] state.
19. Crystallographic refinement. The 1-ns model was refined against the Laue data limited to 1.9 Å resolution to minimize the influence of error in the integrated intensities at higher resolutions, for which I/σ_I is low. All refinement processes used X-PLOR, version 3.851 (29). The crystallographic refinement was restricted to only those residues that, according to inspection of the $|F|_{1\text{ns}} - |F|_{\text{dark}}$ difference Fourier map and of several simulated annealing (SA) omit maps, appear to move in response to photoinitiation. The atomic positions refined are those of the chromophore; the three proximal residues Pro⁶⁹, Cys⁶⁹, and Thr⁷⁰; and the distal side chains of Tyr⁴², Glu⁴⁶, Thr⁵⁰, and Arg⁵². Once these atoms were identified, a difference refinement strategy (30) was used in least-squares minimization of the residual between observed and calculated differences in structure factor amplitudes. This approach gave improved estimates of structural differences by reducing model-error terms. The chromophore refinement parameters incorporate information on the energetics of isomerization of the vinyl bond and charge distribution across the conjugated system of the chromophore (F. Zhou and P. Bash, personal communication). The energy function for rotation about the vinyl bond is given by $E_{\text{DIHEDRAL}} = k_{180}(1 - \cos\delta) + k_0(1 + \cos\delta)$, where δ is the phase shift angle, $k_{180} = 750 \text{ kcal mol}^{-1} \text{ rad}^{-2}$ for the *trans* conformer ($\phi_i = 180^\circ$), and $k_0 = 500 \text{ kcal mol}^{-1} \text{ rad}^{-2}$ for the *cis* conformer ($\phi_i = 0^\circ$). This relationship gives a two-well sinusoidal potential surface for isomerization about the vinyl bond that has wells of different depths. The deeper well is centered at 180° and corresponds to the *trans* conformer; the shallow well is centered at 0° and corresponds to the *cis* conformer. Difference refinement and inspection of SA omit maps were repeated for several occupancy values (10, 15, and 20%) for the extent of photoinitiation in crystals in the range anticipated from microspectroscopic measurements (78). A random 10% sample of the data was retained for use in calculation of R_{free} . Coordinates for the [pR] state of PYP have been deposited in the Brookhaven Protein Data Bank under accession code 2pyr.
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Mutation in Transcription Factor *POU4F3* Associated with Inherited Progressive Hearing Loss in Humans

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The molecular basis for autosomal dominant progressive nonsyndromic hearing loss in an Israeli Jewish family, Family H, has been determined. Linkage analysis placed this deafness locus, *DFNA15*, on chromosome 5q31. The human homolog of mouse *Pou4f3*, a member of the POU-domain family of transcription factors whose targeted inactivation causes profound deafness in mice, was physically mapped to the 25-centimorgan *DFNA15*-linked region. An 8-base pair deletion in the POU homeodomain of human *POU4F3* was identified in Family H. A truncated protein presumably impairs high-affinity binding of this transcription factor in a dominant negative fashion, leading to progressive hearing loss.

The inner ear is a complex structure requiring a large repertoire of genes to orchestrate sound acquisition and vestibular function on many levels, including neuronal

innervation, structural integrity, and mechano-electrical transduction. Although as many as 100 genes may be involved (1), only a small number of genes have been identified that cause nonsyndromic hearing loss: human connexin 26 (*GJB2*) (2), human myosin VIIA (*MYO7A*) (3), human diaphanous (4), and mouse myosin VI (*Myo6*) (5). Another POU-domain transcription factor, *POU3F4* (POU domain, class 3, transcription factor 4), causes human X-linked mixed deafness, *DFN3* (6).

We are studying the genetic basis of progressive hearing loss in an Israeli Jewish family. The family traces its ancestry to Italy and to subsequent migrations through various North African and Middle Eastern countries, including Tunisia, Libya, and Egypt, with branches of the family now living in Israel, the United States, and Belgium (7). Five generations demonstrate autosomal dominant inheritance of progressive deafness (Fig. 1A). The earliest record

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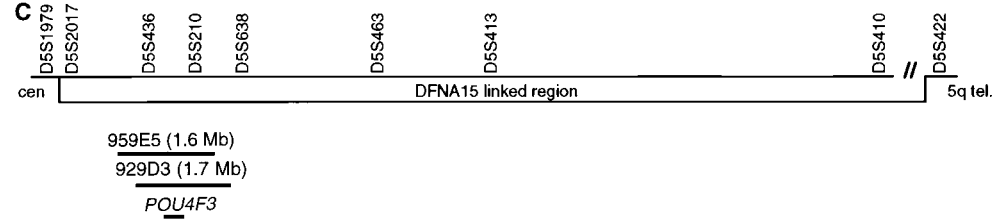
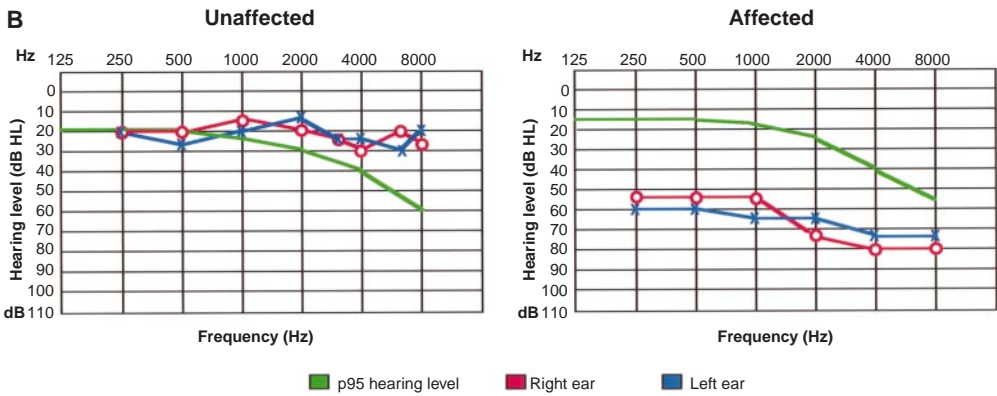
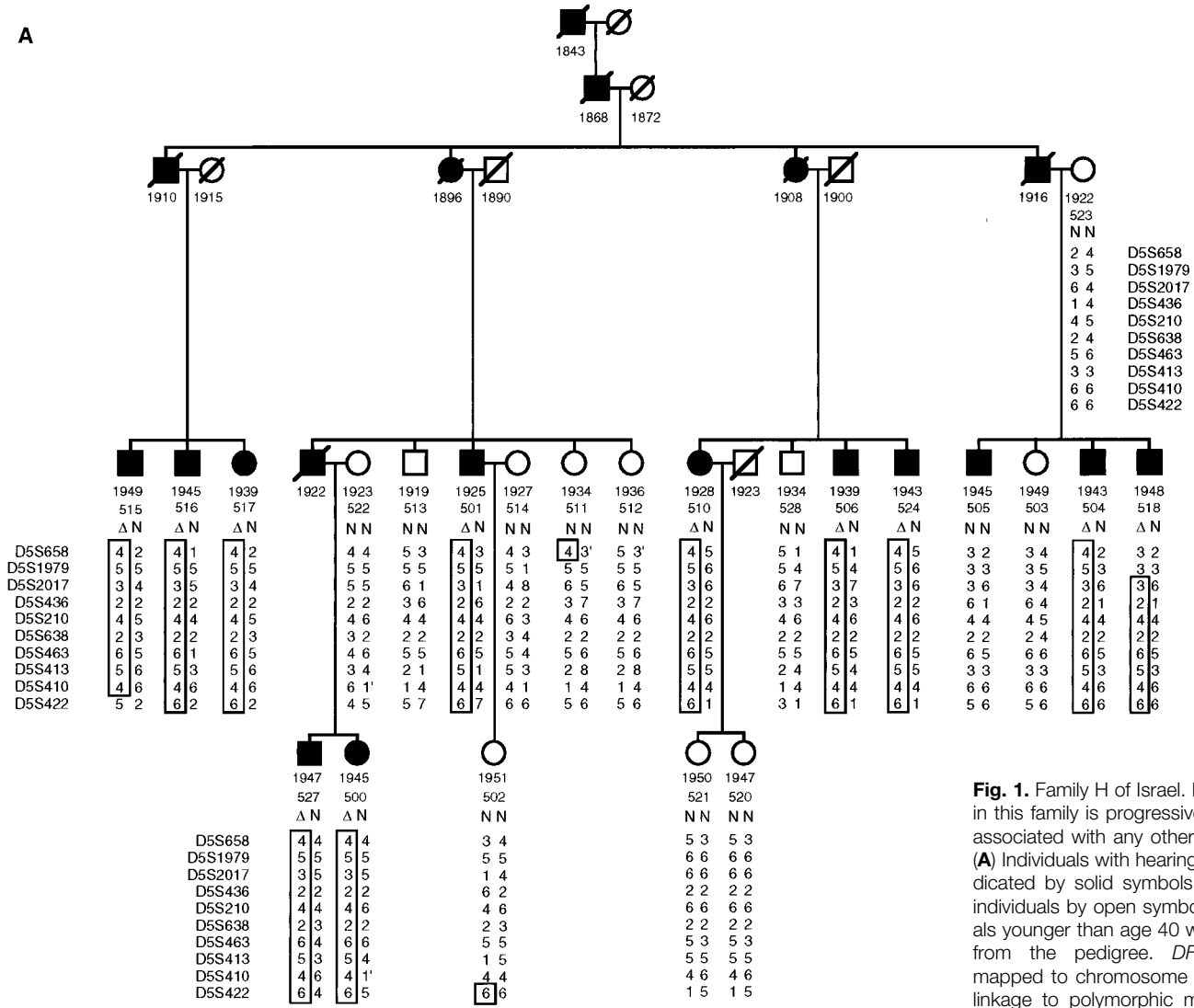


Fig. 1. Family H of Israel. Hearing loss in this family is progressive and is not associated with any other phenotype. (A) Individuals with hearing loss are indicated by solid symbols, unaffected individuals by open symbols. Individuals younger than age 40 were omitted from the pedigree. *DFNA15* was mapped to chromosome 5q31-33 by linkage to polymorphic markers with genotypes indicated for each relative. All affected individuals in the family share the *DFNA15* deletion in *POU4F3*, with the exception of 505, who may represent a phenocopy. All unaffected individuals over age 40 are wild-type at the comparable *POU4F3* site. (B) Representative pure-tone audiograms from two individuals, 511 (unaffected) and 504 (affected). Lines are drawn to show p95 ISO values (8), indicating mean hearing levels for the age and sex of these individuals. (C) Human *POU4F3* on chromosome 5q31 linked to deafness in Family H. Markers D5S1979 and D5S422 define the 25-cM linked region. YACs 959E5 and 929D3 contain *POU4F3*, D5S436, and D5S210.

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of a hearing-impaired family member is from an individual born in 1843 (by family report) in Libya. He had four children, only one of whom was affected. Hearing loss in Family H begins between ages 18 and 30, with a moderate to severe defect in hearing by age 50 (Fig. 1B). On the audiologic examinations, 12 family members were identified as affected with progressive sensorineural hearing loss (8). These 12 individuals and 11 unaffected relatives older than age 40 were available for linkage analysis.

Deafness in Family H was mapped to chromosome 5q31-q33 by linkage analysis. Previously known loci for inherited deafness were excluded by linkage (9). However, lod scores (logarithm of the odds ratio) for linkage of deafness in Family H to *DFNA1* on 5q31 were positive at distant recombination fractions, indicating an independent locus for hearing loss distal to *DFNA1*. The new locus was named *DFNA15* and was localized further to the

25-cM region bounded by D5S1979 and D5S422 (Fig. 1A) (10). Critical meiotic recombination events were detected in affected individuals 518 and 515 and unaffected individuals 511 and 502. Individual 505 is hearing impaired, but he did not inherit any portion of the *DFNA15*-linked haplotype otherwise segregating with deafness in the family (Fig. 1A). Analysis of the maximum-likelihood genetic model for deafness in Family H indicated a rare autosomal dominant allele with full penetrance by age 40 and 0.05 probability of deafness from other causes by age 40 (in other words, 5% phenocopies). Multipoint analysis of linkage included all markers shown in Fig. 1A and yielded negative lod scores for the interval D5S658 to D5S1979 and lod scores of 3.63 for the interval D5S1979 to D5S2017, 4.04 for intervals between D5S2017 and D5S410, and 3.49 for the interval D5S410 to D5S422 (11).

Because few large families have inherited hearing loss, and because of the inacces-

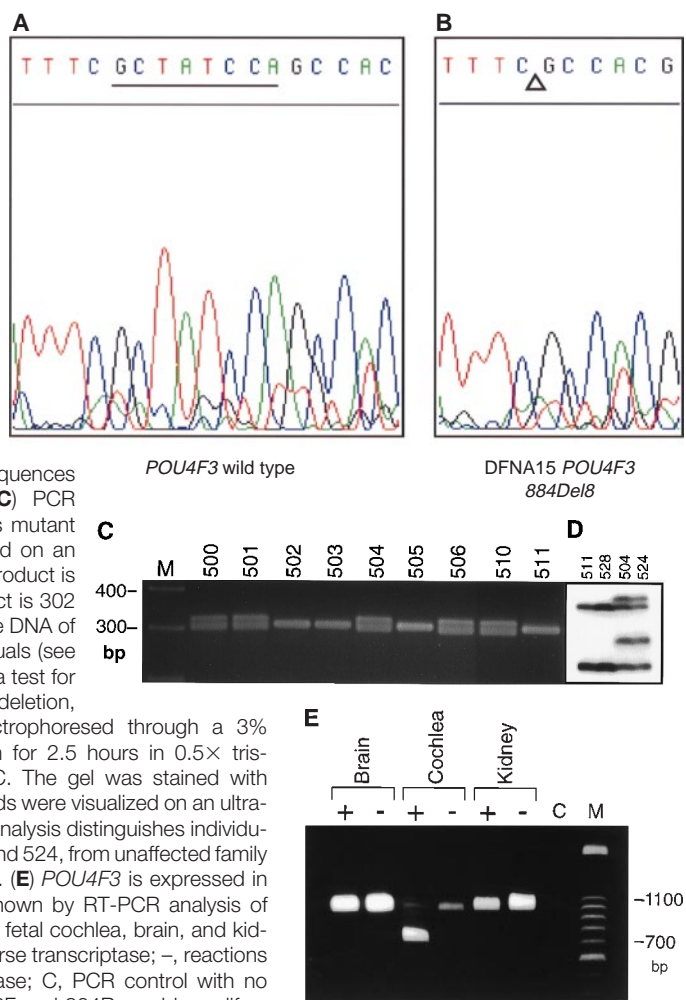
sibility of the human ear, the mouse has proved to be an invaluable model for gaining access to deafness genes and studying human hearing loss. More than 60 mouse mutations exist with defects in the inner ear (12). The structure and function of the inner ear is similar between the two organisms, and the genomic organization is very well conserved. The first genes responsible for autosomal recessive deafness were initially identified in the mouse, demonstrating the value of the mouse for identifying and studying human deafness genes (5, 13).

To identify candidate genes in the *DFNA15*-linked region, we noted that the POU-domain transcription factor *POU4F3* (POU domain, class 4, transcription factor 3; GenBank accession numbers U10060 and U10061) might lie on 5q, on the basis of the localization of mouse *Pou4f3* (also referred to as *Brn3c* and *Brn-3.1*; GenBank accession number S69352) and the homology of human chromosomes 5 and 18 and mouse chromosome 18 (14). *POU4F3* was an excellent candidate for a gene causing human deafness, because targeted deletion of both alleles of *Pou4f3* causes complete deafness in mice (15, 16). To map *POU4F3* more precisely, we synthesized primers for the human *POU4F3* cDNA sequence and used these to amplify pools of the CEPH3 yeast artificial chromosome (YAC) library (Research Genetics). *POU4F3* primers amplified DNA from YACs 959E5 and 929D3 (1610 and 1720 kb, respectively), both of which were also amplified by markers D5S436 and D5S210. Because these markers were linked to deafness in Family H (Fig. 1A), *POU4F3* must lie within the *DFNA15*-linked region (Fig. 1C).

To screen for *POU4F3* mutations in Family H, we designed primers to amplify and sequence the entire coding region; these primers revealed an 8-base pair (bp) deletion in exon 2 (Fig. 2) (17). The predicted result of this deletion is a frame shift beginning at codon 295 and a premature translation stop at position 299. The deletion segregated perfectly with the *DFNA15*-linked haplotype (Fig. 1A); all deaf individuals with the linked haplotype carried the mutation, and all unaffected relatives older than age 40 in Family H carried the wild-type *POU4F3* sequence. However, deaf individual 505 did not inherit either the *DFNA15*-linked haplotype or the *POU4F3* deletion, which suggests that he is a phenocopy of the deafness caused by this deletion of *POU4F3* (8).

To determine whether the 8-bp deletion occurs in the hearing population, we tested 114 unrelated individuals of various North African and Middle Eastern Jewish ancestries for the mutation (18). These individuals were selected to represent the contri-

Fig. 2. Sequence analysis of the *DFNA15* mutation of *POU4F3*. (A) Wild-type *POU4F3* sequenced from genomic DNA of unaffected individuals. The underlined 8-bp sequence is deleted in members of Family H with genetic hearing loss (Fig. 1A). (B) The mutant *DFNA15* allele of *POU4F3* sequenced from genomic DNA of deaf individuals from Family H. *DFNA15* is defined by an 8-bp deletion of *POU4F3*, whose site is based on GenBank sequences U10060 and U10061. (C) PCR products of normal versus mutant alleles of *POU4F3* resolved on an agarose gel. The normal product is 310 bp; the mutant product is 302 bp. PCR products from the DNA of nine representative individuals (see Fig. 1A) are illustrated. As a test for the presence of the 8-bp deletion, PCR products were electrophoresed through a 3% MetaPhor gel at 10 V/cm for 2.5 hours in 0.5× tris-acetate buffer and at 4°C. The gel was stained with ethidium bromide and bands were visualized on an ultraviolet light box. (D) SSCP analysis distinguishes individuals with hearing loss, 504 and 524, from unaffected family H members, 511 and 528. (E) *POU4F3* is expressed in the human cochlea, as shown by RT-PCR analysis of RNA samples from human fetal cochlea, brain, and kidney (+, reactions with reverse transcriptase; -, reactions without reverse transcriptase; C, PCR control with no DNA template). Primers 48F and 804R would amplify a 756-bp product from cDNA and a 1070-bp product from contaminating genomic DNA. The PCR reaction was run on a 1% agarose gel with 100-bp markers (M) (Gibco-BRL).



bution of various ethnic Jewish populations to Family H. Their chromosomes are identified by origin as follows: 60 Moroccan, 54 Libyan, 20 Tunisian, 7 Algerian, 7 Egyptian, 42 Iraqi, 31 Sephardic (originally from Spain, mainly from Italy and the Balkan countries), and 7 Ashkenazic (mainly East European). None of the 228 control chromosomes tested carried the 8-bp deletion in *POU4F3* found in Family H.

POU4F3 is not expressed in human brain, heart, placenta, skeletal muscle, lung, liver, kidney, pancreas, or lymphoblast tissues evaluated by Northern (RNA) blot analysis (19). Nor were any *POU4F3* expressed sequence tags (ESTs) identified in human libraries found in public databases. We examined *POU4F3* RNA expression in human fetal tissues by means of reverse transcription polymerase chain reaction (RT-PCR) with PCR primers from exon 1 and 2 spanning the intron (20). Complementary DNA amplification was only detected in fetal cochlea. This expression pattern corresponds to that observed in the mouse; namely, *Pou4f3* is highly expressed in inner ear hair cells, but only in very defined regions of the brain and not in non-neural tissues including liver, kidney, heart, and skeletal muscle (15, 16, 21). Exclusive *POU4F3* expression in the human cochlea is consistent with the underlying cause of nonsyndromic deafness in Family H.

POU4F3 is a member of the family of POU transcription factors, a group of proteins identified by a well-conserved bipartite domain (22). The POU domain consists of a 75- to 82-amino acid NH₂-terminal region, the POU-specific domain, and a ~60-amino acid COOH-terminal region, the POU homeodomain. A large number of these proteins have been identified in humans, mice, *Drosophila*, *C. elegans*, *Xenopus*, and zebrafish, and are involved in development. In particular, they are known to be involved in terminal differentiation of neuronal cell types. The POU-specific domain is unique to this family of transcription factors, whereas the POU homeodomain is related to other DNA-binding homeodomains involved in developmental regulation. Binding to a 9-bp recognition site, ATAATTAAT, the POU-specific domain contacts the 5' portion of this site and the POU homeodomain contacts the 3' portion (23). However, unlike most homeodomain proteins, the POU homeodomain does not direct high-affinity binding of the POU-domain protein to its DNA target sites on its own, but requires the POU-specific domain for efficient recognition of response elements (24).

Targeted deletion of the *Pou4f3* gene leads to vestibular dysfunction and pro-

found deafness in mice (15, 16). At postnatal day 14, around the time mice begin to hear, neither hair cells nor supporting cells are present. Furthermore, there is extensive neuronal degeneration in inner ear sensory ganglia. These ganglia house the cell bodies of sensory neurons within the cochlea and vestibular apparatus; the hair cells of each portion of the inner ear receive primary afferent endings from these neurons, which send axons through the auditory nerve to the central nervous system (25). As is evident from the *Pou4f3* null mice, this transcription factor is required for the terminal differentiation and trophic support of sensorineural cells.

In Family H, the deletion removes the 3' portion of this POU transcription factor crucial for high-affinity binding to DNA. Because the deletion occurs in the final coding exon, it is possible that the transcript is translated (26). If so, the *POU4F3* mutant peptide would be truncated at 298 amino acids rather than the normal 338 amino acids. The mutant protein would terminate at the end of the first helix, thereby removing the second and third helices of the POU homeodomain. The third helix, referred to as the recognition helix, makes base contacts with the 3' half of the DNA complex subsite in the major groove of the DNA (27). A truncated protein, therefore, may not be able to bind the "right half" of the POU recognition sequence, and thus the binding of this transcription factor would be compromised. The first amino acid lost as a result of the 8-bp deletion in Family H is an isoleucine; this residue is responsible for another member of the class IV POU-domain factors, *Pou4f2*, acting as a repressor (28). Its loss may prevent *POU4F3* from repressing the transcription of target genes crucial for hair cell function. The truncated peptide may also have novel DNA binding properties or may act as a dominant negative mutation by pairing with other POU transcription factors (29). Alternatively, haploinsufficiency of *POU4F3* may down-regulate genes responsible for hair cell survival, causing a progressive loss of hair cells that is clinically significant only after extended time. However, brainstem-evoked responses have been evaluated in *Pou4f3* knockout mice up to 24 months of age. The hearing of +/- animals is indistinguishable from that of +/+ animals (30). These experiments suggest that haploinsufficiency of *POU4F3* is not an explanation for the deafness phenotype in Family H.

Screening for mutations in the *POU4F3* gene is facilitated by its small size, and so it will be straightforward to determine whether mutations in this gene are responsible for deafness in other families. To facilitate test-

ing of *POU4F3* mutations in genomic DNA, we sequenced the genomic region encompassing both *POU4F3* exons, 5' and 3' sequences, and the intron (GenBank accession numbers AF044575 and AF043452). Nearly half the general population loses part or all of their ability to hear by age 80, with a substantial loss in understanding of speech by age 65 (1). Defining the molecular events leading to deafness may form the basis for new ways to help the hearing-impaired population.

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3. Originally, mutations in *MYO7A* were found in a syndromic form of deafness, Usher syndrome type IB [D. Weil *et al.*, *Nature* **374**, 60 (1995)]. Since then, mutations have been found in families with both autosomal dominant and recessive hearing loss [X.-Z. Liu *et al.*, *Nature Genet.* **16**, 188 (1997); X.-Z. Liu *et al.*, *ibid.* **17**, 268 (1997)].
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7. This project was approved by the Israel Ministry of Health Helsinki Committee, by the Human Subjects Division of the Institutional Review Board (IRB) of the University of Washington, and by the IRB of the National Institute of Neurological Disorders and Stroke/NIDCD. Blood samples were obtained after informed consent.
8. Hearing was measured by pure-tone audiometry on all participating relatives of Family H. A complete clinical history of each affected individual was collected to ensure that the hearing loss was not a result of infection, trauma, acoustic trauma, or ototoxic drugs. The hearing loss in this family is sensorineural, with some members exhibiting conductive hearing loss as well. The audiometric curves of all family members were compared with age- and sex-dependent percentile curves [International Organization for Standardization, International Standard ISO 7029 (1984)]. The criterion for hearing impairment in Family H is hearing loss below the 95th percentile (p95) of the reference curves and a hearing threshold greater than 40 dB at 1000 and 2000 Hz (Fig. 1B). The low-frequency hearing loss progressed with increasing age. Three individuals—504, 505, and 516—were tested further, including otologic examination, speech audiometry and immittance testing (tympanometry and acoustic reflexes), auditory evoked potentials [auditory brainstem response (ABR)], and computerized static posturography. The results suggested that all three individuals had some vestibular dysfunction. However, individual 505 also had evidence of otosclerosis, leading to conductive hearing loss. The ABRs of individuals 504 and 516 were normal with respect to latency and shape at thresholds of 95 to 105 dB. The ABR of individual 505 showed prolonged brainstem transmission time, prolonged absolute latencies of the Vth peak, and absence of stapedial reflex; also, the sensorineural hearing loss thresholds were not identical in both ears (at 250 to 1000 Hz, the left-ear component was mild, whereas the right-ear component was moderate to severe). The hearing loss was bilateral in all other affected individuals.

9. Markers for dominant and recessive hearing loss loci are listed on the Hereditary Hearing Loss Homepage (G. Van Camp and R. J. H. Smith, January 1998; Web site, <http://dnalab-www.uia.ac.be/dnalab/hhh>). Blood (20 cm³) was collected from each individual; DNA was made from 10 cm³ of each sample [S. A. Miller, D. D. Dykes, H. F. Polesky, *Nucleic Acids Res.* **16**, 1215 (1988)], and the remaining 10 cm³ was used for establishing lymphoblastoid cell lines [H. Neitzel, *Hum. Genet.* **73**, 320 (1986)].
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17. Primers were designed to amplify the human *POU4F3* gene by means of sequence information for the two coding exons available from GenBank (accession numbers U10060 and U10061) and sequence we obtained on the intron separating the two exons (GenBank accession number AF043452). Sequence of the fragment generated by PCR using the primer pair 629F (5'-CGGCTCTGGCTAATCTCAAG-3') and 939R (5'-AAGGTCCAGTTCTCAGCGA-3') revealed a deletion in affected individuals (Fig. 2, A and B). The deletion was confirmed in all affected Family H members both by screening for single-strand conformation polymorphisms (SSCPs) and by separation of alleles on 3% MetaPhor gels (FMC), using primers spanning the deletion of *POU4F3* (Fig. 2C). Variant bands obtained from SSCP, using PCR primers to amplify the *POU4F3* region that includes the H family deletion, were gel-purified, reamplified, and sequenced as described (4).
18. Primers 629F and 939R surrounding the deletion in affected Family H members were used to amplify genomic DNA. The product was labeled by incorporation of [α -³²P]deoxyguanosine triphosphate during PCR. Each sample was loaded onto a 6% polyacrylamide denaturing gel and electrophoresed at 70 W for 4.5 hours to resolve the 310-bp (wild-type) and 302-bp (Family H mutant allele) fragments. Gels were dried and exposed to x-ray film.
19. Northern blots containing 2 μ g of polyadenylated RNA from 16 different human tissues were hybridized with a portion of the human *POU4F3* cDNA (Multiple Tissue Northern I and II, Clontech) according to the manufacturer's protocol. The probe was a PCR-generated fragment of *POU4F3* exon 2 (nucleotides 629 to 939).
20. Total RNA from human fetal cochlea, brain, and kidney was extracted using the guanidine isothiocyanate method [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. Rutter, *Biochemistry* **18**, 5294 (1979)]. Total RNA (3 μ g) was reverse-transcribed with the SuperScript II kit (Gibco-BRL). Parallel PCR reactions with reverse transcriptase (+RT) and without reverse transcriptase (-RT) were performed to evaluate DNA contamination in RNA samples. Reverse transcriptase (+/- RT) reaction was used as a template for PCR. The following PCR primers spanning the 314-bp intron were used: 48F (5'-TGCAA-GAACCAATCTCC-3') to 804R (5'-GAGCTCTGGCTTGTCTGTCT-3'). A 756-bp product was observed in cochlear RNA, corresponding to the cDNA size. Genomic DNA contamination was present in all samples, as shown by the 1070-bp band observed in all lanes (Fig. 2E). PCR reagents including Taq polymerase (Perkin-Elmer) were used according to the manufacturer's instructions.
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FADD: Essential for Embryo Development and Signaling from Some, But Not All, Inducers of Apoptosis

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FADD (also known as Mort-1) is a signal transducer downstream of cell death receptor CD95 (also called Fas). CD95, tumor necrosis factor receptor type 1 (TNFR-1), and death receptor 3 (DR3) did not induce apoptosis in FADD-deficient embryonic fibroblasts, whereas DR4, oncogenes *ET1A* and *c-myc*, and chemotherapeutic agent adriamycin did. Mice with a deletion in the FADD gene did not survive beyond day 11.5 of embryogenesis; these mice showed signs of cardiac failure and abdominal hemorrhage. Chimeric embryos showing a high contribution of FADD null mutant cells to the heart reproduce the phenotype of FADD-deficient mutants. Thus, not only death receptors, but also receptors that couple to developmental programs, may use FADD for signaling.

CD95 (the Fas antigen) is a death domain-containing receptor of the TNFR family that signals apoptosis to eliminate unwanted, autoreactive lymphocytes (1). Activation of CD95 by either CD95 ligand (CD95L or FasL) or treatment with an agonistic antibody results in receptor aggregation and the rapid recruitment of FADD (Fas-associated death domain protein), a

26-kD cytoplasmic protein with a death domain (2). The interaction of FADD and Fas through their COOH-terminal death domains unmasks the NH₂-terminal death effector domain (DED) of FADD, allowing it to recruit caspase-8 to the Fas signaling complex (3) and thereby activating a cysteine protease cascade leading to cell death.

FADD and its downstream caspase cascade may also participate in signaling by other members of the TNFR family. TRADD (TNFR-1 associated death domain protein), an adapter protein that binds directly to the death domain of TNFR-1, can transduce signals for apoptosis (4). In over-expression systems, FADD is recruited by TRADD to TNFR-1 and DR3 (also called Wsl-1) signaling complexes, and FADD mutants lacking the DED are dominant-negative inhibitors of TNFR-1- and DR3-induced cell death (5, 6).

Outside of cell death signaling and the

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Mutation in Transcription Factor *POU4F3* Associated with Inherited Progressive Hearing Loss in Humans

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