SHORT REPORT

Mutations of *ESPN* cause autosomal recessive deafness and vestibular dysfunction

S Naz, A J Griffith, S Riazuddin, L L Hampton, J F Battey Jr, S N Khan, S Riazuddin, E R Wilcox, T B Friedman

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We mapped a human deafness locus *DFNB36* to chromosome 1p36.3 in two consanguineous families segregating recessively inherited deafness and vestibular areflexia. This phenotype co-segregates with either of two frameshift mutations, 1988delAGAG and 2469delGTCA, in *ESPN*, which encodes a calcium-insensitive actin-bundling protein called espin. A recessive mutation of *ESPN* is known to cause hearing loss and vestibular dysfunction in the *jerker* mouse. Our results establish espin as an essential protein for hearing and vestibular function in humans. The abnormal vestibular phenotype associated with *ESPN* mutations will be a useful clinical marker for refining the differential diagnosis of non-syndromic deafness.

Most congenital non-syndromic hearing loss is inherited as a recessive Mendelian disorder.[1] Forty recessive deafness loci (DFNB) have been mapped and the genes responsible at 20 of these loci have been reported.[2] With Institutional Review Board (IRB) approval and written informed consent we ascertained families PKSN32 and PKSR5A from Punjab, Pakistan. Detailed clinical histories were obtained for affected individuals (ages ranging from 6 to 40 years), who exhibited prelingual, profound, sensorineural hearing loss and independent ambulation delayed beyond 1.5 years of age. Affected members (ages 14, 16, and 39) of family PKSN32 denied vestibular symptoms although caloric testing with electronystagmography (ENG) revealed vestibular areflexia. The four deaf individuals in family PKSR5A reported balance problems, and caloric-ENG testing confirmed vestibular areflexia. Although congenital, profound hearing loss with vestibular areflexia is accompanied by retinitis pigmentosa (RP) in Usher syndrome type 1,[3] affected individuals from PKSN32 or PKSR5A denied nyctalopia or other symptoms of RP. Electroretinography (ERG) on two affected individuals from families PKSN32 (age 14) and PKSR5A (age 19) ruled out the presence of RP.

The deafness segregating in family PKSN32 was not linked to any of the known recessive or dominant deafness loci (Hereditary Hearing Loss Homepage, http://dnalab-www.uia.ac.be/dnalab/hhh/). Therefore, a genome-wide search was performed with the Marshfield Weber 9 marker set. We found a 6 cM region of homozygosity on chromosome 1p36.3 among four affected individuals of this family (fig 1). A maximum two-point LOD score of 4.1 ($\theta = 0$) was obtained with marker *D1S214*, defining a new recessive deafness locus, *DFNB36*. In the second family, PKSR5A, the hearing loss was consistent with linkage to *DFNB36* with a maximum two-point LOD score of 3.4 ($\theta = 0$) for marker *D1S3774* (fig 1).

ESPN, a gene in the *DFNB36* critical interval at 1p36.3, was a good positional candidate because a mutation of *Espn* is known to cause deafness and vestibular dysfunction in the *jerker* mouse.[4] We screened the coding sequence of *ESPN* (GenBank accession number AL136880) by sequencing PCR amplified *ESPN* exons from genomic DNA of affected individuals in the two families. However, the sequencing analysis of *ESPN* was complicated due to the presence of a second *ESPN*-like sequence on chromosome 1p36.13, annotated as *LOC284729* (GenBank accession number AL035288). As we identified frequent frameshift mutations in *LOC284729* disrupting its open reading frame (data not shown) and due to the absence of sequence encoding homologous residues that are important for actin-bundling activity of espin,[5] *LOC284729* appears to be an unprocessed pseudogene. Based upon our analyses of *LOC284729*, this putative pseudogene has been assigned the symbol *ESPNP*. Except for the absence of sequences comparable to exon 1 and exon 13, *ESPNP* has all of the other exons and introns of *ESPN* to which it shows 95% nucleotide sequence identity. *ESPNP* is 10 Mb away from *ESPN* in reverse orientation with respect to *ESPN* and is outside the *DFNB36* linkage interval defined by families PKSN32 (fig 1) and PKSR5A (data not shown).

Intronic primers for amplification of exons 1 and 13 were unique for *ESPN* since *ESPNP* lacks homologous sequences, and intronic primers for amplification of exons 2, 6, 7, 8, 9, and 10 were specific for *ESPN* due to multiple mismatches with *ESPNP* sequence (table 1). However, intronic primers for amplification of other exons were either identical to *ESPN* and *ESPNP* (exons 11 and 12) or failed to discriminate between the mismatches of the two sequences (exons 3, 4, and 5). Therefore, we subcloned the PCR products from exons 3, 4, 5, 11, and 12 and sequenced individual clones. The nucleotide mismatches allowed us to unambiguously assign individual clones to either *ESPN* or *ESPNP*.

We detected two different mutations of *ESPN* segregating with the deafness phenotype in families PKSR5A and PKSN32. In family PKSR5A, affected individuals were homozygous for a 4 bp deletion, 1988delAGAG, in exon 9 (fig 2A), while in family PKSN32 all affected individuals were homozygous for a 4 bp deletion, 2469delGTCA, in exon 13 (fig 2B). The obligate carriers in both families were heterozygous and neither mutation was detected among 150 normal-hearing individuals from Pakistan. The1988delAGAG mutation in exon 9 of *ESPN* was not present in *ESPNP* in deaf individuals of family PKSR5A. *ESPNP* lacks the site of 2469delGTCA mutation in family PKSN32 (fig 2C).

ESPN is predicted to encode an 854 amino acid protein (fig 3) referred to as espin, a name derived from ectoplasmic specialisations (+ in) on the basis of its discovery and localisation in parallel F-actin bundles of ectoplasmic

Abbreviations: ABM, actin-bundling module; ENG, electronystagmography; ERG, electroretinography; IRB, Institutional Review Board; RP, retinitis pigmentosa

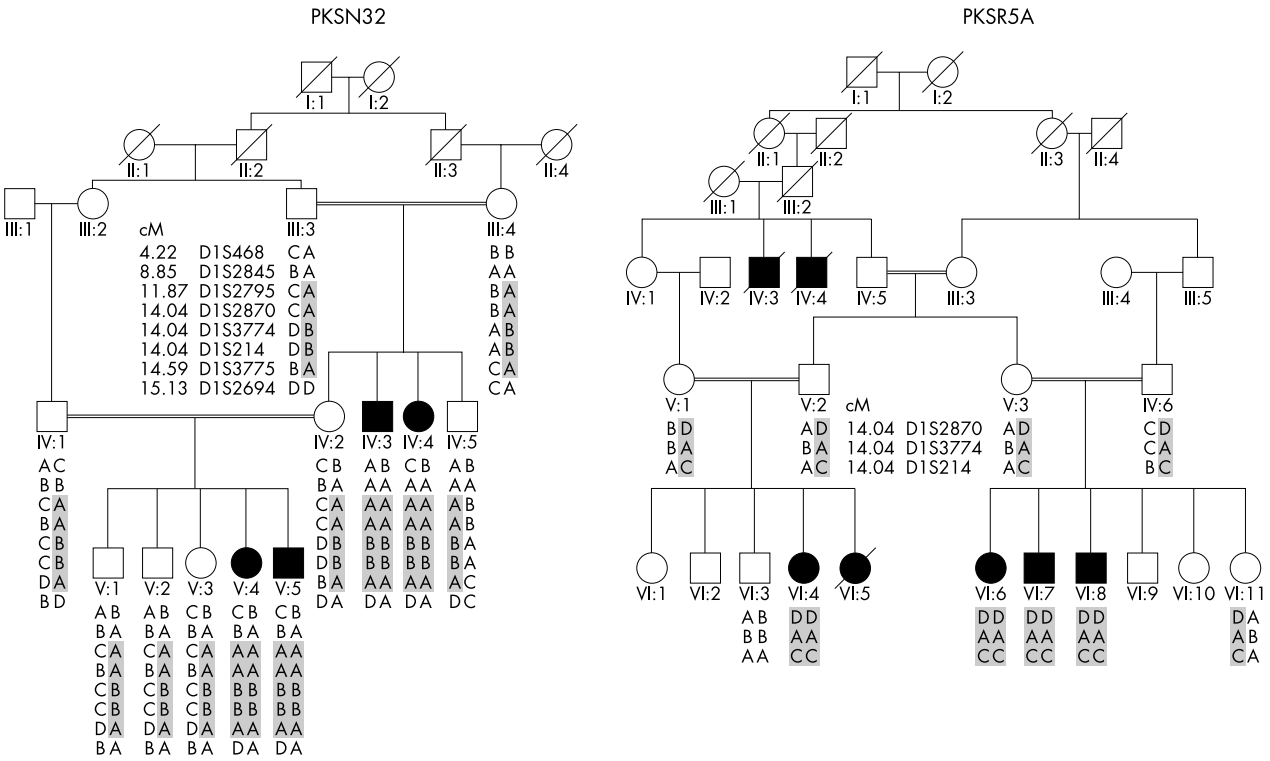

Figure 1 Pedigrees and mapping of *DFNB36*. Families PKSN32 and PKSR5A are shown with the haplotypes of chromosome 1p36.3 STR markers. The grey shaded haplotypes represent the ancestral chromosome harbouring the *ESPN* mutation.

specialisation in testes.[6] Domain prediction algorithms of human espin sequence revealed eight ankyrin-like repeats at the N-terminus, two proline rich regions, a consensus site for ATP or GTP binding (P-loop), which is contained within an actin-binding WH2 motif (amino acids 651–668), and a coiled coil (amino acids 756–831) (figs 2D and 3). There are 66 residues (amino acids 739–804) that show 33% sequence identity to a domain in forked proteins, which are essential for formation of actin bundles in bristles of *Drosophila melanogaster*.[7] Deletion mutagenesis experiments have suggested that espin contains three actin-binding sites (figs 2D and 3).[5 8] Two of the actin-binding sites at the C-terminus constitute the ABM (figs 2D and 3) and are important for espin activity. *Espn* expression constructs lacking either one or both of these actin-binding sites are unable to cross-link actin filaments when transfected into BHK fibroblasts.[5]

The two mutations of *ESPN*, segregating with hearing loss linked to *DFNB36*, cause frameshifts in the *ESPN* translation reading frame. If translated, 1988delAGAG is predicted to

Table 1 Primers for amplification of *ESPN* exons* and flanking intronic sequences

Oligo	Sequence	Size (bp)	Exon(s) amplified
1F	ATTCGAACCCAGTTTTGCTG		
1R	CCACCCACTTCCAGGACTAC	1010	1
2F	AGGAAGGGTGGAGAGATC		
2R	ATGTTGAGTGGGAGCCATTT	859	2
3F	GAGGTCAGACACAGCAGGTG		
5R	AGCGTGGGTTTCCAGTTATG	1954	3–5
6F	GGAACCTGGGTCCTGCTG		
6R	CCTCCCCATGTTTAAGAGCA	552	6
7F	TACGACTCCTGCTCCTCCAGCCACT		
8R	CCCTCTGCAGCCCCTTTCCTAAGC	6909	7 and 8
9F	CCATCCAATCTTGGCTTAGG		
9R	ACTGGTGACAGTGCAGGTGA	377	9
10F	CACAGTGTTCTCAGGCATCG		
10R	GATGGGCTGTGCATCCAG	444	10
11F	AGCTCTGAGGGGGTGTGAC		
12R	TACAAGGGCCAAGAGACAGG	462	11 and 12
13F	CTAGCCCCTCTGTCTTCAGC		
13R	GGTCAGGGAGGGTTTCCAG	439	13

*Sequence corresponds to AL136880.
Nucleotides in each primer mismatched to *ESPNP* sequence are underlined. Amplification conditions and sequences of additional primers for mutational analysis of exons 2, 3, 4, 5, 7, and 8 are available as supplemental material (http://jmg.bmjjournals.com/supplemental/).

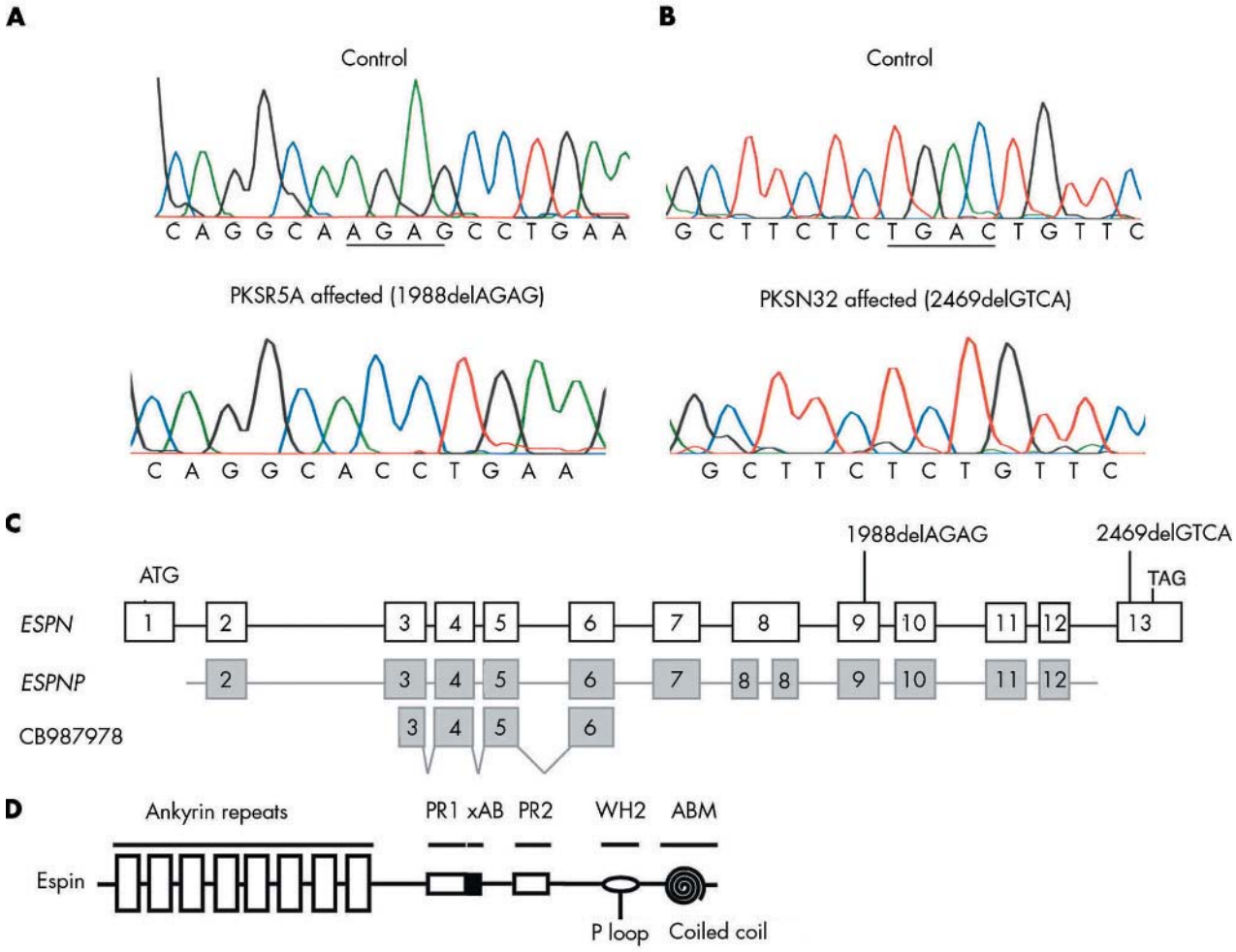

Figure 2 Electropherograms demonstrating *ESPN* mutations and diagrammatic representation of *ESPN* and *ESPNP*. (A) Sequence traces from exon 9 of *ESPN* for control individual VI:3 and affected individual VI:6 in family PKSR5A, showing the wild type sequence and 1988delAGAG, respectively (underlined in the control trace). (B) Sequence traces from exon 13 of *ESPN* for a control and affected individual V:4 in family PKSN32, showing 2469delGTCA (underlined). The traces show the reverse complement strand. (C) Exon/intron structure of *ESPN* (GenBank# AL136880). Exons are represented as numbered boxes, and introns are indicated by lines joining the boxes (not drawn to scale). The locations of mutations found in the sequencing analysis of *ESPN* from the affected individuals in the two families are shown (numbered vertical lines). *ESPNP*, a putative pseudogene, is depicted below *ESPN*. Exons 1 and 13 of *ESPN* are absent and exon 8 is interrupted by an intron. There is only one expressed sequence tag (EST) corresponding to *ESPNP* (GenBank# CB987978), drawn below *ESPNP*. (D) Diagrammatic representation of espin depicting ankyrin repeats encoded by exons 1–4 and the proline rich peptides (PR1 and PR2) encoded by exons 7 and 8. xAB is an actin-binding domain[6] and is encoded by exon 7 of *ESPN*. The WH2 domain (Wiskott Aldrich syndrome homology region 2) containing the P-loop is encoded by exon 9. The coiled coil and other residues important for actin bundling are formed by amino acids encoded by exons 10–13 constituting the actin-bundling module (ABM).

introduce one substituted amino acid after residue 662, followed by a stop codon at nucleotide 1990 and 2469delGTCA is predicted to introduce 27 substituted amino acids after residue 821, followed by a stop codon at nucleotide 2533 resulting in a truncated 844 amino acid protein with 821 correct and 23 substituted residues. These mutations are presumed to result in loss of function of espin. The resulting mutant proteins are predicted to have either no actin-bundling module, (1988delAGAG), or lack one of the C-terminal actin-binding sites (2469delGTCA), which is necessary for espin activity.[5] There is evidence for multiple isoforms of Espn in the mouse[4 5 6 9] and the same may be true in humans as well. However, the mutations associated with *DFNB36* occur in *ESPN* exons, which are known to be present in all reported isoforms of *Espn* in mice.[4 5 7 9]

PCR analysis of human fetal inner ear cDNA revealed expression of *ESPN* in the inner ear (data not shown). In both the cochlea and the vestibule of the mouse inner ear, espin is localised mostly to the stereocilia of hair cells.[4] Stereocilia are specialised microvilli projecting from the apical surfaces of inner ear hair cells and are vital for transduction of sound and for detection of linear and angular acceleration. They contain a densely packed core of parallel bundles of actin filaments.[10] The formation and ordered arrangement of these filaments into bundles requires different actin-binding proteins, which cross-link actin filaments.[11] Multiple actin-binding proteins such as fimbrin, plastin, and espin, are expressed in the inner ear.[11 12] Espin plays a crucial role in cross-linking parallel actin bundles and varying concentrations of espin may determine the extent of elongation and, consequently, the length of parallel actin bundles.[13]

Espin is absent from the stereocilia of *jerker* mice,[4] and consequently by postnatal day 10 the stereocilia are shortened and have reduced stiffness.[14] Within 3 months of birth, a degenerative process leads to the complete loss of all sensory hair cells in the *jerker* mouse.[4 14] In addition to espin,

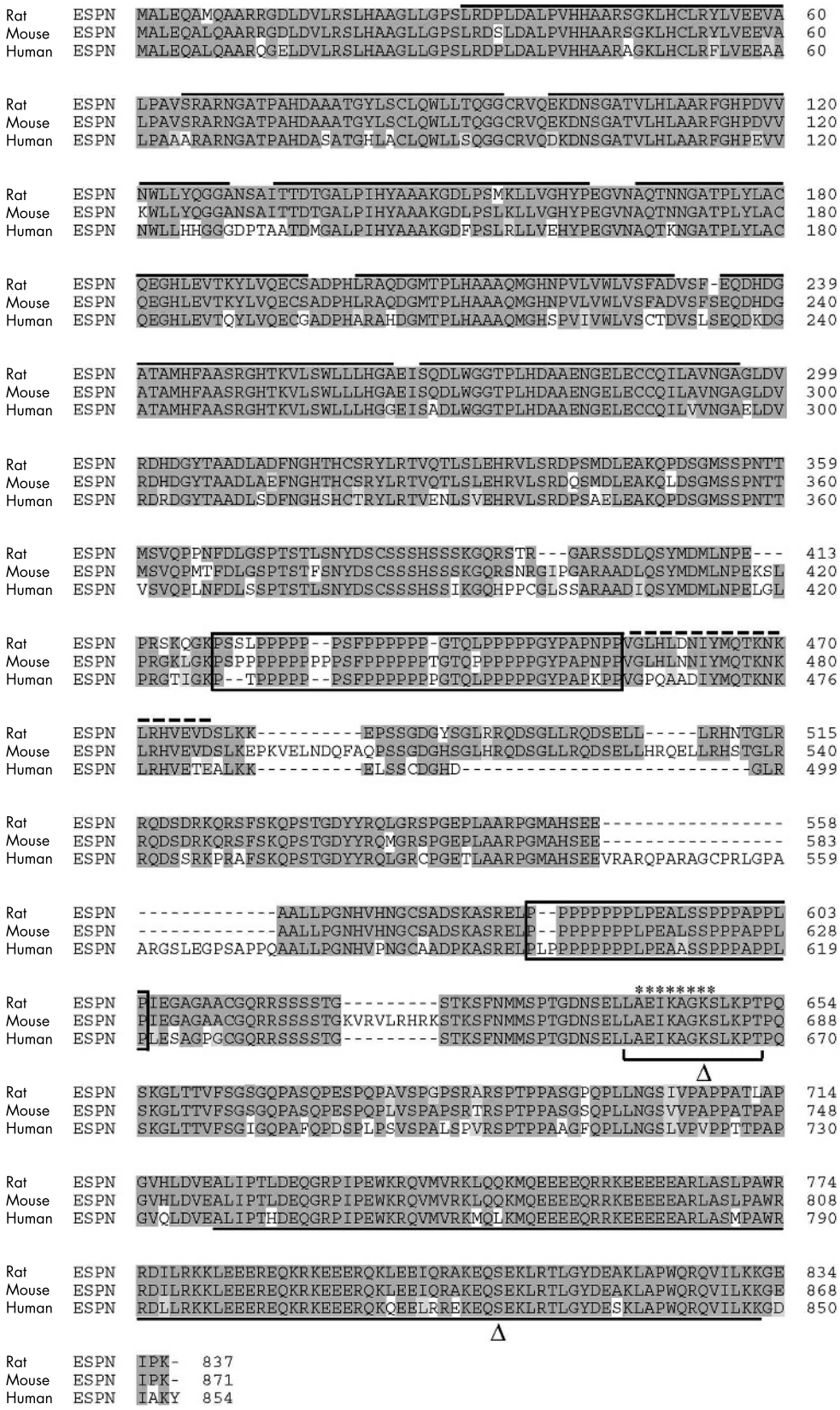

Figure 3 Espin sequence alignment. ClustalW multiple protein alignment of rat, mouse, and human ESPN. Dark shaded residues denote identical amino acids. Light grey shading represents conserved amino acid substitutions and ''-'' indicates a gap in the alignment. Bars on top of amino acid residues at the N-terminus indicate the location of ankyrin repeats. Two proline rich regions are boxed. A dashed line indicates actin-binding amino acids (xAB). Asterisks mark the amino acids corresponding to the P-loop. Amino acids forming the WH2 domain are indicated by a bracket. Amino acids constituting the actin-bundling module, ABM,[5] are underlined. The first amino acid affected by1988delAGAG or 2469delGTCA is indicated by ''Δ''. The amino acids shared by mouse and human espin proteins exhibit 83% identity and 88% similarity. Human and rat *ESPN* are 86% identical and 90% similar.

there are many other cytoskeletal proteins that are necessary for the development and maintenance of stereocilia. Mutations of *ACTG1* encoding γ-actin were recently reported to cause progressive hearing loss in humans.[15] Both β- and γ-actin are present in the stereocilia of auditory hair cells in chicken,[16] raising the possibility that espin interacts with either β-actin, γ-actin, or both in the stereocilia. Moreover, espin has multiple sites for protein–protein interactions, which may serve as a scaffold for assembly of macromolecular complexes important for structure and function of the stereocilia.

Our findings indicate that espin is essential for both hearing and balance in humans. The association of profound deafness and vestibular dysfunction in the absence of other associated phenotypes is unusual, although vestibular dysfunction is often not carefully evaluated or documented for most non-syndromic recessive deafness loci. Vestibular dysfunction has been excluded for 11 DFNB loci: *B1*, *B6, B7/11*, *B12*, *B17*, *B18*, *B21*, *B23*, *B26*, *B29*, and *B30*[17] (see Homepage Hereditary Hearing Loss Homepage for individual references: http://dnalab-www.uia.ac.be/dnalab/hhh/). Affected individuals with mutations of *MYO7A* linked to *DFNB2* have a vestibular phenotype[17] comparable to that associated with *DFNB36* and affected individuals with mutations of *MYO15A* also exhibit signs and symptoms of vestibular dysfunction.[17] Vestibular dysfunction was also reported in a few deaf individuals who have recessive mutations of *MYO6*.[18] The abnormal vestibular phenotype associated with *ESPN* mutations is unusual and will be a useful clinical marker for refining the differential diagnosis of non-syndromic deafness.

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Authors' affiliations

S Naz, S Riazuddin, E R Wilcox, T B Friedman, Section on Human Genetics, LMG, NIDCD, NIH, Rockville, MD 20850, USA
A J Griffith, Section on Gene Structure and Function, LMG, NIDCD, NIH, Rockville, MD, USA
A J Griffith, Hearing Section, NIDCD, NIH, Rockville, MD, USA
L L Hampton, J F Battey Jr, Section on G-protein Coupled Receptors, NINDS, NIH, Bethesda, MD, USA
S N Khan, S Riazuddin, National Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

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Correspondence to: Dr T B Friedman, Laboratory of Molecular Genetics, NIDCD, NIH, Rockville, MD 20850, USA; friedman@nidcd.nih.gov

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