RESEARCH ARTICLE

Mutations of the *RDX* Gene Cause Nonsyndromic Hearing Loss at the *DFNB24* Locus

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Ezrin, radixin, and moesin are paralogous proteins that make up the ERM family and function as cross-linkers between integral membrane proteins and actin filaments of the cytoskeleton. In the mouse, a null allele of *Rdx* encoding radixin is associated with hearing loss as a result of the degeneration of inner ear hair cells as well as with hyperbilirubinemia due to hepatocyte dysfunction. Two mutant alleles of *RDX* [c.1732G>A (p.D578N) and c.1404_1405insG (p.A469fsX487)] segregating in two consanguineous Pakistani families are associated with neurosensory hearing loss. Both of these mutant alleles are predicted to affect the actin-binding motif of radixin. Sequence analysis of *RDX* in the DNA samples from the original DFNB24 family revealed a c.463C>T transition substitution that is predicted to truncate the protein in the FERM domain (F for 4.1, E for ezrin, R for radixin, and M for moesin) (p.Q155X). We also report a more complete gene and protein structure of *RDX*, including four additional exons and five new isoforms of *RDX* that are expressed in human retina and inner ear. Further, high-resolution confocal microscopy in mouse inner ear demonstrates that radixin is expressed along the length of stereocilia of hair cells from both the organ of Corti and the vestibular system. Hum Mutat 28(5), 417–423, 2007.   Published 2007 Wiley-Liss, Inc.[†]

KEY WORDS: deafness; nonsyndromic hearing loss; radixin; ezrin; *RDX*; ERM; cytoskeleton; hair cells; stereocilia

INTRODUCTION

Hearing requires specialized mechanosensory and support cells in the inner ear that have a complex cytoskeletal structure [for review see Frolenkov et al., 2004; Friedman and Griffith, 2003]. Hearing loss in humans can arise from mutations of genes encoding cytoskeleton proteins, including espin (MIM♯ 606351) [Naz et al., 2004; Zheng et al., 2000], harmonin (MIM♯ 605242) [Ahmed et al., 2002], SANS (MIM♯ 607696) [Weil et al., 2003], TRIOBP (MIM♯ 609761) [Riazuddin et al., 2006; Shahin et al., 2006], whirlin (MIM♯ 607928) [Mburu et al., 2003], cadherin 23 (MIM♯ 605516) [Bork et al., 2001], and protocadherin-15 (MIM♯ 605514) [Ahmed et al., 2001, 2003a], and several unconventional myosins [Avraham et al., 1995; Weil et al., 1995; Wang et al., 1998; Ahmed et al., 2003b].

Ezrin (MIM♯ 123900), radixin (MIM♯ 179410), and moesin (MIM♯ 309845), which together comprise the ERM family, are three related proteins in the band 4.1 superfamily that participate in the formation of the membrane-associated cytoskeleton by linking actin filaments and adhesion proteins [Tsukita and Yonemura, 1999]. Genes encoding the ERM protein family are present in all metazoans [Denker and Barber, 2002], and have a conserved structure that includes a FERM (band 4.1, ERM), a central α-domain and a C-terminal actin-binding domain (CTD). ERMs can undergo intramolecular interactions, regulated by phosphatidylinositol 4,5-bisphosphate (PIP_2) binding and

phosphorylation that is thought to either alternatively conceal or expose the CTD [Hoeflich et al., 2003].

In the mouse, a knockout allele of *Rdx* encoding radixin is associated with early postnatal progressive degeneration of cochlear stereocilia and subsequently deafness [Kitajiri et al., 2004]. In this report we provide genetic evidence that wild-type radixin is required for normal sound transduction in humans.

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MATERIALS AND METHODS

Family Enrollment

The Institutional Review Board (IRB) at the National Centre of Excellence in Molecular Biology, Lahore, Pakistan (FWA0-0001758), University of Iowa (IRB199502083), and the NIDCD/NINDS IRB at the National Institutes of Health (OH-93-N-016) approved recruitment of human subjects for this study. Signed informed consent was obtained from all participants of Families PKDF211 and PKDF267 from rural areas of Sindh province, Pakistan, and from the DFNB24 family.

Clinical Evaluation

All members of Families PKDF211 and PKDF267 were subjected to clinical evaluation to rule out syndromic forms of deafness. Pure tone audiometry using air and bone conduction were performed at frequencies 250 Hz, 500 Hz, 1 kHz, 4 kHz, and 8 kHz. Vestibular function was evaluated by using tandem gait and Romberg tests. Funduscopy was performed to exclude an obvious abnormality of the retina. Serum chemistry was evaluated to determine the status of liver and kidney function.

Linkage Analysis

A total of 10 mL of venous blood was obtained and genomic DNA was extracted following a standard protocol [Grimberg et al., 1989]. Short tandem repeat (STR) polymorphic markers were typed for the reported hearing impairment loci (Hereditary Hearing Loss Homepage; http://www.uia.ac.be/dnalab/hhh). Markers were amplified by the PCR on a Gene Amp PCR system 9700 (Applied Biosystems) and were analyzed on an ABI Prism 3100 Genetic Analyzer. The alleles were assigned by means of Genescan and Genotyper software (Applied Biosystems, Foster City, CA).

Candidate Gene Screening

Candidate genes were identified using the University of California–Santa Cruz (UCSC) Genome Bioinformatics web browser (UCSC Genome Bioinformatics; http://genome.ucsc.edu) and selected for mutation screening on the basis of their expression and function in the inner ear. Primers, used for PCR amplification and subsequent sequencing of *RDX* (DQ916738), were designed from the flanking region of each exon using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/). Amplification, sequencing reactions, and mutation analysis were carried out as described [Shabbir et al., 2006].

RDX mutations are numbered based on the cDNA reference sequence DQ916738. The numbering (+1) begins with the A of the translation start codon of *RDX*. Exon 1 ends with nucleotide (nt) −63. Exon 2 begins with nt −64. The translation start codon is in exon 2. Exon 3 begins with nt +13.

Amplification and Cloning of *RDX* Isoforms

Expressed sequence tag (EST) clones BQ186785, CK299903, and BG433367 include sequence of four open reading frames (ORFs) downstream of the reported 3′untranslated region (UTR) of *RDX*. We considered the possibility that these ESTs represent additional but previously unrecognized coding exons of radixin. A forward primer designed from the known exon 1 of *RDX* and a reverse primer from the 3′end of clone BQ186785 and another reverse primer from the 3′end of clone BG433367 (Forward: 5′-CCGGGCAGCCCCGCCTTTTC-3′; Reverse-1: 5′-AACAGCCCAGACAGGAAAGAAATGTTGC-3′ and Reverse-2: 5′-ACCTGCGACAATGATGTCACTTGGA-3′) were used to RT-PCR amplify cDNA and identify the novel isoforms of *RDX*, which were subcloned and fully sequenced. Human retina and inner ear cDNAs were used as template for PCR amplification with LA-Taq polymerase (Takara Bio Mirus, Madison, WI).

Antibodies and Immunocytochemistry

Antisera raised against a synthetic peptide corresponding to amino acid residues 400–409 of human radixin (accession number NP_002897, cat# R 3653; Sigma, St. Louis, MO), was used to immunolocalize radixin in mouse inner ear hair cells. The sequence of the 10 amino acid residues is identical between human and mouse. Fluorescein-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL), and immunocytochemistry was performed as described previously [Belyantseva et al., 2005].

RESULTS

Nonsyndromic Hearing Loss Segregating in Two Pakistani Families Linked to Markers on Chromosome 11q23

We screened approximately 600 families segregating hearing loss for linkage to STR markers at the *DFNB24* locus (Hereditary Hearing Loss Homepage), and found evidence for linkage of markers on chromosome 11q23 to deafness in Families PKDF211 and PKDF267. The inheritance pattern of hearing loss in Families PKDF211 and PKDF267 is consistent with an autosomal recessive trait (Fig. 1). In Family PKDF211 an 18.16-Mb physical interval of marker homozygosity was delimited by D11S917 (96.85 Mb) and D11S908 (114.79 Mb). The maximum two-point log of odds (lod) score for the small Family PKDF211 is 1.71 for markers D11S2017 and D11S4090. In Family PKDF267, meiotic break points provided an interval of 36.39 Mb, bounded by markers D11S918 (78.40 Mb) and D11S908 (114.79 Mb). For deafness segregating in Family PKDF267, a maximum two-point lod score of 3.72 was obtained for D11S901, D11S4175, and D11S898. Assuming that the evidence for linkage of deafness to 11q23 in small Family PKDF211 is not spurious, the haplotype analysis of both families revealed a critical region of 18.16 Mb (11.74 cM) flanked by markers D11S917 and D11S908 (Fig. 2).

All affected individuals in both families displayed prelingual, bilateral, profound sensorineural hearing loss, while vestibular, hepatic, and renal functions were normal. Results of serum chemistry including liver and kidney function tests and glucose and lipid profiles indicated these parameters were within the normal range. Funduscopic examinations of Patient VI:1 (18 years of age) and Patient VI:2 (22 years of age) of Family PKDF211 and Patient IV:2 (37 years of age) of Family PKDF267 (Fig. 1) showed no signs of retinitis pigmentosa.

Mutations of *RDX* Encoding Radixin Are Associated With Nonsyndromic Hearing Loss in Three DFNB24 Families

More than 100 genes are present in our smallest *DFNB24* linkage interval, including five genes that are expressed in the inner ear (Fig. 2; UCSC Genome Bioinformatics: http://genome.ucsc.edu). *RDX*, encoding radixin, was a candidate since it lies in this interval and a radixin-deficient mouse is deaf [Kitajiri et al., 2004]. Two pseudogenes have been reported for *RDX*. *RDXP1*, which is located on chromosome 11p15.4, is an apparent processed pseudogene, whereas *RDXP2* is a truncated version of *RDX*, located at Xp21.3 [Wilgenbus et al., 1993]. To avoid amplification of *RDXP1* and *RDXP2*, we designed primers that specifically
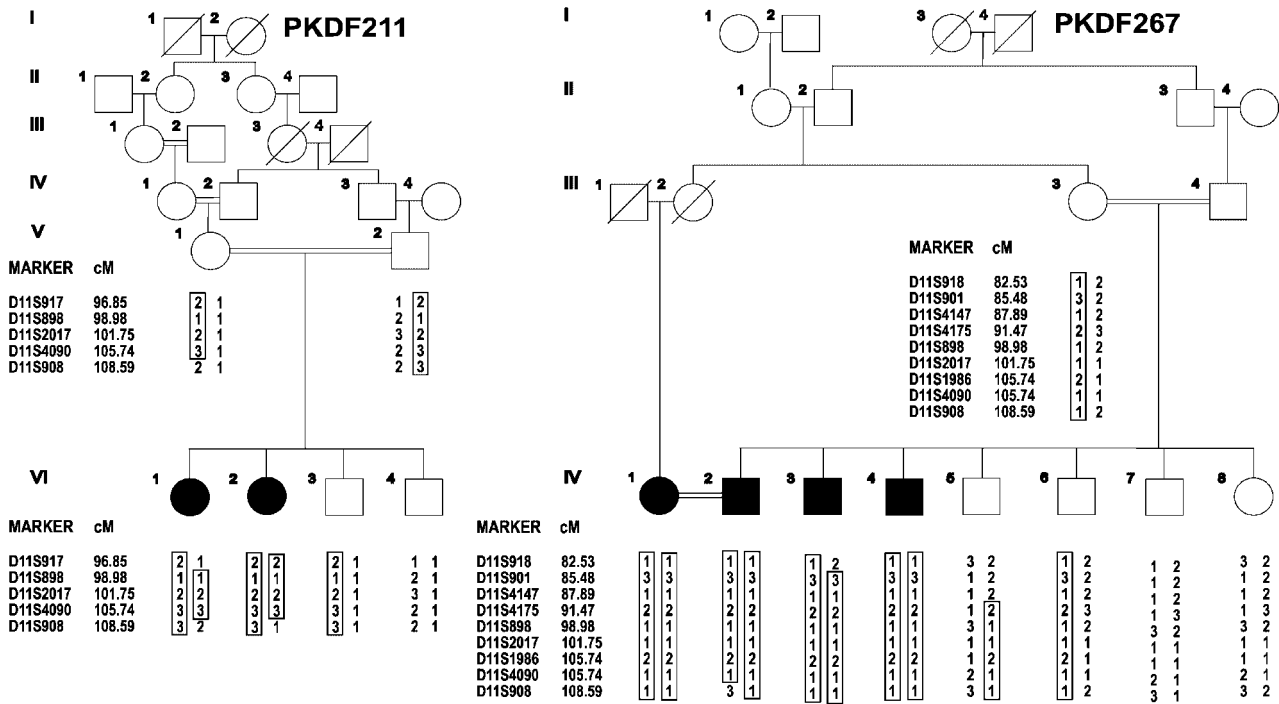
FIGURE 1. Chromosome 11 markers cosegregating with deafness in Families PKDF211 and PKDF267. The linked haplotypes are boxed. The STR markers and their human genetic map positions in centimorgans (cM) according to the Marshfield human genetic map (http://research.marshfieldclinic.org/genetics) are shown on the left of the pedigree. Filled symbols denote profound sensorineural hearing loss. Horizontal double lines indicate a consanguineous marriage.

amplify *RDX*. We sequenced PCR products that included all the coding exons and their splice junctions as well as 5′ and 3′UTRs of *RDX* in affected individuals from Families PKDF211 and PKDF267. A transversion mutation (c.1732G>A) was found in both affected individuals of Family PKDF211. This base pair change results in an amino acid substitution of asparagine for a conserved aspartic acid (p.D578N). Sequence analysis revealed that affected individuals from Family PKDF267 were homozygous for an insertion of 1 nt at position 1404 (c.1404_1405insG) in exon 13 of *RDX*. This mutation is predicted to cause a frameshift in the translational reading frame, resulting in a premature stop codon (p.A469fsX487; Fig. 3A–C). These two alternations (c.1732G>A and c.1404_1405insG) were not found in 200 chromosomes from ethnically- and geographically-matched normally hearing individuals from Sindh province, Pakistan (Table 1).

Since the DFNB24 locus and deafness due to mutations of *RDX* might be allelic disorders, we sequenced *RDX* in affected individuals belonging to the family that originally defined the DFNB24 locus and studied in Richard Smith's laboratory but not previously reported. We found a homozygous transition mutation (c.463C>T) in individuals with DFNB24 hearing impairment. This mutation is predicted to cause a premature truncation of the protein (p.Q155X) in the FERM domain (Fig. 3C; Table 1).

RDX Expresses Six Isoforms in the Human Retina and Inner Ear

Analysis of ESTs in the *RDX* genomic region revealed three clones (BQ186785, CK299903, and BG433367) from a human fetal eye cDNA library with four alternatively spliced exons downstream of the reported 3′UTR of *RDX* (UCSC Genome Bioinformatics; http://genome.cse.ucsc.edu/index.html?org =

Human). Using a forward primer from the known first exon of *RDX* and reverse primers from the 3′end of these EST clones, we amplified five novel isoforms from human inner ear and retina cDNAs. The longest cDNA transcript (isoform b) contains 2,761 bp with an ORF of 1,884 bp and a new 3′UTR of 636 bp (Fig. 3C; accession numbers DQ916738—DQ916742). Following the actin binding domain of radixin, these novel isoforms encode 21 additional amino acids conserved among mammals (Fig. 3D) that show no sequence similarity to other reported proteins, and this sequence is not predicted to encode a recognizable protein domain.

Radixin Is Present Along the Length of the Stereocilia in the Mouse Inner Ear

Radixin immunoreactivity was examined in the organ of Corti and vestibular hair cells of C57BL/6J mice (Fig. 4), and found to be consistent with the reported localization [Kitajiri et al., 2004]. When we examined radixin immunoreactivity at high resolution using immunofluorescence confocal microscopy, radixin was detected along the length of cochlear hair cell stereocilia at postnatal day 30 (P30) with no signal in the cuticular plate (Fig. 4). A similar pattern was observed in hair cells of the crista ampullaris with the greatest signal in the lower portion of stereocilia (Fig. 4).

DISCUSSION

Inner ear hair cell stereocilia have a central core of highly organized parallel filaments of actin, which are held together by different actin-bundling proteins, such as fimbrin and espin [Bartles, 2000]. Radixin expression along the length of the stereocilia suggests that it may also be involved in organizing or anchoring membrane proteins to the central actin filaments. The
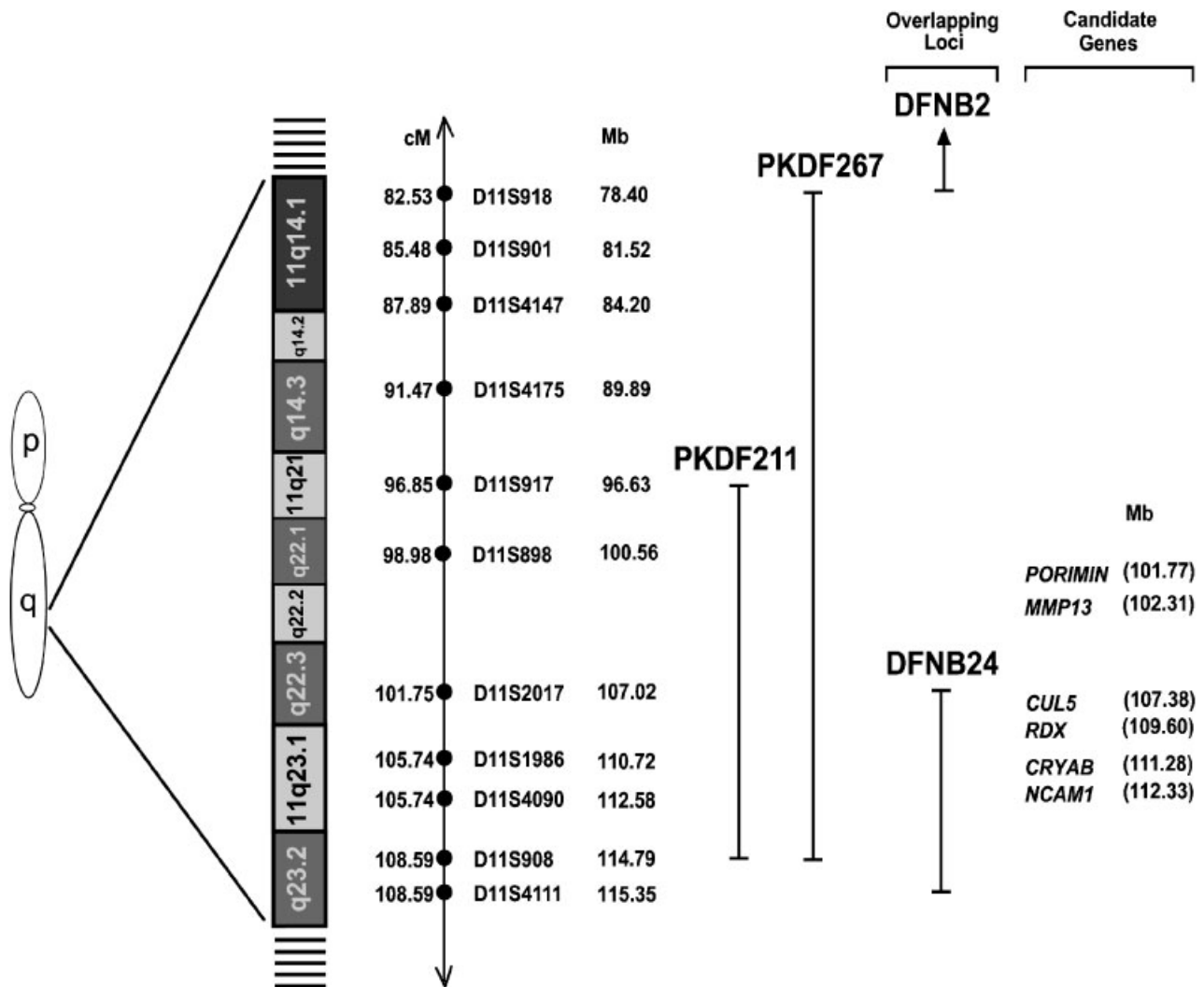
FIGURE 2.   **Ideogram of chromosome 11q14.1–11q23.2, illustrating the linkage intervals in Families PKDF211 and PKDF267 (filled circles: STR markers; solid vertical lines: linkage regions). The linkage interval of *DFNB24* (www.uia.ac.be/dnalab/hhh), the sex averaged recombination distances in centimorgans (cM) (http://research.marshfieldclinic.org/genetics) and the physical distance in megabases (Mb) are indicated along with STR markers. The linkage region from both families combined spans 18.16 Mb flanked by markers D11S917 (96.63 Mb) and D11S908 (114.79 Mb). Cytogenetic localizations of six genes in the linkage interval are shown.**

full length radixin protein has 627 amino acids forming three known functional domains (Fig. 3C), an N-terminal FERM domain, a central helical α-domain, and a CTD. FERM domains localize proteins to the plasma membrane whereas the CTD contains an actin-binding motif [Hoeflich et al., 2003; Turunen et al., 1994; Pestonjamasp et al., 1995]. The helical α-domain plays an active role in radixin protein activation [Ishikawa et al., 2001]. Inactive states of ERM proteins show a low level of binding activity to both membrane and actin. These inactive states are believed to result from a masking mechanism in which the FERM domain binds the CTD to suppress the actin filament and membrane-binding activities [Bretscher et al., 2002; Tsukita and Yonemura, 1999]. Masked ERM molecules are activated when PIP$_2$ binds to the FERM domain [Bretscher et al., 2002; Tsukita and Yonemura, 1999]. Subsequent Rho-induced phosphorylation of radixin Thr564 (located in the C-terminal region) maintains radixin in the active state and regulates its binding to actin [Bretscher et al., 2002; Tsukita and Yonemura, 1999]. Activated ERM molecules join actin filaments to adhesion proteins (e.g., CD43, CD44, ICAM1-3, etc.) in the downstream Rho signaling pathway [Bretscher et al., 2002; Tsukita and Yonemura, 1999]. In hair cell stereocilia, the proteins that interact with radixin remain to be identified.

In the mouse, a radixin deficiency causes deafness but no vestibular dysfunction [Kitajiri et al., 2004]. In this *Rdx$^{-/-}$* mouse, the stereocilia bundle degenerates progressively after P14 [Kitajiri et al., 2004]. In contrast to the organ of Corti, in the vestibular hair cells of *Rdx$^{-/-}$* mice stereocilia do not degenerate and ezrin appeared to compensate for the lose of radixin [Kitajiri et al., 2004]. The radixin knockout mouse is not only deaf but has hyperbilirubinemia [Kikuchi et al., 2002]. Consistent with the hearing and vestibular phenotype of the *Rdx* knockout mouse [Kitajiri et al., 2004], members of Families PKDF211 and PKDF267, homozygous for *RDX* mutant alleles, manifested hearing loss but no obvious vestibular defect. However, we did not detect hyperbilirubinemia in all of the affected individuals from both families. The liver phenotype in the radixin-deficient mouse is thought to be due to mislocalization of the Mrp2 transporter, which binds to the radixin N-terminus but not to the radixin actin binding domain at the C-terminus [Kikuchi et al., 2002].
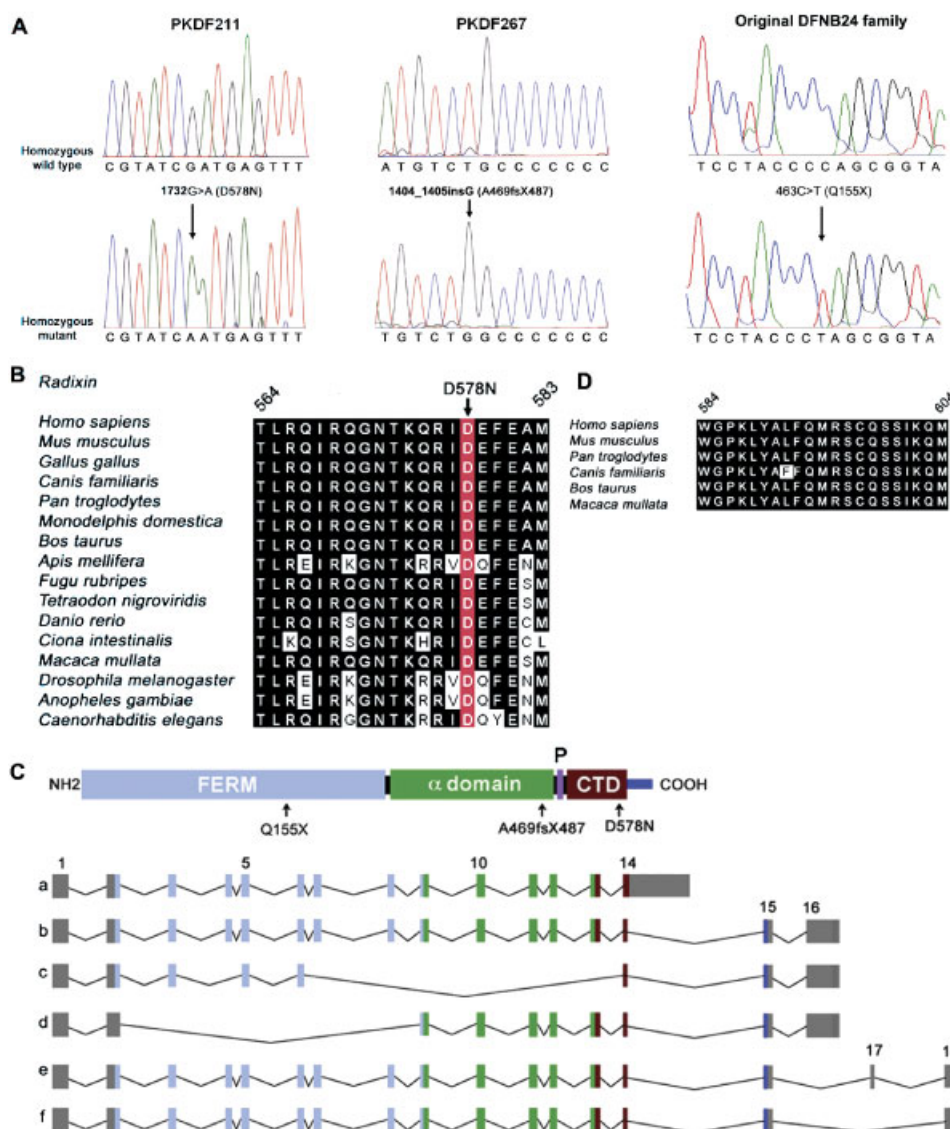
FIGURE 3. Three mutant alleles of *RDX*, a revised gene structure for *RDX* and ClustalW alignment of the tail region of radixin from several species. **A:** Wild-type and mutant alleles from unaffected and affected members of Families PKDF211 and PKDF267, and the original DFNB24 family, respectively. Mutation numbering is based on the cDNA reference sequence DQ916738. **B:** ClustalW multiple sequence alignment of radixin amino acids shows that D578 is conserved across species (shaded background, same amino acids; light background, nonconserved amino acids). **C:** Schematic representation of the protein and isoforms structure for human radixin (reference sequence DQ916738–DQ916742). Domain organization along with amino acid residue numbers and location of p.Q155X, p.A469fsX487, and p.D578N is indicated in the FERM, helical α-domain and CTD, respectively. Below the protein structure is the exonic structure of *RDX* and five novel isoforms of *RDX*. Gray rectangles represent the noncoding region of each isoform. **D:** ClustalW multiple sequence alignment of the 21 newly-identified amino acids of radixin encoded by exon 15. FERM, band 4.1/ezrin/radixin/moesin domain; P, polyproline region.

Mutant alleles found in Families PKDF211 and PKDF267 are predicted to disrupt the actin binding domain of radixin. Identification of three recessive mutations of *RDX* demonstrates the importance of radixin in the human auditory system. The p.D578N substitution is located in the last 34 residues (550–583) of CTD, a conserved actin-binding motif. Aspartic acid (D) is a hydrophilic negatively charged amino acid whereas asparagine (N) is a hydrophilic neutral amino acid and is predicted to alter the actin-binding domain of radixin. The insertion mutation (c.1404_1405insG; p.A469fsX487) in the Family PKDF267 resides at the end of the helical α-domain and is predicted to cause a frameshift, two residues before the polyproline domain, subsequently resulting in a premature stop codon and a truncated

protein of 486 residues. The p.Q155X mutation in the original DFNB24 family resides in exon 5, which is not included in *RDX* isoform d and may suffice in non-inner ear cell types and thus might explain the deafness-only phenotype of p.Q155X. Conversely, exon 5–containing *RDX* transcripts are necessary for inner ear function. In vivo, *RDX* mRNA with either 1404_1405insG or 463C>T mutations may be translated into a truncated protein or could be degraded by the nonsense-mediated decay pathway [Maquat, 2004].

In summary, we have identified three likely pathogenic mutations of *RDX* associated with deafness, segregating as a recessive trait in three consanguineous families. Although the pathogenesis due to the loss of radixin is consistent with

TABLE 1. List of All Nucleotide Changes Found During the Sequencing of *RDX*

Region	Nucleotide changes[a]	Allele frequency[b]	Effect
Exon 5	c.463C>T	0/200	Pathogenic
Exon 13	c.1404_1405insG	0/200	Pathogenic
Exon 14	c.1732G>A	0/200	Pathogenic
Intron 1	c.-64-70delT	20/100	Polymorphism
Intron 2	c.13-115_13-114insT	35/100	Polymorphism
Intron 5	rs36094903:-/T	32/100	Polymorphism
Intron 7	rs2306085:C>T	38/100	Polymorphism
Exon 10	rs2306085:C>T	45/100	Polymorphism
3′ UTR	rs35381725:-/T	25/100	Polymorphism
3′ UTR	3′ UTR (3036delGTGTGT)	15/100	Polymorphism

[a]All changes are numbered according to RefSeq DQ916738. The numbering (+1) begins with the A of the translation start codon of *RDX*. Exon 1 ends with nt -63. Exon 2 begins with nt -64. The translation start codon is in exon 2. Exon 3 begins with nt +13.
[b]All the allele frequencies are given for the second of the two alleles.
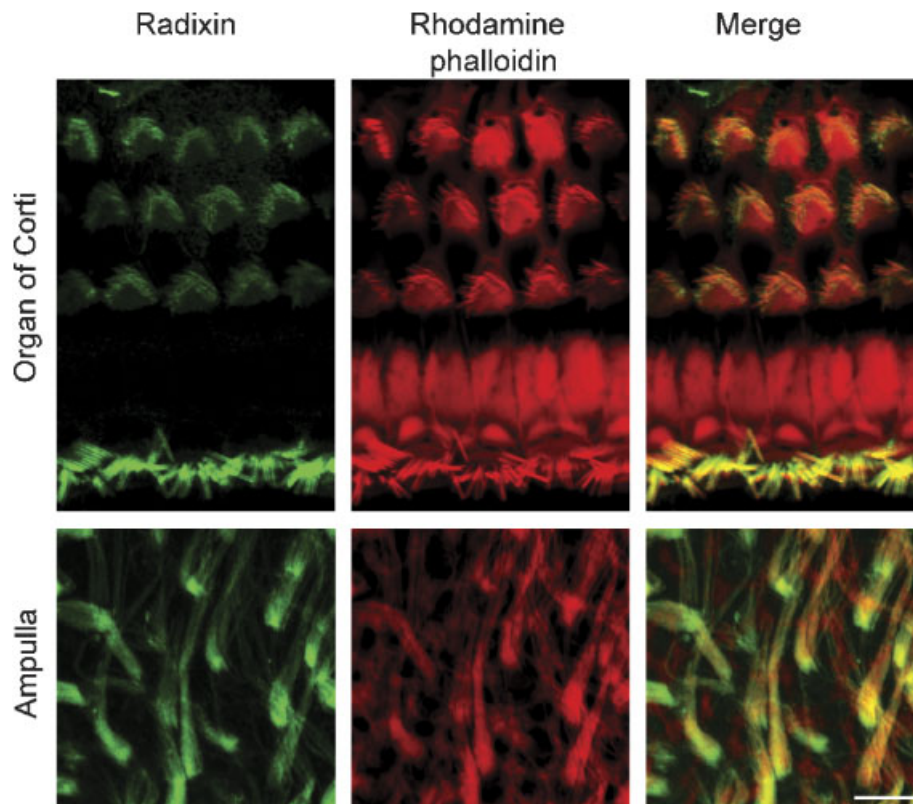rs, reported SNPs.

FIGURE 4. **Immunolocalization and confocal microscopy of radixin in the organ of Corti and vestibular sensory epithelia of P30 C57BL/6J mice. Radixin was detected in the stereocilia of inner and outer hair cells (green channel). Double staining for radixin and F-actin shows colocalization of these proteins in stereocilia (green and red channels together). No radixin immunoreactivity was observed in the kinocilium of developing cochlear and vestibular hair cells. Scale bar: 5 μm.**

observations from the knockout mouse model [Kitajiri et al., 2004], the biochemical role of radixin in normal organ of Corti hair cell stereocilia development and maintenance remains to be determined.

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