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Mutations of *GIPC3* cause nonsyndromic hearing loss DFNB72 but not DFNB81 that also maps to chromosome 19p

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Ethical standards Experiments for this study were performed in Pakistan and in the United States, and comply with the current laws of the country in which they were performed.

Conflict of interest The authors declare that they have no conflict of interest.

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Abstract

A missense mutation of *Gipc3* was previously reported to cause age-related hearing loss in mice. Point mutations of human *GIPC3* were found in two small families, but association with hearing loss was not statistically significant. Here, we describe one frameshift and six missense mutations in *GIPC3* cosegregating with DFNB72 hearing loss in six large families that support statistically significant evidence for genetic linkage. However, *GIPC3* is not the only nonsyndromic hearing impairment gene in this region; no *GIPC3* mutations were found in a family cosegregating hearing loss with markers of chromosome 19p. Haplotype analysis excluded *GIPC3* from the obligate linkage interval in this family and defined a novel locus spanning 4.08 Mb and 104 genes. This closely linked but distinct nonsyndromic hearing loss locus was designated *DFNB81*.

Introduction

We previously mapped a nonsyndromic recessive hearing loss locus *DFNB72* to chromosome 19p13.3 based on analyses of three large consanguineous Pakistani families, PKDF335, PKDF793, and PKDF291, each independently yielding a LOD score of greater than 3.0 (Ain et al. 2007). Assuming locus homogeneity as the least complex explanation of our data, the smallest interval of shared homozygosity within these families spanned 1.16 megabases (Mb) between markers *D19S216* and *D19S1034*, which excluded *GIPC3*. Recently, a missense mutation of *Gipc3* was reported to be associated with age-related sensorineural hearing loss ahl5 and audiogenic seizures in mouse (Charizopoulou et al. 2011). In the same paper, hearing loss (HL) segregating in two small human families was also reported to be due to mutations of *GIPC3*. However, these two human families are not large enough to provide statistically significant evidence of linkage. Thus, the question remains whether mutations of *GIPC3* are associated with HL in humans.

For two of the original DFNB72 families that we reported (PKDF335 and PKDF793), the linkage interval contains 204 genes including *GIPC3*. Additional linkage data and mutation analysis of *GIPC3* now provide evidence of *GIPC3* as the cause of DFNB72 HL. Here, we

report seven homozygous recessive mutations of *GIPC3* associated with mild to profound HL segregating in seven large consanguineous families. Our data also indicate that on chromosome 19p there is another locus for nonsyndromic HL that genetically excludes *GIPC3*. This locus is adjacent to but genetically distinct from *DFNB72* and is designated *DFNB81*.

Materials and methods

Written informed consent was obtained from participants following approval for the study from the Combined Neuroscience Institutional Review Board (IRB OH93-DC-0016) at the National Institutes of Health, Bethesda, MD, USA, the IRBs at the National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan, Quaid-I-Azam University Islamabad, Baylor College of Medicine, and Cincinnati Children's Hospital Research Foundation. Ascertainment and linkage analysis of families PKDF335, PKDF793, and PKDF291 was described previously (Ain et al. 2007). Families DEM4322, PKDF1048, DEM4197, and PKSR22A were ascertained through special education schools from Pakistan while family PKDF1258 contacted us on the recommendation of an audiologist.

Audiological examinations for families PKDF1258, PKDF1048, PKDF335, PKDF793, and PKDF291 were provided by audiologists in Pakistan. For families DEM4322 and DEM4197, audiology was conducted under ambient conditions. Audiograms for family PKSR22A are neither available nor are additional diagnostic assessments of the ear and temporal bone for any of the families. All audiometric data from this study were evaluated by C.C.B. at the NIDCD/NIH. Funduscopic examinations were carried out by ophthalmologists in Pakistan. Tandem gait and Rhomberg tests were performed to evaluate balance.

Genomic DNA was extracted following standard procedures from blood samples donated by members of families newly ascertained for this study. Families PKDF1258, PKDF1048, and PKSR22A were genotyped using short tandem repeat (STR) markers located in the *DFNB72* interval. For family DEM4322, genotyping was performed using the Illumina HumanLinkage-12 panel which contains 6,090 SNP marker loci while for the DEM4197 pedigree, 396 fluorescently labeled STRs were genotyped. For families DEM4322 and DEM4197, genome-wide genotyping was performed at the Center for Inherited Disease Research (CIDR).

To determine if human *GIPC3* mutations are associated with DFNB72 HL, we sequenced the coding region of *GIPC3* in one affected subject from each of the eight families. Primers used to PCR amplify and sequence the coding exons of *GIPC3* were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). DNA sequence of the coding exons of *GIPC3* was determined using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The nucleotide sequence of exon 1 of *GIPC3* has high GC-content (77%) and was amplified using Advantage GC Genomic LA Polymerase Mix (Clontech). Sequencing products were analyzed on an ABI3730 capillary sequencing instrument (Applied Biosystems), and the resulting sequence traces were aligned to the reference sequence using Lasergene 8 (DNASTAR).

Results

Seventeen affected individuals in families DEM4322, PKDF1258, PKDF1048, DEM4197, PKDF335, and PKDF793 had bilateral HL that ranged from mild to profound (Fig. 1a). Bone conduction thresholds, when tested, confirmed the HL as sensorineural. Three affected individuals from family PKDF291 have bilateral, severe to profound mixed HL while the

fourth affected individual has a profound sensorineural HL (Fig. 1b). Hearing loss in these families was not accompanied by obvious vestibular dysfunction, retinal dysfunction, or report of other anomalies. These data suggest that the families are segregating nonsyndromic HL although a yet unrecognized syndrome cannot be ruled out.

We previously defined a 1.16-Mb critical linkage interval for *DFNB72* that extends between markers *D19S216* and *D19S1034* (Ain et al. 2007) (Fig. 2a, b, c). In this study, we used SNP and STR markers to establish linkage between the HL phenotype segregating in additional five Pakistani families and chromosomal region 19p13.3–p13.2. The linkage interval of family PKDF1258 refined the critical *DFNB72* interval to that between markers *D19S209* and *D19S894* (Fig. 2c, gray shaded area). This refined interval spans 1.08 Mb and contains at least 36 genes including *GIPC3*. We sequenced the coding region of *GIPC3* and found one homozygous frameshift and six different homozygous missense mutations in the affected individuals from seven families. The frameshift mutation identified in family PKDF335 arose as a result of a duplication of a guanine nucleotide at position 685 of the mRNA (NM_133261.2). The c.685dupG is predicted to alter the open reading frame by introducing nine missense amino acids followed by a premature stop codon (p.Ala229GlyfsX10). This may render the transcript susceptible to nonsense-mediated mRNA decay (Nicholson et al. 2010). In four DFNB72 families, we identified four homozygous transition mutations of guanine nucleotide (G) to adenine (A) at positions 136, 264, 281, and 767 of the mRNA. These four transition mutations are predicted to substitute Arg for wild-type Gly at position 46, Ile for Met at residue 88, Asp for Gly at position 94, and Asp for Gly at amino acid position 256 of the full length protein (NP_573568.1), respectively. In two DFNB72 families, we detected pyrimidine transitions of cytosine (C) to thymine (T) at positions 565 and 662 of the mRNA that are predicted to replace Arg189 and Thr221 of wild-type GIPC3 with Cys and Ile residues, respectively (Fig. 3a).

The seven homozygous mutations of *GIPC3* cosegregated with HL in the corresponding families and are not present in dbSNP build 130 or in the 1000 Genomes database. We also did not find these nucleotide variants in 572–590 ethnically matched control chromosomes (Table 1). The amino acids affected by the six missense mutations of *GIPC3* reported here are conserved among the three human *GIPC* paralogs and the orthologous genes in Xenopus and Drosophila (Katoh 2002), and among vertebrate *GIPC3* genes (Fig. 3b). The functional consequences of the six missense mutations were evaluated in silico using Mutation Taster (http://www.mutationtaster.org/), SIFT (http://sift.jcvi.org/), and PolyPhen (http://genetics.bwh.harvard.edu/pph/) and were predicted to be pathogenic, disrupting GIPC3 function.

GIPC3 encodes a 312 amino acid protein that contains three predicted low complexity regions and a central conserved PDZ domain named for a motif found in the proteins *P*SD-95, *D*lg, and *Z*O-1 (Katoh 2002; Saitoh et al. 2002). Two of the three low complexity regions are similar in sequence among GIPC family members and are referred to as GIPC homology domains (GH1 and GH2) (Katoh 2002). The GH2 domain of GIPC1 interacts directly with the actin-based molecular motor myosin 6, in which mutations cause HL in humans and mice (Ahmed et al. 2003; Avraham et al. 1995). The frameshift mutation identified in family PKDF335 is located in exon 4 encoding the GH2 domain (Fig. 3c). If the c.685dupG (p.Ala229Gly fsX10) transcript survives nonsense-mediated mRNA decay, it would produce a truncated GIPC3 protein that lacks 85% of the GH2 domain. With one exception (p.Arg189Cys), all of the mutations of *GIPC3* identified to date are located in one of the two GH domains (Fig. 3c).

Interestingly, haplotype analyses in families PKDF1258 and PKDF1048 segregating *GIPC3* mutations did not reveal shared regions of homozygosity with the HL locus identified in

family PKDF291, indicating HL locus heterogeneity on chromosome 19p. Thus, there are at least two closely linked but non-overlapping *DFNB* loci between markers *D19S886* and *D19S916* (Fig. 2c). *GIPC3* is located outside of the linkage interval for HL segregating in family PKDF291 (Fig. 2b, c). Nevertheless, we used genomic DNA of an affected PKDF291 family member and sequenced the six exons of *GIPC3*. As expected, we did not find a pathogenic variant of *GIPC3*. However, we did detect a heterozygous SNP (*rs8113232*; G>A) in exon 2 of *GIPC3*. The parents were homozygous for different alleles of *rs8113232*. Their children are obligate *rs8113232* heterozygotes (Fig. 2b). Together, these data indicate that the cause of HL in family PKDF291 is unrelated to *GIPC3*, and a maximum multipoint LOD score of 3.35 at the marker locus *D19S391* demonstrates a third genetically distinct recessive HL locus on chromosome 19p designated *DFNB81*.

Discussion

A human genome contains deleterious mutations in excess of even H. J. Muller's prediction (Muller 1950). Massively parallel sequencing of exomes or genomes of apparently healthy humans has revealed hundreds of heterozygous and even homozygous nonsense polymorphisms along with thousands of synonymous and missense variants (Li et al. 2010; MacArthur and Tyler-Smith 2010; Yngvadottir et al. 2009). A well-established strategy to distinguish between a benign variant and a pathogenic mutation, especially for heterogeneous disorders such as hearing loss (HL), is to genetically link the phenotype segregating in a large family to a chromosomal interval before attempting to identify a causal mutation (Cavalli-Sforza and King 1986; Dror and Avraham 2009; Friedman and Griffith 2003; Hilgert et al. 2009; Morton and Nance 2006).

The possible involvement of two mutations of *GIPC3* in human nonsyndromic HL was reported, but neither of the two families segregating mutations of *GIPC3* provides a sufficient number of informative meioses to produce a significant LOD score (Charizopoulou et al. 2011). One consanguineous family has two affected individuals (Charizopoulou et al. 2011) and could only yield a maximum LOD score of 1.92. In the second family of Indian origin (Charizopoulou et al. 2011), HL was previously genetically mapped to chromosome 3 and chromosome 19 with the maximum LOD score of 2.78, and both loci were designated *DFNB15* (Chen et al. 1997).

Our data do provide statistically significant evidence of linkage of human HL to mutations of *GIPC3* (Table 1). In addition to describing seven *GIPC3* mutations as the cause of DFNB72 HL, none of which has been previously reported, we also provide evidence of an autosomal recessive HL locus closely linked to but distinct from *DFNB72* (*GIPC3*). The linkage interval for HL segregating in family PKDF291 is bounded by markers *D19S216* and *D19S916* on chromosome 19p and defines an unreported locus designated as *DFNB81* (Human Nomenclature Committee). This interval spans 4.08 Mb and contains 104 genes. The *DFNB81* linkage interval does not include *GIPC3*, and it does not overlap with *DFNB68*, the only other reported recessive HL locus mapped to chromosome 19 with statistically significant evidence of linkage (Fig. 2c) (Santos et al. 2006). Genetically mapping three closely linked nonsyndromic HL loci on chromosome 19 is not unexpected since chromosome 19 has more than double the gene density compared to the genome-wide average (Grimwood et al. 2004). Future studies will take advantage of massively parallel sequencing technologies (Rehman et al. 2010; Walsh et al. 2010) to identify the causative recessive mutation responsible for DFNB81 HL and explore its function in the auditory system.

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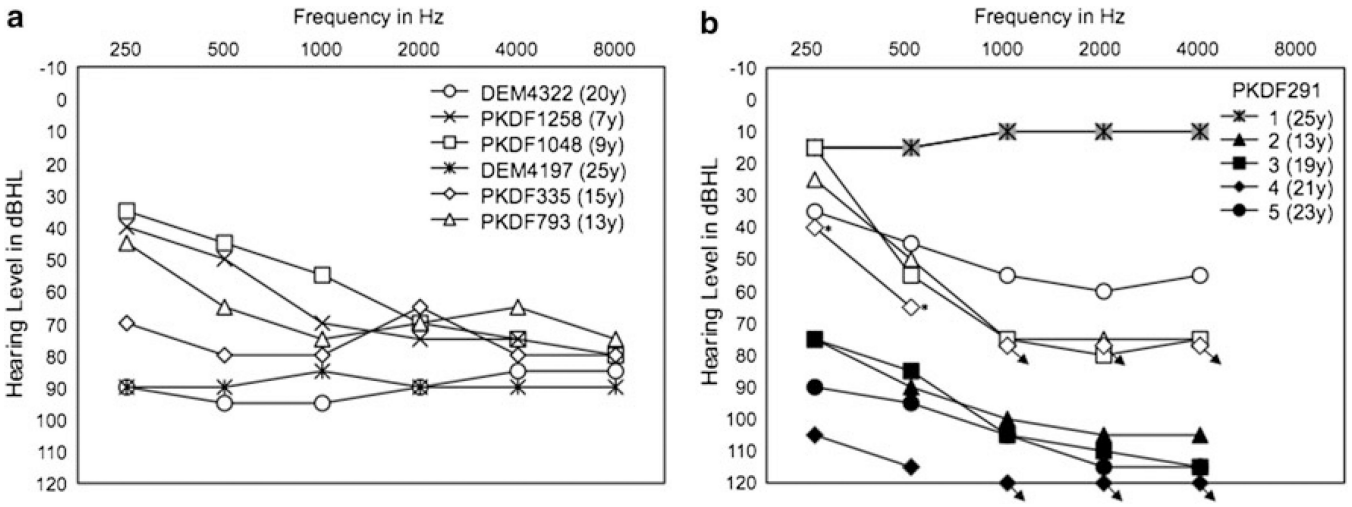

Fig. 1.

a Representative pure-tone air conduction thresholds from an affected individual from each family segregating HL due to mutations of *GIPC3* (*DFNB72*). Hearing loss ranged from mild to severe in families PKDF1258 and PKDF1048, moderate to severe in PKDF335 and PKDF793, and severe to profound in families DEM4322 and DEM4197. All individuals with reported bone-conduction thresholds had sensorineural HL. Two carriers from families PKDF1048 and PKDF793 have normal hearing sensitivity (data not shown). **b** Pure-tone air (*filled symbol*) and bone (*open symbol*) conduction thresholds from members of family PKDF291 that define the *DFNB81* locus. Note that unaffected individual 1 has normal hearing sensitivity, individuals 2, 3, and 5 have severe to profound mixed HL, and individual 4 has a profound sensorineural HL. *Asterisks* indicate response to vibrotactile sensation; *arrows* indicate no response at audiometer output limits

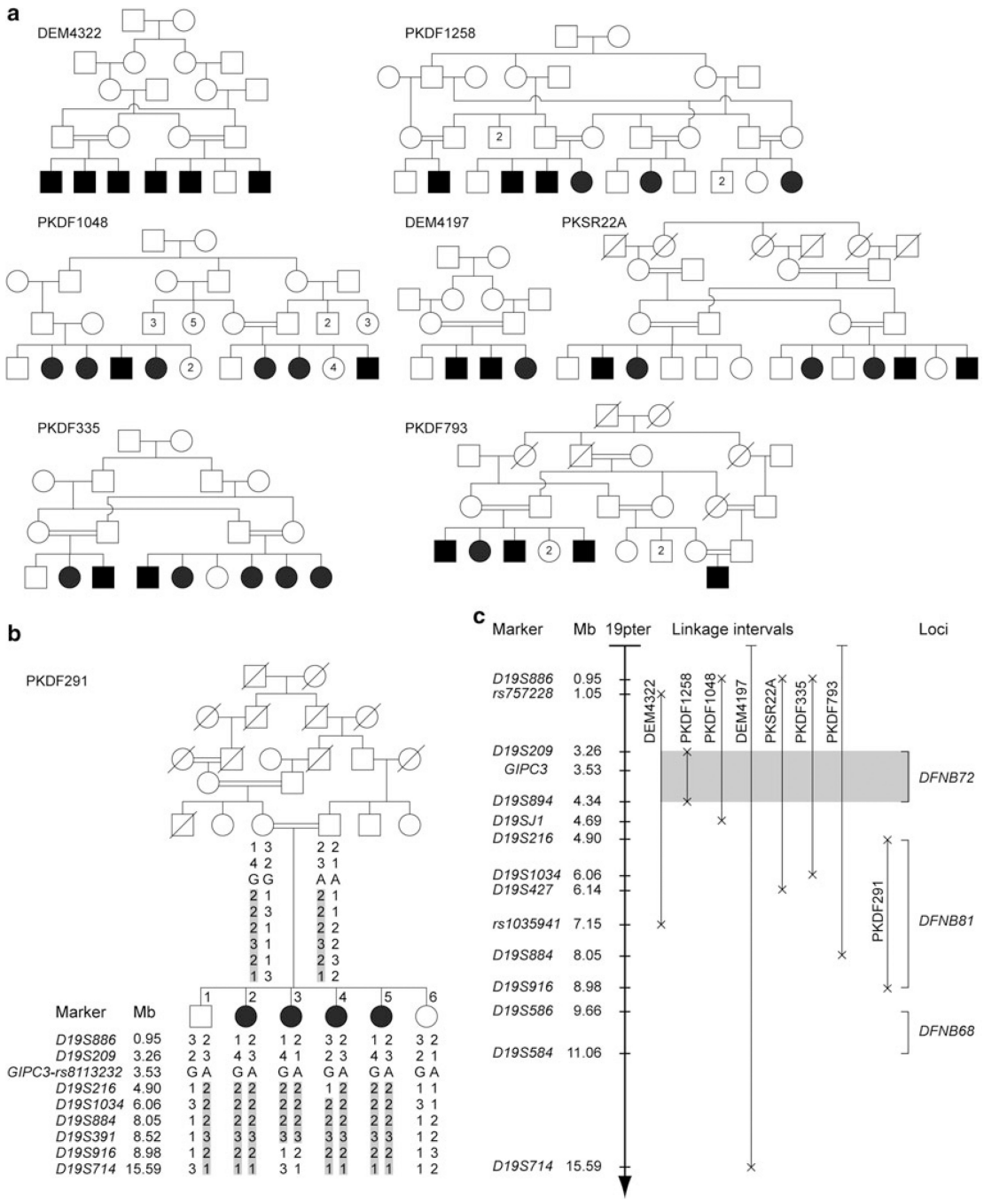

Fig. 2.

a Pedigrees of families segregating deafness genetically mapped to *DFNB72* on chromosome 19p. *Squares* and *circles* denote male and female family members, respectively. *Filled symbols* represent affected individuals. **b** Pedigree and haplotypes of family members of PKDF291 that genetically excludes *GIPC3*. Genotypes of individuals for SNP *rs8113232* located in exon 2 of *GIPC3* are shown. Hearing-impaired individuals are heterozygous for *rs8113232*. **c** Telomeric region of chromosome 19p includes three closely linked hearing loss loci *DFNB72*, *DFNB81*, and *DFNB68*. Regions of homozygosity for each family are represented by *vertical lines* while *crosses* at the ends of *vertical lines* indicate meiotic recombinations. Haplotype analysis (data not shown) of family PKDF1258

refined the *DFNB72* critical interval between markers *D19S209* and *D19S894*. *Gray rectangle* highlights the refined *DFNB72* interval that includes *GIPC3*. Note that the linkage region of family PKDF291 does not overlap with the genomic positions of *GIPC3* or *DFNB68*, defining an unreported deafness locus designated *DFNB81*. Locations of STR markers and *GIPC3* are based on human genome reference sequence NCBI Build 36.1 (hg18). *Mb* megabases

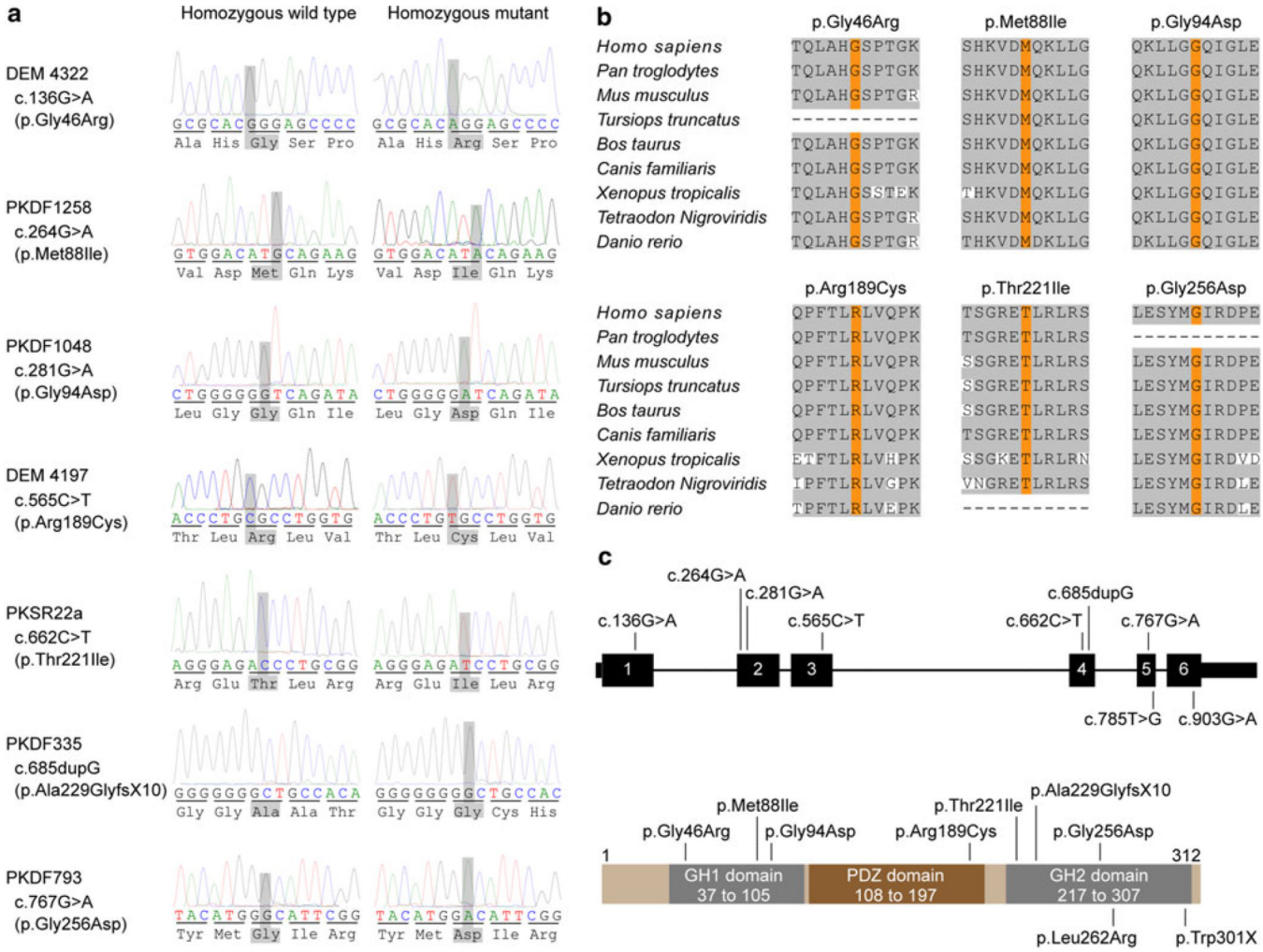

Fig. 3.

a Nucleotide sequence chromatograms from selected regions of *GIPC3* harboring homozygous mutations segregating in seven *DFNB72* families. The mutated nucleotides and predicted altered amino acid residues are highlighted in *gray*. **b** GIPC3 amino acid conservation from nine vertebrates. Conserved amino acids are shaded in *gray* while the amino acids affected due to nucleotide variants identified in our DFNB72 families are highlighted in *orange*. **c** Gene structure (*black rectangles*) and predicted domains of GIPC3 (colored illustration). *Thick rectangles*, *joining lines*, and *thin rectangles* represent exons, introns, and untranslated regions (UTRs), respectively. Locations of seven mutations of *GIPC3* detected in DFNB72 families (*above*) and two previously reported in Charizopoulou et al. (2011) (*below*) are shown for schematics of the gene and protein. Mutation names are based on the full length *GIPC3* transcript (NM_133261.2) and encoded protein (NP_573568.1). *GH* GIPC homology; *PDZ* PSD-95, Dlg, and ZO-1

Table 1

Mutations of *GIPC3*

Family ID	Maximum LOD score[a]	Hearing phenotype	Mutations of *GIPC3*		Control chromosomes
			mRNA NM_133261.2	Protein (score)[h] NP_573568.1	
DEM4322[b]	5.54	Severe to profound	c.136G>A	p.Gly46Arg (0.00)	0/590
PKDF1258[b]	4.06	Mild to severe	c.264G>A	p.Met88Ile (0.00)	0/572
PKDF1048[b]	3.59	Mild to severe	c.281G>A	p.Gly94Asp (0.02)	0/572
DEM4197[b]	2.53	Severe to profound	c.565C>T	p.Arg189Cys (0.00)	0/590
PKSR22A[b]	3.51	Profound[c]	c.662C>T	p.Thr221Ile (0.00)	0/572
PKDF335[d]	3.85	Moderate to severe	c.685dupG	p.Ala229GlyfsX10	0/572
PKDF793[d]	3.71	Moderate to severe	c.767G>A	p.Gly256Asp (0.00)	0/572
Indian family[e]	2.78[f]	Profound	c.785T>G	p.Leu262Arg (0.00)	0/322
W98–042[e]	1.92[g]	Profound	c.903G>A	p.Trp301X	0/312

[a] Multipoint LOD scores are shown for families DEM4322 and DEM4197. Two-point LOD scores are shown for the other families

[b] Newly ascertained DFNB72 families reported in this study

[c] From family history, but no audiological data available

[d] Families that originally defined the *DFNB72* locus (Ain et al. 2007)

[e] Families reported in Charizopoulou et al. (2011)

[f] LOD score reported in Chen et al. (1997)

[g] We calculated two-point LOD score for family W98-042 using *GIPC3* variant c.903G>A as a marker (Charizopoulou et al. 2011). The frequency of A allele was assumed to be 0.001

[h] Effects of amino acid substitutions on protein function using SIFT. Amino acids with probabilities <0.05 are predicted to be deleterious