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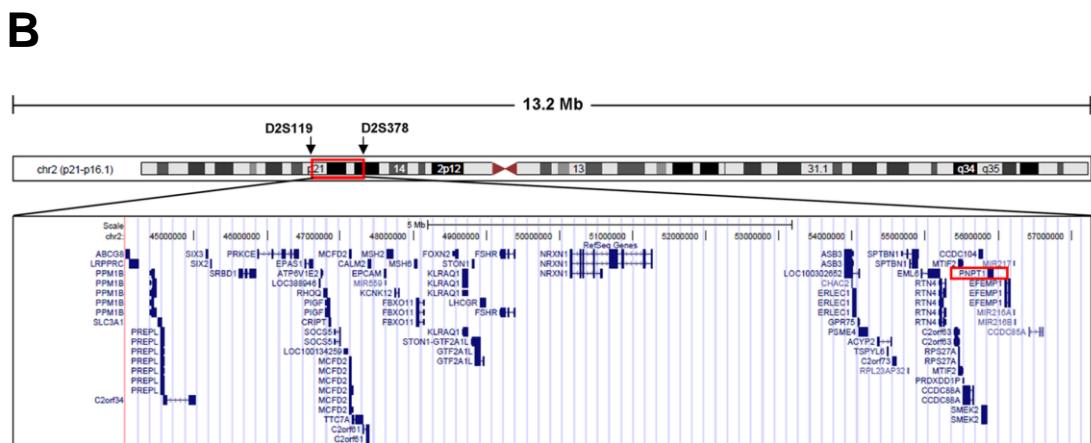
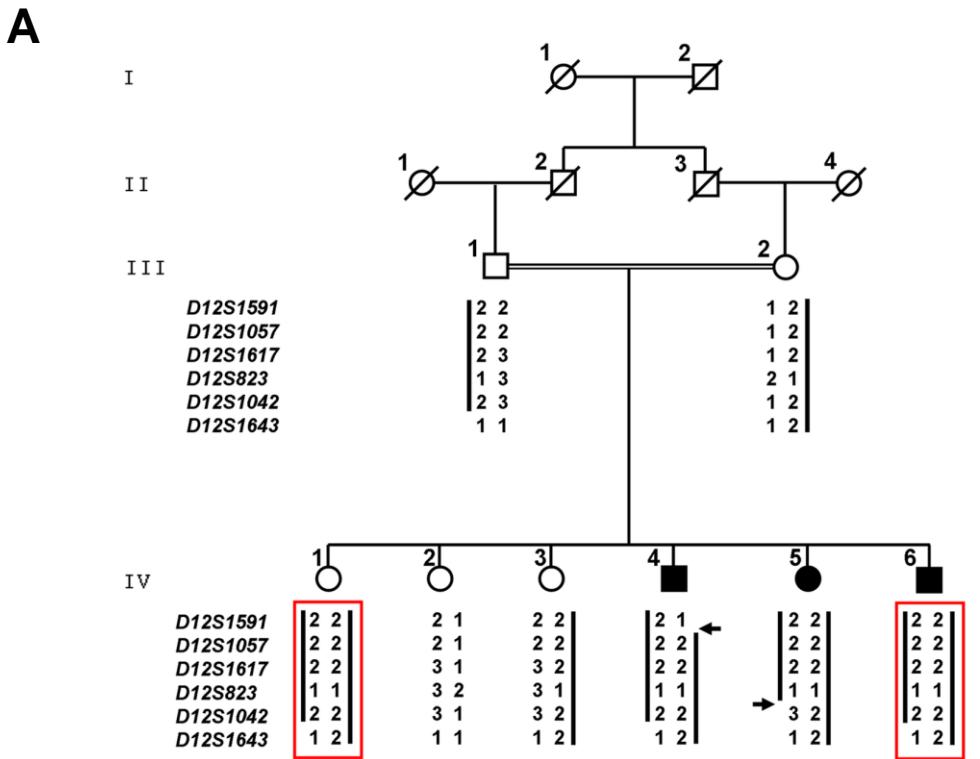
## **Supplemental Data**

### **A Mutation in *PNPT1*, Encoding**

### **Mitochondrial-RNA-Import Protein PNPase,**

### **Causes Hereditary Hearing Loss**

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### **Figure S1. Exclusion of a Putative Locus on Chromosome 12 (A) and Overview of the Critical Region on Chromosome 2p (B)**

(A) Selected microsatellite markers covering the putative chromosome 12 locus were analysed. Haplotype reconstruction revealed that the unaffected subject IV-1 and affected subject IV-6 share identical haplotypes (red boxes). Thus, the locus was excluded as a putative candidate region.

(B) The linked region on the short arm of chromosome 2 is defined by microsatellite markers *D2S119* (telomeric) and *D2S378* (centromeric) and spans approximately 13.2 Mb. *PNPT1* is located close to the centromeric border of the linkage interval. Data was obtained from the UCSC Genome Browser based on Genome Reference Consortium release h37 (February 2009).

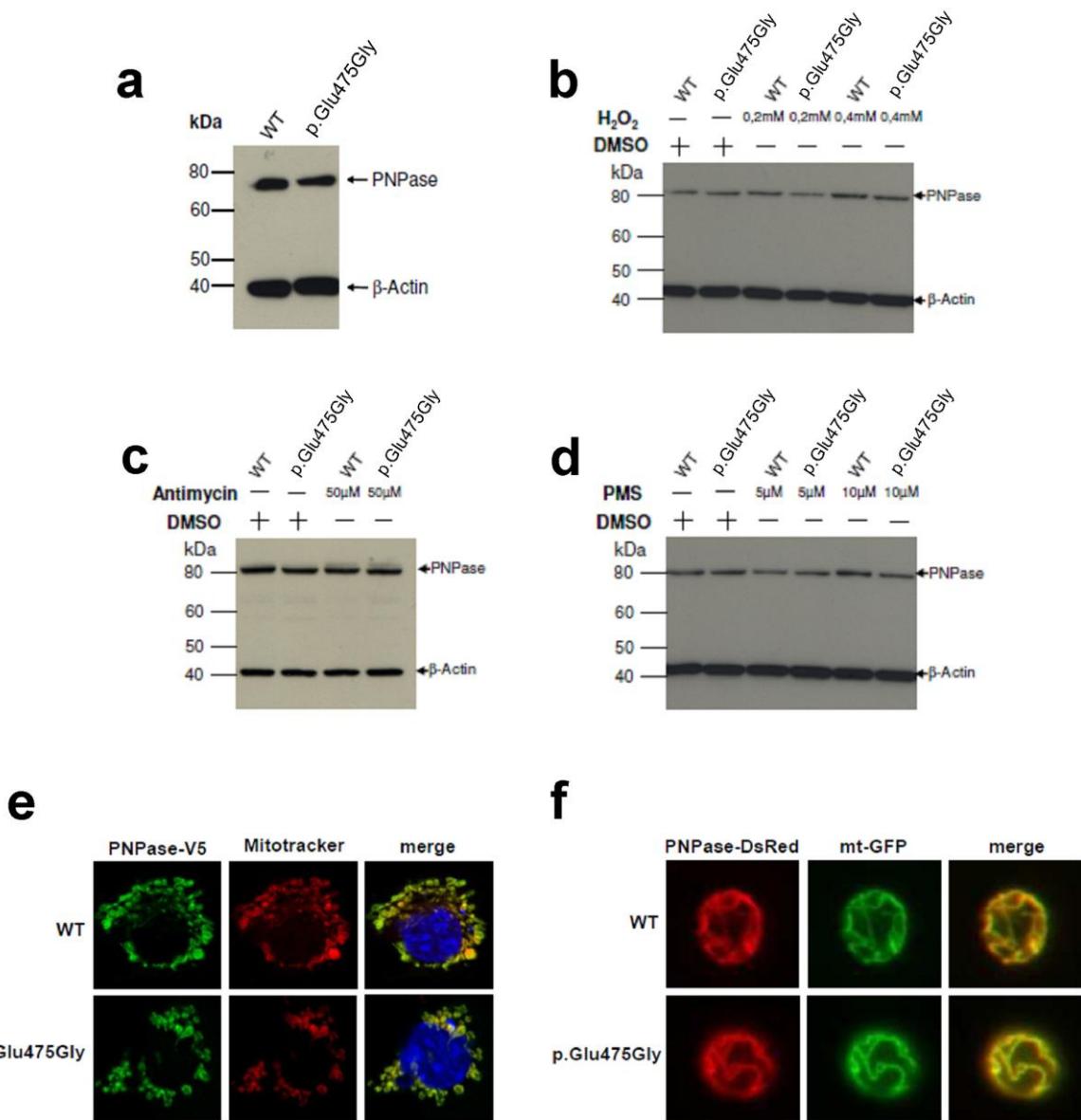
**Figure S2. Identification of *pnpt1* in Zebrafish by RACE-PCR and RT-PCR**

To identify the putative *PNPT1* ortholog in zebrafish, which was not yet annotated in common databases, we performed RACE-PCR (rapid amplification of cDNA ends, 5'/3' RACE 2<sup>nd</sup> generation kit, Roche, Mannheim, Germany) and RT-PCR (OneStep RT-PCR kit, Qiagen, Hilden, Germany) using RNA from zebrafish embryos 48 hours post fertilization (RNA isolation performed with TRIzol reagent, Invitrogen, Darmstadt, Germany). Obtained fragments were analysed by Sanger sequencing and assembled using Seqman software (DNASTAR, Madison, USA). The assembled cDNA sequence contains an open reading frame of 2328 bp and codes for a protein of 776 amino acids (human *PNPT1*: 783 amino acids). The triplet coding for the crucial glutamic acid is highlighted in red. The zebrafish *pnpt1* sequence is available under GenBank accession number JN381023.

human	MAACRYCCSCLRLPLSDGPFLPPIRDRALTQLQVRALWSSAGSRAVADLGNRKEISS	60
zebrafish	----MNVCVCERLMMKSVRMMKMMKTRLCWARVCARG-IQQHSATVTLGDRKLEIST	55
	* * *** : .. : . * : * : . . : . : * : * : **** :	
human	GKLARFADGSAVVQSGDTAVMVTAVSKTKPSQFMPLVVYRQKAAAAGRIPTNYLRRE	120
zebrafish	GKLARFSDGCAVVKSGETSVMVTAVSKSRAAAQFMPLVVYRQKAAAAGRIPTNHLRRE	115
	*****:***.***:***:***:*****:***:*****:*****:*****:****	
human	IGTSDEILTSRIIDRSIRPLFPAGFYDVTQVLNCNLAVDGVNEDVLAINGASVALSLS	180
zebrafish	LGTTDTEILTSRLIDRSIRPLFPAGFYDVTQVMCNIADGVNDPDVLAINGASAALTLS	175
	:**:*.*****:*****:*****:*****:***:***.*****:*****:*****.**:***	
human	DIPWNGPVGAVRIGIIDGEYVNVPRKEMSSSTLNLVVAGAPKSQIVMLEASAENILQQD	240
zebrafish	DIPWNGPIGAVRVGLLDGEFLINPSRSEMTRSSLNLVIAAPSSHVMIEAAAENILQQD	235
	*****:*****:***:***:***:***:***:***:***:***:*****:*****	
human	FCHAIIKGVKYTQQIIQGIQQLVKETGVTKRTPQKLFTPSPPEIVKYTHKLAMERLYAVFT	300
zebrafish	FCHAVKLGVKHTQQIIQSLQQISRDMKISKRS-SRLYTAADMQEHTRLLASDRIYAVFT	294
	****:***:***:*****:***:***:***:***:***:***:***:***:***:*****	
human	DYEHDKVSREAVNKIRLDTEEQLKEKFPEADPYEIIESFNVVAKEVFRSIVLNEYKRC	360
zebrafish	DFTHDKISRDEAINKIRLEAEKIREKFPHAEFPFEVMEAFNSVSKEIFRKLVQYRRCD	354
	*: ***:*****:*****:***:***:***:***:***:***:***:***:***:***	
human	GRDLTLSRNVSCEVDMFKTLHGSALFQRGQTQVLCVTFDSLESGIKSDQVITAINGIKD	420
zebrafish	GRDLTALRNISCEVDVFKPLHGSALFQRGQTQVLCVTFDSLESSLTDVITSALSGVKD	414
	*****:***:*****:***.*****:*****:*****:*****:***:***:***:***	
human	KNFMELHYEFPPYATNEIGKVTGLNRRELGHGALAEKALYPVI PRDFPFTIRVTSEVLESN	480
zebrafish	KNFLLHYEFPPYATNEIGRTGGANRRELGHGALAEKALRPVIPSSFPFTIRVTSEVLESN	474
	***:*****:*****:***.***:*****:*****:*****:*****.*****:*****	
human	GSSSMASACGGSLALMDSGVPISSAVAGVAIGLVTKTDPK-GEIEDYRLLTDILGIEDY	539
zebrafish	GSSSMASVCGGSLALMDAGVPISSPAGVAIGLISEAHPDRPSEIESYRLTDILGIEDY	534
	*****:*****:*****:*****:*****:*****:*****:*****:*****:*****	
human	NGDMDFKIAGTNKGITALQADI KLPGIPIKIVMEAIIQQASVAKKEILQIMNKTISKPRAS	599
zebrafish	NGDMDFKMAGSSKGITALQADV KI PGLPLKIVMEAIIQHATVAKREILGIMGQCISPRSS	594
	*****:***:*****:***:***:*****:***:***:***:***:***:***:***:*	
human	RKENGPVVE TVQVPLSKRAKFVGPGYLNKKLQAETGVTISQVDEETFSVFAPTPSAMHE	659
zebrafish	RNENGPVVENITVPLSRALFIGPGGINLRLQATGVTISQVDEQTFSVFAPTPAAMSE	654
	*:*****:***:***:***:***:***:*****:*****:*****:***:***:***:***	
human	ARDFITEICKDDQEQQLEFGAVYTATITEIRDGVMVKLYPNMTAVLLHNTQLDQRKIKH	719
zebrafish	AQEIIKDTCRDDQEQQLEFGAIYTATITEIRDGVMVKLYPNMSPVLLHNSQLDHKRIQH	714
	*:***:***:***:*****:*****:*****:*****:*****:*****:***:***:***	
human	PTALGLEVGQEIQVKYFGRDPADGRMRMLS RKV LQSPATTVVRTLNDRSSIVMGEPISQSS	779
zebrafish	PSALGLYVGQQIQVKYFGRDPTDGKMRMLS RKV LLSPTATLAKSLSERHSISVGSS----	769
	*:*****:***:*****:***:*****:***:*****:***:***:***:***:***:***	
human	SNSQ 783	
zebrafish	----	

### Figure S3. Alignment of Human and Zebrafish PNPase

The alignment illustrates the high conservation of PNPase amino acid sequence between human and zebrafish. In zebrafish PNPase, the critical glutamic acid (highlighted in red) is conserved and found at position 469 as compared to position 475 in human PNPase. \* indicates fully conserved amino acids, : indicates conservation between residues with strongly similar properties, and . indicates conservation between residues with weakly similar properties.



**Figure S4. Stability and Localization of Recombinant PNPase (WT and p.Glu475Gly)**

(A–D) HEK293T cells were transiently transfected (FuGene HD transfection reagent, Roche, Mannheim, Germany) with mammalian expression vectors (pcDNA3.1) coding for recombinant human PNPase (WT and p.Glu475Gly, respectively). Proteins were isolated and analyzed by Western blotting. Anti-β-actin antibody was used as loading control. Transfected cells were either (A) not treated or incubated with (B) hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), (C) antimycin or (D) phenazine methosulfate (PMS) for 24h. Concentrations as indicated.

(E) COS7 cells were transiently transfected with expression vectors coding for PNPase WT and PNPase p.Glu475Gly, respectively, both in frame with a C-terminal V5-His tag. Mitochondria were stained using Mitotracker CMXROS (Invitrogen, Darmstadt, Germany). Merged images confirm mitochondrial localization for both PNPase WT and PNPase p.Glu475Gly.

(F) Images of yeast cells expressing PNPase recombinant proteins with C-terminal DsRed tag (gateway vector pAG423GPD-ccdB-DsRed, Addgene, Cambridge, USA) are shown. Mitochondrial co-staining was performed via co-transformation of a plasmid coding for GFP combined with a mitochondrial targeting sequence (mt-GFP, Westermann et al., Yeast 16:1421-1427, 2000). As in COS7 cells, recombinant PNPase WT and PNPase p.Glu475Gly localize to mitochondria (merge).



**Figure S5. Multiple Species Alignment of PNPase Protein Sequences**

Multiple alignment of representative PNPase protein sequences was generated by the L-INS-I algorithm of the MAFFT package (Kotah et al., Nucleic acids research 30:3059-3066, 2002). Residues invariant or conservatively substituted in at least 50% of the sequences are rendered on black and grey background, respectively. The altered Glu475 residue is highlighted in red, the Arg/Lys residue that is predicted to form an inter-subunit salt bridge with Glu475 is highlighted in blue.

**Table S1. Protein-Coding Genes within the Linked Region on Chromosome 2**

Gene Name (HGNC)	Protein-Coding Exons	Position on Chromosome 2
<i>LRPPRC</i>	38	44,113,363-44,223,144
<i>PPM1B</i>	5	44,396,000-44,461,741
<i>SLC3A1</i>	10	44,502,597-44,547,959
<i>PREPL</i>	13	44,545,903-44,586,889
<i>C2orf34</i>	11	44,589,043-44,999,729
<i>SIX3</i>	2	45,169,037-45,172,390
<i>SIX2</i>	2	45,232,325-45,236,542
<i>SRBD1</i>	20	45,615,820-45,838,433
<i>PRKCE</i>	15	45,879,043-46,415,128
<i>EPAS1</i>	16	46,524,563-46,613,835
<i>ATP6V1E2</i>	1	46,738,988-46,747,096
<i>RHOQ</i>	5	46,769,867-46,811,825
<i>PIGF</i>	5	46,808,414-46,844,251
<i>CRIP1</i>	5	46,844,325-46,852,880
<i>SOCS5</i>	1	46,926,099-46,989,926
<i>MCFD2</i>	3	47,129,017-47,142,949
<i>TTC7A</i>	20	47,168,313-47,303,274
<i>CALM2</i>	6	47,387,221-47,403,740
<i>EPCAM</i>	9	47,596,287-47,614,165
<i>MSH2</i>	16	47,630,263-47,710,360
<i>KCNK12</i>	2	47,747,917-47,797,470
<i>MSH6</i>	10	48,010,221-48,034,084
<i>FBXO11</i>	23	48,039,990-48,132,814
<i>FOXN2</i>	5	48,541,795-48,606,434
<i>KLRAQ1</i>	21	48,667,908-48,742,524
<i>STON1</i>	3	48,807,763-48,826,025
<i>GTF2A1L</i>	9	48,844,948-48,960,284
<i>LHCGR</i>	11	48,913,921-48,982,880
<i>FSHR</i>	10	49,189,653-49,381,630
<i>NRXN1</i>	21	50,145,644-51,259,674
<i>ASB3</i>	9	53,897,118-54,014,079
<i>CHAC2</i>	3	53,994,929-54,002,287
<i>ERLEC1</i>	14	54,014,068-54,046,495
<i>GPR75</i>	1	54,080,050-54,087,126
<i>PSME4</i>	46	54,091,204-54,197,977
<i>ACYP2</i>	4	54,342,410-54,532,433
<i>C2orf73</i>	5	54,558,071-54,588,714
<i>SPTBN1</i>	35	54,683,454-54,898,582
<i>EML6</i>	41	54,952,149-55,199,154
<i>RTN4</i>	9	55,199,329-55,277,734
<i>C2orf63</i>	12	55,399,687-55,459,449
<i>RPS27A</i>	5	55,459,635-55,462,748
<i>MTIF2</i>	13	55,463,758-55,496,384
<i>CCDC88A</i>	31	55,514,978-55,647,057
<i>CCDC104</i>	10	55,746,740-55,772,216
<i>SMEK2</i>	15	55,775,515-55,844,796
<i>PNPT1</i>	28	55,861,198-55,921,011
<i>EFEMP1</i>	10	56,093,103-56,150,356
<i>CCDC85A</i>	6	56,411,258-56,613,308

Table lists all known protein coding RefSeq Genes between D2S119 and D2S378. Data was taken from UCSC Genome Browser based on Genome Reference Consortium release h37 (February 2009). All protein coding exons and at least 50 bp of flanking sequence were analyzed. Primer sequences are available upon request.