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Supplemental Data

A Mutation in *PNPT1*, Encoding Mitochondrial-RNA-Import Protein PNPase, Causes Hereditary Hearing Loss

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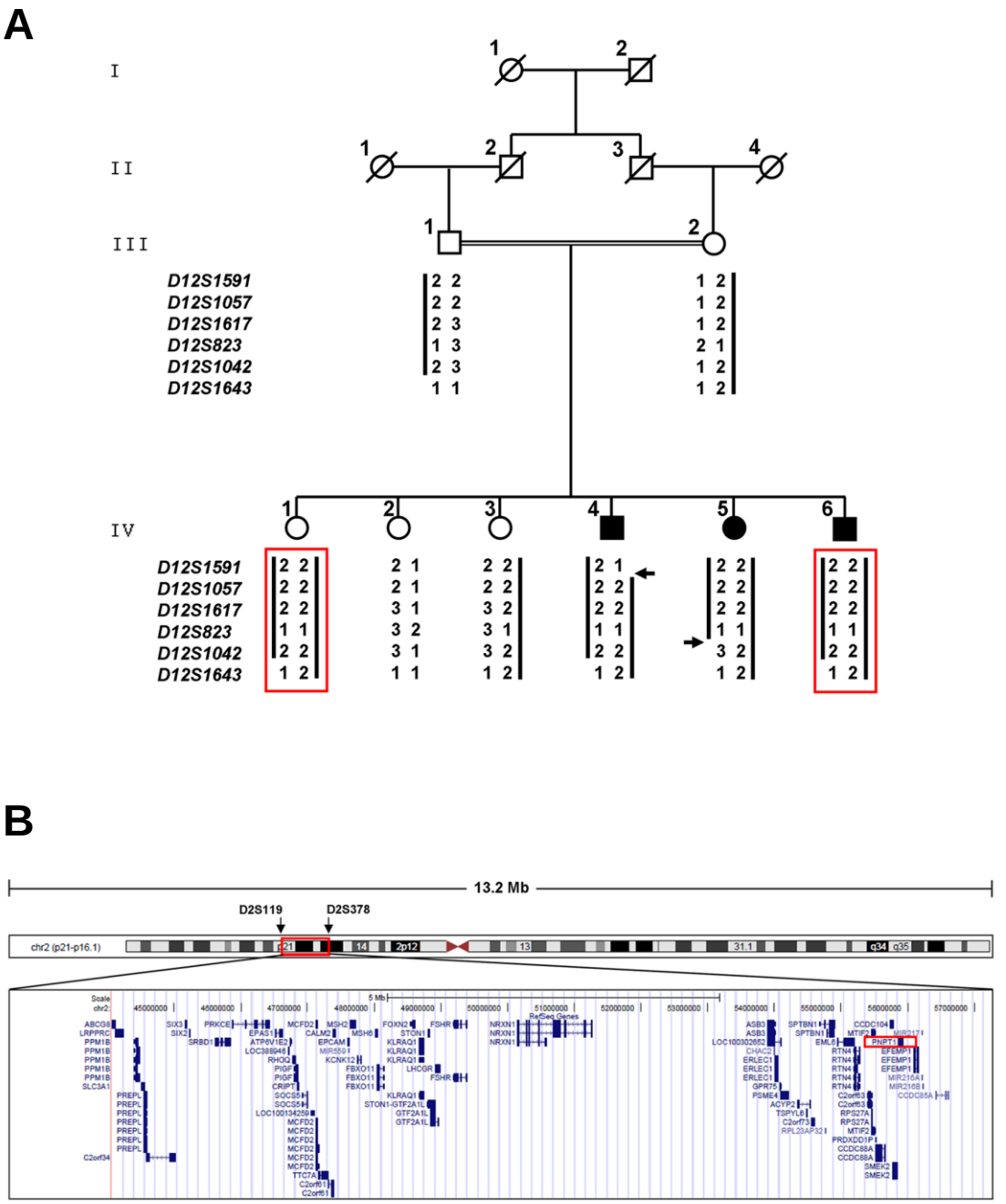

Figure S1. Exclusion of a Putative Locus on Chromosome 12 (A) and Overview of the Critical Region on Chromosome 2p (B)

(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).

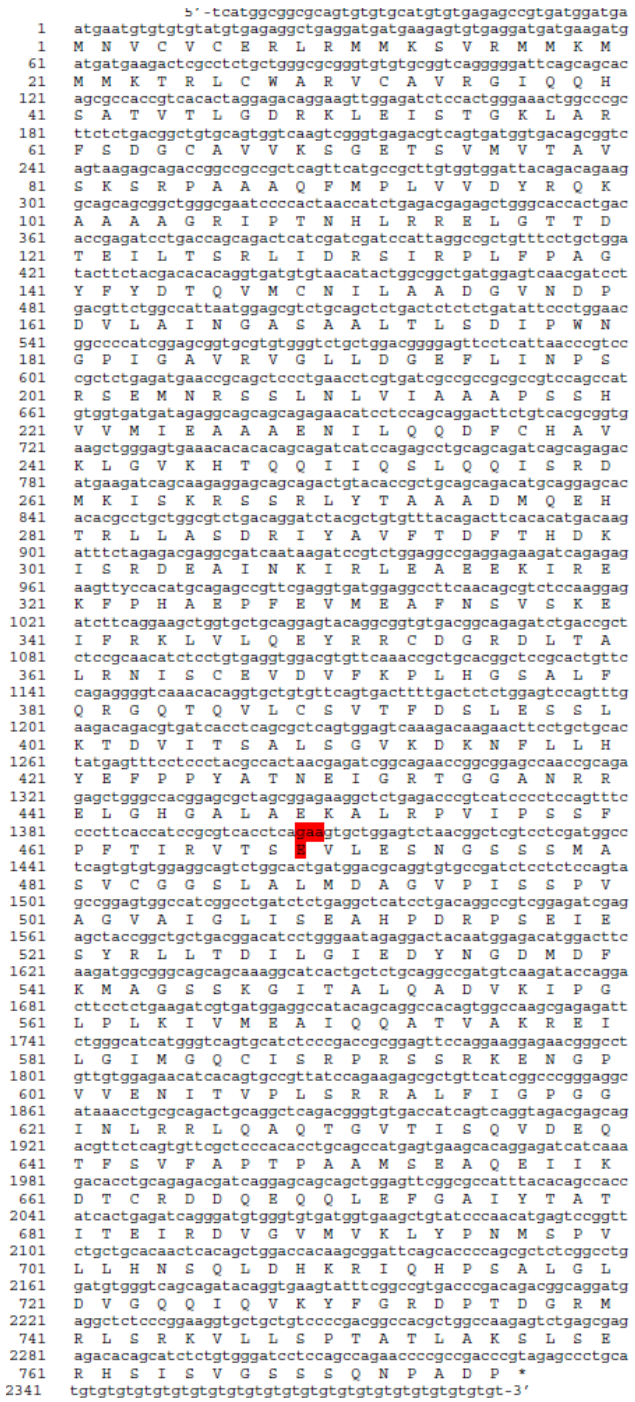

Figure S2. Identification of *pnpt1* in Zebrafish by RACE-PCR and RT-PCR

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 2nd 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.

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.

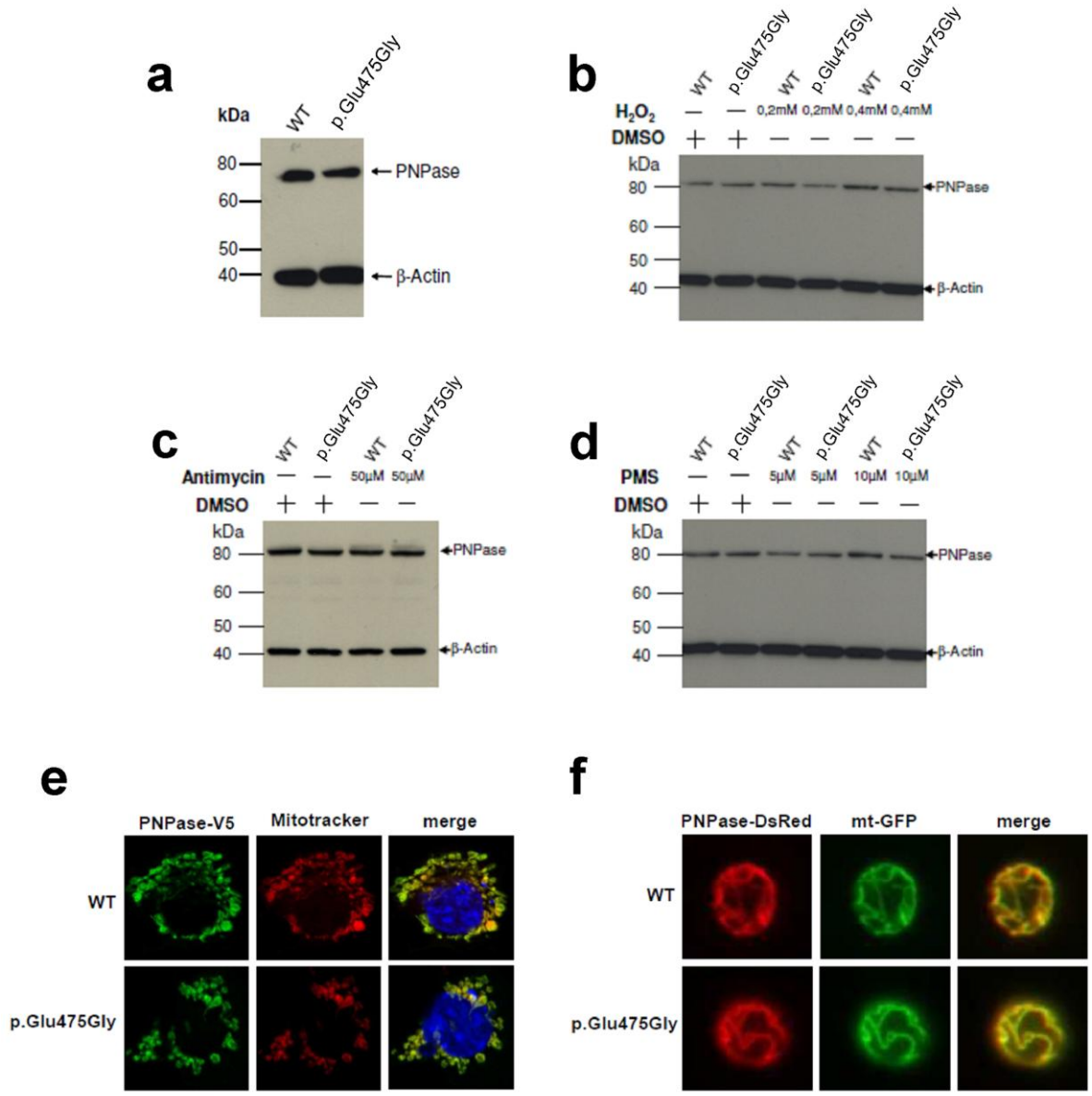

Figure S4. Stability and Localization of Recombinant PNPase (WT and p.Glu475Gly)

(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_2O_2), (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
LRPPRC	38	44,113,363-44,223,144
PPM1B	5	44,396,000-44,461,741
SLC3A1	10	44,502,597-44,547,959
PREPL	13	44,545,903-44,586,889
C2orf34	11	44,589,043-44,999,729
SIX3	2	45,169,037-45,172,390
SIX2	2	45,232,325-45,236,542
SRBD1	20	45,615,820-45,838,433
PRKCE	15	45,879,043-46,415,128
EPAS1	16	46,524,563-46,613,835
ATP6V1E2	1	46,738,988-46,747,096
RHOQ	5	46,769,867-46,811,825
PIGF	5	46,808,414-46,844,251
CRIPT	5	46,844,325-46,852,880
SOCS5	1	46,926,099-46,989,926
MCFD2	3	47,129,017-47,142,949
TTC7A	20	47,168,313-47,303,274
CALM2	6	47,387,221-47,403,740
EPCAM	9	47,596,287-47,614,165
MSH2	16	47,630,263-47,710,360
KCNK12	2	47,747,917-47,797,470
MSH6	10	48,010,221-48,034,084
FBXO11	23	48,039,990-48,132,814
FOXN2	5	48,541,795-48,606,434
KLRAQ1	21	48,667,908-48,742,524
STON1	3	48,807,763-48,826,025
GTF2A1L	9	48,844,948-48,960,284
LHCGR	11	48,913,921-48,982,880
FSHR	10	49,189,653-49,381,630
NRXN1	21	50,145,644-51,259,674
ASB3	9	53,897,118-54,014,079
CHAC2	3	53,994,929-54,002,287
ERLEC1	14	54,014,068-54,046,495
GPR75	1	54,080,050-54,087,126
PSME4	46	54,091,204-54,197,977
ACYP2	4	54,342,410-54,532,433
C2orf73	5	54,558,071-54,588,714
SPTBN1	35	54,683,454-54,898,582
EML6	41	54,952,149-55,199,154
RTN4	9	55,199,329-55,277,734
C2orf63	12	55,399,687-55,459,449
RPS27A	5	55,459,635-55,462,748
MTIF2	13	55,463,758-55,496,384
CCDC88A	31	55,514,978-55,647,057
CCDC104	10	55,746,740-55,772,216
SMEK2	15	55,775,515-55,844,796
PNPT1	28	55,861,198-55,921,011
EFEMP1	10	56,093,103-56,150,356
CCDC85A	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.