Supplementary material for *'Mice with altered KCNQ4 K+ channels implicate sensory outer hair cells in human progressive deafness'* by Kharkovets et al.

SUPPLEMENTARY FIGURES AND TABLE

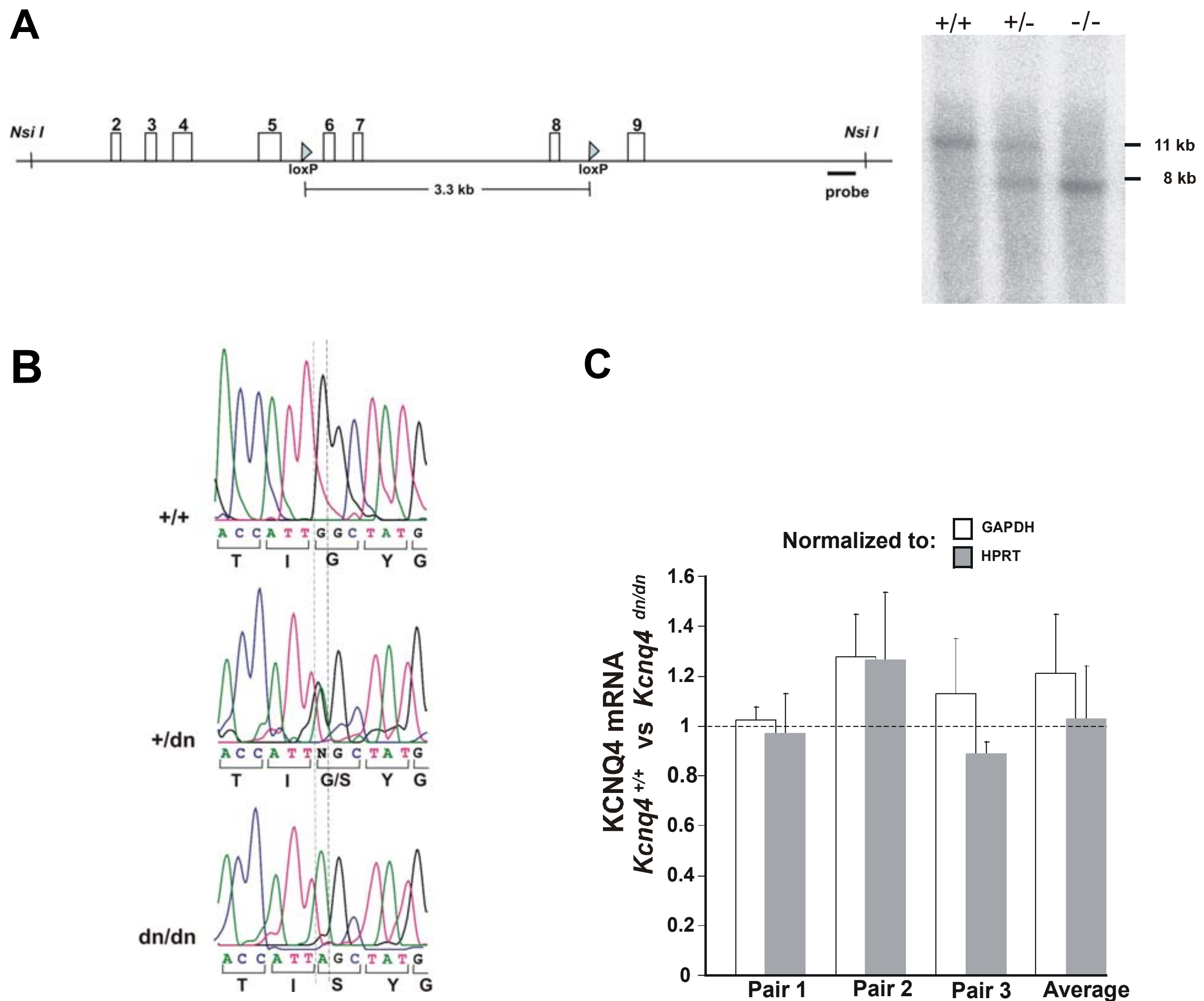

Figure S1. Analysis of modified *Kcnq4* genes. (**A**) Scheme of the floxed mouse *Kcnq4* gene with numbered boxes symbolizing exons (left), and Southern blot analysis of *Nsi* I-cut genomic DNA from WT, *Kcnq4*$^{+/-}$ and *Kcnq4*$^{-/-}$ mice (right) (location of the probe is shown in scheme at left). Expected sizes of hybridizing fragments are 11 kb for WT, and 7.7 kb for the disrupted locus. (**B**) DNA sequences of PCR-amplified pore exons shows the exchange of a G to A on both alleles in *Kcnq4*$^{dn/dn}$ mice (bottom) and on only one allele in *Kcnq4*$^{+/dn}$ mice (middle). This results in an exchange of serine for glycine in the GYG pore signature sequence, resulting in a dominant negative K^+ channel mutant. (**C**) Quantitative real-time RT-PCR analysis of KCNQ4(G286S) mRNA expression in brainstem. In three independent experiments, a *Kcnq4*$^{dn/dn}$ animal was compared to a WT animal. For each pair, the expression was normalized to either GAPDH or HRPT RNA. There is no significant difference in expression levels.

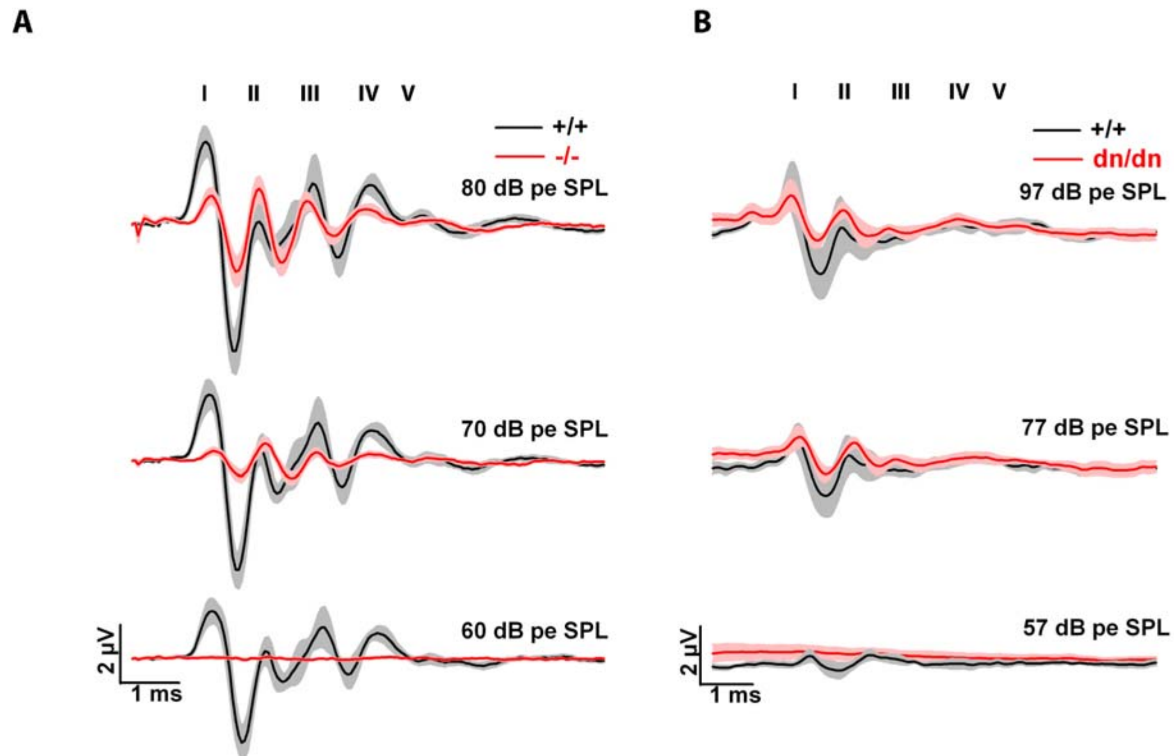

Figure S2. Average auditory brainstem responses of 3-week-old Kcnq4 mutants. (**A**) Auditory brainstem responses evoked by suprathreshold, 30 µs long (high frequency) clicks in 3-week-old in *Kcnq*$^{+/+}$ mice (black, 9 ears) and *Kcnq*$^{-/-}$ mice (red, 6 ears). While in *Kcnq*$^{+/+}$ ABR are clearly visible at 60 dB, they appear only at 70dB in *Kcnq*$^{-/-}$. At 80 dB ABR waves are still smaller in *Kcnq*$^{-/-}$ but already comparable in their latencies to the ABR of *Kcnq*$^{+/+}$ (**B**) Auditory brainstem responses evoked by suprathreshold, 200 µs long (low frequency) clicks (60, 70 and 80 dB pe SPL) in 3-week-old *Kcnq*$^{+/+}$ mice (black, 8 ears) and *Kcnq*$^{dn/dn}$ mice (red, 6 ears).

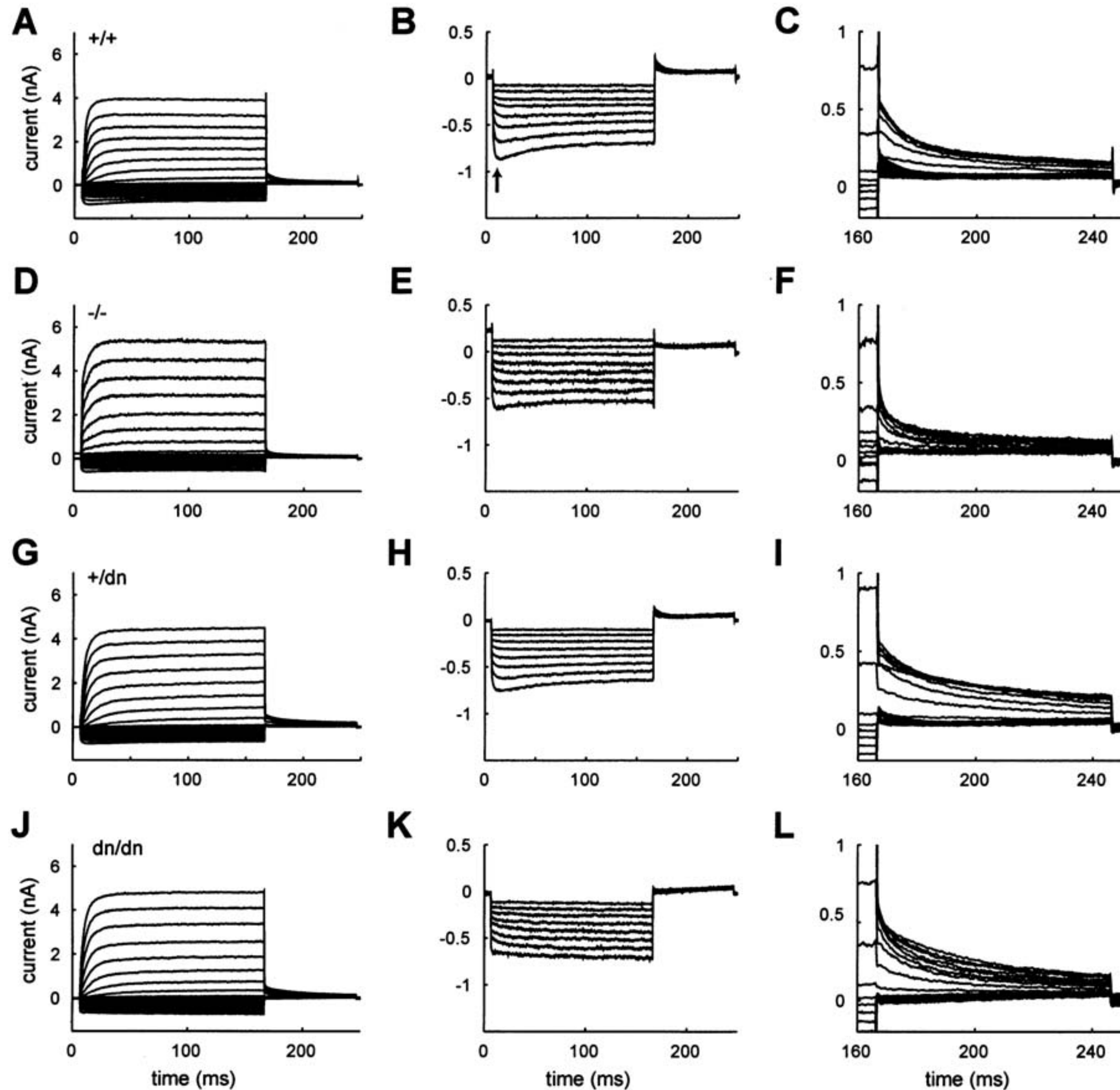

Figure S3. Patch clamp analysis of inner hair cells. Representative whole cell K+ currents from inner hair cells (IHCs) of WT (**A**-**C**), *Kcnq4*$^{-/-}$ (**D**-**F**), *Kcnq4*$^{+/dn}$ (**G**-**I**), and *Kcnq4*$^{dn/dn}$ (**J**-**L**) mice. (**B**, **E**, **H**, **K**) Whole cell currents in response to hyperpolarizing voltage steps (-150 to -80 mV in 10 mV increments). IHCs of *Kcnq4*$^{-/-}$ and *Kcnq4*$^{dn/dn}$ mice showed smaller inward currents in response to hyperpolarizing voltage steps than IHCs of *Kcnq4*$^{+/+}$ and *Kcnq4*$^{+/dn}$ mice. Arrow in (**B**) indicates the peak current at 11 ms after the voltage step which was used to derive the IV curves in Fig. 6N. (**C, F, I, L**) Enlarged view of tail currents are shown in (**A, D, G, J**).

Supplementary Table 1

hair cell type	OHC			IHC
cochlear turn	apical	midbasal	basal	basal
2 weeks				
Kcnq4$^{+/+}$	46/46	74/74	42/42	42/42
Kcnq4$^{+/dn}$	138/138	117/118	112/112	110/110
Kcnq4$^{-/-}$	88/88	74/74	69/69	69/69
4 weeks				
Kcnq4$^{+/+}$	73/73	73/73	73/73	73/73
Kcnq4$^{+/dn}$	32/32	32/32	31/32	32/32
Kcnq4$^{-/-}$	26/26	25/26	18/26	26/26
7 weeks				
Kcnq4$^{+/+}$	44/44	74/74	62/62	60/60
Kcnq4$^{+/dn}$	42/42	44/44	42/42	34/34
Kcnq4$^{-/-}$	73/73	59/59	23/36	33/33
1 year				
Kcnq4$^{+/+}$	129/129	126/128	124/130	117/119
Kcnq4$^{+/dn}$	127/132	15/125	4/154	139/148
Kcnq4$^{-/-}$	87/92	15/144	0/135	128/128

Hair cell degeneration of mice carrying different genotypes
Statistical analysis of the presence of outer hair cells (OHCs) and inner hair cells (IHCs) in apical, midbasal and basal turns of cochlea of the three *Kcnq4* genotypes at different ages. As no degeneration of IHCs could be observed in midbasal or apical turns, only basal turns are shown for IHCs. Given is the number of morphologically intact hair cells / number of organ of Corti sections. As the three OHCs per section of Corti's organ were either all absent or present, they were counted only once. Only morphologically well preserved HE-stained inner ear sections were evaluated. At least 2 animals for each genotype per age were investigated.

SUPPLEMENTARY METHODS

Generation of KCNQ4 mice

Primers for amplifying mouse genomic Kcnq4 sequence: KO1h (5'-ccc tct ctt ctg tag ctg act cat t-3') situated in intron 1; KO9r (5'-agc tcc tga gag tgt ccc ttc tga-3') in intron 10. The genotyping of KO line was performed with three primers: 5'- agc tga tca ccg cct ggt aca tcg -3', 5'- gga gat gcc cag taa ggc gaa gcc-3' and 5'- gag ccc cct ttc cag acc cta c-3' resulting in 622 bp for the WT and 460 bp for the KO allele. For the dominant negative allele we used primer pair 5'-ggc tgt ggg tcc tgt gac cag tcc-3' and 5'- gga ctg ggc aca ggg acc tga cac-3', leading to 258 bp for WT and 298 bp for the mutant allele.

Patch-clamp analysis of hair cells

Cells were clamped from a holding potential of –60 mV to values between –140 mV (OHC) or –150 mV (IHC) and +40 mV in 10 mV increments. A 80 ms lasting step to -50 mV was used to evoke tail currents. Evoked currents were registered by an Axopatch 200A amplifier and analogue/digital converter Digidata Series 1200, sampled at 10 kHz low-pass filtered at 2 kHz and visualized by pClamp 8.1 software.
Activation curves of OHC membrane currents were obtained from tail current analysis. Tail current amplitudes were plotted as a function of the prepulse potential and were fitted with a first order Boltzmann function:

$$I = I_{leak} + I_{max} / (1 + \exp(-(V-V_{1/2})/\alpha))$$

I_{leak} is the leak current amplitude, I_{max} is the amplitude of the fully activated tail current, V is the prepulse voltage, $V_{1/2}$ the voltage at half-maximal activation, and α the slope factor.
Activation curves of IHC membrane currents were obtained from the current amplitudes at 11 ms after the onset of the voltage steps from -140 mV to 40 mV in 10 mV increments. XE991 was diluted in DMSO and added to the extracellular solution from stock solutions to a final concentration of 0.5 µM.

Quantitative real time RT-PCR

Total RNA was prepared from the brainstem of WT and *Kcnq4*$^{dn/dn}$ mice using TRIZOL (Invitrogen) and was digested with RNAse-free DNase (Ambion) before purification with RNeasy columns (Qiagen). cDNA synthesis used SuperScript™ II reverse transcriptase, oligo$(dT)_{15}$ primer, first-strand reaction buffer, 0.1 M DTT, 10 mM dNTP mix and RNaseOUT (Invitrogen). The qRT-PCR was run in an ABI PRISM 7700 Sequence detection System (SDS 2.1 software) using the SYBR green PCR master mix (Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method of relative quantification was applied taking HPRT and GAPDH as reference genes. The analysis was performed on three different mouse pairs as shown in Fig. S1C.

Southern blot

Mouse genomic DNA was digested with *Nsi* I, size fractionated on a 1% agarose gel, and blotted to a nylon membrane using standard procedures. Hybridization was performed with a probe binding to intron 9 (see Fig. S1A) and that was labelled with ^{32}P by random priming. The probe was obtained as a PCR product amplified from mouse genomic DNA using the following primers: 5'-aaactcggcggtggcctg-3 and 5'-tggtccccagagagttgcaaggag-3. The identity of the probe was confirmed by sequencing. The hybridized and washed nylon membrane was exposed to X-ray film (Kodak).