Supplemental Data

Impairment of *SLC17A8* Encoding Vesicular Glutamate Transporter-3, VGLUT3, Underlies Nonsyndromic Deafness DFNA25 and Inner Hair Cell Dysfunction in Null Mice

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Supplemental Subjects and Methods

- Confocal immunocytochemistry -

The detection of Vgluts in wild type cochleas (n=5) was done on 14 µm-thick cryostat sections. Whole-mount cochleas were also prepared for detection of Vglut3, cysteine-string protein and synaptogyrin from *Slc17a8*$^{-/-}$ (n = 4) and *Slc17a8*$^{+/+}$ mice (n = 4).

The dissection, fixation of the cochleas and the immunocytochemical procedures were as described in the main body of the paper. The guinea pig anti-Vglut3, and the rabbit anti-Vglut1 and anti-Vglut2 antibodies have been characterized previously [1; 2]. They were used diluted 1:1000-1:2000. Cysteine-string protein was localized using a rabbit polyclonal diluted 1:500 (Chemicon, Temecula, CA) and synaptogyrin using a mouse monoclonal diluted 1:750 (BD Biosciences, San Diego, CA).

The primary antibodies were revealed using donkey secondaries against rabbit IgG, conjugated to Alexa 568 (Invitrogen, Eugene, OR), against mouse IgG, conjugated to Alexa 488 (Invitrogen), and against guinea pig IgG conjugated to Cy3 (Jackson Immunoresearch, West Grove, PA). They were used diluted 1:400-1:2000.

- Quantitative evaluation of spiral ganglion and lateral efferent endings loss -

Spiral ganglion neuronal quantification. 12 µm-thick cryostat sections were done throughout the entire cochleas of wild type (n = 3) and *Slc17a8*$^{-/-}$ (n = 3) mice. To better visualize neurons in the ganglion, the sections were stained using toluidine blue. To avoid counting the same neurons twice, only 1 section of every sequential 4 were used for quantification using a SAMBA 2005 device coupled to a Zeiss Axioscop microscope (SAMBA Technologies, Meylan, France). Quantitative data

were expressed as the mean number of neurons per mm^2 ± SEM. The Student *t* test was used for the statistical analysis of the data ($p<0.01$).

Supplemental References

1. Gras C, Vinatier J, Amilhon B, Guerci A, Christov C, Ravassard P, Giros B, El Mestikawy S (2005) Developmentally regulated expression of VGLUT3 during early post-natal life. Neuropharmacology 49:901-911
2. Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S (2001) The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. J Neurosci 21:RC181

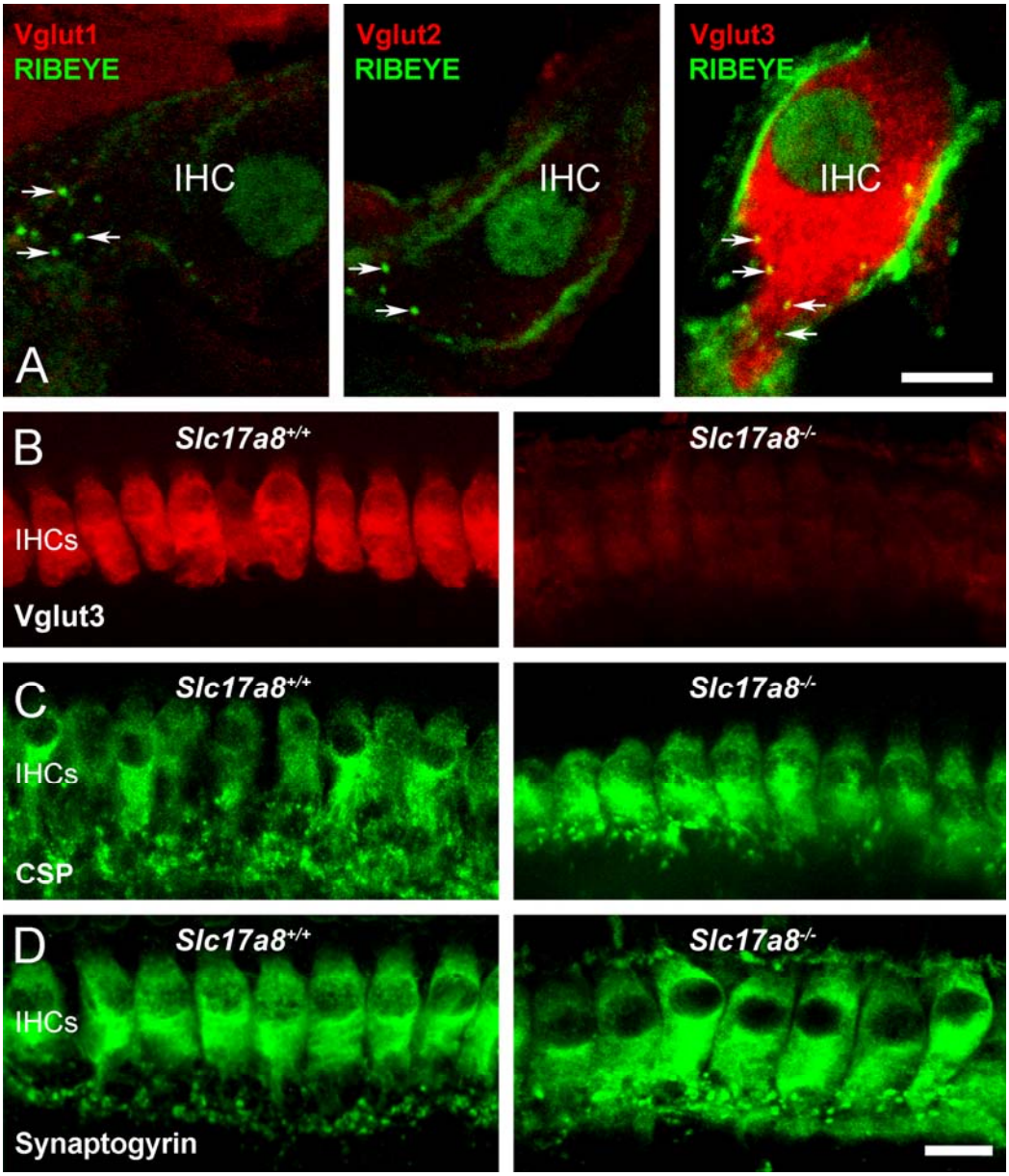

Figure S1. Vglut3, but not Vglut1 and Vglut2 is strongly expressed in the inner hair cell of the cochlea. **(A)** Confocal microscopy of cryostat sections through the basal turn of a wild type mouse cochlea. RIBEYE (green dots) is expressed at the basal pole of the inner hair cells. Only a Vglut3 immunofluorescence can be seen in the hair cells. Neither Vglut1 nor Vglut2 could be detected in the hair cells. Note that the Vglut3 expression mainly affects the lower half of the inner hair cell. **(B-D)** Confocal microscopy of whole-mount preparations of inner hair cells from the basal

cochlear turn of *Slc17a8*$^{+/+}$ and *Slc17a8*$^{-/-}$ mice. The *Slc17a8* deletion completely abolishes the Vglut3 immunostaining **(B)**. In contrast, it has no effect on the expression of the synaptic proteins cysteine-string protein (CSP, **C**) and synaptogyrin (**D**). Scale bar: **A** = 5 µm, **B-D** = 25 µm.

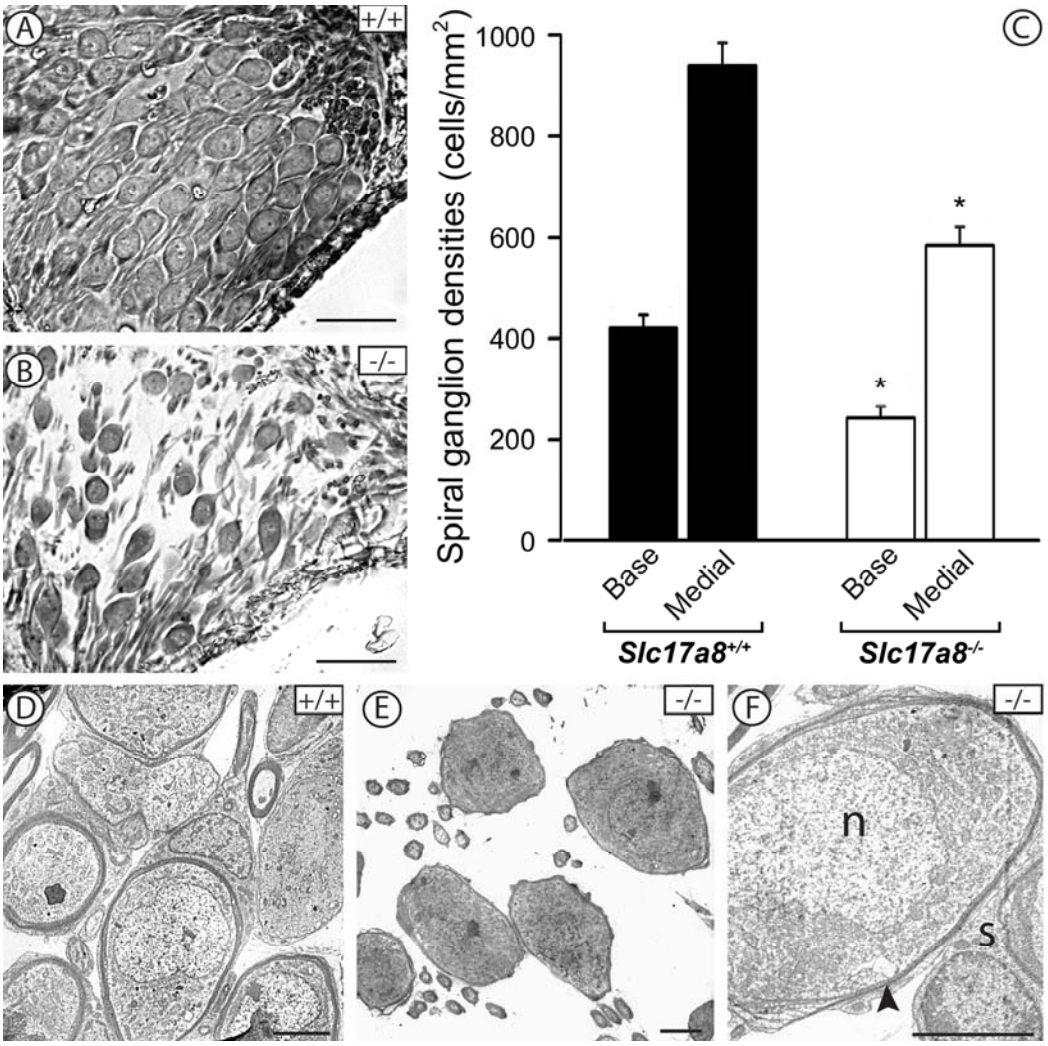

Figure S2. *Slc17a8* deletion leads to a reduction of spiral ganglion neurons. (A-B) Differential interference contrast light microscopy of Epon-embedded semi-thin sections through the spiral ganglion in the basal turn of the cochlea of wild type ($^{+/+}$, **A**) and *Slc17a8* deleted ($^{-/-}$, **B**) mice showing a reduced number of primary auditory neurons. Scale bar: 25 µm. **(C)** Quantitative analysis of the number of spiral ganglion neurons in *Slc17a8*$^{+/+}$ (n = 3 cochleas) and *Slc17a8*$^{-/-}$ mouse cochleas (n = 3 cochleas). A much lower number of neurons could be counted (~40% less) in the basal and medial turns of the *Slc17a8*$^{-/-}$ cochleas, 57.72% and 62.26% remaining neurons, respectively. **(D-F)** Transmission electron micrographs of the spiral ganglion

cells in the cochlear basal turn of wild type ($^{+/+}$, **D**) and *Slc17a8* deleted ($^{-/-}$; **E,F**) mice. **C** and **D**: the neuronal density in the ganglion is greater in the wild type (**C**) than in the *Slc17a8*$^{-/-}$ mice (**D**). **F**: Higher magnification of a ganglion neuron with its satellite cell in a spiral ganglion *Slc17a8*$^{-/-}$ mouse. The neuron shows a regular shape, clear cytoplasm and a round nucleus (n) and is surrounded by several rows of myelin (arrowhead). Scale bars: **C, D** = 40 µm; **F** = 5µm.

Table S1. Primers for DNA sequencing of *SLC17A8*.

Exon	Primer Name	Primer Sequence	Product Size	PCR T(m)
1	SLC17A8.F1	CAAGGTGGTTCTCACACTGG	244	58.1
1	SLC17A8.R1	ACACATTGGCAATACGAAGC	244	58.1
2	SLC17A8.F2	CCCAGAACATCAGTAGTTTTAGCC	564	60.8
2	SLC17A8.R2.3	CGTGCCACTGTACTCTAGCC	564	60.8
3	SLC17A8.F3	TGCTGCTATCCCCAAGTATG	361	58.1
3	SLC17A8.R3	ATTGAGAGCCAGCTATGCAG	361	58.1
4	SLC17A8.F4	TTGTTTCTGGTGCTTAGCTG	241	58.1
4	SLC17A8.R4	GACTGCGCTGTCTCTAGCA	241	58.1
5	SLC17A8.F5	CCTGTGTGAAGAAGCAGCAT	374	60.8
5	SLC17A8.R5.2	CCTCCATGCTCGGCTAATTT	374	60.8
6	SLC17A8.F6	CTTGGTGACCTTCCTCTCCT	351	55.5
6	SLC17A8.R6	AACTCTGAGCCAAACCCATA	351	55.5
7, 8	SLC17A8.F7	TCTGAGTCCCTCAAACCTTG	682	55.5
7, 8	SLC17A8.R8	TGACAGCCAGGCTATTAGGT	682	55.5
9	SLC17A8.F9	ATTCAGGCCTACCCAGCTC	348	58.1
9	SLC17A8.R9	GCTCTGCAGTTCAGTTTACCC	348	58.1
10	SLC17A8.F10	TTCTGCACATGTATCCCTGAA	400	59.5
10	SLC17A8.R10	TTCCTTCTTCCTGGGACTTG	400	59.5
11	SLC17A8.F11	GTAAAGGCTGGGGCAACT	300	58.1

Table S2. Tri- and tetra-nucleotide repeat short tandem repeat polymorphisms (STRPs) and single nucleotide polymorphisms (SNPs) used for linkage disequilibrium analysis.

Mb	D12S/rs#/Contig/SNP[+]	SNP or STR repeat	Location within *SLC17A8*	Pos. in Gene/ Contig	Enzyme (s)	F Primer[*]	R Primer[#]	Anneal T(m) (C°)	Size (bp)
97.223	D12S1063-AC008055	AC	N/A	122026-122219		**CACGACGTTGTAAAACGAC-**ACACAAAGATGAAATTGCCT	**GTTTCTT-**TGGATGAGCCAATTCCTTAA	58.1	194-227
97.392	D12S1706-AC013418	AC	N/A	40825-40969		**CACGACGTTGTAAAACGAC-**CCTATGATTTCCCATCAAGTTT	**GTTTCTT-**ATTATTAGGAGAGCCCTGGG	60.8	119-139
99.117	D12S306-AC010203	AC	N/A	145669-145888		**CACGACGTTGTAAAACGAC-**GTGCTAAATGCACTGTGGC	**GTTTCTT-**TCCCAAGTATCCGGGAC	51.4	200-230
99.128	rs7970382-AC010203	G/T	N/A	157466	T: *Bsr*I	CAGGTGATGCTTAGCTTTGA	CCTCCTGGTTCAAATGATTC	62.2	300
99.176	AC026110 (A)	GATA (12)	N/A	39430-39478		GACTAAATTTGGCCCACAGC	CCCTGGCACTTAGAACAGGA	55.5	236
99.238	AC026110 (B)	AAAT (12)	N/A	102004-102050		GCAGGGAGAATGGCTTGA	GGAGGTTACAAAGAGGCAAATC	55.5	240
99.278	rs11110349-AC026110	C/T	Intron 1	3052/141721	T: *Ase*I	TGCAGGAAGAAAGAAGATGC	CCCTCTTCATGGGGATTTTA	55.5	235
99.298	AC126308 (A)	TAA (11)	Intron 1	10774-10807		TGTCAATGCTCTTTCAGCAG	GGCCTTCCTTCTTCATTCAG	58.1	482
99.305	rs7485480-AC126308	A/G	Intron 2	29926/17405	G: *Dde*I	TTCCACGTCTTGGCTATTGT	CAAGAAGTGTTGGTGAGGATG	55.5	238
99.308	AC126308 (B)	TAT (11)	Intron 2	20069-20102		TCACAAAACTGGCATCTGAA	AATCACTTGAACCCAAGAGG	62.2	492
99.319	rs11568544-AC126308	A/G	Intron 6	44706/32185	A: *Nde*I	GGGTGTTGGTGCAGTACAT	GGGGTAAGTATTTTGTTGACG	55.5	293
99.321	ss104807044-NM139319	T/C	Exon 8	c.951		TCATAAGTGATTGTGTTGCCTTC	ACGGCTCTGATCCTTGCTAA	58	436
99.327	AC126308 (C)	TATG (14)	Intron 9	39410-39464		ACGGTAAGTACTTCAGCTTGTG	CGGGAGAATCACTTGAAAC	55.5	235
99.359	AC126308 (D)	TCCT (20)	N/A	70966-71047		AACACTTAGAATGCCTGGCTA	GCAATGTCTCATTCACCTTCA	55.5	240
99.399	rs11110390-AC010200	C/T	N/A	27634	C: *Nhe*I	CAGGGCAGCATTCTTTCTTA	CAAAGATGGCCACCACAAT	66	228
99.431	AC010200 (A)	AAGG (17)	N/A	60313-60382		GGGATCACCTAAGGTCAGGA	TCAGCAGTTGGTTTCTCAAA	62.2	398
99.440	AC010200 (B)	GAT (43)	N/A	68622-68749		GATTCTCTTCCCAGGTGTCC	AGCCTCCCAGAACAGTGC	55.5	297
99.475	rs35735-AC010200	G/T	N/A	103104	G: *Bst*EII	AGTTTGCGGTGAGCCTAGA	ACCCATCCCTTCCGTAGATA	55.5	482
100.225	D12S1727-AC063948	AC	N/A	14444-14616		**CACGACGTTGTAAAACGAC-**AGTCACCACTGAAAATCCAC	**GTTTCTT-**GAGTGAGACCCCGTAAAAA	53.2	171-181
101.448	D12S1030-AC010202	AC	N/A	136255-136513		**CACGACGTTGTAAAACGAC-**TCCCACATTGACCTATGTAGG	**GTTTCTT-**AGAGTGTAAATGCTACAAGGGC	60.8	243-271

[+]Novel SNPs indicated by letter (A, B, C, D) after BAC in which they were found.
*Forward M13-labeled primer used for PCR indicated by 19 bp sequence attached to 5' end of each forward primer (**bold).**
[#]Pigtail sequence attached to 5' end of reverse primers indicated in **bold**.

Table S3. Age-dependent penetrances for members of Family 1 who are mutation-positive for p.A211V in *SLC17A8*

Age*	Proportion affected	%
20+	17/22	77%
30+	16/20	80%
40+	10/12	83%
50+	6/7	86%
60+	3/4	75%

*indicates subjects of at least that age, eg 20+ refers to age 20 years and older

Table S4. Quantification of the characteristics of the synaptic ribbon of inner hair cells (IHCs) of *Slc17a8*$^{-/-}$ and *Slc17a8*$^{+/+}$ mice.

	***Slc17a8*$^{+/+}$**	***Slc17a8*$^{-/-}$**
Number of investigated cochleas (1 per mouse)	3	6
Number of investigated IHCs	18	30
Total synaptic ribbons observed	19	23
Normal	16*	19
Atypical	3 (2* long and 1 double)	4 (1 long and 3 doubles)
Percent normal/atypical ribbons	69.23% / 30.77%	73.73% / 27.27%
Diameter of vesicles in normal ribbons (nm)	43.77 ± 0.58	43.81 ± 0.59
Number of vesicles in normal ribbon per 100nm dense body length	4.56 ± 0.29	4.84 ± 0.30

*: including 1 ectopic synaptic body

Table S5. Quantification of the confocal immunocytochemistry data from wild type and *Slc17a8*$^{-/-}$ mice

A. Reduction in the number of RIBEYE/CtBP2 immunoreactive dots in the inner hair cells (IHCs) of *Slc17a8*$^{-/-}$ mice (n = 3 $^{+/+}$ and 4 $^{-/-}$ cochleas)

	Number of inner hair cells (IHC)	Total number of RIBEYE-positive dots	RIBEYE-positive dots/IHC (±SEM)	Reduction in *Slc17a8*$^{-/-}$ vs *Slc17a8*$^{+/+}$	*p* value
Slc17a8$^{+/+}$	216	2,754	12.55 ± 0.63		
Slc17a8$^{-/-}$	185	1,240	7.04 ± 0.53	-43.90%	3.56×10^{-8}

B. Reduction in the number of spiral ganglion neurons in the VGLUT3$^{-/-}$ mice (n = 3 $^{+/+}$ and 3 $^{-/-}$ cochleas)

	Number of neurons	Surface (mm2)	Number of neurons/mm2	Reduction in *Slc17a8*$^{-/-}$ vs *Slc17a8*$^{+/+}$	*p* value
Basal turn					
Slc17a8$^{+/+}$	2441	5.814	419.85 ± 25.69		
Slc17a8$^{-/-}$	663	2.736	242.32 ± 22.82	-42.28%	5.39×10^{-6}
Medial turn					
Slc17a8$^{+/+}$	3055	3.249	940.29 ± 43.60		
Slc17a8$^{-/-}$	1118	2.052	544.83 ± 36.36	-42.06%	3.70×10^{-6}

C. Reduction in the number of synaptophysin-positive endings in the inner spiral bundle (ISB) of *Slc17a8*$^{-/-}$ mice (n = 4 $^{+/+}$ and 4 $^{-/-}$ cochleas)

	Number of synaptophysin-(+) endings	Total length of ISB counted (µm)	Number of synaptophysin-(+) endings/µm	Reduction in *Slc17a8*$^{-/-}$ vs *Slc17a8*$^{+/+}$
Basal turn				
Slc17a8$^{+/+}$	1117	557.68	2.003	
Slc17a8$^{-/-}$	286	468.39	0.611	-69.51%
Medial turn				
Slc17a8$^{+/+}$	1916	778.68	2.461	
Slc17a8$^{-/-}$	918	657.52	1.396	-43.26