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Supplemental Data

Mutations in *Grxcr1* Are The Basis for Inner Ear Dysfunction in the Pirouette Mouse

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Figure S1. The *pi* Locus Is Located within a Small Candidate Region on Central Chromosome 5

The first 70 F2 progeny from a (*pi* X CAST/EiJ) F1 intercross were typed for a collection of SSLP markers across a 10 cM region of chromosome 5. An additional 459 F2 mice were typed for SSLP markers flanking the *pi* candidate region. Mice that exhibited recombination events within the region were then typed with additional internal markers. The positions of recombination breakpoints relative to the *pi* locus in unaffected mice were identified by test crosses with known *pi* carrier mice, followed by assessment of behavioral and auditory phenotypes in offspring that inherited the recombinant chromosome.

(A) The allele distribution patterns (ADP) of a set of SSLP markers across a 10 cM region are shown for the first 70 mice from the intercross.

(B) The ADP for the remaining 459 F2 mice are shown. '**X**'s mark the inferred positions of recombination events. Numbers of F2 mice exhibiting the corresponding ADP are indicated.

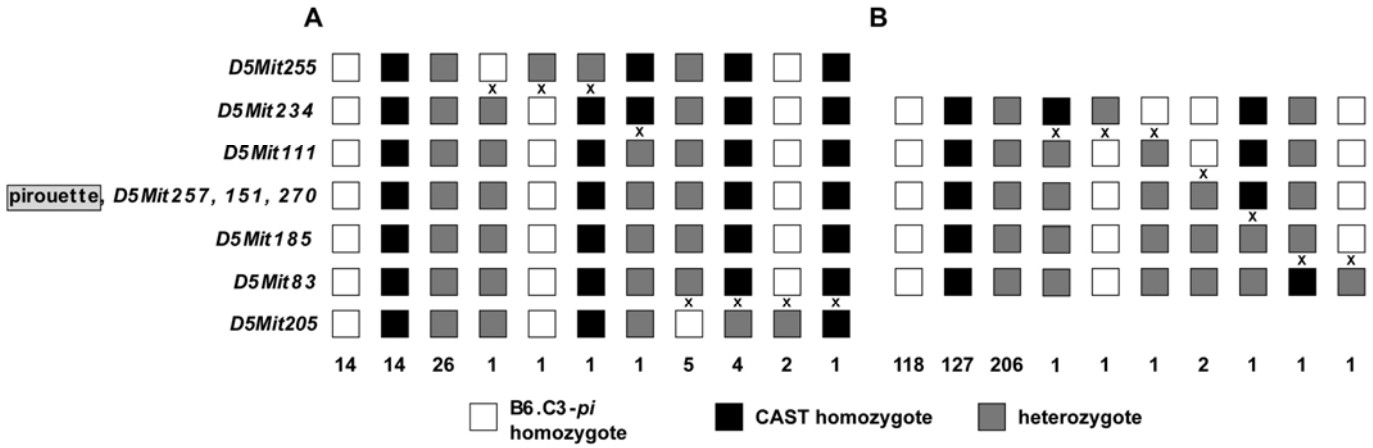

Figure S2. A Common *Grxcr1* Deletion Is Present in the *pi*^{3J} Allele

Products amplified from genomic DNA of control (C, BKS.Cg-m+/+Lepr^{db}/J) and *pi*^{3J}/*pi*^{3J} mice, using eight primer pairs (F through M) designed from sequences upstream of exon 1 and from within intron 1. The deleted region is approximately 200 kb in size. Dashes (--) indicate control PCR reactions lacking template. Positions of molecular size standards are indicated at left in kilobases.

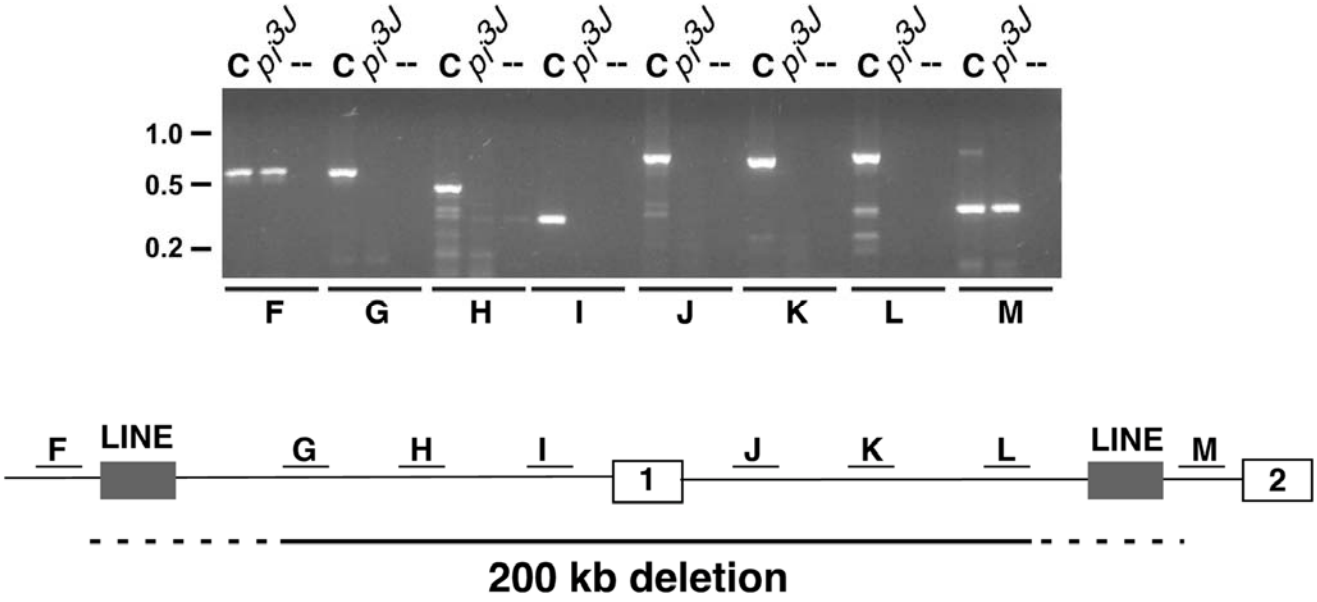

Figure S3. Genomic Rearrangements Indicate Putative Inversion Breakpoints in the Original *pi* Allele

Southern blots containing genomic DNA from control (B6, C57BL/6J; C3H, C3He/FeJ) and *pi/pi* mice were hybridized with probes derived from the *pi* candidate region.

(A) EcoRV-digested DNA hybridized with a probe derived from the 3' end of intron 1.

(B) PmeI-digested DNA hybridized with a 1.1 kb NsiI probe derived from a PAC clone approximately 700 kb telomeric of *Grxcr1*. Arrows indicate fragments with altered size observed in *pi/pi* mice. Positions of molecular size standards are indicated at left in kilobases.

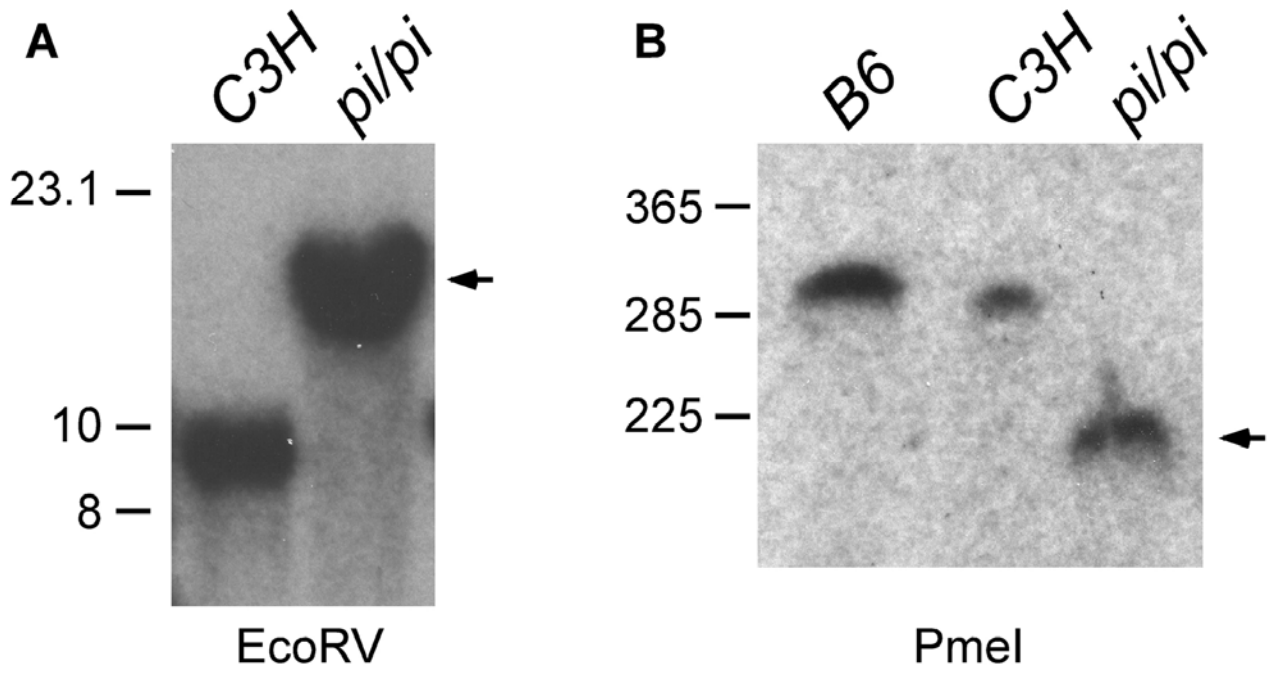

Figure S4. Proteins Related to GRXCR1 Are Present in a Wide Range of Metazoan Species

Alignment of the C-terminal 196 amino acids of GRXCR1 with predicted proteins from a range of metazoan species. The region of similarity with glutaredoxin proteins is indicated with a gray bar, the putative active site cysteine is indicated with '#', and conserved cysteines in the C-terminus are marked with asterisks. Amino acids shaded in blue are identical in each of the sequences; those shaded in black are identical in at least three of the sequences; those shaded in gray are biochemically similar in at least three of the sequences. This domain arrangement is designated as 'KOG2824' in the Conserved Domain Database (Marchler-Bauer, et al., 2003: *Nucleic Acids Res* **31,** 383-7). While glutaredoxins typically contain dual cysteines (CXXC) in their active sites, the single cysteine at the comparable site in GRXCR1 (C156) is completely conserved in this group of metazoan proteins and may retain thiol transferase activity as demonstrated for the omega class of glutathione transferases, which also have a single active site cysteine (Board, et al., 2000: *J Biol Chem* **275,** 24798-806). Although the relative distance between the two groups of four cysteines in the C-terminal regions varies among the proteins, the symmetrical C-X_2-C-X_7-C-X_2-C arrangement of each group is absolutely conserved. Additional predicted proteins exhibit a similar level of similarity to GRXCR1 (data not shown): *A. thaliana* (an additional 14 proteins); *O. sativa* (7); *D. melanogaster* (1); *A. gambiae* (1).

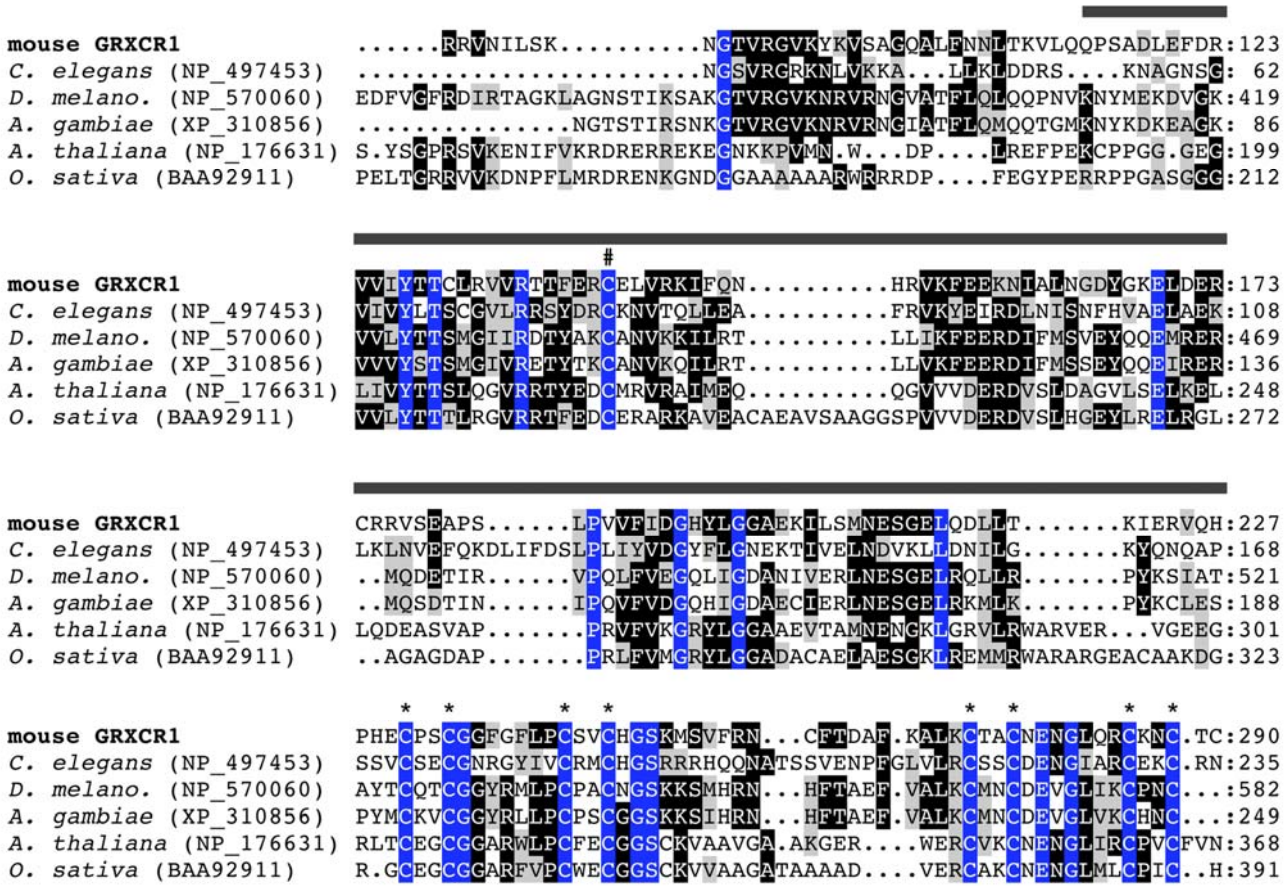
mouse GRXCR1 .....RRVNILSK.....NGTVRGVKYKVSAGQALFNNLTKVLQQPSADLEFDR:123
C. elegans (NP_497453) .....NGSVRGRKNLVKKA...LLKLDDRS....KNAGNSG: 62
D. melano. (NP_570060) EDFVGFRDIRTAGKLAGNSTIKSAKGTVRGVKNRVRNGVATFLQLQQPNVKNYMEKDVGK:419
A. gambiae (XP_310856) .....NGTSTIRSNKGTVRGVKNRVRNGIATFLQMQQTGMKNYKDKEAGK: 86
A. thaliana (NP_176631) S.YSGPRSVKENIFVKRDRERREKEGNKKPVMN.W...DP....LREFPEKCPPGG.GEG:199
O. sativa (BAA92911) PELTGRRVVKDNPFLMRDRENKGNDCGAAAAAARWRRRDP....FEGYPERRPPGASGGG:212
#
mouse GRXCR1 VVIYTTCLRVVRTTFERCELVRKIFQN.....HRVKFEEKNIALNGDYGKELDER:173
C. elegans (NP_497453) VIVYLTSCGVLRRSYDRCKNVTQLLEA.....FRVKYEIRDLNISNFHVAELAEK:108
D. melano. (NP_570060) VVLYTTSMGIIRDTYAKCANVKKIIRT.....LLIKFEERDIFMSVEYQQEMRER:469
A. gambiae (XP_310856) VVVYSTSMGIVRETYTKCANVKQILRT.....LLVKFEERDIFMSSEYQQEIRER:136
A. thaliana (NP_176631) LIVYTTSLQGVRRTYEDCMRVRAIMEQ.....QGVVVDERDVSLDAGVLSELKEL:248
O. sativa (BAA92911) VVLYTTTLRGVRRTFEDCERARKAVEACAEAVSAAGGSPVVVDERDVSLHGEYLRELRGL:272
mouse GRXCR1 CRRVSEAPS.....LPVVFIDGHYLGGAEKILSMNESGELQDLLT.....KIERVQH:227
C. elegans (NP_497453) LKLNVEFQKDLIFDSLPLIYVDGYFLGNEKTIVELNDVKLLDNILG.....KYQNQAP:168
D. melano. (NP_570060) ..MQDETIR.....VPQLFVEGQLIGDANIVERLNESGELRQLLR.....PYKSIAT:521
A. gambiae (XP_310856) ..MQSDTIN.....IPQVFVDGQHIGDAECIERLNESGELRKMLK.....PYKCLES:188
A. thaliana (NP_176631) LQDEASVAP.....PRVFVKGRYLGGAAEVTAMNENGKLGRVLRWARVER...VGEEG:301
O. sativa (BAA92911) ..AGAGDAP.....PRLFVMGRYLGGADACAELAESGKLREMMRWARARGEACAAKDG:323
mouse GRXCR1 PHECPSCGGFGFLPCSVCHGSKMSVFRN...CFTDAF.KALKCTACNENGLQRCKNC.TC:290
C. elegans (NP_497453) SSVCSECGNRGYIVCRMCHGSRRRHQQNATSSVENPFGLVLRCSSCDENGIARCEKC.RN:235
D. melano. (NP_570060) AYTCQTCGGYRMLPCPACNGSKKSMHRN...HFTAEF.VALKCMNCDEVGLIKCPNC...:582
A. gambiae (XP_310856) PYMCKVCGGYRLLPCPSCGGSKKSIHRN...HFTAEF.VALKCMNCDEVGLVKCHNC...:249
A. thaliana (NP_176631) RLTCEGCGGARWLPCFECGGSCKVAAVGA.AKGER....WERCVKCNENGLIRCPVCFVN:368
O. sativa (BAA92911) R.GCEGCGGARFVPCWECGGSCKVVAAGATAAAAD....VERCAKCNENGLMLCPIC..H:391

Figure S5. *Grxcr1* Is Expressed in the Inner Ear

(A) RT-PCR products were amplified from RNA derived from a panel of tissues from wild type adult mice, using *Grxcr1* primers complementary to sequences in exons 1 and 4. *Grxcr1* transcripts containing exons 1 through 4 were present in cochlear RNA (arrow), but absent from the other tissues. We also detected *Grxcr1* transcripts containing exons 2 through 4, but not exon 1, in brain tissue under conditions of increased template concentration or increased PCR cycle number (data not shown). Accordingly, two ESTs (BM938201 and BE955109) associated with this gene are derived from brain tissue. Positions of molecular size standards are indicated at left in kilobases.

(B-C) cRNA probes corresponding to the antisense (B) and control sense (C) strands of the *Grxcr1* transcript were hybridized to mid-modiolar sections through the cochleae of wild-type mice (postnatal day 5). *Grxcr1* transcript signals are localized to the neuroepithelium adjacent to the basilar membrane, at the position of sensory hair cells and their supporting cells (brackets). Blue signals are nuclei counterstained with Hoechst 33258 dye.

Scale bars represent 100 μm (B) and 50 μm (inset).

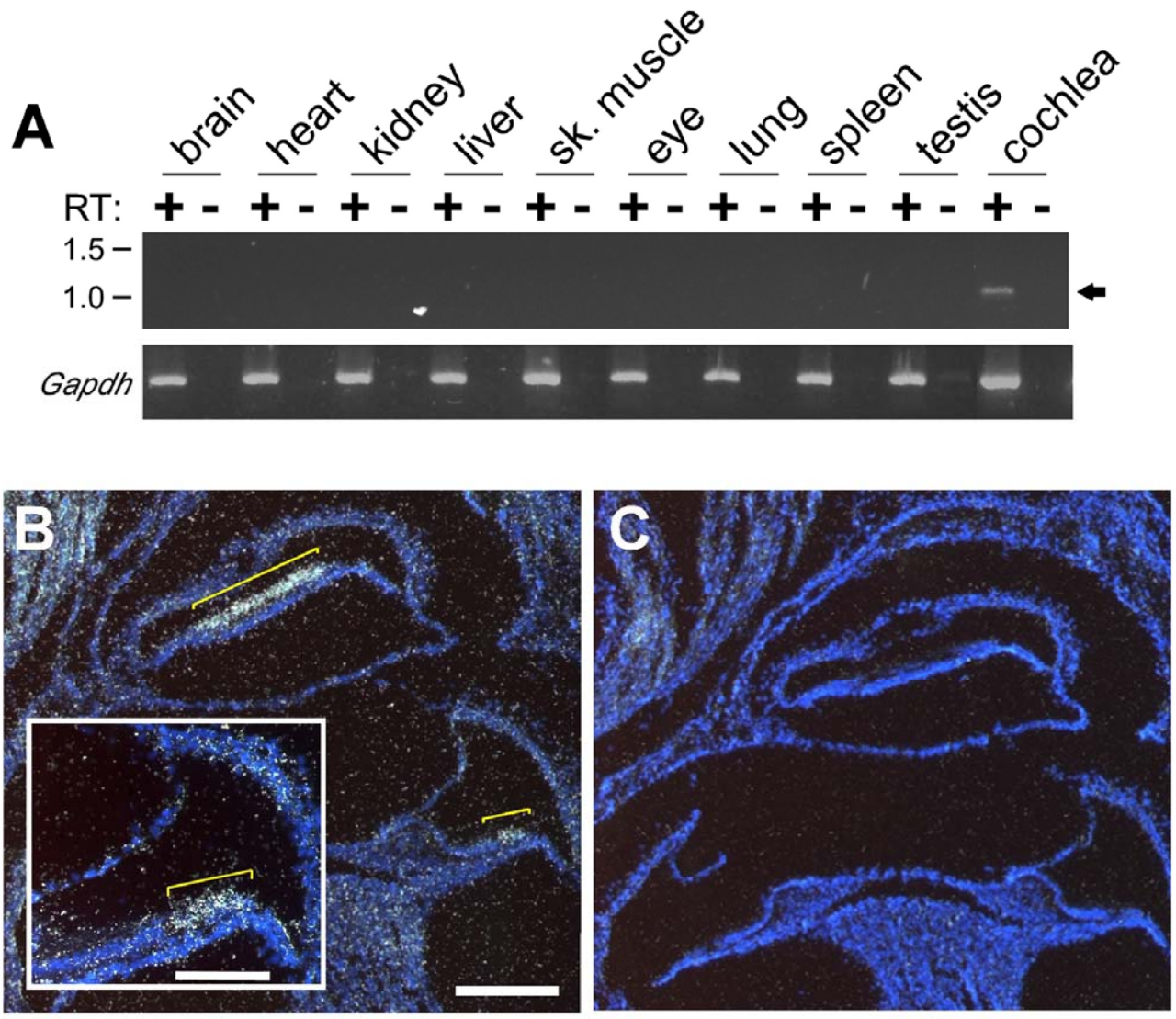
A
brain
heart
kidney
liver
sk. muscle
eye
lung
spleen
testis
cochlea
RT: + - + - + - + - + - + - + - + - + - + -
1.5 –
1.0 –
Gapdh
B
C

Figure S6. Antiserum Raised against a GRXCR1 Peptide Exhibits Reactivity Consistent with Specificity for GRXCR1 Protein

(A-D) Cochleae dissected from *+/pi*^{3J} or *pi*^{3J}*/pi*^{3J} mice at postnatal day 4 were incubated with antiserum raised against a GRXCR1 peptide, followed by a fluorescently tagged secondary antibody (red) and Alexa 488-phalloidin (green). Actin filaments (A) and GRXCR1 immunoreactivity (B) were co-localized (C) in stereocilia of inner and outer hair cells from a control *+/pi*^{3J} mouse. GRXCR1 immunoreactivity appeared in all stereocilia rows including the immature stereocilia of the shortest row (B and C, arrows), which exhibit barely detectable levels of actin. Stereocilia immunoreactivity was absent from an affected *pi*^{3J}*/ pi*^{3J} mouse, which lacks exon 1 that encodes the peptide used for immunization (D). Reactivity was also not observed in cochlear tissue with pre-immune antiserum or with anti-GRXCR1 antiserum after co-incubation with the immunizing peptide (data not shown), supporting specificity of the antiserum for GRXCR1.

(E–J) Cochlear explants cultured from early postnatal, wild-type C57BL/6J mice were transfected with GRXCR1-GFP, then fixed and incubated with anti-GRXCR1 antiserum and a fluorescently-tagged secondary antibody (red). GRXCR1-GFP was localized to the stereocilia bundle of a single transfected outer hair cell (E). This bundle exhibited stronger anti-GRXCR1 immunoreactivity than the bundles of 3 untransfected, neighboring hair cells (F, G). Similarly, GRXCR1-GFP localized to the apical microvilli of three transfected non-sensory cells (H). These microvilli exhibited stronger anti-GRXCR1 immunoreactivity than microvilli of surrounding untransfected cells (I, J). The more prominent signals in transfected cells are consistent with specific reactivity of the antiserum to both endogenous GRXCR1 and over-expressed GRXCR1-GFP fusion protein.

Scale bars represent 5 μm.

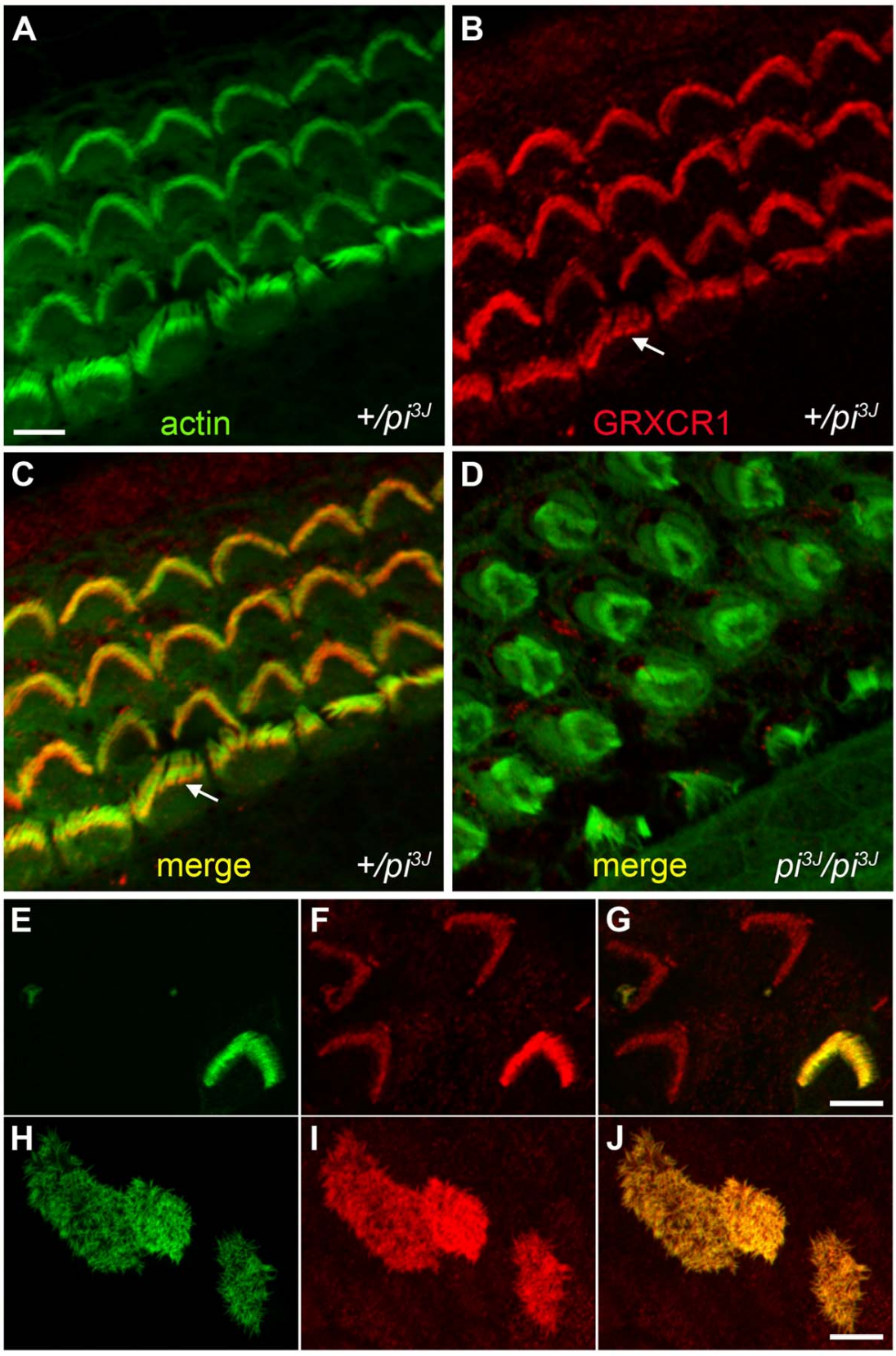
A
actin
+/pi3J
B
GRXCR1
+/pi3J
C
merge
+/pi3J
D
merge
pi3J/pi3J
E
F
G
H
I
J

Figure S7. GRXCR1-GFP Fusion Protein Localizes to Stereocilia and Microvilli in Transfected Explant Cultures

GRXCR1-GFP expression constructs were transfected into cochlear explant cultures from early postnatal wild-type mice.

(A) GRXCR1-GFP (green) co-localizes with actin filaments (red) in stereocilia of an inner hair cell (right). Stereocilia length in GRXCR1-positive hair bundle appeared moderately increased in comparison to the neighboring untransfected hair cell (left).

(B) GRXCR1 co-localizes with actin filaments in the stereocilia bundles of saccular hair cells with no obvious effects on stereocilia dimension.

(C) GRXCR1 localizes to apical microvilli of nonsensory epithelial cells within the organ of Corti. The GRXCR1-positive microvilli are substantially longer than those of neighboring untransfected cells.

(D) Same image as in (C), with increased signal gain to highlight increases in length of GRXCR1-positive microvilli relative to those of neighboring untransfected cells. Note that rhodamine-phalloidin signal in hair cell stereocilia bundles is saturated.

(E) Another example of lengthening of GRXCR1-positive microvilli in non-sensory epithelial cells.

Scale bars represent 5 μm.

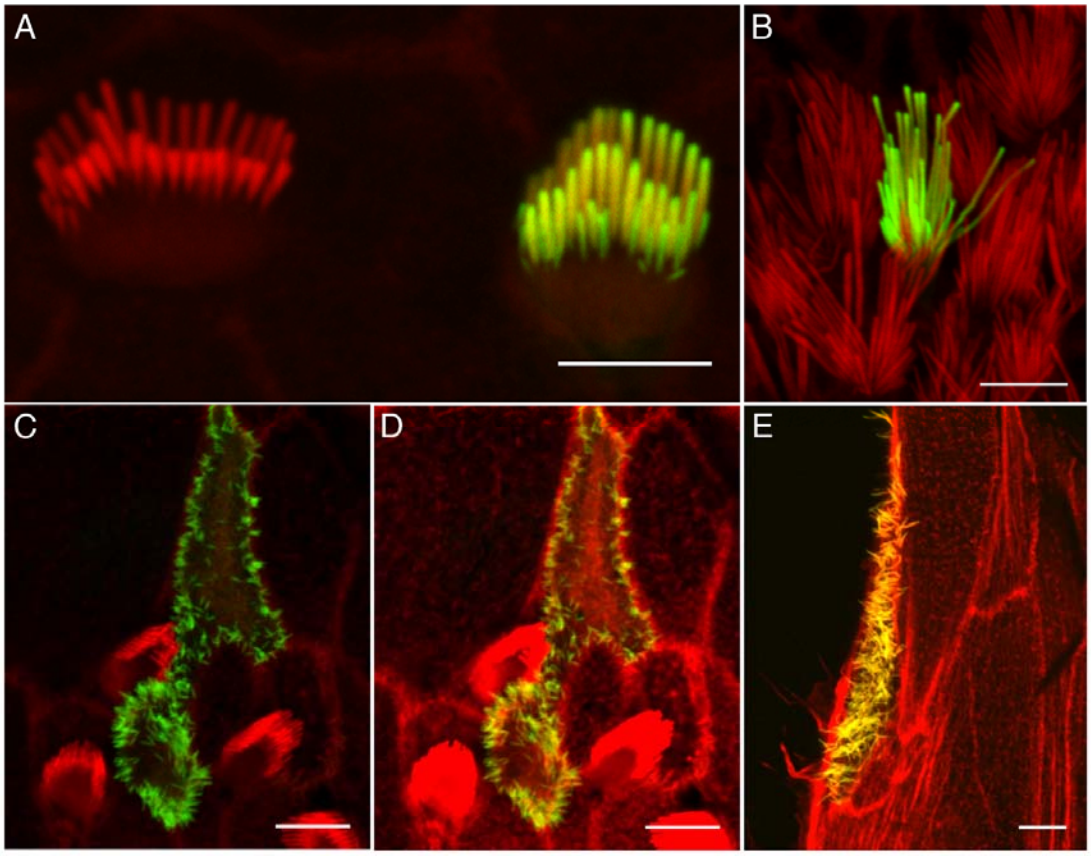
A
B
C
D
E

Table S1. Primer Sequences and Mutation Detection Data

Primer sequences and PCR amplification product sizes, as well as the results of genomic amplification status in deletion analysis of *pi*^{2J} and *pi*^{3J} mutant alleles are provided. Unless noted, all primer sequences are derived from mouse.

	RT-PCR						
A	***Kctd8* RT-PCR**		**FORWARD**		**REVERSE**	**product size, in bp**	
		mf6	5'-TCGCGGAGGTCCATTCCTGAGAGTC-3'	mR3	5'-GGAGCGCTGCCACCACCGACTA-3'	834	Exon 1
		mF2	5'-GCCCAAGGTCACCAAGCAGAACT-3'	mR7	5'-CATATCGCTTCATCCACATTCTTGTCTG-3'		Exon 1-2
B	***Grxcr1* RT-PCR**						
		162559 F2	5' CTGTGGCAAGGGGGATGAACT-3'	162559 R5	5'-TTTGTACTTGACGCCTCTGACTGT'3'	405	Exon 1
		162559 F3	5'-GTCCGGTTTCGAATTGCCTCATC-3'	162559 R4	5'-CCACACGGTCAAATTCCAGGTCAG-3'	375	Exon 1-2
		162559 F4	5'-CAGCTGACCTGGAATTTGACCGT-3'	162559 R6	5'-GGTTAAAAGGTCTTGCAGTTCTCC-3'	290	Exon 2-3
		rat F1	5'-GGTGCTGAGAAAATTTTGTCAATG-3'	162559 R2	5'-TTATCCTTTGTTTAATGAAATGAGGCTTT-3'	294	Exon 3-4
		162559 F2	5'-CTGTGGCAAGGGGGATGAACT-3'	162559 R4	5'-CCACACGGTCAAATTCCAGGTCAG-3'	530	Exon 2-4
		162559 F2	5'-CTGTGGCAAGGGGGATGAACT-3'	162559 R2	5'-TTATCCTTTGTTTAATGAAATGAGGCTTT-3'	990	Exon 1-4
	Genomic PCR						
C	***pi*^{2J}, *pi*^{3J} assay**						**status in pi^{2J}, pi^{3J}**
	5'of *Grxcr1*	5'gen F11	5'-GTTCCTTGCAGTACTGTTGTTATGGTC-3'	5'gen R11	5'-ATGGGGAAGGGATGAAAGAAGTTA-3'	546	present
		5'gen F10	5'-CAGTAGGGGATGTGGTAGGTTCTCAG-3'	5'gen R10	5'-TGTACAGGCTCTTTCTTCTCTAGGTATTTG-3'	541	present
		5'gen F17	5'-TGGTCAAGGCTACATCACAGGTCAGA-3'	5'gen R17	5'-TGGAGAATAAGGCACATGCTGGATAGTAG-3'	563 (F)*	present
		5'gen F16	5'-GGCAATCCCATCATGTCTAAGTCTCCAG-3'	5'gen R16	5'-CTTCTGAGGTATCCATGATGCCAACTTTC-3'	187 (A)	present
		5'gen F15	5'-CCCCCTGGTGTGACTGTTTTCTATC-3'	5'gen R15	5'-AGTCTAATTGCTTTGGCCTGTGAGTTTG-3'	372 (B)	absent
		5'gen F14	5'-TCCATAAATGAGTGAGCATATCCTAACGAG-3'	5'gen R14	5'-CAGCTTTGTATTGATTTCTATGGGTCTTC-3'	289	absent
		5'gen F13	5'-GACCCCTGACTACTTTTCCATGATTACTGA-3'	5'gen R13	5'-CATTTGCTTCTCTGTCTATTTGCTTCCTTAT-3'	799	absent
		5'gen F12	5'-CCTGGGCTTTGTTTAGTATGTGCTGTT-3'	5'gen R12	5'-CTGCCCACTTCACTTTCCTGTTT-3'	252	absent
		5'gen F9	5'-GTGGCCACTGCTGCTGCTTCTTT-3'	5'gen R9	5'-ATTCCCCATTGCTTATACACGTTCACTTTA-3'	514	absent

	5'gen F8	5'-GTTTCATTGCGAGGATTTCCACATTTCT-3'	5'gen R8	5'-AGCCATTGCCAGCATCTCACTTCA-3'	553 (G)	absent
	5'gen F7	5'-TAGGGCCAGTAACAGGGGTCAGAAC-3'	5'gen R7	5'-GGTCCCCTCAATAGTCCATCCACAT-3'	370	absent
	5'gen F6	5'-ATGGGACTCTAGGGAATTTTGGTTTA-3'	5'gen R6	5'-AAGAGGTGGTTGGGGCTGTTTACTG-3'	560	absent
	5'gen F5	5'-ACATGTCAAGTGCCGGGGAGAAG-3'	5'gen R5	5'-CAATGGGTTACTGGGATGGATA-3'	465 (H)	absent
	5'gen F4	5'-GGTGCCAGCCAGAGGAAAACT-3'	5'gen R4	5'-AGGAGGTAGGGCTAGAGACAGGAAG-3'	417	absent
	5'gen F3	5'-TCCTTCTGTTATGCCCACTGACTACT-3'	5'gen R3	5'-CATGAGCCCTACTGGAAGATGATACT-3'	315 (I)	absent
	5'gen F1	5'-GATGCCCTGTGAGTTTTGATGCCTT-3'	5'gen R1	5'-AAACAGTCCATATGGGGCTGACACC-3'	410	absent
	5'gen F2	5'-TGAACTCACAGCAGTTGTTGTTGGC-3'	5'gen R2	5'-GTCCTCTGTGCCTTCTCGGTGTGT-3'	575	absent
exon1	162559 F2	5' CTGTGGCAAGGGGGATGAACT-3'	162559 R5	5'-TTTGTACTTGACGCCTCTGACTGT'3'	405 (C)	absent
intron 1	Intron 1 F1	5'-ACTCCAGTTTCCTCCCTCCGTCTTC-3'	intron 1 R1	5'-CCTGGTAGTGATAATGGGTGTGATGGA-3'	617	absent
	Intron 1 F2	5'-AATTTCTGTTTCTATTGCCTATTCTATTTCTAA-3'	intron 1 R2	5'-TTATATGCCCAAAACCTGAGACCTG-3'	696 (J)	absent
	Intron 1 F3	5'-GAATTAGACACAGGGGCTGGATAAACG-3'	intron 1 R3	5'-GCAGGGAGGCAATGAAGTTGAAGAGT-3'	416	absent
	Intron 1 F4	5'-GTTCAGGGCTCCCTATGCTTCAAGTC-3'	intron 1 R4	5'-ACTGGGGAATTTCCTGCAAACGG-3'	455	absent
	Intron 1 F5	5'-GGCTGGCCCACTTTTATTCTTTATCACCT-3'	intron 1 R5	5'-GACAAAACCCCACAGGCATACCGAAAAC-3'	673 (K)	absent
	Intron 1 F6	5'-TGTTTTCTCCACTGCCCCTCCTATG-3'	intron 1 R6	5'-TGTTATTTATTCATTTCTGCGTCTTCAA-3'	482	absent
	Intron 1 F7	5'-TTTAAAGGTATTGGGCAACACGCTCA-3'	intron 1 R7	5'-GCACAGCCCTCTTCTTGGAACTCG-3'	429	absent
	Intron 1 F8	5'-AAATCTAATGGCTGTTGTGGACAATGC-3'	intron 1 R8	5'-GCGGGCTCTTATACTGTTATGCAATGAT-3'	700 (L)	absent
	intron 1 F10	5'-TGCCAGATTGTGTTATGGGTTCTTAGTTC-3'	intron 1 R10	5'-GTGCCCTTTCTGGAGCCTGGTATC-3'	378	absent
	intron 1 F11	5'-ATGTGGGATATAATTTGGTAAGGAAGCA-3'	intron 1 R11	5'-GAACCCCAGCACCTCACTATTGT-3'	142	absent
	intron 1 F12	5'-CCTGTGTCCCTCTGTACCTCTGCTGAAC-3'	intron 1 R12	5'-TAGACAGAAAGGGAGGAAATGGGTATGA-3'	201	absent
	intron 1 F13	5'-TGGCCATGAAACACAGGACAGG-3'	intron 1 R13	5'-GAGGCCCATTTCTGACATTTGTG-3'	210 (D)	absent
	intron 1 F15	5'-AAGAGTGGAGCTGTTGGGAAGTAGTAT-3'	intron 1 R15	5'-GGTTTAATTGCTTTGGGCCTATGATG-3'	198 (E)	present
	intron 1 F16	5'-AATAATTATGTTTGACACCAGTAGGGTCTC-3'	intron 1 R16	5'-AGCAAGTAAGCTAAAATCCCAGAAGCC-3'	178	present
	intron 1 F9	5'-CACGCCTCCTCCCACCCCACTT-3'	intron 1 R9	5'-TACTAAAACCAGCCAGCCAGAACTTCACTACATT-3'	354 (M)	present

D **hybridization probes**

pi	intron1 F6	5'-TGTTTTCTCCACTGCCCCTCCTATG-3'	intron 1 R6	5'-TGTTATTTATTCATTTCTGCGTCTTCAA-3'	482
pi^{tde}	intron1 F6	5'-TGTTTTCTCCACTGCCCCTCCTATG-3'	intron 1 R6	5'-TGTTATTTATTCATTTCTGCGTCTTCAA-3'	482

pi^{tg370}	intron 2 F11	5'-AAGGCTACCATACCAACAACGAATAAGG-3'	intron 2 R11	5'-CACAACAATAAAGAAACTGGGGAGAG-3'	650

E SSCP analysis

Atp8a1	5'-GGCTCAACCACCAGTCAATCAAC-3'	5'-CAAACGCCTGGAAATGCTCAAA-3'	334
Kctd8	5'-CCTGACCTGTGGTTCTTTTGTGTAG-3'	5'-AAAGCTCTTTGCCATTTCCTTCA-3'	230

F 3' RACE

primary *Grxcr1* primer	162559 F2	5'-CTGTGGCAAGGGGGATGAACT-3'
secondary *Grxcr1* primer	162559 F5	5'-TGGTACAGTCAGAGGCGTCAAGTA-3'

G Human GRXCR1

Exon 1	5'- TGATGTTAGTTTCACAGGAGTGC-3'	5'- GAGGCTTGCTTTAACTGGAGAA-3'	597
Exon 2	5'- TCTGTATCCCAATTACAGATGTTG-3'	5'- TCCAATCCCTTTGGTTTGAG-3'	550
Exon 3	5'- TGTCTTCTTCTCTTTGGCATCC-3'	5'- TCACTCTAGCTGCAACAAACCT-3'	400
Exon 4	5'- CCACTCACAGTTCAGAAAGACC-3'	5'- TTAGAAACCAGGGTCTAGCACA-3'	498

*letters in parentheses correspond to assay designations in Figure 2A and Figure S2