

**Angulin proteins ILDR1 and ILDR2 regulate alternative pre-mRNA splicing through binding to splicing factors TRA2A, TRA2B, or SRSF1**

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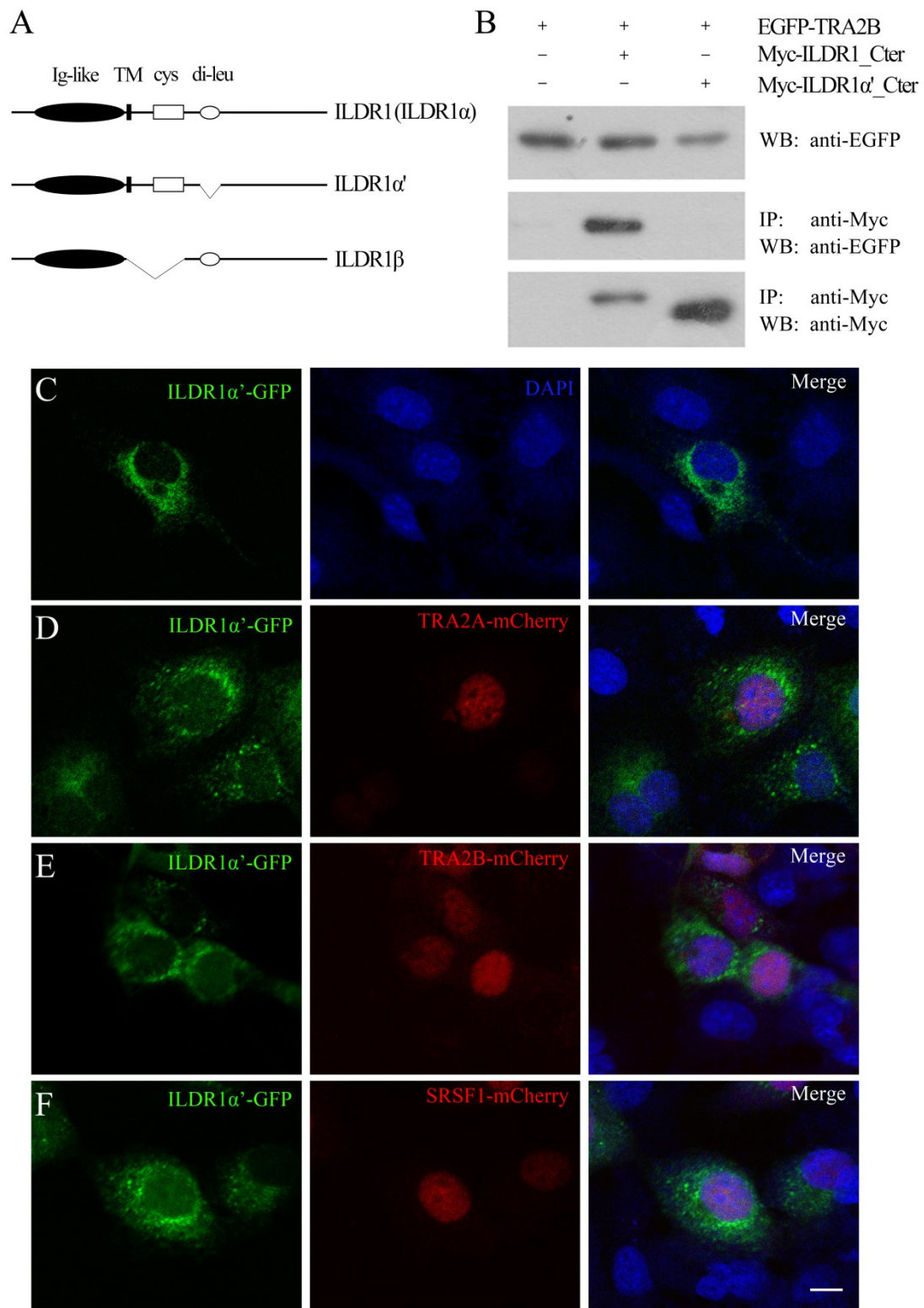
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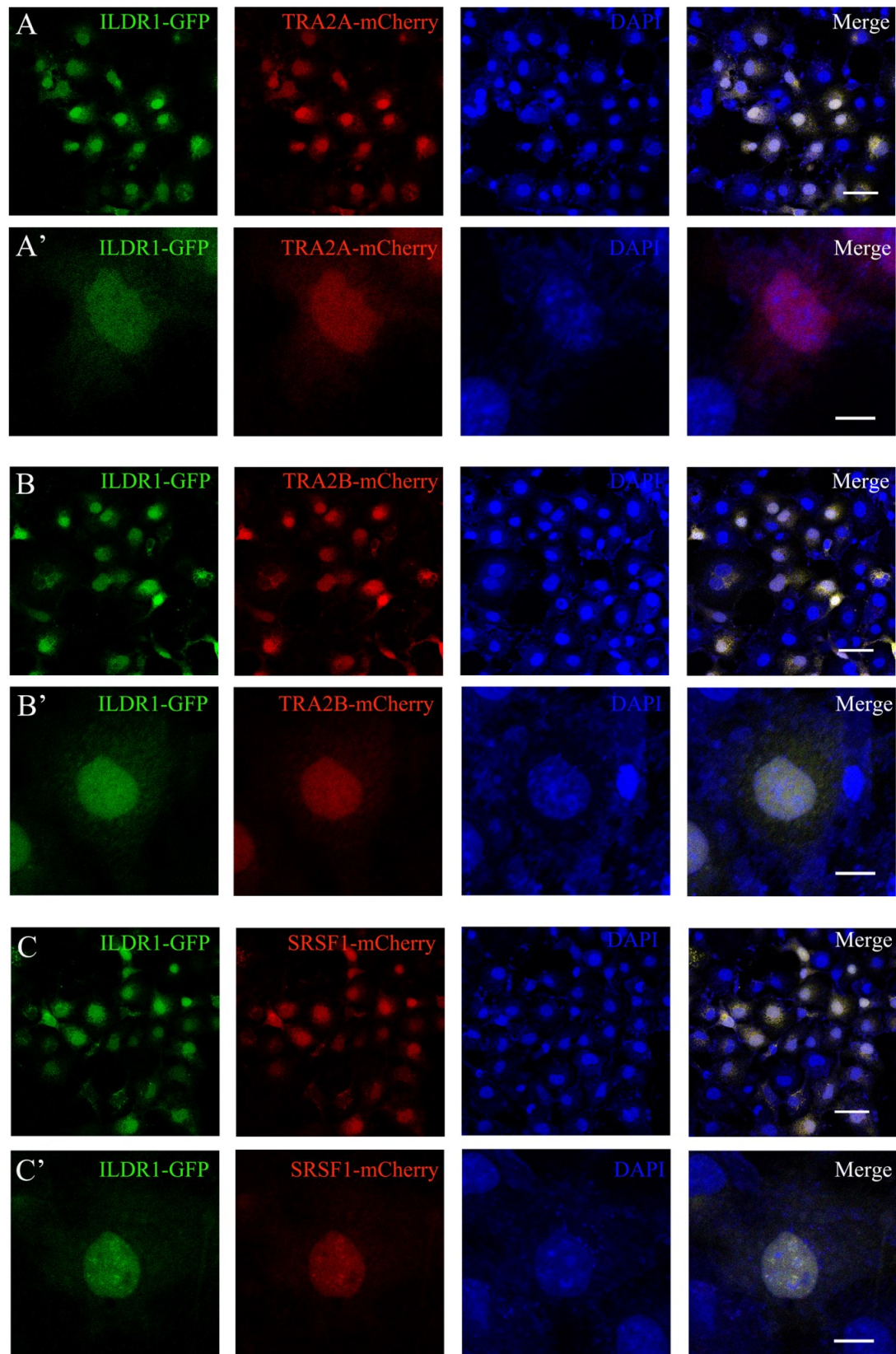
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**Key words:** ILDR1; ILDR2; alternative pre-mRNA splicing; RS protein; yeast two-hybrid;  
hearing loss



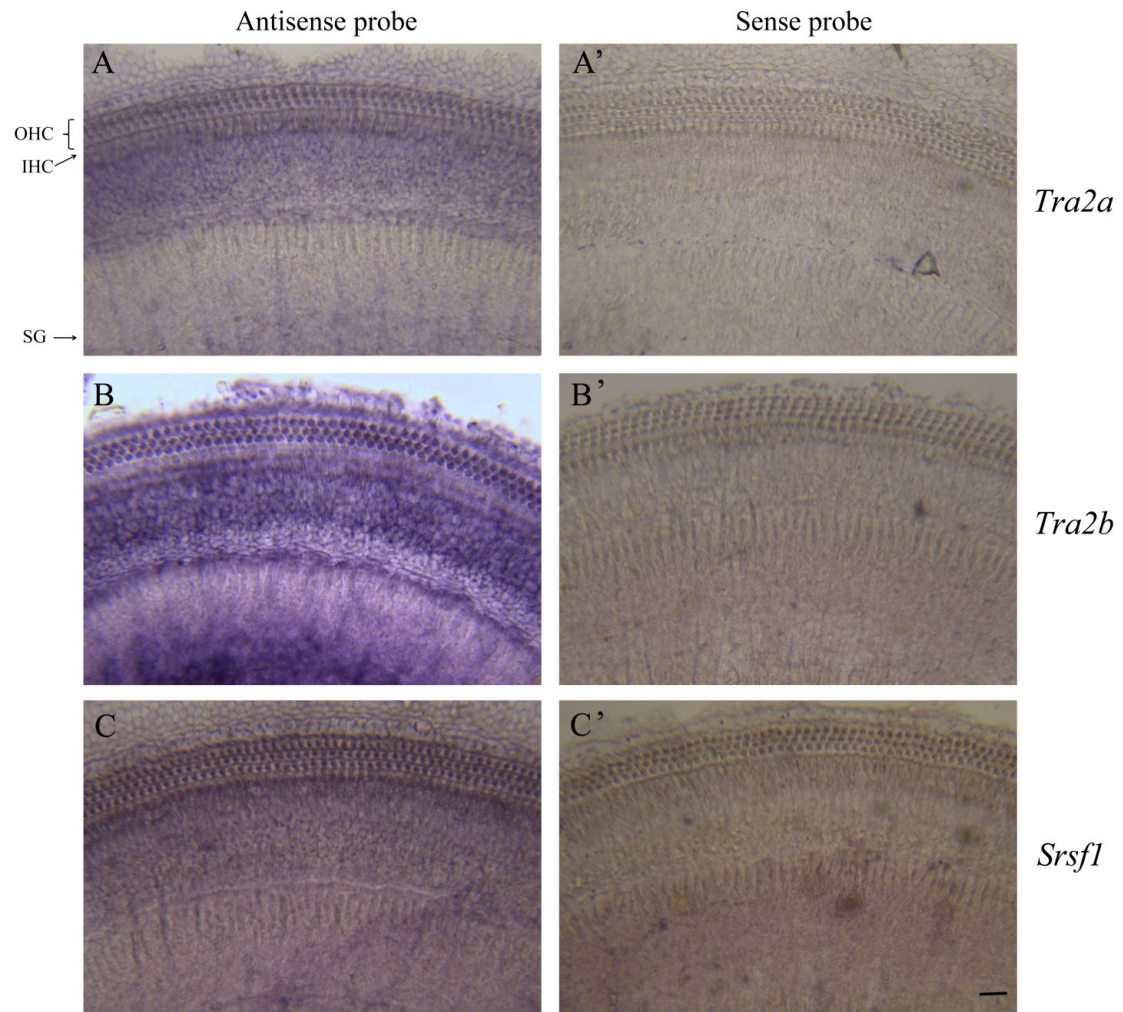
**Supplemental figure S1. ILDR1 $\alpha'$  does not interact with TRA2B.** (A) Schematic drawing of the domain structures of three *Mus musculus* ILDR1 variants, ILDR1 $\alpha$ , ILDR1 $\alpha'$ , and ILDR1 $\beta$ . (B) Western blots showing that EGFP-tagged TRA2B was co-immunoprecipitated with Myc-tagged ILDR1 (ILDR1 $\alpha$ ) cytoplasmic fragment, but not ILDR1 $\alpha'$  cytoplasmic fragment. Expression

vectors were transfected into HEK293T cells to express epitope-tagged proteins, and cell lysis were subject to immunoprecipitation. IP indicates antibody used for immunoprecipitation and WB indicates antibody used for detection. (C) When overexpressed in COS-7 cells, ILDR1 $\alpha$ '-GFP localizes in the cytoplasm. (D-F) When cotransfected with TRA2A-mCherry, TRA2B-mCherry, or SRSF1-mCherry, ILDR1 $\alpha$ '-GFP remains in the cytoplasm, whereas the splicing factors localize in the nuclei. Expression vectors were transfected into COS-7 cells to express epitope-tagged proteins. Nuclei were stained with DAPI. Scale bar: 10  $\mu$ m.

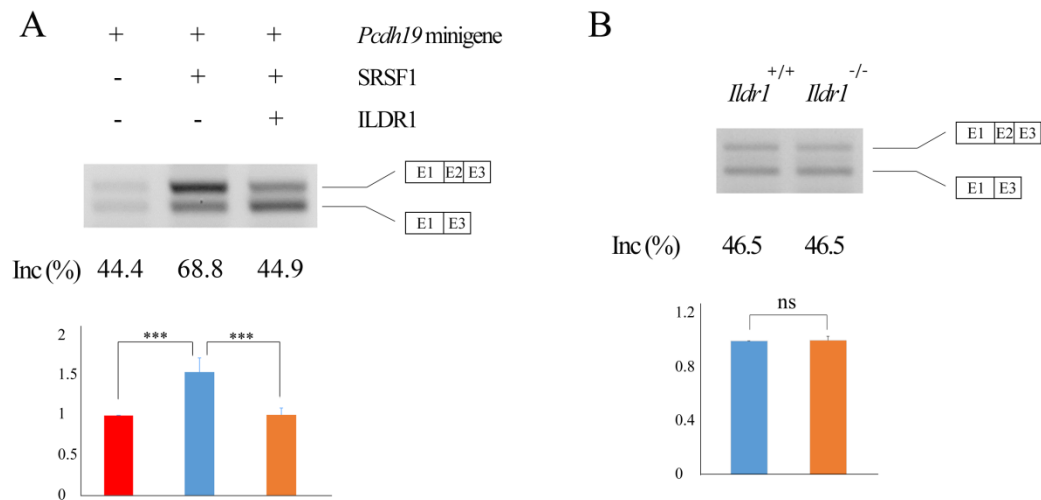


**Supplemental figure S2. ILDR1 translocates into the nuclei when TRA2A, TRA2B, or SRSF1 is present. (A) and (A') ILDR1-GFP translocates into the nuclei when TRA2A-mCherry**

is present. **(B)** and **(B')** ILDR1-GFP translocates into the nuclei when TRA2B-mCherry is present. **(C)** and **(C')** ILDR1-GFP translocates into the nuclei when SRSF1-mCherry is present. Expression vectors were transfected into COS-7 cells to express epitope-tagged proteins. Nuclei were stained with DAPI. Scale bars: 50  $\mu\text{m}$  in (A), (B) and (C), 10  $\mu\text{m}$  in (A'), (B') and (C').



**Supplemental figure S3. Expression pattern of *Tra2a*, *Tra2b*, and *Srsf1* in the mouse cochlea examined with whole-mount *in situ* hybridization.** Cochlea of postnatal day 8 (P8) mice were dissected and hybridized with *Tra2a* (A), *Tra2b* (B), and *Srsf1* (C) antisense probes. *Tra2a* (A'), *Tra2b* (B'), and *Srsf1* (C') sense probes were used as negative controls. Abbreviations: OHC, outer hair cells; IHC, inner hair cells; SG, spiral ganglion. Scale bar: 15  $\mu$ m.



**Supplemental figure S4. *Protocadherin 19* (*Pcdh19*) pre-mRNA splicing is affected by ILDR1.**

(A) RT-PCR revealed that exon 2 of *Pcdh19* minigene is subjected to alternative splicing (lane 1).

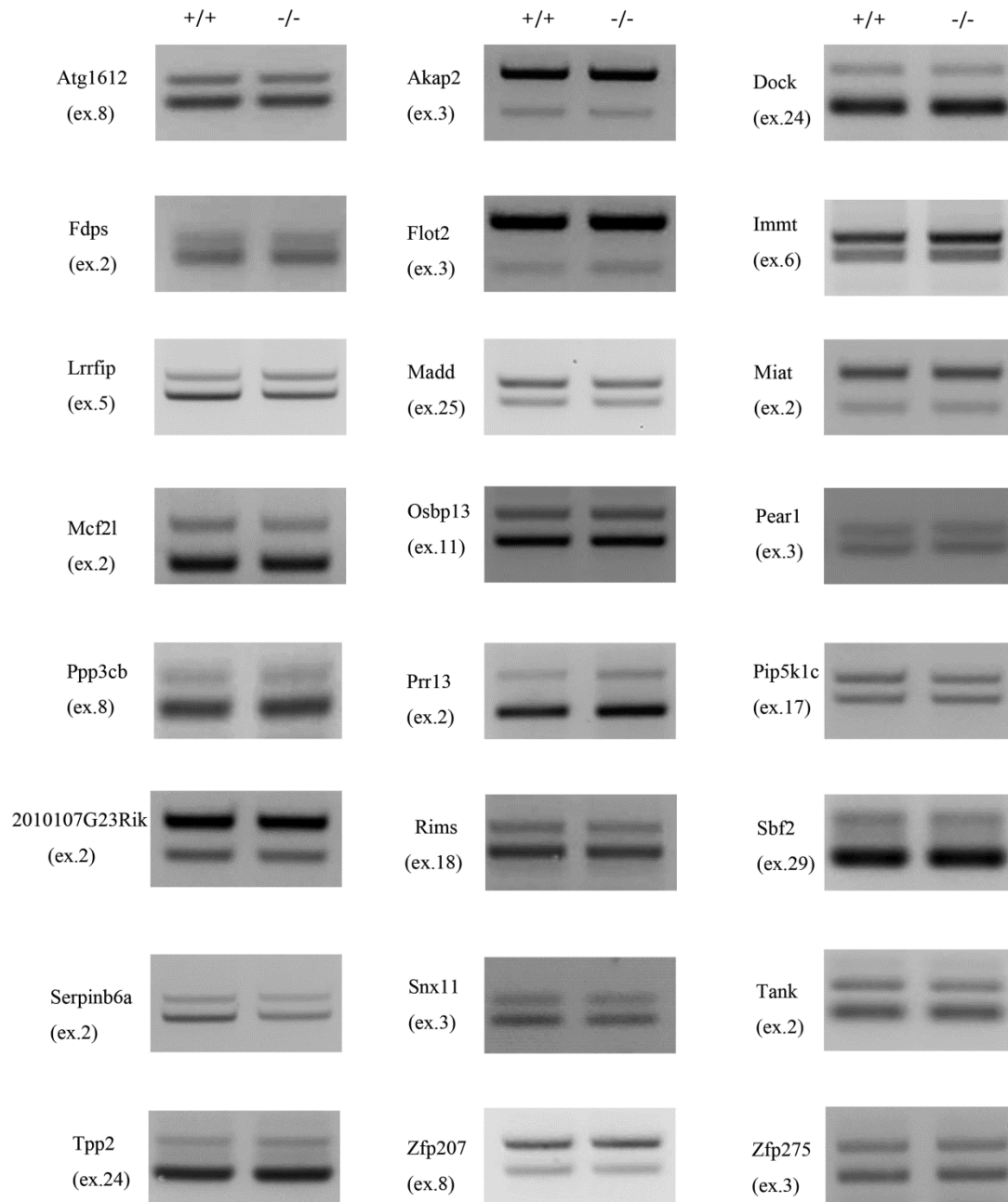
The inclusion of exon 2 was enhanced by SRSF1 (lane 2), whose effect was inhibited when

ILDR1 was present (lane 3). (B) The alternative splicing of *Pcdh19* exon 2 was not affected in

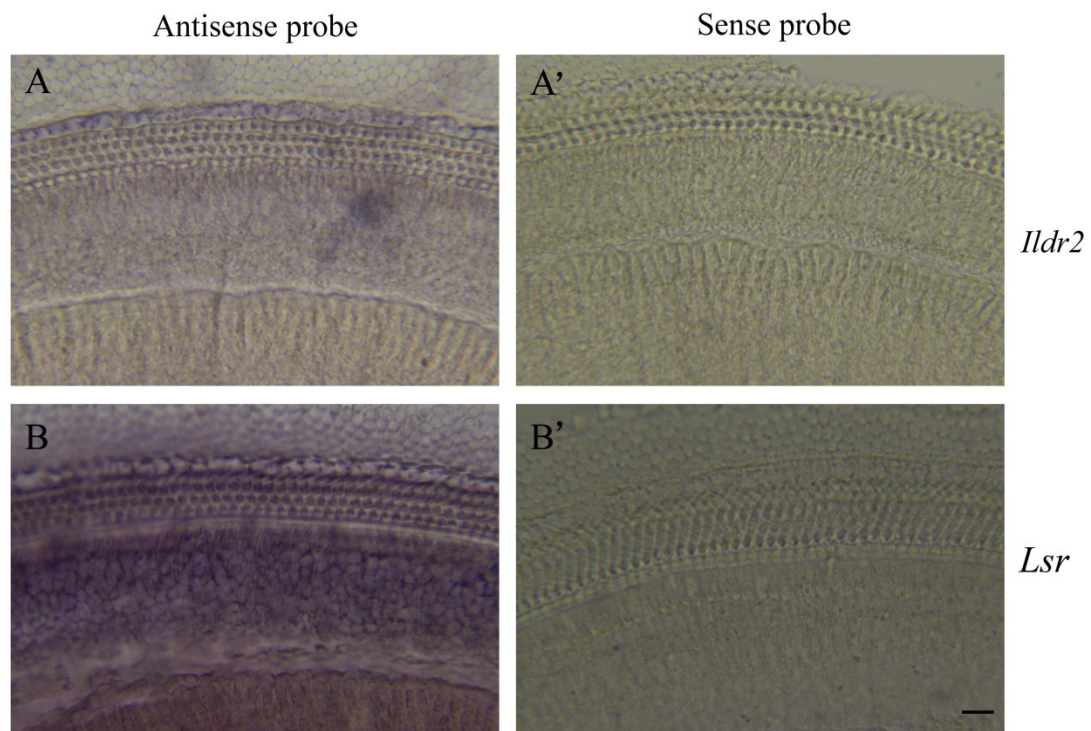
*Ilchr1* knockout mice. The relative exon inclusion rate was calculated from three independently

performed experiments. The differences between groups were determined by Student's t-test. \*\*\*

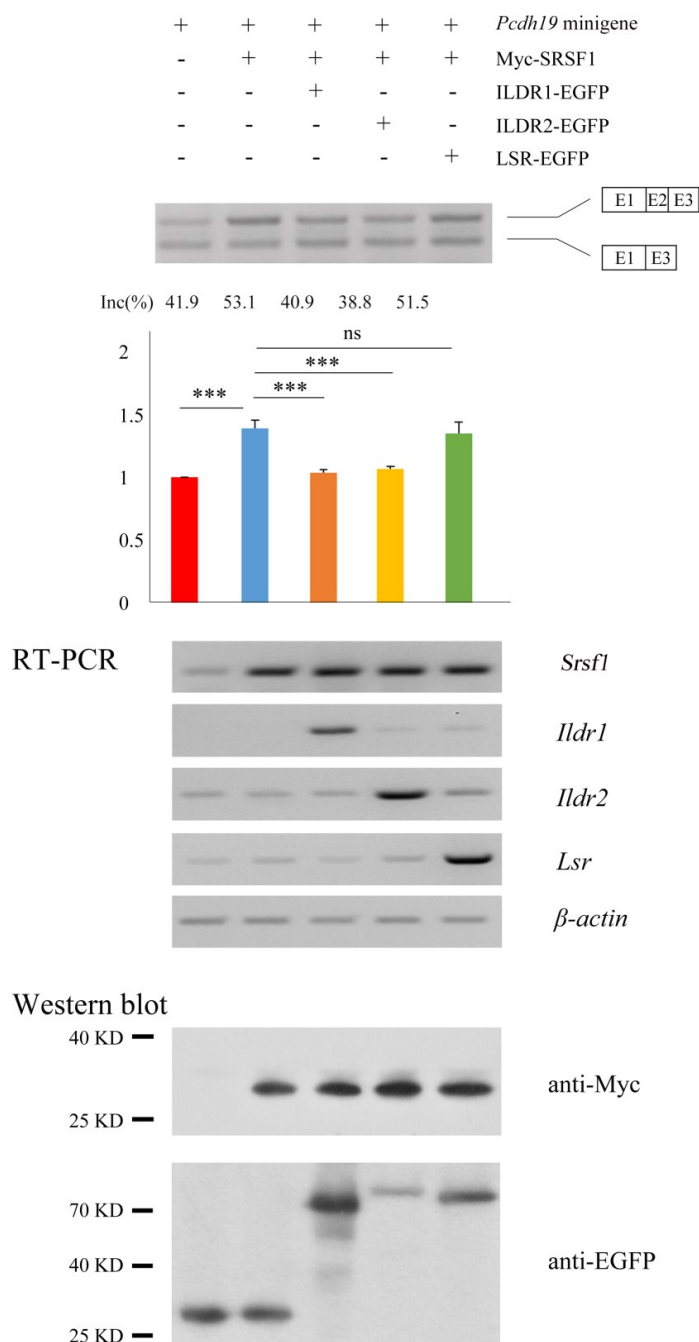
P<0.001; ns, not significant.



**Supplemental figure S5. Pre-mRNA alternative splicing was not affected in *Ildr1* knockout mice.** Total RNA of P0 mice inner ear was extracted and pre-mRNA alternative splicing of twenty-four genes was evaluated by RT-PCR.

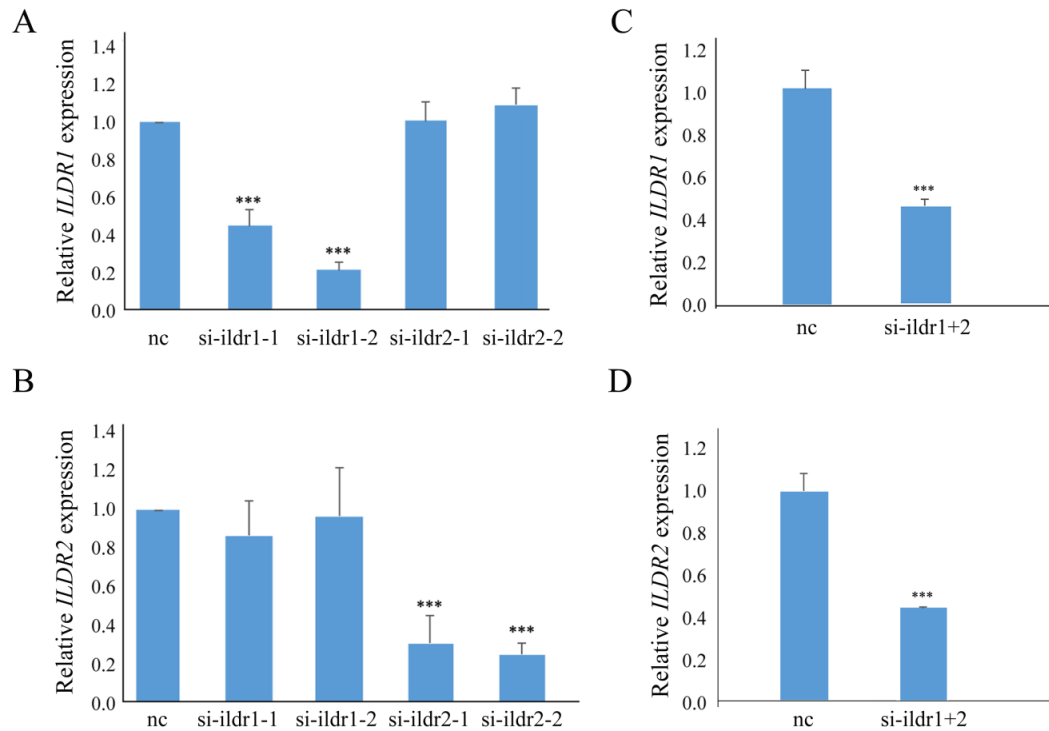


**Supplemental figure S6. Expression of *Ildr2* and *Lsr* in the mouse cochlea examined with whole-mount *in situ* hybridization.** Cochlea of postnatal day 8 (P8) mice were dissected and hybridized with *Ildr2* (A) or *Lsr* (B) antisense probes. *Ildr2* (A') or *Lsr* (B') sense probes were used as negative control. Scale bar: 15  $\mu$ m.

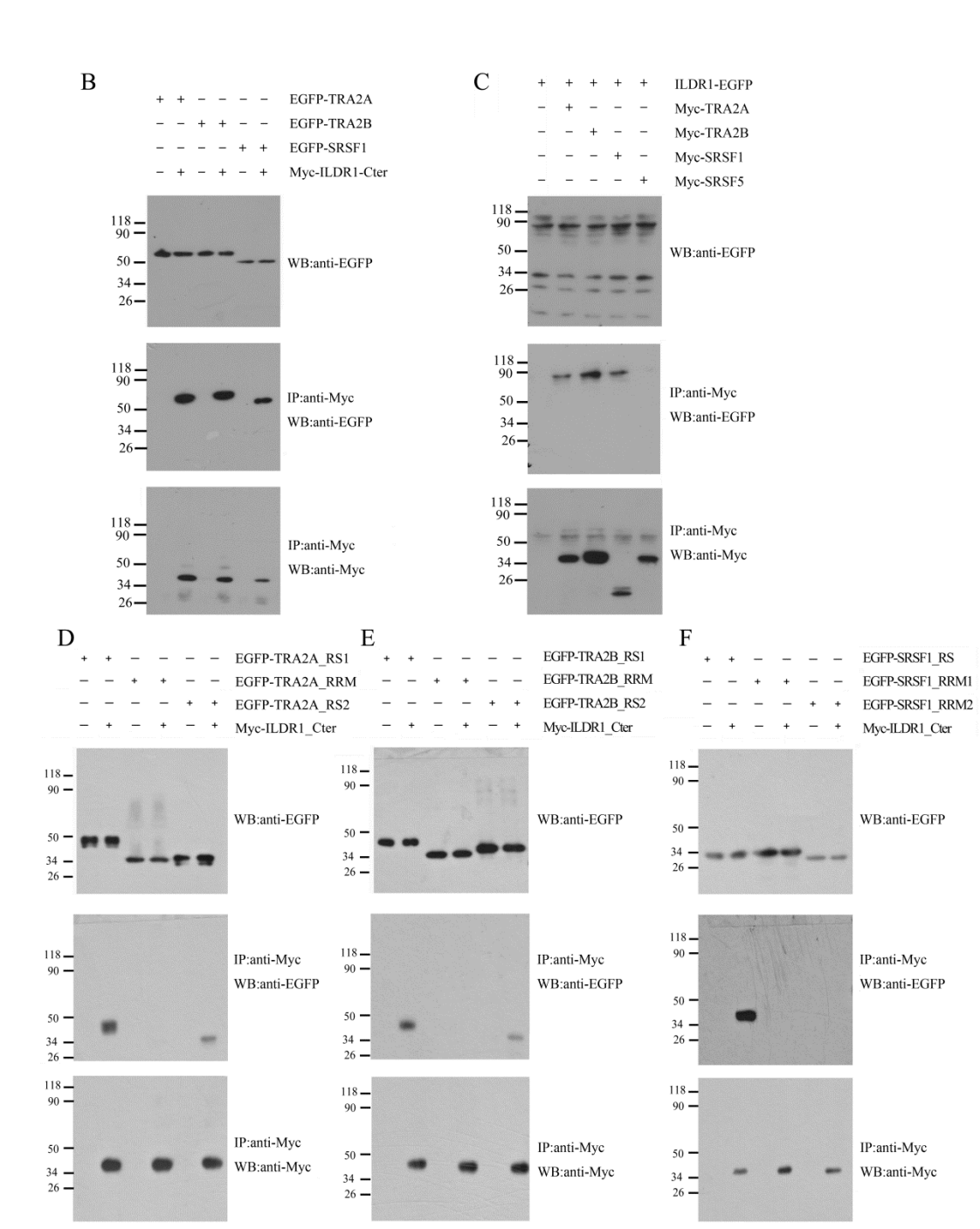


**Supplemental figure S7. ILDR2 affects *Protocadherin 19* (*Pcdh19*) pre-mRNA splicing.**

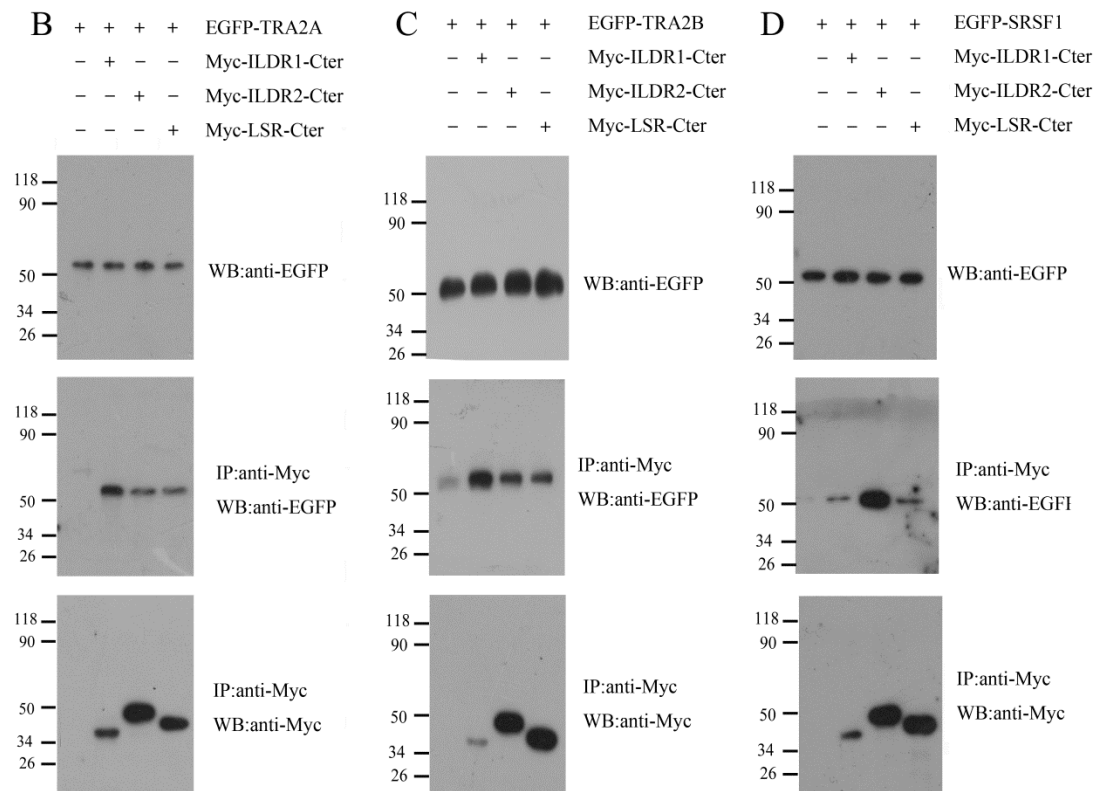
RT-PCR revealed that exon 2 of *Pcdh19* minigene is subjected to alternative splicing (lane 1). The inclusion of exon 2 was enhanced by SRSF1 (lane 2), whose effect was inhibited by ILDR1 (lane 3) or ILDR2 (lane 4), but not LSR (lane 5). The level of overexpression was examined via RT-PCR and Western blot. The relative exon inclusion rate was calculated from three independently performed experiments. The differences between groups were determined by Student's t-test. \*\*\*  $P < 0.001$ ; ns, not significant.



**Supplemental figure S8. Examination of the siRNAs against *ILDR1* and *ILDR2* in HEK293T cells.** (A) and (B) The efficiency and specificity of siRNAs against *ILDR1* and *ILDR2* was examined by quantitative PCR. (C) and (D) The efficiency of combined siRNAs (si-ildr1-2 and si-ildr2-2) was examined by quantitative PCR. The relative expression level was calculated from three independently performed experiments. The differences between groups were determined by Student's t-test. \*\*\* P<0.001.



**Supplemental figure S9. Uncropped blots corresponding to figure 1.**



**Supplemental figure S10. Uncropped blots corresponding to figure 5.**

**Supplementary table S1. RT-PCR primers used in splicing examination.**

| <b>Gene Name</b> | <b>Oligonucleotide 5'&gt;3'</b> | <b>product (bp)</b> |
|------------------|---------------------------------|---------------------|
| Atg16l2-E8-F     | TGTGAGCATCAGCGAGATCC            | 223/158             |
| Atg16l2-E8-R     | CCCAACCGAGTGACCTCTTC            |                     |
| Akap2-E3-F       | TCAAGGAAGCAGCGGACTTT            | 385/206             |
| Akap2-E3-R       | CGGAAGTCACCTTGCAAGACA           |                     |
| Dock6-E24-F      | CCATACTGTGGGGCTGATCC            | 212/119             |
| Dock6-E24-R      | AGCTGGAAGAAGAACCAGGC            |                     |
| Fdps-E2-F        | TGCACGCTCAGCTTTTAGGT            | 407/181             |
| Fdps-E2-R        | CCAGCTCCTTCTCAGTCAGC            |                     |
| Flot2-E3-F       | GGTCTCAGGAGGCTGTTGTG            | 232/141             |
| Flot2-E3-R       | GTCTCTACGTCTCACAGCG             |                     |
| Immt-E6-F        | GAGACACTCCAGCTTCAGCA            | 302/205             |
| Immt-E6-R        | TGCAGCATTCTGGGCTGTAA            |                     |
| Lrrfip1-E5-F     | GCATCTCTGGGTGGGACTTC            | 278/206             |
| Lrrfip1-E5-R     | TCCAGCAGCATGTCCTTCAG            |                     |
| Madd-E25-F       | GCTACAAGCGCCATCTTTGG            | 284/220             |
| Madd-E25-R       | CCCTGGTCCATACCCATTCC            |                     |
| Miat-E2-F        | GAGGACTCCCCCAGTTCTGT            | 236/188             |
| Miat-E2-R        | TTGTGCCAAAGCAGTGA CTC           |                     |
| Mcf2l-E26-F      | GAAGGCTACGTGAGCTCATCG           | 239/123             |
| Mcf2l-E26-R      | GAGTGGGATGTTTTGCTCCAG           |                     |
| Osbp13-E11-F     | TACTTCGCTTTAAGGTCGGC            | 347/238             |
| Osbp13-E11-R     | AGCAGAACTTCCTGGGCATC            |                     |
| Pear1-E3-F       | CCTGAAGGCCGTCGGAAG              | 178/104             |
| Pear1-E3-R       | GGGACAAAGTGGCATTGCAG            |                     |
| Ppp3cb-E12-F     | GGAGTGTTGGCTGGAGGACG            | 193/95              |
| Ppp3cb-E12-R     | ATGTGGTGGAGAGAATCCTCG           |                     |

|                    |                        |         |
|--------------------|------------------------|---------|
| Prr13-E2-F         | GTGCGAACCCAGACTGAGAA   | 269/151 |
| Prr13-E2-R         | GTGGACAGGCAGGATTGGAA   |         |
| Pip5k1c-E17-F      | CTGCCAGCCAGGCCTCAG     | 372/294 |
| Pip5k1c-E17-R      | AATCTTGCTGCCCCAACAGG   |         |
| 2010107G23Rik-E2-F | CCCGGGAACCGTGCG        | 217/138 |
| 2010107G23Rik-E2-R | TGCTACTTCTGTGCTGGGTG   |         |
| Rims-E18-F         | GCCGAATGGATAGACACCGT   | 333/153 |
| Rims-E18-R         | TTGTCTGGACAGACCCGGTA   |         |
| Sbf2-E29-F         | TCTCACTGTCAGACCAGCAC   | 261/166 |
| Sbf2-E29-R         | GCGGCTTGTCGTTTCAAGAG   |         |
| Serpinb6a-E2-F     | TCAGACTCCTCCCGACTCTC   | 432/264 |
| Serpinb6a-E2-R     | CCCCCATGAAGACCATAGCC   |         |
| SNX11-E3-F         | TGGACGGGACAGGTTTTCTG   | 215/155 |
| SNX11-E3-R         | AGGAGCCTTCATTCTGCACC   |         |
| Tank-E2-F          | AGCTACTTCCGGTTGCAGTC   | 398/193 |
| Tank-E2-R          | TATCCATGCATGCCTGTCGG   |         |
| TPP2-E24-F         | CCTTGCAGGCTCCTTGACAT   | 221/183 |
| TPP2-E24-R         | TGAGATCGCGTAATGCTTCAGT |         |
| Zfp207-E8-F        | TCTTTTCCCCAGTGCTGGAC   | 285/192 |
| Zfp207-E8-R        | ATTGAAGCTGCTGGCTTTGC   |         |
| Zfp275-E3-F        | CGGGTGAGACCTTCCCTACA   | 247/155 |
| Zfp275-E3-R        | GCTCCGGGTTCAAACCTGAGA  |         |