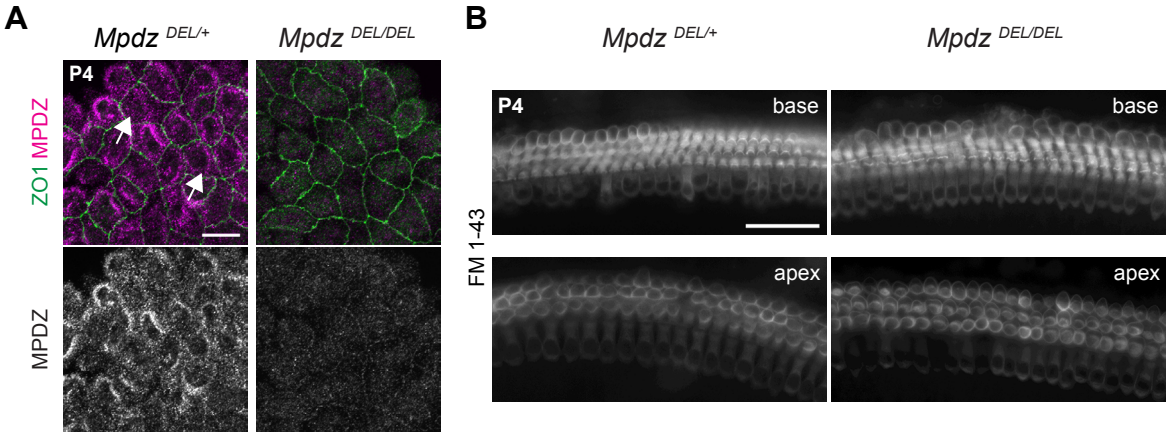

Fig. S1. A) MPDZ immunolabeling (magenta) in choroid plexus epithelial cells at P4 (4th ventricle/hindbrain; 1 µm projections). MPDZ signal at the apical membrane near the ZO1-labeled apical junction (arrows) is absent in *Mpdz* mutants. **B)** FM 1-43 uptake in HCs at P4. Despite hair bundle defects (see Fig. 1C), HCs in *Mpdz* mutants are able to load FM 1-43 in a comparable manner to control HCs. Scale bars are 10 µm (A), 25 µm (B).

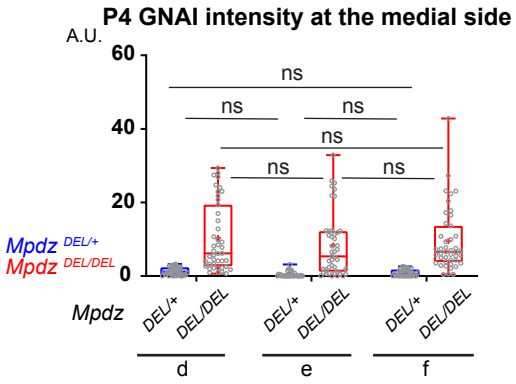

Fig. S2. GNAI enrichment across the medial side of the hair bundle in P4 OHCs. The medial apex is divided in 3 regions of equal width (d, e, f) and GNAI signal is measured by region. *Mpdz*$^{DEL/+}$ N=3, n=34 OHC; *Mpdz*$^{DEL/DEL}$ N=3, n=43 OHC (Two-way ANOVA with Sidak's multiple comparison test; *Mpdz*$^{DEL/+}$*:* d vs e P=0.9423; e vs f P=0.9911; d vs f P=0.9930; *Mpdz*$^{DEL/DEL}$*:* d vs e P=0.4144; e vs f P=0.7904; d vs f P=0.9216; ns, not significant.).

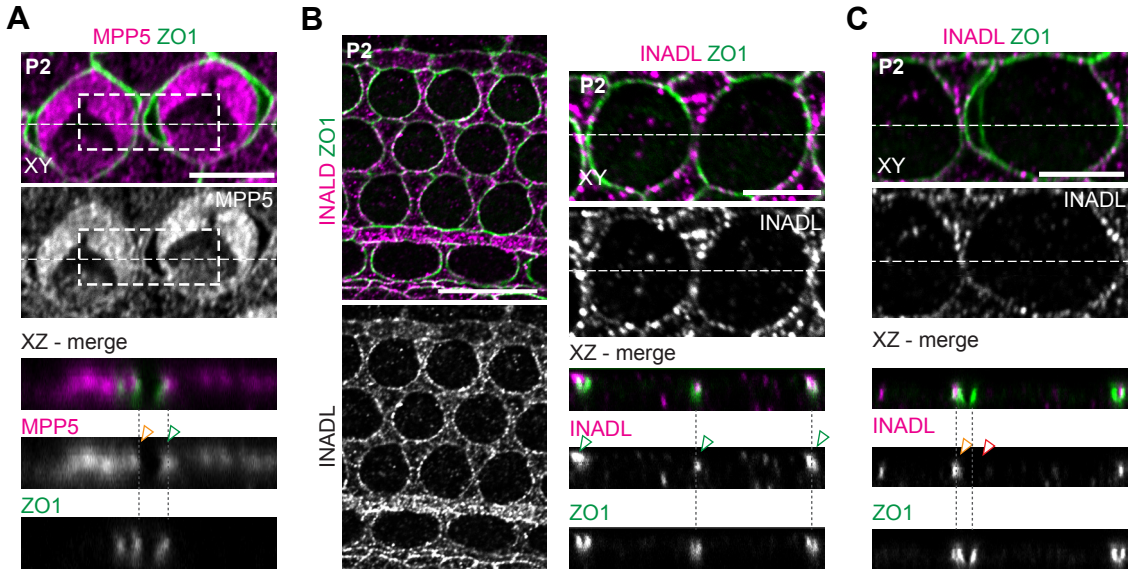

Fig. S3. MPP5 and INADL are enriched at the apical junction in support cells. **A)** MPP5 immunolabeling (magenta) in flat (XY) and projection (XZ) views for two OHC3 at P2 (wild-type; cochlear mid). We took advantage of TCA fixation artifacts where the apical membrane of the HC and neighboring support cell became separated. MPP5 is enriched at both the HC (green arrowhead) and support cell (orange arrowhead) apical junctions. **B)** INADL immunolabeling (magenta) in flat (left panels and XY) and projection (XZ) views of the auditory epithelium at P2 (wild-type; cochlear mid). INADL is not detected at the HC apical membrane, but co-enriched with ZO1 at the HC-support cell junctions (green arrowheads). **C)** INADL immunolabeling (magenta) in flat (XY) and projection (XZ) views of two OHC3 at P2 (cochlear mid) with the same TCA fixation artifacts as in (A). INADL is only enriched at the support cell apical junction (orange arrowhead) and absent at the HC junction (red arrowhead). Scale bars are 5μm (A, B right, C) and 10μm (B, left).

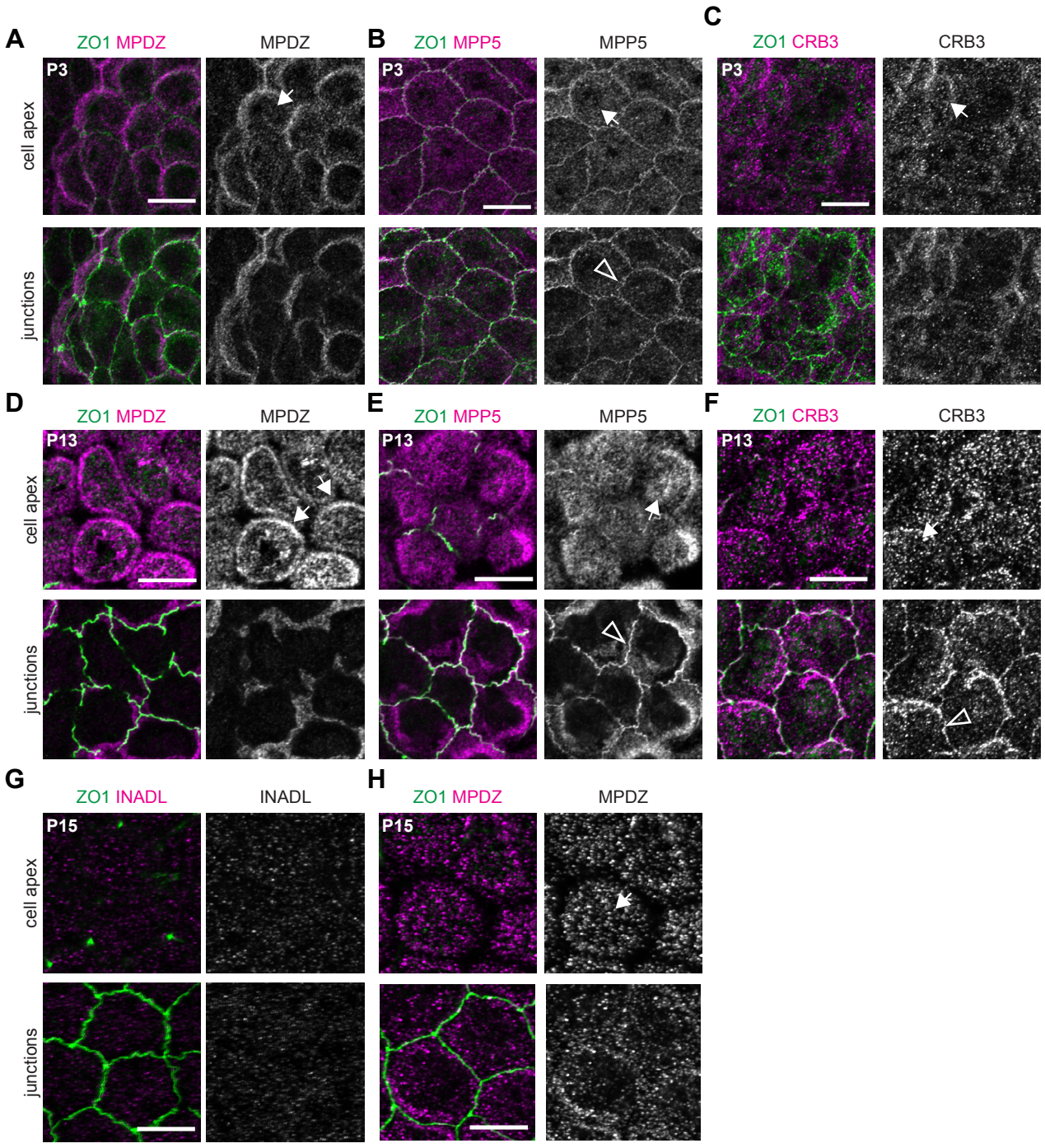

Fig. S4. MPDZ, MPP5 and CRB3 localization in epithelial cells of the hindbrain choroid plexus. The choroid plexus (4th ventricle/hindbrain) is immunolabeled at P3 (A-C), P13 (D-F) or P15 (G-H). Top panels show a projection of the most apical Z slices in a stack ("cell apex", 0.7 (A-F) or 1 (G-H) µm depth) to visualize the apical membrane. Bottom panels show a projection of lower apical Z slices in a stack ("junctions", same depths as "cell apex") to visualize the apical junctions. MPDZ is enriched at the apical membrane, where it accumulates preferentially near the junctions (arrows). MPDZ does not co-localize with ZO1 at apical junctions. In contrast, MPP5 and CRB3 (at P13) co-localize with ZO1 at apical junctions (unfilled arrowheads), in addition to sharing a similar pattern as MPDZ at the apical membrane (arrows). INADL is not detected either at the apical membrane or at apical junctions (H). Scale bars are 10µm.

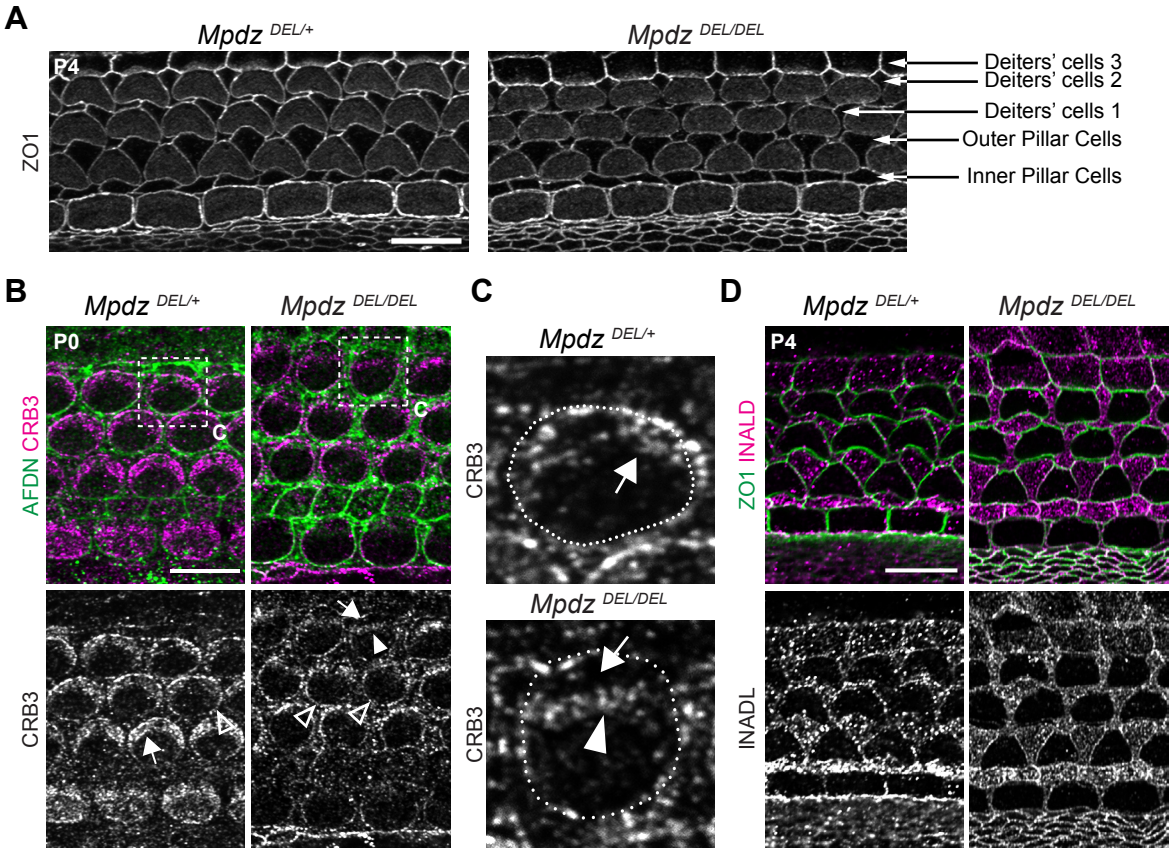

Fig. S5. MPDZ is required for the correct distribution of CRB3 at the hair cell apical membrane. **A)** ZO1 labeling of apical junctions in the auditory epithelium at P4 (cochlear base). In absence of MPDZ, the auditory epithelium maintains a normal pattern of intercalation between the apical domains of HCs and support cells, suggesting that junctional integrity is not significantly affected. **B-C)** CRB3 labeling at P0 (cochlear base). In absence of MPDZ, CRB3 is lost at the bare zone (arrow) and ectopically found in the hair bundle (filled arrowhead). CRB3 is retained at apical junctions (unfilled arrowheads). Note how CRB3 behaves similarly to MPP5 (see Figure 4D-E). The boxed regions in (B) are magnified in (C). **D)** INADL labeling at P4 (cochlear base). INADL enrichment remains unchanged in absence of MPDZ, as expected from the absence of INADL signal in HCs (see Supp. Fig. 3B-C). Scale bars are 10μm.

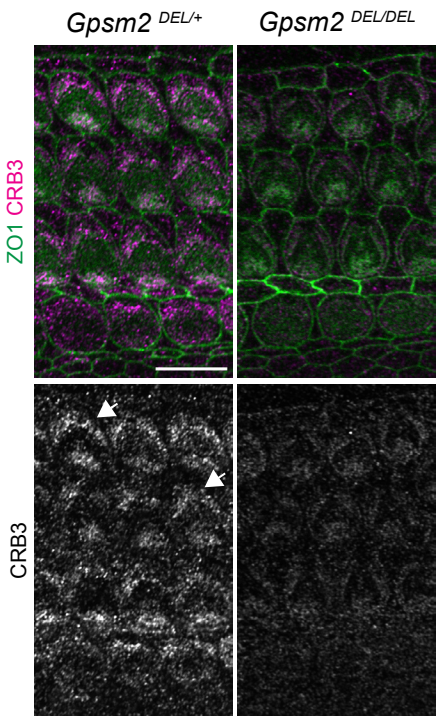

Fig. S6. CRB3 enrichment at the hair cell apical membrane is greatly reduced in *Gpsm2* mutants. CRB3 labeling (magenta) at P4 (cochlear apex). CRB3 enrichment is greatly reduced at the HC apical surface, and lacks polarization (preferential enrichment) at the lateral bare zone (arrows) in absence of GPSM2. Scale bar is 10μm.

Table S1. Comparison of *Mpdz* mouse strains including genetic background, hydrocephalus phenotype and lifespan.

	Our Data	Feldner et al., EMBO Mol Med 2017		Yang et al., EMBO Mol Med 2019
Mpdz mouse allele	*Mpdz em1(IMPC)J/Mmucd*	*MpdzΔ*	*Mpdz Gt(XG734)Byg* (From Milner et al, Addict Biol 2015)	
exons targeted and protein truncation	indel in exon 6 > truncation after 1st PDZ domain	floxed exons 4-5 > truncation before all 13 PDZ domains	gene trap in exons 11-12 > truncation after 3rd PDZ domain	
background	C57BL/6N	C57BL/6 (sub-strain unknown)	C57BL/6 (sub-strain unknown)	C57BL/6J
hydrocephalus	not observed	seen by P21	from P3	from P4
survival rate of homozygotes	no death	median 25 days	median 20 days	21 days
frequency of homozygote obtained				
from het x het crosses	genotyping after P21: 20% (N=145)	at birth (P0): 25%	P1 - P10: 20%	at birth (P0): 9%
from het x hom crosses	genotyping from P0 to P21: 43% (N=430)			

Table S2. Source data file.

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