The American Journal of Pathology, Vol. 192, No. 5, May 2022

The American Journal of
PATHOLOGY
ajp.amjpathol.org

NEUROPATHOLOGY

Collapsin Response Mediator Protein 1 (CRMP1) Is Required for High-Frequency Hearing

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Accepted for publication January 11, 2022.

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Collapsin response mediator protein 1 (CRMP1), also known as dihydropyrimidinase-related protein 1, participates in cytoskeleton remodeling during axonal guidance and neuronal migration. In cochlear hair cells, the assembly and maintenance of the cytoskeleton is of great interest because it is crucial for the morphogenesis and maintenance of hair cells. Previous RNA sequencing analysis found that *Crmp1* is highly expressed in cochlear hair cells. However, the expression profile and functions of CRMP1 in the inner ear remain unknown. In this study, the expression and localization of CRMP1 in hair cells was investigated using immunostaining, and was shown to be highly expressed in both outer and inner hair cells. Next, the stereocilia morphology of *Crmp1*-deficient mice was characterized. Abolishing CRMP1 did not affect the morphogenesis of hair cells. Interestingly, scanning electron microscopy detected hair cell loss at the basal cochlear region, an area responsible for high-frequency auditory perception, in *Crmp1*-deficient mice. Correspondingly, an auditory brainstem response test showed that mice lacking CRMP1 had progressive hearing loss at high frequencies. In summary, these data suggest that CRMP1 is required for high-frequency auditory perception. *(Am J Pathol 2022, 192: 805–812; https://doi.org/10.1016/j.ajpath.2022.01.011)*

Hair cells in the inner ear are highly specialized cells responsible for converting sound-evoked vibrations into electrical signals that propagate to the central nervous system. Protruding from the apical surface of hair cells, the mechanosensory hair bundle has rows of stereocilia that increase in height in a staircase-like fashion.[1] As the mechanosensory organelle of hair cells, hair bundles have a unique structure and protein composition, including mechanotransduction channels localized near the tips of stereocilia,[2] enabling them to convert mechanical stimuli into electrical signals.[3]

The cDNA of murine collapsin response mediator protein 1 (CRMP1) was first cloned and characterized approximately 3 decades ago.[4] *Crmp1* is widely expressed in the cochlea, retina, and brain during development.[4,5] In neuronal cells, CRMP1 colocalizes with actin in the growth cone and is essential for cytoskeletal reorganization and neurite outgrowth.[6] In prostate cancer cells, CRMP1 regulates the polymerization of F-actin, likely via an interaction with Wiskott-Aldrich syndrome protein family member 1,[7] which accelerates actin nucleation and actin filament elongation.[8,9] Although *Crmp1* is enriched in cochlear hair cells,[5] its functions in auditory perception remain unknown.

In this study, the expression and localization of CRMP1 in the murine cochlea was investigated. Next, the extent to which deletion of CRMP1 affects stereocilia morphogenesis and auditory perception was studied by characterizing *Crmp1* null mutant mice. The results showed that CRMP1 is not required for the morphogenesis of stereocilia during development; instead, it is required for hair cell maintenance at the basal turn of the cochlea in adult mice.

Supported by National Institute on Deafness and Other Communication Disorders grant DC017147, Royal National Institute for Deaf People Discovery Research Grant, and Indiana University School of Medicine startup funding (B.Z.).

Disclosures: None declared.

https://doi.org/10.1016/j.ajpath.2022.01.011

Materials and Methods

Animal Care and Genotyping

Crmp1 null mutant mouse (C57BL/6NCrl-*Crmp1*$^{em1(IMPC)Mbp}$/*Mmucd*; MGI:6430667), in which exon 3 of *Crmp1* was deleted, was obtained from Mutant Mouse Resource & Research Centers (Bethesda, MD). The frameshift caused by the deletion of exon 3 is predicted to generate an early stop codon after the amino acid in position 40. To avoid potential off-target mutations induced by clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), mutant mice were backcrossed with wild-type C57/BL6J mice. To genotype *Crmp1* mutant mice, the following primers were used: 5′-CTGCTGCTGCCTCACTCAGTGAC-3′, 5′-GCAGGTAAGTGTTGACATCAATGCC-3′, and 5′-CAGTCTGAACTGGTTCAGGGTGC-3’. To genotype pups of different sexes, the following primers targeting the Y chromosome were used: 5′-TCCCAGAATCGGTGCTCCTTA-3′, and 5′-CGCCCCCTCACGTCACTCCTT-3’. All animal experiments were conducted following protocols approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. Both male and female mice were used in this study. At least three mice per genotype in each experiment were analyzed. Age-matched, wild-type mice were used as controls.

Immunostaining

Cochlear whole-mount staining was performed as described.[10–12] In brief, cochleae were dissected and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Samples then were washed three times in Hank’s balanced salt solution (cat. 14175103; Thermo Fisher Scientific, Carlsbad, CA) and incubated in blocking buffer (Hank’s balanced salt solution containing 5% bovine serum albumin, 1% goat serum, and 0.5% Triton X-100 (Fisher Scientific, Hampton, NH) for 20 minutes. Samples then were incubated with the following primary antibodies overnight at 4°C: anti-CRMP1 (cat. HPA035640; Millipore-Sigma, St. Louis, MO), antitaperin (cat. HPA020899; Millipore-Sigma), anti-espin (cat. SC-393469; Santa Cruz, Dallas, TX), anti−myosin 7a (cat. 25-6790; Proteus BioSciences, Ramona, CA), and Alexa Fluor 568−phalloidin (cat.A12380; Thermo Fisher Scientific). After being washed, the samples were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (cat. A11070; Thermo Fisher Scientific) or Alexa Fluor 488 goat anti-mouse secondary antibody (cat. A11017; Thermo Fisher Scientific) at room temperature for 2 hours. Then, the samples were washed and mounted using Prolong Diamond Antifade Mountant (cat. P36971; Thermo Fisher Scientific). Stacked images (Z step, approximately 0.17 μm) then were acquired

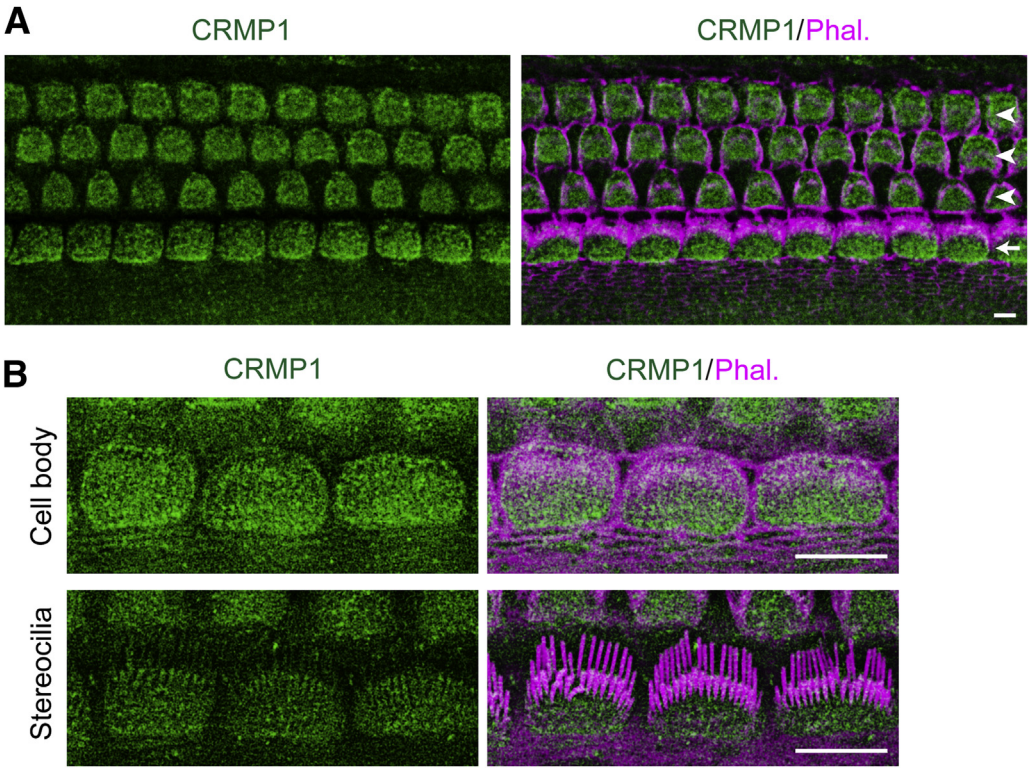

Figure 1 Collapsin response mediator protein 1 (CRMP1) is highly expressed in murine hair cells. **A:** Cochlear explants were dissected from postnatal day 5 wild-type C57/BL6J mice and costained with CRMP1 antibody (green) and phalloidin (Phal.) (magenta) to show hair cells. CRMP1 is highly expressed in both inner hair cells (**arrow**) and outer hair cells (**arrowheads**). **B:** Cochlear explants dissected from postnatal day 7 wild-type C57/BL6J mice were costained with CRMP1 antibody (green) and phalloidin (magenta) to show stereocilia or the cell bodies of hair cells. CRMP1 is localized mainly in the cytoplasm. A weak signal also was detected in the stereociliary region. Scale bars = 5 μm.

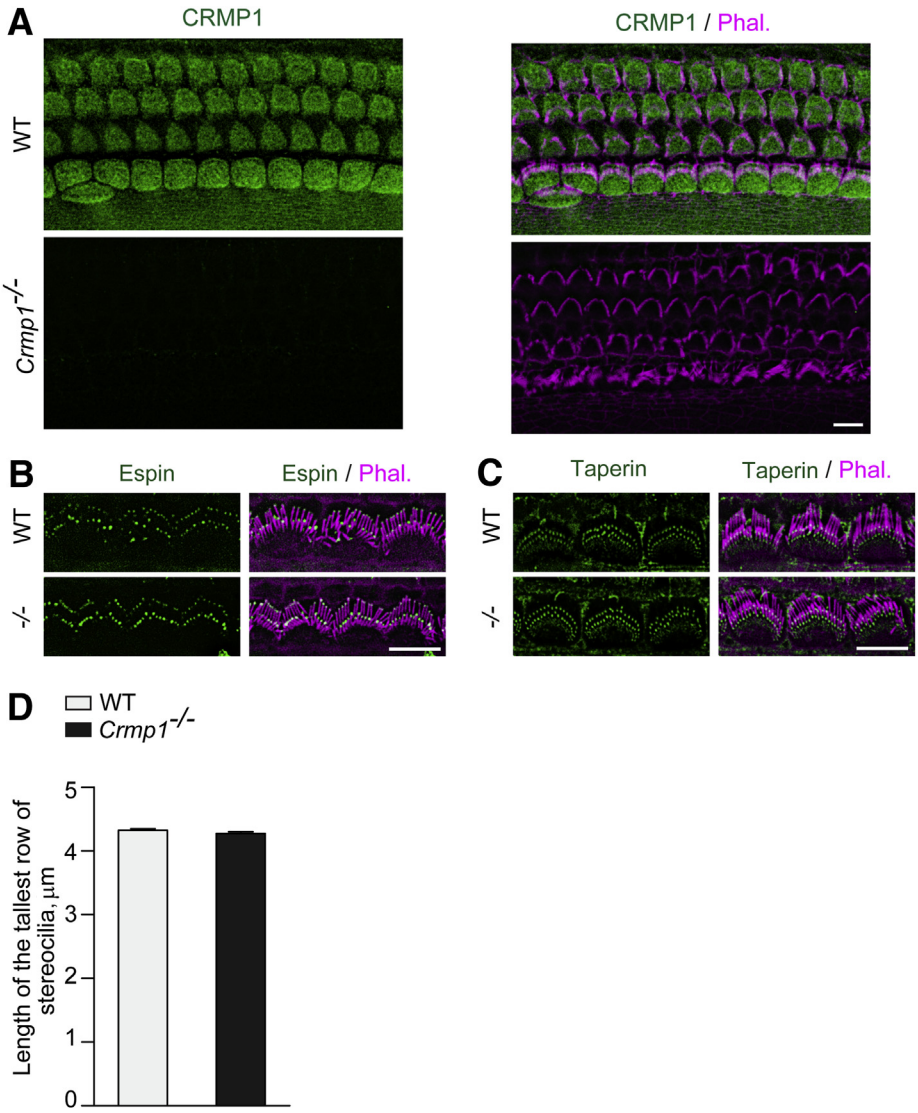

Figure 2 *Crmp1*-deficient hair cells have a relatively normal morphology during development. **A:** Cochlear explants dissected from postnatal day 5 wild-type (WT) C57/BL6J and *Crmp1* mutant mice were costained with the collapsin response mediator protein 1 (CRMP1) antibody (green) and phalloidin (Phal.) (magenta) to show hair cells. Note the absence of a signal in the *Crmp1* mutant cochlear epithelium. **B** and **C:** Cochlear explants dissected from postnatal day 5 wild-type and *Crmp1* mutant mice were stained with espin antibody (**B**) and taperin antibody (**C**). **D:** Length of the tallest row of stereocilia of inner hair cells. More than 40 inner hair cells in each group were measured. Note that there was no significant difference between wild-type and *Crmp1* mutant mice (two-tailed unpaired *t*-test). Data are represented as the means ± SEM. Scale bars = 5 µm.

by a DM6 FS automated deconvolution microscope (Leica, Buffalo Grove, IL) using a 100× objective (Leica HCX PL APO 100×/1.40-0.70 OIL). The length of the tallest row of stereocilia of inner hair cells was measured as described previously[10] using ImageJ software version 1.53a (NIH, Bethesda, MD; *https://imagej.nih.gov/ij*). At least three mice per genotype were analyzed. Age-matched wild-type mice were used as controls.

Scanning Electron Microscopy

The experiments were performed as previously described.[10–12] Middle and basal portions of cochleae from adult mice were dissected according to a published protocol.[13,14] Samples were fixed in a fixative containing 2.5% glutaraldehyde, 4% formaldehyde, 0.05 mmol/L HEPES buffer pH 7.2, 10 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, and 0.9% NaCl for 1 hour at room temperature. Cochleae then were dissected to remove the stria vascularis, Reissner's membrane, and tectorial membrane. Samples were postfixed by immersion for 1 day in the same fixative at 4°C and then in 1% OsO_4 for 1 hour. Samples then were dehydrated in ethanol, critical point dried using Autosamdri-815A (Tousimis, Rockville, MD), mounted on aluminum stubs, and coated with gold. Then, the samples were viewed on a JEOL 7800F scanning electron microscope (JEOL, Akishima, Tokyo, Japan). The width of the tallest row of stereocilia of inner hair cells was measured as described

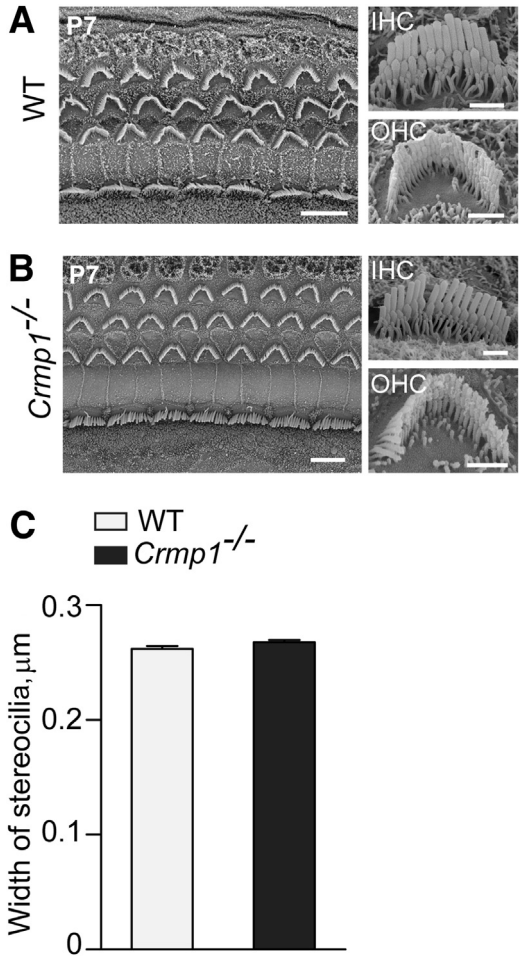

Figure 3 Morphology of postnatal hair cells analyzed by scanning electron microscopy. **A** and **B:** Whole mounts from the cochlear middle turn of wild-type (WT) (**A**) and *Crmp1* mutant mice (**B**) at the age of postnatal day 7 (P7) were analyzed by scanning electron microscopy. Note the well-organized stereocilia in *Crmp1* mutant mice. **C:** Width of the tallest row of stereocilia of inner hair cells from wild-type and *Crmp1*-deficient mice. More than 25 inner hair cells in each group were measured and no significant difference was detected by the two-tailed unpaired *t*-test. Data are represented as the means ± SEM. Scale bars: 5 μm (**A** and **B**, **left**); 1 μm (**A** and **B**, **right**). IHC, inner hair cell; OHC, outer hair cell.

previously[15,16] using ImageJ software version 1.53a (NIH, Bethesda, MD; *https://imagej.nih.gov/ij*). Note that only the width of stereocilia with approximately perpendicular orientation was measured.[16] Seven postnatal day 7 *Crmp1* mutant mice including three males and four females, five 2-month-old *Crmp1* mutant mice including three males and two females, and six 4-month-old *Crmp1* mutant mice including four males and two females were analyzed. No sex-based difference was observed. In each experiment, at least three age-matched, wild-type mice were used as controls.

Auditory Brainstem Response Measurement

Auditory brainstem responses (ABRs) of mice were recorded using the TDT Bioacoustic system 3 and BioSigRZ software (Tucker-Davis Technologies, Alachua, FL) as described.[10–12] In brief, mice were anesthetized and kept warm on a thermostatic pad. The recording and reference electrodes were inserted under each animal's skin at the vertex and ipsilateral ear, while the grounding electrode was inserted under each animal's skin near the tail. A speaker was placed 5-cm away from the ear of the mouse. Tone stimuli were presented 21 times per second, and a total of 512 responses was averaged at each frequency and level combination. The intensity of the sound stimulus was started at 90 dB sound pressure level and decreased in 10 dB sound pressure level stepwise to a subthreshold level.[10,12] ABR thresholds for pure tone were tested at 4, 8, 12, 16, 20, 24, 28, and 32 kHz. Five 3-week-old *Crmp1* mutant mice including 4 males and 1 female, seven 2-month-old *Crmp1* mutant mice including four males and three females, and six 4-month-old *Crmp1* mutant mice including four males and two females were used in this study. No sex-based difference was observed.

Statistical Analysis

All of the data in this study are shown as the means ± SEM. The Kolmogorov-Smirnov test was used first to determine the normality of the data distribution. Then a two-tailed unpaired *t*-test was used to compare stereocilia length and width, and evaluate the thresholds of click ABR in wild-type and *Crmp1* mutant mice. Two-way analysis of variance was used to determine statistically significant differences in pure-tone thresholds in wild-type and *Crmp1* mutant mice. Significant differences in each frequency were determined by the Bonferroni *post hoc* test.

Results

CRMP1 Is Highly Expressed in Murine Cochlear Hair Cells

Prior studies show that *Crmp1* mRNA is enriched in cochlear hair cells.[5] To determine the expression profile of CRMP1 in the inner ear, cochlear epithelia of postnatal day 5 C57/BL6J wild-type mice were dissected and fixed. Immunostaining was performed using anti-CRMP1 antibodies. Consistent with the mRNA expression profile,[5] CRMP1 protein was highly expressed in both outer hair cells (OHCs) and inner hair cells (IHCs) (Figure 1A). At higher magnification, CRMP1 was localized mainly in the cytoplasm (Figure 1B). A weak CRMP1 signal diffusing along the stereocilia also was detected (Figure 1B).

Crmp1 Mutant Hair Cells Have Normal Morphology during Development

CRMP1 is required for cortical neuron migration and neurite morphogenesis.[6,17] The study sought to determine the function of CRMP1 in the cochlear development and

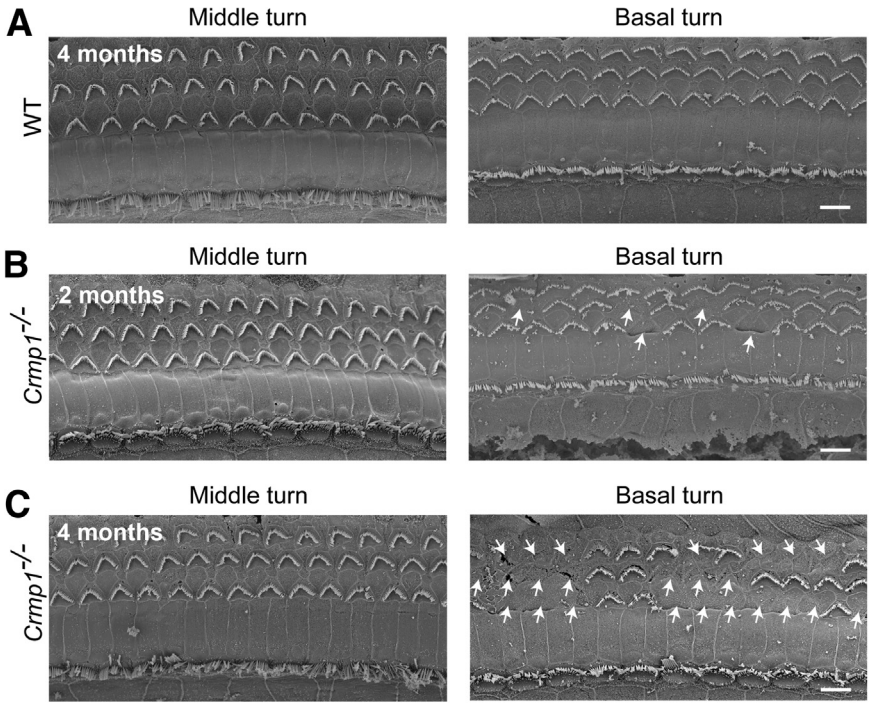

Figure 4 Morphology of adult hair cells analyzed by scanning electron microscopy. **A:** Whole mounts from the cochlear middle and basal regions of 4-month-old wild-type (WT) mice were analyzed by scanning electron microscopy. **B** and **C:** Whole mounts from the middle regions (corresponding to sensitivity to tones 8-22 kHz) and basal regions (corresponding to sensitivity to tones 22-32 kHz) of the cochlea of *Crmp1* mutant mice at the ages of 2 months (**B**) and 4 months (**C**) were analyzed by scanning electron microscopy. The stereocilia in the middle turn of the cochlea were well organized in both wild-type and mutant hair cells. Note the outer hair cell (OHC) loss (**arrows**) in *Crmp1* mutant mice evidenced by loss of stereociliary bundles. Scale bars = 5 μm.

morphogenesis of hair cells. *Crmp1* mutant mice were generated at Mutant Mouse Resource & Research Centers using a CRISPR/Cas9 system. To determine whether *Crmp1* mutant mouse was a null mutant, whole-mount immunostaining was performed. A strong immunostaining signal was obtained from the wild-type cochlea. Remarkably, no signal was detected in the *Crmp1* mutant (Figure 2A). This result suggests that this mutant mouse was a *Crmp1* null mutant.

Whether *Crmp1* mutant mice have defects in the morphogenesis of stereocilia, which are filled with a paracrystalline array of actin filaments, was investigated next. Phalloidin staining of whole-mounted postnatal day 5 sensory epithelia in *Crmp1* mutant mice showed three well-organized rows of OHCs and one row of IHCs (Figure 2A). At higher magnification, stereocilia were arranged in a staircase-like pattern in both wild-type and mutant hair cells (Figure 2, B and C). Next, the localization of espin, an actin-binding protein localized at the distal end of stereocilia and essential for morphogenesis of stereocilia,[18–20] and taperin, an actin-binding protein localized at the proximal end of stereocilia and that regulates actin cytoskeleton in stereocilia,[10,21] was studied using whole mount immunostaining. No significant difference in either protein between wild-type and *Crmp1*-deficient hair cells was observed (Figure 2, B and C). In addition, using taperin as a marker of the stereociliary base,[10] the length of the tallest row of stereocilia of IHCs was measured. No significant difference in stereociliary length between wild-type and *Crmp1*-deficient mice was observed (Figure 2D).

Scanning electron microscopy was performed to characterize the stereociliary structure in further detail. Consistent with fluorescence microscopy (Figure 2), *Crmp1*-deficient cochlear epithelia showed three well-organized rows of OHCs and one row of IHCs on postnatal day 7 (Figure 3, A and B). At higher magnification, stereocilia in both OHCs and IHCs were generally arranged in a staircase-like pattern (Figure 3, A and B). In addition, the width of the tallest row of stereocilia of IHCs was measured. No significant difference was observed in stereocilia length (Figure 3C). These results suggest that the stereocilia of *Crmp1* mutant hair cells are minimally affected at early developmental stages.

Adult *Crmp1* Mutant Mice Show Hair Cell Loss at the Basal Cochlear Region

The study next addressed whether CRMP1 is required for hair cell maintenance in adult mice. Scanning electron microscopy was performed to analyze sensory epithelia in adult mice. *Crmp1* mutant mice at the ages of 2 months and 4 months had regular V-shaped hair bundles in the middle turn of the cochlea (Figure 4). In the basal turn of the cochlea, OHC loss, evidenced by loss of stereocilia, was observed in the 2-month-old mutant mice (Figure 4B). Remarkably, in the basal turn of the cochlea, *Crmp1* mutant mice at the age of 4 months lost more than 35% of hair cells, most of which were OHCs (Figure 4C and Supplemental Figure S1A). Previous studies found that hair cells can lose their hair bundle and survive as bundleless cells for at least 1 week.[22] To confirm the hair cell death in *Crmp1* mutant mice, antibody against myosin 7a was used to visualize cell bodies of the hair cells. Indeed, there was robust OHC loss (Supplemental Figure S1B) in the basal turn of the cochlea dissected from 4-month-old mutant mice.

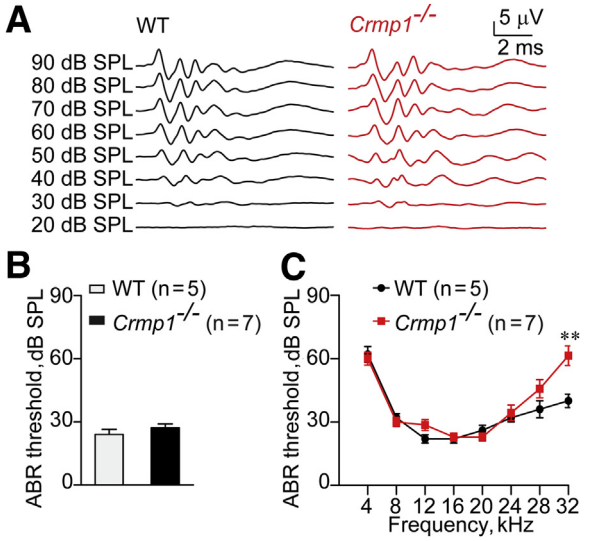

Figure 5 Analysis of hearing function of 2-month-old animals by auditory brainstem response (ABR). **A:** Representative traces of ABRs to click stimuli in wild-type (WT) (**black traces**) and *Crmp1*-deficient mice (**red traces**). **B:** ABR thresholds for click stimuli. No significant difference between wild-type and *Crmp1* mutant mice was detected by the two-tailed unpaired *t*-test. Data are represented as the means ± SEM. **C:** ABR thresholds for pure tones. Data are represented as the mean ± SEM. $P < 0.01$ between WT and *Crmp1* mutant mice by two-way analysis of variance. Note the significantly increased hearing thresholds at 32 kHz in the *Crmp1* mutant. $^{**}P < 0.01$ by Bonferroni *post hoc* test. SPL, sound pressure level.

These results suggest that CRMP1 is required for hair cell maintenance in adult mice.

CRMP1 Deficiency Leads to Progressive High-Frequency Hearing Loss

The basal turn of the cochlea is known to be responsible for high-frequency auditory perception.[23] To investigate whether *Crmp1*-deficient mice have high-frequency hearing loss, the ABR was measured in mice at the age of 3 weeks. Five *Crmp1* mutant mice and eight wild-type mice were analyzed. The thresholds of click ABR in *Crmp1* mutant mice were not significantly higher (Supplemental Figure S2A). Interestingly, ABR thresholds for pure tones of various frequencies showed hearing loss at 32 kHz (Supplemental Figure S2B). Next, auditory functions in seven 2-month-old *Crmp1*-deficient mice and five age-matched, wild-type mice were analyzed. The thresholds of click ABR were not significantly higher in *Crmp1* mutant mice (Figure 5, A and B), whereas ABR thresholds for pure tones showed a hearing loss at 32 kHz in 2-month-old mutant mice (Figure 5C). Auditory functions in six 4-month-old *Crmp1* mutant mice were measured next. Thresholds of click ABR were still not affected significantly (Figure 6A). Remarkably, recording ABRs in response to pure tones showed that *Crmp1* mutant mice had significantly increased hearing thresholds at high frequencies ranging from 24 to 32 kHz (Figure 6B). These results suggest that CRMP1 deficiency leads to progressive high-frequency hearing loss in mice.

Discussion

Crmp1 is enriched in cochlear hair cells in newborn mice.[5] CRMP1 plays an essential role in neuronal development by regulating the actin cytoskeleton.[6] Thus, we hypothesized that CRMP1 is critical for the development and morphogenesis of hair cells. Immunostaining and scanning electron microscopy showed that deleting CRMP1 had no observable effect on the morphogenesis of stereociliary bundles. CRMP2, the paralog of CRMP1 with more than 70% amino acid sequence identity, is also highly expressed in postnatal hair cells.[24,25] In neuronal cells, CRMP1 and CRMP2 have different functions.[6] However, it is possible that these two proteins have similar functions in hair cells and that CRMP1 deficiency is compensated partly by CRMP2. Thus, it will be of interest to characterize CRMP1 and CRMP2 double-knockout mice and investigate the extent to which collapsin response mediator proteins are involved in hair cell morphogenesis.

Crmp1 mutant mice have progressive OHC loss at the basal cochlear turn and subsequent progressive high-frequency hearing loss, suggesting that CRMP1 is important for hair cell maintenance in adult mice. The mechanisms underlying *Crmp1* deletion-induced hair cell loss are still unclear. Actin dynamics in adult hair cells are crucial for hair cell maintenance. In adult hair cells, actin incorporation in the distal end of stereocilia is required for stereocilia maintenance.[26] It is possible that CRMP1 plays a part in hair cell maintenance by regulating the actin cytoskeleton in adult hair cells. In addition, traumatic noise induces depolymerization of actin filaments, resulting in the death of hair cells and increases in hearing thresholds.[27] Because hair cells at the basal cochlear turn are more

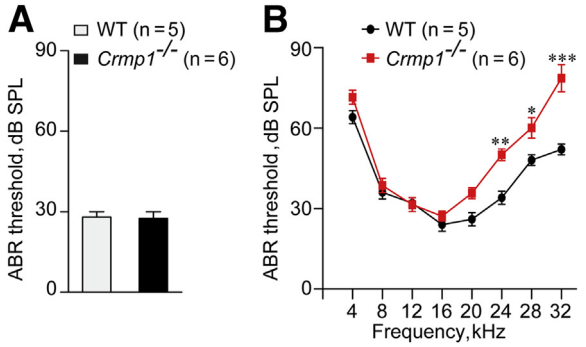

Figure 6 Analysis of hearing function of 4-month-old animals by auditory brainstem response (ABR). **A:** ABR thresholds for click stimuli. No significant difference between wild-type (WT) and *Crmp1* mutant mice was detected by the two-tailed unpaired *t*-test. **B:** ABR thresholds for pure tones. Thresholds were increased significantly in *Crmp1* mutant mice at high frequencies ranging from 24 to 32 kHz. Data are represented as the means ± SEM. $P < 0.01$ between WT and *Crmp1* mutant mice by two-way analysis of variance. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by Bonferroni *post hoc* test. SPL, sound pressure level.

susceptible to noise, it will be of interest to study the extent to which CRMP1 is involved in noise-induced hearing loss in the future. In addition, screening interacting proteins of CRMP1 and investigating the extent to which those binding partners are required for auditory perception will be informative.

A previous study found that reducing plasma membrane calcium-transporting ATPase 2 (PMCA2) expression decreases CRMP1 levels and promotes the death of spinal cord neurons through an unknown mechanism.[28] PMCA2, which is highly expressed in OHCs, is essential for auditory perception by extruding calcium from hair cells and regulating intracellular calcium levels.[29] PMCA2-null mutant mice have profound hearing loss across all the tested frequencies.[30] Similar to *Crmp1*-deficient mice, PMCA2-null mutant mice have minimally affected stereocilia morphology during development and subsequent robust hair cell loss, especially at the basal turn of the cochlea, in adult mice.[31] It is possible that CRMP1/CRMP2 might be downstream proteins of PMCA2 in hair cells and partially involved in PMCA2-mutation–induced hair cell death and subsequent hearing loss. In this study, no difference in PMCA2 expression in *Crmp1* mutant mice compared with wild-type mice was observed (data not shown). It will be of interest to study the expression and localization of CRMP1 in PMCA2 mutant hair cells. PMCA2-null mutant mice will be characterized to address this possibility. In addition, the $Cdh23^{G753A}$ single-nucleotide polymorphism in C57BL/6J ancestral strains leads to age-related progressive hearing loss.[32] Interestingly, studies have shown that the $Cdh23^{G753A}$ single-nucleotide polymorphism interacts with *Atp2b2*, encoding the PMCA2 protein, to modify auditory perception.[31] Because CRMP1 and PMCA2 have been found in the same signaling pathway in spinal cord neurons,[28] it is possible that the $Cdh23^{G753A}$ single-nucleotide polymorphism might exacerbate the hearing loss caused by CRMP1 deletion, as it does in PMCA2 mutants.[31] To test this hypothesis and extensively investigate the underlying mechanisms of high-frequency hearing loss caused by CRMP1 deletion, it would be interesting to cross *Crmp1* mutant mice with $Cdh23^{Ahl+}$ mice, which bear a normal functioning *Cdh23* allele and are not susceptible to age-related hearing loss,[33] and evaluate their auditory function.

In summary, this study shows that mice lacking CRMP1 have robust hair cell loss at the basal turn of the cochlea and that CRMP1 is required for high-frequency auditory perception in adult mice.

Author Contributions

J.L., C.L., and B.Z. performed the experiments and wrote the manuscript; B.Z. conceptualized and supervised the study.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2022.01.011*.

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